

Effects of therapeutic antibodies and ubiquitination on dimerization and down-regulation of EGF receptor and ErbB2

By

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Oslo, September 2012

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TABLE OF CONTENTS

ABBREVIATIONS	2
PUBLICATIONS INCLUDED	3
INTRODUCTION	4
Background	4
ErbB protein architecture	4
Signaling	7
Endocytosis	9
Clathrin-dependent endocytosis	11
Clathrin-independent endocytosis	12
Ubiquitin in receptor endocytosis	14
Functional differences between ErbB proteins	15
ErbB proteins in cancer initiation, progression and treatment	16
Molecular targeting of ErbB proteins in cancer	18
AIMS OF THE STUDY	28
SUMMARY OF PAPERS	30
Paper I	30
Paper II	31
Paper III	32
Paper IV	32
METHODOLOGICAL CONSIDERATIONS	34
Cell lines	34
Transient transfection	35
Small interfering RNA	35
Immunological methods	36
¹²⁵ I-EGF binding assay	37
Chemical inhibitors	37
GENERAL DISCUSSION	39
Effects of anti-EGFR antibodies on receptor dimerization and internalization	39
Antibody-induced down-regulation by macropinocytosis	42
Targeting the endocytosis deficient ErbB2	44
REFERENCES	48

ABBREVIATIONS

ADCC	Antibody-dependent cellular cytotoxicity
ATP	Adenosine triphosphate
AP-2	Adaptor protein 2
C-tail	Cytoplasmic tail
CCP	Clathrin-coated pit
CCV	Clathrin-coated vesicle
CHIP	Carboxyl terminus Hsc70-interacting protein
EEA1	Early endosomal antigen 1
EMA	European Medicines Evaluation Agency
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ESCRT	Endosomal Sorting Complexes Required for Transport
Fc	Fragment crystallizable
FDA	Food and Drug Administration
GRB2	Growth factor receptor-bound protein 2
HNSCC	Head and neck squamous-cell carcinomas
HSP	Heat-shock protein
HSC	Heat shock cognate
IgG	Immunoglobulin G
mAbs	monoclonal antibodies
MAPK	Mitogen-activated protein kinase
MVBs	Multivesicular bodies
NSCLC	Non-small cell lung cancer
NRGs	Neuregulins
PAE	Porcine Aortic Endothelial
PI3K	Phosphatidylinositol 3-kinase
RAB	Ras-associated binding
SHP1	protein-tyrosine phosphatase 1
Sos	Son of sevenless
siRNA	Short interfering RNA
TKIs	Tyrosine kinase inhibitors
TGF α	Transforming growth alpha
Ub	Ubiquitin
UIM	Ubiquitin interaction motif
VEGF	Vascular endothelial growth factor

PUBLICATIONS INCLUDED

Paper I **Christian Berger**, Ute Krengel, Espen Stang, Ernesto Moreno, and Inger Helene Madshus

Nimotuzumab and cetuximab block ligand-independent EGF receptor signaling efficiently at different concentrations

Journal of Immunotherapy, 2011 Sep;34(7):550-5.

Paper II **Christian Berger**, Inger Helene Madshus and Espen Stang

Cetuximab in combination with anti-human IgG antibodies efficiently down-regulates the EGF receptor by macropinocytosis

Experimental Cell Research (In press)

Paper III Juliana Bentes Hughes, **Christian Berger**, Marianne Skeie Rødland, Max Hasmann, Espen Stang, and Inger Helene Madshus

Pertuzumab increases epidermal growth factor receptor down-regulation by counteracting epidermal growth factor receptor-ErbB2 heterodimerization

Molecular Cancer Therapeutics, 2009 Jul;8(7):1885-92.

Paper IV Tram Thu Vuong, **Christian Berger**, Vibeke Bertelsen, Espen Stang and Inger Helene Madshus

Preubiquitinated chimeric ErbB2 is constitutively endocytosed and subsequently degraded in lysosomes

Experimental Cell Research (Submitted)

INTRODUCTION

Background

Cell growth and development depends on regulatory molecules, such as growth factors. Growth factors bind to growth factor receptors at the surface of cells. One of the first growth factors to be isolated and described was shown to stimulate proliferation of epidermal cells and was thus named epidermal growth factor (EGF) (4). A decade later, the EGF receptor (EGFR) was identified (5). EGFR is part of the ErbB family of proteins, which includes 4 members: the EGFR (ErbB1), ErbB2 (HER2/Neu), ErbB3 and ErbB4. The importance of the ErbB proteins during embryogenesis and growth control is exemplified by studies of knock-out mice. Mice lacking EGFR die prematurely due to respiratory failure (6), while embryos lacking ErbB2 die due to defective cardiac development (7). Besides their normal physiological role, the ErbB proteins are linked to cancer, and there is sequence homology between the EGFR and the viral oncogene v-ErbB (8). Several studies have reported overexpression of ErbB proteins in tumors (9-14). Consequently, ErbB proteins are molecular targets for cancer therapy. Over 20 years of drug development has resulted in the clinical approval of several ErbB inhibitors. Unfortunately, only a subgroup of cancer patients responds to anti-ErbB therapeutics (reviewed in (1)). Gaining insight into processes underlying ErbB protein function should result in development of new drugs.

ErbB protein architecture

Like most growth factor receptors, the ErbBs are single-pass transmembrane proteins with an approximate size of 180-kDa. They are expressed in various cells of epithelial, mesenchymal and neuronal origin (15). Due to their enzymatic capacity, the transfer of phosphate groups to tyrosine residues, the ErbB proteins (ErbB1-4) are categorized as the subclass I of the receptor tyrosine kinase superfamily. All members of the ErbB family have a common molecular architecture comprising an extracellular ligand binding region, a transmembrane region and an intracellular region with a tyrosine kinase domain flanked by a juxtamembrane domain and a cytoplasmic tail (C-tail) (Figure 1). The modular

structure of the extracellular part can be described with an I-II-III-IV nomenclature reflecting four distinct protein domains of two different types; leucine rich repeat (LRR)

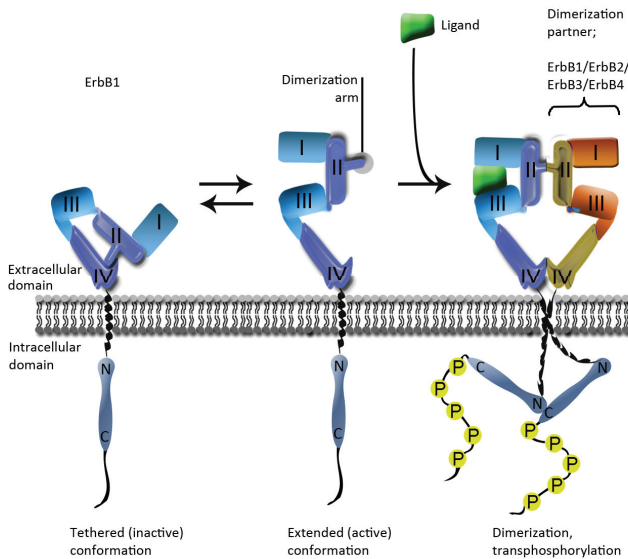


Figure 1. General structure and conformational states of ErbB proteins. All ErbB proteins, except ErbB2, can adopt the tethered receptor conformation. Ligand binding stabilizes the extended conformation, where the dimerization arm is exposed and the receptors can transphosphorylate each other upon homo- or heterodimerization. P=phosphotyrosine

domains I and III and cysteine rich (CR) domains II and IV. LRR motifs are generally involved in protein-protein interactions (16). Accordingly, domains I and III each contribute with a ligand binding surface, with low and high ligand interaction affinities, respectively.

Disulfide-bonded cysteines in domain II expose a loop (a β -hairpin termed the “dimerization arm”)

that reaches across the dimer interface in order to interact with the domain II-loop of another ErbB (reviewed in (17)). At the intracellular level, the kinase domain has a bilobular structure (N- and C-terminal lobes). A regulatory loop within the kinase can adopt an open configuration permitting access to adenosine triphosphate (ATP) in a cleft enclosed by the two lobes, enabling transfer of phosphate to acceptor tyrosines (18). The C-tail is a regulatory region, targeted for phosphorylation at tyrosine residues either by intrinsic (autophosphorylation) or neighbouring N-terminal lobes (transphosphorylation).

ErbB proteins may adopt distinct conformational states. In the tethered (also called inactive) conformation, the extracellular domains II and IV form an intramolecular tether

where the dimerization arm is occluded. This closes the intracellular ATP-regulatory loop, forming a kinase domain with moderate catalytic activity (19, 20). In the extended (active) conformation, domains II and IV do not form contacts, the dimerization arm is exposed, and the kinase domain becomes accessible to activation. Once two monomers are in the extended conformation, dimerization (pairing) can occur between two receptors of the same type (homodimerization) or between two different ErbB members (heterodimerization). Extracellular dimerization leads to asymmetric kinase interactions: the C-terminal lobe of one kinase domain interacts with the N-terminal lobe of the other. This stabilizes the asymmetric kinase dimer and elevates the catalytic activity (20). The activated kinase can then transphosphorylate the dimerizing partners C-tail (see Figure 1).

The active and inactive conformations are in equilibrium on the cellular surface, where up to ~20% of EGFRs have been reported to exist in an extended conformation in the absence of added ligand (21), and several reports have demonstrated a basal level of ligand-independent ErbB activity. This especially applies to cells overexpressing ErbB proteins (22-25). Binding of ligand, however, strongly shifts the equilibrium towards the extended conformation.

Structural variations exist between ErbB proteins. For the “orphan” receptor ErbB2, no known ligand has yet been identified. Crystallization studies revealed the presence of specific residues in domains I and III of ErbB2, interfering with ligand binding (26). Additionally, the disulfide-bonded cysteines in domain II have a different orientation in ErbB2 (26). The dimerization arm of ErbB2 is therefore constantly projected outwards, “locking” the orphan ErbB2 in an extended conformation. ErbB3, on the other hand, has been suggested to be unable to bind ATP due to amino acid substitutions in the tyrosine kinase domain (27).

Sequence variation between the ligand binding regions of EGFR, ErbB3, and ErbB4 results in binding to specific sets of extracellular ligands. The EGF family of ligands includes EGF, transforming growth factor- α (TGF α) and amphiregulin, which bind to EGFR. β -cellulin, heparin-binding EGF (HB-EGF) and epiregulin interacts with both EGFR and ErbB4. The neuregulins (NRGs) (NRG1 and NRG2) bind both ErbB3 and ErbB4, while NRG3 and NRG4 bind to ErbB4 only (28).

Signaling

Activation of ErbB proteins may result in a variety of biological outcomes, both physiological and pathological. Activated ErbB dimers generate cellular responses by initiating intracellular signaling pathways. Accumulating knowledge has transformed the view on the ErbB network from a simple linear pathway into a complex multilayered network, tightly regulated by positive and negative feedback (29, 30). The specificity and magnitude of ErbB-mediated signaling is determined by several factors: cellular context, the identity of the ligand, the identity of the dimerization partners, positive and negative regulators of the receptors, and most importantly the various proteins which use phosphotyrosines in the C-tail as docking sites to associate with and recruit a series of downstream signal transducers (31).

The two key signaling pathways activated by the ErbB proteins are the mitogen-activated protein kinase (MAPK) (also named Erk 1/2) pathway, controlling gene transcription, cell-cycle progression, and cell proliferation as well as the phosphatidylinositol 3` kinase B (PI3K) pathway, which promotes anti-apoptotic signaling and cell survival (1) (Figure 2A). It was recently suggested that activated EGFRs may also induce apoptosis, reflecting the complexity of the ErbB network (32).

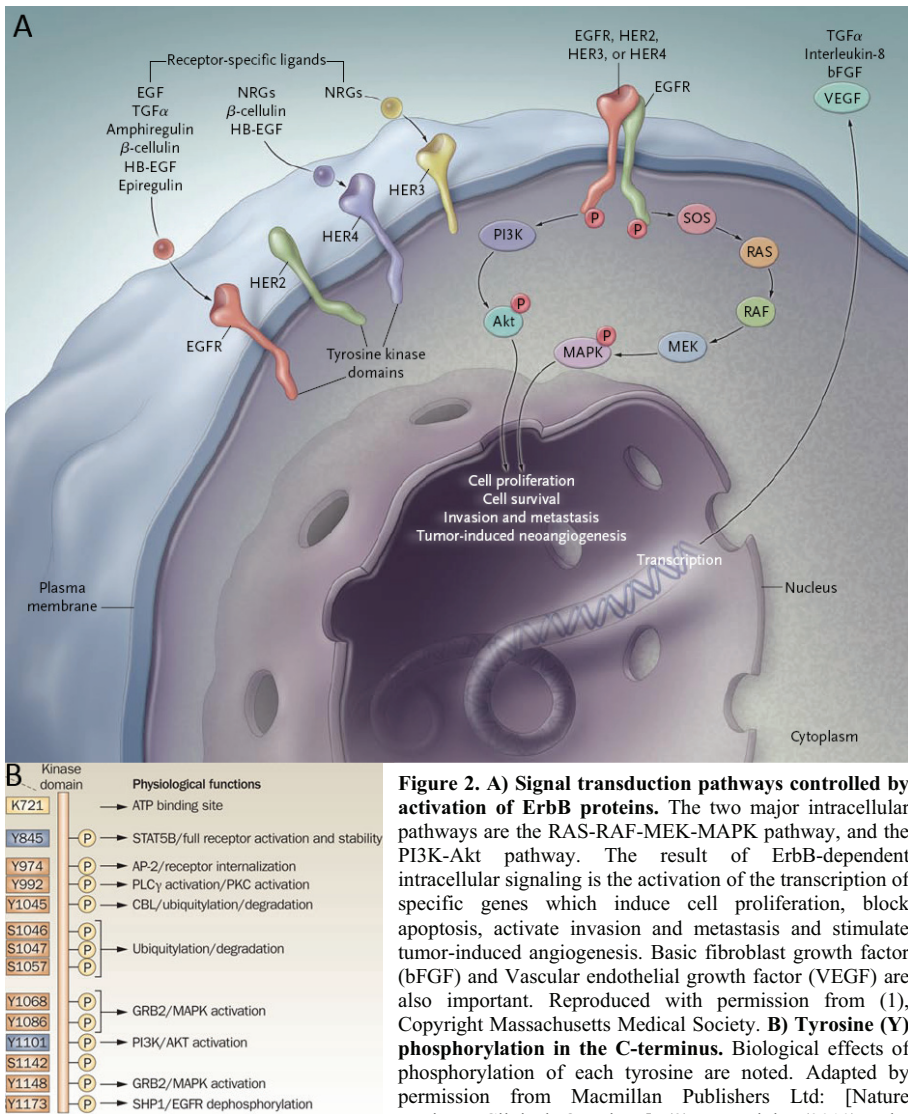


Figure 2. A) Signal transduction pathways controlled by activation of ErbB proteins. The two major intracellular pathways are the RAS-RAF-MEK-MAPK pathway, and the PI3K-Akt pathway. The result of ErbB-dependent intracellular signaling is the activation of the transcription of specific genes which induce cell proliferation, block apoptosis, activate invasion and metastasis and stimulate tumor-induced angiogenesis. Basic fibroblast growth factor (bFGF) and Vascular endothelial growth factor (VEGF) are also important. Reproduced with permission from (1), Copyright Massachusetts Medical Society. **B) Tyrosine (Y) phosphorylation in the C-terminus.** Biological effects of phosphorylation of each tyrosine are noted. Adapted by permission from Macmillan Publishers Ltd: [Nature Reviews Clinical Oncology] (3), copyright (2010). The figure legends are modified.

Phosphorylation of the C-tail dictates the specificity of the cellular response by binding various adaptor proteins (3) (Figure 2B). Phosphorylation of tyrosine 1068 or 1086 in the EGFR, for example, recruits the adaptor protein Growth factor receptor-bound protein 2 (GRB2) which activates the Ras exchange factor Son of Sevenless (Sos) to initiate a

signal amplification cascade (RAS-RAF-MEK-MAPK) leading to MAPK (Erk 1/2) activation. Phosphorylation of tyrosine residue 1173, in contrast, activates a negative feedback loop by binding the protein-tyrosine phosphatase 1 (SHP1), leading to attenuation of ErbB-mediated MAPK (Erk 1/2) activity (33).

Endocytosis

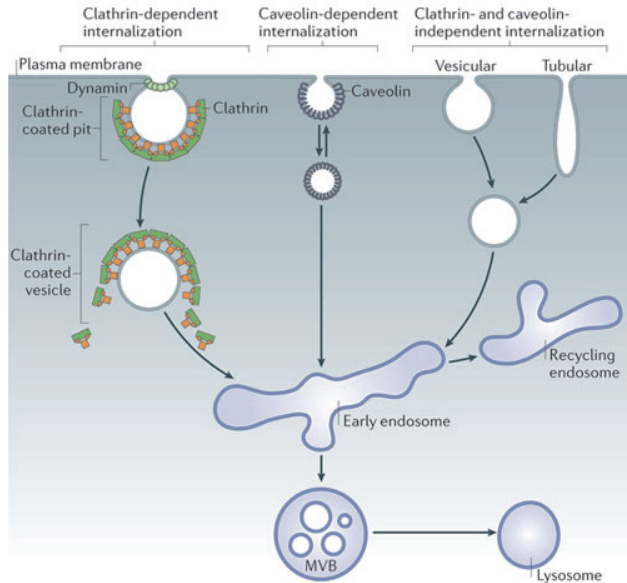
Sustained ErbB-signaling can lead to carcinogenesis (reviewed in (28, 34)). An important way by which cells regulate ErbB signaling is endocytosis; the uptake of extracellular material and membrane components into the cell interior. Reducing the number of receptors from the surface desensitizes the cell to external stimuli. Endocytosis is therefore a major negative feedback loop that prevents excessive ligand-induced signaling. It is becoming evident that receptor signaling and endocytosis are interactive processes (reviewed in (35)). The former initiates the latter, but endocytosis also regulates the type and intensity of signaling. Accumulation of activated receptors in intracellular compartments is sometimes necessary to achieve full signal activation. The compartments can act as signaling platforms, switching on and off various signals (reviewed in (36)).

Multiple pathways of endocytosis have been identified and classified according to the cellular proteins that control them, their morphological characteristics, sensitivity to chemical inhibitors, and their functional difference of internalizing and routing specific cargo (reviewed in (37)). Common to all pathways is the ability to internalize membrane proteins, lipids, nutrients, extracellular fluid or ligands by invagination of the plasma membrane into a budding structure pinching off to form vesicle (Figure 3). Vesicles derived from the various pathways fuse with sorting compartments called early endosomes. These structures are characterized by an acidic environment due to proton pumps in the membrane and are highly dynamic, being capable of undergoing homotypic fusion (reviewed in (38)). Further sorting of receptors to other compartments is mediated by key protein and lipid components present in early endosomes, such as the Ras-associated binding (Rab) proteins (39). These GTPases bind in their active state to intracellular membranes and recruit Rab effectors to exert their roles in budding, fusion and motility of intracellular vesicles. One such effector is the Early Endosomal Antigen-1

(EEA1) which mediates fusion between early endosomes. Because of its specific localization, EEA1 is the most common marker used for early endosomes (38). If the receptors are to be sorted for recycling, the membranes of early endosomes undergo extensive tubulation, and receptors are inserted into such tubuli and sent back to the plasma membrane (37). Routing of receptors for degradation, on the other hand, requires the presence of specific sorting signals conjugated to the C-tails (reviewed in (40)). These signals promote the generation of microdomains that concentrate the receptors into inner vesicles in endosomes, instead of delivery to the tubulating, recycling membranes. The inner vesicles are formed by inward budding of the limiting endosomal membrane, thus named intraluminal vesicles. Cargo is delivered to intraluminal vesicles via a series of Endosomal Sorting Complexes Required for Transport (ESCRT) complexes (ESCRT-I to -III) (reviewed in (41)). ESCRT-0 is the first protein recruited to early endosomes by interaction with sorting signals of receptors, leading to retention of cargo within microdomains in early endosomes. ESCRT-I-III complexes are then sequentially recruited (42). Sorting signals recruit ESCRT-I to late endosomal membranes, where ESCRT-II and ESCRT-III subsequently bind, leading to intraluminal vesicle formation and generation of multivesicular bodies (MVBs). Fusion of MVBs with lysosomes completes the receptor degradation route.

The two major ways by which ErbB proteins can internalize are clathrin-dependent and clathrin-independent endocytosis (2) (see Figure 3 for an overview).

Figure 3.
Clathrin-dependent and -independent internalization pathways. Internalized cargo is trafficked into early endosomes, where it is sorted either back to the cell surface (via recycling endosomes) or into other compartments for degradation. Endosomes that are not recycled mature into late endosomes and multivesicular bodies (MVBs), which fuse with lysosomes harboring proteolytic enzymes, resulting in degradation of internalized cargo. Figure legend is modified. Reprinted with permission from Macmillan Publishers Ltd: [Nature Reviews Molecular Cell Biology] (2), copyright (2011).



Clathrin-dependent endocytosis

Membrane invagination during clathrin-mediated endocytosis is characterized and driven by oligomerization of the structural protein clathrin into a protein coat (Figure 3). Formation of the budding structure, called a clathrin-coated pit (CCP), is initiated by adaptor proteins which interact both with cargo and clathrin to promote CCP assembly (43). The adaptor protein complex 2 (AP2) is recruited to the plasma membrane to function as a key mediator of cargo selection and coat formation (reviewed in (44)). Binding motifs in AP2 interact with and recruit clathrin to initiate polymerization of the clathrin triskelia. AP2 is a heterotetrameric complex consisting of four subunits: α and β 2 adaptins, μ 2 and σ 2 (45). It interacts with signaling receptors by binding to tyrosine-based and dileucine motifs (46). Tyr974 has been identified as the AP2 binding motif in EGFR (see Figure 2B), but interaction of these motifs with AP2 does not seem to be important for clathrin-dependent endocytosis of the EGFR (47).

Since the plasma membrane is rigid and stabilized by the cytoskeleton, invagination is an energetically demanding process. Actin polymerization is considered to be necessary to modulate membrane curvature. So is the presence of additional adaptor proteins, including Epsin and Eps15 (48, 49). Epsin and Eps15 can link cargo, such as modified EGFR, to clathrin, and thereby aid in curving the membrane into a CCP (48). As the CCP invaginates further, the GTPase dynamin is recruited to the vesicle neck by BAR domain-containing proteins (reviewed in (50)). Dynamin pinches off the vesicle from the membrane upon GTP hydrolysis, releasing it into the cell as a clathrin-coated vesicle (CCV) (2). The final step of CCV progression involves vesicle uncoating. The ATPase heat shock cognate 70 (HSC70) releases clathrin and the adaptor proteins into the cytosol, ready to be reused (51). The naked vesicle and its cargo fuse with, or mature into, early endosomes.

Clathrin-independent endocytosis

Clathrin-independent endocytosis was first suggested to exist over 20 years ago (52). Since then, several clathrin-independent pathways have been characterized. It should be noted that the endocytic routes of internalization are closely related and are often hard to separate. Some studies even imply that similar cellular proteins control the different pathways (53-55). The different routes are not mutually exclusive, but rather interact with each other. Inhibition of one route may result in upregulation of another in the same cell. They can act in parallel, with the relative proportions of each depending on cell type and cargo context. Ligand-bound EGFR is primarily internalized clathrin-dependently, however, increase in ligand concentration can lead to a substantial fraction of receptors being endocytosed through a clathrin-independent route (56). Some characteristics, including morphological traits and protein dependency, are considered as specific markers for the different pathways.

Caveolin-dependent endocytosis

Caveolae means small caves, and these structures are flask-shaped invaginations of the plasma membrane. The vesicles are enriched with and dependent on the protein caveolin, whose expression itself is sufficient to drive formation of the typical flask-shaped

caveolae (57). Formation and function of caveolae is also dependent on the cytoplasmic coat component cavin (58). The model predicts that caveolin initially oligomerizes to form membrane domains enriched in phosphatidylserine and cholesterol. Cavin then recognizes and binds these domains, stabilizing the membrane curvature of caveolae. The requirement for actin and the vesicle scission GTPase dynamin has been implicated in caveolin-dependent endocytosis (reviewed in (59)). Whether caveolae may or may not pinch off from the membrane is currently unclear, and it is suggested that they normally are immobile structures at the plasma membrane (reviewed in (52)).

Clathrin- and caveolin-independent endocytosis

Endocytosis can also take place in the absence of clathrin and caveolin, as exemplified by viral entry of SV40 into cells (60). Several subtypes of this pathway have been identified, but the common denominator is cholesterol for clustering of lipid-anchored proteins into lipid microdomains at the plasma membrane. These highly ordered domains are often called “lipid rafts” and are considered to play a role in receptor trafficking and signaling (61). While cholesterol depletion disrupts this pathway in general, some of the subtypes can be distinguished by dynamin dependence. The CLIC/GEEC pathway, for example, is not inhibited by overexpression of mutant dynamin (59). It is hypothesized that membrane scission is driven by a synergy between cholesterol and actin instead of dynamin to produce vesicles from either tubular or vesicular budding structures (Figure 3).

Macropinocytosis

Macropinocytosis is bulk uptake whereby large areas of the plasma membrane, extracellular fluid, nutrients and antigens are internalized. This pathway is associated with numerous processes, such as cell migration, tumor progression, antigen presentation, and pathogen entry (reviewed in (62)). It is initiated when the actin cytoskeleton rearranges to form ruffled membrane extensions that fuse with themselves or back with the plasma membrane, trapping cargo and extracellular components between these sites. The entire region is then internalized into vesicles that are considerably larger than CCVs. They are called macropinosomes and represent an early endosomal stage also positive for EEA1 (63). Although the uptake of cargo is considered to be non-selective,

the process occurs in response to specific stimuli, such as growth factors (64). Stimulation with high concentrations of EGF initiates macropinocytosis in the human epidermoid carcinoma cell line A431 (65-67). Also in other cell types circular dorsal membrane ruffles, typical for macropinocytosis, are formed in response to strong EGFR stimulation. This has been shown to concentrate EGFRs at these regions and induce rapid internalization of a large portion of the receptors from the surface, independently of traditional vacuoles such as CCVs and caveolae (68).

Although internalization by macropinocytosis was discovered early, the molecules that control it and its relation to other pathways are not clear. Membrane ruffling, mediated by rearrangement of actin filaments, is considered a prerequisite for macropinocytosis. However, several studies indicate that ruffling and EGF-stimulated macropinocytosis are independent cellular processes (reviewed in (37)).

Ubiquitin in receptor endocytosis

The processes that underlie down-regulation of ErbB proteins include internalization by any of the endocytic pathways described above and subsequent subcellular degradation. The best characterized sorting signal for routing receptors to the degradation pathway is ubiquitin (Ub), a 76-amino acid protein that can affect a variety of cellular processes (69). Ub is conjugated to the ϵ -amino group of a lysine residue in a target protein in a three-step cascade called ubiquitination. First, an Ub-activating enzyme (E1) activates Ub in an ATP-dependent reaction. Ub is then transferred to the active site of a Ub-conjugating enzyme (E2). Finally, a Ub-protein ligase (E3) catalyzes the transfer of Ub from E2 to the substrate. As Ub itself carries several conserved acceptor lysines (Lys6-11-27-29-33-48-63), Ub can itself be ubiquitinated, and different Ub-conjugation patterns can arise (reviewed in (70)). The distinct ubiquitination patterns imply specific consequences for the modified substrate. Conjugation of a single Ub to a protein (monoubiquitination), or conjugation of multiple single Ubs to several lysines in the substrate (multiple monoubiquitination), can regulate endocytosis, lysosomal routing, meiosis and chromatin remodeling. The substrate can also be modified with Ub chains of various length and linkages (polyubiquitination) (reviewed in (71)). Lys48-linked polyubiquitination, consisting of a minimal unit of four Ubs, targets proteins for degradation in the 26S

proteasome. Lys63-linked Ub-chains are among others implicated in kinase activation, endocytosis and DNA-repair (70).

Ubiquitinated receptors are recognized by proteins harboring ubiquitin-interaction motifs (UIMs). The adaptor proteins epsin1 and Eps15 contain UIMs and thereby efficiently interact with and recruit ubiquitinated ErbB proteins to CCPs (72). Ubiquitin chains are recognized by ESCRT sorting complexes, and it is well-known that ubiquitination is required as sorting signal for routing ErbB proteins to intraluminal vesicles and lysosomes (40). However, it is still debated whether ubiquitination is necessary for the internalization step of receptor endocytosis (73-75). Our group has recently demonstrated that polyubiquitination of the EGFR is sufficient to induce constitutive endocytosis of the EGFR (76). For the other ErbB-members, the effect of Ub on receptor internalization and endocytosis is less well characterized. A recent study has however demonstrated that Lys48- and Lys63-linked polyubiquitination is important in trafficking and degradation of ErbB2 (77).

Functional differences between ErbB proteins

EGFR is the prototypical member of the ErbB family. Upon binding ligand, it readily adopts the extended conformation, it forms homo- or hetero- dimers, activates the intracellular kinase and is endocytosed. Important functional differences do however exist between the ErbB proteins.

ErbB2 has a constantly exposed dimerization arm and is the preferred dimerization partner of the other ErbB members (78). But it is unclear whether ErbB2 is capable of forming homodimers. While some papers suggest that ErbB2 cannot participate in homodimerization (79), others show ErbB2 in homodimers upon receptor overexpression (80). ErbB2 is capable of dimerizing with any of the other receptors and once activated upon dimerization, it serves as a potent amplifier of the PI3K and the MAPK pathways (81). ErbB2 seems to interact with more phosphotyrosine binding proteins than the other members (82). Furthermore, ErbB2 is generally considered to be endocytosis deficient and is normally concentrated at the plasma membrane in nonmanipulated cells (83, 84). When overexpressed, it is also shown to retain dimerizing

partners at the plasma membrane (85). Receptors that heterodimerize with ErbB2, including EGFR, have inhibited endocytosis (85-87). These functional characteristics cause sustained proliferative signaling, making ErbB2 an important target for pharmaceutical inhibition.

ErbB3s catalytical activity is severely reduced when compared to the other ErbBs. It has been suggested to be either “kinase dead” due to its inability to bind ATP and its lack of intrinsic tyrosine kinase activity (27, 88), or to be capable of nucleotide binding with weak kinase activity (89). However, its C-terminal kinase lobe readily interacts in an asymmetric manner to activate the N-terminal kinase activity of the dimerization partner. One such binary relationship between the orphan ErbB2 and the kinase-impaired ErbB3 is suggested to be the most active ErbB pair with respect to proliferative potential (90).

ErbB4 is the least studied member of the ErbB proteins. It has a complex biology, as compared to the other ErbBs. It can exist as four alternatively spliced variants, with the four isoforms displaying different functional properties (91). ErbB4 readily forms heterodimers and promotes MAPK/PI3K signaling.

ErbB proteins in cancer initiation, progression and treatment

Cancer is a multi-step process, initiated when genetic changes transform normal cells into neoplastic cells. Such alterations are termed “driver mutations”, driving the induction of oncogene functions or the loss of tumor suppressor functions. The resulting subclone of abnormal cells has a growth advantage in the local tissue environment. Although ErbB proteins are often mutated in tumors, driver mutations directly affecting ErbB genes are rare (92). ErbB proteins play important roles during tumor progression.

Tumor progression occurs when some of the neoplastic cells acquire additional mutations, giving them selective advantages for expansion and growth. Expansion of the mutant clones is a step which occurs repetitively, with each round being triggered by acquired genetic or epigenetic alterations (93). Genome instability is therefore a hallmark of cancer which enables a normal cell to develop the characteristic traits of a cancer cell, as described by Hanahan *et al* (34, 94). Sustained proliferative signaling, mediated primarily by growth factors and their receptors, is essential for clonal expansion and is claimed to be the most fundamental trait of a cancer cell (94). In order to sustain the

accelerated growth and expansion of tumor cells, many cancers increase their growth-promoting signaling by several alternative mechanisms, such as activation of autocrine loops, overexpression of growth factor receptors which induces ligand-independent signaling (22), or constitutive signaling in absence of ligands upon mutations in receptors or downstream mediators. Autocrine production of NRG1, TGF α or EGF is associated with increased cancer cell proliferation and reduced patient survival (95-97). Overexpression of EGFR is found in head and neck squamous-cell carcinomas (HNSCC), non-small cell lung cancer (NSCLC), ovarian and other tumor types and is correlated with higher proliferation and reduced survival (98). Overexpression of ErbB2 is reported in breast, ovarian, gastric, bladder, and other carcinomas (99-101) and is suggested to result in an excess of ErbB2-mediated signalling by inducing formation of ErbB2 heterodimers and the spontaneous formation of ErbB2 homodimers (102). Co-expression of ErbB3 promotes cell survival in tumors overexpressing ErbB2, where ErbB2 activates the PI3K pathway by interaction with GRB2 (102, 103). EGFR is often mutated in glioblastomas (104). The most common EGFR mutation is the constitutively active EGFRvIII variant (105), which is also reported in breast, lung and ovarian tumors (106). EGFR with mutations in the kinase domain is shown to activate anti-apoptotic pathways in cancer cells, such as the PI3K pathway (107). ErbB2 mutation has been reported in NSCLC (108), and associated with constitutive phosphorylation and activation of ErbB2 and EGFR (109). Mutations in the ErbB downstream signal transducer RAS (K-RAS mutations) is found in up to 25% of human cancers (92).

Increased proliferative signaling and mutations associated with elevated growth normally triggers cellular death by apoptosis. Cancer cells evade this barrier by resisting apoptosis. The most common strategy is by mutations in the tumor suppressor gene p53, leading to a loss of DNA damage sensor function which would otherwise initiate apoptosis (34, 110). Another strategy is anti-apoptotic signaling via the PI3K pathway, which can be activated by the ErbB proteins (see Figure 2A).

Following clonal expansion, some neoplastic cells have the capability of detaching from the primary tumor mass and entering nearby lymphatic and blood vessels (local invasion). These cells harbor metastatic potential; spreading of cells from the primary

tumor to distant organs, and their continuous growth (111). Several mechanisms underlie the hallmark of invasion. These are: loss of the cell- cell adhesion, increased motility and secretion of proteases aiding in penetration of vessel walls (94). It has been suggested that growth factors and their receptors play critical roles during this step of cancer progression in carcinomas. EGF promotes the expression of E-cadherin repressors, while overexpression of ErbB2 potentiates cellular invasion by enhancing secretion of proteases and down-regulating protease inhibitors (reviewed in (92)).

Some malignant cells survive the circulation and adhere to vascular endothelial cells of distant organs. They penetrate the vessel walls by mechanisms similar to invasion, and enter the parenchyma of the distant tissue (extravasation). Here, the cancer cells adapt to the foreign microenvironment and form micrometastases. The continued growth of micrometastases into secondary tumors completes the process of cancer progression, which again relies on sustained proliferation by the production of growth factors and their receptors (111). It also depends on the supply of oxygen and nutrients by the growth of new blood vessels- the capability of inducing angiogenesis. In tumors, EGFR signaling can activate production of the proangiogenic factor VEGF (112, 113) (see Figure 2A).

Molecular targeting of ErbB proteins in cancer

Increased knowledge in the field of cancer research has changed clinical oncology with respect to improved prognosis and therapy. Many of the molecules involved in cancer progression have been pin-pointed and used as pharmaceutical targets, ranging from DNA itself to proteins (reviewed in (94)). The development and approval of molecular targeted therapeutics, has resulted in clinical use along with classical treatment modalities such as surgery, chemotherapy and radiotherapy. Some examples of early and recently developed molecular drugs are growth factor receptor inhibitors (114) and epigenetically acting drugs (such as azacytidine) (115). Human carcinomas frequently express active ErbB proteins, and EGFR was the first growth factor receptor to be used as molecular target in cancer therapy (116).

Inhibitors targeting ErbB proteins are generally categorized in two major groups: monoclonal antibodies (mAbs) and tyrosine kinase inhibitors (TKIs). Many of these agents have already been approved for treatment of metastatic cancers by several agencies worldwide, including the Food and Drug Administration (FDA) and the European Medicines Evaluation Agency (EMA), (summarized in Table 1, table based on (3, 102, 117-119)). Several new classes of anti-ErbB drugs are currently undergoing clinical trials, such as heat-shock protein (HSP) inhibitors (17-AAG) and antibody-chemotherapy conjugates (see Table 1). Other strategies are also underway, some of which are targeting other components of the ErbB network besides the receptors themselves (120).

Table 1: ErbB targeting inhibitors

Inhibitor	Type	Target	Mechanism	Approval/Clinical trial
<i>mAbs</i>				
Cetuximab C225 Erbix [®]	Human -mouse Chimeric mAb	ErbB1	Inhibits ligand binding, activation of ADCC	Metastatic colorectal cancer that is refractory to chemotherapy (irinotecan) Locally or regionally advanced HNSCC in combination with radiotherapy
Nimotuzumab h-R3 TheraCIM [®]	Human mAb	ErbB1	Inhibits ligand binding, activation of ADCC	Not yet approved by FDA/EMA Ongoing phase II/III trials for HNSCC, NSCLC, glioblastoma and pancreatic cancer
Panitumumab ABX-EGF Vectibix [®]	Human mAb	ErbB1	Inhibits ligand binding, activation of ADCC	Metastatic colorectal cancer in combination with or following chemotherapy (fluoropyrimidine, oxaliplatin and irinotecan)
Trastuzumab Herceptin [®]	Human mAb	ErbB2	Inhibits receptor signaling, activation of ADCC	Metastatic breast cancer
Trastuzumab-DM1	mAb- cytotoxic conjugate	ErbB2	Targeted delivery of a antimicrotubule agent (DM1)	Ongoing phase III trial for breast cancer
Pertuzumab 2C4 Omnitarg [®]	Human mAb	ErbB2	Inhibits receptor dimerization, activation of ADCC	Ongoing phase II/III trials for ovarian and breast cancer Completed trial for breast cancer in combination with trastuzumab and chemotherapy (docetaxel)
<i>TKIs</i>				
Erlotinib OSI-774 Tarceva [®]	ATP mimic	ErbB1	Inhibits receptor kinase activity	NSCLC Advanced pancreatic cancer in combination with chemotherapy (gemcitabine)
Gefitinib ZD1839 Iressa [®]	ATP mimic	ErbB1	Inhibits receptor kinase activity	Locally advanced metastatic NSCLC
Lapatinib GW572016 Tykerb [®]	ATP mimic	ErbB1 ErbB2	Inhibits receptor kinase activity	Metastatic breast cancer in combination with chemotherapy (capecitabine)
<i>HSP inhibitors</i>				
17-AAG Tanespimycin [®]	Ansamycin derivative	HSP90	Inhibits ErbB2 stability, degradation	Phase II/III trials for breast cancer and multiple myeloma

Abbreviations: mAb monoclonal antibody, ADCC antibody-dependent cellular cytotoxicity, TKI tyrosine kinase inhibitor, Hsp heat shock protein.

Monoclonal antibodies

Multiple mechanisms underlie the antitumor activity of anti-ErbB mAbs (reviewed in (1)). First, the antibody can suppress receptor signaling by inhibition of ligand binding or interference with receptor dimerization. Second, the Fragment crystallizable (Fc) region can recruit and initiate immune effector functions such as antibody-dependent cellular cytotoxicity (ADCC), leading to tumor cell lysis. Third, binding of the antibody can induce receptor internalization and degradation. This can be strongly enhanced upon using pairs of noncompetitive mAbs (121). The combination of two mAbs presumably forms large receptor-antibody complexes at the cell surface that internalize into the cytosol and are eventually routed to lysosomes for degradation (114, 122). The rate of internalization has been suggested to be proportional to the size of the complex, but the endocytic pathway by which mAb combinations internalize ErbBs has not been clearly described (123). The effect of antibody combination, however, has been confirmed to synergistically inhibit tumor growth (124, 125).

Cetuximab

Cetuximab is a chimeric mouse-human IgG1 mAb. The mouse progenitor of Cetuximab, M225, was one of the first anti-ErbB antibodies to be developed (126). Cetuximab was later found to be capable of inducing all the antitumor characteristics described above for anti-ErbB mAbs, and was approved for treatment of metastatic colorectal cancer in 2004. It specifically interacts with the ligand-binding surface of EGFR with higher affinity than EGF and TGF α , thereby antagonizing ligands (127). An additional consequence upon binding to domain III, is that the Fab region of the antibody is clashing with domain I, thereby sterically preventing the receptor from adopting the active conformation (128). This counteracts ligand-independent dimerization and downstream signaling (129), which usually occurs in cells that overexpress EGFR. Another important consequence of Cetuximab binding is that it induces internalization of EGFR (130). This is demonstrated to happen in a different manner than ligand-induced internalization and trafficking of EGFR. The process itself was slower, and it occurred without stimulating receptor phosphorylation. Furthermore, the antibody-receptor complex was primarily recycled instead of routed to lysosomes (131). Cetuximab was also demonstrated to relocalize the

EGFR to the nucleus (132). Unfortunately, such receptor routing could have negative consequences, since nuclear EGFR has been associated with poor clinical outcome (133, 134). Finally, Cetuximab has also been demonstrated to induce ADCC (135, 136).

The most common side effect associated with inhibition of EGFR activity is skin rash. This reaction is considered to be a marker of clinical response to the specific drug and probably reflects the extent of EGFR blockade achieved in the tumor (137-139).

Nimotuzumab

Another example of a mAb targeting EGFR is the humanized IgG1 Nimotuzumab. Like Cetuximab, Nimotuzumab binds the EGFR domain III and displays similar mechanisms of action by blocking ligand binding and oncogenic signaling. Also, the similar effects were observed with respect to increased apoptosis, and inhibition of proliferation and angiogenesis (140, 141). Like Cetuximab, Nimotuzumab also has the capacity of activating ADCC, which is not surprising since the Fc-region of both antibodies is human IgG1 (142). However, it shows unique functional characteristics, some of which seem beneficial compared to Cetuximab. These include the absence of skin rash (143). One explanation for this could be that its clinical dose is lower than its toxic dose based on the ten times lower affinity compared to Cetuximab (144). Another explanation for the lack of skin rash is based on a structural difference: while Cetuximab binds in a way that inhibits domain I from approaching domain III and thereby interferes with the active EGFR conformation, X-ray crystallography suggested that Nimotuzumab could allow domain I to approach domain III and therefore permit the EGFR to adopt the active conformation while simultaneously blocking EGF binding (145). Nimotuzumab could therefore be less toxic because it could allow basal levels of ligand-independent EGFR signaling.

Another difference between the antibodies is that Cetuximab is a human-mouse chimera, while Nimotuzumab is a fully humanized mAb. The mouse component of Cetuximab could provoke a more general allergic reaction and thereby contribute to the severe skin rash which was not observed in case of humanized Nimotuzumab (146, 147). Indeed, the murine progenitor of Nimotuzumab (Ior/egf/r3) has been shown to provoke allergic reactions (147). However, the systemic inhibition by anti-EGFR inhibitors is

considered to provoke skin rash due to specific EGFR inhibition (28). Also anti-EGFR TKIs were shown to negatively affect cells of the human epidermis upon specific inhibition of EGFR activity (148), again arguing that it is the difference in the level of EGFR inhibition that is primarily responsible for the different side effects observed for Nimotuzumab and Cetuximab.

Such mechanistic and functional differences are important to clarify when evaluating the therapeutic potential of new drugs. Nimotuzumab is currently not approved by the FDA nor the EMEA, but is undergoing late clinical trials (see Table 1) and is approved by several countries for treatment of HNSCC and glioblastoma (117).

Trastuzumab

Trastuzumab is a humanized mAb that binds the extracellular region of ErbB2 at domain IV (149). It is approved by the FDA for treatment of metastatic breast cancer (see Table 1). Trastuzumab was also demonstrated to inhibit signaling pathways (PI3K), angiogenesis and cause cell cycle arrest (102, 150). It was suggested to also inhibit ErbB2 signaling by interfering with ErbB2-EGFR dimerization (151). In addition, the antibody causes ADCC (152).

Pertuzumab

ErbB2 overexpression provides strong growth advantage to tumor cells and promotes spontaneous formation of active ErbB2 homo- and hetero –dimers (31). The resulting signaling units are potent (90) and associated with poor clinical outcome (153). Besides Trastuzumab, several other ErbB2 targeting antibodies are being tested. Pertuzumab is a fully humanized mAb that binds domain II of ErbB2 (154). Many of the contact residues that Pertuzumab occludes are homologous to EGFR and ErbB3 residues necessary for receptor dimerization. Binding of Pertuzumab sterically prevents interaction between the dimerization arms (154). Pertuzumab therefore prevents ErbB2 from engaging in dimerization and signaling with the other ErbBs (155). It has also been suggested to affect ligand-independent dimerization in cancer cells overexpressing ErbB2 (156). The ability to initiate immune effector mechanisms is also important for the antitumor activity of Pertuzumab (157). Pertuzumab is currently in the end of a Phase III breast cancer trial. In combination with Trastuzumab and chemotherapy, it was demonstrated to significantly

extend the progression-free survival (158, 159). One possible contribution to the promising results of combining Trastuzumab and Pertuzumab could be enhanced down-regulation of ErbB2. As described above, two mAbs targeting distinct epitopes (Trastuzumab binding to domain IV and Pertuzumab binding to domain II), could form large receptor-antibody complexes that again could be internalized and degraded.

Tyrosine kinase inhibitors

TKIs are low molecular weight synthetic compounds that are designed to mimic ATP and to bind the kinase domain of ErbB proteins. These inhibitors compete with ATP binding and thereby block the magnesium-ATP-binding cleft, either reversibly or irreversibly. The outcome is block of signal transduction of both the MAPK and the PI3K pathways. Erlotinib and Gefitinib are anilinoquinazoline-based reversible inhibitors specific for EGFR, while Lapatinib is a thiazolylquinazoline-based reversible inhibitor with dual specificity (for EGFR and ErbB2 (3)). The quinazoline AG1478 is another TKI that could have therapeutic potential (160, 161). It binds to EGFR and has been shown to constitutively inhibit the active EGFR kinase (162, 163).

Heat shock protein (HSP) inhibitors

Another class of anti-ErbB agents is the HSP inhibitors. HSPs are molecular chaperones that are necessary for the conformational folding and maturation of various proteins. ErbB2 is a client of the chaperone HSP90 which recognizes the receptor as hyperactive and conformationally unstable. It stabilizes the active conformation of ErbB2 at the plasma membrane. HSP90 stabilizes the mature form of the receptor, probably by stabilizing its association with the kinase domain (164, 165). Disrupted interaction with HSP90 leads to endocytosis and down-regulation of ErbB2 (166, 167). The naturally occurring Ansamycin Geldanamycin, has been found to interrupt the interaction of HSP90 with ErbB2 by association with the ATP/ADP-binding pocket of HSP90. It then adopts a shape similar to that of ADP and prevents the chaperone from adopting its stabilizing conformation. Since Geldanamycin is toxic and displays chemical instability, the less toxic derivative, 17-AAG, was developed, and is currently in clinical trials (168). 17-AAG induces degradation of ErbB2, growth arrest and apoptosis (169). The exact mechanism involved in HSP90 disruption of ErbB2 degradation is not yet clear. It is

however well established that Geldanamycin or 17-AAG treatment leads to ubiquitination of ErbB2 (170, 171). ErbB2 may then undergo clathrin-dependent endocytosis and be routed to lysosomes for degradation (172). The Ub ligases CHIP (carboxyl terminus Hsc70-interacting protein) and/or Cullin5 are probably involved in this process (173, 174). It is still debated whether the ubiquitination is required as a signal for receptor endocytosis, or whether it serves as a signal for proteasomal degradation of ErbB2.

Resistance to ErbB-targeted therapeutics

It should be noted that most anti-ErbB drugs show modest cancer cell toxicity when administered alone (monotherapy) (reviewed in (1)). Instead, their antitumor efficiency becomes evident when combined with the classical cancer treatment modes (combination therapy). They sensitize tumors to cytotoxic agents or to radiotherapy by several functions (reviewed in (3)); blocking prosurvival pathways and activation of autocrine loops which may otherwise enable micrometastases to develop clones resistant to radio-/chemo-therapy (92), inducing cell cycle arrest, (140, 175), preventing cancer cells from evading apoptosis by inhibition of anti-apoptotic signaling (176), and sensitize cancer cells to radiotherapy by suppressing radiation-induced DNA repair (134).

Several mechanisms are responsible for the low responsiveness to ErbB targeting drugs as monotherapy (1, 28, 177). Aberrant signaling due to intrinsic or acquired mutations mediates resistance to ErbB antagonists. K-RAS mutations cause resistance to Cetuximab, Panitumumab, Gefitinib and Erlotinib due to constitutive activation of the MAPK pathway independently of EGFR (1). An acquired mutation in the EGFR kinase domain (T90M) causes a conformational change in the ATP-binding cleft, interfering with binding of Erlotinib and Gefitinib (3). This again leads to resistance in patients that initially respond to the TKIs.

An additional mechanism that explains resistance is positive and negative feedback regulation. Cancer cells can resist an ErbB specific inhibitor by activation of positive feedback loops, including up-regulation of an alternative ErbB member or initiation of autocrine loops. By doing so they compensate for the signaling dependency of one ErbB protein, to which the drug is targeted, via activation of alternative ErbB

dimers. A recent study reported that resistance to Cetuximab can be acquired through increased ErbB2 signaling (178). The cancer cells bypass EGFR by amplification of ErbB2 or up-regulation of heregulin, which can result in increased ErbB2-ErbB3 heterodimerization. The authors further demonstrated that colorectal cancer patients that have acquired resistance to Cetuximab have ErbB2 amplification or high levels of heregulin. Nonfunctioning of negative feedback loops is another way to overcome the effect of inhibitors. Endocytosis, a major negative feedback regulatory mechanism that normally prevents excessive signaling, is often defective in cancer (reviewed in (114)). Cancer cells can also negatively affect endocytosis of ErbB proteins by ErbB2 overexpression. As previously described, ErbB2 is endocytosis deficient and promotes spontaneous heterodimerization upon overexpression. The resulting dimers are retained at the plasma membrane and cause increased proliferative signaling.

The recent insight into how cancer cells resist ErbB targeted therapeutics explains the need for new strategies (3). One emerging strategy is to simultaneously target multiple receptors and pathways. This can be obtained by using combinations of anti-ErbB mAbs and/or TKIs (28, 114). An obvious drawback is the limited knowledge and availability of inhibitors to ErbB3 and ErbB4, when compared to the more well characterized inhibitors of EGFR and ErbB2 (see Table 1).

Dimerization between ErbB3 and ErbB2 is believed to form the strongest signaling unit of all ErbB pairs (90). ErbB3 is expressed in breast and ovarian tumors and is implicated in the tumorigenesis of lung and prostate tumors (reviewed in (102)). It is involved in both ligand-independent and ligand-dependent oncogenic signaling by interaction with ErbB2, EGFR and/or heregulin (179). However, no therapeutics that specifically target ErbB3 have yet been approved for clinical use. The lack of ErbB3 inhibition may contribute to the resistance to TKIs and mAbs that target EGFR and/or ErbB2 in lung and breast cancer cells (102). Activation of ErbB3 to compensate for EGFR/ErbB2 inhibition is another example of positive feedback regulation, representing major challenges for the use of drugs targeting single pathways..

ErbB4 is reported to be mutated in various carcinomas, including gastric, colorectal, NSCLC and breast cancers (reviewed in (91, 180)). ErbB4 is further reported to have oncogenic effects in malignant melanomas (180). However, ErbB4 expression in

breast cancer patients was demonstrated to be associated with favorable outcome (91). ErbB4 has also been suggested to function as a tumor suppressor in certain cancers. The contradictory roles of ErbB4 expression/activity may also be explained by the four alternatively spliced variants of ErbB4, with the four isoforms displaying different biological functions (91). A few experimental mAbs towards the ErbB4 isoforms have been developed and demonstrated to block cancer cell growth *in vitro*. However, the physiological and pathological role of ErbB4 needs further study.

AIMS OF THE STUDY

Members of the ErbB family of growth factor receptors are expressed in various tissues, where they mediate cellular proliferation, differentiation and migration during embryogenesis and adulthood. Receptor activation occurs via ligand binding and receptor dimerization, which leads to cellular responses by initiation of intracellular signaling cascades that control genetic expression. The process of ErbB activation is tightly regulated by both positive and negative feedback mechanisms. Upon activation, the receptors become subject to covalent modifications, including phosphorylation and/or ubiquitination, to precisely regulate the level of signaling. Endocytosis is an example of a negative feedback mechanism that primarily shuts down signaling by down-regulating the receptors. Aberrant ErbB activity has been linked to carcinogenesis and is associated with poor patient survival. EGFR is often mutated in glioblastomas or overexpressed in HNSCC, NSCLC, and ovarian cancer, while ErbB2 is found to be overexpressed in breast, ovarian, gastric, bladder, and other carcinomas (92). Mutations and overexpression of the ErbB proteins leads to elevated receptor activation by several mechanisms; increased ligand affinity, spontaneous dimerization in absence of ligand, and constitutive kinase activity. ErbB2 is particularly efficient in formation of spontaneous dimers upon overexpression. Since ErbB2 is considered to be endocytosis deficient (84), ErbB2 also increases receptor signaling by retaining dimerization partners at the plasma membrane (85). This is an example of how the feedback loops that control normal ErbB protein signaling are frequently dysregulated in cancers. Consequently, the ErbB proteins have become attractive molecular targets for drugs that specifically inhibit each of the events necessary for receptor activation. Several types of ErbB antagonists have shown promising *in vitro* effects by blocking receptor activation or inducing receptor down-regulation. However, most of them show limited *in vivo* anti-tumor effects when administered alone, and are therefore mainly used in combination with cytotoxic compounds or radiotherapy. This warrants the need for novel strategies that could increase the ability of existing drugs to down-regulate carcinogenic ErbB proteins. Three antibodies that target EGFR or ErbB2 are currently approved, and multiple new ones are being evaluated in clinical trials. Similarly, three kinase inhibitors that block EGFR

and/or ErbB2 are approved for treatment of metastatic cancers. A main problem of using such molecular targeted therapeutics is that systemic inhibition of EGFR produces toxic side effects. This has been proposed to be due to inhibition of the physiological role of the EGFR (28). Skin rash is the most common side effect of EGFR antagonists, but the extent of this varies between the employed inhibitors. Insight into mechanism underlying reduced systemic toxicity that has been observed for some of the novel anti-ErbB agents is necessary for evaluating the use of such new agents. Yet another category of anti-ErbB drugs are the HSP inhibitors, where the Geldanamycin derivate 17-AAG is in clinical trials. We have in the present study used monoclonal antibodies with the aim to compare the ability of two mAbs to counteract ligand binding and signaling of EGFR. Mechanism responsible for mAb-induced endocytic down-regulation of EGFR and investigations of how mAbs target ErbB2 and affect ErbB2 dimerization and endocytic down-regulation of EGFR should clearly be better defined. The role of ubiquitination in endocytosis and down-regulation of ErbB2 by HSP inhibitors, such as 17-AAG has to some extent been investigated, and it should be clarified whether ubiquitination itself is sufficient to induce endocytosis of ErbB2 and whether 17-AAG-induced down-regulation of ErbB2 is due to ubiquitination. In particular, the aim of the present study was set to investigate whether;

- Cetuximab differs from Nimotuzumab with respect to inhibition of EGF binding, EGFR dimerization and EGFR signaling in cells that overexpress EGFR.
- Cetuximab in combination with a secondary antibody can efficiently down-regulate EGFR by increased endocytosis in cells that overexpress the EGFR.
- Pertuzumab disrupts dimerization of ErbB2-EGFR heterodimers and consequently affects down-regulation of EGFR in cells overexpressing EGFR and ErbB2.
- Ubiquitination itself is sufficient to induce endocytic down-regulation of ErbB2, by using a chimeric preubiquitinated ErbB2 that is compared to wild-type ErbB2 targeted with 17-AAG.

SUMMARY OF PAPERS

Paper I

Nimotuzumab and Cetuximab block ligand-independent EGF receptor signaling efficiently at different concentrations

An antibody depends on sufficient binding affinity/avidity to exert inhibition of cancer cell proliferation. Effective binding may block ligand binding and/or receptor dimerization, induce receptor down-regulation by endocytosis and activate ADCC. The two monoclonal antibodies Nimotuzumab and Cetuximab both bind the extracellular region of EGFR and inhibit kinase activation by blocking ligand binding. Previous studies had demonstrated that Nimotuzumab has lower binding affinity than Cetuximab, and this was proposed to explain the low clinical side effects observed for Nimotuzumab, which provokes a lower degree of skin rash, a typical marker for systemic inhibition of EGFR signaling. In addition to the different binding affinities, a structural difference was recently suggested to be responsible for the different *in vivo* effects, arguing that both antibodies block ligand binding efficiently, but that Nimotuzumab does so while permitting the active receptor conformation and thereby allowing a basal level of downstream signaling. In ***paper I*** we investigated if Nimotuzumab functionally differs from Cetuximab in cells overexpressing EGFR. The antibodies were compared with respect to their ability to block EGF binding, EGFR-ErbB2 dimerization, and downstream Erk activation. Our data showed that Nimotuzumab inhibits basal EGFR dimerization as well as downstream signaling, but only at concentrations above those needed in case of Cetuximab. The different concentrations required corresponded to the previously described differences in binding affinity, again supporting the view that it is the low binding of Nimotuzumab that is responsible for the small side effects and not the difference in the ability to inhibit basal level of dimerization and signaling.

Paper II

Cetuximab in combination with anti-human IgG antibodies efficiently down-regulates the EGF receptor by macropinocytosis

Cetuximab increases the antitumor efficiency of radiotherapy and chemotherapy and is approved for combination therapy in colorectal cancer and head and neck squamous-cell carcinomas. Cetuximab binds specifically to EGFR and inhibits binding of ligands to the EGFR. An additional mechanism of action that has been demonstrated for Cetuximab is induction of EGFR endocytosis. The antibody-induced internalization is slow, and only small amounts of receptor are degraded. Recently, several studies have focused on enhancing antibody induced down-regulation of ErbB proteins by combination with other monoclonal antibodies. This was shown to synergistically inhibit tumor growth. However, the effect that a combination of antibodies has on EGFR activation, endocytosis, trafficking and degradation remains unclear. In **paper II** we tested whether a secondary antibody that binds Cetuximab increased Cetuximab-induced endocytosis of EGFR in cells overexpressing the receptor. The combination of antibodies resulted in efficient EGFR degradation, which was more rapid when compared to degradation by high levels of EGF. The antibodies induced EGFR phosphorylation and ubiquitination, but kinase activity was not required for antibody-induced internalization of the EGFR. In contrast to EGF, the antibody combination induced EGFR down-regulation in absence of Erk activation. Furthermore, internalization induced by the antibodies was dependent on actin, but not clathrin and dynamin. We demonstrated that the internalization could be blocked upon disruption of actin filaments by using latrunculin B or by the macropinocytosis inhibitor amiloride. In conclusion, we demonstrate that a combination of antibodies can induce rapid and efficient endocytic down-regulation of EGFR by macropinocytosis, in the absence of proliferative signaling.

Paper III

Pertuzumab increases epidermal growth factor receptor down-regulation by counteracting epidermal growth factor receptor-ErbB2 heterodimerization

Overexpression of ErbB2 is frequently found in human breast cancer and other carcinomas. When overexpressed, ErbB2 promotes spontaneous formation of active ErbB2 heterodimers. The dimers mediate strong and uncontrolled signaling that is associated with poor patient survival. Our group has previously shown that ErbB2 retains EGFR at the plasma membrane. Pertuzumab is a humanized monoclonal antibody directed against ErbB2s dimerization arm. In ***paper III*** we studied the effect of Pertuzumab on EGFR-ErbB2 dimerization and on endocytic down-regulation of the EGFR. Our results showed that in cells over-expressing EGFR and ErbB2, we could detect formation of spontaneous ligand-independent EGFR-ErbB2 dimers as well as of ligand-induced EGFR-ErbB2 heterodimers. Interestingly, this dimerization was efficiently counteracted upon incubation with Pertuzumab. The amount of EGF-induced EGFR homodimers was increased upon treatment with Pertuzumab, and incubation with Pertuzumab increased ligand-induced internalization and degradation of EGFR. Altogether, this paper shows that Pertuzumab is a potent heterodimerization inhibitor, and that disruption of EGFR-ErbB2 dimers results in increased internalization and down-regulation of EGFR. Over time, this will probably decrease the level of signaling EGFR-ErbB2 complexes at the plasma membrane.

Paper IV

Preubiquitinated chimeric ErbB2 is constitutively endocytosed and subsequently degraded in lysosomes

Unlike the other ErbB proteins, ErbB2 is considered to be endocytosis deficient and is normally concentrated at the plasma membrane. Stabilization of ErbB2 at the plasma membrane is believed to be the result of a stable interaction with HSP90. Incubation with

the HSP90 inhibitor 17-AAG interrupts the association between HSP90 and ErbB2, and it is well established that 17-AAG treatment leads to ubiquitination and efficient down-regulation of ErbB2. Whether ubiquitination itself is sufficient to induce internalization and degradation of ErbB2 is not yet clear. Our group has recently demonstrated that polyubiquitination of EGFR is sufficient to promote constitutive endocytosis of EGFR. To investigate whether Ub directs internalization and degradation of ErbB2, we have in *paper IV* constructed and used a chimeric preubiquitinated ErbB2 containing full-length ErbB2 and a C-terminally appended tetra-Ub chain (ErbB2-Ub₄). In contrast to wild-type ErbB2, the fusion protein was found to be constitutively endocytosed without the need for 17-AAG. We further showed that internalization of the ErbB2-Ub₄ construct depended on clathrin, and overexpression of truncated versions of the clathrin adaptor proteins epsin1 and Eps15 reduced internalization. The construct was observed to be constitutively modified with both Lys63- and Lys48-linked polyUb chains which are in several cases functional signals for endocytosis and degradation, respectively. Like for 17-AAG- treated ErbB2, ErbB2-Ub₄ was spontaneously internalized into early endosomes, followed by routing to late endosomes and lysosomes. Degradation was confirmed, and we demonstrated that the ubiquitinated protein was more efficiently degraded than was wild-type ErbB2. Our data argue that ErbB2 is generally localized to the plasma membrane, but that ubiquitination is sufficient to induce clathrin-dependent endocytosis and lysosomal degradation of the ErbB2.

METHODOLOGICAL CONSIDERATIONS

Cell lines

The present studies were performed in cultured cell lines. Cell lines are easy to work with, provide large variation regarding the type and expression levels of ErbB proteins, and are readily accessible for manipulation with drugs and various reagents. Cell lines are susceptible to mycoplasma infection and accumulation of genotypic or phenotypic changes, and due to this, our cell lines were routinely analyzed and kept in culture for a limited number of passages. The use of cell lines in cancer research is based on the assumption that they to some extent are similar to both nonperturbed and neoplastic cells. Cultured cells require serum, since they are not supplied by blood. High interstitial fluid pressure in solid tumors represents a barrier for delivery of high molecular weight compounds (181). This applies to therapeutic antibodies, and especially to a combination of antibodies that would further increase their molecular size (paper II). In addition, the *in vivo* environment might obscure binding of an antibody (e.g. anti-human IgG used in paper II) to tumor cells by containing antigens that are otherwise not present in cultured cells. Neoplastic cells are continuously supplied with growth factors by an extensive capillary system. Lack of this supply in cultured cell lines might be argued to contribute to an artificial system, especially when analyzing growth factor signaling. However, the possibility to remove growth factor ligands from cultured cells is necessary when the aim of the study is to detect changes in ligand-independent receptor interactions. In paper I, stably transfected porcine aortic endothelial (PAE) cells were used since they do not express endogenous ErbB proteins. In addition, the cells were starved to remove ligands and terminate further signal-transduction initiated by ligands in the medium. However, we can not be sure if the cells are secreting autocrine factors after starvation. The use of cells expressing only EGFR (PAE.EGFR) enabled us to study the effect of Cetuximab and Nimotuzumab on ligand-independent receptor dimerization and signaling that otherwise could be masked by the presence of ErbB ligands or other ErbB proteins. For the same reason, it can be argued that the lack of endogenous proteins and/or high expression levels of receptors could lead to results that would normally not be observed

in human cancer cells overexpressing ErbB proteins. However, the PAE cell lines used in our studies are well characterized, and they stably express EGFR at a high level and demonstrate activation and internalization of EGFR comparable to that of human cells endogenously expressing the EGFR (182, 183).

Transient transfection

An additional advantage of using isolated cells or cell lines is the ability to easily introduce and investigate modified versions of proteins, including mutant versions or protein domains, by transient transfection. In paper IV, we based our study on ErbB2 which was modified with a linear chain of four ubiquitins. The ErbB2-Ub₄ construct was transiently expressed in PAE cells prior to each experiment. One limitation to this approach is that transient transfection can result in very high expression of the encoded protein. ErbB2 homodimerization is suggested to be density dependent (184), and high ErbB2 expression may induce artificial receptor interactions which could again lead to enhanced internalization. The internalization of ErbB2-Ub₄ was always compared with transiently transfected wild-type ErbB2. However, transfection with wild-type ErbB2 could have resulted in higher ErbB2 expression levels, as compared to ErbB2-Ub₄, since wild-type ErbB2 is believed to be endocytosis resistant. An additional limitation is that it is difficult to secure constant expression levels, since protein expression upon transient transfection varies from experiment to experiment.

Small interfering RNA

Small interfering RNA (siRNA) is a widely used tool to suppress the expression of a protein and thereby study the effect of incubation with or without the protein of interest. Besides down-regulating the expression of a certain protein, introduction of RNA may produce nonspecific side effects in the cell. This is why we always compared the effect of clathrin knock-down with control cells that were transfected with scrambled negative control siRNA having similar length and identical concentration to the siRNA against clathrin heavy chain.

Immunological methods

Several immunologically based methods were used to detect changes in ErbB protein levels, ErbB protein interactions, ErbB protein -modifications, and ErbB protein-localization. These methods rely on recognition of the antibody used towards the antigen of interest. One problem that is frequently encountered is the misleading information on specificity of an antibody that is provided by manufacturers. This is particularly problematic when making conclusions about a specific protein that has high sequence similarity to other proteins, such as ErbB proteins. Antibodies that are assumed to bind one specific ErbB protein usually recognize other ErbB proteins additionally. All anti-ErbB antibodies that were used for western blotting were tested for specificity by detecting immunoreactivity in cells that expressed other ErbB proteins than the antibody-targeted receptor. In particular, the lack of antibody specificity towards EGFR limited our options in paper I to directly study EGFR dimerization upon cross-linking EGFR and ErbB2. Isolation of dimers was achieved by immunoprecipitation. The immunoprecipitation was based on linking antibodies to pre-coupled protein A or protein G magnetic beads. To confirm that the antibody was precipitating the antigen and not the beads themselves by non-specific interactions, a sample with only beads and no antibody was included as negative control. Cross-linking of proteins with the non-cleavable chemical BS³ was used in paper I and III to identify interactions between ErbB proteins upon dimerization. One pitfall that is important to be aware of when using BS³ is that it binds all cell surface proteins since BS³ is membrane impermeable. BS³ non-specifically links all amine-containing targets. This also includes anti-ErbB antibodies, which were added to cells before incubation with BS³ and could have created receptor-antibody complexes at the plasma membrane. If the antibody-receptor complex was too large to enter a 6% gradient gel, this could explain the reduction of 380 kDa bands upon antibody stimulation, as seen in paper I and III. However, even two ErbB proteins in complex with one IgG antibody should be small enough in size to enter a 6% gel, and no bands above 380 kDa were detected during these experiments.

Confocal microscopy was used to detect the cellular localization of ErbB proteins, ligands, and/or anti-ErbB antibodies. This provides the possibility to identify a detailed

localization of proteins in single cells. However, cell-to-cell variations are frequent, and it is important to objectively analyze a large number of cells in a sample before preparing a representative image. To identify the nature of compartments containing the protein of interest, double staining was used, and colocalization was investigated. Several considerations must be kept in mind when using combinations of primary and secondary antibodies for analysis of colocalization. A merged image must be generated from two images taken in the same z-plane. Even so, a protein may appear to be localized within an intracellular compartment if it displays strong plasma membrane fluorescence in the same z-plane as the intracellular vesicle, and care should be taken when interpreting colocalization images. Choosing the right antibody combinations and the correct instrument settings are crucial to avoid cross excitation and bleedthrough. One obvious requirement is to use primary antibodies of different species, to prevent cross-reactions. To check for cross-reactions of secondary antibodies in double stained samples, a negative control lacking one of the primary antibodies was always included for each new antibody combination. Otherwise, antibody cross-reactions could result in overlapping fluorescence and mistakenly be interpreted as colocalization.

¹²⁵I-EGF binding assay

Use of iodinated EGF provides a sensitive way to quantitatively measure ligand-binding, -internalization, -recycling, and -degradation. 1 ng of ¹²⁵I-EGF was used to measure EGF-induced EGFR internalization. The 1 ng concentration is too low to saturate clathrin-dependent endocytosis (56), and this amount of EGF is therefore considered to be efficiently internalized.

Chemical inhibitors

Multiple inhibitors were used throughout this work to investigate the consequence of blocking the function of certain proteins. Although application of chemical inhibitors is a powerful and commonly used approach to achieve this, they often block more than one target and can be toxic at high concentrations and thereby produce nonspecific side effects. One example is the kinase inhibitor AG1478 which was used in paper I and II to block EGFR kinase activity. It competitively inhibits the ATP binding site in the kinase

domain and is considered to be highly potent and specific for EGFR (185). Although being specific towards an ErbB kinase domain, several studies have reported that it also inhibits ErbB2, and our group has recently noticed that AG1478 is also a potent inhibitor of the ErbB3 kinase activity (186). This would represent a problem when studying the effect of EGFR inhibition in cells also expressing other ErbB proteins. For the purpose of the present work, AG1478 was used in cells expressing EGFR only, and conclusions could be made since we only studied inhibition of EGFR and not the other ErbB members. For other experimental settings, this emphasizes that one should be cautious even when using inhibitors that have previously been reported to be specific. One possibility is to test for potential off-target effects. Although the proton pump inhibitor amiloride is reported to selectively block macropinocytosis (62), we, in paper II, also studied endocytosis of transferrin to confirm that amiloride at the concentration used, did not inhibit clathrin-dependent endocytosis.

GENERAL DISCUSSION

Effects of anti-EGFR antibodies on receptor dimerization and internalization

Nimotuzumab has shown promising antitumor efficiency both *in vitro* and *in vivo*, particularly in combination with chemo- and radiation –therapy (117, 142, 144). Comparative studies involving its counterpart, the worldwide approved Cetuximab, are important when evaluating the therapeutic potential of the antibody. Such studies may reveal molecular mechanisms that explain functional differences observed for these two mAbs in the clinic. The major argument in favor of Nimotuzumab is that it does not provoke severe skin toxicity (143). One explanation for this is that Nimotuzumab, in contrast to Cetuximab, blocks ligand binding while simultaneously permitting a basal level of EGFR signaling (145). However, our results show that, apart from having effect at different concentrations, the two antibodies did not appear to differ mechanistically with respect to inhibiting ligand-binding, blocking basal and ligand-induced dimerization and Erk activity (Paper I). This is in agreement with the reduced affinity of Nimotuzumab when compared to Cetuximab (144) and not the lack of inhibition of basal EGFR activation (145), that is responsible for the different *in vivo* effects. Indeed, the concentration of Nimotuzumab used in Paper I was increased compared to Cetuximab by a factor of ten, corresponding to differences in affinity. Our study was performed on cells expressing the intact receptor, and this might explain why the effects differ from those suggested by crystal structures of antibody/receptor fragments in solution. In contrast to our results, You *et al.* showed that treatment with Nimotuzumab did not significantly affect the level of EGFR, Erk, and Akt phosphorylation in tumor biopsies (187). However, as noted by the authors, the immunohistochemical staining intensities for these proteins were low and may not have been sensitive enough to detect small changes in the basal level of EGFR activity. In addition, the lack of EGFR signaling inhibition and the low anti-tumor activity in response to increasing amounts of Nimotuzumab could be attributed to the low affinity of the antibody.

A recent study showed that Cetuximab and Nimotuzumab differ not only in affinity, but also in avidity (188). In contrast to Cetuximab, Nimotuzumab requires bivalent binding and therefore a high level EGFR expression for stable attachment to cells. If bivalent binding did occur in our study, it could have been responsible for the observed disruption of active EGFR dimers since bivalent antibody binding has been shown to prevent receptor dimerization due to steric constraints on the receptor (189, 190). One potential difference between the PAE.EGFR cells used in paper I and cells used in other studies is the EGFR expression level. Only in cells with high (H125 cells) and very high (A431 cells) EGFR expression levels, i.e. in cells with an EGFR density at the plasma membrane high enough to allow bivalent binding did Nimotuzumab and Cetuximab bind with the same efficiency (188). Since we had to increase the concentration of Nimotuzumab to obtain equal inhibitory effects as for Cetuximab, this could suggest that the PAE.EGFR cells did not have high enough concentration of EGFR at the plasma membrane for Nimotuzumab to bind bivalently. PAE.EGFR cells have been well characterized and express EGFR at levels corresponding to H125 cells (182, 191). and the cells should therefore stably bind Nimotuzumab. However, it should be noted that receptor levels in these studies are measured per cell, and not per area. The surface areas of H125 and PAE cells have different EGFR densities even though the receptor expression levels per cell are similar. The lack of formation of large complexes in the size range of two receptors with one IgG antibody upon crosslinking, (see also Immunological Methods in the Methodological Considerations chapter) supports the notion that the EGFR density is too low to allow bivalent binding of anti-EGFR antibodies in PAE.EGFR cells.

Both antibodies are capable of eliciting ADCC (135, 142), which was not investigated in the present study due to methodological limitations. This is important to address when proposing explanations for functional differences between antibodies that share the same Ig subclass (IgG1), which is known to be the main activating class of ADCC (192).

Based on our *in vitro* data comparing Nimotuzumab and Cetuximab with respect to their capacity to inhibit ligand binding, receptor dimerization and downstream activation of Erk, we suggest the following model;

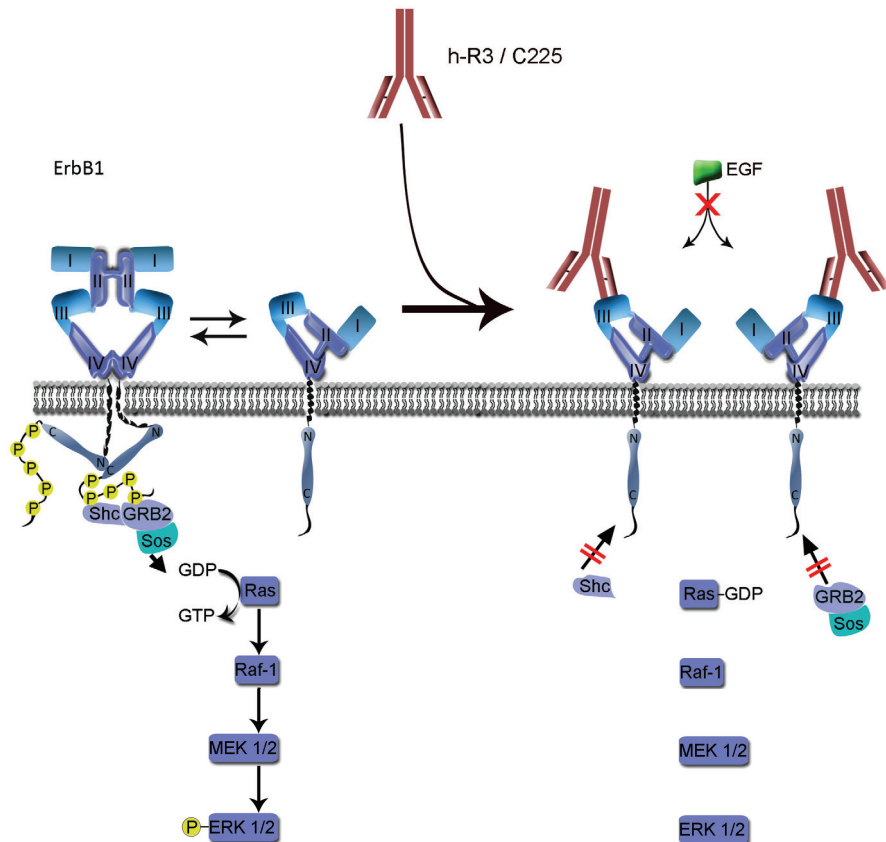


Figure 4. Effect of Nimotuzumab (h-R3) and Cetuximab (C225) on ErbB1 dimerization and signaling. In absence of ligand, ErbB1 can adopt the active receptor conformation and engage in dimers that mediate basal levels of ligand-independent downstream signaling (phosphorylation of Erk 1/2). Nimotuzumab and Cetuximab bind the ErbB1 domain III with different affinity and block ligand binding accordingly. Both antibodies inhibit ligand-dependent and ligand-independent ErbB1 dimerization by promoting the inactive receptor conformation. This reduces the basal level of Erk activity.

An additional mechanism of action that contributes to the antitumor activity of anti-ErbB mAbs is receptor endocytosis. The ability of Cetuximab and Nimotuzumab (data not shown for Nimotuzumab) to internalize and route EGFR was investigated, but no significant difference between the antibodies was observed, and it was difficult to make precise conclusions due to limited sensitivity of the internalization method that was

chosen. Instead, a new approach to increase the observed antibody-induced internalization was investigated in detail in Paper II.

Antibody-induced down-regulation by macropinocytosis

Cetuximab has previously been demonstrated to induce internalization of EGFR (130), which is considered an important mechanism that may contribute to antitumor activity if the receptor becomes efficiently down-regulated by degradation (1). However, Cetuximab-induced internalization is slow, and a fraction of EGFR can be routed to the nucleus instead of to lysosomes (131, 132), a process that is associated with negative outcome since nuclear EGFR has been associated with poor clinical outcome (133, 134). Combining Cetuximab with other mAbs is shown to efficiently down-regulate EGFR by inhibiting recycling (121). The mechanism of action is proposed to be antibody-induced clustering of the receptor at the plasma membrane followed by efficient internalization and degradation in lysosomes (114). mAb combinations are reported to significantly enhance the antitumor effect of Cetuximab *in vivo*, independently of functions mediated by the antibodies Fc regions (ADCC) (125).

In an attempt to increase Cetuximab-induced EGFR internalization, we did a sequential incubation with Cetuximab followed by an anti-human IgG antibody (Paper II). The antibody combination was observed to induce large clusters of EGFR at the plasma membrane, followed by rapid EGFR internalization and degradation. Internalized EGFR-antibody complexes localized to large early endosomal compartments, as confirmed by confocal- and electron –microscopy. The vesicle size corresponded to macropinosomes, since they are considerably larger than CCVs. This suggested that macropinocytosis was responsible for mediating EGFR-antibody internalization.

Macropinocytosis is a form of bulk uptake that non-selectively internalizes extracellular material. The fluid phase marker dextran was used to confirm that the antibody combination was internalized from the plasma membrane. Although often stated in the literature (193), dextran is not specific for macropinocytosis. This was noticed in our control experiments when observing vesicular colocalization of dextran and fluorescent EGF at concentrations typical for clathrin-dependent endocytosis (15 ng) (data not shown). After confirming that the antibody combination internalized EGFR

independently of clathrin and dynamin, we next used latrunculin B to investigate actin dependency. Inhibition of actin polymerization by latrunculin B resulted in efficient block of endocytosis, arguing that the internalization was actin- dependent and characteristic for macropinocytosis, since rearrangement of the actin cytoskeleton is required for internalization by macropinocytosis. To confirm this, we next used amiloride, which is considered to be a more selective blocker of macropinocytosis (65, 194). Incubation with amiloride also blocked antibody-induced EGFR internalization efficiently.

The antibody-induced vesicles resembled MVBs and were positive for both EEA1 and the late endosome/lysosome marker LAMP1, in accordance with degradation data. Some studies suggest that macropinosomes do not mature into late endosomes and MVBs (reviewed in (62)). EGF-induced macropinosomes in A431 cells are shown to be positive for EEA1 (63), but they are recycled to the plasma membrane instead of maturing into MVBs. Yet other studies, using other cell lines than A431, showed that EGF-induced macropinosomes undergo maturation into late endosomes and fuse with lysosomes (195). This is in agreement with our data, suggesting that macropinosomes undergo maturation. Sorting of EGF-EGFR complexes into MVBs is believed to depend on EGFR ubiquitination and interaction with ESCRT complexes on the limiting membrane of endosomes (196). We found that the antibody combination induced ubiquitination of the EGFR, and upon internalization, the EGFR-antibody complexes initially colocalized with EEA1 and later with LAMP1. Immuno-EM further demonstrated efficient sorting into MVBs, all strongly suggesting routing to late endosomes/lysosomes.

PI3K activity is suggested to be required for macropinocytosis, since PI3K activates GTPases that are involved in rearrangement of actin filaments. Amiloride is proposed to inhibit macropinocytosis by inhibiting these GTPases (194). We therefore investigated PI3K activity, but could not observe any activation of the downstream effector Akt (data not shown) upon treatment with the antibody combination, despite efficient inhibition of macropinocytosis by amiloride. Although the antibody combination was found to phosphorylate EGFR at several tyrosine residues, it did not induce downstream Erk activation. One possible reason for this is that activation at tyrosine residue 1173 in EGFR is known to recruit the tyrosine phosphatase SHP1 that can

attenuate ErbB-mediated Erk activity (33). Additionally, macropinocytosis was not affected when the kinase activity of EGFR was blocked. Our results thus suggest that other mechanisms than PI3K and EGFR kinase activity are involved in antibody-induced macropinocytosis. This is in agreement with previous reports showing that the murine version of Cetuximab in combination with other anti-EGFR mAbs down-regulate the EGFR without activation of the receptor or components of downstream signaling pathways (MAPK and PI3K) (121). One possible mechanism for antibody-induced macropinocytosis could be that internalization of EGFR depends on receptor interactions rather than kinase activity (197).

Altogether, our data show that a combination of antibodies can efficiently down-regulate EGFR by induction of macropinocytosis that culminates in lysosomal degradation of the cargo. The process is probably initiated via clustering of EGFR into large receptor-antibody complexes. The steric cross-linking by the antibodies is most likely responsible for the observed activation of EGFR, perhaps by orienting the receptor kinase domains into such close proximity that some asymmetric kinase interactions can take place. However, the internalization did not depend on this activation. Incubation with antibodies induced a considerably lower EGFR phosphorylation compared to incubation with high concentrations (60 ng) of EGF (data not shown). But antibody combination induced a much more rapid receptor degradation than did the ligand. EGF at high concentrations has been shown to induce macropinocytosis (65), at which point the clathrin-dependent endocytosis of EGFR is probably saturated in these cell lines (196). Both ligand- and antibody-induced degradation was similar upon incubation for 4 h, suggesting that whatever pathways were involved, they may have become saturated. As pointed out, the endocytic pathways are probably not mutually exclusive, and it can very well be that the antibody combination initiated several parallel routes, but primarily macropinocytosis since only inhibition of this pathway efficiently blocked the antibody-induced internalization.

Targeting the endocytosis deficient ErbB2

ErbB2 is normally located at the plasma membrane, where its overexpression is associated with spontaneous receptor dimerization (31) and poor clinical outcome (153).

One model suggests that the high surface expression of ErbB2 is due to increased recycling of the receptor when compared to the other ErbB members (87, 198). Another model argues that ErbB2 is endocytosis deficient, either due to the presence of signals that retain ErbB2 at the cell surface or the lack of signals that promote rapid internalization (83, 196). This model is supported by studies showing that internalization of EGFR is negatively affected upon heterodimerization with ErbB2 (83, 85). Interaction with HSP90 may also be responsible for retaining ErbB2 at the plasma membrane. The present work consistently supports the notion that ErbB2 is endocytosis deficient and thereby also negatively affects internalization of the EGFR. In paper III we show that Pertuzumab disrupts ligand-independent EGFR-ErbB2 dimers, counteracts ligand-induced EGFR-ErbB2 dimerization and thereby increases EGF-induced internalization and degradation of EGFR. Our data confirm that Pertuzumab did not affect recycling of EGFR in these cells, but that it rather prevents EGFR from dimerizing with ErbB2 at the plasma membrane. Eventually, Pertuzumab will probably reduce the sustained proliferative signaling emerging from EGFR-ErbB2 complexes at the plasma membrane. These results contribute to the understanding of mechanisms involved in Pertuzumab activity.

Different concentrations of EGF were used throughout these experiments, ranging from 1 ng/ml for the internalization assay to 60 ng/ml for the recycling and degradation assays. As pointed out above, different ligand concentrations could induce different endocytic pathways varying in extent and efficiency of internalization.

In paper IV, no endocytosis of wild-type ErbB2 could be observed unless the cells were incubated with 17-AAG, supporting the view that ErbB2 is endocytosis deficient. It has been proposed that ErbB2 is normally endocytosed and recycled, but that 17-AAG down-regulates surface ErbB2 by increasing degradative sorting rather than increasing endocytosis (199). However, our confocal experiments could not detect internalized ErbB2 in absence of 17-AAG. It should be noted that wild-type ErbB2 was found to be constitutively degraded when cells were incubated with cycloheximide, suggesting that some internalization of wild-type ErbB2 occurred although ErbB2 could not be detected in endosomes. As previously suggested, this could be due to high expression of ErbB2

when using transient transfections. This could potentially induce artificial receptor interactions and result in internalization.

It is well established that 17-AAG treatment leads to ubiquitination and down-regulation of ErbB2 (200). It is also suggested that fragmentation of ErbB2, possibly as a result of ubiquitination, precedes its degradation (199). Whether cleavage is required for endocytosis of ErbB2 is not clear (166, 201), and our group has previously demonstrated that geldanamycin induces endocytosis and lysosomal degradation of full-length ErbB2 (172). This is in accordance with our current results, showing that C-terminal cleavage is not required for endocytosis of ErbB2 (Paper IV). Furthermore, we show that ubiquitination itself is sufficient to induce internalization and degradation of ErbB2. This is in accordance with a previous paper from our group where the same type of ubiquitin chain was found to induce internalization of the EGFR (76). Our unpublished data show that, as for ErbB2-Ub₄, the appended ubiquitin chain was also sufficient to induce degradation of EGFR-Ub₄ (data not shown). However, in contrast to EGFR-Ub₄, ErbB2-Ub₄ was found to be modified by both Lys48- and Lys63-linked Ub chains. Furthermore, wt ErbB2 was shown to be ubiquitinated on Lys48- and Lys63 upon incubation with 17-AAG. A recently published study has demonstrated that Lys48- and Lys63-linked polyubiquitination is important for trafficking and degradation of ErbB2 (77). Whether it is the appended ubiquitin chain itself, or the induced polyubiquitination that is responsible for internalization and degradation of ErbB2-Ub₄ is therefore not clear. One limitation is that ErbB2-Ub₄ may not be biologically relevant. The patterns of poly-Ub chains that are generated naturally by cells are probably different from the appended tetra-Ub chain in the ErbB2-Ub₄ construct. However, our results clearly show that a linear chain of four ubiquitins is sufficient to induce events leading to clathrin-dependent and kinase-independent endocytosis and subsequent degradation of ErbB2.

ErbB2 is probably endocytosis deficient due to stable association with Hsp90. 17-AAG targets HSP90, and our results show that in contrast to ErbB2-Ub₄, wild type ErbB2 can only be internalized upon incubation with 17-AAG, further suggesting that ErbB2 is endocytosis deficient upon association with HSP90. Our results also show that endocytosis of ErbB2-Ub₄ increases upon incubation with 17-AAG, suggesting that HSP90 can stabilize also ErbB2-Ub₄ at the plasma membrane. Incubation with 17-AAG

disrupts the stabilization of ErbB2 and enhances ErbB2-Ub₄ internalization, probably by inducing further ubiquitination of the receptor.

The co-chaperone of HSP90, HSP70, is known to be involved in internalization, lysosomal delivery and degradation of client proteins (202), including plasma membrane-localized ErbB2 (200). HSP70 was stably associated only with ErbB2-Ub₄, but inefficiently associated with wild type ErbB2 (data not shown), suggesting that this co-chaperone more efficiently recognized the appended Ub chain both in the absence and presence of 17-AAG. Co-chaperones have been demonstrated to facilitate internalization and degradation of target proteins (202). This suggests that ErbB2 is endocytosis-resistant when associated with Hsp90, but not with Hsp70. 17-AAG has previously been shown to induce exchange of Hsp90 by Hsp70, further inducing ubiquitination of ErbB2 by CHIP (200, 203).

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Paper I

Christian Berger, Ute Krengel, Espen Stang,
Ernesto Moreno, and Inger Helene Madshus.

Nimotuzumab and cetuximab block ligand-independent EGF
receptor signaling efficiently at different concentrations.

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Paper II

Christian Berger, Inger Helene Madshus, and Espen Stang.

Cetuximab in combination with anti-human IgG antibodies efficiently
down-regulates the EGF receptor by macropinocytosis.

Experimental Cell Research (In press)

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Cetuximab in combination with anti-human IgG antibodies efficiently down-regulates the EGF receptor by macropinocytosis

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Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; CHC, clathrin heavy chain; CLIC: Clathrin-independent carrier; EGFR, epidermal growth factor receptor; EEA1, early endosome antigen 1; C225, ESCRT: endosomal sorting complex required for transport; Cetuximab; mAbs, monoclonal antibodies; MVBs, multivesicular bodies; PAE cells, Porcine Aortic Endothelial cells; RT, room temperature

Abstract

The monoclonal antibody C225 (Cetuximab) blocks binding of ligand to the epidermal growth factor receptor (EGFR). In addition, it is known that incubation with C225 induces endocytosis of the EGFR. This endocytosis has previously been shown to be increased when C225 is combined with an additional monoclonal anti-EGFR antibody. However, the effects of antibody combinations on EGFR activation, endocytosis, trafficking and degradation have been unclear. By binding a secondary antibody to the C225-EGFR complex, we here demonstrate that a combination of antibodies can

efficiently internalize and degrade the EGFR. Although the combination of antibodies activated the EGFR kinase and induced ubiquitination of the EGFR, the kinase activity was not required for internalization of the EGFR. In contrast to EGF-induced EGFR down-regulation, the antibody combination efficiently degraded the EGFR without initiating downstream proliferative signaling. The antibody-induced internalization of EGFR was found not to depend on clathrin and/or dynamin, but depended on actin polymerization, suggesting induction of macropinocytosis. Macropinocytosis may cause internalization of large membrane areas, and this could explain the highly efficient internalization of the EGFR induced by combination of antibodies.

Key words : EGFR down-regulation, macropinocytosis, Cetuximab, signaling

Introduction

The ErbB family consists of four closely related members: epidermal growth factor (EGF) receptor (EGFR, also known as ErbB1 or HER1), ErbB2 (HER2/Neu), ErbB3 (HER3) and ErbB4 (HER4). Overexpression of the EGFR and/or its ligands is associated with increased cellular proliferation and resistance to apoptosis in a number of human epithelial cancers, such as head and neck-, ovarian-, cervical-, gastric-, colorectal- and breast cancer [1], and several new treatment modalities targeting the EGFR are in preclinical and clinical trials. Important approaches in neutralizing EGFR signaling are tyrosine kinase inhibitors and monoclonal antibodies (mAbs). While kinase inhibitors mimic ATP and bind to the intracellular kinase domain, antibodies target the extracellular part of the receptor, thereby preventing ligand binding, conformational activation and/or receptor dimerization [2-4]. Activating ligands bind to domains I and III within the extracellular region of the EGFR. These domains are β helix leucine rich repeat (LRR)-

like domains of ~160 amino acids each [5]. Ligand binding induces EGFR dimerization and activation, as well as endocytosis and down-regulation by both clathrin-dependent and clathrin-independent pathways (reviewed in [6]). A number of different clathrin-independent endocytic pathways have been identified, including among others, caveolin- and flotillin-associated endocytosis, clathrin- and caveolae-independent endocytosis, the CLIC/GEEC (clathrin-independent carrier/glycosylphosphatidylinositol-anchored protein-enriched endosomal compartments) pathway, and macropinocytosis (for recent reviews see [7-12]). Macropinocytosis is readily induced upon activation the EGFR as well as other members of the ErbB family [6, 9, 12-14], and does like CLICs [15], account for a major uptake of fluid and turnover of plasma membrane. However, while the CLIC pathway is constitutive [15], macropinocytosis is a signal dependent process [11, 12].

Several anti-EGFR mAbs have been approved for cancer treatment, and C225 (Cetuximab, Erbitux), which is a human/mouse chimeric antibody, has proven clinically effective, especially when combined with chemotherapy or radiation [16]. C225 binds to domain III of the EGFR and inhibits binding of ligand [17]. C225 also has cytotoxic effect by inducing antibody-dependent cellular cytotoxicity (ADCC) and can counteract tumor growth through several different mechanisms (reviewed in [3, 16, 18]). One important way to counteract carcinogenesis is induction of receptor down-regulation. The mouse anti-EGFR antibody mAb-225, from which C225 was engineered, has been shown to induce endocytosis of the EGFR [19, 20]. The endocytic down-regulation was strongly increased when mAb-225 was combined with another noncompetitive anti-EGFR antibody [21]. It should however also be recognized that C225 has been shown to induce

nuclear localization of the EGFR [22], a condition often associated with poor clinical outcome (reviewed in [23]).

Combination of antibodies can result in formation of large receptor-antibody complexes at the cell surface. The rate of endocytosis of antibody-receptor complexes has been proposed to be proportional to the size of the complex [24], and incubation with a combination of antibodies has furthermore been shown to increase EGFR down-regulation due to inhibited endosomal recycling of the receptor [21]. We have in the current study investigated how C225 alone and in combination with a polyclonal anti-human IgG antibody internalized and degraded the EGFR. Our present results suggest that the anti-human IgG antibody efficiently enhanced C225-induced endocytosis of the EGFR. Furthermore, the antibody combination used was found to internalize the EGFR by macropinocytosis and to result in efficient EGFR degradation.

Materials and Methods

Materials

Human recombinant EGF was from Bachem (Budendorf, Switzerland). Mouse ¹²⁵I-EGF was from Perkin-Elmer, Inc. (Waltham, MA, USA). Alexa 647-conjugated EGF, lysine-fixable Oregon Green 488-conjugated dextran (MW 10 kD), Rhodamine-conjugated Phalloidin, TO-PRO-3 and AG1478 were from Life Technologies Ltd (Paisley, UK). PD153035 was from Tocris Bioscience (Ellisville, MO, USA). Protein G-coupled magnetic beads were from Invitrogen (Carlsbad, CA, USA). All other reagents were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted.

Antibodies

Cetuximab (C225, Erbitux) was from Merck KGaA (Darmstadt, Germany). Goat anti-early endosomal antigen 1 (EEA1) and mouse anti-ubiquitin antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit anti-donkey IgG, anti-hemagglutinin (HA) tag, anti-LAMP1, anti-KDEL and anti-tubulin antibodies were from Abcam plc (Cambridge, UK). Sheep anti-EGFR antibody (to the intracellular part) was from Fitzgerald Industries International, Inc. (Acton, MA, USA). Rabbit anti-phospho-Erk (pThr202/pTyr204) and mouse anti-phospho-EGFR (pTyr1068) antibodies were from Cell Signaling Technology, Inc. (Danvers, MA, USA). Mouse anti-phospho-EGFR (pTyr1173) antibody was from Millipore (Billerica, MA, USA), and mouse anti-clathrin heavy chain (CHC) antibody was from BD Biosciences (Heidelberg, Germany). Non-conjugated rabbit anti-sheep IgG, Rhodamine Red-X-conjugated donkey anti-human IgG, peroxidase-conjugated donkey anti-rabbit -, anti-mouse, anti-sheep -, Alexa Fluor 488-conjugated donkey anti-goat -, Cy2-conjugated donkey anti-rabbit -, Cy2-conjugated

donkey anti-sheep -, Cy5-conjugated donkey anti-sheep - and Rhodamine Red-X-conjugated donkey anti-rabbit IgG antibodies were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Alexa Fluor 647-conjugated donkey anti-goat, Alexa Fluor 647-conjugated donkey anti-rabbit and Alexa Fluor 488-conjugated goat anti-rabbit antibodies were from Life Technologies Ltd.

Cell Culture and Treatment

Porcine Aortic Endothelial cells stably transfected with a plasmid encoding human wild-type EGFR (PAE.EGFR) was provided by Alexander Sorkin, University of Pittsburgh, USA. These cells (expressing $1 - 4 \times 10^5$ receptors) [25] were grown in Ham's F-12 medium (Lonza, Basel, Switzerland) supplemented with 10% (vol/vol) fetal bovine serum, 0.5x penicillin-streptomycin mixture (Lonza) and 400 µg/ml G418 sulphate (Invitrogen). A431 cells from the American Type Culture Collection (Rockville, MD, USA) were grown in Dulbecco's modified Eagle's medium (DMEM) (sodium bicarbonate, 3.7 g/l, and high glucose, 4.5 mg/l) (BioWittaker, Walkersville, MD, USA) containing 10% (vol/vol) fetal bovine serum, 2 mM L-glutamine (Lonza) and 0.5× Penicillin-Streptomycin mixture (Lonza). HeLa cells and MCF-7 cells were grown in DMEM with 2 mM L-glutamine. C225, anti-human IgG, AG1478, PD153035, latrunculin B, amiloride, Oregon Green 488-conjugated dextran, Alexa 647-conjugated EGF and/or nonlabelled EGF were added to cells in minimal essential medium (MEM) (Life Technologies Ltd) without bicarbonate and with 0.1% (wt/vol) bovine serum albumin (BSA). For treatment with C225 only, cells were incubated with C225 (5 µg/mL) for 30 min on ice, washed with ice-cold phosphate-buffered saline (PBS) and chased at 37°C in MEM [0.1% (wt/vol) BSA] for the indicated time periods. For incubation with

antibody combinations, cells were first incubated with C225 (5 $\mu\text{g/mL}$ in all experiments except for experiments presented in Fig. S9) for 30 min on ice, washed with ice-cold PBS and subsequently incubated with anti-human IgG (25 $\mu\text{g/mL}$) for 30 min on ice before being washed with ice-cold PBS and chased at 37°C in MEM [0.1% (wt/vol) BSA]. Control cells were incubated as described above with MEM [0.1% (wt/vol) BSA] only in the absence of antibodies. In experiments investigating degradation, the cells were incubated and chased in MEM [0.1% (wt/vol) BSA] with 25 $\mu\text{g/ml}$ cycloheximide (CHX).

Transfection of cells

To knock down CHC, PAE cells were transfected twice with siRNA with a 48 hours interval using Lipofectamine 2000 (Life Technologies Ltd) according to the manufacturer's recommendations. The target sequence GCAAUGAGCUGUUUGAAGA [26] was synthesized by Life Technologies Ltd. Control cells were transfected with Silencer Negative control #1 from Applied Biosystems (Carlsbad, CA, USA). The pcDNA3.1-HA-Dynamin1-K44A plasmid (encoding K44A dynamin lacking GTPase activity) was provided by Professor Sandra Schmid, and cells were transfected using Lipofectamine 2000 according to the manufacturer's recommendations. Cells were analyzed approximately 20 hours upon transfection.

Western blotting

Cells were washed 3 times with ice-cold PBS before being lysed and subjected to SDS-PAGE and immunoblotting as previously described [27]. Proteins were detected using Pierce SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) and a

Kodak Image Station 4000R (Carestream Health, Inc., Rochester, NY, USA). All experiments were repeated three times.

Internalization of ^{125}I -EGF

Upon incubation of cells, as described in figure legends, internalization of ^{125}I -EGF was measured essentially as previously described [28]. Internalized ^{125}I -EGF was estimated as the ratio of internalized to surface-localized cpm.

Immunoprecipitation

Upon incubation as described in figure legends, cells were lysed in preheated (100 °C) 1% SDS in PBS, incubated at 100°C for 5 min and chilled on ice before homogenization, using a QIA-shredder column (QIAGEN, Valencia, CA, USA). To precipitate the EGFR, sheep anti-EGFR antibody was first coupled to protein G-coupled magnetic beads, in 0.1 M phosphate buffer, pH 8, with 0.05% Triton X-100, at room temperature for 1 h. Antibody-coupled beads were dissolved in 2x immunoprecipitation (IP) buffer: 2% (vol/vol) Triton X-100, 0.5% (wt/vol) sodium deoxycholate, 1% (wt/vol) BSA, 2 mM EDTA, 40 mM NaF, 6 mM NEM, 1:100 (vol/vol) protease inhibitor and phosphatase inhibitor cocktail (P8340 and P5726) before the antibody-coupled beads and cell lysates were gently mixed for 1 h at 4°C. The beads were then washed with 1x IP buffer (50% 2x IP buffer + 50% SDS [1%] in PBS) and eluted in 2x sample buffer [27]. The immunoprecipitated EGFR and total cell lysates (TCL) were subsequently subjected to SDS-PAGE and immunoblotting.

Immunocytochemistry and confocal microscopy

Upon incubation with the indicated reagents, cells grown in 60 mm dishes (Sarstedt AG & Co., Nümbrecht, Germany) were washed once with PBS and fixed with ice-cold

ethanol for 10 min or 4% paraformaldehyde (PFA) (Reidel-de Haën, Seelze, Germany) in Soerensen's phosphate buffer for 10 min at room temperature (RT). Cells were then washed 3 times with PBS before anti-quenching with 50 mM NH_4Cl for 10 min at RT and washing twice with PBS. PFA-fixed cells were permeabilized using Triton X-100 (0.1% vol/vol in PBS) for 10 min at RT. During incubation with the anti-LAMP1 antibody, 0.1% (vol/vol) Saponine (Merck KGaA) was used for permeabilization. Saponine was also included in buffers used in the following labeling steps. Cells were washed with PBS and blocked with BSA (1% wt/vol in PBS) for 30 min before incubation with primary antibodies for 1 h at RT. Samples were washed 3 times with PBS before incubation with secondary antibodies for 30 min at RT. For actin staining, cells were incubated with 33 nM Rhodamine-conjugated Phalloidin in PBS for 30 min at RT following immunolabeling. Nuclei were stained with 0.5 μM TO-PRO-3 in PBS for 2 h at RT under humid conditions followed by immunolabeling. The cells were mounted using Dako fluorescent mounting medium (Glostrup, Denmark) and examined by confocal microscopy analysis (Leica TCS SP; Leica Microsystems AG, Wetzlar, Germany and Olympus FluoView FV1000; Olympus Corporation, Tokyo, Japan). The images were processed using Adobe Photoshop CS2. All experiments were repeated three times.

Immuno electron microscopy

Cells incubated as described in figure legends, were prepared for immuno electron microscopy (immuno-EM) as described [29, 30]. Thawed cryo-sections were labeled as described in figures to legends, and bound antibodies were visualized using protein A gold (purchased from G. Posthuma, Utrecht, The Netherlands). Sections were examined using a Tecnai G² Spirit TEM (FEI, Eindhoven, The Netherlands) equipped with a

Morada digital camera using iTEM (SIS) software (Soft Imaging Solutions, Muenster, Germany). Images were processed using Adobe Photoshop CS2.

Results

C225-induced endocytosis of the EGFR is enhanced upon antibody-induced crosslinking of the C225-EGFR complex

Binding of C225 to the EGFR has been shown to promote receptor internalization by a yet undefined pathway [19, 20, 22]. To track C225-EGFR internalization, porcine aortic endothelial (PAE) cells stably transfected with a plasmid encoding the EGFR (PAE.EGFR cells) were incubated with C225 on ice, chased for 1 h at 37°C, fixed and subsequently labeled with a fluorochrome-conjugated anti-human IgG antibody (Fig. 1A, upper panel). The labeling demonstrated that although the majority of C225 remained localized to the plasma membrane even after chase at 37°C, a small amount of C225 localized to EEA1-positive vesicles, indicating some, but not a very efficient, antibody-induced internalization of the EGFR.

Incubation with two noncompetitive anti-EGFR antibodies in combination has, when compared to incubation with a single antibody, been demonstrated to more efficiently induce EGFR down-regulation [21, 24]. The increased down-regulation was reported to be a result of increased clustering and decreased recycling of the antibody-EGFR complex [21]. Also, aggregation of a mouse anti-EGFR antibody-EGFR complex, induced by incubation with a secondary anti mouse IgG antibody, was shown to increase antibody-induced EGFR degradation [24]. To study if this was also the case upon incubation with C225, we incubated PAE.EGFR cells with C225 on ice, followed by incubation with a donkey-anti human IgG antibody on ice before chase at 37°C (later

referred to as the combined-antibody incubation). Contrary to when cells were incubated with C225 alone, where the majority of antibody seemed to remain randomly distributed over the plasma membrane (Fig. 1A, upper panel), the labeling in cells incubated with both C225 and anti-human IgG localized to what appeared to be large clusters at the plasma membrane and/or intracellular vesicles (Fig. 1A, lower panel). The pattern was the same throughout the z-axis supporting a vesicular localization. The endosomal nature of these compartments was confirmed by partial colocalization with EEA1 (Fig. 1A) upon 1 h chase, and LAMP1 upon prolonged chase (Fig. 1B), as well as by partial colocalization with the fluid-phase endocytosis marker dextran (Fig. S1). This suggests that binding of a secondary IgG antibody to C225 enhanced C225-induced internalization of the EGFR.

To confirm that the EGFR was endocytosed along with the antibodies, cells incubated with C225 in combination with anti-human IgG were, upon fixation, labeled with an antibody recognizing the intracellular domain of the EGFR. Confocal microscopy analysis demonstrated co-localization between anti-human IgG and EGFR, confirming that the antibody complex remained associated with the EGFR (Fig. 1C). Cells were also incubated with anti-human IgG alone as a control for nonspecific antibody binding. Incubation with anti-human IgG only did not result in a fluorescence signal, nor did it induce EGFR internalization (data not shown).

To characterize the combined-antibody induced clustering and internalization of EGFR in more detail, PAE.EGFR cells incubated with or without C225 and donkey anti-human IgG on ice followed by chase at 37°C for various times, were prepared for immuno electron microscopy (immuno-EM). In cells not incubated with antibodies, labeling for

the EGFR appeared as single gold particles that seemed to be randomly distributed along the plasma membrane (Fig. S2A). Double labeling using antibodies recognizing the intracellular part of EGFR and donkey IgG respectively, demonstrated that the combined-antibody incubation induced clustering of the EGFR at the plasma membrane already upon incubation on ice (Fig. S2B). Upon 30 min chase at 37°C, the size of the EGFR-C225-donkey anti-human IgG clusters was increased, and labeling also localized to what morphologically resembled early endosomes (Fig. 2A-B). However, in the same specimen, what appeared to be tubular plasma membrane invaginations showing labeling for EGFR and donkey IgG, were frequently observed (Fig. 2A and S3C). Such invaginations may, depending on the angle of sectioning, on the micrographs not only appear as tubular plasma membrane invaginations, but probably also as intracellular cisternal or vesicular compartments, making an exact discrimination between plasma membrane- and endosome-localized clusters difficult (see Fig. 2A). Whether these apparent tubules really represent tubular invaginations or folding of larger plasma membrane areas is also difficult to determine (see also Fig. S4). Some labeling did, however, also localize to compartments with the morphology of late multivesicular endosomes (multivesicular bodies (MVBs)) (Fig. 2C). Upon prolonged chase, the labeling localizing to intraluminal vesicles of MVBs increased (Fig. 2E-F). This, together with the above described localization to EEA1 and LAMP1 positive compartments (see Fig. 1), confirmed that the combined-antibody incubation induced internalization and endosomal localization of EGFR. It should, however, be noted that strong plasma membrane labeling was observed even upon 3 h chase (Fig. S3E and G). What was also striking upon chase at all time points and to a smaller extent directly upon

incubation on ice, was a strong labeling for donkey anti-human IgG along narrow intercellular spaces (Fig. 3 and Fig. S3). In the same area, anti-EGFR antibody labeled the plasma membrane in each of the neighboring cells. This suggests that donkey anti-human IgG and possibly also to some extent C225 alone, in addition to cross-linking EGFRs directly next to each other at the plasma membrane had a zipper effect, cross-linking EGFRs on large plasma membrane domains both within the same cell, and between neighboring cells (see also Fig. S4).

PAE.EGFR cells, which do not express endogenous proteins belonging to the EGFR family, were used to avoid effects of EGFR heterodimerization. To investigate whether the observed effect is general and to what extent it depends on the EGFR expression level, we repeated the internalization experiments using cell lines expressing EGFR at different levels. Incubation of A431 cells, which express high amounts of EGFR, with C225 alone or in combination with anti-human IgG, showed similar results to what was observed in PAE.EGFR cells, suggesting that the effects are general, at least for cells expressing high levels of EGFR (Fig. S5). Also HeLa cells, expressing EGFR at intermediate levels, showed endosomal localization of the donkey anti-human IgG antibody upon the combined-antibody incubation (Fig. S6A). MCF-7 cells however, expressing EGFR at a low level, showed only very limited localization of anti-human IgG in endosomes (Fig. S6B). Altogether, this demonstrates that the effect of the combined antibody incubation is general, but varies depending on the EGFR expression level. The latter is in line with the suggestion that the size of antibody-receptor lattices formed at the cell surface determines the endocytosis efficiency [24].

Antibody-induced internalization of the EGFR is clathrin-independent

Previous studies have not addressed the molecular mechanisms involved in antibody-induced endocytosis of the EGFR in detail. Ligand-induced internalization of EGFR is mostly clathrin-dependent, but also clathrin-independent pathways have been identified (reviewed in [14]). While low concentrations of EGF induced clathrin-dependent endocytosis, high concentrations of EGF could induce clathrin-independent endocytosis and/or macropinocytic or dorsal ruffles-mediated internalization of the EGFR [14, 31-33]. Although immuno-EM demonstrated that the combined-antibody incubation caused clustering of EGFR mainly on non-coated plasma membrane regions, some clusters also localized to regions containing coated pits, and labeling was also observed in what appeared to be coated vesicles (Fig. 4A). To investigate whether antibody-induced EGFR internalization was clathrin-dependent, cells were transfected with siRNA to clathrin heavy chain (CHC). Western blotting was used to confirm efficient clathrin knock down (see Fig. S7A). As expected, knock down of CHC was found to efficiently inhibit endocytosis of fluorescently labeled EGF (Fig. S7B). Internalization of C225 was, however, not inhibited in CHC knock down cells, neither when cells were incubated with the C225 alone (Fig. 4B), nor when incubated with C225 in combination with anti-human IgG (Fig. 4C). These findings strongly suggest that antibody-induced endocytosis of the EGFR is clathrin-independent.

Antibody-induced internalization of the EGFR is dynamin-independent

EGF-induced formation of dorsal ruffles, which leads to massive internalization of the EGFR, is clathrin-independent, but does depend on dynamin-2 [31]. Likewise, internalization of ErbB2 induced by the combination of two anti-ErbB2 antibodies was previously demonstrated to be dynamin-dependent [24]. To examine the role of dynamin

in the combined-antibody-induced internalization of EGFR, PAE.EGFR cells were transiently transfected with a dominant negative dynamin mutant (K44A), previously demonstrated to block endocytosis of EGF [34]. In cells expressing the HA-tagged K44A dynamin, internalization of EGF (15 ng/ml) into early endosomes was blocked (Fig. S8). However, C225 in combination with anti-human IgG was internalized and localized to EEA1-positive vesicles both in cells with and without expression of K44A dynamin, demonstrating that the combined-antibody-induced internalization of EGFR was dynamin-independent (Fig. 5).

Antibody-induced internalization of the EGFR occurs by macropinocytosis

To further dissect mechanisms involved in antibody-induced EGFR endocytosis, we addressed potential actin dependency. As expected, preincubation of PAE.EGFR cells with the actin polymerization inhibitor latrunculin B [35] resulted in complete depolymerization of actin filaments (Fig. 6A). When cells were incubated with C225 in combination with anti-human IgG, internalization of the antibodies was inhibited upon incubation with latrunculin (Fig. 6B). Compared to in control cells, where the antibodies were visible only in discrete clusters or vesicles, the antibodies were found to be retained at the plasma membrane upon incubation with latrunculin, suggesting that actin-mediated macropinocytosis is responsible for the observed internalization.

To further investigate a potential role of macropinocytosis in antibody-induced EGFR internalization, PAE.EGFR cells were exposed to amiloride. Amiloride, which is an inhibitor of Na^+/H^+ exchange, has been widely used as inhibitor of macropinocytosis [12] and was recently demonstrated to block macropinocytosis due to reduced submembranous pH and inhibition of GTPases required for actin polymerization [36].

When cells were incubated with amiloride for 2 h prior to incubation with C225 and anti-human IgG, antibody-induced internalization of EGFR was inhibited (Fig. 7A). Instead of being localized to vesicles, the anti-human IgG (and thus also C225 and the EGFR) remained at the plasma membrane. To confirm that clathrin-mediated endocytosis was not affected by amiloride, internalization of ^{125}I -EGF at the concentration of 1 ng/ml was measured (Fig. 7B). Internalization of EGF was found to be efficient also in the presence of amiloride. Altogether, these data show that the combined-antibody-induced internalization of EGFR required actin polymerization (Fig. 6 and 7), but not clathrin (Fig. 4), nor dynamin (Fig. 5). This strongly suggested that macropinocytosis is responsible for the internalization.

EGFR phosphorylation induced by C225 and anti-human IgG is not required for internalization of the EGFR

Ligand-induced internalization of the EGFR is generally considered to depend on dimerization-induced activation of the kinase domain followed by transphosphorylation of tyrosines in the cytoplasmic tail of the EGFR [6, 37]. However, also dimerization-dependent, but kinase-independent, ligand-induced endocytosis of the EGFR has been demonstrated [38]. Endocytosis mediated by dorsal ruffles was found to depend on EGFR phosphorylation [31], while endocytosis induced by the combination of two noncompetitive anti-EGFR antibodies occurred without detectable EGFR phosphorylation [21]. We have recently demonstrated that incubation with C225 alone inhibited basal Erk activation downstream of the EGFR [39]. To study whether this was also the case when C225 was combined with the anti-human IgG antibody, PEA.EGFR cells were incubated with or without C225 in combination with anti-human IgG. EGFR

phosphorylation, and downstream signaling was investigated using antibodies to phospho-Tyr1173, phospho-Tyr1086, and phospho-Tyr1068 in EGFR, and to phospho-Erk, respectively. As previously demonstrated [39], C225 alone did not affect the basal level of EGFR phosphorylation, but basal Erk activity was clearly inhibited (data not shown). However, when C225 was combined with anti-human IgG, EGFR was significantly phosphorylated at tyrosine residues 1173 and 1086 (data not shown), while Tyr 1068 was only slightly phosphorylated (Fig. 8A). Activation of Erk was to some extent inhibited when compared to control levels.

Since C225 in combination with anti-human IgG induced EGFR phosphorylation, we examined whether EGFR kinase activity was required to mediate the combined-antibody induced EGFR internalization. To inhibit EGFR kinase activity, PAE.EGFR cells were incubated with either of the EGFR specific kinase inhibitors AG1478 and PD153035, both prior to and upon incubation with the antibodies. Antibody-induced phosphorylation of EGFR was efficiently counteracted by the kinase inhibitors (Fig. 8A). Internalization of the EGFR-antibody complex, was, however, not affected by AG1478 or PD153035 (Fig. 8B), demonstrating that EGFR kinase activity and phosphorylation of EGFR was not required for antibody-induced endocytosis.

C225 in combination with anti-human IgG induces ubiquitination of the EGFR

The immuno-EM studies showed that EGFR-C225-donkey anti-human IgG complexes were efficiently sorted to intraluminal vesicles of MVBs (see Fig. 2). Since sorting of EGF-EGFR complexes into MVBs is believed to depend on EGFR ubiquitination and interaction with ESCRT complexes on the limiting membrane of endosomes [6], we investigated to what extent incubation with C225 alone or in combination with anti-

human IgG induced EGFR ubiquitination. Cells were incubated with C225 alone or C225 in combination with anti-human IgG and chased for 30 min at 37°C. Incubation with EGF for 10 min at 37°C was used as a positive control. Immunoprecipitation of the EGFR and Western blotting using an antibody to ubiquitin demonstrated that the antibody combination, but not C225 alone, induced efficient EGFR ubiquitination (Fig. 9).

C225 in combination with anti-human IgG induces rapid and efficient degradation of EGFR in a C225 concentration dependent manner

Since the EGFR was efficiently internalized and localized to a LAMP1 positive compartment upon the combined-antibody incubation, we investigated how efficiently the antibody combination degraded the EGFR when compared to degradation induced by high concentrations of EGF (60 ng/ml). Incubation with antibodies or with EGF both resulted in degradation of the EGFR (Fig. 10). Degradation induced by the antibodies was, however, more efficient than degradation induced by incubation with EGF. Incubation with the combination of antibodies resulted in EGFR degradation already upon a 2 h chase, while continuous incubation with EGF gave a similar effect upon 4 h chase. Based on our previous study showing that C225 at a concentration of 5 µg/ml, most efficiently inhibited binding of EGF [39], this concentration was used in all experiments described up to now. However, since the endocytosis rate of antibody-receptor complexes has been proposed to be proportional to the size of the complex [24], we also investigated to what extent C225 at lower concentrations induced internalization and degradation of EGFR. Incubation with decreasing concentrations of C225, but with the same concentration of anti-human IgG as used for previous experiments, demonstrated that the internalization and degradation was indeed depending on the C225

concentration (Fig. S9). While 5 $\mu\text{g/ml}$ caused efficient down-regulation of EGFR from the plasma membrane (Fig S9A) and efficient EGFR degradation (Fig. S9C), the effect was gradually decreased upon incubation with decreasing concentrations of C225. As the size of the antibody-receptor complex most likely depends on the EGFR expression level, we also compared what effect the combined-antibody incubation had on degradation of the EGFR in cells with varying levels of EGFR expression. In line with the internalization experiments shown in Fig. S6, the combination of 5 $\mu\text{g/ml}$ C225 and anti-human IgG induced EGFR degradation in HeLa cells, but not MCF-7 cells (Fig. S9D-E).

Discussion

The antibody C225 (Cetuximab, Erbitux) has been approved for clinical use, but use of C225 has so far produced modest response when used as single agent in patients with colorectal cancer [40, 41], with head and neck cancer [42] and with non-small cell lung cancer [43]. The clinical benefit seems to increase when C225 is combined with chemotherapy or radiation therapy [16]. For such reasons, C225 is rarely used therapeutically as single agent. Several strategies have been used to increase the therapeutic potential of anti-receptor antibodies. With respect to ErbB2, a combination of antibodies has been shown to increase antibody-induced inhibition of tumor growth [44, 45], possibly as an effect of antibody-induced internalization and degradation of ErbB2 [45]. However, the exact molecular mechanisms leading to antibody-induced down-regulation of ErbB-proteins have so far not been fully clarified. To investigate how antibodies can induce internalization and degradation of the EGFR, we used C225 alone or C225 combined with an anti-human IgG antibody. Microscopy analysis demonstrated that binding of anti-human IgG to the C225-EGFR complexes strongly enhanced C225-

induced receptor internalization, thus supporting the notion that aggregation of C225-EGFR complexes increases the internalization efficiency and/or inhibits recycling. Our results further demonstrate that the combination of antibodies induced internalization of EGFR in a clathrin-independent manner. The internalization was also dynamin-independent, which in addition to clathrin-coated pits, rules out caveolae as a possible internalization pathway (reviewed in [8]). Morphologically the anti-human IgG-C225-EGFR positive domains showed several similarities to CLICs [15], but in contrast to the CLIC/GEEC pathway which is constitutive [15], the pathway investigated in the current study was clearly induced and depending on extensive antibody-induced crosslinking of the EGFR. Disruption of actin filaments by latrunculin, however, efficiently blocked endocytosis, arguing that the internalization was actin-dependent. Furthermore, internalization was blocked upon preincubation with amiloride. Amiloride is widely used as an inhibitor of macropinocytosis, and suppression with amiloride was in a recent review suggested to define macropinocytosis [11]. Altogether, although we can not fully exclude alternative pathways, such as CLICs, our results suggest that antibody-mediated aggregation induces internalization of the EGFR by macropinocytosis. Macropinocytosis was originally considered a non-regulated process, but although all details are currently not understood, it is clear that the remodeling of the cytoskeleton leading to macropinocytosis is highly regulated (reviewed in [12]). Activation of PI3-kinase is important for activation of the GTPases Rac1 and Cdc42 which are involved in actin remodeling and are inhibited upon incubation with amiloride [36]. However, even though we found that the combined-antibody incubation induced phosphorylation of the EGFR, antibody-induced macropinocytosis occurred also when this phosphorylation was blocked

by EGFR kinase inhibitors. This is consistent with previous reports, demonstrating that down-regulation of EGFR by combination of the antibody mAb-225 (the murine version of C225) and other monoclonal antibodies was independent of EGFR kinase activity [21]. The exact molecular events involved in antibody-induced macropinocytosis thus remain undefined.

The fate of macropinosomes has been reported to vary, possibly depending both on how macropinocytosis was initially induced and on the cell type studied. While EGF-induced macropinosomes in HEK293 cells has been shown to fuse with EEA1 positive early endosomes, mature to late endosomes and finally fuse with lysosomes [46], macropinosomes induced by EGF in A431 cells showed limited fusion with early endosomes and no maturation into late endosomes [47, 48]. Since sorting of the EGFR into MVBs is believed to depend on ubiquitin-mediated interaction with the ESCRT complexes (reviewed in [6]), such differences may be due to varying degree of EGFR ubiquitination. In our present study, we found that the combined-antibody incubation induced ubiquitination of the EGFR, and upon internalization, the EGFR-antibody complexes initially colocalized with EEA1 and later with LAMP1. Immuno-EM further demonstrated efficient sorting into MVBs, all strongly suggesting routing to late endosomes/lysosomes. Consistently, the EGFR was found to be degraded upon the combined-antibody incubation. When compared to ligand-induced degradation of the EGFR, antibody-induced clustering induced a more rapid degradation. This difference was clear upon 2 h chase, but upon prolonged chase, the degradation was more or less the same. The different efficiency of EGFR degradation can probably in part be explained by the different efficiency of internalization. Clathrin-dependent endocytosis of the EGFR is

saturable [6], and the limited size of each coated pit may possibly also limit the number of EGFR molecules that can be internalized at any time. Macropinocytosis will on the other hand include large plasma membrane areas containing clustered EGFRs and thus allow internalization of a large number of receptors in a single event. It should, however, be noted that our electron microscopy analysis indicated that also macropinocytosis could be a saturable process. We found that even upon 3 h chase, large EGFR-C225-donkey anti human IgG complexes were retained at the plasma membrane. This, together with the apparent crosslinking between C225-EGFR positive membrane areas on neighboring cells (probably strongly inhibiting internalization of EGFR) could explain why the extent of EGF-induced and antibody-induced degradation of EGFR was more or less similar upon 4 h chase. Altogether, our results show that extensive cross-linking of EGFR at the plasma membrane can induce efficient macropinocytosis-mediated down-regulation of the EGFR. The possible zipper effect introduced by the secondary antibody could on single cells be an important mechanical force driving plasma membrane invagination prior to scission and internalization. However, as this zipper effect also appears to crosslink neighboring cells, it might have a negative effect on EGFR down-regulation in confluent cell cultures and, importantly, also *in vivo*.

Conflict of interest

The authors confirm that there are no conflicts of interest.

Acknowledgements

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Figure Legends

Figure 1: C225-induced internalization of EGFR is increased when combined with

anti-human IgG. (A) PAE.EGFR cells were either incubated with C225 only (C225) on ice, chased for 1 h at 37°C, fixed and stained using Rhodamine Red-X-conjugated anti-human IgG and goat anti-EEA1 antibodies followed by Alexa Fluor 488-conjugated donkey anti-goat antibody or incubated with C225 followed by Rhodamine Red-X-conjugated anti-human IgG (C225+IgG) on ice, chased for 1 h at 37°C, fixed and stained using goat anti-EEA1 antibody followed by Alexa Fluor 488-conjugated donkey anti-goat antibody and TO-PRO-3 for nuclear counterstaining. Scale bar, 20 µm. **(B)** PAE.EGFR cells were incubated with C225 and Rhodamine Red-X-anti-human IgG (C225+IgG) on ice, chased for 4 h at 37°C, fixed and stained using rabbit anti-LAMP1 antibody followed by Alexa Fluor 488-conjugated goat anti-rabbit antibody and TO-PRO-3. Scale bar, 20

μm . **(C)** PAE.EGFR cells were incubated with C225 and Rhodamine Red-X-anti-human IgG (C225+IgG) on ice, chased for 1 h at 37°C, fixed and stained using sheep anti-EGFR antibody followed by Cy5-conjugated donkey anti-sheep antibody. Scale bar, 20 μm . Lower images show higher magnification of framed areas. Scale bar, 5.44 μm .

Figure 2: Combined-antibody incubation induces EGFR clustering and endocytosis.

PAE.EGFR cells, sequentially incubated with C225 and donkey-anti human IgG on ice before chase at 37°C for 30 min (**A-C**), 1 h (**D-E**) or 3 h (**F**), were prepared for cryo-immuno-EM. Thawed cryosections were double-labeled using a sheep antibody to the intracellular part of EGFR followed by rabbit anti-sheep IgG and 10 nm protein A gold, rabbit anti-human IgG and 15 nm protein A gold. Labeling was localized to the plasma membrane (**A** and **D**), in typical early endosomes (e.e.) (**B** and **D**), and in MVBs at different stages of formation (**C**, **E** and **F**). Whether the area labeled with * in **A** represents an early endosome, or a plasma membrane invagination is unclear. Bar 100 nm.

Figure 3: Combined-antibody incubation induces cross-linking of EGFR clusters on neighboring cells.

PAE.EGFR cells, sequentially incubated with C225 and donkey-anti human IgG on ice before chase at 37°C for 1 h, were prepared for cryo-immuno-EM. Thawed cryosections were double-labeled using a sheep antibody to the intracellular part of EGFR followed by rabbit anti-sheep IgG and 10 nm protein A gold and rabbit anti-human IgG and 15 nm protein A gold. **B** shows a high magnification picture of the framed area in **A**. Bars: A 1 μm ; B 100 nm.

Figure 4: Antibody-induced endocytosis of the EGFR is clathrin-independent. (A)

PAE.EGFR cells were sequentially incubated with C225 and donkey-anti human IgG on

ice and either fixed directly (left panel) or upon 30 min chase at 37°C (right panel). Thawed cryosections were double-labeled using a sheep antibody to the intracellular part of the EGFR followed by rabbit anti-sheep IgG and 10 nm protein A gold and rabbit anti-human IgG and 15 nm protein A gold. c.p.: coated pit; c.v.: coated vesicle. Bars 100 nm.

(B-C) PAE.EGFR cells were transfected with Silencer Negative control siRNA (Control) or CHC siRNA (Clathrin KD) prior to incubation with antibodies. **(B)** Cells incubated with C225 only were chased for 1 h, fixed and stained using Rhodamine Red-X-conjugated anti-human IgG and goat anti-EEA1 antibodies followed by Alexa Fluor 488-conjugated donkey anti-goat antibody. **(C)** Cells incubated with C225 and Rhodamine Red-X-anti-human IgG in combination (C225+IgG) were chased for 1 h, fixed and stained using goat anti-EEA1 antibody followed by Alexa Fluor 488-conjugated donkey anti-goat antibody and TO-PRO-3 for nuclear counterstaining. Scale bar, 20 μ m.

Figure 5: Internalization of EGFR induced by the antibody combination is dynamin independent. PAE.EGFR cells, transfected with a plasmid encoding HA-tagged K44A dynamin, were incubated with C225 and Rhodamine Red-X-anti-human IgG (IgG) on ice, chased for 1 h, fixed and stained using rabbit anti-HA and goat anti-EEA1 antibodies followed by Cy2-conjugated donkey anti-rabbit and Alexa Fluor 647-conjugated donkey anti-goat antibodies. The right image represents a merge of Rhodamine Red-X-anti-human IgG localization and staining for EEA1. Scale bar, 20 μ m.

Figure 6: Internalization of EGFR induced by the antibody combination is dependent on actin. **(A)** PAE.EGFR cells were preincubated with or without (Control) latrunculin B (10 μ g/ml) for 30 min at 37°C. Cells were then fixed with ethanol and stained using Rhodamine-conjugated Phalloidin and TO-PRO-3. **(B)** Cells preincubated

with or without latrunculin were subsequently incubated with C225 and Rhodamine Red-X-anti-human IgG, chased for 1 h, fixed with ethanol and stained using goat anti-EEA1 antibody. For cells treated with latrunculin, this drug was present also during incubation with antibodies and during the chase period. Scale bar, 20 μ m.

Figure 7: The combined-antibody incubation induces EGFR internalization by macropinocytosis. (A) PAE.EGFR cells, preincubated with or without (Control) amiloride (1 mM) for 2 h at 37°C, were subsequently incubated with C225 and Rhodamine Red-X-anti-human IgG, chased for 1 h in the presence of amiloride (1 mM) and fixed. Scale bar, 10 μ m. (B) Cells preincubated with or without (Control) amiloride (1 mM) for 2 h at 37°C, were subsequently incubated with 1 ng/ml 125 I-EGF at 37°C for the times indicated. The ratio of internalized to surface-localized 125 I-EGF (mean \pm SE of three independent experiments with four parallels) was plotted as a function of time.

Figure 8: Activation of EGFR, induced by the combined-antibody incubation, is not required for EGFR internalization. PAE.EGFR cells preincubated with or without (Control) AG1478 (1 μ M) or PD153035 (5 μ M) for 1 h at 37°C, were incubated with or without C225 and Rhodamine Red-X-anti-human IgG (C225+IgG) and subsequently chased for 1 h in the presence or absence of AG1478 or PD153035. (A) Cell lysates were subjected to SDS-PAGE using two parallel 10% gels and immunoblotting with anti-EGFR-phospho-tyrosine antibody (pTyr1173 or pTyr1068), anti-phospho-Erk (pErk) and anti-Tubulin antibodies. (B) Fixed cells were stained using goat anti-EEA1 antibody followed by Alexa Fluor 488-conjugated donkey anti-goat antibody and TO-PRO-3. Scale bar, 10 μ m.

Figure 9: The combination of antibodies induces EGFR ubiquitination. PAE.EGFR cells were either incubated with or without (Control) C225 alone or with the antibody combination (C225+IgG) and subsequently chased for 30 min at 37 °C, or incubated with EGF (60 ng/ml) for 10 min at 37 °C. The cell lysates were subjected to immunoprecipitation under denaturing conditions using sheep anti-EGFR antibody. The immunoprecipitated material was then analyzed by Western blotting, using antibody to ubiquitin. The membranes were stripped and subsequently reblotted using sheep anti-EGFR antibody. Total cell lysates (TCL) were immunoblotted using sheep anti-EGFR antibody, and anti-Tubulin antibody was used as loading control.

Figure 10: The antibody combination degrades EGFR with high efficiency. PAE.EGFR cells were either incubated with or without (Control) the antibody combination (C225+IgG) and subsequently chased for 2 or 4 h in presence of CHX, or incubated with EGF (60 ng/ml) for 2 or 4 h in presence of CHX. Cell lysates were subjected to SDS-PAGE using a 10% gel and immunoblotted with sheep anti-EGFR antibody. Anti-Tubulin antibody was used as loading control.

Highlights

- Cetuximab induced endocytosis of EGFR increases upon combination with anti-human IgG
- Antibody combination causes internalization of EGFR by macropinocytosis
- Antibody-induced internalization of EGFR is independent of EGFR kinase activity
- Antibody combination may have a zipper effect and cross-link EGFRs on neighboring cells

Figure 1

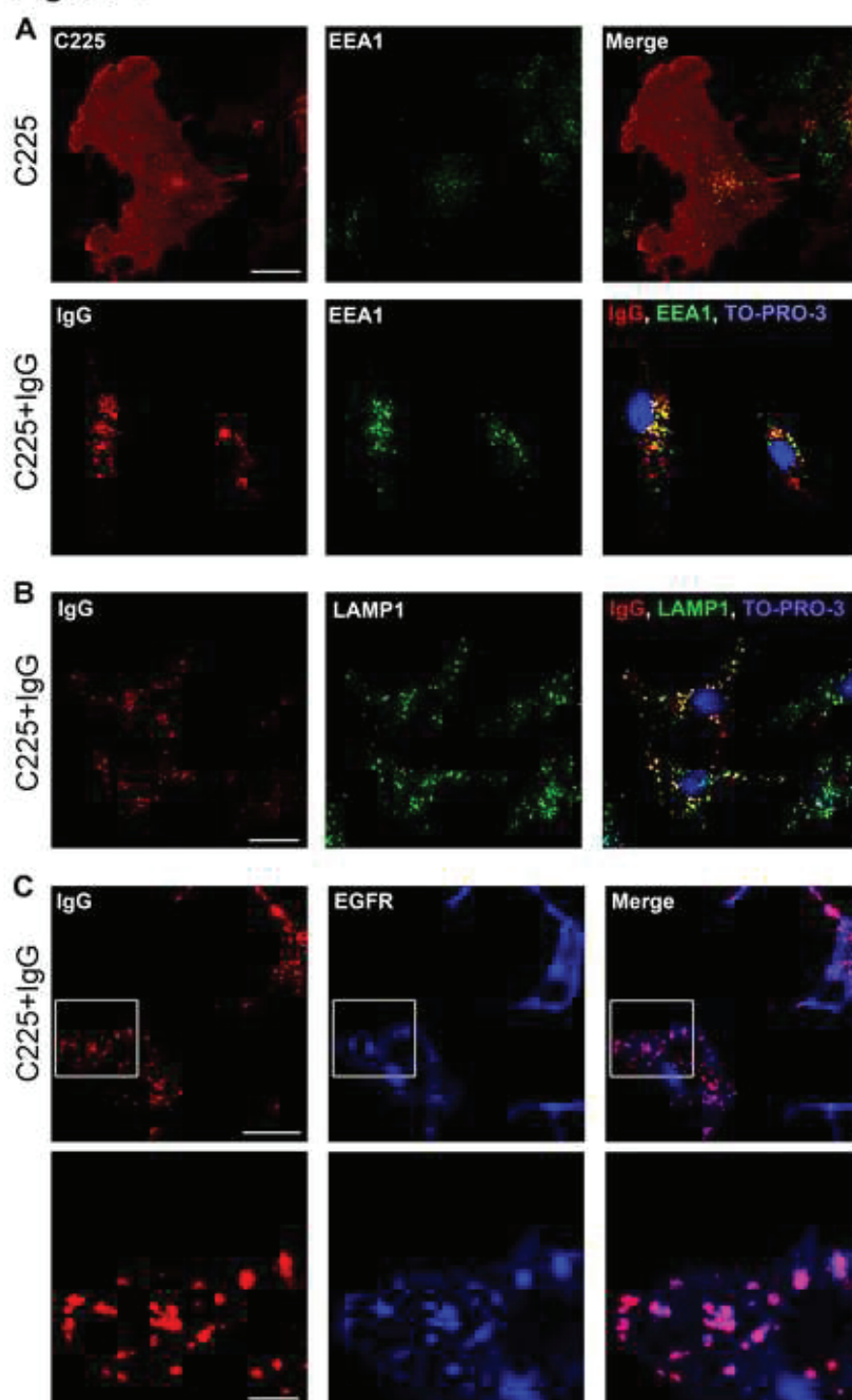
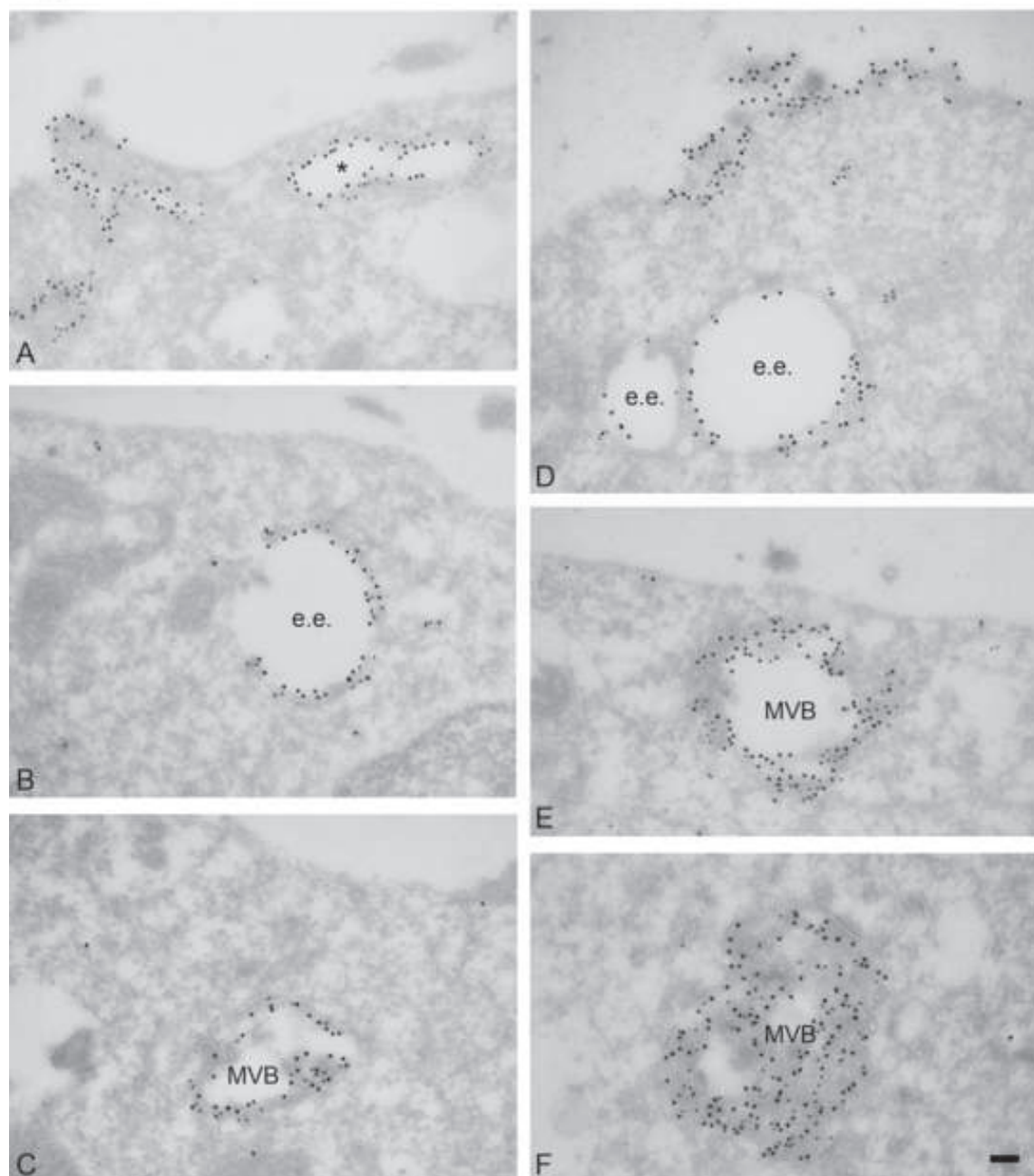


Figure 2



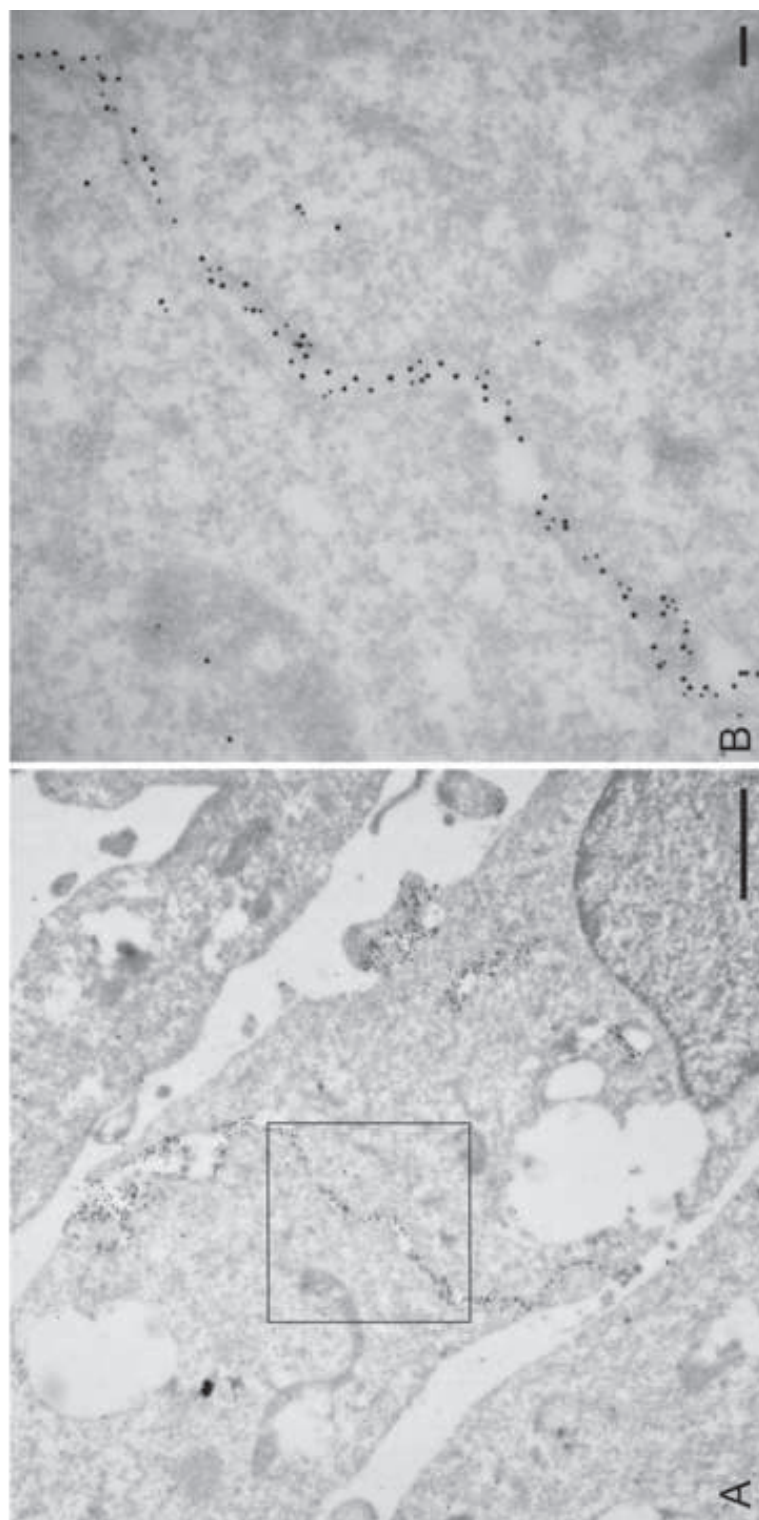


Figure 3

Figure 4

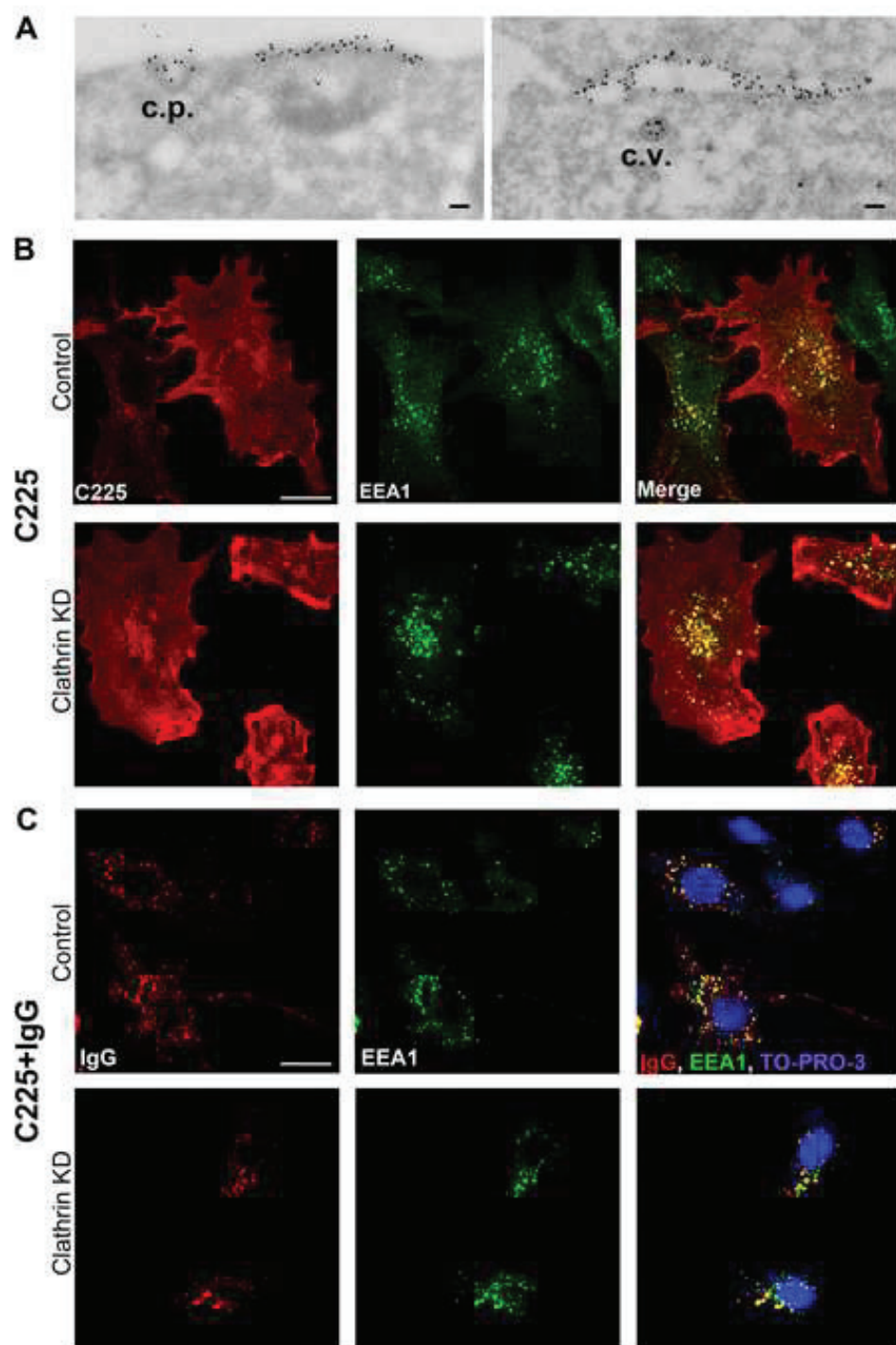


Figure 5

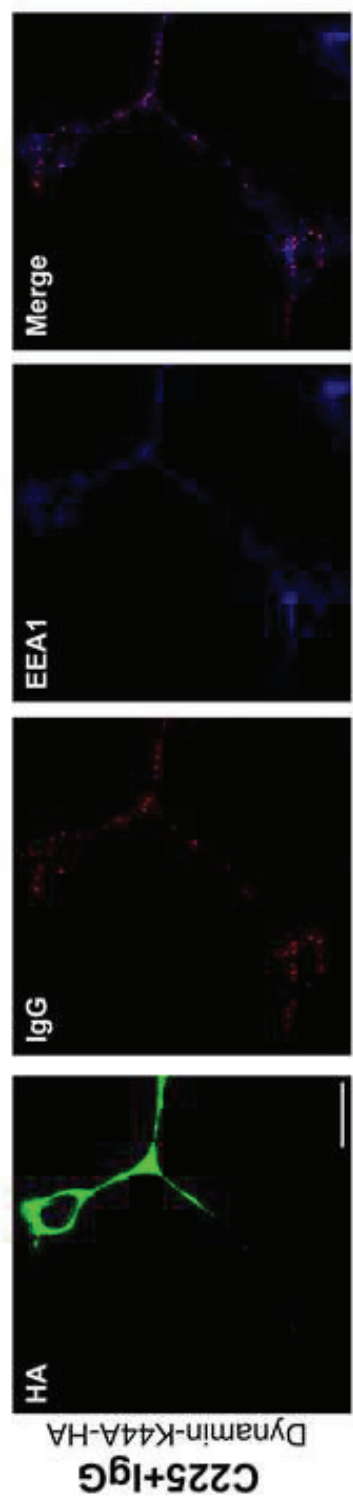


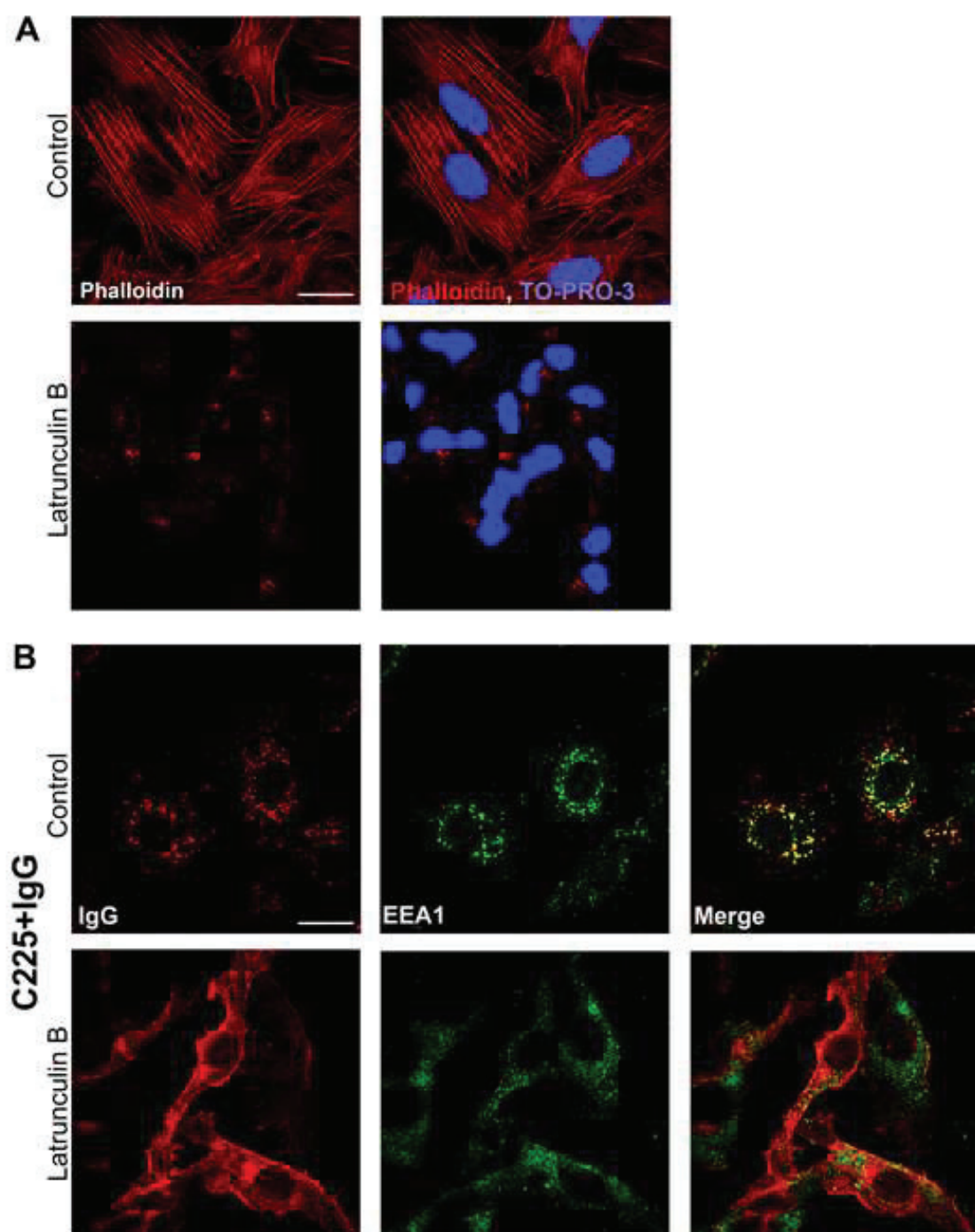
Figure 6

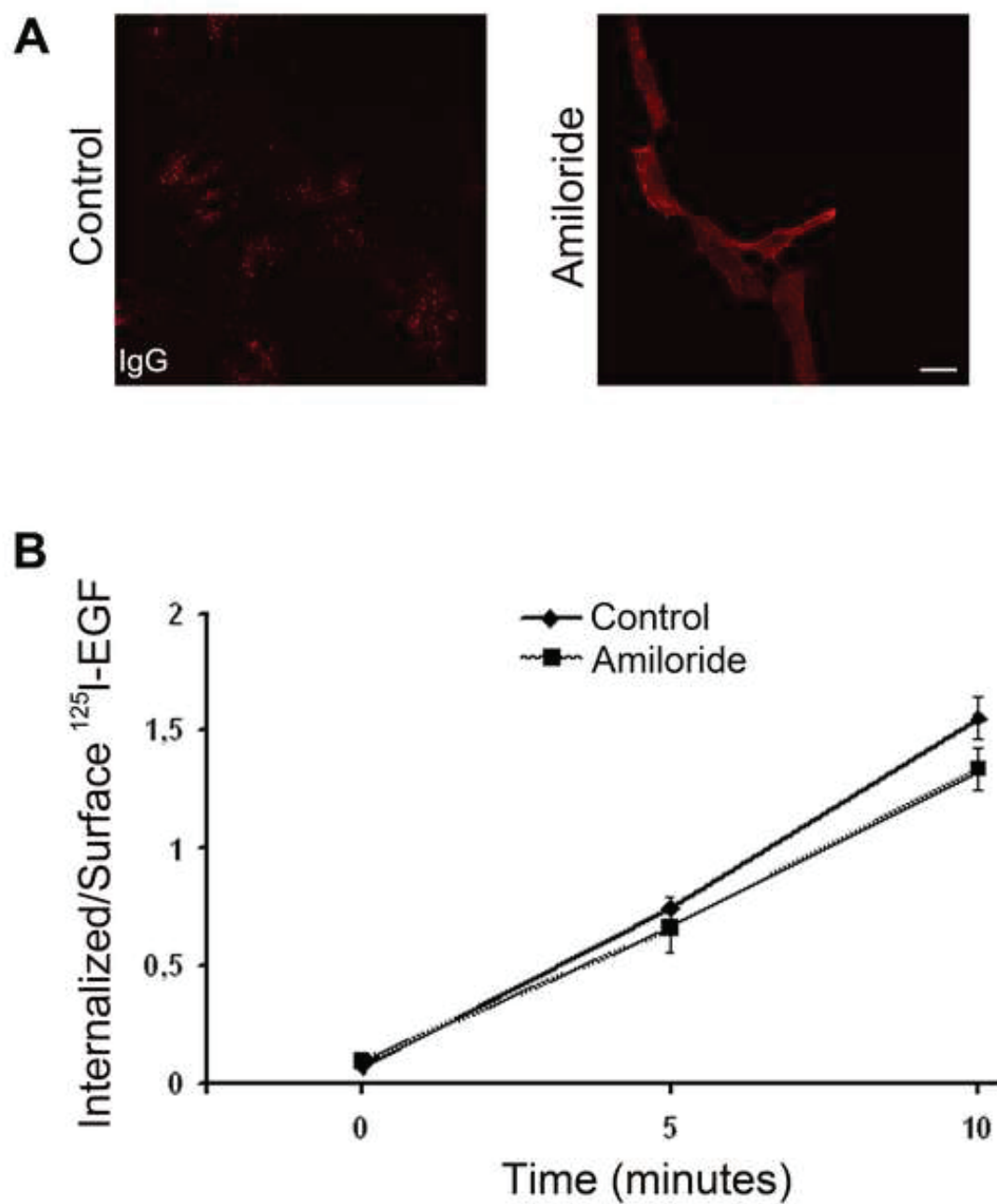
Figure 7

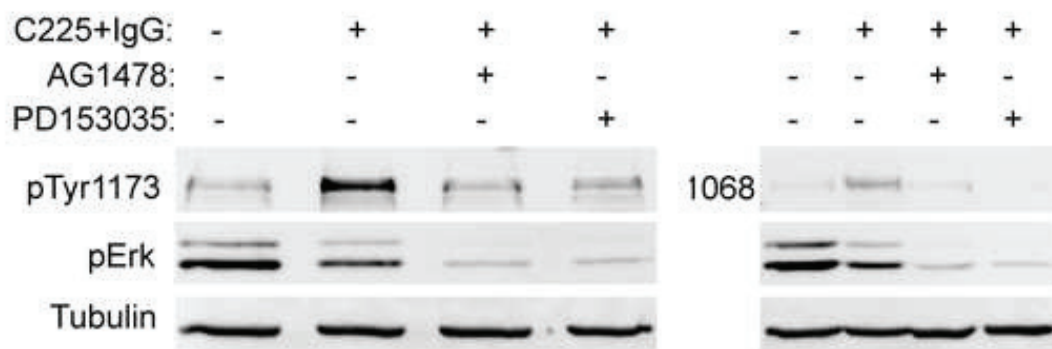
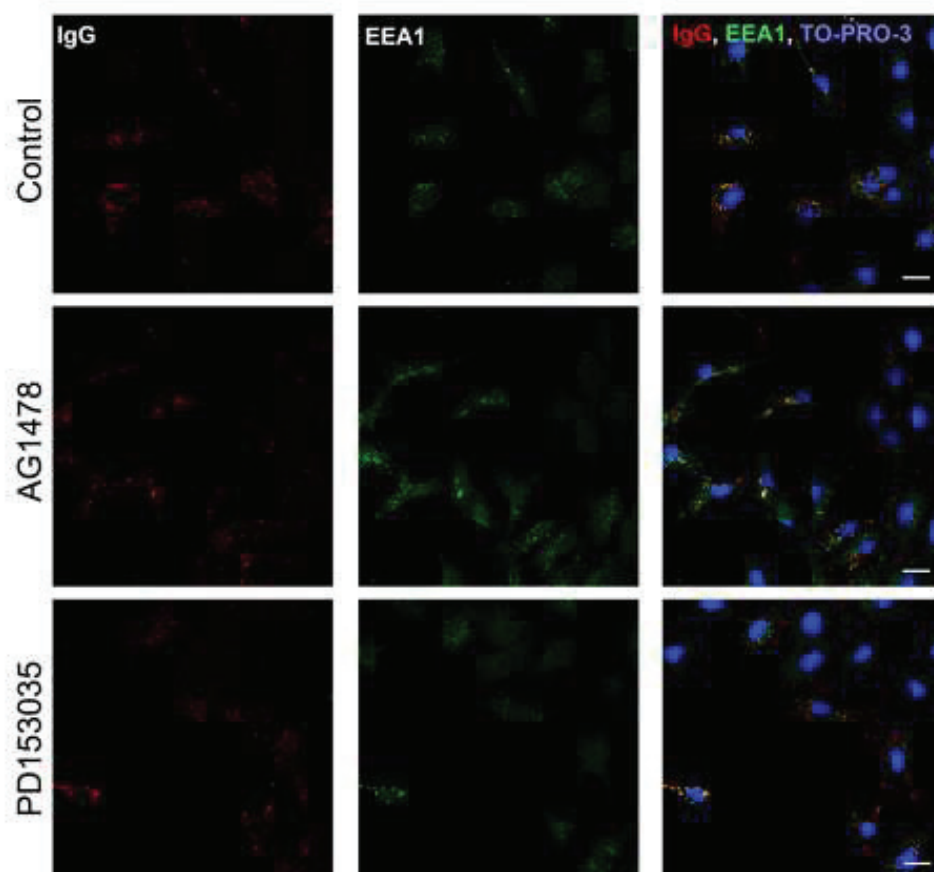
Figure 8**A****B**

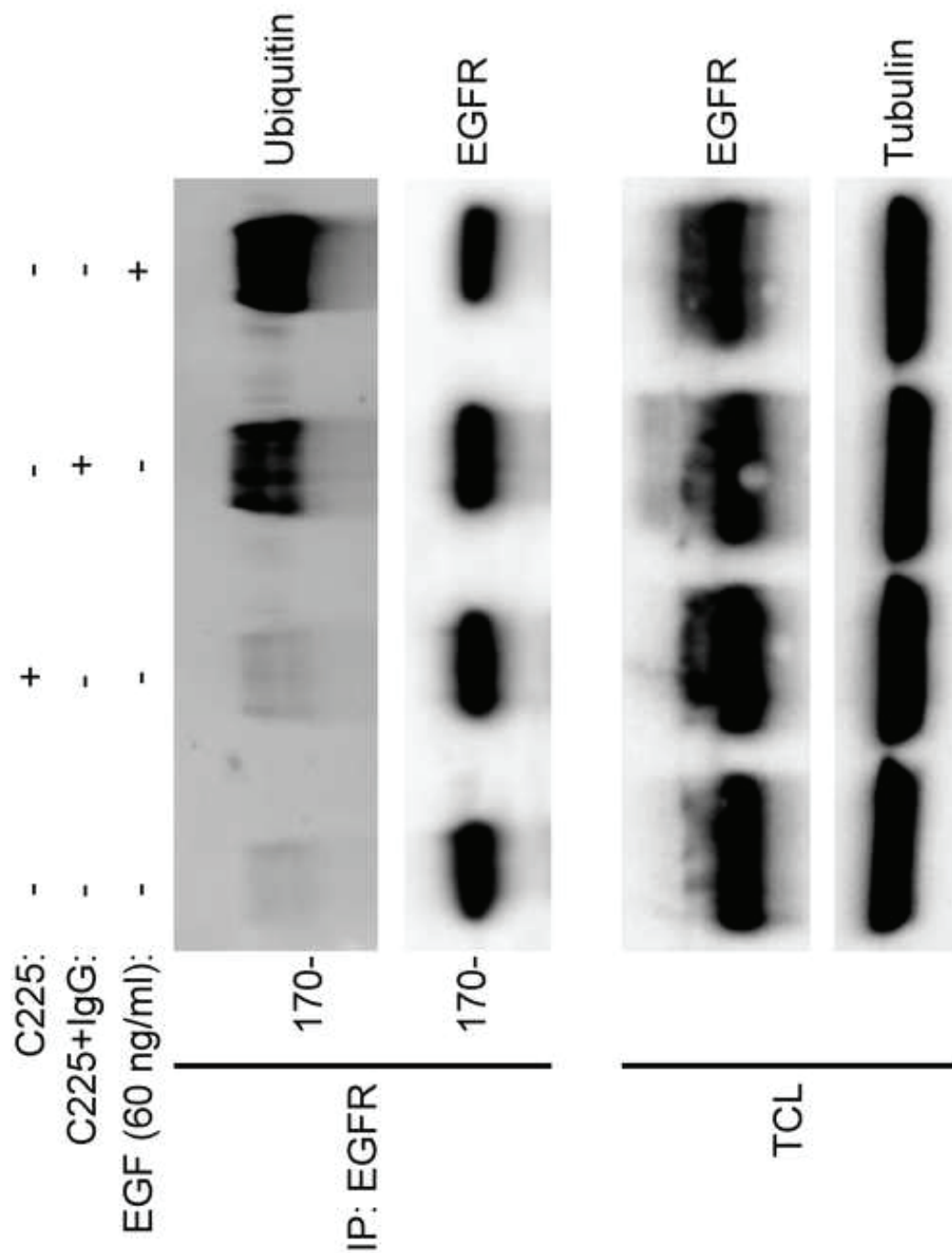
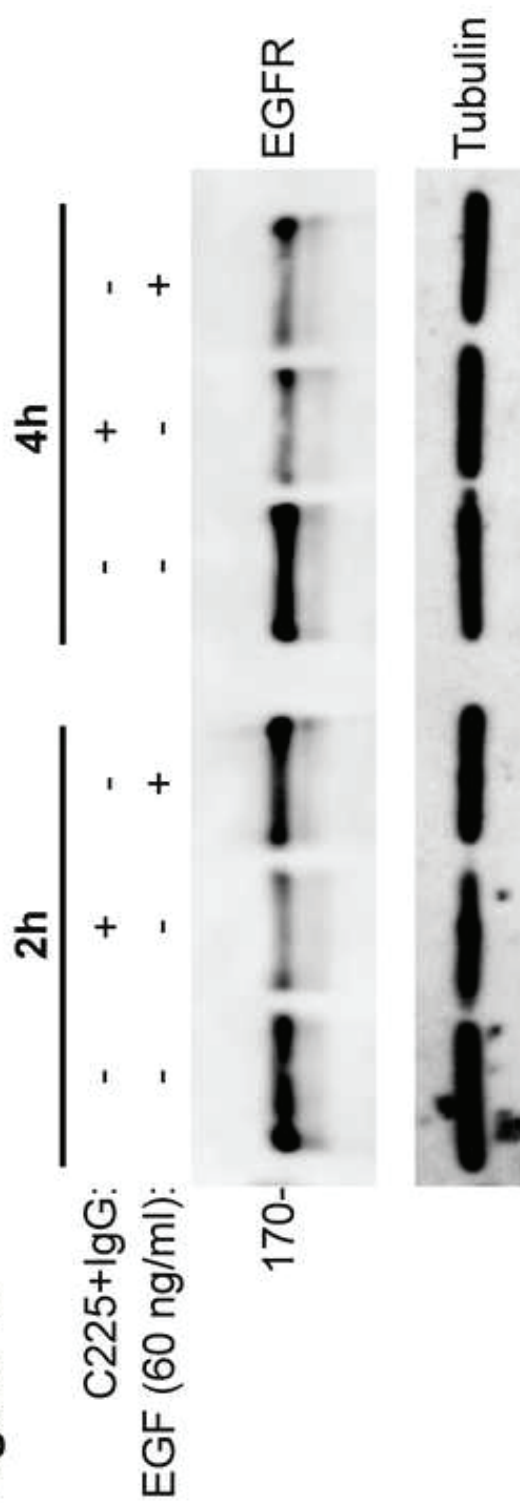
Figure 9

Figure 10

Supplementary information

Supplementary Figure S1: Antibody combination internalizes surface-localized fluorescent dextran. PAE.EGFR cells were incubated with C225 and Rhodamine Red-X-anti-human IgG (C225+IgG) in presence of Oregon Green 488-conjugated dextran (2 mg/ml), chased for 1 h in presence of dextran (2 mg/ml) and fixed. Scale bar, 10 μ m.

Supplementary Figure S2: Combined-antibody incubation induces clustering of EGFR at the plasma membrane. **A)** PAE.EGFR cells not incubated with antibodies were prepared for immuno-EM and labeled with sheep-anti EGFR followed by rabbit-anti sheep and 10 nm protein A gold. **B)** PAE.EGFR cells, sequentially incubated with C225 and donkey-anti human IgG on ice, were prepared for cryo-immuno-EM. Thawed cryosections were double labeled using a sheep antibody to the intracellular part of EGFR followed by rabbit anti-sheep IgG and 10 nm protein A gold, and rabbit anti-human IgG and 15 nm protein A gold. Bars 100 nm.

Supplementary Figure S3: Combined-antibody incubation induces clustering of EGFR and cross-linking of neighboring cells. PAE.EGFR cells, sequentially incubated with C225 and donkey-anti human IgG on ice, followed by chase at 37°C for 30 min (**A-D**) or 3 h (**E-I**) were prepared for cryo-immuno-EM. Thawed cryosections were double labeled using a sheep antibody to the intracellular part of EGFR followed by rabbit anti-sheep IgG and 10 nm protein A gold, and rabbit anti-human IgG and 15 nm protein A

gold. **B**, **C** and **D** show high magnification pictures of the framed areas in **A**; **F**, **H** and **I** show high magnification pictures of the framed areas in **E** and **G**, respectively. Bars 100 nm.

Supplementary Figure S4: Combined-antibody incubation may have a zipper effect.

Schematic drawings showing different variations of antibody induced patching of EGFR and possible cross-linking of plasma membrane sheets. **A)** The lines indicating the plasma membrane (p.m.) may represent either the membrane on separate areas of a single cell, or the plasma membranes of two different cells. The left side illustrates how C225 on its own can crosslink neighboring EGFRs, and possibly also EGFRs on separate, but closely positioned membrane domains. The right side illustrates how anti-human IgG in different ways can crosslink neighboring EGFR-bound C225 molecules both within one membrane domain and on closely positioned membrane domains. **B)** Within the same cell cross-linking induced the combined-antibody incubation may have a zipper effect leading to the formation of either tubular invaginations (left side), or folding of larger membrane sheets (right side). Pale colors indicate antibodies localized in the back. **C)** The combined-antibody incubation may have a zipper effect leading to the cross-linking of plasma membrane domains on neighboring cells.

Supplementary Figure S5: Antibody-induced receptor internalization is efficient in cells with high expression of EGFR. (A) A431 cells were incubated with C225 on ice,

chased for 1 h at 37°C, fixed and stained using Rhodamine Red-X-anti-human IgG. **(B)** A431 cells were incubated with C225 followed by Rhodamine Red-X-anti-human IgG on

ice before chase for 1 h at 37°C. **(C)** A431 cells, incubated as in **B**, were upon fixation stained using sheep anti-EGFR antibody followed by Cy5-conjugated donkey anti-sheep antibody. Scale bars, 20 μ m. Lower images in **C** show higher magnification of framed areas. Scale bar, 4.55 μ m.

Supplementary Figure S6: The degree of EGFR internalization upon combined-antibody incubation varies depending on the EGFR expression level. HeLa cells **(A)** and MCF-7 cells **(B)** were incubated with the antibody combination (IgG), chased for 1 h, fixed and stained using goat anti-EEA1 antibody followed by Alexa Fluor 488-conjugated donkey anti-goat antibody and TO-PRO-3 for nuclear counterstaining. Scale bars, 10 μ m.

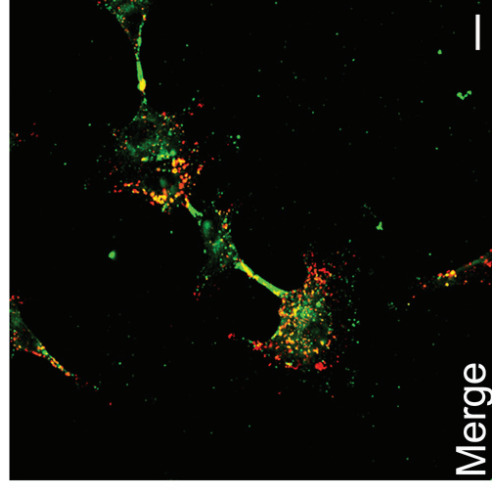
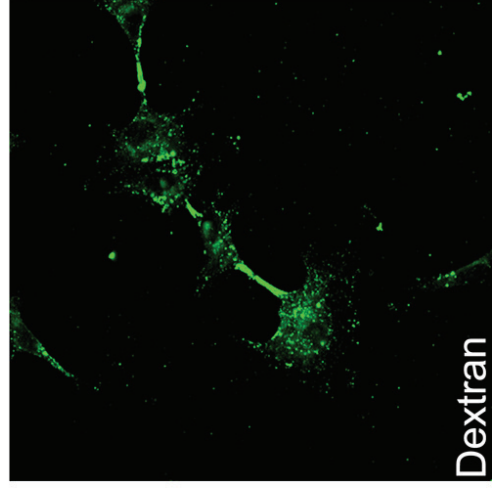
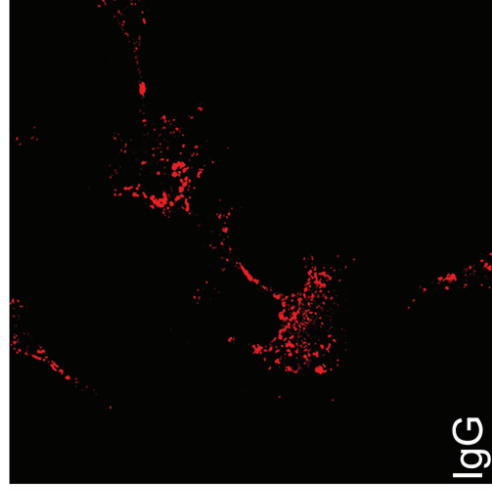
Supplementary Figure S7: Knock-down of CHC blocks internalization of EGF. PAE.EGFR cells were transfected with Silencer Negative control siRNA (Control) or CHC siRNA (Clathrin KD). **(A)** Cell lysates were subjected to SDS-PAGE and immunoblotting with anti-CHC antibody. Anti-Tubulin antibody was used as loading control. **(B)** Cells were incubated with Alexa 647-conjugated EGF (15 ng/ml) for 15 min at 37°C, fixed and stained using goat anti-EEA1 antibody followed by Alexa Fluor 488-conjugated donkey anti-goat antibody. Scale bar, 20 μ m.

Supplementary Figure S8: Transfection of cells with HA-K44A-Dynamin1 reduced internalization of EGF. PAE.EGFR cells were transfected with plasmid encoding HA-tagged K44A dynamin before incubation with Alexa 647-conjugated EGF (15 ng/ml) for

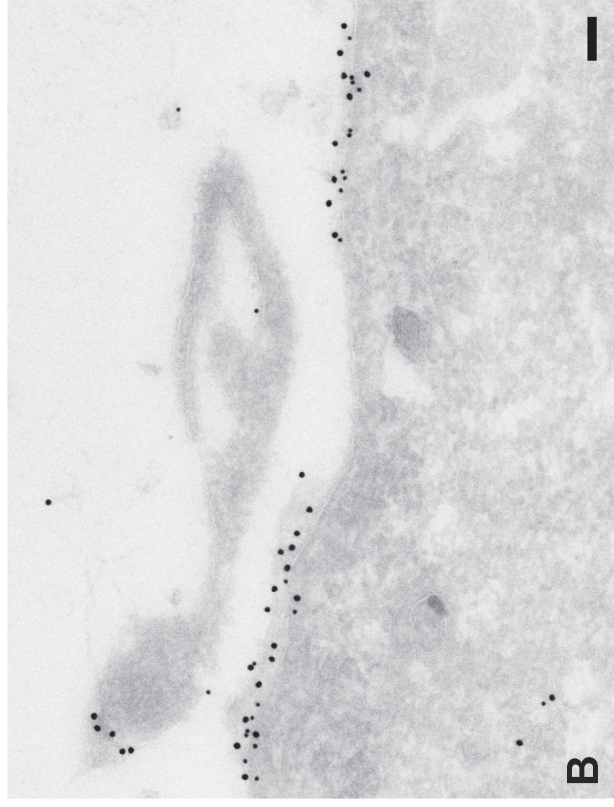
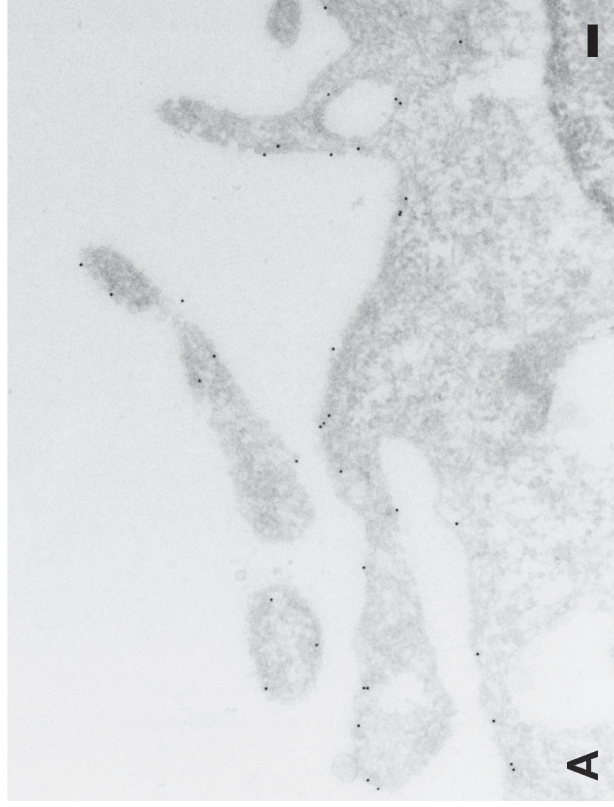
15 min at 37°C. Cells were then fixed and stained using rabbit anti-HA tag and goat anti-EEA1 antibodies followed by Rhodamine Red-X-conjugated donkey anti-rabbit and Alexa Fluor 488-conjugated donkey anti-goat antibodies. Scale bar, 20 μ m.

Supplementary Figure S9: The effect of the combined-antibody incubation on EGFR internalization and degradation varies depending on C225 concentration and on EGFR expression levels. (A-B) PAE.EGFR cells were incubated with the antibody combination (IgG) with decreasing concentrations of C225 (as indicated in figures), chased for 1 h, fixed and either **(A)** stained using goat anti-EEA1 antibody followed by Alexa Fluor 488-conjugated donkey anti-goat antibody, or **(B)** stained using sheep anti-EGFR antibody followed by Cy2-conjugated donkey anti-sheep antibody. TO-PRO-3 was used for nuclear counterstaining. Scale bars, 10 μ m. **(B)** The confocal settings and image analysis were kept constant to demonstrate the gradually increasing labeling of EGFR at the plasma membrane upon decreasing C225 concentrations. **(C)** PAE.EGFR cells were incubated with or without the antibody combination (C225+IgG) using decreasing concentrations of C225 (as indicated in figures) and subsequently chased for 2 h in the presence of CHX. Cell lysates were subjected to SDS-PAGE using a 10% gel and immunoblotted with sheep anti-EGFR antibody. Anti-Tubulin antibody was used as loading control. **(D-E)** HeLa cells **(D)** and MCF-7 cells **(E)** were incubated with or without 5.0 μ g/ml C225 followed by anti-human IgG (C225+IgG), and subsequently chased for 2 h in presence of CHX, or incubated with EGF (60 ng/ml) for 2 h in presence of CHX. Cell lysates were subjected to SDS-PAGE using a 10% gel and immunoblotted with sheep anti-EGFR antibody. Anti-Tubulin antibody was used as loading control.

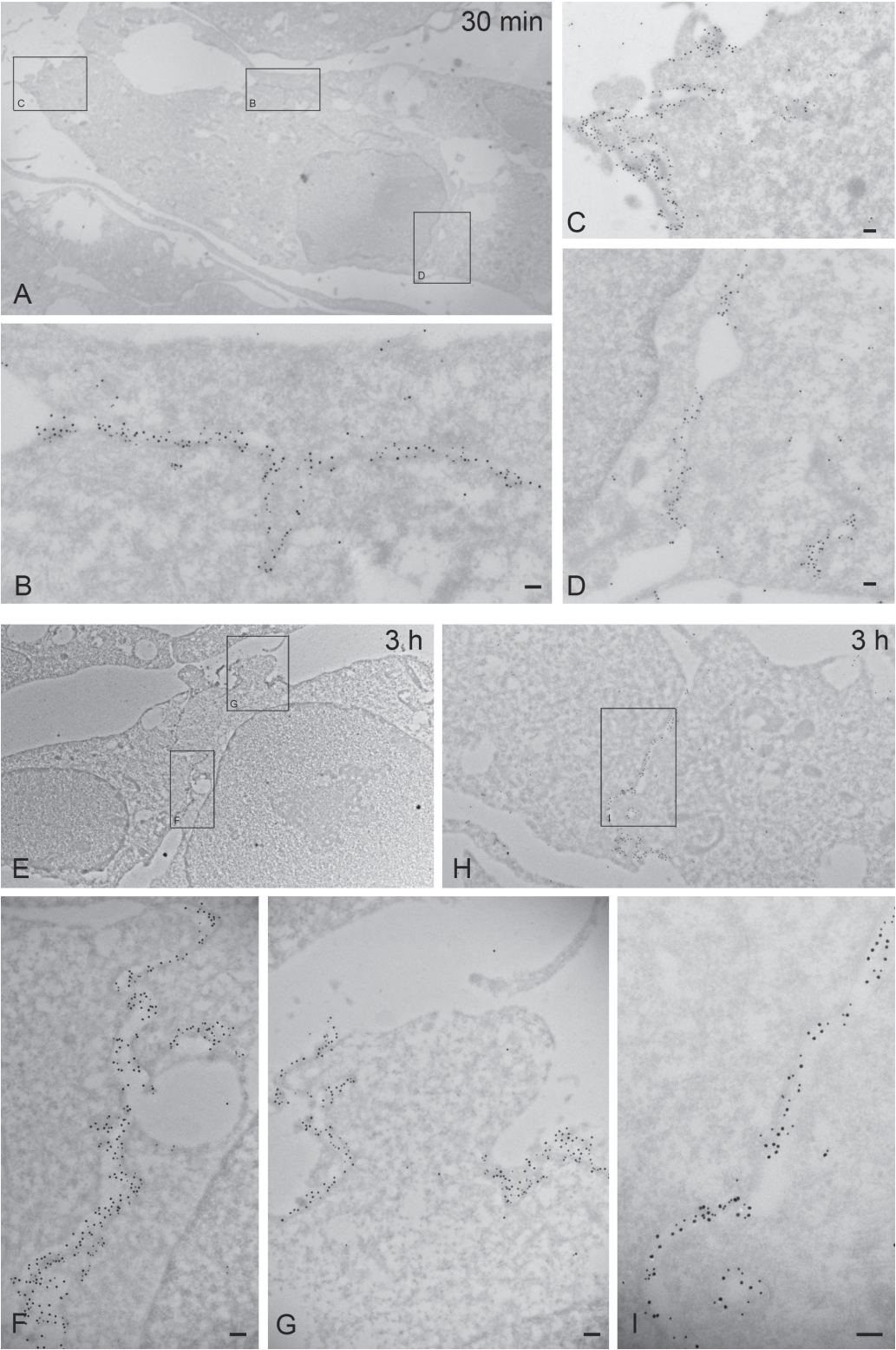
Supplementary Figure S1



Supplementary Figure S2

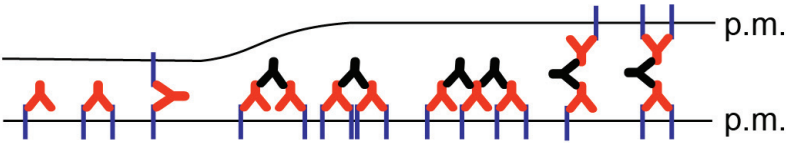


Supplementary Figure S3

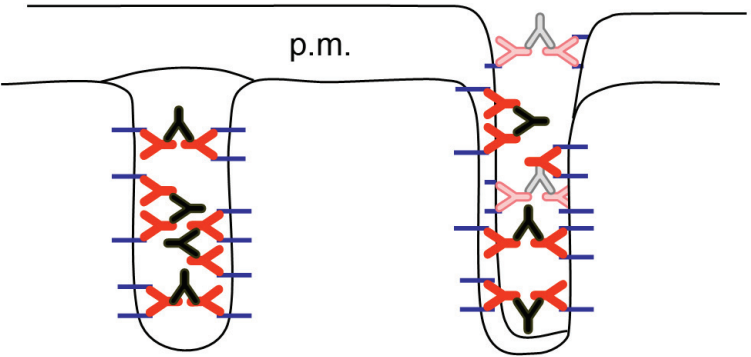


Supplementary Figure S4

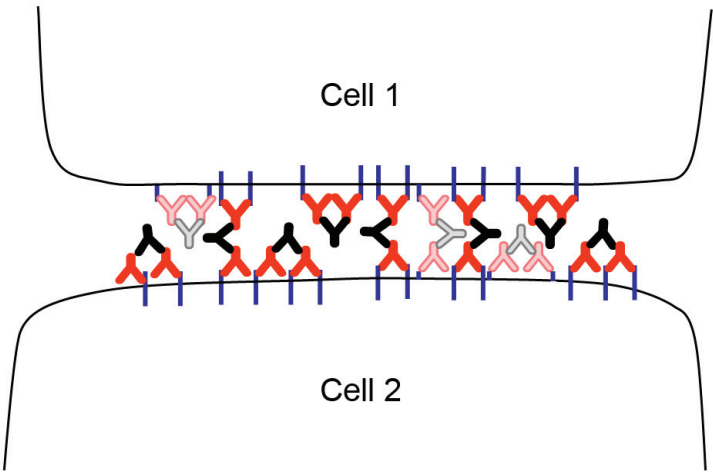
A



B

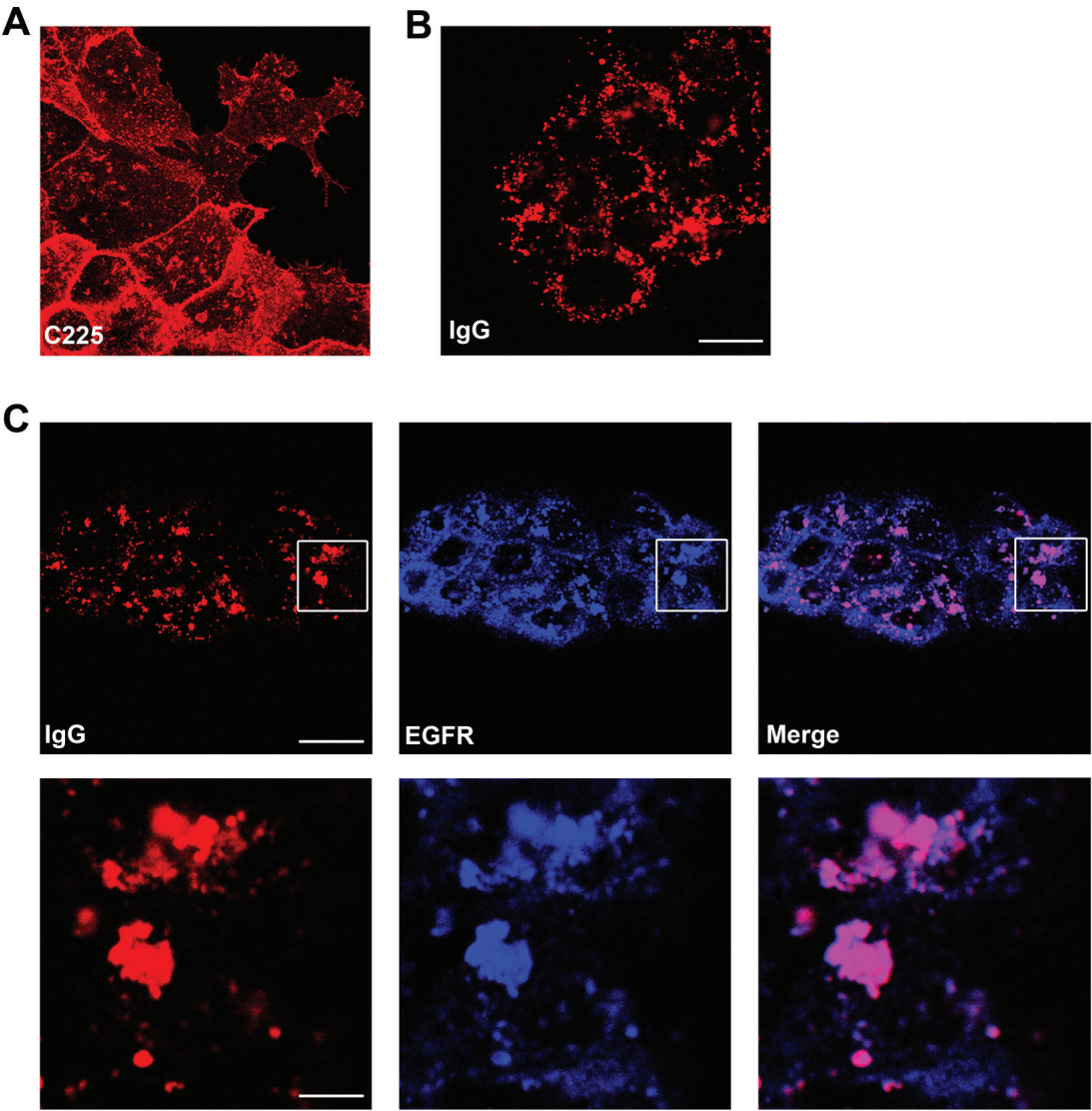


C



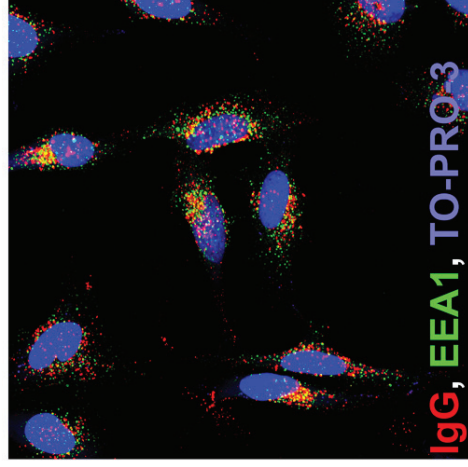
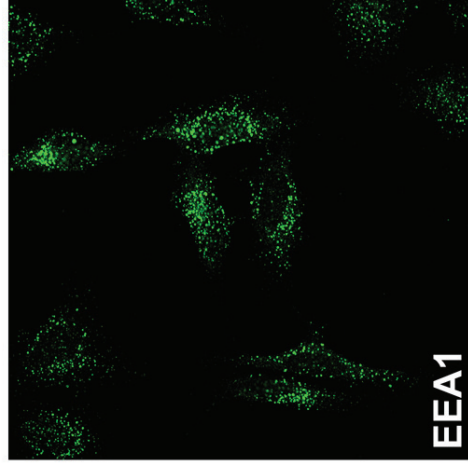
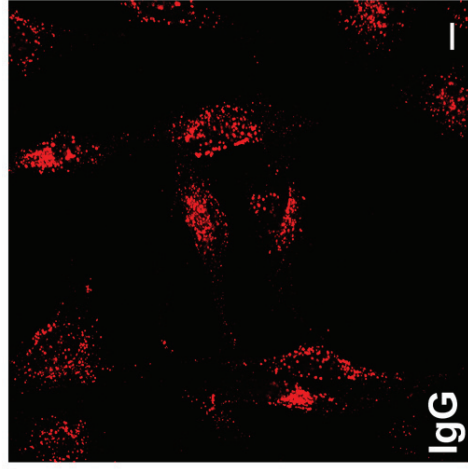
EGFR: | C225:   Anti-human IgG:  

Supplementary Figure S5

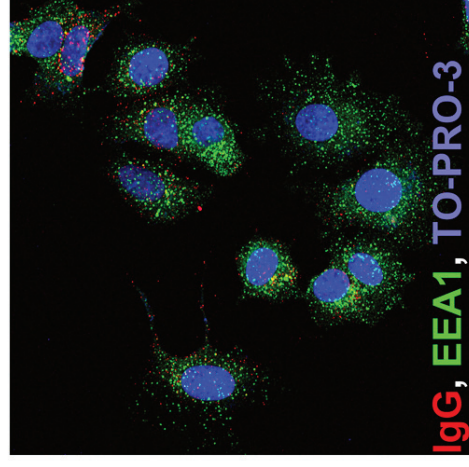
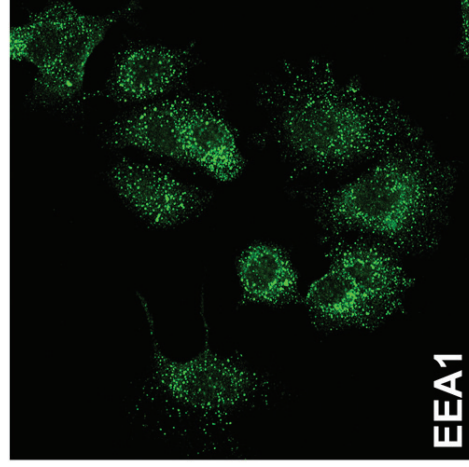
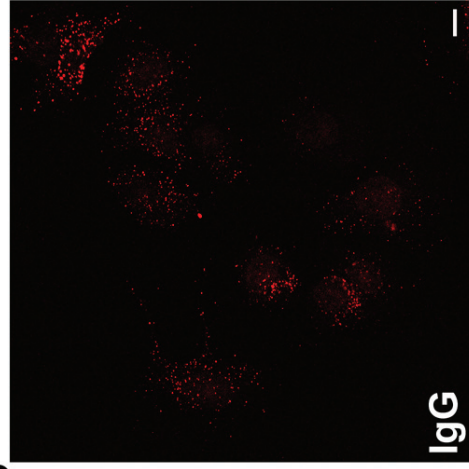


Supplementary Figure S6

A

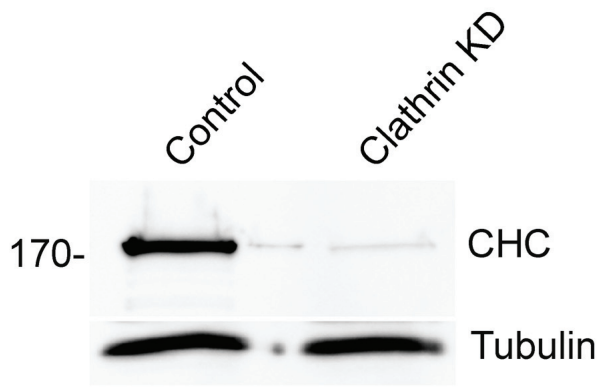


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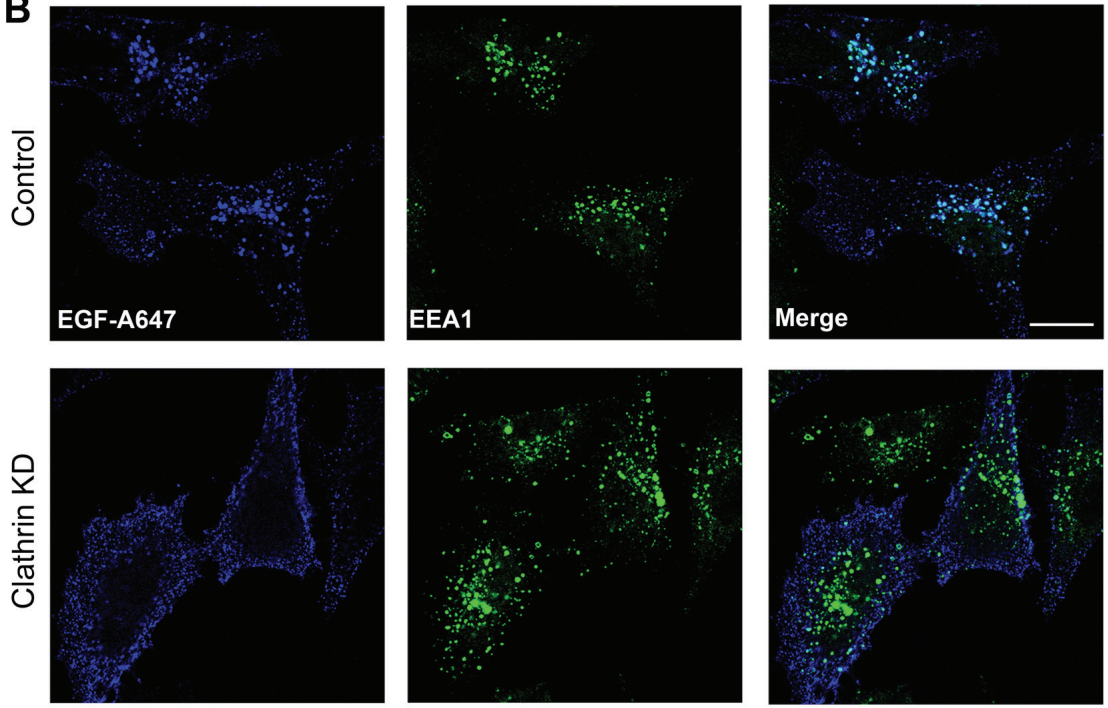


Supplementary Figure S7

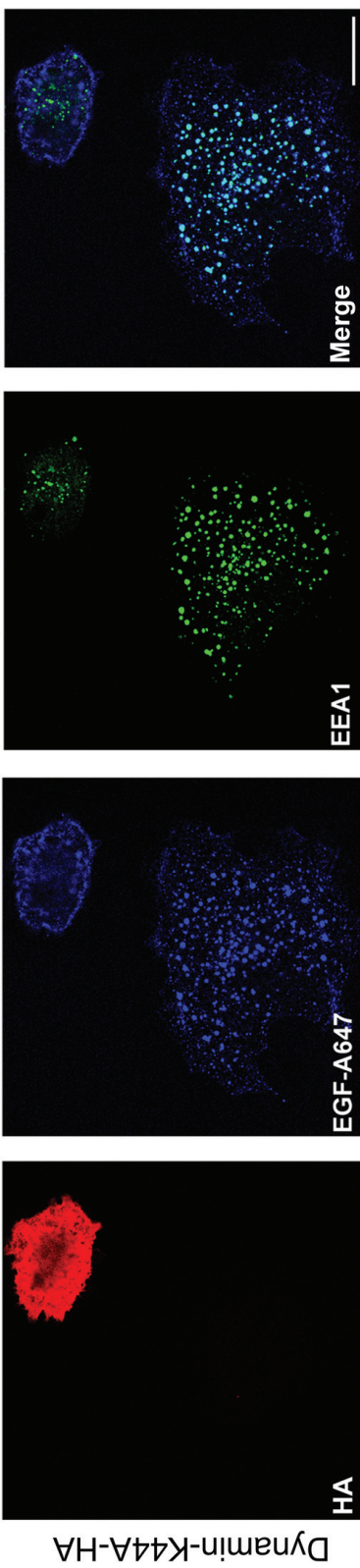
A



B



Supplementary Figure S8



A

5 μ g
0.5 μ g
0.05 μ g
0.005 μ g

lgG
EEA1
lgG, EEA1, TO-PRO-3

B

5 μ g
0.5 μ g
0.05 μ g
0.005 μ g

lgG
EGFR
lgG, EGFR, TO-PRO-3

C

C225 (μ g)+lgG: - 5 0.5 0.05 0.005

170- EGFR
Tubulin

D

C225+lgG: - + -
EGF (60 ng/ml): - - +

170- EGFR
Tubulin

E

C225+lgG: - + -
EGF (60 ng/ml): - - +

170- EGFR
Tubulin

E

C225-IgG:	-	+	-
EGF (60 ng/ml):	-	-	+
170-			
	EGFR		
	Tubulin		

Paper III

Juliana Bentes Hughes, **Christian Berger**, Marianne Skeie Rødland,
Max Hasmann, Espen Stang, and Inger Helene Madshus.

Pertuzumab increases epidermal growth factor receptor down-regulation by
counteracting epidermal growth factor receptor-ErbB2 heterodimerization.

Molecular Cancer Therapeutics, 2009 Jul;8(7):1885-92.

Paper IV

Tram Thu Vuong, **Christian Berger**, Vibeke Bertelsen,
Espen Stang, and Inger Helene Madshus.

Preubiquitinated chimeric ErbB2 is constitutively endocytosed
and subsequently degraded in lysosomes.

Experimental Cell Research (Submitted)

Preubiquitinated chimeric ErbB2 is constitutively endocytosed and subsequently degraded in lysosomes

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Abbreviations: CHC, clathrin heavy chain; EGFR, epidermal growth factor receptor; EEA1, early endosome antigen 1; ESCRT, endosomal sorting complex required for transport; GA, geldanamycin; Hsp90, Heat shock protein 90; LAMP1, lysosomal-associated membrane protein 1; PAE cells, Porcine Aortic Endothelial cells; RTK, receptor tyrosine kinase; Ub, ubiquitin; UIM, ubiquitin interacting motif

Abstract

The oncoprotein ErbB2 is endocytosis-deficient, probably due to its interaction with Heat Shock Protein 90. We previously demonstrated that clathrin-dependent endocytosis of ErbB2 is induced upon incubation of cells with Ansamycin derivatives, such as geldanamycin and its derivative 17-AAG. We have previously demonstrated that a preubiquitinated chimeric EGFR (EGFR-Ub₄) is constitutively endocytosed in a clathrin-dependent manner. We now demonstrate that also an ErbB2-Ub₄ chimera is endocytosed constitutively and clathrin-dependently. Upon expression, the ErbB2-Ub₄ was further ubiquitinated, and by Western blotting, we demonstrated that ErbB2-Ub₄ nucleates and enables formation of both Lys48-linked and Lys63-linked polyubiquitin chains. This is in contrast to EGFR-Ub₄, where no further ubiquitination was observed. ErbB2-Ub₄ was constitutively internalized and eventually sorted to late endosomes and lysosomes where the fusion protein was degraded. ErbB2-Ub₄ was not cleaved prior to internalization. Interestingly, over-expression of Ubiquitin Interaction Motif -containing dominant negative fragments of the clathrin adaptor proteins epsin1 and Eps15 negatively affected endocytosis of ErbB2. Altogether, this argues that ubiquitination is sufficient to induce clathrin-mediated endocytosis and lysosomal degradation of the otherwise plasma membrane localized ErbB2. Also, it appears that C-terminal cleavage is not required for endocytosis.

Key words: ErbB2, endocytosis, signaling, ubiquitination, kinase activity

Introduction

The epidermal growth factor receptor (EGFR) family consists of four receptor tyrosine kinases (RTKs): EGFR/ErbB1, ErbB2, ErbB3 and ErbB4. Aberration of signaling in human disease has in many instances been ascribed to altered function or expression of ErbB proteins, and over-expression and/or activating mutations of ErbB proteins are frequently associated with cancer. This especially applies to breast and ovarian carcinoma and to colon-, prostate-, non small cell lung- and pancreatic cancer. The ErbB proteins have similar overall structure. However, they behave in different ways with respect to ligand-induced activation and down-regulation [1]. Down-regulation by endocytosis and subsequent degradation counteracts growth promoting and anti-apoptotic signaling. Understanding mechanisms involved in down-regulation of ErbB proteins should pave the way towards rational design of new drugs. Mechanistic insight has already resulted in ErbB-interacting agents undergoing clinical trials. Activation of ErbB proteins depends on dimerization, which is normally ligand-dependent, but possibly also ligand-independent when ErbB proteins are over-expressed [2]. ErbB2 is an orphan receptor, which due to its constitutively exposed dimerization arm is incapable of binding ligand [3]. ErbB2 is over-expressed in a number of human malignancies, and over-expression is associated with poor clinical outcome [4]. Due to the pre-exposed dimerization arm, ErbB2 readily interacts with other ErbB proteins (EGFR, ErbB3 and ErbB4) both in presence and absence of growth factors. By heterodimerization, ErbB2 plays a dominant role in mediating the malignant phenotype [5]. Also, ErbB2 signals poor prognosis due to its efficient interaction with other RTKs, such as c-Met and with the cytosolic kinase Src [6, 7].

ErbB2 is normally concentrated at the plasma membrane and hardly observed in endosomes in non-manipulated cells. We and others have considered ErbB2 to be endocytosis deficient [8-10]. We have previously demonstrated that when ErbB2 is over-expressed, EGFR-ErbB2 heterodimerization causes retention of EGFR at the plasma membrane [8]. Furthermore, we have demonstrated that when cells are incubated with the anti-ErbB2 antibody Pertuzumab, EGFR-ErbB2 heterodimers dissolve, and in the presence of EGF, the free EGFRs form homodimers that are rapidly endocytosed [11]. This suggests that ErbB2 upon heterodimerization has a tethering function, inhibiting EGFR endocytosis. At the plasma membrane, ligands

that interact with EGFR can induce formation of EGFR-ErbB2 heterodimers and cause sustained proliferative signaling compared to signaling from EGFR homodimers. This pinpoints ErbB2 as an important treatment target [12].

ErbB2 is a Heat shock protein 90 (Hsp90) client, and Hsp90 stabilizes ErbB2 at the plasma membrane, probably by direct interaction with ErbB2 [13, 14]. Endocytic down-regulation of ErbB2 is induced when the interaction with Hsp90 is interrupted by the Hsp90-interacting Ansamycin geldanamycin (GA) [15-17]. The Hsp90-interacting agents, such as GA and the GA-derivatives 17-AAG and 17-DMAG bind to the ATP/ADP-binding pocket of Hsp90 with higher affinity than the nucleotide, thereby replacing it. This inhibits the chaperone function of Hsp90 and leads to degradation of Hsp90 client proteins. 17-AAG has less general toxicity than GA and is now in clinical trials [18, 19]. However, the exact molecular mechanisms whereby Hsp90 inhibition leads to degradation of Hsp90 client proteins have not been worked out. It is clear that ErbB2 is ubiquitinated as a result of incubation with GA [20], and it has been assumed that the ubiquitin (Ub) ligases CHIP (carboxyl terminus Hsc70-interacting protein) and/or Cullin5 is involved in GA-induced internalization and degradation of ErbB2 [21-23]. However, whether ubiquitination serves as an endocytosis signal or as a signal for proteasomal cleavage of ErbB2 is unclear [15, 16, 24]. We have in the following investigated how constitutive ubiquitination affects ErbB2 endocytosis and degradation. This has been addressed by use of a chimeric preubiquitinated ErbB2 containing full-length ErbB2 and a C-terminally appended tetra-Ub chain (ErbB2-Ub₄).

We recently demonstrated that a tetra-Ub chain could mediate clathrin-dependent endocytosis of the EGFR regardless of kinase activity [25], and we have in the current study investigated and compared ubiquitination, endocytosis and degradation of wt ErbB2 and ErbB2-Ub₄. Interestingly, we now demonstrate that also in case of ErbB2 is a tetra-Ub chain capable of inducing endocytosis and degradation of the fusion protein. Like in case of EGFR-Ub₄, ErbB2-Ub₄ was found to be endocytosed via clathrin-coated pits in the absence of kinase activity. The ErbB2 chimera was also demonstrated to be further ubiquitinated by attachment of Lys48- and Lys63-linked Ub chains. To this end, we therefore conclude that ubiquitination enhances both endocytosis and degradation of ErbB2.

Materials and Methods

Materials

17-AAG was from Tocris Bioscience. Protein A-coupled magnetic beads were from Invitrogen (Carlsbad, CA, USA). Other chemicals were from Sigma-Aldrich Corporation (St. Louis, MO, USA) unless otherwise noted.

Antibodies

Mouse anti-ErbB2 antibody (clone TAB250 to the extracellular part) and rabbit anti-ErbB2 antibody (PAD: Z4881 to the intracellular part) were from Invitrogen. Mouse anti-ErbB2 antibody (clone 42 to the extracellular part), and mouse anti-CHC antibody were from BD Biosciences (Heidelberg, Germany), goat anti-ErbB2 antibody (to the extracellular part) was from R&D Systems (Minneapolis; MN, USA), mouse anti-ErbB2 (Ab-3 to the intracellular part) was from Calbiochem – Merck Biosciences (Beeston, Nottingham, UK), and rabbit anti-phospho-ErbB2 (pY1248) antibody was from Millipore (Billerica, MA, USA). Rabbit anti-tubulin, rabbit anti-lysosomal-associated membrane protein 1 (LAMP1) and rabbit anti-Myc antibodies were from Abcam plc (Cambridge, UK). Mouse anti-Ub and goat anti-Early endosomal antigen 1 (EEA1) antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Alexa Fluor 488-conjugated goat anti-rabbit and Alexa Fluor 647-conjugated donkey anti-goat antibodies were from Invitrogen. Rhodamine RedX-conjugated donkey anti-mouse, DyLight 488-conjugated donkey anti-goat and peroxidase-conjugated donkey anti-rabbit, -mouse, and -human antibodies were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Human anti-Lys63-linked polyUb antibody (Apu3.A8) and human anti-Lys48-linked polyUb antibody (Apu2.07) were provided by Genentech, Inc. (South San Francisco, CA, USA).

Cell culture and treatment

Porcine Aortic Endothelial (PAE) cells were grown in Ham's F-12 medium with L-glutamine (Lonza Group Ltd., Basel Switzerland), containing 0.5× Penicillin-Streptomycin mixture (Lonza Group, Ltd.) and 10% (v/v) Fetal Bovine Serum (FBS) at 37 °C with 5% CO₂. During experiments, cells were incubated with the indicated

compounds either in minimal essential medium (MEM; without bicarbonate) (Invitrogen) with 0.1% BSA, or in Ham's F-12 medium if experiments lasted for more than 4 h. In experiments investigating receptor degradation, the cells were incubated with 25 µg/ml cycloheximide.

Plasmids

The pcDNA3.1-ErbB2 was described earlier [8]. Generation of pcDNA3.1/Hygro(+)-ErbB2-Ub₄ was performed by Mutagenex Inc. (Piscataway, NJ, USA). In brief, the full-length wt ErbB2 was amplified by PCR from pRK5-HER2-GFP (a gift from Andrew Chantry, University of East Anglia, Norwich, United Kingdom) using the primers 5'AAGCTTGCCACCATGGAGCTGGCGGCCTTGTGCC3' and 5'CCTAGGCACTGGCAGTCCAGACCCAGG3' containing *HindIII* and *AvrII* restriction sites, respectively. The PCR product was then ligated into pcDNA3.1/Hygro(+)-4xUb vector generated from pcDNA3.1/Hygro-EGFR-Ub₄ (described in [25]), in which the EGFR part was excised from the plasmid using restriction enzymes *HindIII* and *AvrII*. It should be noted that the second *AvrII* restriction site at position 6822 in the vector was site-directly mutated for the purpose of having a unique *AvrII* restriction site. The ErbB2-Ub₄ construct was sequenced to confirm that no mutations had been introduced during PCR and cloning. The pcDNA3.1-Myc-Epsin1 ENTH-UIIM was described earlier [26]. pEGFP-C2-Eps15 DIIIΔ2 was a gift from Alexandre Benmerah (Université Paris Descartes, Paris, France).

Transfection of cells

Transient transfection with plasmids as indicated in figure legends was performed using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's recommendations. The transfected cells were analyzed approximately 20 h upon transfection. For knock-down of clathrin heavy chain (CHC), the cells were transfected twice with siRNA with a 48 h interval using Lipofectamine™ 2000. The target sequence was GCAAUGAGCUGUUUGAAGA [27], and the siRNA duplexes were synthesized and annealed by Invitrogen. The control siRNA cells were transfected with Silencer Negative control #1 from Applied Biosystems (Carlsbad, CA, USA).

Western blotting

Cells were incubated as described in figure legends before being lysed and analyzed by SDS-PAGE and subsequently subjected to immunoblotting as previously described [8]. The reactive proteins were visualized by using Pierce SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL, USA), and the chemiluminescence signals were detected by Kodak Image Station 400R (Carestream Health, Inc., Rochester, NY, USA). Restore PLUS Western Blot Stripping Buffer (Thermo Scientific) was used according to the manufacturer's recommendations for stripping of membranes.

Co-immunoprecipitation

Upon transfection and incubation as described in figure legends, cells were lysed in ice-cold lysis buffer (20 mM HEPES, pH 7.2, 2 mM $MgCl_2$, 100 mM NaCl, 0.1 mM EDTA, 0.1% Triton X-100, 5 mM NEM, with 1:100 (v/v) of protease inhibitor cocktail and phosphatase inhibitor cocktail 2 (P8340 and P5726)). The lysates were then added to protein A-coupled magnetic beads precoupled to rabbit antibody to ErbB2 in 0.1 M phosphate buffer, pH 8, with 0.05% Triton X-100, at room temperature for 1 h. Antibody-coupled magnetic beads and cell lysates were gently mixed for 1 h at 4°C. The beads were washed four times with lysis buffer before being eluted in 2× sample buffer [8] at 95°C for 5 min. The eluted proteins and total cell lysates were subsequently subjected to SDS-PAGE and immunoblotting.

Analysis of ErbB2 ubiquitination

To study ubiquitination of ErbB2 and ErbB2-Ub₄, cells were lysed in preheated (100 °C) 1% SDS in PBS, incubated at 100°C for 5 min and chilled on ice before homogenization, using a QIA-shredder column (QIAGEN, Valencia, CA, USA). The lysates were added to protein A-coupled magnetic beads precoupled to mouse anti-ErbB2 antibody (clone 42) as described above. The beads were dissolved in 2x immunoprecipitation (IP) buffer: 2% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 1% (w/v) BSA, 2 mM EDTA, 40 mM NaF, 6 mM NEM, 1:100 (v/v) protease inhibitor and phosphatase inhibitor cocktail. Antibody-coupled magnetic beads and cell lysates were gently mixed for 1 h at 4°C. The beads were then washed

with 1x IP buffer (50% 2x IP buffer + 50% SDS [1%] in PBS) and eluted in 2x sample buffer [8]. Immunoprecipitated ErbB2 was analyzed by immunoblotting with antibody to Ub or antibodies specifically targeting Lys63- or Lys48-linked polyUb chains.

Immunocytochemistry and confocal microscopy

Cells were grown in 60 mm CELL⁺ culture dishes (Sarstedt AG & Co., Nümbrecht, Germany) or on 12 mm coverslips (Menzel-Gläser, Braunschweig, Germany) and transfected with the appropriate plasmids (as described in figure legends). After incubation with the indicated reagents, cells were washed with PBS and fixed in preheated (37 °C) 4% paraformaldehyde (PFA) (Reidel-de Haën, Seelze, Germany) in Soerensen's phosphate buffer for 10 min. Cells were then washed three times in PBS before quenching of background fluorescence in 50 mM NH₄Cl for 10 min at room temperature followed by washing twice with PBS. Fixed cells were permeabilized using 0.1% Triton X-100 in PBS. In experiments staining with anti-LAMP1 antibody, 0.1% saponine (Merck KGaA, Darmstadt, Germany) was used instead of Triton X-100 for cell permeabilization and included in all buffers in the following staining procedure. Nonspecific binding of antibodies was blocked by preincubation with 1% BSA in PBS for 30 min before incubation with primary antibody (diluted in 1% BSA in PBS) for 1 h. Samples were washed with PBS before incubation with a secondary antibody (diluted in 1% BSA in PBS) for 30 min. The cells were mounted using Dako fluorescent mounting medium (Glostrup, Denmark) and examined using confocal microscopy (TCSXP; Leica, Wetzlar, Germany). The images were processed using Adobe Photoshop CS2.

Results

17-AAG induces ubiquitination and internalization of ErbB2

It has previously been reported that ErbB2 is ubiquitinated as a result of incubation with GA, and it has been assumed that Ub is necessary for GA-induced internalization and degradation of ErbB2 [13, 22]. We confirmed ubiquitination and internalization of ErbB2 upon incubation with 17-AAG in PAE cells that do not express endogenous ErbB proteins, but were transfected to express wt ErbB2. Cell lysates from cells incubated with or without 17-AAG were subjected to immunoprecipitation with an antibody to ErbB2 before the precipitated material was analyzed by Western blotting

with an anti-Ub antibody. As demonstrated in Fig. 1A, ubiquitination of ErbB2 was efficient only upon 17-AAG incubation. We further used confocal microscopy analysis to study the localization of wt ErbB2 in the presence or absence of 17-AAG. A clear vesicular localization of ErbB2 was only observed in 17-AAG incubated cells. These data confirm that 17-AAG induces ubiquitination and internalization of ErbB2.

To investigate whether ubiquitination as such facilitates endocytic down-regulation, we constructed a chimeric protein consisting of full-length ErbB2 and four linearly connected Ubs (ErbB2-Ub₄) (Fig. 2A). The string of Ubs attached to the C-terminus of ErbB2 is identical to the one we previously attached to the C-terminus of EGFR [25]. Western blot analysis of immunoprecipitated ErbB2 from PAE cells transiently transfected with plasmids encoding wt ErbB2 or ErbB2-Ub₄ showed that the molecular weight was increased for ErbB2-Ub₄ when compared to wt ErbB2. The fusion protein was also readily recognized by an antibody to Ub (Fig. 2B).

ErbB2-Ub₄ is constitutively modified with Lys63- and Lys48-linked polyUb chains

We further studied ubiquitination of wt ErbB2 and ErbB2-Ub₄ in PAE cells expressing wt ErbB2 or ErbB2-Ub₄ upon incubation with or without 17-AAG. The cell lysates were subjected to immunoprecipitation with an antibody to ErbB2 before the precipitated material was analyzed by Western blotting with an anti-Ub antibody. As demonstrated in Fig. 1A and 3A, a strong high molecular weight smear was observed for wt ErbB2 in the presence of 17-AAG, confirming 17-AAG-induced ubiquitination of ErbB2. However, in the case of ErbB2-Ub₄, a smear was observed both in the absence and presence of added 17-AAG (Fig. 2B and 3A). Although the ubiquitination increased upon incubation with 17-AAG, the ErbB2-Ub₄ appeared to be further ubiquitinated regardless of incubation with 17-AAG. The extra Ub chains on ErbB2-Ub₄ could arise from ubiquitination of the pre-appended tetra-Ub and/or of ErbB2 itself. The constitutive ubiquitination could suggest that ErbB2-Ub₄ is recognized by the quality control machinery of the cell, which is responsible for ubiquitination of unfolded proteins upon recognizing exposed hydrophobic residues [28]. To study the nature of the polyUb chains on wt ErbB2 and ErbB2-Ub₄, we immunoprecipitated ErbB2 (wt and -Ub₄) and performed Western blotting using antibodies recognizing Lys48-linked or Lys63-linked Ub chains. Under all conditions,

we found that the ubiquitinated ErbB2 was recognized by antibodies to both Lys48- and Lys63-linked Ub chains (Fig. 3B).

ErbB2-Ub₄ localizes constitutively to early and late endosomes

In order to investigate whether the appended Ub string affects the subcellular localization of ErbB2, we used confocal microscopy analysis to study the localization of wt ErbB2 and of ErbB2-Ub₄ in absence or presence of 17-AAG. Interestingly, ErbB2-Ub₄ was observed to localize to intracellular vesicles in the absence of 17-AAG (Fig. 4A, lower left panel), in contrast to wt ErbB2, which mainly localized to the plasma membrane under similar conditions (Fig. 4A, upper left panel). However, when 17-AAG was added, the amount of ErbB2 positive vesicles increased both in cells expressing wt ErbB2 and in cells expressing ErbB2-Ub₄ (Fig. 4A upper and lower right panels). These findings argue that while wt ErbB2 localizes to endosomes only upon incubation with 17-AAG, ErbB2-Ub₄ is internalized constitutively, and further that internalization of ErbB2-Ub₄ can be enhanced upon incubation with 17-AAG. To confirm the endosomal nature of the vesicles where ErbB2 and ErbB2-Ub₄ accumulated, we labeled cells with antibodies recognizing endosome-associated proteins. Antibodies marking early endosomes (anti-EEA1) and antibodies marking late endosomes and lysosomes (anti-LAMP1) were used. Upon incubation of cells expressing wt ErbB2 with 17-AAG, ErbB2 was observed in both early and late endosomes (Fig. 4B). ErbB2-Ub₄ was, however, observed to localize to both EEA1 and LAMP1 positive compartments even in the absence of 17-AAG (Fig. 4C). This again argues that ubiquitination of ErbB2 triggers endocytosis and translocation of ErbB2 to late endosomes and lysosomes.

ErbB2-Ub₄ is not cleaved prior to internalization

Previous studies have demonstrated that the intracellular C-terminal part of ErbB2 can be cleaved upon incubation with GA. This cleavage depended on proteasomal activity and did apparently occur at the plasma membrane. It is however not clear whether cleavage is in fact required for endocytosis of ErbB2 as such [15, 16, 24]. To investigate whether the constitutive internalization of ErbB2-Ub₄ depends on a Ub-induced C-terminal cleavage, we used anti-ErbB2 antibodies specifically recognizing either the ErbB2 extracellular domain or the very C-terminal part of ErbB2 and studied the localization of wt ErbB2 and ErbB2-Ub₄ in cells incubated with or without

17-AAG. In line with data presented above, ErbB2-Ub₄ demonstrated a constitutive vesicular localization, while wt ErbB2 showed vesicular staining only upon incubation with 17-AAG (Fig. 5). Importantly, both antibodies showed the same labeling pattern, confirming our previous conclusion that ErbB2 is not cleaved prior to internalization [16]. Based on these data, it seems that ubiquitination of ErbB2 primarily is a signal for endocytic sorting and not a signal for proteasome-mediated cleavage of the ErbB2 C-terminus.

ErbB2-Ub₄ is internalized clathrin-dependently

We previously demonstrated that ErbB2 could be endocytosed via clathrin-coated pits upon incubation with GA [16]. To investigate whether ErbB2-Ub₄ is also internalized via clathrin-coated pits, PAE cells were transfected with siRNA to clathrin heavy chain (CHC) before being transfected with plasmids encoding wt ErbB2 or ErbB2-Ub₄. As demonstrated in Fig. 6A, knock-down of CHC was efficient. Consistent with previous reports, siRNA-mediated down-regulation of CHC efficiently blocked 17-AAG-induced endocytosis of wt ErbB2 (Fig. 6B). Additionally, we observed a lack of vesicular staining for ErbB2 in CHC-depleted cells expressing ErbB2-Ub₄. These data argue that the endocytosis of ErbB2-Ub₄ is clathrin-dependent. Block of ErbB2-Ub₄ internalization was observed both in cells incubated with and without 17-AAG, suggesting that both constitutive and 17-AAG-induced endocytosis of ErbB2-Ub₄ is clathrin-dependent.

Overexpression of UIM-containing fragments of epsin1 and Eps15 inhibits internalization of wt ErbB2 and ErbB2-Ub₄

We have recently demonstrated that the adaptor protein epsin 1 interacts with the ubiquitinated EGFR via its ubiquitin interacting motifs (UIMs) and that this interaction promotes translocation of EGFR into central parts of clathrin-coated pits [26]. Furthermore, our recent data also demonstrated that both epsin 1 and the adaptor protein Eps15 constitutively interacted with EGFR-Ub₄ [25]. In order to investigate whether epsin1 and Eps15 are also involved in clathrin-mediated endocytosis of wt ErbB2 and ErbB2-Ub₄, we made use of truncated versions of epsin 1 and Eps15. The ENTH-UIM part of epsin 1 (Epsin1 ENTH-UIM) or the C-terminal part of Eps15 containing two UIMs (Eps15DIIIΔ2) were co-expressed with either wt ErbB2 or ErbB2-Ub₄ before the cells were analyzed by confocal microscopy. Co-expression of

Epsin1 ENTH-UIIM or Eps15DIIIΔ2 and ErbB2-Ub₄ was found to reduce the vesicular ErbB2-Ub₄ localization otherwise observed in cells expressing ErbB2-Ub₄ only (Fig. 7A). The same effect was observed in 17-AAG-stimulated cells co-expressing wt ErbB2 and Epsin1 ENTH-UIIM or Eps15DIIIΔ2 (Fig. 7B). Together, these results suggest that the adaptor proteins epsin 1 and Eps15 recruit ubiquitinated ErbB2 to clathrin coated pits from where ErbB2 is endocytosed.

Kinase activity is not required for endocytic down-regulation of ErbB2-Ub₄

To investigate whether ErbB2 kinase activity is required for translocation of ErbB2-Ub₄ to coated pits, we inhibited the ErbB2 kinase using the kinase inhibitor AG879 (Fig. 8A). Immunofluorescence confocal microscopy analysis of cells expressing ErbB2-Ub₄ demonstrated that kinase activity was not required in order to induce endocytosis of ErbB2-Ub₄ (Fig. 8B). Neither did inhibition of the ErbB2 kinase activity affect co-localization of ErbB2-Ub₄ with LAMP1-positive endosomes (Fig. 8C). This argues that ErbB2 kinase activity is not required for ubiquitin-mediated internalization of ErbB2-Ub₄ or for the transport of ErbB2-Ub₄ along the endocytic pathway to late endosomes and lysosomes.

ErbB2-Ub₄ is efficiently degraded upon endocytosis

Since ErbB2-Ub₄ was efficiently transported to LAMP1-positive compartments both in the presence and absence of 17-AAG, we investigated to what extent ErbB2-Ub₄ was constitutively degraded. The remaining ErbB2-Ub₄ and wt ErbB2 in cells incubated with cycloheximide for increasing time periods were analyzed by Western blotting using an antibody to the extracellular part of ErbB2. Our data demonstrated a more efficient degradation of ErbB2-Ub₄ when compared to wt ErbB2 (Fig. 9). The difference in degradation efficiency increased with increased incubation time, and upon incubation for 4 h, ErbB2-Ub₄ was barely detectable.

Discussion

The endocytosis-promoting effect of ubiquitination on protein internalization has been demonstrated for several transmembrane proteins, including the EGFR [reviewed in 29]. We have previously demonstrated that a chimeric preubiquitinated EGFR (EGFR-Ub₄) was internalized in a constitutive manner without requirement for kinase

activity [25]. This argues that ubiquitination as such can induce endocytosis. ErbB2 is generally restricted to the plasma membrane and appears to be endocytosis-resistant [reviewed in 30, 31]. The inhibition of ErbB2 endocytosis is probably mediated by its interaction with Hsp90, since inhibition of Hsp90 by Ansamycins (GA or 17-AAG) induced endocytosis and degradation of ErbB2 [32]. It is assumed that ubiquitination is involved in ErbB2 down-regulation upon incubation with Hsp90-interacting agents [21]. The impact of ubiquitination on down-regulation of ErbB2 has, however, not been directly investigated, and it has been unclear whether ubiquitination of ErbB2 would be sufficient to induce its down-regulation from the plasma membrane. To address whether ubiquitination as such can induce ErbB2 endocytosis, we engineered a tetra-Ub-containing fusion protein, ErbB2-Ub₄, and investigated the effect of appending the tetra-Ub string on ubiquitination, endocytosis and degradation of ErbB2.

Our current data clearly show that ErbB2-Ub₄ was endocytosed constitutively and kinase-independently in a clathrin-dependent manner. ErbB2-Ub₄ was observed to localize to EEA1-positive and LAMP1-positive endosomes, and appears to use the same endocytic pathway as ErbB2 upon incubation with GA or GA-derivatives (our current results and [16]). Additionally, labeling with an antibody recognizing the C-terminal part of ErbB2 demonstrated that ErbB2-Ub₄ was not cleaved prior to endocytosis.

The impaired endocytosis of ErbB2 could potentially also be explained by rapid recycling of ErbB2, and incubation with GA has been reported to induce down-regulation of ErbB2 by diverting endocytosed ErbB2 from a recycling to a degradative pathway [33]. Endocytosed ubiquitinated cargo is recognized by the endosomal sorting complex required for transport (ESCRT) machinery [reviewed in 34]. This could suggest that ubiquitination of ErbB2 is not a signal for endocytosis as such, but rather a signal for interaction with the ESCRT machinery and sorting towards late endosomes and lysosomes. Based on our current results we cannot exclude this, but in contrast to ErbB2-Ub₄, wt ErbB2 was not observed in endosomes unless the cells were incubated with 17-AAG. Furthermore, endocytosis of both ubiquitinated ErbB2 (our current data) and ubiquitinated EGFR [25], was inhibited by dominant negative fragments of the Ub-binding, clathrin-coated pit localized adaptor

proteins Eps15 and epsin 1. This argues that ubiquitination is involved in initial transport of EGFR and ErbB2 to clathrin-coated pits.

Our current data also demonstrated that incubation with 17-AAG induced strong ubiquitination of wt ErbB2, and Western-blotting experiments showed that ErbB2 was modified by both Lys48- and Lys63-linked Ub chains. ErbB2-Ub₄ was also modified by Lys48- and Lys63-linked Ub chains, even in the absence of 17-AAG. Given this scenario, we cannot conclude as to whether or not the endocytosis and sorting to degradative compartments solely depends on the appended Ub₄ chain or whether Lys63-linked polyUb chains are responsible for down-regulation of the ErbB2-Ub₄ chimera. However, the finding that EGFR-Ub₄ was constitutively endocytosed [25] and degraded (our unpublished results) argue that the Ub₄ string is sufficient to induce clathrin-dependent endocytosis as well as ESCRT-mediated sorting and degradation.

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Figure Legends

Fig. 1. ErbB2 was ubiquitinated and internalized upon incubation with 17-AAG.

PAE cells transiently transfected with a plasmid encoding wt ErbB2 were incubated with or without 3 μ M 17-AAG for 1 h at 37 °C. A) Cell lysates were subjected to immunoprecipitation under denaturing conditions using mouse anti-ErbB2 antibody (clone 42). The immunoprecipitated material was then analyzed by Western blotting using an anti-ubiquitin (Ub) antibody. The membrane was stripped and subsequently reblotted for ErbB2 using rabbit anti-ErbB2 antibody. Total cell lysates (TCL) were immunoblotted with rabbit anti-ErbB2 antibody, demonstrating the expression of ErbB2 and also blotted with anti-Tubulin antibody as loading control. B) Fixed cells were immunostained with mouse anti-ErbB2 antibody (clone TAB250) followed by Rhodamine Red-X-conjugated anti-mouse antibody. Scale bar, 10 μ m.

Fig. 2. Characterization of the ErbB2-Ub₄ chimera.

A) Schematic picture of the ErbB2-Ub₄ construct (See Materials and Methods for details) EC: Extracellular domain. TM: Transmembrane domain. IC: Intracellular domain Ub: Ubiquitin. B) PAE cells were transiently transfected with plasmids encoding wt ErbB2 or ErbB2-Ub₄. Cell lysates were prepared and subjected to immunoprecipitation under denaturing conditions using a mouse anti-ErbB2 antibody (clone 42). The immunoprecipitated material was then analyzed by Western blotting using an antibody to ubiquitin (Ub), and the membrane was reblotted using rabbit anti-ErbB2 antibody.

Fig. 3. ErbB2-Ub₄ was modified by Lys63- and Lys48-linked polyubiquitin chains upon expression.

PAE cells transiently transfected with plasmids encoding wt ErbB2 or ErbB2-Ub₄ were incubated with or without 3 μ M 17-AAG for 1 h at 37 °C. The cell lysates were subjected to immunoprecipitation under denaturing conditions using mouse anti-ErbB2 antibody (clone 42). The immunoprecipitated material was then analyzed by Western blotting using antibodies to ubiquitin (Ub) (A), or antibodies recognizing Lys48- or Lys63-linked polyUb chains (B). The membranes were stripped and subsequently reblotted using rabbit anti-ErbB2 antibody. Total cell lysates (TCL)

were immunoblotted using rabbit anti-ErbB2 antibody, demonstrating the expressed amounts of ErbB2/ ErbB2-Ub₄, and were also blotted with anti-Tubulin antibody as loading control.

Fig. 4. ErbB2-Ub₄ was constitutively localized to early and late endosomes.

A) PAE cells transiently transfected with plasmids encoding wt ErbB2 or ErbB2-Ub₄ were incubated with or without 3 μ M 17-AAG for 2 h at 37 °C. The cells were then fixed and immunostained with mouse anti-ErbB2 antibody (clone TAB250) and goat anti-EEA1 or rabbit anti-LAMP1 antibodies. Only staining for ErbB2 is demonstrated. B) PAE cells transiently transfected with wt ErbB2 were incubated with 3 μ M 17-AAG as in A). The cells were fixed and immunostained with mouse anti-ErbB2 antibody (clone TAB250) and goat anti-EEA1 or rabbit anti-LAMP1 antibodies followed by Rhodamine Red-X-conjugated (red) anti-mouse and Alexa Flour 647-conjugated (green) anti-goat or Alexa Fluor 488-conjugated (green) anti-rabbit antibodies. C) PAE cells transiently transfected with ErbB2-Ub₄ were fixed and immunostained as in B). It should be noted that some of the cells demonstrated in A) are also demonstrated in B) and C). Scale bar, 10 μ m.

Fig. 5. Neither wt ErbB2, nor ErbB2-Ub₄, was cleaved prior to internalization.

PAE cells transiently transfected with plasmids encoding wt ErbB2 or ErbB2-Ub₄ were incubated with or without 3 μ M 17-AAG for 2 h at 37 °C. The cells were then fixed and immunostained with goat anti-ErbB2 antibodies to the extracellular part (e.c.) and mouse anti-ErbB2 (Ab-3) to the intracellular part (i.c.) followed by DyLight 488-conjugated donkey anti-goat (green) and Rhodamine RedX-conjugated donkey anti-mouse (red) antibodies. Scale bars are indicated on micrographs.

Fig. 6. Endocytosis of wt ErbB2 and ErbB2-Ub₄ was clathrin-dependent.

PAE cells were transfected with CHC siRNA or Silencer Negative control siRNA as described in Materials and Methods. Approximately 20 h prior to experiments, the cells were transfected with plasmids encoding wt ErbB2 or ErbB2-Ub₄. A) Lysates of cells transfected with siRNA and wt ErbB2 or ErbB2-Ub₄ were analyzed by Western blotting, using antibodies to CHC and Tubulin (loading control). B) siRNA transfected cells expressing wt ErbB2 or ErbB2-Ub₄ were incubated with or without

3 μ M 17-AAG for 2 h at 37 °C, fixed, and immunostained with mouse anti-ErbB2 antibody (clone TAB250) before confocal microscopy analysis. Scale bar, 20 μ m.

Fig. 7. Endocytosis of wt ErbB2 and ErbB2-Ub₄ was inhibited upon overexpression of UIM-containing dominant negative epsin1 and Eps15 fragments.

PAE cells were transiently transfected with plasmids encoding wt ErbB2 (B) or ErbB2-Ub₄ (A) together with plasmids encoding the Myc-tagged ENTH-UIM domain of epsin 1 (Epsin1 ENTH-UIM) or EGFP-tagged C-terminal domain of Eps15 containing the two UIMs (Eps15DIII Δ 2). Cells expressing wt ErbB2 were incubated with 3 μ M 17-AAG for 1 h at 37 °C, while cells expressing ErbB2-Ub₄ were incubated in MEM only. The cells were fixed and immunostained with mouse anti-ErbB2 (clone TAB250) antibody alone (cells expressing ErbB2 + Eps15DIII Δ 2), or with mouse anti-ErbB2 (clone TAB250) and rabbit anti-Myc antibodies (cells expressing ErbB2 + Epsin1ENTH-UIM) prior to incubation with fluorescently labeled secondary antibodies. The cells were then analyzed by confocal microscopy. Doubly transfected cells are indicated with asterisks. Scale bars, 10 μ m.

Fig. 8. Endocytosis of ErbB2-Ub₄ was independent of ErbB2 kinase activity.

A) PAE cells transiently transfected with plasmids encoding ErbB2-Ub₄ were incubated with or without the ErbB2 kinase inhibitor AG879 (50 μ M) for 6 h at 37 °C. Cell lysates were then subjected to Western blotting using antibody to ErbB2-phosphotyrosine 1248 (pY1248) and mouse anti-ErbB2 antibody (clone 42). Tubulin was used as loading control. B-C) PAE cells transiently expressing ErbB2-Ub₄ were incubated with AG879 as in A). The cells were subsequently fixed and double stained with mouse anti-ErbB2 (clone TAB250) and EEA1 antibodies (B), or with mouse anti-ErbB2 (clone TAB250) and LAMP1 antibodies (C) before confocal microscopy analysis. Corner insets show higher magnification of the framed areas. Scale bar, 10 μ m.

Fig. 9. ErbB2-Ub₄ was degraded more efficiently than was wt ErbB2.

PAE cells transiently transfected with wt ErbB2 or with ErbB2-Ub₄ plasmids were incubated at 37 °C in medium containing cycloheximide for the times indicated. The cells were lysed, and aliquots of each lysate were subjected to Western blotting with

antibodies to the extracellular domain of ErbB2 (mouse anti-ErbB2 antibody, clone 42) and Tubulin as loading control.

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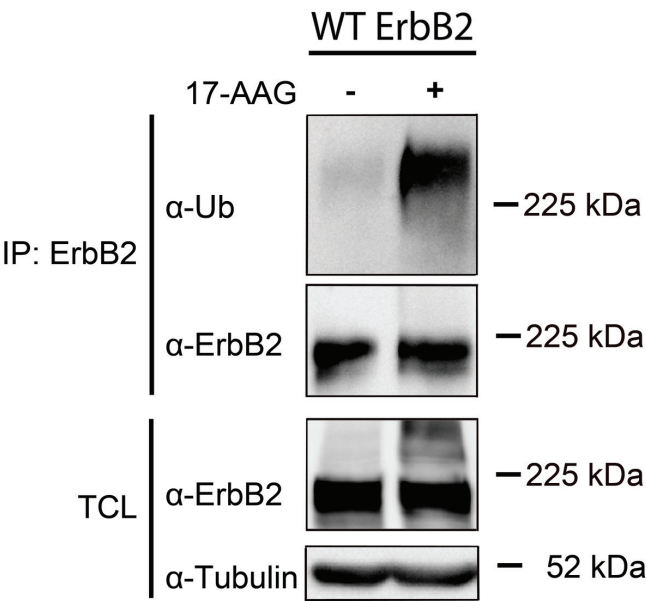
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FIGURE 1

A



B

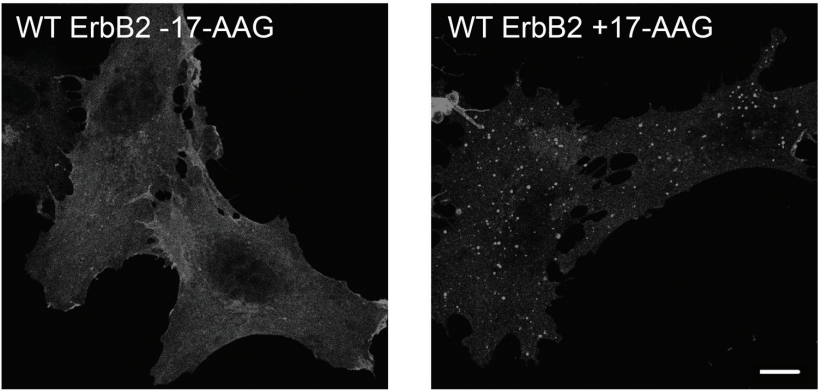
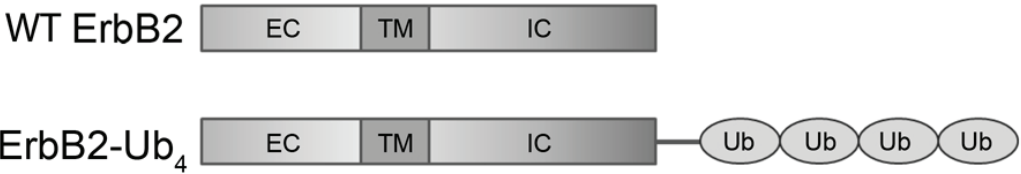


FIGURE 2

A



B

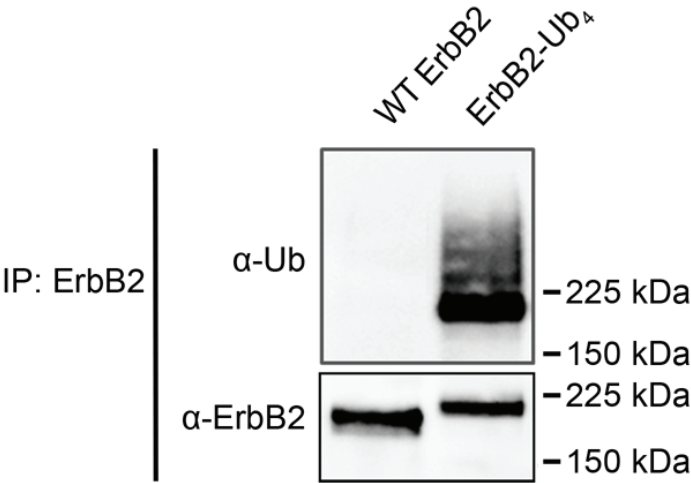
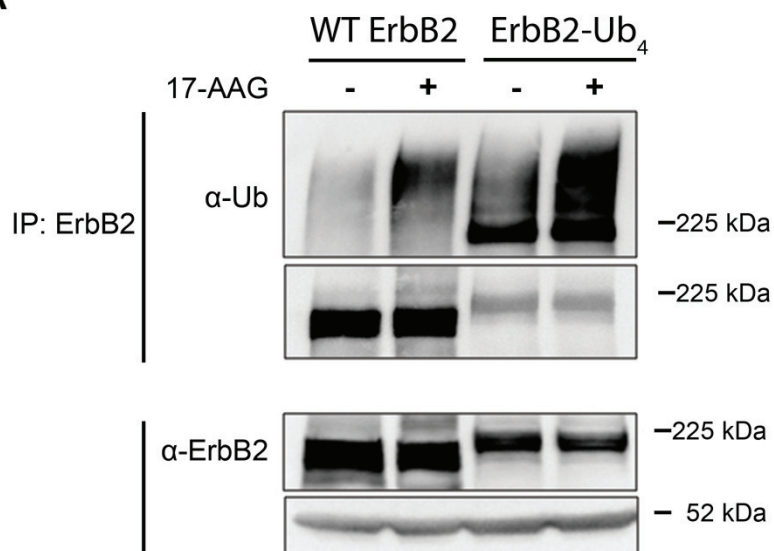


FIGURE 3

A



B

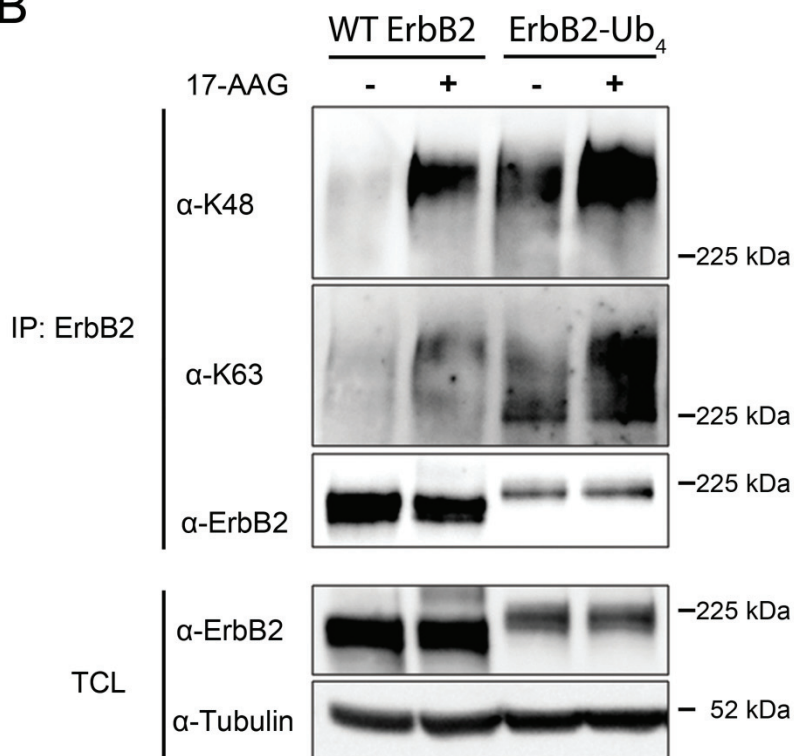
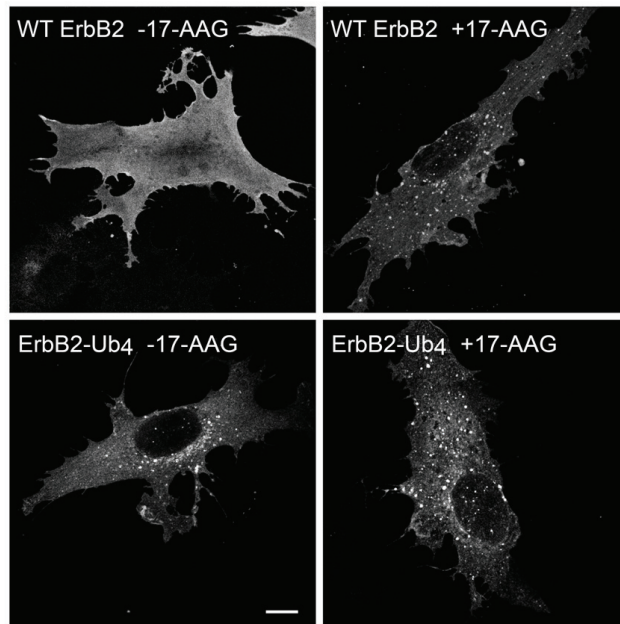
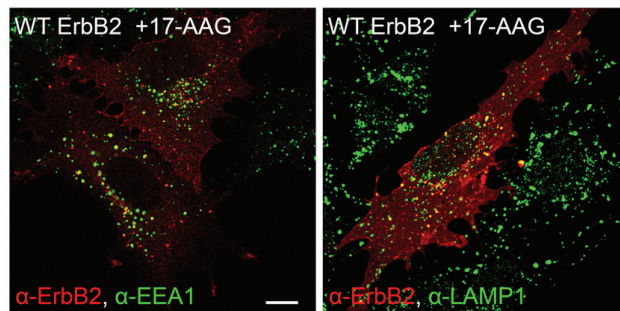


FIGURE 4

A



B



C

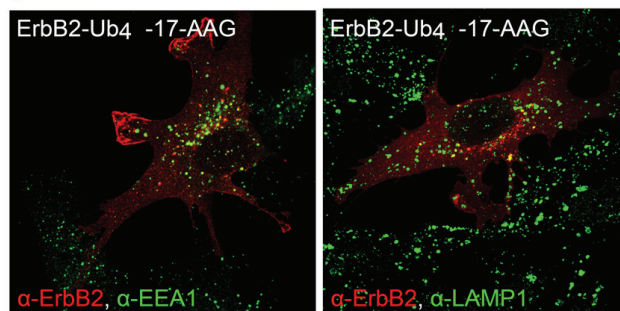


FIGURE 5

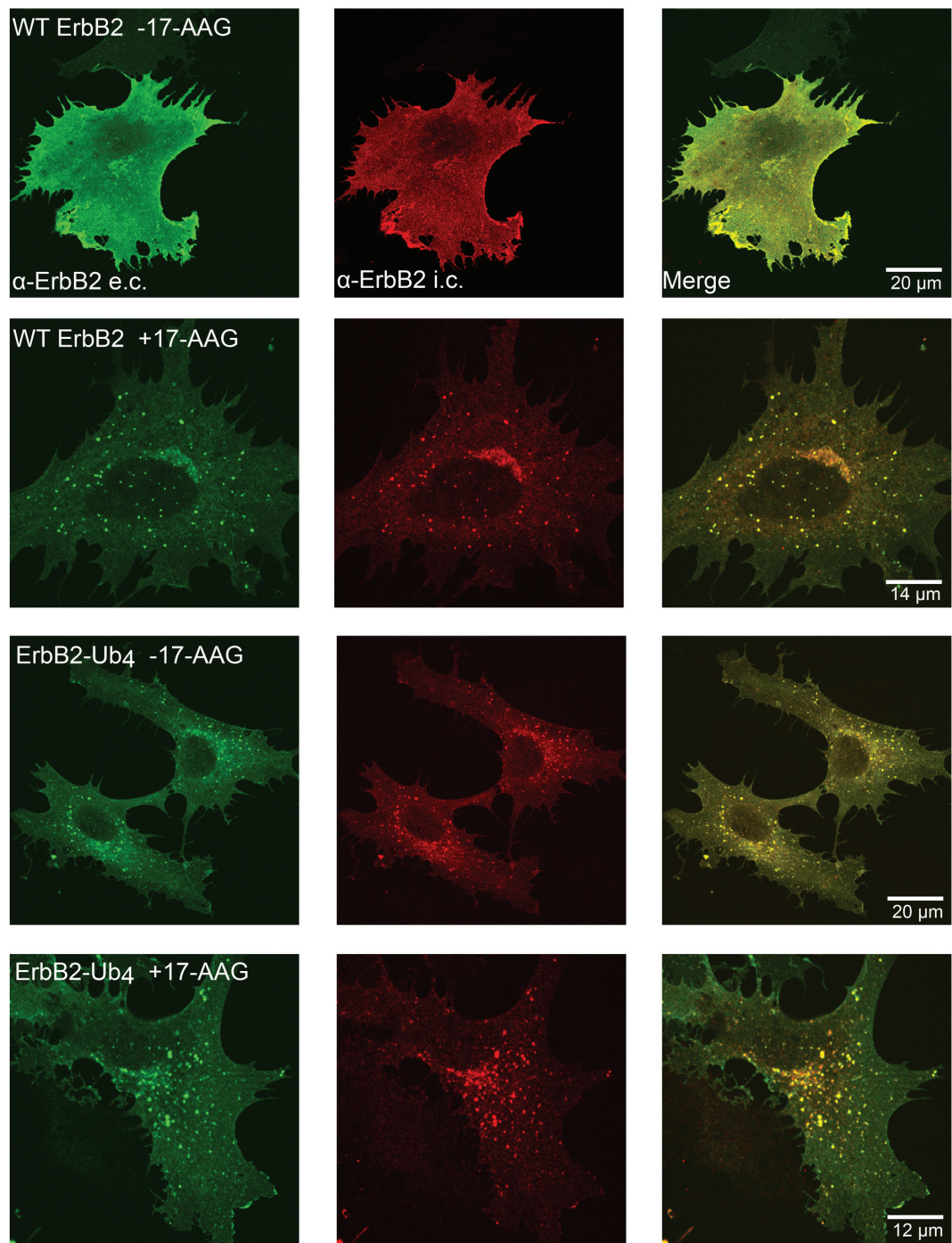
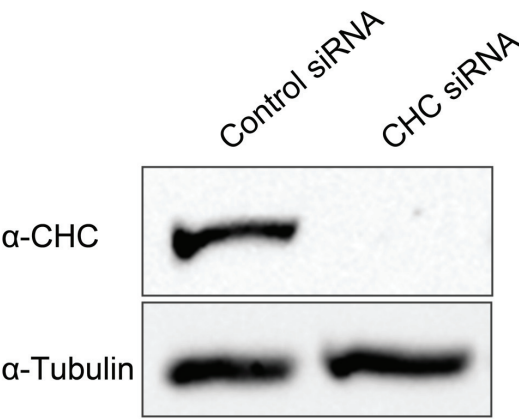


FIGURE 6

A



B

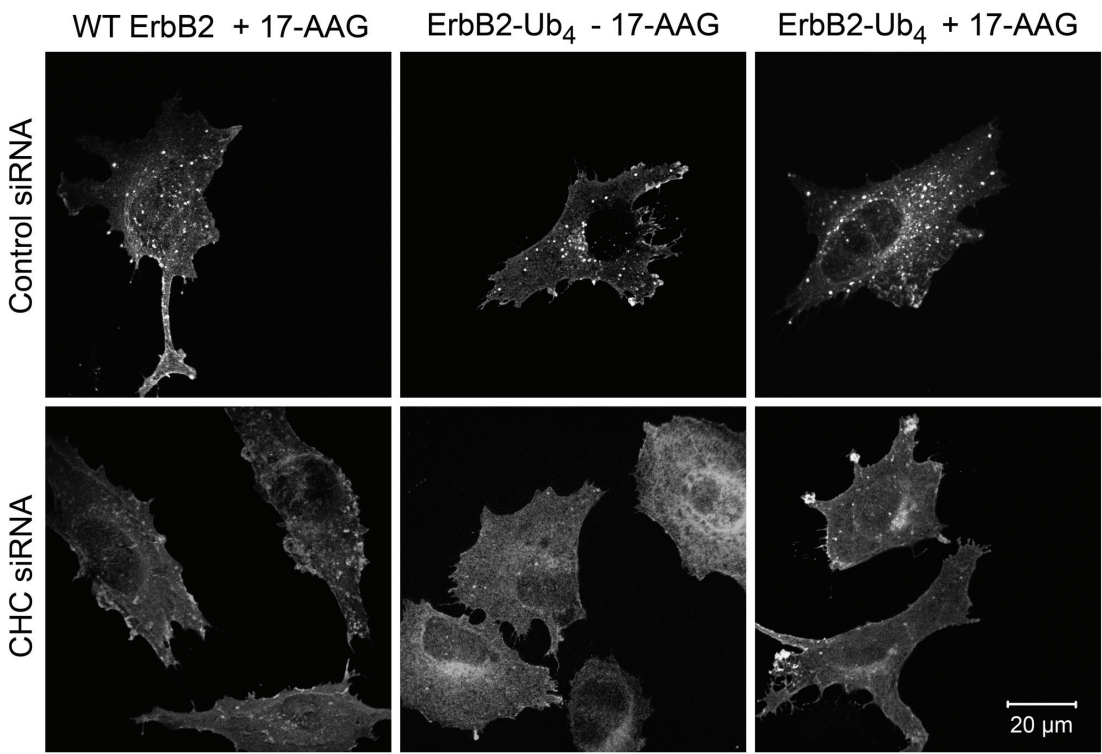


FIGURE 7

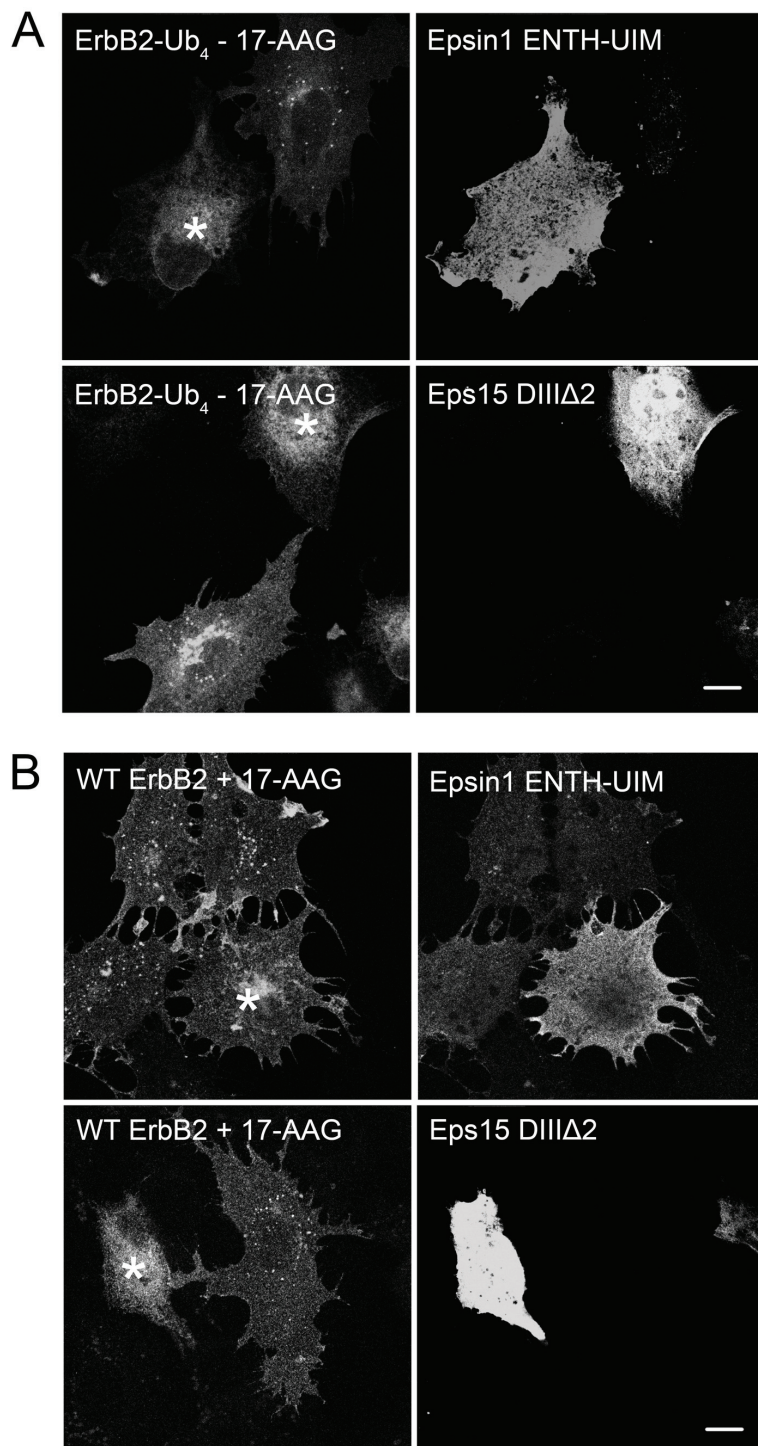
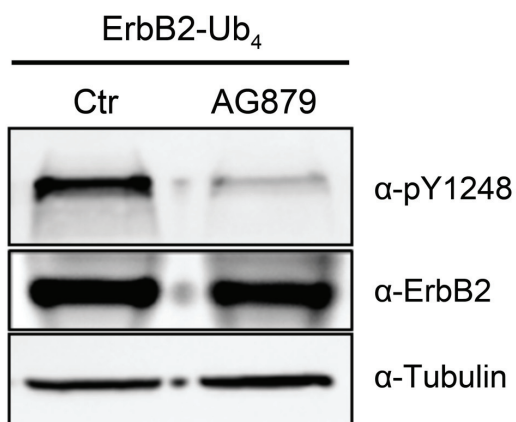
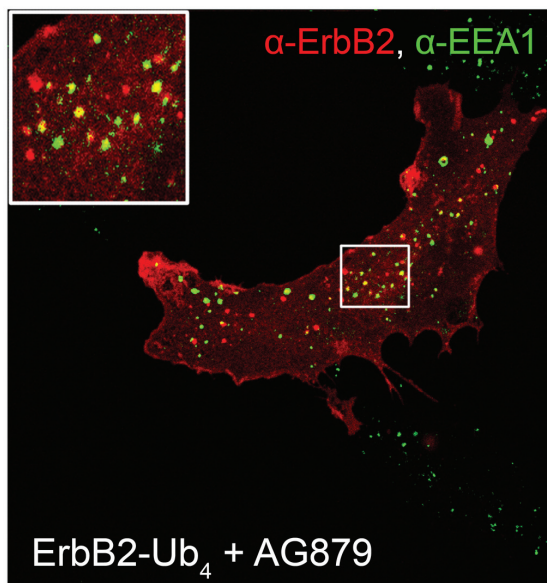


FIGURE 8

A



B



C

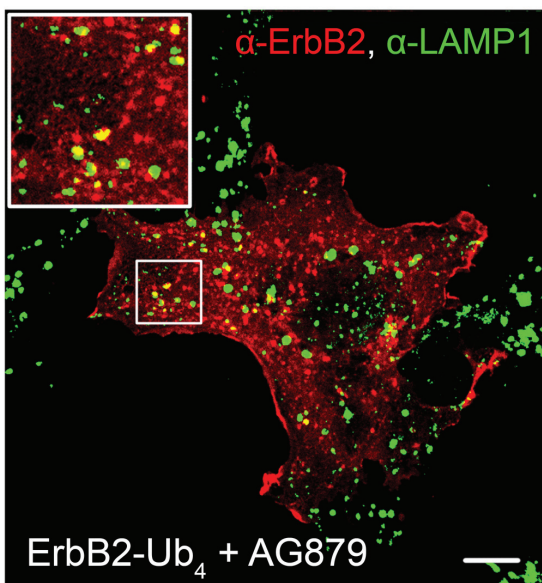


FIGURE 9

