Endocytic downregulation of the oncoproteins ErbB2 and ErbB3

By

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2015

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Series of dissertations submitted to the
Faculty of Medicine, University of Oslo
No. 2137

ISBN 978-82-8333-160-8
ISSN 1501-8962

Cover: Hanne Baadsgaard Utigard
Printed in Norway: 07 Media AS – www.07.no

ACKNOWLEDGMENTS

The work presented in this thesis was funded by the Norwegian Cancer Society, to whom I express my sincere gratitude.

I would like to thank my supervisors Prof. Inger Helene Madshus, Dr. Espen Stang, Dr. Vibeke Bertelsen and Prof. Henrik S Huitfeldt. Throughout the course of my studies they proved to be generous with both their time, their experience and guidance, without which this work would not have been possible. At all times they were willing to provide insight, advice, discussion and constructive criticism which has proved invaluable to me during my studies.

I am sincerely grateful to the members of my group, both past and present, specifically Małgorzata M. Sak for sharing with me her extensive knowledge of laboratory techniques, Anne Marthe Fosdahl for her support, both professionally and socially and Marianne S. Rødland who was always willing to assist in so many ways. Markus Dietrich and Filip Nikolaysen contributed to my time in the group in so many ways and to them I extend a warm and sincere thank you. I am also extremely grateful to Camilla Raiborg for her help her time and her genuine, positive enthusiasm. Sincere gratitude is also due to all my co-authors for their contributions without which this work would not have been possible. To all the members of the Department of Pathology who are too numerous to mention, I thank you all for a memorable four years which was rich in both professional and social aspects and for many fond memories which I will carry with me in the future. In a similar vein, I extend heartfelt thanks to my office and laboratory friends who made the last four years so pleasant and who contributed in so many ways to a positive and friendly environment.

Last but not least, I wish to express my deepfelt gratitude to my family and friends whose constant encouragement, confidence and support in so many ways facilitated my progress over the past four years and helped me through the tough times.

Oslo, August 2015
Monika Szymańska

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Abbreviations

AP-2	Adaptor protein 2
Cbl	Casitas B-lineage lymphoma
CCP	Clathrin coated pit
CCV	Clathrin coated vesicle
CIE	Clathrin- independent endocytosis
CLASP(s)	Clathrin Associated Sorting Protein(s)
CME	Clathrin- mediated endocytosis
DUB	Deubiquitinating enzyme
EE	Early endosome
EEA1	Early endosomal antigen 1
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EH	Epsin homology
ENTH	Epsin N-terminal homology
ESCRT	Endosomal Sorting Complexes Required for Transport
EGFP	Enhanced green fluorescent protein
Hrs	Hepatocyte growth factor regulated tyrosine kinase substrate
IgG	Immunoglobulin G
ILV	Intraluminal vesicle
Immuno-EM	Immuno-electron microscopy
LE(s)	Late endosome(s)
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
mAb	Monoclonal antibody
mAb mix	Monoclonal antibodies mixture
MAPK	Mitogen-activated protein kinase
MHC-I	Major histocompatibility complex class I
MVB(s)	Multivesicular body(ies)
NRG(s)	Neuregulin(s)
PAE	Porcine Aortic Endothelial
PI3K	Phosphatidylinositol 3-kinase

PIP2	Phosphatidylinositol 4,5-bisphosphate
PRD	Proline rich domain
PTB	Phosphotyrosine binding
Rab	Ras-associated binding
RNAi	RNA interference
RTK	Receptor tyrosine kinase
SH	Src homology
siRNA	Short interfering RNA
Tf	Transferrin
TfR	Transferrin receptor
TGF α	Transforming growth factor alpha
TGN	*Trans*-Golgi network
UIM	Ubiquitin interacting motif

Papers Included

Paper I

Sak,M.M., Szymanska,M., Bertelsen,V., Hasmann, M., Madshus,I.H. and Stang,E.

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

Carcinogenesis, 34, 2031-8, May 2013

Paper II

Szymanska, M., Fosdahl, A.M., Raiborg, C., Dietrich, M. Liestøl, K., Stang, E. and Bertelsen, V.

Interaction with epsin 1 regulates the constitutive clathrin-dependent internalization of ErbB3.

Manuscript

Paper III

Szymanska, M., Fosdahl, A.M., Nikolaysen, F., Pedersen, M.W., Grandal, M.M., Stang, E. and Bertelsen V.

The combination of two antibodies recognizing non-overlapping epitopes of ErbB2 induces ErbB2 ubiquitination and internalization in an ErbB2 kinase activity dependent manner.

Manuscript

Introduction

The eukaryotic super-family of protein kinases represents unique, strictly organized and precisely regulated molecules that play an important role in nearly every aspect of cell biology. In the human genome, about 2% of genes encode for more than 500 distinct kinases (Manning et al, 2002). There are two main classes of kinases: tyrosine kinases (TKs) and serine–threonine kinases (STKs), both TKs and STKs can be membrane-bound and localized free in the cytoplasm and in the nucleus. There are 90 members of the tyrosine kinase class, 58 of them being receptor tyrosine kinases (RTKs). All RTKs share a similar molecular structure, including extracellular ligand-binding domain and cytoplasmic region that contains the tyrosine kinase domain. In brief, upon the binding of growth factor, cytokines and hormones to the receptor, the intracellular signalling pathways controlling cell cycle progression, development, differentiation, transcription and apoptosis are activated (reviewed in Lahiry et al, 2010; Roskoski, 2014). Moreover, mutations and aberrant regulation of RTKs can cause constant signalling and can lead to development of a variety of pathological conditions including autoimmune and cardiovascular diseases, diabetes inflammation and cancer. The RTKs are divided into 20 subfamilies, one subfamily being the EGFR (epidermal growth factor receptor) family (also called the ErbB family) which is a family of receptors for growth factors. Excessive activation of ErbB proteins has been reported to be a major contributor to cancer development and progression (reviewed in Yarden & Pines, 2012). Shutting down the signaling may be achieved by downregulating an activated receptor by inducing its internalization and degradation. The study presented in this work aimed at increasing the understanding of the mechanisms controlling the internalization of ErbB2 and ErbB3.

Members of the EGFR/ErbB protein family

Structure, activation, signaling

The EGFR or ErbB family consists of four members: EGFR (also called ErbB1 or HER1), ErbB2 (also called Neu or HER2), ErbB3 (also called HER3) and ErbB4 (also called HER4). The receptors are expressed in neuronal, epithelial and mesenchymal cells in different tissues where they play key roles in cell differentiation, growth and survival and they have been shown to be involved in development, proliferation and differentiation (reviewed in Olayioye et al, 2000). ErbB proteins localize mainly to plasma membrane, except ErbB3, which has been found at the plasma membrane and constitutively in intracellular vesicles (Sak et al, 2012). All ErbB proteins have a large, highly glycosylated extracellular region of approximately 620 amino acids that can be subdivided into four domains (I-IV) (Figure 1). Domains I and III serve as the primary ligand-binding regions. The cysteine-rich domains II and IV are important for receptor dimerization where domain II serves as the dimerization arm and domain IV plays an auto-inhibitory role by interacting with domain II and keeping the dimerization arm hidden. A single transmembrane domain links the extracellular region to the approximately 540-amino-acid intracellular region of the receptor that contains a tyrosine kinase domain as well as a carboxy-terminal tail (C-tail) of approximately 230 amino acids (Lemmon et al, 2014; Ward et al, 1995). The kinase domain has a bi-lobular structure composed of N- and C-terminal lobes which upon ligand binding and dimerization, adopt an asymmetric conformation where the N-lobe of one kinase domain is interacting with the C-lobe of the other kinase domain (Zhang et al, 2006). The C-tail has a regulatory role and its residual tyrosines undergo phosphorylation either by intrinsic (autophosphorylation) or neighbouring kinase domain (transphosphorylation).

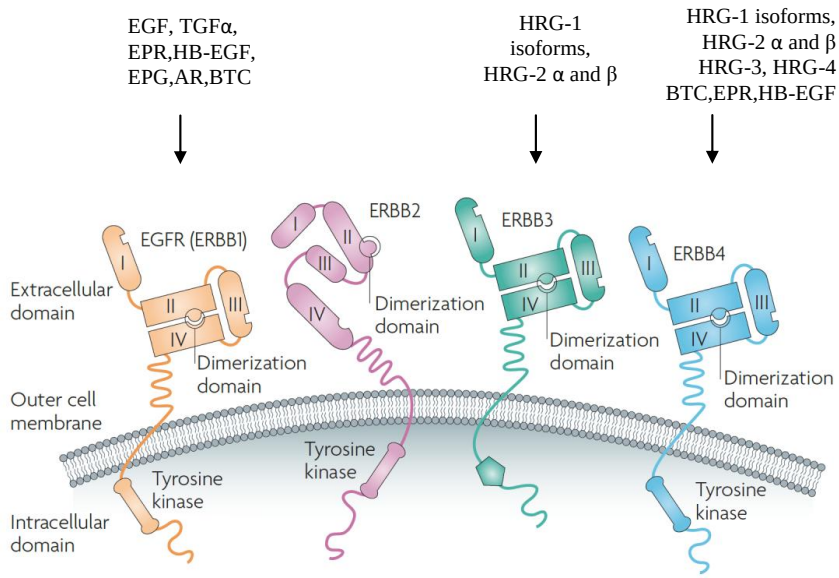


Figure 1. ErbB proteins and their ligands. ErbB proteins share a similar structure. They are composed of the extracellular domain,a transmembrane segment and intracellular tyrosine kinase domain. Sub-domains I and III in the extracellular part of the receptor are responsible for ligand binding and sub-domains II and IV are involved in dimerization. In contrast to other ErbBs, ErbB2 doesn't bind any known soluble ligands and it exists in a constitutively open conformation, while other ErbB receptors exist in a "closed" conformation in the absence of ligands. The kinase domain is flanked by tyrosine authophosphorylation sites and ErbB3 has marginal kinase activity compared with other ErbB receptors. Abbreviations: epidermal growth factor (EGF), transforming growth factor alpha (TGFα), epiregulin (EPR), heparin-binding EGF-like growth factor (HB-EGF), epigen (EPG), amphiregulin (AR), betacellulin (BTC), heregulin (HRG) also known as neuregulin (NRG), tomoregulin (TR). Adapted by permission from Nature Publishing Group: Nature Reviews Cancer (Baselga & Swain, 2009). The figure legend is modified.

Although all ErbB proteins are very similar in their structure (Figure 1), each family member also has unique characteristics. EGFR, the first ErbB protein to be discovered (Carpenter et al, 1978), is a 175-kDa protein, and glycosylation of its extracellular domain has been shown to affect ligand binding and association with other ErbB proteins (Tsuda et al, 2000; Whitson et al, 2005; Zhen et al, 2003). ErbB2 is a 180-kDa protein which does not bind any known soluble ligands. Based on crystallographic studies it was shown that specific residues present in domain I and III in the extracellular part of ErbB2 interfere with ligand binding and, importantly, the extracellular domain has a conformation with the dimerization arm constitutively open (Garrett et al, 2003). This unique structure of domain II makes ErbB2 the

preferable partner for heterodimerization with other members of ErbB family and when ErbB2 is overexpressed it can self-associate and form functional homodimers (Ghosh et al, 2011). ErbB3 is a 180-kDa glycoprotein with 10 potential N-linked glycosylation sites (Sithanandam & Anderson, 2008). ErbB4, a 180-kDa protein, is the least characterized member of the ErbB family. It is distinctive among ErbB proteins because it can undergo proteolytic processing after ligand binding. The resulting intracellular fragment can then enter the nucleus and regulate gene transcription (Fiaturi et al, 2014; Sardi et al, 2006). Alternative splicing of the mRNA generates two isoforms of ErbB4 that differ in their extracellular juxtamembrane regions and their sensitivity to proteolytic cleavage (Elenius et al, 1997). There are 11 growth factors in the EGF-like family (Figure 1) (reviewed in Roskoski, 2014). EGFR is regulated by at least seven different ligands, each contains an EGF-like domain that is responsible for receptor binding and activation (Harris, 2003; Lemmon et al, 2014). There are two ligands that bind to ErbB3 and seven ligands binding to ErbB4 (Figure 1), but there is no known soluble ligand that bind to ErbB2 (reviewed in Roskoski, 2014). All of the ligands of the ErbB family are produced as membrane-bound precursor proteins (Buonanno & Fischbach, 2001; Harris, 2003) which are cleaved by cell-surface proteases, members of the ADAM (a disintegrin and metalloproteases) family, to yield the active growth factor species (Lemmon et al, 2014; Roskoski, 2014). The ErbB ligands may act in an autocrine fashion on the same cell from which they were released, in a juxtacrine fashion on an adjacent cell, or in a paracrine fashion on a nearby cell, but they all act over short distance (reviewed in Roskoski, 2014).

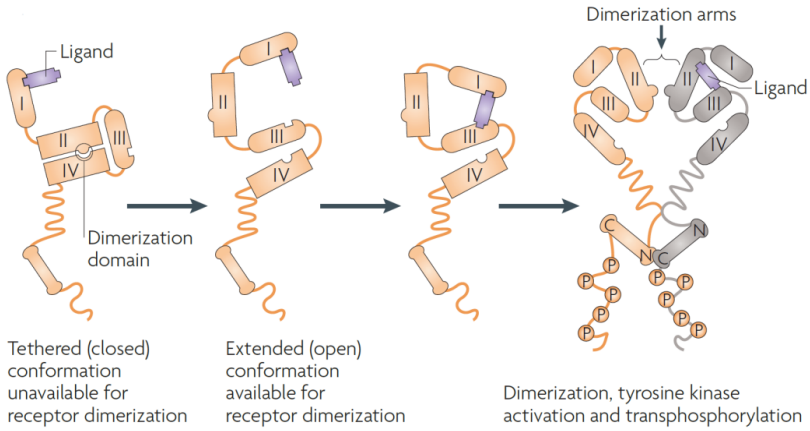
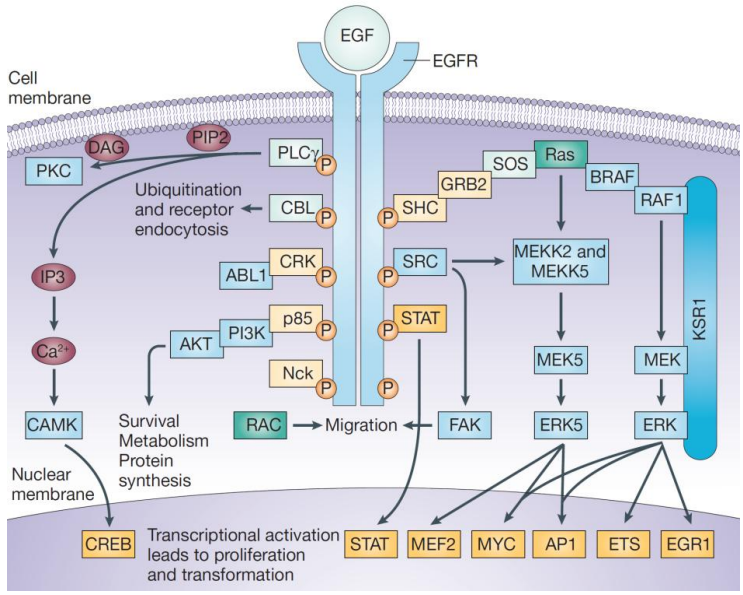


Figure 2. Activation of the ErbB proteins. Upon ligand binding the extracellular domain of the ErbB receptors undergoes conformational changes leading to exposure of the dimerization arm (sub-domain II), receptor dimerization, activation of the tyrosine kinase domain and phosphorylation of the tyrosine residues in the C-terminal tail. Adapted by permission from Nature Publishing Group: Nature Reviews Cancer (Baselga & Swain, 2009). The figure legend is modified.

In general, binding a ligand to the extracellular domain of the receptor allows for stabilization of extended conformation leading to receptor homo- or hetero-dimerization followed by auto- or transphosphorylation of the dimerization partners and initiation of signaling pathways (Figure 2) (Garrett et al, 2002; Lemmon et al, 2014; Ogiso et al, 2002; Schlessinger, 2002). Under physiological conditions, ErbB2 homodimers are believed to be non-functional, but overexpression of ErbB2 has been found to form functional homodimers (Ghosh et al, 2011). Domain I in ErbB3 is a major contributor in ligand binding, as opposed to EGFR where the domain III is dominant. ErbB3's ligands bind with higher affinity than EGFR's (Kani et al, 2005). The extracellular domain of ErbB3 retains ligand binding even at acidic endosomal pH (in both the extended and locked conformations) (Kani et al, 2005; reviewed in Sithanandam & Anderson, 2008). ErbB3 was for a long time considered to be kinase-impaired due to the lack of several non-conserved regions in its kinase domain (Kraus et al, 1989; reviewed in Sithanandam & Anderson, 2008). Recent data do, however, show that ErbB3 is capable of binding ATP and when clustered at the plasma membrane it can transautophosphorylate its intracellular region (Shi et al, 2010). Activity of the kinase domain of ErbB3 also appears to be sufficient to phosphorylate other ErbB proteins in heterodimer complexes (Sak et al, 2012; Shi et al, 2010).

A



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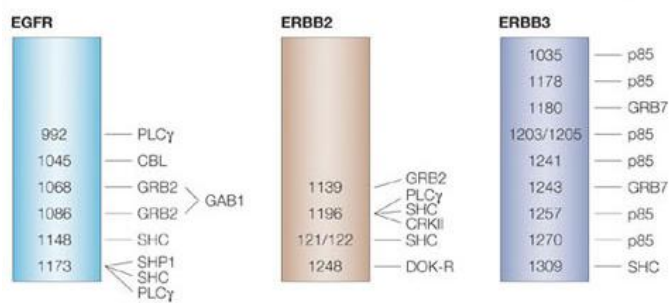

Figure 3. (A) The ErbB signaling pathways. Ligand binding to EGFR induces its dimerization and activation, leading to recruitment of multiple signaling molecules and activation of the following pathways: the PI3K/Akt, Ras/Raf/Mek/Erk, PLC/PKC and STAT and generating cellular responses including: cellular proliferation, migration and survival. Kinases are shown in blue, scaffolds are dark blue, adaptor proteins are yellow, G proteins are green and transcription factors are orange. Adapted by permission from Nature Publishing Group: Nature Reviews Cancer (Kolch & Pitt, 2010). The figure legend is modified. **(B) Phosphorylation sites of the EGFR, ErbB2 and ErbB3 C-terminal tails.** Schematic representation of the main autophosphorylation sites in EGFR, ErbB2 and ErbB3 and of the signaling molecules associated with these sites. Adapted by permission from Nature Publishing Group: Nature Reviews Cancer (Hynes & Lane, 2005). The figure legend is modified.

Signaling network activation by ErbB proteins depends on several factors: cellular context, receptor expression, type and availability of the ligands and the presence and interplay between various signaling and adaptor proteins. Ligand binding and receptor dimerization results in phosphorylation of tyrosine residues at specific sites in the C-terminal tails of the ErbB proteins (Figure 3B), which serve as docking sites for downstream signal transducers containing Src homology (SH2) or phosphotyrosine binding (PTB) domains (Yarden & Sliwkowski, 2001). This leads to recruitment of various downstream effectors and activation of a variety of signalling pathways controlling cell proliferation, differentiation, survival, migration and apoptosis. ErbB proteins mainly activate the phospholipase C gamma (PLCγ), the signal transducer and activator of transcription (STAT) pathway, the phosphatidylinositol 3-kinase (PI3K)-Akt and the mitogen activated protein kinase (MAPK) pathways (Hynes & Lane, 2005) (Figure 3A). ErbB3 contains 6 docking sites for direct binding of the p85 subunit of PI3K (Hellyer et al, 1998) and to some extent this is the reason for ErbB3's oncogenic potency. ErbB2 has been shown to activate MAPK pathway through Grb2, Shc, downstream of kinase related (DOK-R) and CRK (Dankort et al, 2001) as well as PLCγ (reviewed in Hynes & Lane, 2005; Marone et al, 2004).

Endocytosis

Endocytosis is a general term describing multiple pathways (Figure 4) by which cells control the uptake of nutrients and regulate the composition of the plasma membrane. Overall, the endocytic pathways can be divided into phagocytosis (or "cell eating") and pinocytosis (or "cell drinking"). Phagocytosis is limited to phagocytes and is used for the uptake and degradation of solid particles, bacteria, and viruses. Pinocytosis takes place in all cell types and is typically used to internalize fluid surrounding the cell. Pinocytosis can be further divided into different pathways defined by specific molecular regulators, the presence or absence of coat proteins (clathrin and caveolin), and dynamin dependency.

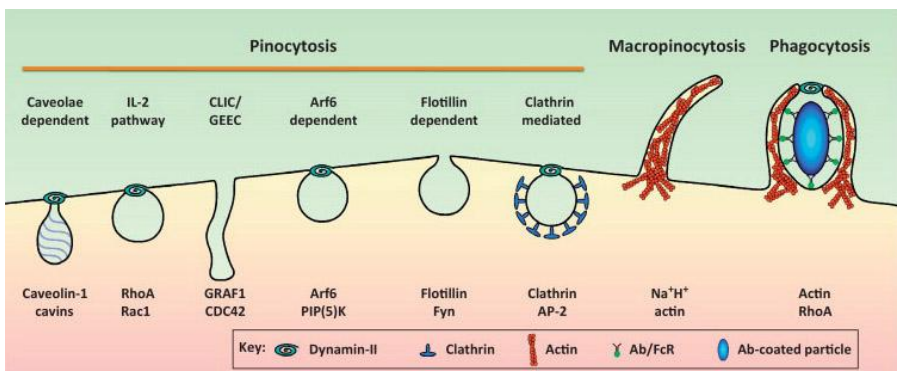

Figure 4. Endocytic pathways. The endocytic activities executed by cells are classified into pinocytosis, macropinocytosis, or phagocytosis. The various pinocytic mechanisms are classified according to their cellular requirements, i.e., dynamin or actin. For each mechanism, the major players are listed. Abbreviations: AP-2, adaptor protein 2; RhoA, Ras homolog gene family member A; Rac1, Ras-related C3 botulinum toxin substrate 1; GRAF1, GTPase regulator associated with focal adhesion kinase-1; CDC42, cell division control protein 42 homolog; Arf6, ADP ribosylation factor 6; PIP(5)K, phosphatidyl-inositol-5-phosphate kinase; Fyn, a member of the protein tyrosine kinase oncogene family; Ab, antibody; FcR, fragment constant receptor; IL-2, interleukin-2; CLIC, clathrin-independent carriers; GEEC, GPI-anchored-protein-enriched endosomal compartment. Adapted by permission from Elsevier: Trends in Microbiology (Mercer & Greber, 2013). The figure legend is modified.

Endocytosis is facilitated by invagination of the plasma membrane leading to the internalization of the membrane proteins, lipids, nutrients, and extracellular fluid. These cargo molecules are carried by vesicles which form by pinching off from the

plasma membrane (reviewed in McMahon & Boucrot, 2011). Vesicles derived from the plasma membrane fuse with early endosomes (EE), which are common sorting station for most of the endocytic mechanisms. Internalized cargo can be recycled back to the plasma membrane or sorted to different intracellular destinations, such as the trans-Golgi network (TGN) or to late endosomes (LEs)/lysosomes and designated for degradation. EE exhibit structural, compositional and functional heterogeneity. The structure of EE is characterized by the presence of morphologically distinct sub-sub-domains that have different functions i.e. tubular extensions that will give rise to recycling endosomes, and large intraluminal vesicles (ILVs) that in the process of endosome maturation (Huotari & Helenius, 2011) will form multivesicular bodies (MVBs), which in turn give rise to exosomes or will be involved in degradative pathway (Figure 5) (reviewed in Jovic et al, 2010). The process of maturation is characterized by, amongst other things: membrane components exchange, endosomal fission, the formation of additional ILVs, decrease in luminal pH, the acquisition of lysosomal components and morphological alterations (reviewed in Huotari & Helenius, 2011).

The sorting of various cargos is mediated by proteins and lipid components that are present in EE (reviewed in Jovic et al, 2010). One group of endocytic regulators constitute Ras-associated binding (Rab) proteins, which are small GTP-binding proteins that regulate endosome fusion and motility (reviewed in Stenmark, 2009). The Rab proteins, including Rab5 and Rab4, localize to the intracellular membranes in their active state and recruit other proteins, known as Rab effectors (Grosshans et al, 2006). One of the Rab5 effectors, the early endosomal antigen-1 (EEA1), is the most commonly used marker for EEs. The membrane of EE undergoes extensive tubulation whereby receptors that are sorted into the newly-formed tubular membranes recycle back to the cell surface via a “fast recycling” or “slow recycling” pathway (reviewed in Jovic et al, 2010). Rab4 is localized to EE and sorts receptors via “fast recycling” pathway back to the plasma membrane. Rab11 is known to associate primarily with perinuclear recycling endosomes and has been shown to regulate recycling of endocytosed proteins via “slow recycling” pathway (reviewed in Grant & Donaldson, 2009; and Jovic et al, 2010). Additionally, endosomes specialized for intracellular signaling are characterized by the presence of signaling effectors such as APPL1 and APPL2 (Adaptor protein containing PH domain, PTB

domain and Leucine zipper motif). APPL1 and APPL2 are associated with a subset of Rab5-positive EE but are not present on organelles in the canonical recycling and degradative pathways, and have been suggested to function primarily in signal transduction (reviewed in Jovic et al, 2010; Miaczynska et al, 2004).

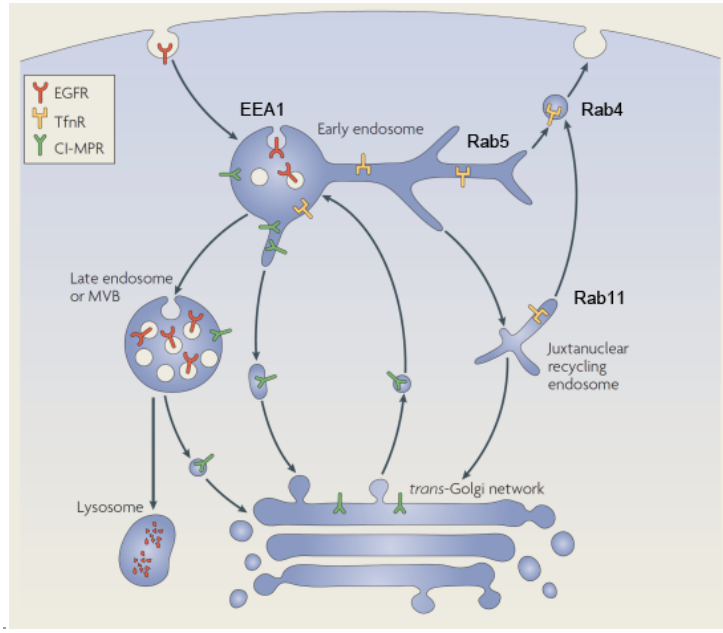

Figure 5. Endosomal sorting. Following internalization, cation-independent mannose 6-phosphate receptor (CI-MPR), transferrin receptor (TfnR) and epidermal growth factor receptor (EGFR), among other receptors, enter the early endosome, where they are segregated into separate trafficking itineraries by a series of sorting events. TfnR is recycled back to the plasma membrane either through a fast recycling route or, more slowly, through the juxtanuclear endocytic recycling compartment. By contrast, EGFR is retained within the limiting membrane of the early endosome prior to undergoing ESCRT (endosomal sorting complex required for transport)-mediated sorting into intraluminal vesicles of the early endosome. These vesicles mature into late endosomes or multivesicular bodies (MVBs) that become competent and fuse with lysosomes, leading to EGFR degradation. Finally, CI-MPR is sorted for retrieval back to the *trans*-Golgi network through both early and late endosomal pathways. Adapted by permission from Nature Publishing Group: Nature Reviews Molecular Cell Biology from (Cullen, 2008). Illustration and figure legend are modified according to (Grant & Donaldson, 2009).

The sorting of receptors for degradation depend on the presence of specific signals in their C-terminal tails, such as ubiquitination (discussed in the next paragraphs). Ubiquitinated cargo is captured by the endosomal sorting complex for transport (ESCRT) machinery and sorted into ILVs of MBV. The ESCRT machinery consists

of four sub-complexes: ESCRT-0, -I, -II and -III and accessory factors (reviewed in Hurley & Hanson, 2010; Raiborg & Stenmark, 2009). The first protein complex recruited to the EEs is ESCRT-0, which is composed of the two interacting proteins: Hrs (hepatocyte-growth-factor-reulated tyrosine-kinase substrate) and STAM 1/2 (signal transducing adaptor molecule). Hrs and STAM bind to ubiquitinated cargo and to clathrin and together with Eps15 (epidermal growth factor receptor pathway substrate 15) stabilize the association with cargo (Bache et al, 2003b). Furthermore, Hrs interacts with the Tsg101 subunit of the ESCRT-I, thus recruiting it to LEs (Bache et al, 2003a). Next, ESCRT-II binds to ESCRT I and recruits ESCRT-III, leading to inward vesicle budding which is required for MVB maturation. MVBs eventually fuse with the lysosomes leading to receptor degradation (Henne et al, 2011; Williams & Urbe, 2007).

Early endosomes can, in addition to the functions mentioned above, serve as sorting stations for the retrograde transport of cargo from EE to TGN. Retromer machinery is a multimeric complex composed of subcomplexes of SNX (sorting nexin) proteins that are preferentially recruited to maturing EE. The main function of retromer complex is to recognize cargo concentrated in the EE and facilitate its sorting to the appropriate destination. (Bonifacino & Hurley, 2008; Cullen, 2008; Seaman, 2012; Wassmer et al, 2007).

Interestingly, recent studies have devoted a lot of attention to the occurrence of contact sites between endosomes and the endoplasmic reticulum (ER). Besides the cytosolic phosphatases also the ER-localized phosphatase, PTP1B, was shown to dephosphorylate activated EGFR in the endosomal membrane (reviewed in Raiborg et al, 2015). Additionally, contact sites between the endosome and ER have been reported to form before endosomal fission on sites marked by a retromer-associated protein, indicating that endosome-ER contact sites may indirectly mediate endosomal sorting (reviewed in Raiborg et al, 2015). However some aspects in this field remain still unresolved and further elucidation of the matter could provide a clearer overview.

Clathrin-dependent endocytosis

The clathrin-mediated endocytosis (CME) is used by all known eukaryotic cells and its first described physiological function was to selectively internalize receptors that are carrying ligands. Ligand-induced CME of many receptor (such as RTKs and G-protein coupled receptors) often results in lysosomal degradation. However, CME of certain receptor can also occur constitutively, in the absence of their nutrient ligands, like for transferrin receptor (TfR) and low density lipoprotein receptor (LDLR), where upon internalization these receptors are recycled back to the plasma membrane (reviewed in McMahon & Boucrot, 2011).

Clathrin has been identified as the major protein forming the lattice-like coat around vesicles, which were characterized as "vesicles in a basket" (Rosenbluth & Wissig, 1964). On the cytosolic side of the plasma membrane, the clathrin-coated vesicle (CCV) cycle proceeds in five steps: initiation, cargo selection, coat assembly, scission and uncoating (Figure 6) (reviewed in Kirchhausen et al, 2014; McMahon & Boucrot, 2011; Traub, 2009).

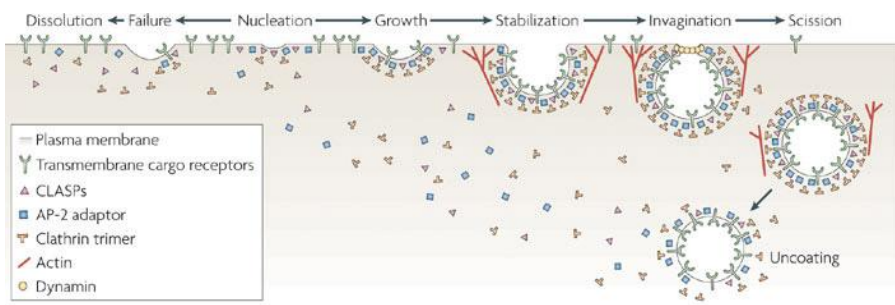

Figure 6. The clathrin-coated pit cycle. The formation of clathrin-coated vesicles proceeds through a series of steps: assembly of coat components at a designated site on the plasma membrane, membrane bending and cargo concentration to generate a coated pit, membrane scission and uncoating. Clathrin is recruited by AP‑2 and together with accessory proteins termed CLASPs (clathrin‑associated sorting proteins), such as epsin 1, Eps15, ARH and Dab2 cluster cargo molecules within the forming vesicle. Successful cargo capture is monitored, in part, by dynamin to prevent abortive dissolution. Clathrin-coated pit is pinched-off from the membrane due to action of dynamin and free, coated vesicle quickly moves away from the plasma membrane and is rapidly uncoated. Re‑entry of coat components into the soluble pool allows additional rounds of coat cycle. Adapted by permission from Nature Publishing Group: Nature Reviews Molecular Cell Biology (Traub, 2009). Figure legend is modified.

Initiation of vesicle formation involves membrane invagination induced by adaptor proteins that are recruited to the site and drive budding of the clathrin-coated pit (CCP). Clathrin has a triskelion (three-legged) shape and is formed by three heavy chain subunits, each with an associated light chain subunit (Figure 7A) (reviewed in Kirchhausen et al, 2014; Liu et al, 1995). Clathrin does not bind directly to the membrane or to cargo, but relies on interaction with cytosolic adaptor protein complexs such as AP-2 (adaptor protein complex 2) and other accessory proteins (reviewed in Traub, 2009). AP-2 is a highly conserved, stable complex composed of four subunits: the ~100 kDa α-subunit, the ~100 kDa β2-subunit, the 50 kDa μ2-subunit and the 17 kDa σ2-subunit (Figure 7A) (Matsui & Kirchhausen, 1990; Traub, 2009). For the α-subunit, two isoforms α-1 and α-2, also known as α_A and α_C, have been identified (Ball et al, 1995). Upon phosphorylation of the μ2-subunit by AAK1 (AP-2 associated kinase 1), AP-2 is recruited to the plasma membrane where it functions as a key regulator of the maturing CCPs (Collins et al, 2002; reviewed in Traub, 2009). AP-2 can bind directly to endocytic motifs present in the cytoplasmic tail of cargo through its μ2-subunit and σ2-subunit, or indirectly through its α- and β2-subunit appendage domains. AP-2 binds to inositol lipids (PIP_2; PIP_2 is a general term for Phosphatidylinositol 4,5-bisphosphate or PtdIns(4,5)P_2) in the plasma membrane through α- and μ2-subunits and to clathrin heavy chain (CHC) through the β2-subunit.

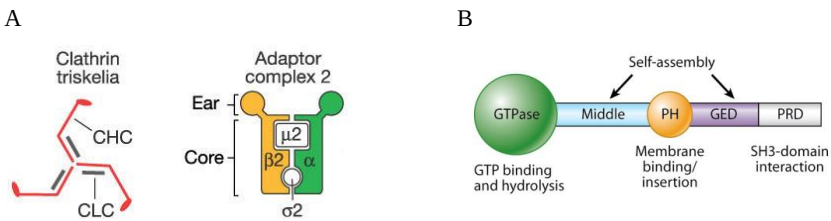

Figure 7. (A) Structure of the clathrin triskelion and AP-2 complex. Clathrin triskelion is represented as a trimer of articulated legs and each leg is composed of one heavy chain (CHC) and one light chain (CLC). The heavy chains are joined at their C-terminal ends at a common hub, from which the legs spread (Ferguson et al, 2008). AP-2 is a heterotetramer complex composed of two large 100 kDa α- and β-subunits and two smaller, the 50 kDa μ2-subunit and the 17 kDa σ2-subunit and α- and β2-subunit appendage domains(Matsui & Kirchhausen, 1990). Adapted by permission from Nature Publishing Group: Nature (Conner & Schmid, 2003). The figure and figure legend is modified. **(B) Structure of the dynamin.** Dynamin consists of five functionally distinct domains, as indicated. Abrreviations: pleckstrin homology (PH), GTPase effector domain (GED), proline-rich domain (PRD). Adapted by permission from Annual Reviews: Annual Review of Cell and Development Biology (Schmid & Frolov, 2011).The figure and figure legend are modified.

However, recent studies demonstrated the existence of a so-called "putative nucleation module", which assembles at the plasma membrane prior to AP-2. The "putative nucleation module" is composed of Eps15 and FCHo 1 and FCHo 2 proteins (F-BAR domain-containing Fer/Cip4 homology domain only 1 and 2; F-BAR (Bin-Amphiphysin-Rvs)), which can "sense" and bind to low curvatures in order to drive CCP formation (Henne et al, 2010; McMahon & Boucrot, 2011; Reider et al, 2009; Stimpson et al, 2009). Proteins in the nucleation module recruit AP-2 which selects and binds to cargo together with other accessory proteins (described in more detail in the next paragraphs).

CCP formation involves clathrin triskelia polymerization into hexagons and pentagons which stabilizes curvature and reorganizes accessory adaptor proteins localization, such as Eps15, to the edge of the coated pit (Kazazic et al, 2009; Tebar et al, 1996). Constriction of its neck is further facilitated by clathrin polymerization, bringing the surrounding membrane to a closed position. Vesicle scission is induced by polymerization of the dynamin through GTP, but the precise mechanism of the membrane fission is not clear yet (reviewed in McMahon & Boucrot, 2011). Dynamin is member of a protein family which is characterized by a highly homologous N-terminal GTPase domain as well as an α-helical middle domain and GED (GTPase effector domain) (Figure 7B). Dynamin also encodes a lipid-binding PH (pleckstrin homology) domain and a C-terminal PRD domain that mediates protein interactions with SH3 domain-containing binding partners (reviewed in Schmid & Frolov, 2011). Dynamin is reported to play a dual role in CME, both as a regulator and/or fidelity monitor during the early steps of endocytosis and facilitating fission of CCV from the plasma membrane (reviewed in Schmid & Frolov, 2011). The vesicle scission is aided by recruitment of amphiphysin, another accessory protein (reviewed in Ahmed et al, 2010), which binds to dynamin 2 and is responsible for membrane bending. Other proteins that preferentially bind to the curvatures at the neck of the pits and to dynamin to facilitate vesicle fission are endophilin and sorting nexin 9 (SNX9).

The CCV is now irreversibly released into the interior of the cell. Uncoating, the last step, is initiated by synaptojanin, the lipid phosphatase that dephosphorylates PIP_2 into PI(4)P and helps to release adaptor and accessory proteins such as AP-2, epsin and AP-180 (described in next paragraph). Subsequent binding of ATPase heat shock cognate 70 (HSC70) and its cofactor, auxilin (or cyclin G-associated kinase (GAK) in

non-neuronal tissues) leads to disassembly of the clathrin coat from its lattice arrangement back to triskelia (Schlossman et al, 1984; Taylor et al, 2011). During the uncoating step, clathrin and other proteins are recycled back to the cytosol, where they can be re-used in another CCP formation cycle. The nascent vesicles undergo further trafficking within the cell.

Involvement of the actin cytoskeleton in CME is still debated and it seems to be cell-type dependent. In general, actin does not appear to be required in the early steps of CCP formation in mammalian cells but it can support the invagination, elongation, scission and movement of the CCV during the ingestion of larger cargoes (Cureton et al, 2009). Actin can associate with clathrin through interaction with Hip1R (huntingtin-interacting protein 1-related), (Chen & Brodsky, 2005; Engqvist-Goldstein et al, 2001; Legendre-Guillemin et al, 2005). Actin can also be recruited to the budding vesicle following association of the actin-related complex 2/3 (Arp 2/3) mediated by cortactin or by N-WASP (Wiskott-Aldrich syndrome protein) (Higgs, 2001; McMahon & Boucrot, 2011; Uruno et al, 2001).

Sorting signals and endocytic adaptors in CME

A wide range of activated transmembrane receptors that are internalized in a clathrin-dependent manner carry different, unrelated sorting signals in their intracellular part, ranging from short peptide to whole proteins that are attached to cargoes (Traub, 2009). Several cargo sorting motifs that are recognized by different subunits of AP-2 have been identified, such as the YXXØ motif which binds to the μ2-subunit and is present in, among others, the cytoplasmic tail of TfR, and the acidic di-leucine [DE]XXXL[LIM] sequence which binds to the α-σ2 hemicomplex (reviewed in Sorkin, 2004; Traub, 2009). AP-2 was previously considered to be an obligatory adaptor for all clathrin-mediated endocytosis. Previous reports demonstrated that depletion of the AP-2 complex leads to reduced uptake of TfR, a classical marker for CME (Hinrichsen et al, 2003; Motley et al, 2003; Traub, 2009). It has also been suggested that AP-2 may not be stoichiometrically required for coat assembly, but it may have a more cargo-selective function in CME (Conner & Schmid, 2003). It thus seems that interaction with AP-2 is not always necessary for cargo recognition during CME and a variety of endocytic adaptors, termed CLASPs (clathrin associated sorting proteins), have been recognized to bind to different cargo sorting signals (Sorkin, 2004; Traub, 2009). CLASPs are usually mono- or dimeric proteins and

most of them contain domains that are able to bind to both cargo, lipid, clathrin and AP-2, but some are more specialized and interact either with cargo, lipids, clathrin or AP-2 (reviewed in Reider & Wendland, 2011) (see Table 1). One example could be recruitment of the LDLR that relies on recognition of its FXNPXY motif (Sallusto et al, 1995), by adaptor proteins Dab2 and ARH. Dab2 and ARH proteins belong to the PTB domain containing subfamily of CLASPs and are cargo-specific adaptors, which interact with AP-2 and clathrin, recruiting LDLR to clathrin coated pits (reviewed in Reider et al, 2009; Sorkin, 2004; Traub, 2009).

Table 1. Endocytic adaptor proteins and their interactions*

Adaptor protein	Lipid binding	Cargo binding	Clathrin binding	Accessory protein binding	Adaptor type
Mammalian adaptor proteins					
AP-2	+	+	+	+	Heterotetramer
Epsin1, epsin2	+	+	+	+	CLASP
ARH, NUMB, DAB2	+	+	+	+	CLASP
β-arrestin	+	+	+	+	CLASP
AP180/CALM	+		+	+	CLASP
Stonin2		+		+	CLASP
Eps15		+		+	CLASP
FCHO1, FCHO2	+			+	Muniscin

* Adapted with permission from Company of Biologists Ltd. (Reider & Wendland, 2011). Table is modified.

Other examples of CLASPs are epsin1 and Eps15 which bind selectively to ubiquitinated cargo through tandemly arrayed UIMs (ubiquitin-interacting motifs) (reviewed in Traub, 2009). Epsin is an evolutionary well conserved protein with many homologues in lower species, like Ent1 and Ent2 that are found in yeast (De Camilli et al, 2002). Epsin 1 and its isoforms (epsin 2a, epsin 2b) are expressed in all vertebrates in many tissues and cells while epsin 3 is specifically expressed in keratinocytes induced by type I collagen (Spradling et al, 2001; Tessneer et al, 2013).

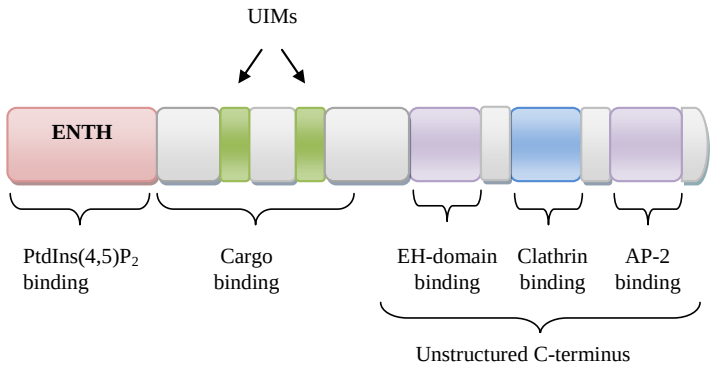

Figure 8.Structure of epsin 1. The Epsin N-Terminal Homology (ENTH) domain is localized at the NH_2-terminus of epsin and it major function is to induce membrane curvature. ENTH domain is followed by two or three ubiquitin-interacting motifs (UIMs), depending on splice variations, which bind to poly-ubiquitinated cargo. The C-terminal part of epsin is unstructured but it is characterized by the presence of multiple DPW motifs, shown as binding sites for AP-2, the "clathrin boxes", which are responsible for binding to clathrin and NPF motifs, which are responsible for binding to EH-domain containing proteins, such as Eps15. Adapted by permission from Nature Publishing Group: Nature Reviews Molecular Cell Biology (Wendland, 2002). Illustration and legend are modified.

Epsin contains an evolutionary conserved domain of approximately 150 amino acids referred to as the ENTH domain (Epsin N-Terminal Homology), located at the N-terminus (Ford et al, 2002). The ENTH domain has a compact α-helical structure and is known to induce membrane curvature. It has been shown that binding of the ENTH domain to PIP_2 is sufficient for stable membrane association (Itoh et al, 2001). The ENTH domain is followed by two or three UIMs, depending on splice variations (Hofmann & Falquet, 2001), while the COOH-terminal region of epsin is involved in recruiting components of the clathrin coat (Figure 8) (reviewed in Wendland, 2002). The C-terminal part of epsin is unstructured but it is characterized by the presence of multiple DPW motifs, which function as binding site for AP-2 (Legendre-Guillemin et al, 2004; Wendland, 2002). Epsin also binds to clathrin through "clathrin boxes", consisting of hydrophobic and acidic residues, and to Eps15 through the NPF motif (Chen et al, 1999; Salcini et al, 1999). Epsin 1 has been shown to function as a cargo-specific adaptor protein for EGF-induced internalization of EGFR but not of transferrin (Kazazic et al, 2009). However, the role of epsin 1 as a cargo specific

adaptor for EGFR is debated, since other reports found that epsin 1 is not needed for EGFR endocytosis (Huang et al, 2004). Epsin 1 is also required for the clathrin-mediated entry of the influenza virus (Chen & Zhuang, 2008) and ubiquitinated MHC-I (Major histocompatibility complex class I) (Duncan et al, 2006). The biochemical properties of epsin establish its role as a cargo specific adaptor protein, but epsin also has a general function in CME and in plasma membrane remodeling (Boucrot et al, 2012; Ford et al, 2002; Hurley & Wendland, 2002). A recent study (Messa et al, 2014) suggested that the epsin proteins are essential in coordination of the clathrin-coat formation from its early to late stages where it links actin with clathrin-coated pit progression from shallow to deeply invaginated structures.

Ubiquitination as a signal for endocytosis

Ubiquitin is the major signal for internalization of most cargoes studied in yeast. However in mammalian cells, the role of ubiquitin in endocytosis is more complicated since not only the cargo is ubiquitinated, but also the endocytic adaptors and it has been shown that multiple pathways could be involved in internalization of ubiquitinated receptors (reviewed in Acconcia et al, 2009; and Madshus & Stang, 2009). Ubiquitin-mediated internalization of transmembrane receptors, such as RTKs, requires precise molecular machinery that will recognize the ubiquitinated cargo and further mediate the entrance into degradative pathway.

Ubiquitin is a small, highly conserved protein composed of 76 amino acids, which can be covalently attached to lysine (Lys) residues of target proteins in a 3 step process utilizing: Ub-activating (E1), Ub-conjugating (E2) and Ub-ligating (E3) enzymes (Hershko & Ciechanover, 1998; Komander, 2009; Ye & Rape, 2009). It was also reported that ubiquitin can be attached to cargo directly by the E2 enzyme (Hoeller et al, 2007). Given that ubiquitin itself contains seven Lys residues it could also be a substrate for ubiquitination. Monoubiquitination occurs when a single ubiquitin molecule is added to the target protein (Hicke & Dunn, 2003), alternatively, single ubiquitin molecules can bind to several Lys residues, giving rise to multimonoubiqitination (Haglund et al, 2003; Haglund & Dikic, 2005; Komander & Rape, 2012). Ubiquitin can be also added to the target protein in the form of polyubiquitin chains (Peng et al, 2003), which leads to the formation of structures with different properties depending on how the chains are assembled. It has been

demonstrated that chains are built sequentially, beginning with the substrate-attached ubiquitin. The formation of the chain can occur not only through the seven internal Lys residues on ubiquitin, but also through the ubiquitin N-terminus (Behrends & Harper, 2011; Clague & Urbe, 2010; Metzger et al, 2012; Vijay-Kumar et al, 1987). Polyubiquitin chains can be homogenous or mixed, linear or branched, with conformation depending on the type of Lys-linkage (reviewed in Komander & Rape, 2012). Polyubiquitination via Lys48 and Lys63 are the best characterized (Hicke et al, 2005) (Haglund & Dikic, 2005) and it is well established that attachment of polyubiquitin via Lys48 targets proteins for proteosomal degradation (reviewed in Hershko & Ciechanover, 1998) while Lys63-linked ubiquitin chains are implicated in DNA repair, endocytosis, lysosomal degradation, protein translation, and cell signaling regulation (reviewed in Haglund & Dikic, 2005; and Komander & Rape, 2012).

There are three distinct classes of E3 ligases that have been identified to stimulate transfer of ubiquitin and ubiquitin-like proteins through either a direct or an indirect mechanism. The E3 ligases that mediate the direct transfer of ubiquitin from E2 to substrate include: HECT (the homologous to the E6AP carboxyl terminus) domain family (Rotin & Kumar, 2009), RING (really interesting new gene) and RING-related E3s, finger proteins, and members of the U-box family (Deshaies & Joazeiro, 2009; and reviewed in Metzger et al, 2012). The RING finger E3 ubiquitin ligase Cbl (Casitas B-lineage lymphoma) (Thien & Langdon, 2001) was shown to regulate ubiquitination of EGFR by binding directly to phosphorylated receptor tyrosine 1045, or indirectly through Grb2, and leading to degradation of EGFR (Levkowitz et al, 1999; Levkowitz et al, 1998; Madshus & Stang, 2009; Waterman et al, 1999b). Cbl can also bind to ErbB2 but the binding is not efficient enough to target ErbB2 for degradation. ErbB2 downregulation has been shown to be mediated by the U-box E3 ligase-CHIP (containing terminus Hsc 70- interacting protein) and RING E3 ligase-Cullin5 (Xu et al, 2002). ErbB3 and ErbB4 cannot bind Cbl but it has been shown that Nrdp1 (neuregulin receptor degradation pathway protein 1), which is also a RING E3 ligase, mediates receptor ubiquitination and suppresses the levels of these receptors in the absence of added growth factor (reviewed in Carraway, 2010; Diamonti et al, 2002). NEDD4 (neural precursor cell expressed, developmentally downregulated 4) and Icht, which belong to HECT E3 ligases, were shown to be involved in mediating downregulation of ErbB4 (Carraway, 2010; Rotin & Kumar,

2009). Ubiquitination can be reversed due to the action of deubiquitylating enzymes (DUBs) that recycle ubiquitin to the cytoplasmic pool (Komander et al, 2009). Two endosome-localized DUBs have been identified: AMSH (associated molecule with the SH3-domain of STAM) and UBPY (ubiquitin-specific processing protease Y). AMSH is a member of the JAMM/MPN+ family of metalloproteases (Cope et al, 2002; Maytal-Kivity et al, 2002), which have been shown to negatively regulate EGFR sorting to the lysosomes (McCullough et al, 2004). UBPY, also known as USP8, is a cysteine protease of the UBP/USP family (ubiquitin-specific processing protease) and has been suggested to regulate cellular ubiquitin levels and entry into S-phase (Naviglio et al, 1998). USP8 has been proposed to be involved in regulation of ErbB2 (Meijer & van Leeuwen, 2011) and ErbB3 (Cao et al, 2007) trafficking, but involvement of USP8 in EGFR lysosomal degradation is not clear (reviewed in Maly & Macrae, 2014).

Clathrin-independent endocytosis

It is now clear that mammalian cells, in addition to classical clathrin-dependent endocytosis, utilize various other types of endocytic mechanisms, collectively termed clathrin-independent endocytosis (CIE). Some of these endocytic pathways are closely related and interact with each other, therefore inhibition of one pathway can up-regulate some others which in physiological conditions would play less important roles. These pathways employ different sets of proteins, but some studies demonstrate that similar, if not the same, proteins can participate in various endocytic events.CIE, besides macropinocytosis, include dynamin- dependent mechanisms (RhoA and caveolin-caveolae/lipid raft dependent) and dynamin-independent mechanisms (Cdc42 dependent and Arf6 dependent, also called the CLIC/GEEC pathway (CLIC, clathrin-independent carrier; GEEC, GPI-AP (glycosyl-phosphatidylinositol-anchored proteins) enriched early endosomal compartment) (reviewed in Mayor et al, 2014; Sandvig et al, 2011).

Macropinocytosis

Macropinocytosis is the non-selective uptake of large areas of plasma membrane, nutrients, antigens and solute macromolecules. Macropinocytosis can be transiently stimulated by growth factors (Wang et al, 2010a) and it depends on proteins like: Rac, Pak1, Cdc42, CtBP1/BARS, Arf6 (Kerr & Teasdale, 2009) and sorting nexins (Sallusto et al, 1995) but it remains relatively inactive in most cell types (reviewed in Kerr & Teasdale, 2009; Sandvig et al, 2011). The most common form of macropinocytosis, usually occurring at cell edge, is actin-mediated, dynamin-independent membrane ruffling. It was also demonstrated that dynamin-dependent formation of the macropinosome could be initiated at circular-dorsal ruffles. Macropinosomes are non-coated, large in size, usually > 0.2μm in diameter, endocytic vesicles, which ultimately deliver their content to LEs/ lysosomes or recycle it back to the plasma membrane. Multiple viruses (HIV-1), bacteria (for example *Salmonella* spp.) and protozoa (*Leishmania amazonesis*) modify and use macropinocytosis as the entry pathway into the host cells (reviewed in Lim & Gleeson, 2011). The immune system uses macropinocytosis as a means of sampling the environment for the presence of antigens. The antigen-presenting cells capture the antigens and deliver them to MHC class II-positive compartments, for degradation into peptides followed by recycling to the plasma membrane as MHC II-peptide complexes (Sallusto et al, 1995). Macropinocytosis was also suggested for uptake of large plasma membrane areas containing clusters of EGFR and anti-receptor antibodies thus allowing internalization of a large number of receptors in a single event (Berger et al, 2012).

Caveolin-mediated endocytosis

Caveolae are a special type of lipid rafts (sub-domains of the plasma membrane containing high concentration of cholesterol and glycoshingolipids), with a characteristic morphology of flask-shape invaginations of the plasma membrane enriched in sphingolipids, cholesterol and glycosyl phosphatidyl (GPI)-anchored proteins (Conner & Schmid, 2003; Mayor & Pagano, 2007). Caveolae are present at the surface of many mammalian cell types and are dependent on protein caveolin 1. It has been demonstrated that the process of caveolar budding requires Src-family kinases, dynamin and local actin polymerization (Sverdlov et al, 2007). Formation of the caveolae also depends on a complex of peripheral membrane proteins, termed

cavins (cavin 1, -2, -3) (Hansen & Nichols, 2010). Caveolin initially aggregates at the plasma membrane, cavin 3 forms a complex with cavin 1 and caveolin, stabilizing caveolae by linking it to actin cytoskeleton through myosin-1c (Briand et al, 2011; Hansen & Nichols, 2010; Parton & del Pozo, 2013). The endocytic potential of caveolae is controversial and it is currently being investigated (dicussed in Mayor et al, 2014). Current evidence suggests that caveolae can detach from the plasma membrane to form caveolar endocytic carriers. The majority of them are immobile, whereas a minority display a fast "kiss-and-run" behavior (Pelkmans & Zerial, 2005) and will reach the EE and then return back to the cell surface (reviewed in Gong et al, 2008; Mayor et al, 2014).

Clathrin- and caveolin-independent endocytosis

Several pathways that do not require coat proteins, such as clathrin and caveolin, have been identified in recent years. Clathrin- and caveolin-independent pathways are induced and regulated by different set of proteins, giving cells greater flexibility in response to the extracellular stimuli. The first receptor described to follow clathrin- and caveolin-independent pathway was IL-2 (Interleukin 2). IL-2 internalization is Rho-A dependent and dynamin-independent (Lamaze et al, 2001). Flotillin 1 and flotillin 2 are marker proteins for another clathrin- and caveolin-independent internalization pathway (Glebov et al, 2006). Flotillins are a family of proteins that cluster preferentially in specific, cholesterol rich membrane microdomains (Rivera-Milla et al, 2006), and whose budding occurs rarely and depends on Fyn kinase (Riento et al, 2009). It has been proposed that flotillins are involved in the uptake of lipid-associated receptors and some lipids by aggregating them outside an invaginated membrane that give rise to vesicles (Frick et al, 2007; reviewed in Sandvig et al, 2011). Flotillin1-dependent uptake of GPI-linked proteins and cholera toxin B was reported to be dynamin-independent (Glebov et al, 2006), but basolateral uptake of GPI-anchored proteins was found to be dependent on flotillin 2 and dynamin (Ait-Slimane et al, 2009). The internalization pathway of GPI-linked proteins involving flotillin 1 and flotillin 2 has been investigated more recently (Frick et al, 2007; Glebov et al, 2006), but previously it has been suggested that GPI-linked proteins were internalized by Arf1- and Cdc42-dependent pathway (Sabharanjak et al, 2002). The internalization of GPI-linked proteins has been observed to occur through tubular intermediates, termed CLICs (clathrin-independent carriers)

(Kirkham & Parton, 2005; Sabharanjak et al, 2002) that do not require dynamin activity for scission from the plasma membrane (Sabharanjak et al, 2002). It still remains unclear how the plasma membrane is bent into tubular structures or what proteins participate in detaching it from the cell surface, but it has been proposed that tubular budding structures are pinched off from the membrane due to synergy between actin and cholesterol (Howes et al, 2010).

Endocytic downregulation of ErbBs

Endocytosis is an important way of regulating ErbB-mediated signaling where activated receptors are internalized and either recycled back to the plasma membrane or targeted for lysosomal degradation, resulting in the termination of signaling. However, several studies have demonstrated that even when the ligand-bound receptor is localized to intracellular endosomal compartments, it can still serve as an active signaling platform (reviewed in Hupalowska & Miaczynska, 2012; Pyrzynska et al, 2009). Indeed, in the case of EGFR, it has also been reported that there are functional differences in the quality of the signaling from the EGFR localized on the endosomes and at the plasma membrane. Whereas EGFR localized at the cell surface was reported to mediate proliferative signaling, the EGFR on the endosomes was observed to activate apoptosis (Burke et al, 2001; Hyatt & Ceresa, 2008; Rush et al, 2012; Wang et al, 2002).

Endocytic downregulation of EGFR

Endocytosis of activated EGFR was one of the first ligand-induced endocytic pathways to be discovered and became a prototypical model system for all RTKs. The turnover of EGFR (or the half-life of the protein) in cells expressing moderate levels of this the receptor varies in the range of 6-10 h (Beguinot et al, 1984; Stoscheck & Carpenter, 1984b), reaching up to 24 h in cells overexpressing EGFR (Stoscheck & Carpenter, 1984a). EGFR can undergo internalization and recycling in the absence of ligand but the rate of its internalization is slow. It has been shown that EGFR is internalized via CME (Carpentier et al, 1982; Hanover et al, 1984). However there are conflicting results regarding the requirement of AP-2 for endocytosis of EGFR. Some reports show that endocytosis of EGFR is inhibited in the absence of AP-2 (Huang et al, 2004; Johannessen et al, 2006), while others

(Conner & Schmid, 2003; Motley et al, 2003) state that the entry of EGF can proceed independently of AP-2. Additionally, EGFR has been reported to undergo internalization via CIE (Hinrichsen et al, 2003; Sigismund et al, 2005) and in some cell lines activation of EGFR has been shown to increase membrane dynamics and ruffling leading to macropinocytic uptake of EGFR (Chinkers et al, 1979; Orth et al, 2006), but the kinetic rates were much slower that for those observed for CME (reviewed in Sorkin & Goh, 2008). The type of bound ligands can regulate the intracellular sorting and downregulation of EGFR. Upon internalization and acidification of the endosomes EGF remains bound to EGFR (Sorkin & Waters, 1993), whereas TGF-α dissociates from the receptor at much higher pH (Ebner & Derynck, 1991). Consequently, binding of EGF targets EGFR for the degradative pathway due to prolonged phosphorylation and ubiquitination while TGFα-induced activation results in recycling of the receptor back to the cells surface (Longva et al, 2002). Moreover it was demonstrated that while EGF and TGFα induce CME, HB-EGF and BTC induce CIE (Henriksen et al, 2013) and target EGFR to lysosomes (Roepstorff et al, 2009). In addition, the involvement of ubiquitination in endocytosis of EGFR has been discussed. Some studies showed that ubiquitination of EGFR is not necessary for its internalization, especially at low doses of EGF, which do not induce detectable EGFR ubiquitination (Sigismund et al, 2005). On the contrary, other studies demonstrated that EGFR is ubiquitinated also at low concentrations of EGF, and that epsin 1 interacts with ubiquitinated EGFR (Kazazic et al, 2009). Our group discovered that ubiquitination of EGFR occurs already at the plasma membrane (Stang et al, 2000) and later reports showed that upon stimulation with EGF, EGFR becomes multimono- and polyubiquitinated, the latter mainly via Lys63- but also via Lys48-linked polyubiquitin chains (Huang et al, 2006; Umebayashi et al, 2008). CIE was also postulated to be an endocytic route used for downregulation of activated EGFR. Sigismund *et al.* reported that at high concentrations of EGF, EGFR was found to co-localize with caveolin 1, but it not necessarily indicated that internalization of EGFR occurs through caveolae (Sigismund et al, 2005). However, Kazazic *et al.* found that high concentrations of EGF do not recruit EGF-EGFR into caveolae nor increase the basal levels of caveolar endocytosis and that despite the concentration of EGF, CME is the main pathway used for EGFR internalization (Kazazic et al, 2006). Later, Sigismund *et al.* (Sigismund et al, 2008) found that CIE

of EGFR is in fact independent of caveolin 1 and further demonstrated that at low concentrations of EGF, CME plays a major role in recycling EGFR to the cell surface, and only a minor role in degradation. On the other hand, they reported that at high concentrations of EGF, CME is still operating, but a major portion of EGFR is ubiquitinated and internalized through CIE, which in turn leads to its efficient degradation, protecting the cell from overstimulation (Sigismund et al, 2008). It has been suggested that these differences could arise due to the different cell lines used by different groups. In some cell lines that express moderate or low levels of EGFR, CME is the main pathway used to internalize EGFR, at both low and high concentrations of EGF (Kazazic et al, 2009). However, in cases of EGFR overexpression and high doses of the ligand, CME becomes saturated (Lund et al, 1990; Sorkin & Goh, 2008; Wiley, 1988) and it has been proposed that CIE takes over the uptake of EGF (Sigismund et al, 2008; Sigismund et al, 2005; Sorkin & Goh, 2008).

Endocytic downregulation of ErbB2

Early studies showed that the kinase domain of ErbB2 is activated and phosphorylated but that ErbB2 is less efficiently internalized than EGFR due to its C-terminal domain that either does not possess all the signals required for rapid internalization or contains a signal that inhibits rapid internalization (Sorkin et al, 1993). The precise mechanisms for inefficient endocytosis of ErbB2 are still debated (reviewed in Bertelsen & Stang, 2014). Later reports have shown that, on the one hand, ErbB2 undergoes slow internalization and rapid recycling (Austin et al, 2004) but on the other hand, it is retained at the plasma membrane where it is concentrated on cellular protrusions (Hommelgaard et al, 2004) and in lipid rafts (Nagy et al, 2002) and only minor amounts of ErbB2 were found in endosomal compartments, even when overexpressed (Lerdrup et al, 2006; Longva et al, 2005). It has been shown that ErbB2 interacts with lipid-raft associated flotillins and that this interaction stabilizes ErbB2 at the cell surface (Pust et al, 2013). In addition, it has been shown that ErbB2 is stabilized at the plasma membrane by the Cdc37-Hsp90 complex through direct interactions with the catalytic domain of the receptor (Raja et al, 2011). Hsp90 (Heat shock protein complex 90) is a major chaperone involved in quality control of newly synthesized proteins (Taipale et al, 2010) and the specific interactions between Hsp90 and their clients are regulated by various co-chaperones,

such as Cdc37 (Pearl, 2005). One way to inhibit Hsp90 is by the use of GA (Geldanamycin) or its derivatives, such as 17AAG (17-allylaminogeldanamycin), which also downregulates ErbB2 (Austin et al, 2004; Lerdrup et al, 2006; Pedersen et al, 2008). The study of Austin *et al.* (Austin et al, 2004) concluded that the GA-induced inhibition of Hsp90 caused downregulation of ErbB2 by inhibiting recycling and increasing lysosomal sorting, but not by increased internalization. However, later reports show that GA and 17AAG induce internalization of ErbB2, mainly through CME (Cortese et al, 2013; Pedersen et al, 2008; Vuong et al, 2013). Baulida *et al.* (Baulida et al, 1996) showed that ErbB2 does not interact with the adaptor complex AP-2 however, this does not exclude interaction with other CLASPs. The studies of both Lerdrup *et al.* (Lerdrup et al, 2006) and Pedersen *et al.* (Pedersen et al, 2008) showed that Hsp90 inhibitors can induce caspase- and/or proteasome-mediated cleavage of ErbB2. Later report of Lerdrup *et al.* (Lerdrup et al, 2007) demonstrated that upon incubation with GA, the C-terminus of ErbB2 is cleaved and is accompanied by proteasome-dependent endocytosis of ErbB2, which is in contrast to the studies of Pedersen *et al*. who demonstrated that upon GA treatment, ErbB2 is internalized as a full length protein. However, these studies are in agreement claiming that the overall GA-induced degradation of ErbB2 depends on proteasomal activity. Moreover, Pedersen *et al.* found that proteosomal activity is required for transport of ErbB2 from the limiting membrane to inner vesicles of MVBs but that ErbB2 itself is not the proteosomal target (Pedersen et al, 2008). Furthermore, GA-treatment was early reported to induce ubiquitination of ErbB2 (Mimnaugh et al, 1996) and later on it has been shown to disrupt ErbB2-Hsp90 complex with simultaneous recruitment of Hsp70 (Xu et al, 2001) along with the ubiquitin E3 ligases CHIP and Cullin5 (Xu et al, 2002; Zhou et al, 2003) to ErbB2. Recently our group (Vuong et al, 2013) found that an ErbB2-ubiquitin chimera (ErbB2-Ub_4) was constitutively endocytosed and degraded via CME which supports the role of ubiquitination as a signal for endocytosis of ErbB2. Furthermore, the internalization of ErbB2-Ub_4 as well as wild-type ErbB2 has been shown to depend on epsin 1 and Eps15 (Vuong et al, 2013). The Cbl proteins (reviewed in Schmidt & Dikic, 2005), which are involved in ligand-induced EGFR ubiquitination and downregulation, were also reported to bind to ErbB2 (Carraway, 2010; Sorkin & Goh, 2008). However, binding of Cbl to ErbB2 was reported to be inefficient in induction of ubiquitination and degradation of ErbB2 (Citri et al, 2003).

Endocytic downregulation of ErbB3

Little is known about endocytosis of ErbB3 when compared to EGFR and ErbB2. ErbB3 has relatively short half-life, estimated to be between 2,5 and 3,5 hours (Cao et al, 2007; Warren et al, 2006; Waterman et al, 1998). ErbB3 was thought to be endocytosis impaired since it doesn't bind to AP-2 (Baulida & Carpenter, 1997; Baulida et al, 1996). However, later studies demonstrated low rate of ligand-dependent ErbB3 internalization (Baulida & Carpenter, 1997). The studies of Waterman *et al.* showed that, upon stimulation with ligand, ErbB3 was endocytosed and recycled back to the cell surface instead of being degraded (Waterman et al, 1999a). The reason for lack of lysosomal sorting was reported to be due to missing sorting signals in the C-terminal tail of ErbB3. Further on, the study of Warren *et al.* demonstrated that binding of the full length HRG, heregulin-extracellular domain (HRG-ECD), composed of EGF-like domain and the N-terminal IgG-like domain, sterically interfere with the formation of larger order oligomers from dimers and disrupts ErbB3 oligomers at the plasma membrane leading to enhanced downregulation of ErbB3. However, the mechanisms underlying the disruption of higher order complexes were not investigated. Furthermore, they reported that the short version of HRG, composed of EGF-like domain only, is both necessary and sufficient for initial binding and activation of ErbB2-ErbB3 heterodimers (Warren et al, 2006). The study of Cao *et al.* demonstrated that cellular levels of ErbB3 can be suppressed by factors, such as HRG, which controls stability of Nrdp1 which in turn mediates ubiquitination of ErbB3. They further reported that HRG stimulation leads to stabilization of USP8, which deubiquitinates Nrdp1 and thus targets ErbB3 for ligand-induced degradation (Cao et al, 2007). Besides HRG-induced internalization, ErbB3 was also shown to localize to intracellular vesicles in the absence of added ligand (Lemoine et al, 1992; Prigent et al, 1992; Sak et al, 2012), and more recently the constitutive internalization of ErbB3 was found to occur via CME (Sak et al, 2012). However, the exact mechanisms and requirement for specific adaptor proteins still remains under investigation. Previously, the study of Diamonti *et al.* (Diamonti et al, 2002) reported that Nrdp1 is the main component responsible for maintaining steady-state levels of ErbB3 by regulating constitutive internalization of ErbB3 and directing it to lysosomal degradation. However, it has also been demonstrated that Nrdp1 associates with ErbB3 and stimulates its ubiquitination and degradation by proteasomes (Qiu & Goldberg, 2002). Recently another E3 ligase, NEDD4, has been

reported to interact with ErbB3 in a ligand-independent manner, and when NEDD4 was inhibited by siRNA, the levels of ErbB3 were elevated, leading to increased ErbB3-mediated signaling (Huang et al, 2015). It was also demonstrated that ErbB3 in its nascent, not mature form was found to interact with Hsp90 (Gerbin & Landgraf, 2010). The recent report of Asp *et al.* (Asp et al, 2014) reported that GA-induced inhibition of Hsp90 caused ErbB3 degradation, but no studies so far have demonstrated direct, physical interaction between Hsp90 and full length ErbB3. Moreover, the study of Asp *et al.* demonstrated that ErbB3, like ErbB2, is stabilized at the cell surface by interaction with flotillins (Asp et al, 2014).

ErbB proteins in cancer

The ErbB proteins regulate cellular growth, migration, and survival under normal physiological conditions but it has also been demonstrated that ErbB proteins can play important roles during tumor progression. ErbB proteins are implicated in many human cancers in many ways. Point mutations and overexpression of the receptors or overproduction of ligands can cause constitutive activation or defective degradation of the ErbB proteins (Hynes & MacDonald, 2009; Tebbutt et al, 2013). Overexpression of EGFR due to gene amplification was shown to be involved in the pathogenesis and progression of different types of carcinoma including: lung, breast, head and neck, ovarian, gastric cancers (reviewed in Yarden & Pines, 2012). Mutations in EGFR, most frequently deletion in the extracellular domain, which is denoted EGFRvIII, are present in many cancer types (Sugawa et al, 1990). EGFRvIII is constitutively active and upon internalization it is recycled back to the cell surface, escaping ubiquitination and lysosomal degradation, leading to sustained oncogenic signaling (Grandal et al, 2007). Sustained signaling through EGFR is also caused by overexpression of ErbB2 and was shown to inhibit endocytosis and degradation of EGFR (Haslekas et al, 2005). Interestingly, it has also been reported that EGFR can localize to other organelles, for example it can be translocated from the plasma membrane to the nucleus through a retrograde pathway from the Golgi to ER (Wang et al, 2010b). EGFR, but also ErbB2 have been found in the nucleus to function as transcriptional regulators of cancer-related genes and promote cancer growth (reviewed in Han & Lo, 2012).

Dimerization of one of ErbB member with other RTKs may enhance its oncogenic activity. Heterodimerization of ErbB2 and ErbB3 activates the PI3K/Akt signaling pathway through ErbB3 and triggers proliferation and survival of human cancers. This pathway plays an important role in the development of various human cancers, including ErbB2-overexpressing breast cancer (Holbro et al, 2003; Lee-Hoeflich et al, 2008), prostate cancer (Jathal et al, 2011), ovarian cancer (Mills & Yarden, 2010) and non-small cell lung cancer (NSCLC) (Engelman et al, 2007; Huang et al, 2013). In addition to full length ErbB2, truncated forms of ErbB2 (p95HER2) that are generated by ADAM-mediated proteolytic cleavage or during translation from internal translation codons (Tse et al, 2012; Zagozdzon et al, 2011), have been reported to be present in a subgroup of ErbB2-positive cancers (reviewed in Bertelsen & Stang, 2014; Christianson et al, 1998). The oncogenic function of ErbB3 is mostly mediated through overexpression and interaction with EGFR or ErbB2. ErbB3 is overexpressed in several cancer types, including pancreatic, breast, ovarian and gastric cancers (reviewed in Sithanandam & Anderson, 2008). Although it has been demonstrated that ErbB3 overexpression is associated with poor prognosis in patients with breast cancer, it has also been reported that ErbB3 overexpression may be a positive prognostic factor (reviewed in Koutras et al, 2010). A naturally occurring secreted form of the ErbB3 receptor, p85-soluble ErbB3 has been shown to be a potent negative regulator of heregulin-stimulated ErbB2, ErbB3, and ErbB4 activation (Lee et al, 2001).

ErbB-targeted therapeutics

The increased expression of growth factor receptors during tumorigenesis can be used as pharmaceutical targets for targeted therapies in treatment of human cancers. Targeted therapeutics, when used in combination with standard therapy, can help to improve clinical anti-tumor response to chemotherapy by blocking ligand binding and shutting down the signaling that occurs through growth factor receptors (Weiner et al, 2010). The frequent activation of ErbB family members in cancer makes them attractive targets for design of such therapeutics (overview in Figure 9). Several ErbB agents have been developed to target mostly EGFR and ErbB2 (Figure 9), since these two receptors have the best-defined roles in cancer, and are approved for the treatment of various solid tumors (reviewed in Sliwkowski & Mellman, 2013). Therapeutics targeting ErbB proteins are divided into two main classes: monoclonal

antibodies (mAbs) that bind to extracellular domain, and low molecular weight tyrosine kinase inhibitors (TKIs) that bind to intracellular tyrosine domain. Another class of agents targeting ErbB proteins include heat-shock protein (Hsp) inhibitors, such as 17AAG and antibody-drug conjugates (ADCs), such as T-DMI (trastuzumab conjugated to maytansine derivative (DM1)) (reviewed in Sliwkowski & Mellman, 2013). Also other small molecule inhibitors that bind to ErbB signaling molecules exist (reviewed in Hynes & MacDonald, 2009) (overview in Figure 9).

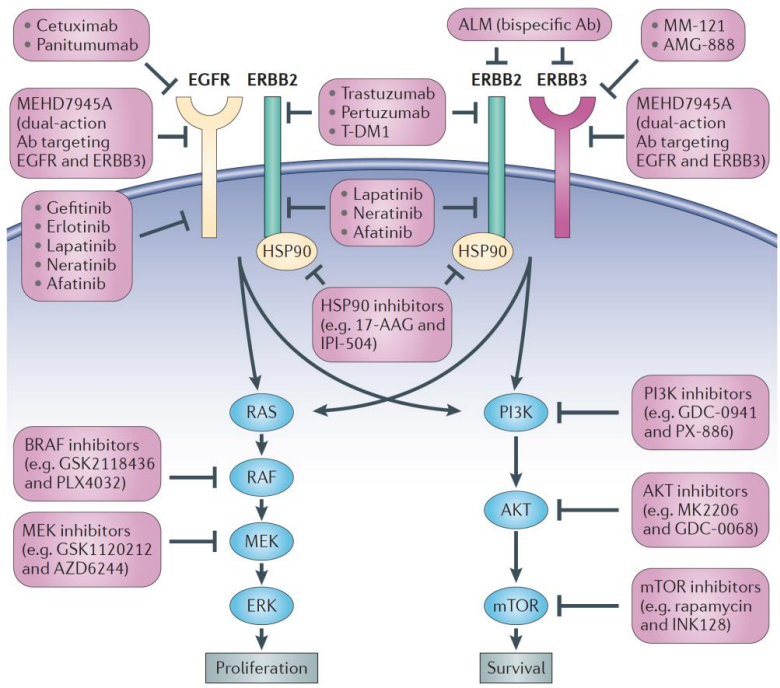

Figure 9. The ErbB signaling- targeted therapeutics. Overview of monoclonal antibodies (mAb) and tyrosine kinase inhibitors (TKIs) specifically targeting the ErbB family members (described in more detail in the text). Adapted by permission from Nature Publishing Group: Nature Reviews Cancer (Yarden & Pines, 2012). Figure legend is modified.

Monoclonal antibodies

One of the most effective strategies in targeted therapies involves the use of recombinant proteins, usually mAbs that target proteins which are expressed at the cell surface. mAbs that are used in therapy are genetically engineered by combining genes from multiple sources of B lymphocytes (mostly mouse and human), human immune cells, or coupled phage or yeast display libraries (reviewed in Sliwkowski &

Mellman, 2013). The adopted approach makes it possible to avoid the immune response in humans, which otherwise could be triggered by the use of antibodies produced in mice. Mechanisms that underlie the antitumor activity of mAbs (Figure 10) include binding to the extracellular part of the specific receptor, which in turn can interfere with ligand binding and dimerization with other receptors. Other antibodies can, upon binding to the certain receptor, induce its internalization and degradation (Zhou et al, 2011). Moreover, it has been shown that combination of a minimum of two antibodies with non-competitive binding sites enhanced the efficient internalization of the targeted receptor (Ben-Kasus et al, 2009; Hughes et al, 2012; Pedersen et al, 2015). Furthermore, mAbs can trigger antibody-dependent cellular cytotoxicity (ADCC) and recruit immune cells, such as NK cells, macrophages, and neutrophils, leading to the apoptosis of tumor cells (reviewed in Weiner et al, 2010) (Figure 10).

Anti-EGFR monoclonal antibodies

EGFR was one of the first growth factor receptors used as a target in cancer therapy (Gschwind et al, 2004). Cetuximab (Erbitux®) is a human-mouse chimeric IgG1 mAb that binds to domain III in the extracellular part of inactive EGFR preventing the receptor from adopting the open conformation (Li et al, 2005) and blocking binding of the ligand. It has been demonstrated that binding of cetuximab can induce internalization of EGFR (Jaramillo et al, 2006; Sunada et al, 1986) and induce ADCC (Kimura et al, 2007). Cetuximab is approved for the treatment of wild type *K-RAS* EGFR-expressing metastatic colorectal cancer. Nimotuzumab (TheraCIM®), a humanized IgG1 mAb that binds to EGFR domain III, has been shown to display similar mechanisms of action to cetuximab, while only partially inducing ADCC (reviewed in Friedlander et al, 2008). Panitumumab (Vectibix™) is a fully human IgG2 mAb that binds to EGFR's domain III with higher affinity than cetuximab, preventing binding of the ligand. Panitumumab inhibits EGF-dependent tumor cell activation, EGFR tyrosine phosphorylation, and cell proliferation but is less effective in mediating ADCC than cetuximab (Astsaturov et al, 2006; and reviewed in Sliwkowski & Mellman, 2013). Panitumumab is approved for treatment of multiple EGFR-expressing human solid tumors, with wild type *K-RAS* (Weiner et al, 2008) (overview in Figure 9).

Anti-ErbB2 monoclonal antibodies

Monoclonal antibodies that are approved for treatment of ErbB2-positive breast cancer include trastuzumab (Herceptin®) and pertuzumab (Perjeta®), (see overview Figure 9 for an overview) (reviewed in Sliwkowski & Mellman, 2013). Trastuzumab is a humanized IgG1 mAb that binds to the extracellular domain IV of ErbB2 (Carter et al, 1992). However the exact mechanisms of its anti-tumor activity are not completely understood. In the early reports trastuzumab has been reported to prevent dimerization of ErbB2 with other ErbB proteins which resulted in reduced signaling from ErbB2 (Klapper et al, 1997). Later it has been observed that trastuzumab inhibited the PI3K signaling pathway, caused cell cycle arrest and inhibited proliferation (Sliwkowski et al, 1999), and induce ADCC (Weiner & Adams, 2000). On one hand it has been suggested that trastuzumab induces internalization and degradation of ErbB2 (Baselga et al, 2001), but on the other hand, others did not observe trastuzumab-induced internalization of ErbB2 (Longva et al, 2005). Trastuzumab was also reported to block metalloprotease cleavage of ErbB2, which prevents formation of p95HER2, a truncated form of ErbB2 (Molina et al, 2001). Contrary to trastuzumab, anti-tumor activities of pertuzumab, another therapeutic antibody against ErbB2, are better characterized. Pertuzumab is a fully humanized IgG1 mAb that binds to domain II of ErbB2 preventing its dimerization with other receptors (Franklin et al, 2004). It has been shown that pertuzumab together with trastuzumab and 17AAG induced efficient internalization and degradation of ErbB2. Moreover, the combination of pertuzumab, trastuzumab and 17AAG increased the inhibitory effect on Akt activation of each of these agents (Hughes et al, 2012). Pertuzumab was demonstrated to inhibit ligand-induced ErbB2-EGFR heterodimerization and to facilitate EGF-induced internalization and degradation of EGFR in cells overexpressing ErbB2 (Hughes et al, 2009). In paper I we have observed similar effects of action of pertuzumab on HRG-induced ErbB2-ErbB3 heterodimerization, where pertuzumab has been shown to prevent ErbB2-ErbB3 heterodimers formation and inhibit ErbB3 phosphorylation in cells overexpressing ErbB2. In addition, it has been reported that pertuzumab blocked HRG-induced ErbB2-ErbB3 heterodimerization leading to reduced phosphorylation of ErbB3 and MAPK and Akt signaling, thus inhibiting growth of cancer cells (Sakai et al, 2007). Pertuzumab was also shown to mediate ADCC (Yamashita-Kashima et al, 2011). mAbs targeting ErbB2 have proven to be efficient in the treatment of ErbB2-positive

breast cancer patients. Dual blockage of ErbB2 by two mAbs has been shown to be active in early breast cancer and late-stage metastatic breast cancer and produced an overall better clinical outcome (reviewed in Sliwkowski & Mellman, 2013). The combination of two anti-ErbB2 mAbs together with conventional methods, such as chemotherapy, is even more efficient in the treatment of ErbB2-positive breast cancer patients than each agent alone. Pertuzumab is approved in combination with trastuzumab and docetaxel for the treatment of patients with ErbB2-positive metastatic breast cancer without prior treatment with anti-ErbB2 therapy for metastatic disease. Moreover, this combination was also approved for neoadjuvant therapy in ErbB2-positive patients with locally advanced, inflammatory, or early stage breast cancer (Baselga et al, 2012; Maly & Macrae, 2014; Moasser & Krop, 2015). In addition, mAbs can also be used as vehicles for the selective delivery of cytotoxic agents to tumors. One example is T-DM1 which is composed of trastuzumab (T) conjugated to maytansine derivative (DM1) (Lambert & Chari, 2014). It has been proposed that a trastuzumab conjugate simply uses ErbB2-overexpressing cells as a target for the delivery of a potent cytotoxic agent, which is released only after limited digestion of the entire antibody (Lewis Phillips et al, 2008). T-DM1, which has recently gained approval for use, was reported to prolong progression-free survival in late-stage ErbB2-positive breast cancer patients for whom other treatments have failed (reviewed in Sliwkowski & Mellman, 2013).

Anti-ErbB3 monoclonal antibodies

ErbB3 is one of the most potent activators of the PI3K/Akt pathway and is upregulated and transphosphorylated in several forms of cancer, in particular following treatment with EGFR and ErbB2 inhibitors. In past years, several mAbs targeting ErbB3 have been developed. MM-121, is a human IgG2 mAb that binds specifically to ErbB3 and is able, directly or indirectly, to block ligand–induced receptor heterodimerization with ErbB2, and to inhibit HRG-induced ErbB3 and Akt phosphorylation (reviewed in Aurisicchio et al, 2012). However, MM-121 is mainly active in cancers with ligand-dependent activation (Schoeberl et al, 2010). Currently, MM-121 is being assessed in Phase I and II clinical trials in breast cancer, non-small cell lung cancer, ovarian cancer as well as other solid tumor cancers (reviewed in Aurisicchio et al, 2012). AMG 888, a human anti-ErbB3 mAb, has been also shown to block ErbB3-induced Akt and Erk signaling and to inhibit growth of several tumor

cell lines alone, but it proved to work better in combination with other ErbB family inhibitors (reviewed in Aurisicchio et al, 2012). TK-A3 and TK-A4, both IgG1 mouse anti-ErbB3 mAbs, have been shown to modulate the growth rate of different cancers. TK-A3 and TK-A4 were found to inhibit receptor recycling to the cell surface, block ligand binding and induce receptor internalization and degradation (reviewed in Aurisicchio et al, 2012; Belleudi et al, 2012). Another interesting example of mAbs is MEHD7945A, which is a human dual specific anti-ErbB3 and anti-EGFR mAb that has been shown to inhibit EGFR- and ErbB3-mediated signaling *in vitro* and *in vivo* and to induce ADCC (Schaefer et al, 2011). However, it has been found to act mainly by blocking ligand binding and setting the receptors in a closed conformation (reviewed in Aurisicchio et al, 2012). Analogous bi-specific antibody, MM-111 that binds simultaneously to both ErbB2 and ErbB3, has been shown to block signaling and tumor growth (McDonagh et al, 2012).

Tyrosine Kinase Inhibitors and other inhibitors

The second major class of therapeutics targeting ErbB family members is low molecular weight tyrosine kinase inhibitors (TKIs) that compete reversibly or irreversibly with ATP binding to the kinase domain of ErbB proteins (overview in Figure 9). Only a few specific TKIs to receptor tyrosine kinase family have been developed for the treatment of cancer patients. Although, it has been shown that TKIs have numerous, partly severe side effects, TKIs serve as additional options in second-third- and/or fourth-line therapy (Eckstein et al, 2014). Gefitinib (Iressa®) (Herbst et al, 2004) and erlotinib (Tarceva®) (Minna & Dowell, 2005) are reversible, selective EGFR-TKIs that preferentially bind the kinase in the active conformation. Anti-tumor effects of gefitinib and erlotinib have been investigated in many tumors overexpressing EGFR and most trials failed to show potent clinical effects. However there are some exceptions, as for patients with non-small cell lung cancer that contain mutations in the kinase domain of EGFR, who responded well to EGFR-TKIs (reviewed in Tebbutt et al, 2013). Skin rash is one of the most common adverse effects associated with the inhibition of EGFR and this reaction is considered as a biomarker of EGFR inhibition. Although, is not universally accepted as a clinical response, patients who develop rashes tend to more likely exhibit therapeutic effects (Saif & Kim, 2007; and reviewed in Sliwkowski & Mellman, 2013). There are no specific ErbB2-TKIs, but lapatinib (Tykerb/Tyverb®), afatinib (Gilotrif™) and

neratinib are TKIs that inhibits both EGFR and ErbB2. Lapatinib is a reversible TKI that binds to kinase-inactive conformation of EGFR. Lapatinib has been shown to inhibit EGF-stimulated phosphorylation of Akt in ErbB2-overexpressing human breast cancer cell line and EGF-stimulated phosphorylation of EGFR and Akt in human head and neck cancer cells that overexpress EGFR (reviewed in Tebbutt et al, 2013). It has also been demonstrated that lapatinib can induce apoptosis of tumor cells *in vitro* and *in vivo* in xenografted mice with cell lines over-expressing EGFR and ErbB2 (reviewed in Hojjat-Farsangi, 2014; Rusnak et al, 2001; Xia et al, 2002).

In addition to TKIs, several small-molecule inhibitors targeting Hsp90 (Figure 9) are now in clinical investigation in a wide range of cancer types (reviewed in Jhaveri et al, 2012). Hsp90 is an important target in cancer therapy because many of its clients constitute oncoproteins, such as ErbB2, with important functions in the development and promotion of cancer. GA, originally pursued as an antibiotic, was later shown to bind directly to Hsp90 and interfere with Hsp90-v-src heterocomplex formation (Whitesell et al, 1994). GA competes with ATP in binding to the N-terminal domain of Hsp90, which results in inhibition of Hsp90 activity followed by ubiquitination and degradation of its clients (Pearl et al, 2008). GA is not suitable as a clinical agent because of its poor solubility, limited *in vivo* stability and significant hepatotoxicity in animals (Neckers, 2006), but it serves as a "model" molecule for other agents that are now in clinical trials (reviewed in Jhaveri et al, 2012). 17AAG a less toxic derivative of GA is an Hsp90 inhibitor that has been extensively studied for targeting oncoproteins. It was demonstrated that 17AAG inhibits cell growth and induces apoptosis (Munster et al, 2002; Solit et al, 2002). Currently, there are approximately 15 other, besides 17AAG, Hsp90 inhibitors in clinical trials (Barrott & Haystead, 2013).

Resistance to ErbB-targeted therapies

Resistance to therapies targeting ErbB proteins is currently a common limitation of such treatment. There are two types of resistance: intrinsic resistance that includes the factors that exist before treatment and acquired resistance that develops during treatment of tumor cells (reviewed in Holohan et al, 2013). Numerous mechanisms of acquired resistance have been reported, such as loss of tumor suppressors, additional mutations and overexpression of oncoproteins, such as ErbB proteins and their

ligands, and other signal receptors. Loss of expression or inactivation of PTEN (phosphatase and tension homolog) was found to be one of the reasons for acquired resistance to trastuzumab, lapatinib and gefitinib (Wheeler et al, 2010). Formation of EGFR-ErbB3 and ErbB2-ErbB3 heterodimers was shown to accelerate proliferation of cancer cells and failed to respond to targeted therapies against EGFR and ErbB2 due to compensatory activation of ErbB3-dependent signaling pathways. Cetuximab, gefitinib and erlotinib are ineffective in patients with *K-RAS* mutations because these anti-EGFR drugs cannot suppress EGFR-independent MAPK survival signaling (reviewed in Ciardiello & Tortora, 2008; Tsujioka et al, 2010). In addition, acquired resistance to EGFR targeted therapy is caused by development of an additional *EGFR* mutation, T790M, which causes structural changes in the tyrosine kinase domain of EGFR preventing erlotinib and gefitinib from binding to EGFR (Pao et al, 2005).

Unfortunately, 50-60% of metastatic breast cancer patients appear resistant to trastuzumab (Romond et al, 2005). The mechanisms of trastuzumab resistance include activation of alternative signaling from other ErbB proteins, insulin-like growth factor receptor (Liu et al, 2011), activation of PI3K/Akt/mTOR or loss of PTEN (Saal et al, 2005), overexpression of c-MET (Shattuck et al, 2008), up-regulation of Src activity (Rexer & Arteaga, 2012) or MUC4 (Mukhopadhyay et al, 2011), increased VEGF (vascular endothelial growth factor) expression (Yang et al, 2002) and expression of the p95 isoform of HER2 (Gallardo et al, 2012). p95HER2 is expressed in nearly 30% of ErbB2-positive breast cancers and despite lacking an extracellular domain, p95HER2 still triggers proliferative downstream signaling events and forms potent heterodimers due to its active kinase region (Arribas et al, 2011). The absence of the extracellular domain eliminates mAb-targeted therapies and constitutes a cause of resistance to such drugs. However, p95HER2 remains susceptible to kinase inhibition and the use of TKIs serves as an important way to overcome antibody resistant therapies (Schroeder et al, 2014).

There are several ways to enhance efficiency of inhibition of ErbB proteins and to overcome the acquired resistance to ErbB-targeted therapies:

1) Dual targeting of extra- and intracellular parts of ErbB proteins that will enhance their inhibition (more in Figure 10a). It was also shown that simultaneous use of both mAb and TKIs alters receptor conformation and glycosylation (Figure 10c). Gan *et al.* (Gan et al, 2007) reported that upon treatment with a TKI targeting

EGFR (AG1478) the post-translational glycosylation of the EGFR is disrupted leading to trafficking of immature, high mannose forms of EGFR to the cell surface where it contributes to increased mAb 806 binding and may increase ADCC activity (reviewed in Tebbutt et al, 2013).

2) Targeting more than one receptor by dual mAbs that will prevent heterodimerization. For example, bi-specific monoclonal antibody, MM-111 was designed to target cancer cells with acquired resistance to anti-ErbB2 therapy. MM-111 binds to both ErbB2 and ErbB3, and form inactive trimmers and inhibits *in vitro* HRG-induced phosphorylation and cell proliferation. However, as a single agent MM-111 is unable to fully block ErbB2-ErbB3 heterodimerization (Aurisicchio et al, 2012). On the other hand MM-111 seemed to increase efficiency of trastuzumab and lapatinib (McDonagh et al, 2012). It has been shown recently that trastuzumab in combination with lapatinib (also illustrated in Figure 10b) inhibited *in vitro* and xenograft growth of human breast cancer cell lines significantly more than either agent alone (reviewed in Tebbutt et al, 2013). Likewise, the combination of cetuximab and gefitinib was shown to have synergistic *in vitro* anti-proliferative activity against the A431 cancer cell line (Bos et al, 1997).
3) The use of mAbs in combination with TKIs, that will increase surface receptor and engage ADCC (Cavazzoni et al, 2012) (and described earlier and illustrated in Figure 10d), which is probably the most important clinical activity (reviewed in Tebbutt et al, 2013).

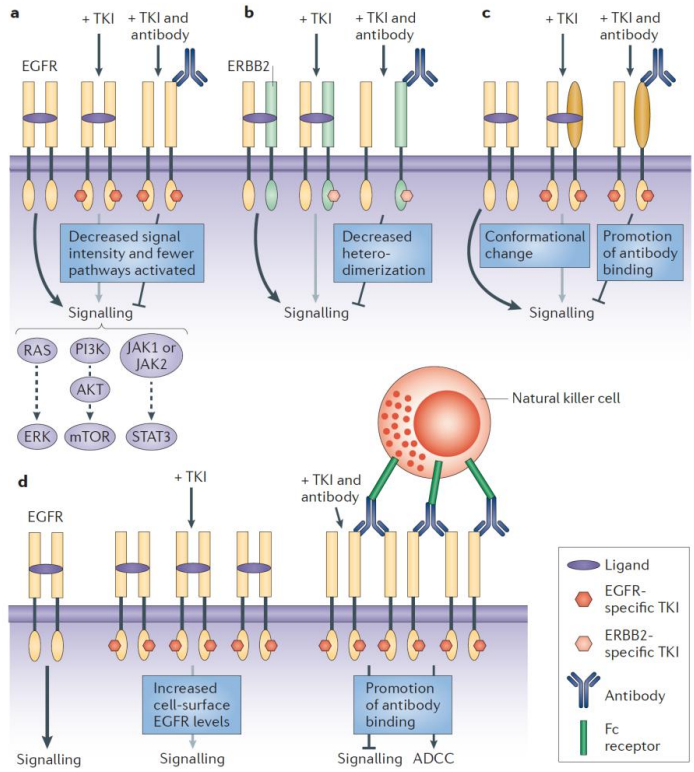

Figure 10. Enhanced anti-tumor mechanisms mediated by dual EGFR therapy. (a) Decreased signal intensity. The combination of an EGFR-targeted antibody with the TKI inhibits EGFR activation by preventing ligand binding and EGFR dimerization. This is probably the main mechanism associated with the use of dual therapeutics. **(b) Decreased receptor heterodimerization**. Upon heterodimerization of EGFR and ErbB2, intensity and diversity of signalling increases when compared with that from EGFR homodimers. The addition of a TKI specific for either receptor reduces signalling from the heterodimer but does not directly prevent dimerization. Adding an EGFR-targeted or ErbB2-targeted antibody in combination with the TKI directly reduces heterodimerization and prevents downstream signalling. **(c) Induction of a conformational change in the receptor.** EGFR-specific TKIs can change the conformation of EGFR (shown as dark yellow ovals), increasing the binding of certain EGFR-targeted antibodies and thereby making them more efficient at inhibiting the receptor. **(d) Induction of antibody-dependent cell cytotoxicity (ADCC).** TKIs directed towards EGFR cause retention of the receptor on the cell surface, resulting in increased levels of cell-surface EGFR-targeted antibody leading to interaction and activation of natural killer cells more efficiently, stimulating a stronger ADCC response to the cancer cell. Thick black arrows indicate strong signalling, thin grey arrows indicate weak signalling, inhibiting arrows indicate no signalling and dashed arrows indicate multiple steps. Abbreviations: JAK, Janus kinase; STAT, signal transducer and activator of transcription. Adapted by permission from Nature Publishing Group: Nature Reviews Cancer (Tebbutt et al, 2013). Figure legend is modified.

Aims of the Study

The overall aim of this thesis was to investigate and elaborate upon our understanding of the molecular mechanisms underlying endocytic downregulation of the ErbB proteins. A specific focus of the work was directed towards the involvement of cargo-specific adaptors in CME of ErbB3, the effects of ErbB2 on downregulation of ErbB3 and the effects of mAbs on endocytic downregulation of ErbB2. These research strands were intended to contribute to an overall better understanding of the regulatory mechanisms underpinning constitutive, ligand- or antibody-induced internalization of ErbB2 and ErbB3.

Paper I

The ErbB proteins are attractive targets in the development of drugs that can specifically interfere with molecules involved in tumorgenesis. mAbs that bind to the extracellular domain of the ErbB proteins have been shown to block ligand binding, receptor dimerization and activation as well as induce receptor downregulation. ErbB2 is considered endocytosis resistant and is the preferred dimerization partner for other ErbB family members. In our previous study we have shown that overexpression of ErbB2 and heterodimerization with EGFR inhibited EGF-induced internalization of EGFR causing a prolonged signaling. However, upon incubation with pertuzumab heterodimerization was inhibited and the EGF-induced downregulation of EGFR was enhanced (Hughes et al, 2009). The aim of the study presented in paper I was to investigate to what extent ErbB2 also inhibits endocytosis and downregulation of ErbB3, and whether pertuzumab has an effect on constitutive or ligand-induced signaling and downregulation of ErbB3 in cells with high levels of ErbB2.

Paper II

Internalization of ErbB proteins constitutes an important mechanism in shutting-down the signaling from activated receptors. We have previously shown that ErbB3 is internalized constitutively in a clathrin-dependent manner (Sak et al, 2012). ErbB3 does, however, not bind to AP-2 and in the study presented in paper II the aim was to identify other CLASPs which may be involved in recruiting ErbB3 into CCPs.

Paper III

It has been demonstrated that most mAbs have limited *in vivo* anti-tumor effects when administrated alone. The combination of two or more antibodies does however, often induce downregulation of the ErbB proteins. Still, little is known about the molecular mechanisms causing and regulating antibody-induced internalization and/or degradation of ErbB proteins. The aim of the study presented in paper III was to investigate the ability of a mixture of two mAbs to induce ErbB2 internalization and degradation. We examined the involvement of clathrin-dependent and clathrin-independent pathways, as well as the role of antibody-induced phosphorylation and ubiquitination in endocytic downregulation of ErbB2.

Summary of Papers

Paper I

Sak, M.M., Szymanska, M., Bertelsen, V., Hasmann, M., Madshus, I.H. and Stang, E.

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

Carcinogenesis, *34*, 2031-8, May 2013

Overexpression of ErbB2 contributes to its tumor-promoting functions and is associated with poor patient prognosis. ErbB2 is thought to be endocytosis resistant and thus concentrated at the plasma membrane and is a preferred dimerization partner for other ErbB proteins, causing constitutive signaling and promoting cell growth and anti-apoptotic effects. Of special interest are ErbB2-ErbB3 heterodimers, which constitute the most potent oncogenic unit. In this study we investigated the effect of ErbB2 expression on ErbB3 downregulation and signaling, upon stimulation with or without the ErbB3 ligands HRG-β1 and HRG-β1 ECD and assessed for the effects of the anti-ErbB2 antibody pertuzumab in cell lines co-expressing ErbB3 with different amounts of ErbB2. We found efficient phosphorylation of and signaling from ErbB3 only in cell lines co-expressing ErbB2, supporting the notion that efficient activation of ErbB3 depends on heterodimerization. Our flow cytometry data and confocal image analyses showed that overexpression of ErbB2, in the absence of ligand, did not inhibit ErbB3 internalization. We further investigated the degradation of ErbB3 in cell lines which co-express different levels of ErbB2. Our data indicate that incubation with HRG leads to heterodimerization of ErbB2 and ErbB3, which in turn prevents endocytosis and degradation of ErbB3. We next characterized the effects of pertuzumab on ligand-induced ErbB3 phosphorylation and degradation in cell lines co-expressing ErbB2. Our data strongly suggest that upon incubation with pertuzumab ligand-induced formation of ErbB2-ErbB3 heterodimers is inhibited, thus allowing efficient degradation of ligand-bound ErbB3 also in cell lines overexpressing ErbB2.

Paper II

Szymanska, M., Fosdahl, A.M., Raiborg, C., Dietrich, M., Liestøl, K., Stang, E. and Bertelsen, V.

Interaction with epsin 1 regulates the constitutive clathrin-dependent internalization of ErbB3.

Manuscript

ErbB3 has been demonstrated to be constitutively internalized in a clathrin-dependent manner, but it has also been shown that it doesn't interact with AP-2, the main adaptor complex localized to the CCPs. However other CLASPs can also recruit cargo to the coated-pits, and in this study we performed a high-throughput siRNA screen to identify adaptor proteins required for constitutive internalization of ErbB3. We knocked down 25 CLASPs know to participate in CME as well as components of the AP-2 complex. CHC and dynamin 2 served as strong and milder positive controls, respectively, to help to identify a broad spectrum of hits. We employed an automatic fluorescent microscope and identified several proteins that, upon knock-down, interfered with the extent of ErbB3 internalization. We found that knock-down of epsin 1 inhibited the level of ErbB3 internalization to the same level as knock-down of CHC. We further confirmed that ErbB3 is constitutively ubiquitinated and that the ubiquitination increases upon ligand binding. Our co-immunoprecipitation data further showed that ErbB3 interacted with an epsin 1 mutant containing membrane and ubiquitin binding domains only. Thus, our data suggest that ErbB3 can interact with epsin 1 in an ubiquitination dependent manner and that both constitutive and ligand-induced CME of ErbB3 is regulated through interaction with epsin 1.

Paper III

Szymanska, M., Fosdahl, A.M., Nikolaysen, F., Pedersen, M.W., Grandal, M.M., Stang, E. and Bertelsen V.

The combination of two antibodies recognizing non-overlapping epitopes of ErbB2 induces ErbB2 ubiquitination and internalization in an ErbB2 kinase activity dependent manner.

Manuscript

Although ErbB2 is mainly localized to the plasma membrane, there are several ways to downregulate it, for example by the use of Hsp90 inhibitors or by exposure to mAbs, which can induce internalization and degradation of ErbB2. In this study we have investigated the mechanism of action of two mAbs binding to ErbB2 and assessed for their ability to induce downregulation of ErbB2 in cell lines overexpressing the receptor. We show by confocal microscopy that the mAb mixture induced internalization of ErbB2 and its localization to EEs and to LEs/lysosomes. Additionally, we demonstrate that the mAb mixture caused degradation of ErbB2. Furthermore, we investigated the molecular mechanisms causing downregulation of ErbB2 and showed that the mAb mixture induced ErbB2 phosphorylation and ubiquitination. Moreover, we demonstrated that inhibition of the ErbB2 kinase activity by lapatinib reduced antibody-induced ubiquitination, internalization and degradation of ErbB2. We also studied the endocytic pathways that are involved in antibody-induced internalization of ErbB2 and concluded that the mAb mixture promotes internalization of ErbB2 in a dynamin 2 and actin-dependent manner.

Methodological Considerations

Cell lines

Biological experiments are most often performed with cultured, immortalized cell lines because they are readily available and provide large variation in type and expression levels of different proteins. Cell lines are accessible for manipulation with gene silencing, protein overexpression and various inhibitors and therapeutics. However there are several disadvantages to the use of cell lines e.g. they have adapted to growth in culture where they are prone to genotypic and phenotypic changes as well as to mycoplasma infections. Therefore, we have routinely performed analysis for mycoplasma infections and kept all cell lines in culture for limited number of passages. For the current study, we have selected human cancer cell lines that express various levels of ErbB proteins and porcine aortic endothelial (PAE) cells. PAE cells, which do not express ErbB proteins endogenously (Malassagne et al, 1998), were stably transfected with plasmids encoding different ErbB proteins alone or in various combinations.

The focus of paper I was to study how ErbB2-ErbB3 heterodimerization affects trafficking of ErbB3 and how pertuzumab affects heterodimerization and degradation of ErbB3 in PAE cells expressing ErbB2 and ErbB3. In addition, we have investigated the ligand-induced phosphorylation of ErbB3 and activation of downstream signaling in PAE cell lines expressing ErbB3 only or in combination with either EGFR or ErbB2. PAE cell lines constitute a very useful tool to study the molecular mechanisms underlying receptor internalization and signalling. PAE cells are easy to transfect with siRNA and plasmids and, due to their flat morphology, make a good model for microscopy based studies. PAE cells do however, constitute a rather artificial system, since they do not express the ErbB proteins endogenously and are transfected to express receptors originating from a different species. PAE cells expressing EGFR only (PAE.EGFR) have been characterized before (Carter & Sorkin, 1998). Different PAE cell lines: PAE.EGFR.ErbB3, PAE.ErbB2.ErbB3 (Haslekas et al, 2005; Pedersen et al, 2009), PAE.ErbB3 (Sak et al, 2012) have been validated and extensively used in our current and previous work. To confirm our findings observed in PAE cells, we have also used human breast cancer cell lines:

MCF-7 and SK-BR-3, which express comparable amounts of ErbB3 but varying levels of ErbB2.

In paper II we have used MCF-7, a breast adenocarcinoma cell line expressing moderate levels of ErbB3 and very low levels of ErbB2 and EGFR. This allowed us to study ligand-independent internalization of ErbB3 without the influence of overexpression of other ErbB proteins. We also made use of HeLa cells, which have been extensively used in similar studies, especially regarding EGFR. HeLa cells do not express ErbB3 at detectable levels, but the ease of co-transfecting HeLa cells made it possible to study of protein- protein interactions between ErbB3 and epsin 1, which were not possible to achieve in MCF-7 due to the combination of low levels of endogenous ErbB3 and transient interaction between ErbB3 and epsin 1.

In paper III we have used OE-19, a human oesophageal carcinoma that contains a 100-fold ErbB2 gene amplification (Dahlberg et al, 2004). This cell line facilitated the study of the effects of novel therapeutic antibodies in ErbB2-dependent cancer cell lines. This is very important factor in our studies, since it appears that a subset of breast and gastric cancers with ErbB2 amplification become resistant to the existing treatment. In addition, (papers I and III) we have used other cell lines overexpressing ErbB2, such as SK-BR-3 cells (mammary gland/ breast adenocarcinoma), and NCI-N87 cell line (gastric carcinoma), which also express moderate levels of ErbB3 and EGFR.

Chemical inhibitors

The use of chemical inhibitors is a powerful tool to investigate the potential role of certain proteins in cellular processes but also has many disadvantages. In this work different, chemical inhibitors have been used to study internalization pathways, for example monensin, amiloride and cytochalasin D (paper II). In order to check the effect the various drugs had on clathrin-mediated endocytosis, we carried out a dose-response assay measuring internalization of radioactive transferrin. Cytochalasin D, a drug that acts on actin cytoskeleton (Sampath & Pollard, 1991), primarily targeting phagocytosis and macropinocytosis, was used at concentrations that were reported to disrupt actin polymerization without causing unspecific side effects (Mortensen & Larsson, 2003; Stuart & Brown, 2006). Monensin, a monovalent ion-selective ionophore, was reported to inhibit the Na^+/H^+ antiporter, in consequence neutralize the acidic intracellular compartments such as the trans Golgi apparatus, lysosomes,

and certain endosomes (Mollenhauer et al, 1990). Monensin was also shown to inhibit exit of internalized receptors and other endocytic cargo from sorting endosomes and the endocytic recycling compartment (Stein et al, 1984; Tran et al, 2003). Amiloride is reported to be a strong inhibitor of epithelial Na^+ channel and a weaker inhibitor of Na^+/H^+ exchanger (Kleyman & Cragoe, 1988). Another inhibitor used in our work (paper III) is lapatinib, a potent ErbB2 and EGFR dual tyrosine kinase inhibitor (Xia et al, 2002). Lapatinib competes with ATP for binding to the kinase domain of both ErbB2 and EGFR. Titration of lapatinib was performed in order to determine its minimal working concentration which inhibited EGF-induced phosphorylation of EGFR and ErbB2.

Transient transfections

Transient transfection with plasmids is a method of introducing foreign genes or genes that are otherwise expressed only in small amounts, or mutated versions of a gene of interest, without incorporation into the genome. The transfection is transient not only because the genes are not incorporated into the genome, but because genetic material, i.e. the plasmid, can be lost during cell division and under the influence of environmental factors. This method enables the study of interaction between proteins that are not otherwise expressed in a particular cell line, or where endogenous levels are so low that it is impossible to detect it or efficiently precipitate the interacting complexes with the available antibodies. By transfection of different mutants that will compete with endogenous proteins it is possible to study specific cellular processes. The use of deletion mutants also allows studying functions of a particular protein domains. Moreover, this method allows the use of tagged proteins, which are easier to precipitate due to more efficient and more specific antibodies. Because it was not possible to co-precipitate endogenous ErbB3 and epsin 1 in MCF-7, we co-transfected HeLa cells with two plasmids simultaneously to raise the levels of both proteins (paper II). We used plasmids encoding EGFP tagged deletion mutant of epsin 1 and wild-type ErbB3, for their better co-precipitation and detection under microscope. However, there are some limitations of this method i.e. some cells will overexpress the encoded protein to an extent that can change its normal localization in the cell, alter trafficking and cause unwanted protein aggregation. Conversely, not all cells will be transfected with the desired DNA. In the case of double transfections and single-transfected cells can be easily distinguished and immediately compared

when visualized under the microscope. We have used the low transfection efficiency to our benefit when studying the effects of EGFP- tagged dominant-negative mutant of dynamin 2 on antibody-induced internalization of ErbB2 (paper III) and when analyzing the effects of EGFP-ENTH-UIM epsin 1 on ErbB3 internalization (paper II). When analyzing the cells under the confocal microscope we could then easily identify and distinguish transfected from non-transfected cells and compare the cells directly in one image.

RNA Interference

RNA interference (RNAi), discovered by Fire and Mello (Fire et al, 1998), is post-transcriptional processes of mRNA degradation used by different organisms to control protein expression. The most common way to inhibit protein expression is the introduction of small interfering RNA (siRNA). siRNA is a synthetic oligonucleotide duplex composed of 19 complementary base pairs and 2-nucleotide 3′ overhangs. When siRNA is introduced into a cell, it is first unwound into two single-stranded RNAs, the guide strand which binds to RNA-induced silencing complex (RISC) and the passenger strand which is degraded. Next, RISC is directed to the specific mRNA and, by complementary base- pairing of the guide strand with the sequence in a mRNA molecule, induces cleavage by argonaute, the catalytic component of the RISC complex. The effects of the siRNAs are transient, especially in actively dividing cells. While siRNA provides the opportunity to inhibit the expression of specific protein in a variety of cell lines, its utility is limited to cells that are simple to transfect. In our screen (paper II) we have tested different human cell lines for ease of transfection with different siRNAs and different lipid reagents and assessed for the specificity in protein knock-down in addition to cell viability and cytotoxicity. One of the disadvantages of RNAi methodology are off-target effects, which occur when an siRNA sequence is not specific for the protein of interest and consequently it can bind to, and reduce levels of, many other unwanted mRNAs. In our study we have used 4 individual sequences for each protein and 4 individual non-targeting (random sequence, not present in target genome) siRNA sequences together with siRNA to housekeeping genes as negative controls to ensure that the observed effects are due to the knock-down of the particular protein. While optimizing the assay we have also tested different concentrations of siRNA to find the lowest effective concentration such that the risk of targeting unwanted mRNAs was minimized. It is worth noting

that mammalian cells, as a protective mechanism, will actively respond to the introduction of double-stranded RNA by triggering the interferon response pathway, which in turn can cause off-target effects. The motifs of some siRNAs that stimulate interferon have not yet been identified, so caution should be exercised when interpreting results. RNAi is considered to be a powerful tool in recent biological research for the study of gene functions and also as a novel strategy in therapies.

High-throughput screening (HTS)

Due to its selectivity in silencing protein expression and robustness, RNAi technology is currently used in small and large-scale screens that systematically shut down the protein of interest and identify the role of specific proteins in particular cellular processes. RNAi has been adopted for functional genomics, endocytic pathway analysis, and drug target validation experiments and is now being used in high-throughput experiments with large numbers of siRNAs. Because of the benefits of HTS, which include its simplicity, rapidity, low cost, and high efficiency, we have implemented this technology in assessing the cellular response to siRNA knock-down. In paper II we used siRNA to knock-down 31 selected proteins involved in CME and checked the effect on internalization of ErbB3. Upon knock-down of the selected protein, an antibody to extracellular part of ErbB3 was bound on ice and subsequently "chased" at 37°C. The localization of the anti-ErbB3 antibody was detected using secondary antibodies conjugated to the fluorescent dye. Nuclei stained with Hoechst were used for the automatic cell recognition and automatic image acquisition and assessment of the cells phenotype were used to identify statistically significant 'hits'. To prevent false positives and ensure the overall reliability of results, we have implemented appropriate statistical procedures, which took all the small variations in the fixation and staining protocol, as well as phenotypic variability between plates, into consideration. Using this approach, we were able to identify cargo specific adaptors involved in clathrin-mediated internalization of ErbB3.

Wide-field fluorescent microscopy and confocal microscopy

Wide-field fluorescence microscopy is a frequently used fluorescence imaging technique that relies on the simultaneous illumination of the whole sample. In paper II we have used an automatic wide-field fluorescent microscope to collect data from the thousands of cells. In this simplified approach, cells were detected based on

nuclei staining while internalized ErbB3 "spots" were automatically assigned to a particular cell and their number and intensity were counted. One of the biggest challenges encountered while using automatic microscope was to ensure that the surface in each well of a 96-well plate was covered with a uniform, flat layer of adherent cells. During scanning of the plate by the microscope, the objective was moving from one well to another and for each image the focus had to be set on given objects, in this instance the cell nuclei. Whenever there was an empty spot in a well, focus was lost and the recorded images were of poor quality, making it impossible to analyze the cells. Therefore, we took a great care to ensure that confluence of the cells on the day of image acquisition is in not less that 60% and not more than 90%.

Confocal microscopy enables visualization within both living and fixed cells and tissues with increased optical resolution and contrast. Confocal microscopy takes advantage of the spatial pinhole and point illuminations to eliminate out-of-focus light and increase resolution in x and y planes. In papers I and III we have primarily used confocal microscope to analyze in more detail the nature of intracellular structures, especially when studying the colocalization of the receptors with markers of early or late endosomes. For each experiment many cells were analyzed to correct for individual cell variations and each experiment was performed minimum three times to account for technical variations in the staining protocol. A number of important points must be kept in mind when working with confocal microscopes and caution should be taken when interpreting the results. All merged images must be taken in the same z-plane. Sometimes plasma membrane proteins may appear to colocalize with markers of intracellular compartments especially when the protein is overexpressed and generates a strong fluorescent signal. In this case a good solution could be to use so called superresolution microscopes, such as STED (stimulated emission depletion), STORM (stochastic optical reconstruction microscopy) or electron microscope. When using combinations of secondary antibodies conjugated to different dyes, a bleed-through caused by overlapping excitation or/and emission light can occur, which in turn can lead to misinterpretation of the data. To minimize this problem we have taken images of different fluorochromes sequentially, where the dye with the longest emissions was scanned first and the dye with the shortest emissions was scanned last. To avoid masking the specimen signal and to avoid the false-positive signal we have quenched, with ammonium chloride, autofluorescent signal arising from aldehyde groups present in the fixative. Finally, all presented

images were taken below saturation level for each fluorochrome and processed with Adobe Photoshop or Image J to adjust brightness and contrast.

Immunological detection

Immunological detection is based on antigen epitope- antibody interaction and it depends on antibody specificity and the state of the protein (native/denaturated) as well as on antibody concentration, incubation time and temperature. The protein of interest is detected due to binding of the antigen specific "primary" antibody, which, in the next step, is detected by the specific "secondary" antibody. To facilitate visualization of the protein, enzymes or fluorescent dyes can be bound to the secondary antibodies (indirect detection), as well as to primary antibodies (direct detection). The direct detection method has a few advantages such as the reduced number of steps in the staining protocol and avoidance of antibody cross-reactivity or non-specificity, but also is less sensitive in terms of signal amplification. By using an indirect detection method, signal amplification can be increased because several secondary antibodies may bind to a single primary molecule. The biggest challenge when working with immuno-based detection methods is to use primary antibodies that are specific for protein of interest (discussed in the below paragraphs).

Detection of the ErbB proteins, their localization and interaction with other proteins was based on an indirect method utilizing fluorescent dyes conjugated to the secondary antibodies. To study the localization of two (or three) proteins in the same cell by confocal microscopy, double (or triple) staining was applied. When combining two or more primary antibodies in one setup, we used antibodies from different species. To make sure that there was no cross-reaction between multiple antibodies, a control tests lacking one of the primary antibody or using the primary antibody raised in different species than the secondary antibody, were always performed at the beginning of each new experiment. Also, when performing double (and triple) staining it is important to use secondary antibodies conjugated to varicolored dyes, which emission lights do not overlap.

Another application of the antibody-based detection method used most frequently in this work is western blot. Western blot is a technique designed to detect proteins present in lysates from different cells and tissues. Proteins in the lysates are denaturated and separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylaminde gel electrophoresis) according to their size. Next, proteins are transferred to a

membrane, typically nitrocellulose or PVDF, where they are detected by specific antibodies. The specificity of the antibodies in recognizing different ErbB proteins was tested in PAE cell lines that express only one member of the ErbB family. To this end, a novel system for gel electrophoresis was applied, Mini-PROTEAN TGX Precast Gels from Bio-Rad™. This innovative method enabled faster protein separation and transfer to a nitrocellulose membrane, which was usually accomplished in less than 1 h. Proteins were detected by the use of secondary antibodies conjugated to horseradish peroxidase (HRP), which produced chemiluminescence that was recorded by camera. Images, which were acquired below saturation level, were processed by Image Lab 4.1 software (Bio-Rad) and subjected to quantification based on pixel intensity. To account for variations between samples, all the values were normalized to the protein- loading control that was run on the same gel in parallel with the protein of interest. In should be mentioned that densitometric analysis can be inaccurate, as it may be affected by the size and intensity of the band and background noise. Therefore, the western blot image that was quantified was always presented along with the quantification.

Studies of protein-protein interaction by IP and Co-IP

When analyzing particular protein-protein interactions it is important to make sure that all non-interacting proteins will be excluded from the sample. Isolation of proteins of interest can be achieved by immunoprecipitation (IP) and co-IP. In papers II and III, to properly precipitate proteins from the cell lysates, antibodies recognizing the protein of interest were pre-bound to magnetic beads coupled with Protein A or Protein G. Next, precipitates were eluted in the SDS-buffer to ensure the proper denaturation of proteins as well as antibodies which will dissociate from the beads and can be analyzed by western blotting. In a control sample, beads without pre-bound antibodies were incubated with the cell lysates, to control for unspecific interaction between beads and proteins present in the cell lysates. When analyzing ubiquitination of ErbB3 (paper II) and ErbB2 (paper III) cells were subjected to hot (96˚C) SDS-based lysis, which caused protein denaturation but did not destroy covalent bonds between ubiquitin molecules and receptor. When analyzing interaction of ErbB3 and epsin 1, co-IP method was used. In this case, cells were handled with precaution and lysis was performed, in a buffer without strong denaturating agents and with addition of phosphates and protease inhibitors to avoid

dissociation of protein complexes. To ensure that active enzymes did not cause denaturation and degradation of proteins, cells and cell lysates were kept on ice and always washed in buffers containing inhibitor cocktails.

Internalization of radioactive transferrin (^{125}I-Tf)

Transferrin is internalized in clathrin-dependent manner only. Internalization of radioactive labeled Tf was used as a quantitative assay to confirm the specificity of siRNA silencing of the major components of the clathrin-mediated endocytosis and chemical inhibition of CIE. Internalization of radioactive labeled Tf constitutes very sensitive and quantitative method of detection of even small amounts of intracellularly localized ligand. However, one needs to remember to handle the radioactive solutions and hazardous waste with precaution.

Immuno-electron microscopy

In paper III we have employed immuno-electron microscopy (immuno-EM) to study antibody- induced internalization of ErbB2 in more detail. Immuno-EM offers several advantages over light-based microscopy, having higher resolution than confocal microscope and allowing electron dense protein coats at the plasma membrane and internal vesicles of multivesicular bodies to be detected without using specific markers. As for other antibody based techniques, immuno-EM is depending on antibody specificity and labeling efficiency. Since the labeling efficiency differs for each antibody and each antigen, and also depends on the size of the colloidal gold used for labeling, a direct comparison of labeling intensities is not possible.

General Discussion

The oncogenic potency of the ErbB2-ErbB3 heterodimer has recently gained an increased interest due to several findings showing that ErbB3, in addition to mediate anti-apoptotic signaling, is responsible for acquired resistance to anti-EGFR and anti-ErbB2 therapies (reviewed in Telesco et al, 2011). Upregulation of ErbB3 is commonly seen in various malignancies, such as colorectal carcinoma, gastric, ovarian, prostate, bladder and breast cancers. In some human breast cancers ErbB3 is upregulated, both at mRNA and protein levels, and this seems to be associated with metastasis, tumor size, and risk of local recurrence (Huotari & Helenius, 2011). Furthermore, Begnami *et al*., found that, in gastric carcinomas, ErbB3 was the most common partner for dimerization, especially with ErbB2, and both receptors were shown to be the significant predictors of poor survival (Begnami et al, 2011). Therefore, studies investigating molecular factors regulating dimerization and downregulation of ErbB2 and ErbB3, which in turn will help to understand mechanisms of drug-resistance and develop new or improve existing targeted therapeutics, are of great importance. Previous studies from our group (Sak et al, 2012) demonstrated that ErbB3 is constitutively internalized through CME, and our current study (paper II) shows that epsin 1 plays a crucial role as cargo-specific adaptor for ErbB3. The study also demonstrated that ErbB3 is constitutively ubiquitinated and that the ubiquitination is enhanced in the presence of ligand, which is in agreement with other studies showing that ubiquitination regulates plasma membrane expression of ErbB3 both in the presence or absence of the ligand (Cao et al, 2007; Diamonti et al, 2002; Huang et al, 2015). Our data show an interaction between ErbB3 and an epsin 1 mutant containing its UIMs, but lacking its clathrin and AP-2 binding regions, furthermore suggests that epsin 1 regulates ErbB3 by recruiting ubiquitinated ErbB3 into coated pits. Such a mechanism has not previously been shown for ErbB3, but is in line with our previous studies on epsin 1 as adaptor in CME of ubiquitinated EGFR and ErbB2 (Bertelsen et al, 2011; Kazazic et al, 2009; Vuong et al, 2013). Our group has ongoing investigations focusing on unraveling the mechanisms for endosomal sorting and degradation of ErbB3. In the future it will be interesting to explore the role of intracellular localized ErbB3, especially with respect to existence and duration of ErbB3-mediated signaling.

ErbB3 has been considered to be kinase-inactive but in line with previous finding (Shi et al, 2010) we report that in fact ErbB3 has a weak kinase activity and is able to form homodimers. However, we also show that co-expression of ErbB3 along with ErbB2, clearly increased ligand-induced activation of ErbB3 and downstream signaling, supporting that ErbB3 heterodimerization is important for efficient signal transduction.

In order to inhibit the activity of ErbB proteins, highly specific and fully inactivating therapeutics have to be used (Tebbutt et al, 2013). One strategy involves the use of mAbs that target ErbB proteins and can prevent ligand binding and/or receptor dimerization and/or decrease surface expression of the targeted receptor. We have previously demonstrated that pertuzumab counteracted the inhibitory effect of ErbB2 on EGF-induced degradation of the EGFR in cells overexpressing ErbB2 (Hughes et al, 2009). Pertuzumab was also reported to inhibit HRG-dependent morphogenesis *in vitro* and to induce tumor regression in ligand-dependent breast cancer xenograft model (Lee-Hoeflich et al, 2008). In paper I we show that upon incubation with pertuzumab HRG-induced phosphorylation of ErbB3 and downstream signaling were reduced, which supports the notion that pertuzumab inhibits ligand-induced ErbB2-ErbB3 heterodimerization. Pertuzumab inhibited ligand-induced downstream signaling even in cell lines expressing low levels of ErbB2. Thus, our findings indicate that the use of pertuzumab can be beneficial also in treatment of cancer patients with ErbB3 positive tumors showing low ErbB2 expression.

The molecular mechanisms of action of therapeutic antibodies are complex and not fully understood. Clinical activity of single antibodies, such as pertuzumab and trastuzumab is limited but when these two agents were combined they showed synergistic anti-tumor effects (Gianni et al, 2012). In preclinical studies single mAbs were reported to induce internalization of ErbB2 (Klapper et al, 2000), but several other studies have demonstrated that a mixture of two or three mAbs is much more effective in downregulation of ErbB2 (Ben-Kasus et al, 2009; Friedman et al, 2005; Pedersen et al, 2015). In line with this, we detected (paper III) efficient downregulation of ErbB2 upon treatment with the mAb mixture but not upon single mAbs. Whether mAbs induce internalization or inhibit recycling of the targeted receptors is still debated. Our data support the notion that incubation with the mAb mixture caused internalization of ErbB2, since treatment with monensin, a recycling inhibitor, resulted in only minimal intracellular localization of ErbB2, while

treatment with mAb mixture had much more pronounced effects. In addition we show that the mAb mixture induced efficient ubiquitination of ErbB2. While ubiquitination of ErbB2 upon Hsp90 inhibitors is reported to be a result of recruitment of Hsp70 along with ubiquitin ligases, a different mechanism seems to be responsible for the antibody-induced ErbB2 ubiquitination since we found no recruitment of Hsp70 to ErbB2 upon incubation with the antibodies. With respect to internalization of EGFR, Cbl-induced ubiquitination was shown to recruit EGFR into CCPs (Bertelsen et al, 2011; Stang et al, 2004). Recruitment of Cbl to phosphorylated Tyr1045 of EGFR can be either direct or indirect, via Grb2 (Huang & Sorkin, 2005; Sorkin & Goh, 2008). In our current study we show that upon incubation with the mAb mixture, ErbB2 becomes efficiently phosphorylated in general and specifically to some degree at Tyr1112 of ErbB2. This site, which corresponds to Tyr1045 in EGFR, has previously been shown to be a binding site for c-Cbl (Klapper et al, 2000). However, since we do not get any inhibitory effect upon knock down of c-Cbl or Cbl-b our results indicate that antibody-induced ubiquitination of ErbB2 is not depending on Cbl. Hence, the ubiquitin ligase machinery accountable for the antibody-induced ubiquitination of ErbB2 remains to be identified.

Ubiquitination can, among others, be a signal for endocytosis. Whether it is a signal for CME or CIE or both is however discussed. For ubiquitinated EGFR both pathways seem to be involved (Madshus & Stang, 2009; Sigismund et al, 2005; Sorkin & Goh, 2008). A previous study from our group did however demonstrate that a chimeric pre-ubiquitinated ErbB2 was internalized in a clathrin-dependent manner (Vuong et al, 2013). In the case of antibody-induced internalization of ErbB2 we found that both CME and CIE are involved. To what extent the internalization depends on ubiquitination is uncertain since we were not able to specifically inhibit ubiquitination without also inhibiting ErbB2 phosphorylation. In a previous study we found that the combination of antibodies forming complexes with EGFR efficiently downregulated the receptor primarily by induction of macropinocytosis (Berger et al, 2012). Our current data can thus support the view that antibody-induced crosslinking of receptors, like ErbB2, at the plasma membrane in itself is sufficient to initiate CIE. The study of Orth *et al.* (Orth et al, 2006) demonstrated that a large portion of activated EGFR can be internalized from the plasma membrane through circular dorsal ruffles. This pathway requires EGFR kinase activity, and it depends on PI3K, actin and dynamin 2. Moreover, they show that this pathway occurs independently

and in parallel to CME, which is in agreement with our observations that both CME and CIE are involved in antibody-induced internalization of ErbB2 and that dynamin 2- and actin-dependent CIE appears to be the major pathway.

Another strategy of targeting ErbB proteins in therapy involves the use of TKIs. Interestingly, we found that upon inhibiting ErbB2 kinase activity, the antibody-induced internalization and degradation of ErbB2 were inhibited. Thus, our results imply that kinase activity is a required step for antibody-induced downregulation of ErbB2. However, we do not know whether ErbB2 kinase activity is needed for recruitment of ubiquitin ligases or for downstream activation of PI3K and accumulation of its major lipid product, PIP_3 leading to e.g. actin rearrangement facilitating CIE uptake of antibody-ErbB2 complexes.

From a therapeutic point of view the different effects of mAbs alone compared to mAbs in combination with TKIs are interesting. One may speculate that on one hand, treatment with mAbs alone will reduce cell surface levels of targeted receptor leading to decreased oncogenic signaling. On the other hand, a TKI induced increase of inactive ErbB2 at the plasma membrane would enhance binding of the therapeutic antibodies and thus induce stronger ADCC. Based on this our current results indicate that TKIs may enhance the therapeutic effects of mAbs (also discussed in Scaltriti et al, 2009) however, further studies on synergistic effect of mAbs and TKIs are required.

Overall, by employing basic science, this work gives insight into the molecular mechanism involved in regulation of ErbB2 and ErbB3, two oncoproteins that are very important targets in rational design of anti-cancer therapeutics.

References

Acconcia F, Sigismund S, Polo S (2009) Ubiquitin in trafficking: the network at work. *Experimental cell research* **315:** 1610-1618

Ahmed S, Bu W, Lee RT, Maurer-Stroh S, Goh WI (2010) F-BAR domain proteins: Families and function. *Commun Integr Biol* **3:** 116-121

Ait-Slimane T, Galmes R, Trugnan G, Maurice M (2009) Basolateral internalization of GPI-anchored proteins occurs via a clathrin-independent flotillin-dependent pathway in polarized hepatic cells. *Molecular biology of the cell* **20:** 3792-3800

Arribas J, Baselga J, Pedersen K, Parra-Palau JL (2011) p95HER2 and breast cancer. *Cancer research* **71:** 1515-1519

Asp N, Pust S, Sandvig K (2014) Flotillin depletion affects ErbB protein levels in different human breast cancer cells. *Biochimica et biophysica acta* **1843:** 1987-1996

Astsaturov I, Cohen RB, Harari PM (2006) EGFR-targeting monoclonal antibodies in head and neck cancer. *Curr Cancer Drug Targets* **6:** 691-710

Aurisicchio L, Marra E, Roscilli G, Mancini R, Ciliberto G (2012) The promise of anti-ErbB3 monoclonals as new cancer therapeutics. *Oncotarget* **3:** 744-758

Austin CD, De Maziere AM, Pisacane PI, van Dijk SM, Eigenbrot C, Sliwkowski MX, Klumperman J, Scheller RH (2004) Endocytosis and sorting of ErbB2 and the site of action of cancer therapeutics trastuzumab and geldanamycin. *Molecular biology of the cell* **15:** 5268-5282

Bache KG, Brech A, Mehlum A, Stenmark H (2003a) Hrs regulates multivesicular body formation via ESCRT recruitment to endosomes. *The Journal of cell biology* **162:** 435-442

Bache KG, Raiborg C, Mehlum A, Stenmark H (2003b) STAM and Hrs are subunits of a multivalent ubiquitin-binding complex on early endosomes. *The Journal of biological chemistry* **278:** 12513-12521

Ball CL, Hunt SP, Robinson MS (1995) Expression and localization of alpha-adaptin isoforms. *Journal of cell science* **108 (Pt 8):** 2865-2875

Barrott JJ, Haystead TA (2013) Hsp90, an unlikely ally in the war on cancer. *The FEBS journal* **280:** 1381-1396

Baselga J, Albanell J, Molina MA, Arribas J (2001) Mechanism of action of trastuzumab and scientific update. *Semin Oncol* **28:** 4-11

Baselga J, Cortes J, Kim SB, Im SA, Hegg R, Im YH, Roman L, Pedrini JL, Pienkowski T, Knott A, Clark E, Benyunes MC, Ross G, Swain SM, Group CS (2012) Pertuzumab plus trastuzumab plus docetaxel for metastatic breast cancer. *N Engl J Med* **366:** 109-119

Baselga J, Swain SM (2009) Novel anticancer targets: revisiting ERBB2 and discovering ERBB3. *Nature reviews Cancer* **9:** 463-475

Baulida J, Carpenter G (1997) Heregulin degradation in the absence of rapid receptor-mediated internalization. *Experimental cell research* **232:** 167-172

Baulida J, Kraus MH, Alimandi M, Di Fiore PP, Carpenter G (1996) All ErbB receptors other than the epidermal growth factor receptor are endocytosis impaired. *The Journal of biological chemistry* **271:** 5251-5257

Begnami MD, Fukuda E, Fregnani JH, Nonogaki S, Montagnini AL, da Costa WL, Jr., Soares FA (2011) Prognostic implications of altered human epidermal growth factor receptors (HERs) in gastric carcinomas: HER2 and HER3 are predictors of poor outcome. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **29:** 3030-3036

Beguinot L, Lyall RM, Willingham MC, Pastan I (1984) Down-regulation of the epidermal growth factor receptor in KB cells is due to receptor internalization and subsequent degradation in lysosomes. *Proceedings of the National Academy of Sciences of the United States of America* **81:** 2384-2388

Behrends C, Harper JW (2011) Constructing and decoding unconventional ubiquitin chains. *Nat Struct Mol Biol* **18:** 520-528

Belleudi F, Marra E, Mazzetta F, Fattore L, Giovagnoli MR, Mancini R, Aurisicchio L, Torrisi MR, Ciliberto G (2012) Monoclonal antibody-induced ErbB3 receptor internalization and degradation inhibits growth and migration of human melanoma cells. *Cell Cycle* **11:** 1455-1467

Ben-Kasus T, Schechter B, Lavi S, Yarden Y, Sela M (2009) Persistent elimination of ErbB-2/HER2-overexpressing tumors using combinations of monoclonal antibodies: relevance of receptor endocytosis. *Proceedings of the National Academy of Sciences of the United States of America* **106:** 3294-3299

Berger C, Madshus IH, Stang E (2012) Cetuximab in combination with anti-human IgG antibodies efficiently down-regulates the EGF receptor by macropinocytosis. *Experimental cell research* **318:** 2578-2591

Bertelsen V, Sak MM, Breen K, Rodland MS, Johannessen LE, Traub LM, Stang E, Madshus IH (2011) A chimeric pre-ubiquitinated EGF receptor is constitutively endocytosed in a clathrin-dependent, but kinase-independent manner. *Traffic* **12:** 507-520

Bertelsen V, Stang E (2014) The Mysterious Ways of ErbB2/HER2 Trafficking. *Membranes (Basel)* **4:** 424-446

Bonifacino JS, Hurley JH (2008) Retromer. *Current opinion in cell biology* **20:** 427-436

Bos M, Mendelsohn J, Kim YM, Albanell J, Fry DW, Baselga J (1997) PD153035, a tyrosine kinase inhibitor, prevents epidermal growth factor receptor activation and inhibits growth of cancer cells in a receptor number-dependent manner. *Clinical cancer research : an official journal of the American Association for Cancer Research* **3:** 2099-2106

Boucrot E, Pick A, Camdere G, Liska N, Evergren E, McMahon HT, Kozlov MM (2012) Membrane fission is promoted by insertion of amphipathic helices and is restricted by crescent BAR domains. *Cell* **149:** 124-136

Briand N, Dugail I, Le Lay S (2011) Cavin proteins: New players in the caveolae field. *Biochimie* **93:** 71-77

Buonanno A, Fischbach GD (2001) Neuregulin and ErbB receptor signaling pathways in the nervous system. *Current opinion in neurobiology* **11:** 287-296

Burke P, Schooler K, Wiley HS (2001) Regulation of epidermal growth factor receptor signaling by endocytosis and intracellular trafficking. *Molecular biology of the cell* **12:** 1897-1910

Cao Z, Wu X, Yen L, Sweeney C, Carraway KL, 3rd (2007) Neuregulin-induced ErbB3 downregulation is mediated by a protein stability cascade involving the E3 ubiquitin ligase Nrdp1. *Mol Cell Biol* **27:** 2180-2188

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

Carpentier JL, Gorden P, Anderson RG, Goldstein JL, Brown MS, Cohen S, Orci L (1982) Co-localization of 125I-epidermal growth factor and ferritin-low density lipoprotein in coated pits: a quantitative electron microscopic study in normal and mutant human fibroblasts. *The Journal of cell biology* **95:** 73-77

Carraway KL, 3rd (2010) E3 ubiquitin ligases in ErbB receptor quantity control. *Semin Cell Dev Biol* **21:** 936-943

Carter P, Presta L, Gorman CM, Ridgway JB, Henner D, Wong WL, Rowland AM, Kotts C, Carver ME, Shepard HM (1992) Humanization of an anti-p185HER2 antibody for human cancer therapy. *Proceedings of the National Academy of Sciences of the United States of America* **89:** 4285-4289

Carter RE, Sorkin A (1998) Endocytosis of functional epidermal growth factor receptor-green fluorescent protein chimera. *The Journal of biological chemistry* **273:** 35000-35007

Cavazzoni A, Alfieri RR, Cretella D, Saccani F, Ampollini L, Galetti M, Quaini F, Graiani G, Madeddu D, Mozzoni P, Galvani E, La Monica S, Bonelli M, Fumarola C, Mutti A, Carbognani P, Tiseo M, Barocelli E, Petronini PG, Ardizzoni A (2012) Combined use of anti-ErbB monoclonal antibodies and erlotinib enhances antibody-dependent cellular cytotoxicity of wild-type erlotinib-sensitive NSCLC cell lines. *Molecular cancer* **11:** 91

Chen C, Zhuang XW (2008) Epsin 1 is a cargo-specific adaptor for the clathrin-mediated endocytosis of the influenza virus. *Proceedings of the National Academy of Sciences of the United States of America* **105:** 11790-11795

Chen CY, Brodsky FM (2005) Huntingtin-interacting protein 1 (Hip1) and Hip1-related protein (Hip1R) bind the conserved sequence of clathrin light chains and thereby influence clathrin assembly in vitro and actin distribution in vivo. *The Journal of biological chemistry* **280:** 6109-6117

Chen H, Slepnev VI, Di Fiore PP, De Camilli P (1999) The interaction of epsin and Eps15 with the clathrin adaptor AP-2 is inhibited by mitotic phosphorylation and enhanced by stimulation-dependent dephosphorylation in nerve terminals. *The Journal of biological chemistry* **274:** 3257-3260

Chinkers M, McKanna JA, Cohen S (1979) Rapid induction of morphological changes in human carcinoma cells A-431 by epidermal growth factors. *The Journal of cell biology* **83:** 260-265

Christianson TA, Doherty JK, Lin YJ, Ramsey EE, Holmes R, Keenan EJ, Clinton GM (1998) NH2-terminally truncated HER-2/neu protein: relationship with shedding of the extracellular domain and with prognostic factors in breast cancer. *Cancer research* **58:** 5123-5129

Ciardiello F, Tortora G (2008) EGFR antagonists in cancer treatment. *N Engl J Med* **358:** 1160-1174

Citri A, Skaria KB, Yarden Y (2003) The deaf and the dumb: the biology of ErbB-2 and ErbB-3. *Experimental cell research* **284:** 54-65

Clague MJ, Urbe S (2010) Ubiquitin: same molecule, different degradation pathways. *Cell* **143:** 682-685

Collins BM, McCoy AJ, Kent HM, Evans PR, Owen DJ (2002) Molecular architecture and functional model of the endocytic AP2 complex. *Cell* **109:** 523-535

Conner SD, Schmid SL (2003) Regulated portals of entry into the cell. *Nature* **422:** 37-44

Cope GA, Suh GS, Aravind L, Schwarz SE, Zipursky SL, Koonin EV, Deshaies RJ (2002) Role of predicted metalloprotease motif of Jab1/Csn5 in cleavage of Nedd8 from Cul1. *Science* **298:** 608-611

Cortese K, Howes MT, Lundmark R, Tagliatti E, Bagnato P, Petrelli A, Bono M, McMahon HT, Parton RG, Tacchetti C (2013) The HSP90 inhibitor geldanamycin perturbs endosomal structure and drives recycling ErbB2 and transferrin to modified MVBs/lysosomal compartments. *Molecular biology of the cell* **24:** 129-144

Cullen PJ (2008) Endosomal sorting and signalling: an emerging role for sorting nexins. *Nature reviews Molecular cell biology* **9:** 574-582

Cureton DK, Massol RH, Saffarian S, Kirchhausen TL, Whelan SP (2009) Vesicular stomatitis virus enters cells through vesicles incompletely coated with clathrin that depend upon actin for internalization. *PLoS pathogens* **5:** e1000394

Dahlberg PS, Jacobson BA, Dahal G, Fink JM, Kratzke RA, Maddaus MA, Ferrin LJ (2004) ERBB2 amplifications in esophageal adenocarcinoma. *The Annals of thoracic surgery* **78:** 1790-1800

Dankort D, Jeyabalan N, Jones N, Dumont DJ, Muller WJ (2001) Multiple ErbB-2/Neu Phosphorylation Sites Mediate Transformation through Distinct Effector Proteins. *The Journal of biological chemistry* **276:** 38921-38928

De Camilli P, Chen H, Hyman J, Panepucci E, Bateman A, Brunger AT (2002) The ENTH domain. *FEBS letters* **513:** 11-18

Deshaies RJ, Joazeiro CA (2009) RING domain E3 ubiquitin ligases. *Annu Rev Biochem* **78:** 399-434

Diamonti AJ, Guy PM, Ivanof C, Wong K, Sweeney C, Carraway KL, 3rd (2002) An RBCC protein implicated in maintenance of steady-state neuregulin receptor levels. *Proceedings of the National Academy of Sciences of the United States of America* **99:** 2866-2871

Duncan LM, Piper S, Dodd RB, Saville MK, Sanderson CM, Luzio JP, Lehner PJ (2006) Lysine-63-linked ubiquitination is required for endolysosomal degradation of class I molecules. *The EMBO journal* **25:** 1635-1645

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

Eckstein N, Roper L, Haas B, Potthast H, Hermes U, Unkrig C, Naumann-Winter F, Enzmann H (2014) Clinical pharmacology of tyrosine kinase inhibitors becoming generic drugs: the regulatory perspective. *J Exp Clin Cancer Res* **33:** 15

Elenius K, Corfas G, Paul S, Choi CJ, Rio C, Plowman GD, Klagsbrun M (1997) A novel juxtamembrane domain isoform of HER4/ErbB4. Isoform-specific tissue distribution and differential processing in response to phorbol ester. *The Journal of biological chemistry* **272:** 26761-26768

Engelman JA, Zejnullahu K, Mitsudomi T, Song Y, Hyland C, Park JO, Lindeman N, Gale CM, Zhao X, Christensen J, Kosaka T, Holmes AJ, Rogers AM, Cappuzzo F, Mok T, Lee C, Johnson BE, Cantley LC, Janne PA (2007) MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. *Science* **316:** 1039-1043

Engqvist-Goldstein AE, Warren RA, Kessels MM, Keen JH, Heuser J, Drubin DG (2001) The actin-binding protein Hip1R associates with clathrin during early stages of endocytosis and promotes clathrin assembly in vitro. *The Journal of cell biology* **154:** 1209-1223

Ferguson ML, Prasad K, Boukari H, Sackett DL, Krueger S, Lafer EM, Nossal R (2008) Clathrin triskelia show evidence of molecular flexibility. *Biophys J* **95:** 1945-1955

Fiaturi N, Castellot JJ, Jr., Nielsen HC (2014) Neuregulin-ErbB4 signaling in the developing lung alveolus: a brief review. *Journal of cell communication and signaling* **8:** 105-111

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391:** 806-811

Ford MG, Mills IG, Peter BJ, Vallis Y, Praefcke GJ, Evans PR, McMahon HT (2002) Curvature of clathrin-coated pits driven by epsin. *Nature* **419:** 361-366

Franklin MC, Carey KD, Vajdos FF, Leahy DJ, de Vos AM, Sliwkowski MX (2004) Insights into ErbB signaling from the structure of the ErbB2-pertuzumab complex. *Cancer Cell* **5:** 317-328

Frick M, Bright NA, Riento K, Bray A, Merrified C, Nichols BJ (2007) Coassembly of flotillins induces formation of membrane microdomains, membrane curvature, and vesicle budding. *Curr Biol* **17:** 1151-1156

Friedlander E, Barok M, Szollosi J, Vereb G (2008) ErbB-directed immunotherapy: antibodies in current practice and promising new agents. *Immunol Lett* **116:** 126-140

Friedman LM, Rinon A, Schechter B, Lyass L, Lavi S, Bacus SS, Sela M, Yarden Y (2005) Synergistic down-regulation of receptor tyrosine kinases by combinations of mAbs: implications for cancer immunotherapy. *Proceedings of the National Academy of Sciences of the United States of America* **102:** 1915-1920

Gallardo A, Lerma E, Escuin D, Tibau A, Munoz J, Ojeda B, Barnadas A, Adrover E, Sanchez-Tejada L, Giner D, Ortiz-Martinez F, Peiro G (2012) Increased signalling of EGFR and IGF1R, and deregulation of PTEN/PI3K/Akt pathway are related with trastuzumab resistance in HER2 breast carcinomas. *British journal of cancer* **106:** 1367-1373

Gan HK, Walker F, Burgess AW, Rigopoulos A, Scott AM, Johns TG (2007) The epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor AG1478 increases the formation of inactive untethered EGFR dimers. Implications for combination therapy with monoclonal antibody 806. *The Journal of biological chemistry* **282:** 2840-2850

Garrett TP, McKern NM, Lou M, Elleman TC, Adams TE, Lovrecz GO, Kofler M, Jorissen RN, Nice EC, Burgess AW, Ward CW (2003) The crystal structure of a truncated ErbB2 ectodomain reveals an active conformation, poised to interact with other ErbB receptors. *Molecular cell* **11:** 495-505

Garrett TP, McKern NM, Lou M, Elleman TC, Adams TE, Lovrecz GO, Zhu HJ, Walker F, Frenkel MJ, Hoyne PA, Jorissen RN, Nice EC, Burgess AW, Ward CW (2002) Crystal structure of a truncated epidermal growth factor receptor extracellular domain bound to transforming growth factor alpha. *Cell* **110:** 763-773

Gerbin CS, Landgraf R (2010) Geldanamycin selectively targets the nascent form of ERBB3 for degradation. *Cell Stress Chaperones* **15:** 529-544

Ghosh R, Narasanna A, Wang SE, Liu S, Chakrabarty A, Balko JM, Gonzalez-Angulo AM, Mills GB, Penuel E, Winslow J, Sperinde J, Dua R, Pidaparthi S, Mukherjee A, Leitzel K, Kostler WJ, Lipton A, Bates M, Arteaga CL (2011) Trastuzumab has preferential activity against breast cancers driven by HER2 homodimers. *Cancer research* **71:** 1871-1882

Gianni L, Pienkowski T, Im YH, Roman L, Tseng LM, Liu MC, Lluch A, Staroslawska E, de la Haba-Rodriguez J, Im SA, Pedrini JL, Poirier B, Morandi P, Semiglazov V, Srimuninnimit V, Bianchi G, Szado T, Ratnayake J, Ross G, Valagussa P (2012) Efficacy and safety of neoadjuvant pertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): a randomised multicentre, open-label, phase 2 trial. *The Lancet Oncology* **13:** 25-32

Glebov OO, Bright NA, Nichols BJ (2006) Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells. *Nature cell biology* **8:** 46-54

Gong Q, Huntsman C, Ma D (2008) Clathrin-independent internalization and recycling. *J Cell Mol Med* **12:** 126-144

Grandal MV, Zandi R, Pedersen MW, Willumsen BM, van Deurs B, Poulsen HS (2007) EGFRvIII escapes down-regulation due to impaired internalization and sorting to lysosomes. *Carcinogenesis* **28:** 1408-1417

Grant BD, Donaldson JG (2009) Pathways and mechanisms of endocytic recycling. *Nature reviews Molecular cell biology* **10:** 597-608

Grosshans BL, Ortiz D, Novick P (2006) Rabs and their effectors: achieving specificity in membrane traffic. *Proceedings of the National Academy of Sciences of the United States of America* **103:** 11821-11827

Gschwind A, Fischer OM, Ullrich A (2004) The discovery of receptor tyrosine kinases: targets for cancer therapy. *Nature reviews Cancer* **4:** 361-370

Haglund K, Di Fiore PP, Dikic I (2003) Distinct monoubiquitin signals in receptor endocytosis. *Trends in biochemical sciences* **28:** 598-603

Haglund K, Dikic I (2005) Ubiquitylation and cell signaling. *The EMBO journal* **24:** 3353-3359

Han W, Lo HW (2012) Landscape of EGFR signaling network in human cancers: biology and therapeutic response in relation to receptor subcellular locations. *Cancer Lett* **318:** 124-134

Hanover JA, Willingham MC, Pastan I (1984) Kinetics of transit of transferrin and epidermal growth factor through clathrin-coated membranes. *Cell* **39:** 283-293

Hansen CG, Nichols BJ (2010) Exploring the caves: cavins, caveolins and caveolae. *Trends in cell biology* **20:** 177-186

Harris R (2003) EGF receptor ligands. *Experimental cell research* **284:** 2-13

Haslekas C, Breen K, Pedersen KW, Johannessen LE, Stang E, Madshus IH (2005) The inhibitory effect of ErbB2 on epidermal growth factor-induced formation of clathrin-coated pits correlates with retention of epidermal growth factor receptor-ErbB2 oligomeric complexes at the plasma membrane. *Molecular biology of the cell* **16:** 5832-5842

Hellyer NJ, Cheng K, Koland JG (1998) ErbB3 (HER3) interaction with the p85 regulatory subunit of phosphoinositide 3-kinase. *The Biochemical journal* **333 (Pt 3):** 757-763

Henne WM, Boucrot E, Meinecke M, Evergren E, Vallis Y, Mittal R, McMahon HT (2010) FCHo proteins are nucleators of clathrin-mediated endocytosis. *Science* **328:** 1281-1284

Henne WM, Buchkovich NJ, Emr SD (2011) The ESCRT pathway. *Dev Cell* **21:** 77-91

Henriksen L, Grandal MV, Knudsen SL, van Deurs B, Grovdal LM (2013) Internalization mechanisms of the epidermal growth factor receptor after activation with different ligands. *PLoS One* **8:** e58148

Herbst RS, Fukuoka M, Baselga J (2004) Gefitinib--a novel targeted approach to treating cancer. *Nature reviews Cancer* **4:** 956-965

Hershko A, Ciechanover A (1998) The ubiquitin system. *Annu Rev Biochem* **67:** 425-479

Hicke L, Dunn R (2003) Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Annu Rev Cell Dev Biol* **19:** 141-172

Hicke L, Schubert HL, Hill CP (2005) Ubiquitin-binding domains. *Nature reviews Molecular cell biology* **6:** 610-621

Higgs H (2001) Branching out: cortactin stabilizes actin networks generated by the Arp2/3 complex. *Trends in biochemical sciences* **26:** 219

Hinrichsen L, Harborth J, Andrees L, Weber K, Ungewickell EJ (2003) Effect of clathrin heavy chain- and alpha-adaptin-specific small inhibitory RNAs on endocytic accessory proteins and receptor trafficking in HeLa cells. *The Journal of biological chemistry* **278:** 45160-45170

Hoeller D, Hecker CM, Wagner S, Rogov V, Dotsch V, Dikic I (2007) E3-independent monoubiquitination of ubiquitin-binding proteins. *Molecular cell* **26:** 891-898

Hofmann K, Falquet L (2001) A ubiquitin-interacting motif conserved in components of the proteasomal and lysosomal protein degradation systems. *Trends in biochemical sciences* **26:** 347-350

Hojjat-Farsangi M (2014) Small-molecule inhibitors of the receptor tyrosine kinases: promising tools for targeted cancer therapies. *Int J Mol Sci* **15:** 13768-13801

Holbro T, Beerli RR, Maurer F, Koziczak M, Barbas CF, 3rd, Hynes NE (2003) The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation. *Proceedings of the National Academy of Sciences of the United States of America* **100:** 8933-8938

Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG (2013) Cancer drug resistance: an evolving paradigm. *Nature reviews Cancer* **13:** 714-726

Hommelgaard AM, Lerdrup M, van Deurs B (2004) Association with membrane protrusions makes ErbB2 an internalization-resistant receptor. *Molecular biology of the cell* **15:** 1557-1567

Howes MT, Mayor S, Parton RG (2010) Molecules, mechanisms, and cellular roles of clathrin-independent endocytosis. *Current opinion in cell biology* **22:** 519-527

Huang F, Khvorova A, Marshall W, Sorkin A (2004) Analysis of clathrin-mediated endocytosis of epidermal growth factor receptor by RNA interference. *The Journal of biological chemistry* **279:** 16657-16661

Huang F, Kirkpatrick D, Jiang X, Gygi S, Sorkin A (2006) Differential regulation of EGF receptor internalization and degradation by multiubiquitination within the kinase domain. *Molecular cell* **21:** 737-748

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

Huang S, Li C, Armstrong EA, Peet CR, Saker J, Amler LC, Sliwkowski MX, Harari PM (2013) Dual targeting of EGFR and HER3 with MEHD7945A overcomes acquired resistance to EGFR inhibitors and radiation. *Cancer research* **73:** 824-833

Huang Z, Choi BK, Mujoo K, Fan X, Fa M, Mukherjee S, Owiti N, Zhang N, An Z (2015) The E3 ubiquitin ligase NEDD4 negatively regulates HER3/ErbB3 level and signaling. *Oncogene* **34:** 1105-1115

Hughes JB, Berger C, Rodland MS, Hasmann M, Stang E, Madshus IH (2009) Pertuzumab increases epidermal growth factor receptor down-regulation by counteracting epidermal growth factor receptor-ErbB2 heterodimerization. *Mol Cancer Ther* **8:** 1885-1892

Hughes JB, Rodland MS, Hasmann M, Madshus IH, Stang E (2012) Pertuzumab Increases 17-AAG-Induced Degradation of ErbB2, and This Effect Is Further Increased by Combining Pertuzumab with Trastuzumab. *Pharmaceuticals (Basel)* **5:** 674-689

Huotari J, Helenius A (2011) Endosome maturation. *The EMBO journal* **30:** 3481-3500

Hupalowska A, Miaczynska M (2012) The new faces of endocytosis in signaling. *Traffic* **13:** 9-18

Hurley JH, Hanson PI (2010) Membrane budding and scission by the ESCRT machinery: it's all in the neck. *Nature reviews Molecular cell biology* **11:** 556-566

Hurley JH, Wendland B (2002) Endocytosis: driving membranes around the bend. *Cell* **111:** 143-146

Hyatt DC, Ceresa BP (2008) Cellular localization of the activated EGFR determines its effect on cell growth in MDA-MB-468 cells. *Experimental cell research* **314:** 3415-3425

Hynes NE, Lane HA (2005) ERBB receptors and cancer: the complexity of targeted inhibitors. *Nature reviews Cancer* **5:** 341-354

Hynes NE, MacDonald G (2009) ErbB receptors and signaling pathways in cancer. *Current opinion in cell biology* **21:** 177-184

Itoh T, Koshiba S, Kigawa T, Kikuchi A, Yokoyama S, Takenawa T (2001) Role of the ENTH domain in phosphatidylinositol-4,5-bisphosphate binding and endocytosis. *Science* **291:** 1047-1051

Jaramillo ML, Leon Z, Grothe S, Paul-Roc B, Abulrob A, O'Connor McCourt M (2006) Effect of the anti-receptor ligand-blocking 225 monoclonal antibody on EGF receptor endocytosis and sorting. *Experimental cell research* **312:** 2778-2790

Jathal MK, Chen L, Mudryj M, Ghosh PM (2011) Targeting ErbB3: the New RTK(id) on the Prostate Cancer Block. *Immunol Endocr Metab Agents Med Chem* **11:** 131-149

Jhaveri K, Taldone T, Modi S, Chiosis G (2012) Advances in the clinical development of heat shock protein 90 (Hsp90) inhibitors in cancers. *Biochimica et biophysica acta* **1823:** 742-755

Johannessen LE, Pedersen NM, Pedersen KW, Madshus IH, Stang E (2006) Activation of the epidermal growth factor (EGF) receptor induces formation of EGF receptor- and Grb2-containing clathrin-coated pits. *Mol Cell Biol* **26:** 389-401

Jovic M, Sharma M, Rahajeng J, Caplan S (2010) The early endosome: a busy sorting station for proteins at the crossroads. *Histol Histopathol* **25:** 99-112

Kani K, Park E, Landgraf R (2005) The extracellular domains of ErbB3 retain high ligand binding affinity at endosome pH and in the locked conformation. *Biochemistry* **44:** 15842-15857

Kazazic M, Bertelsen V, Pedersen KW, Vuong TT, Grandal MV, Rodland MS, Traub LM, Stang E, Madshus IH (2009) Epsin 1 is involved in recruitment of ubiquitinated EGF receptors into clathrin-coated pits. *Traffic* **10:** 235-245

Kazazic M, Roepstorff K, Johannessen LE, Pedersen NM, van Deurs B, Stang E, Madshus IH (2006) EGF-induced activation of the EGF receptor does not trigger mobilization of caveolae. *Traffic* **7:** 1518-1527

Kerr MC, Teasdale RD (2009) Defining macropinocytosis. *Traffic* **10:** 364-371

Kimura H, Sakai K, Arao T, Shimoyama T, Tamura T, Nishio K (2007) Antibody-dependent cellular cytotoxicity of cetuximab against tumor cells with wild-type or mutant epidermal growth factor receptor. *Cancer Sci* **98:** 1275-1280

Kirchhausen T, Owen D, Harrison SC (2014) Molecular structure, function, and dynamics of clathrin-mediated membrane traffic. *Cold Spring Harbor perspectives in biology* **6:** a016725

Kirkham M, Parton RG (2005) Clathrin-independent endocytosis: new insights into caveolae and non-caveolar lipid raft carriers. *Biochimica et biophysica acta* **1746:** 349-363

Klapper LN, Vaisman N, Hurwitz E, Pinkas-Kramarski R, Yarden Y, Sela M (1997) A subclass of tumor-inhibitory monoclonal antibodies to ErbB-2/HER2 blocks crosstalk with growth factor receptors. *Oncogene* **14:** 2099-2109

Klapper LN, Waterman H, Sela M, Yarden Y (2000) Tumor-inhibitory antibodies to HER-2/ErbB-2 may act by recruiting c-Cbl and enhancing ubiquitination of HER-2. *Cancer research* **60:** 3384-3388

Kleyman TR, Cragoe EJ, Jr. (1988) Amiloride and its analogs as tools in the study of ion transport. *J Membr Biol* **105:** 1-21

Kolch W, Pitt A (2010) Functional proteomics to dissect tyrosine kinase signalling pathways in cancer. *Nature reviews Cancer* **10:** 618-629

Komander D (2009) The emerging complexity of protein ubiquitination. *Biochem Soc Trans* **37:** 937-953

Komander D, Clague MJ, Urbe S (2009) Breaking the chains: structure and function of the deubiquitinases. *Nature reviews Molecular cell biology* **10:** 550-563

Komander D, Rape M (2012) The ubiquitin code. *Annu Rev Biochem* **81:** 203-229

Koutras AK, Fountzilas G, Kalogeras KT, Starakis I, Iconomou G, Kalofonos HP (2010) The upgraded role of HER3 and HER4 receptors in breast cancer. *Crit Rev Oncol Hematol* **74:** 73-78

Kraus MH, Issing W, Miki T, Popescu NC, Aaronson SA (1989) Isolation and characterization of ERBB3, a third member of the ERBB/epidermal growth factor receptor family: evidence for overexpression in a subset of human mammary tumors. *Proceedings of the National Academy of Sciences of the United States of America* **86:** 9193-9197

Lahiry P, Torkamani A, Schork NJ, Hegele RA (2010) Kinase mutations in human disease: interpreting genotype-phenotype relationships. *Nature reviews Genetics* **11:** 60-74

Lamaze C, Dujeancourt A, Baba T, Lo CG, Benmerah A, Dautry-Varsat A (2001) Interleukin 2 receptors and detergent-resistant membrane domains define a clathrin-independent endocytic pathway. *Molecular cell* **7:** 661-671

Lambert JM, Chari RV (2014) Ado-trastuzumab Emtansine (T-DM1): an antibody-drug conjugate (ADC) for HER2-positive breast cancer. *Journal of medicinal chemistry* **57:** 6949-6964

Lee-Hoeflich ST, Crocker L, Yao E, Pham T, Munroe X, Hoeflich KP, Sliwkowski MX, Stern HM (2008) A central role for HER3 in HER2-amplified breast cancer: implications for targeted therapy. *Cancer research* **68:** 5878-5887

Lee H, Akita RW, Sliwkowski MX, Maihle NJ (2001) A naturally occurring secreted human ErbB3 receptor isoform inhibits heregulin-stimulated activation of ErbB2, ErbB3, and ErbB4. *Cancer research* **61:** 4467-4473

Legendre-Guillemin V, Metzler M, Lemaire JF, Philie J, Gan L, Hayden MR, McPherson PS (2005) Huntingtin interacting protein 1 (HIP1) regulates clathrin assembly through direct binding to the regulatory region of the clathrin light chain. *The Journal of biological chemistry* **280:** 6101-6108

Legendre-Guillemin V, Wasiak S, Hussain NK, Angers A, McPherson PS (2004) ENTH/ANTH proteins and clathrin-mediated membrane budding. *Journal of cell science* **117:** 9-18

Lemmon MA, Schlessinger J, Ferguson KM (2014) The EGFR family: not so prototypical receptor tyrosine kinases. *Cold Spring Harbor perspectives in biology* **6:** a020768

Lemoine NR, Barnes DM, Hollywood DP, Hughes CM, Smith P, Dublin E, Prigent SA, Gullick WJ, Hurst HC (1992) Expression of the ERBB3 gene product in breast cancer. *British journal of cancer* **66:** 1116-1121

Lerdrup M, Bruun S, Grandal MV, Roepstorff K, Kristensen MM, Hommelgaard AM, van Deurs B (2007) Endocytic down-regulation of ErbB2 is stimulated by cleavage of its C-terminus. *Molecular biology of the cell* **18:** 3656-3666

Lerdrup M, Hommelgaard AM, Grandal M, van Deurs B (2006) Geldanamycin stimulates internalization of ErbB2 in a proteasome-dependent way. *Journal of cell science* **119:** 85-95

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

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

Lewis Phillips GD, Li G, Dugger DL, Crocker LM, Parsons KL, Mai E, Blattler WA, Lambert JM, Chari RV, Lutz RJ, Wong WL, Jacobson FS, Koeppen H, Schwall RH, Kenkare-Mitra SR, Spencer SD, Sliwkowski MX (2008) Targeting HER2-positive breast cancer with trastuzumab-DM1, an antibody-cytotoxic drug conjugate. *Cancer research* **68:** 9280-9290

Li S, Schmitz KR, Jeffrey PD, Wiltzius JJ, Kussie P, Ferguson KM (2005) Structural basis for inhibition of the epidermal growth factor receptor by cetuximab. *Cancer Cell* **7:** 301-311

Lim JP, Gleeson PA (2011) Macropinocytosis: an endocytic pathway for internalising large gulps. *Immunology and cell biology* **89:** 836-843

Liu B, Fan Z, Edgerton SM, Yang X, Lind SE, Thor AD (2011) Potent anti-proliferative effects of metformin on trastuzumab-resistant breast cancer cells via inhibition of erbB2/IGF-1 receptor interactions. *Cell Cycle* **10:** 2959-2966

Liu SH, Wong ML, Craik CS, Brodsky FM (1995) Regulation of clathrin assembly and trimerization defined using recombinant triskelion hubs. *Cell* **83:** 257-267

Longva KE, Blystad FD, Stang E, Larsen AM, Johannessen LE, Madshus IH (2002) Ubiquitination and proteasomal activity is required for transport of the EGF receptor to inner membranes of multivesicular bodies. *The Journal of cell biology* **156:** 843-854

Longva KE, Pedersen NM, Haslekas C, Stang E, Madshus IH (2005) Herceptin-induced inhibition of ErbB2 signaling involves reduced phosphorylation of Akt but not endocytic down-regulation of ErbB2. *Int J Cancer* **116:** 359-367

Lund KA, Opresko LK, Starbuck C, Walsh BJ, Wiley HS (1990) Quantitative analysis of the endocytic system involved in hormone-induced receptor internalization. *The Journal of biological chemistry* **265:** 15713-15723

Madshus IH, Stang E (2009) Internalization and intracellular sorting of the EGF receptor: a model for understanding the mechanisms of receptor trafficking. *Journal of cell science* **122:** 3433-3439

Malassagne B, Taboit F, Conti F, Batteux F, Atia N, Chereau C, Conjeaud H, Theron MC, Attal J, Braet F, Houdebine LM, Calmus Y, Houssin D, Weill B (1998) A newly established porcine aortic endothelial cell line: characterization and application to the study of human-to-swine graft rejection. *Experimental cell research* **238:** 90-100

Maly JJ, Macrae ER (2014) Pertuzumab in Combination with Trastuzumab and Chemotherapy in the Treatment of HER2-Positive Metastatic Breast Cancer: Safety, Efficacy, and Progression Free Survival. *Breast cancer : basic and clinical research* **8:** 81-88

Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S (2002) The protein kinase complement of the human genome. *Science* **298:** 1912-1934

Marone R, Hess D, Dankort D, Muller WJ, Hynes NE, Badache A (2004) Memo mediates ErbB2-driven cell motility. *Nature cell biology* **6:** 515-522

Matsui W, Kirchhausen T (1990) Stabilization of clathrin coats by the core of the clathrin-associated protein complex AP-2. *Biochemistry* **29:** 10791-10798

Mayor S, Pagano RE (2007) Pathways of clathrin-independent endocytosis. *Nature reviews Molecular cell biology* **8:** 603-612

Mayor S, Parton RG, Donaldson JG (2014) Clathrin-independent pathways of endocytosis. *Cold Spring Harbor perspectives in biology* **6**

Maytal-Kivity V, Reis N, Hofmann K, Glickman MH (2002) MPN+, a putative catalytic motif found in a subset of MPN domain proteins from eukaryotes and prokaryotes, is critical for Rpn11 function. *BMC Biochem* **3:** 28

McCullough J, Clague MJ, Urbe S (2004) AMSH is an endosome-associated ubiquitin isopeptidase. *The Journal of cell biology* **166:** 487-492

McDonagh CF, Huhalov A, Harms BD, Adams S, Paragas V, Oyama S, Zhang B, Luus L, Overland R, Nguyen S, Gu J, Kohli N, Wallace M, Feldhaus MJ, Kudla AJ, Schoeberl B, Nielsen UB (2012) Antitumor activity of a novel bispecific antibody that targets the ErbB2/ErbB3 oncogenic unit and inhibits heregulin-induced activation of ErbB3. *Mol Cancer Ther* **11:** 582-593

McMahon HT, Boucrot E (2011) Molecular mechanism and physiological functions of clathrin-mediated endocytosis. *Nature reviews Molecular cell biology* **12:** 517-533

Meijer IM, van Leeuwen JE (2011) ERBB2 is a target for USP8-mediated deubiquitination. *Cell Signal* **23:** 458-467

Mercer J, Greber UF (2013) Virus interactions with endocytic pathways in macrophages and dendritic cells. *Trends in microbiology* **21:** 380-388

Messa M, Fernandez-Busnadiego R, Sun EW, Chen H, Czapla H, Wrasman K, Wu Y, Ko G, Ross T, Wendland B, De Camilli P (2014) Epsin deficiency impairs endocytosis by stalling the actin-dependent invagination of endocytic clathrin-coated pits. *eLife* **3:** e03311

Metzger MB, Hristova VA, Weissman AM (2012) HECT and RING finger families of E3 ubiquitin ligases at a glance. *Journal of cell science* **125:** 531-537

Miaczynska M, Christoforidis S, Giner A, Shevchenko A, Uttenweiler-Joseph S, Habermann B, Wilm M, Parton RG, Zerial M (2004) APPL proteins link Rab5 to nuclear signal transduction via an endosomal compartment. *Cell* **116:** 445-456

Mills GB, Yarden Y (2010) The rebirth of a phoenix: ovarian cancers are addicted to ErbB-3. *Cancer Cell* **17:** 217-218

Mimnaugh EG, Chavany C, Neckers L (1996) Polyubiquitination and proteasomal degradation of the p185c-erbB-2 receptor protein-tyrosine kinase induced by geldanamycin. *The Journal of biological chemistry* **271:** 22796-22801

Minna JD, Dowell J (2005) Erlotinib hydrochloride. *Nature reviews Drug discovery* **Suppl:** S14-15

Moasser MM, Krop IE (2015) The Evolving Landscape of HER2 Targeting in Breast Cancer. *JAMA oncology*

Molina MA, Codony-Servat J, Albanell J, Rojo F, Arribas J, Baselga J (2001) Trastuzumab (herceptin), a humanized anti-Her2 receptor monoclonal antibody, inhibits basal and activated Her2 ectodomain cleavage in breast cancer cells. *Cancer research* **61:** 4744-4749

Mollenhauer HH, Morre DJ, Rowe LD (1990) Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity. *Biochimica et biophysica acta* **1031:** 225-246

Mortensen K, Larsson LI (2003) Effects of cytochalasin D on the actin cytoskeleton: association of neoformed actin aggregates with proteins involved in signaling and endocytosis. *Cell Mol Life Sci* **60:** 1007-1012

Motley A, Bright NA, Seaman MN, Robinson MS (2003) Clathrin-mediated endocytosis in AP-2-depleted cells. *The Journal of cell biology* **162:** 909-918

Mukhopadhyay P, Chakraborty S, Ponnusamy MP, Lakshmanan I, Jain M, Batra SK (2011) Mucins in the pathogenesis of breast cancer: implications in diagnosis, prognosis and therapy. *Biochimica et biophysica acta* **1815:** 224-240

Munster PN, Marchion DC, Basso AD, Rosen N (2002) Degradation of HER2 by ansamycins induces growth arrest and apoptosis in cells with HER2 overexpression via a HER3, phosphatidylinositol 3'-kinase-AKT-dependent pathway. *Cancer research* **62:** 3132-3137

Nagy P, Vereb G, Sebestyen Z, Horvath G, Lockett SJ, Damjanovich S, Park JW, Jovin TM, Szollosi J (2002) Lipid rafts and the local density of ErbB proteins influence the biological role of homo- and heteroassociations of ErbB2. *Journal of cell science* **115:** 4251-4262

Naviglio S, Mattecucci C, Matoskova B, Nagase T, Nomura N, Di Fiore PP, Draetta GF (1998) UBPY: a growth-regulated human ubiquitin isopeptidase. *The EMBO journal* **17:** 3241-3250

Neckers L (2006) Chaperoning oncogenes: Hsp90 as a target of geldanamycin. *Handb Exp Pharmacol***:** 259-277

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

Olayioye MA, Neve RM, Lane HA, Hynes NE (2000) The ErbB signaling network: receptor heterodimerization in development and cancer. *The EMBO journal* **19:** 3159-3167

Orth JD, Krueger EW, Weller SG, McNiven MA (2006) A novel endocytic mechanism of epidermal growth factor receptor sequestration and internalization. *Cancer research* **66:** 3603-3610

Pao W, Miller VA, Politi KA, Riely GJ, Somwar R, Zakowski MF, Kris MG, Varmus H (2005) Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. *PLoS Med* **2:** e73

Parton RG, del Pozo MA (2013) Caveolae as plasma membrane sensors, protectors and organizers. *Nature reviews Molecular cell biology* **14:** 98-112

Pearl LH (2005) Hsp90 and Cdc37 -- a chaperone cancer conspiracy. *Curr Opin Genet Dev* **15:** 55-61

Pearl LH, Prodromou C, Workman P (2008) The Hsp90 molecular chaperone: an open and shut case for treatment. *The Biochemical journal* **410:** 439-453

Pedersen MW, Jacobsen HJ, Koefoed K, Dahlman A, Kjaer I, Poulsen TT, Meijer PJ, Nielsen LS, Horak ID, Lantto J, Kragh M (2015) Targeting Three Distinct HER2 Domains with a Recombinant Antibody Mixture Overcomes Trastuzumab Resistance. *Mol Cancer Ther* **14:** 669-680

Pedersen NM, Breen K, Rodland MS, Haslekas C, Stang E, Madshus IH (2009) Expression of epidermal growth factor receptor or ErbB3 facilitates geldanamycin-induced down-regulation of ErbB2. *Mol Cancer Res* **7:** 275-284

Pedersen NM, Madshus IH, Haslekas C, Stang E (2008) Geldanamycin-induced down-regulation of ErbB2 from the plasma membrane is clathrin dependent but proteasomal activity independent. *Mol Cancer Res* **6:** 491-500

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

Peng J, Schwartz D, Elias JE, Thoreen CC, Cheng D, Marsischky G, Roelofs J, Finley D, Gygi SP (2003) A proteomics approach to understanding protein ubiquitination. *Nature biotechnology* **21:** 921-926

Prigent SA, Lemoine NR, Hughes CM, Plowman GD, Selden C, Gullick WJ (1992) Expression of the c-erbB-3 protein in normal human adult and fetal tissues. *Oncogene* **7:** 1273-1278

Pust S, Klokk TI, Musa N, Jenstad M, Risberg B, Erikstein B, Tcatchoff L, Liestol K, Danielsen HE, van Deurs B, Sandvig K (2013) Flotillins as regulators of ErbB2 levels in breast cancer. *Oncogene* **32:** 3443-3451

Pyrzynska B, Pilecka I, Miaczynska M (2009) Endocytic proteins in the regulation of nuclear signaling, transcription and tumorigenesis. *Mol Oncol* **3:** 321-338

Qiu XB, Goldberg AL (2002) Nrdp1/FLRF is a ubiquitin ligase promoting ubiquitination and degradation of the epidermal growth factor receptor family member, ErbB3. *Proceedings of the National Academy of Sciences of the United States of America* **99:** 14843-14848

Raiborg C, Stenmark H (2009) The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. *Nature* **458:** 445-452

Raiborg C, Wenzel EM, Stenmark H (2015) ER-endosome contact sites: molecular compositions and functions. *The EMBO journal* **34:** 1848-1858

Raja SM, Clubb RJ, Ortega-Cava C, Williams SH, Bailey TA, Duan L, Zhao X, Reddi AL, Nyong AM, Natarajan A, Band V, Band H (2011) Anticancer activity of Celastrol in combination with ErbB2-targeted therapeutics for treatment of ErbB2-overexpressing breast cancers. *Cancer Biol Ther* **11:** 263-276

Reider A, Barker SL, Mishra SK, Im YJ, Maldonado-Baez L, Hurley JH, Traub LM, Wendland B (2009) Syp1 is a conserved endocytic adaptor that contains domains involved in cargo selection and membrane tubulation. *The EMBO journal* **28:** 3103-3116

Reider A, Wendland B (2011) Endocytic adaptors--social networking at the plasma membrane. *Journal of cell science* **124:** 1613-1622

Rexer BN, Arteaga CL (2012) Intrinsic and acquired resistance to HER2-targeted therapies in HER2 gene-amplified breast cancer: mechanisms and clinical implications. *Crit Rev Oncog* **17:** 1-16

Riento K, Frick M, Schafer I, Nichols BJ (2009) Endocytosis of flotillin-1 and flotillin-2 is regulated by Fyn kinase. *Journal of cell science* **122:** 912-918

Rivera-Milla E, Stuermer CA, Malaga-Trillo E (2006) Ancient origin of reggie (flotillin), reggie-like, and other lipid-raft proteins: convergent evolution of the SPFH domain. *Cell Mol Life Sci* **63:** 343-357

Roepstorff K, Grandal MV, Henriksen L, Knudsen SL, Lerdrup M, Grovdal L, Willumsen BM, van Deurs B (2009) Differential effects of EGFR ligands on endocytic sorting of the receptor. *Traffic* **10:** 1115-1127

Romond EH, Perez EA, Bryant J, Suman VJ, Geyer CE, Jr., Davidson NE, Tan-Chiu E, Martino S, Paik S, Kaufman PA, Swain SM, Pisansky TM, Fehrenbacher L, Kutteh LA, Vogel VG, Visscher DW, Yothers G, Jenkins RB, Brown AM, Dakhil SR, Mamounas EP, Lingle WL, Klein PM, Ingle JN, Wolmark N (2005) Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. *N Engl J Med* **353:** 1673-1684

Rosenbluth J, Wissig SL (1964) The Distribution of Exogenous Ferritin in Toad Spinal Ganglia and the Mechanism of Its Uptake by Neurons. *The Journal of cell biology* **23:** 307-325

Roskoski R, Jr. (2014) The ErbB/HER family of protein-tyrosine kinases and cancer. *Pharmacological research : the official journal of the Italian Pharmacological Society* **79:** 34-74

Rotin D, Kumar S (2009) Physiological functions of the HECT family of ubiquitin ligases. *Nature reviews Molecular cell biology* **10:** 398-409

Rush JS, Quinalty LM, Engelman L, Sherry DM, Ceresa BP (2012) Endosomal accumulation of the activated epidermal growth factor receptor (EGFR) induces apoptosis. *The Journal of biological chemistry* **287:** 712-722

Rusnak DW, Lackey K, Affleck K, Wood ER, Alligood KJ, Rhodes N, Keith BR, Murray DM, Knight WB, Mullin RJ, Gilmer TM (2001) The effects of the novel, reversible epidermal growth factor receptor/ErbB-2 tyrosine kinase inhibitor, GW2016, on the growth of human normal and tumor-derived cell lines in vitro and in vivo. *Mol Cancer Ther* **1:** 85-94

Sabharanjak S, Sharma P, Parton RG, Mayor S (2002) GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway. *Dev Cell* **2:** 411-423

Saif MW, Kim R (2007) Incidence and management of cutaneous toxicities associated with cetuximab. *Expert Opin Drug Saf* **6:** 175-182

Sak MM, Breen K, Ronning SB, Pedersen NM, Bertelsen V, Stang E, Madshus IH (2012) The oncoprotein ErbB3 is endocytosed in the absence of added ligand in a clathrin-dependent manner. *Carcinogenesis* **33:** 1031-1039

Sakai K, Yokote H, Murakami-Murofushi K, Tamura T, Saijo N, Nishio K (2007) Pertuzumab, a novel HER dimerization inhibitor, inhibits the growth of human lung cancer cells mediated by the HER3 signaling pathway. *Cancer Sci* **98:** 1498-1503

Salcini AE, Chen H, Iannolo G, De Camilli P, Di Fiore PP (1999) Epidermal growth factor pathway substrate 15, Eps15. *Int J Biochem Cell Biol* **31:** 805-809

Sallusto F, Cella M, Danieli C, Lanzavecchia A (1995) Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J Exp Med* **182:** 389-400

Sampath P, Pollard TD (1991) Effects of cytochalasin, phalloidin, and pH on the elongation of actin filaments. *Biochemistry* **30:** 1973-1980

Sandvig K, Pust S, Skotland T, van Deurs B (2011) Clathrin-independent endocytosis: mechanisms and function. *Current opinion in cell biology* **23:** 413-420

Sardi SP, Murtie J, Koirala S, Patten BA, Corfas G (2006) Presenilin-dependent ErbB4 nuclear signaling regulates the timing of astrogenesis in the developing brain. *Cell* **127:** 185-197

Scaltriti M, Verma C, Guzman M, Jimenez J, Parra JL, Pedersen K, Smith DJ, Landolfi S, Ramon y Cajal S, Arribas J, Baselga J (2009) Lapatinib, a HER2 tyrosine kinase inhibitor, induces stabilization and accumulation of HER2 and potentiates trastuzumab-dependent cell cytotoxicity. *Oncogene* **28:** 803-814

Schaefer G, Haber L, Crocker LM, Shia S, Shao L, Dowbenko D, Totpal K, Wong A, Lee CV, Stawicki S, Clark R, Fields C, Lewis Phillips GD, Prell RA, Danilenko DM, Franke Y, Stephan JP, Hwang J, Wu Y, Bostrom J, Sliwkowski MX, Fuh G, Eigenbrot C (2011) A two-in-one antibody against HER3 and EGFR has superior inhibitory activity compared with monospecific antibodies. *Cancer Cell* **20:** 472-486

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

Schlossman DM, Schmid SL, Braell WA, Rothman JE (1984) An enzyme that removes clathrin coats: purification of an uncoating ATPase. *The Journal of cell biology* **99:** 723-733

Schmid SL, Frolov VA (2011) Dynamin: functional design of a membrane fission catalyst. *Annu Rev Cell Dev Biol* **27:** 79-105

Schmidt MH, Dikic I (2005) The Cbl interactome and its functions. *Nat Rev Mol Cell Biol* **6:** 907-918

Schoeberl B, Faber AC, Li D, Liang MC, Crosby K, Onsum M, Burenkova O, Pace E, Walton Z, Nie L, Fulgham A, Song Y, Nielsen UB, Engelman JA, Wong KK (2010) An ErbB3 antibody, MM-121, is active in cancers with ligand-dependent activation. *Cancer research* **70:** 2485-2494

Schroeder RL, Stevens CL, Sridhar J (2014) Small molecule tyrosine kinase inhibitors of ErbB2/HER2/Neu in the treatment of aggressive breast cancer. *Molecules* **19:** 15196-15212

Seaman MN (2012) The retromer complex - endosomal protein recycling and beyond. *Journal of cell science* **125:** 4693-4702

Shattuck DL, Miller JK, Carraway KL, 3rd, Sweeney C (2008) Met receptor contributes to trastuzumab resistance of Her2-overexpressing breast cancer cells. *Cancer research* **68:** 1471-1477

Shi F, Telesco SE, Liu Y, Radhakrishnan R, Lemmon MA (2010) ErbB3/HER3 intracellular domain is competent to bind ATP and catalyze autophosphorylation. *Proceedings of the National Academy of Sciences of the United States of America* **107:** 7692-7697

Sigismund S, Argenzio E, Tosoni D, Cavallaro E, Polo S, Di Fiore PP (2008) Clathrin-mediated internalization is essential for sustained EGFR signaling but dispensable for degradation. *Dev Cell* **15:** 209-219

Sigismund S, Woelk T, Puri C, Maspero E, Tacchetti C, Transidico P, Di Fiore PP, Polo S (2005) Clathrin-independent endocytosis of ubiquitinated cargos. *Proceedings of the National Academy of Sciences of the United States of America* **102:** 2760-2765

Sithanandam G, Anderson LM (2008) The ERBB3 receptor in cancer and cancer gene therapy. *Cancer gene therapy* **15:** 413-448

Sliwkowski MX, Lofgren JA, Lewis GD, Hotaling TE, Fendly BM, Fox JA (1999) Nonclinical studies addressing the mechanism of action of trastuzumab (Herceptin). *Semin Oncol* **26:** 60-70

Sliwkowski MX, Mellman I (2013) Antibody therapeutics in cancer. *Science* **341:** 1192-1198

Solit DB, Zheng FF, Drobnjak M, Munster PN, Higgins B, Verbel D, Heller G, Tong W, Cordon-Cardo C, Agus DB, Scher HI, Rosen N (2002) 17-Allylamino-17-demethoxygeldanamycin induces the degradation of androgen receptor and HER-2/neu and inhibits the growth of prostate cancer xenografts. *Clinical cancer research : an official journal of the American Association for Cancer Research* **8:** 986-993

Sorkin A (2004) Cargo recognition during clathrin-mediated endocytosis: a team effort. *Current opinion in cell biology* **16:** 392-399

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

Sorkin A, Goh LK (2008) Endocytosis and intracellular trafficking of ErbBs. *Experimental cell research* **314:** 3093-3106

Sorkin A, Waters CM (1993) Endocytosis of growth factor receptors. *BioEssays : news and reviews in molecular, cellular and developmental biology* **15:** 375-382

Spradling KD, McDaniel AE, Lohi J, Pilcher BK (2001) Epsin 3 is a novel extracellular matrix-induced transcript specific to wounded epithelia. *The Journal of biological chemistry* **276:** 29257-29267

Stang E, Blystad FD, Kazazic M, Bertelsen V, Brodahl T, Raiborg C, Stenmark H, Madshus IH (2004) Cbl-dependent ubiquitination is required for progression of EGF receptors into clathrin-coated pits. *Molecular biology of the cell* **15:** 3591-3604

Stang E, Johannessen LE, Knardal SL, Madshus IH (2000) Polyubiquitination of the epidermal growth factor receptor occurs at the plasma membrane upon ligand-induced activation. *The Journal of biological chemistry* **275:** 13940-13947

Stein BS, Bensch KG, Sussman HH (1984) Complete inhibition of transferrin recycling by monensin in K562 cells. *The Journal of biological chemistry* **259:** 14762-14772

Stenmark H (2009) Rab GTPases as coordinators of vesicle traffic. *Nature reviews Molecular cell biology* **10:** 513-525

Stimpson HE, Toret CP, Cheng AT, Pauly BS, Drubin DG (2009) Early-arriving Syp1p and Ede1p function in endocytic site placement and formation in budding yeast. *Molecular biology of the cell* **20:** 4640-4651

Stoscheck CM, Carpenter G (1984a) Characterization of the metabolic turnover of epidermal growth factor receptor protein in A-431 cells. *Journal of cellular physiology* **120:** 296-302

Stoscheck CM, Carpenter G (1984b) Down regulation of epidermal growth factor receptors: direct demonstration of receptor degradation in human fibroblasts. *The Journal of cell biology* **98:** 1048-1053

Stuart AD, Brown TD (2006) Entry of feline calicivirus is dependent on clathrin-mediated endocytosis and acidification in endosomes. *J Virol* **80:** 7500-7509

Sugawa N, Ekstrand AJ, James CD, Collins VP (1990) Identical splicing of aberrant epidermal growth factor receptor transcripts from amplified rearranged genes in human glioblastomas. *Proceedings of the National Academy of Sciences of the United States of America* **87:** 8602-8606

Sunada H, Magun BE, Mendelsohn J, MacLeod CL (1986) Monoclonal antibody against epidermal growth factor receptor is internalized without stimulating receptor phosphorylation. *Proceedings of the National Academy of Sciences of the United States of America* **83:** 3825-3829

Sverdlov M, Shajahan AN, Minshall RD (2007) Tyrosine phosphorylation-dependence of caveolae-mediated endocytosis. *J Cell Mol Med* **11:** 1239-1250

Saal LH, Holm K, Maurer M, Memeo L, Su T, Wang X, Yu JS, Malmstrom PO, Mansukhani M, Enoksson J, Hibshoosh H, Borg A, Parsons R (2005) PIK3CA mutations correlate with hormone receptors, node metastasis, and ERBB2, and are mutually exclusive with PTEN loss in human breast carcinoma. *Cancer research* **65:** 2554-2559

Taipale M, Jarosz DF, Lindquist S (2010) HSP90 at the hub of protein homeostasis: emerging mechanistic insights. *Nature reviews Molecular cell biology* **11:** 515-528

Taylor MJ, Perrais D, Merrifield CJ (2011) A high precision survey of the molecular dynamics of mammalian clathrin-mediated endocytosis. *PLoS Biol* **9:** e1000604

Tebar F, Sorkina T, Sorkin A, Ericsson M, Kirchhausen T (1996) Eps15 is a component of clathrin-coated pits and vesicles and is located at the rim of coated pits. *The Journal of biological chemistry* **271:** 28727-28730

Tebbutt N, Pedersen MW, Johns TG (2013) Targeting the ERBB family in cancer: couples therapy. *Nature reviews Cancer* **13:** 663-673

Telesco SE, Shih AJ, Jia F, Radhakrishnan R (2011) A multiscale modeling approach to investigate molecular mechanisms of pseudokinase activation and drug resistance in the HER3/ErbB3 receptor tyrosine kinase signaling network. *Molecular bioSystems* **7:** 2066-2080

Tessneer KL, Cai X, Pasula S, Dong Y, Liu X, Chang B, McManus J, Hahn S, Yu L, Chen H (2013) Epsin Family of Endocytic Adaptor Proteins as Oncogenic Regulators of Cancer Progression. *Journal of cancer research updates* **2:** 144-150

Thien CB, Langdon WY (2001) Cbl: many adaptations to regulate protein tyrosine kinases. *Nature reviews Molecular cell biology* **2:** 294-307

Tran DD, Russell HR, Sutor SL, van Deursen J, Bram RJ (2003) CAML is required for efficient EGF receptor recycling. *Dev Cell* **5:** 245-256

Traub LM (2009) Tickets to ride: selecting cargo for clathrin-regulated internalization. *Nature reviews Molecular cell biology* **10:** 583-596

Tse C, Gauchez AS, Jacot W, Lamy PJ (2012) HER2 shedding and serum HER2 extracellular domain: biology and clinical utility in breast cancer. *Cancer Treat Rev* **38:** 133-142

Tsuda T, Ikeda Y, Taniguchi N (2000) The Asn-420-linked sugar chain in human epidermal growth factor receptor suppresses ligand-independent spontaneous oligomerization. Possible role of a specific sugar chain in controllable receptor activation. *The Journal of biological chemistry* **275:** 21988-21994

Tsujioka H, Yotsumoto F, Shirota K, Horiuchi S, Yoshizato T, Kuroki M, Miyamoto S (2010) Emerging strategies for ErbB ligand-based targeted therapy for cancer. *Anticancer Res* **30:** 3107-3112

Umebayashi K, Stenmark H, Yoshimori T (2008) Ubc4/5 and c-Cbl continue to ubiquitinate EGF receptor after internalization to facilitate polyubiquitination and degradation. *Molecular biology of the cell* **19:** 3454-3462

Uruno T, Liu J, Zhang P, Fan Y, Egile C, Li R, Mueller SC, Zhan X (2001) Activation of Arp2/3 complex-mediated actin polymerization by cortactin. *Nature cell biology* **3:** 259-266

Vijay-Kumar S, Bugg CE, Cook WJ (1987) Structure of ubiquitin refined at 1.8 A resolution. *J Mol Biol* **194:** 531-544

Vuong TT, Berger C, Bertelsen V, Rodland MS, Stang E, Madshus IH (2013) Preubiquitinated chimeric ErbB2 is constitutively endocytosed and subsequently degraded in lysosomes. *Experimental cell research* **319:** 32-45

Wang JT, Kerr MC, Karunaratne S, Jeanes A, Yap AS, Teasdale RD (2010a) The SNX-PX-BAR family in macropinocytosis: the regulation of macropinosome formation by SNX-PX-BAR proteins. *PLoS One* **5:** e13763

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

Wang YN, Wang H, Yamaguchi H, Lee HJ, Lee HH, Hung MC (2010b) COPI-mediated retrograde trafficking from the Golgi to the ER regulates EGFR nuclear transport. *Biochemical and biophysical research communications* **399:** 498-504

Ward CW, Hoyne PA, Flegg RH (1995) Insulin and epidermal growth factor receptors contain the cysteine repeat motif found in the tumor necrosis factor receptor. *Proteins* **22:** 141-153

Warren CM, Kani K, Landgraf R (2006) The N-terminal domains of neuregulin 1 confer signal attenuation. *The Journal of biological chemistry* **281:** 27306-27316

Wassmer T, Attar N, Bujny MV, Oakley J, Traer CJ, Cullen PJ (2007) A loss-of-function screen reveals SNX5 and SNX6 as potential components of the mammalian retromer. *Journal of cell science* **120:** 45-54

Waterman H, Alroy I, Strano S, Seger R, Yarden Y (1999a) The C-terminus of the kinase-defective neuregulin receptor ErbB-3 confers mitogenic superiority and dictates endocytic routing. *The EMBO journal* **18:** 3348-3358

Waterman H, Levkowitz G, Alroy I, Yarden Y (1999b) The RING finger of c-Cbl mediates desensitization of the epidermal growth factor receptor. *The Journal of biological chemistry* **274:** 22151-22154

Waterman H, Sabanai I, Geiger B, Yarden Y (1998) Alternative intracellular routing of ErbB receptors may determine signaling potency. *The Journal of biological chemistry* **273:** 13819-13827

Weiner LM, Adams GP (2000) New approaches to antibody therapy. *Oncogene* **19:** 6144-6151

Weiner LM, Belldegrun AS, Crawford J, Tolcher AW, Lockbaum P, Arends RH, Navale L, Amado RG, Schwab G, Figlin RA (2008) Dose and schedule study of panitumumab monotherapy in patients with advanced solid malignancies. *Clinical cancer research : an official journal of the American Association for Cancer Research* **14:** 502-508

Weiner LM, Surana R, Wang S (2010) Monoclonal antibodies: versatile platforms for cancer immunotherapy. *Nat Rev Immunol* **10:** 317-327

Wendland B (2002) Epsins: adaptors in endocytosis? *Nature reviews Molecular cell biology* **3:** 971-977

Wheeler DL, Dunn EF, Harari PM (2010) Understanding resistance to EGFR inhibitors-impact on future treatment strategies. *Nat Rev Clin Oncol* **7:** 493-507

Whitesell L, Mimnaugh EG, De Costa B, Myers CE, Neckers LM (1994) Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. *Proceedings of the National Academy of Sciences of the United States of America* **91:** 8324-8328

Whitson KB, Whitson SR, Red-Brewer ML, McCoy AJ, Vitali AA, Walker F, Johns TG, Beth AH, Staros JV (2005) Functional effects of glycosylation at Asn-579 of the epidermal growth factor receptor. *Biochemistry* **44:** 14920-14931

Wiley HS (1988) Anomalous binding of epidermal growth factor to A431 cells is due to the effect of high receptor densities and a saturable endocytic system. *The Journal of cell biology* **107:** 801-810

Williams RL, Urbe S (2007) The emerging shape of the ESCRT machinery. *Nature reviews Molecular cell biology* **8:** 355-368

Xia W, Mullin RJ, Keith BR, Liu LH, Ma H, Rusnak DW, Owens G, Alligood KJ, Spector NL (2002) Anti-tumor activity of GW572016: a dual tyrosine kinase inhibitor blocks EGF activation of EGFR/erbB2 and downstream Erk1/2 and AKT pathways. *Oncogene* **21:** 6255-6263

Xu W, Marcu M, Yuan X, Mimnaugh E, Patterson C, Neckers L (2002) Chaperone-dependent E3 ubiquitin ligase CHIP mediates a degradative pathway for c-ErbB2/Neu. *Proceedings of the National Academy of Sciences of the United States of America* **99:** 12847-12852

Xu W, Mimnaugh E, Rosser MF, Nicchitta C, Marcu M, Yarden Y, Neckers L (2001) Sensitivity of mature Erbb2 to geldanamycin is conferred by its kinase domain and is mediated by the chaperone protein Hsp90. *The Journal of biological chemistry* **276:** 3702-3708

Yamashita-Kashima Y, Iijima S, Yorozu K, Furugaki K, Kurasawa M, Ohta M, Fujimoto-Ouchi K (2011) Pertuzumab in combination with trastuzumab shows significantly enhanced antitumor activity in HER2-positive human gastric cancer xenograft models. *Clinical cancer research : an official journal of the American Association for Cancer Research* **17:** 5060-5070

Yang W, Klos K, Yang Y, Smith TL, Shi D, Yu D (2002) ErbB2 overexpression correlates with increased expression of vascular endothelial growth factors A, C, and D in human breast carcinoma. *Cancer* **94:** 2855-2861

Yarden Y, Pines G (2012) The ERBB network: at last, cancer therapy meets systems biology. *Nature reviews Cancer* **12:** 553-563

Yarden Y, Sliwkowski MX (2001) Untangling the ErbB signalling network. *Nature reviews Molecular cell biology* **2:** 127-137

Ye Y, Rape M (2009) Building ubiquitin chains: E2 enzymes at work. *Nature reviews Molecular cell biology* **10:** 755-764

Zagozdzon R, Gallagher WM, Crown J (2011) Truncated HER2: implications for HER2-targeted therapeutics. *Drug Discov Today* **16:** 810-816

Zhang X, Gureasko J, Shen K, Cole PA, Kuriyan J (2006) An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. *Cell* **125:** 1137-1149

Zhen Y, Caprioli RM, Staros JV (2003) Characterization of glycosylation sites of the epidermal growth factor receptor. *Biochemistry* **42:** 5478-5492

Zhou H, Zha Z, Liu Y, Zhang H, Zhu J, Hu S, Shen G, Cheng L, Niu L, Greene MI, Teng M, Liu J (2011) Structural insights into the down-regulation of overexpressed p185(her2/neu) protein of transformed cells by the antibody chA21. *The Journal of biological chemistry* **286:** 31676-31683

Zhou P, Fernandes N, Dodge IL, Reddi AL, Rao N, Safran H, DiPetrillo TA, Wazer DE, Band V, Band H (2003) ErbB2 degradation mediated by the co-chaperone protein CHIP. *The Journal of biological chemistry* **278:** 13829-13837