Ubiquitination in Control of EGF Receptor Endocytosis

by

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Series of dissertations submitted to the
Faculty of Medicine, University of Oslo
No. 727

ISBN 978-82-8072-774-9

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Printed in Norway: AiT e-dit AS, Oslo, 2009.

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ACKNOWLEDGEMENTS

The present work has been carried out at the Laboratory for Molecular and Cellular Cancer Research, Institute of Pathology, Faculty Division Rikshospitalet, University of Oslo, during the period 2003-2008. I am grateful for the financial support for this project which was mainly provided by The Norwegian Cancer Society.

First I want to express my gratitude to my supervisor Professor Inger Helene Madshus and to my co-supervisor Dr. Espen Stang for giving me the opportunity to work in your group and in such an interesting field of research. I truly appreciate your guidance, support and enthusiasm, and for always being available for questions and discussions whenever it was needed. Inger Helene, I also want to thank you for providing additional financial support enabling me to complete this study and for believing in me. I'm happy for getting the opportunity to continue to work in you group. Espen, I truly appreciate your beautiful EM-experiments. They have been extremely valuable for this work. It is always a great relief when important results can be confirmed by your lovely pictures. I will also thank you for your patience and for all help with big things and small –but invaluable– details all the way.

This work would not have been performed without the help of countless generous helpful people. Sincere thanks to

- All the co-authors of the papers included in this thesis. Without your contribution, this study would of course not have been possible.
- Lene E. Johansen for always sharing your knowledge and being willing to help out and take time to answer my endless questions.
- Marianne S. Rødland for providing assistance in numerous ways at all stages, I really appreciate your help and friendship.
- Malgorzata M. Sak for your great enthusiasm and for being so sweet and hard-working. The effort you put in to help me finish this work is priceless.
- Kamilla Breen and Michael V. Grandal for reading this thesis at various stages and providing invaluable feedback

I am grateful to all my former and present colleagues at the institute and in the Madshus' group. Without such a pleasant and friendly environment, this work would at many stages have been unbearable. Particularly, I want to mention and thank Maja Kazazic and Lene M. Grøvdal for being fantastic colleagues, friends and traveling partners during this period, Michael V. Grandal for all fruitful discussions, for creating an amusing working environment in our lab during your stay and for being a good friend and my music soul-mate, Camilla Stormo for being the sweetest master student, a good friend and for all your questions which made me a better researcher. Finally, Anders Sandvik and Alexander Erofeev are thanked for being so nice office mates. Anders, having you around in the office all weekends and late nights made the last year much easier.

Nina Marie Pedersen and Kamilla Breen, thank you both for being wonderful friends. Your presence has been very important to me during this period. You have followed me all the way through all hard and good times; I can not imagine how I could have managed to fulfill this work without you being there for me. Thank you!

Thanks also to my friends outside the laboratory and my family. Thank you all for all the good times we have shared, for support and encouragement. My family is especially appreciated for helping out with my family life, your help made it possible for me to concentrate on my work whenever needed. A special and warm thank to 'my two boys' Pål-André and Mads, for unconditional support and for your endless patience, generosity, encouragement and love.

Oslo, August 2008

Vibeke Bertelsen

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ABBREVIATIONS

AP-2	Adaptor protein 2
EEA1	Early endosomal antigen 1
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EH	Epsin homology
ENTH	Epsin N-terminal homology
ESCRT	Endosomal Sorting Complexes Required for Transport
GFP	Green fluorescent protein
GGA(s)	Golgi-associated, γ-adaptin homologue(s)
GH	Growth hormone
GHR	Growth hormone receptor
GPI	Glycosyl phosphatidylinositol
Hrs	Hepathocyte growth factor regulated tyrosine kinase substrate
Immuno-EM	Immuno-electron microscopy
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
MAPK	Mitogen-activated protein kinase
MHC-I	Major histocompatibility complex class I
MVB(s)	Multivesicular body(ies)
PAE	Porcine Aortic Endothelial
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PLC	Phospholipase C
PLD	Phospholipase D
PRD	Proline rich domain
PTB	Phosphotyrosine binding
RNAi	RNA interference
RTK	Receptor tyrosine kinase
SH	Src homology
siRNA	Short interfering RNA
STAM	Signal-transducing adaptor molecule
STAT	Signal transducer and activator of transcription
Tf	Transferrin
TfR	Transferrin receptor
TGFα	Transforming growth factor alpha
TGN	*Trans*-Golgi network
TULA	T-cell ubiquitin ligand
UIM	Ubiquitin interacting motif

PAPERS INCLUDED

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

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

Experimental Cell Research, Vol 313, 1696-1709, May 2007.

Paper II Espen Stang, Frøydis D. Blystad, Maja Kazazic, **Vibeke Bertelsen**, Tonje Brodahl, Camilla Raiborg, Harald Stenmark, and Inger Helene Madshus

Cbl-dependent ubiquitination is required for progression of EGF Receptors into clathrin-coated pits.

Molecular Biology of the Cell, Vol 15, 3591-3604, August 2004.

Paper III Maja Kazazic*, **Vibeke Bertelsen***, Ketil Winther Pedersen, Tram Thu Vuong, Michael Vibo Grandal, Marianne Skeie Rødland, Linton M. Traub, Espen Stang, and Inger Helene Madshus
* These authors contributed equally to this work

Epsin recruits ubiquitinated EGF receptors into clathrin-coated pits.

Revised manuscript submitted (Traffic)

Paper IV **Vibeke Bertelsen**, Malgorzata Magdalena Sak, Kamilla Breen, Espen Stang, and Inger Helene Madshus.

Cbl and Ubiquitin promote translocation of EGF Receptor into clathrin-coated pits.

Manuscript

1 INTRODUCTION

Cells are continuously supplied with information from other cells and the microenvironment, and this information is often in the form of signaling molecules like hormones, cytokines and growth factors. It is important that the interpretation of this information is correct to maintain homeostasis, normal growth and development, as well as cell survival. Signaling molecules affect the target cell by binding to receptors localized to the plasma membrane, and the receptors then translate the signals from extracellular stimuli to intracellular messages. One major family of receptors for growth factors is the family of receptor tyrosine kinases (RTKs) which consists of approximately 20 different subclasses. To avoid excessive cell growth, it is essential to attenuate the signaling downstream of such growth factor receptors. One way of attenuating signaling is down-regulation of activated receptors by endocytosis. In this work, the focus has been on endocytic down-regulation of the epidermal growth factor receptor (EGFR), which belongs to the ErbB family of receptor tyrosine kinases.

1.1 THE ERBB FAMILY OF RECEPTOR TYROSINE KINASES

The ErbB family comprises the subclass I of the receptor tyrosine kinases. There are four members of this family: EGFR (also called ErbB1 or HER1), ErbB2 (also called Neu or HER2), ErbB3 (also called HER3) and ErbB4 (also called HER4). These ErbB proteins are expressed in cells of epithelial, mesenchymal and neuronal origin, and they are shown to be involved in development, proliferation and differentiation (reviewed in Olayioye et al. 2000). The different ErbB proteins have approximately 40 % sequence similarity (Stein and Staros 2000) and share the same overall protein structure. They contain a highly glycosylated extracellular domain with a ligand-binding region and a dimerization loop, a transmembrane domain, and a cytoplasmic domain with tyrosine kinase activity (reviewed in Olayioye et al. 2000; Burgess et al. 2003). Although they exhibit structural similarity, the different ErbB proteins differ with respect to a variety of properties, such as ligand-binding, kinase activity, and signaling pattern and are thus thought to have distinct physiological roles.

The ErbB proteins are activated upon ligand binding. Binding of ligand induces a conformational change in the extracellular domain of the ErbB proteins causing the dimerization loop to be exposed and thereby allowing the ErbB proteins to form dimers, either homodimers or heterodimers. All high-affinity ligands of the ErbB family of receptors belong to the epidermal growth factor (EGF) family of growth factors as they all have an EGF-like domain. Nearly all are synthesized as transmembrane precursors, and mature soluble ligands are produced by extracellular cleavage by metalloproteases (reviewed in Harris et al. 2003). The ligands differ in their specificity. Some ligands bind exclusively to one member of the ErbB family, whereas others can bind and activate more than one receptor (as summarized in Figure 1). Despite the large number of ligands identified for EGFR, ErbB3 and ErbB4, no ligand has been identified for ErbB2 (Olayioye et al. 2000). The extracellular domain of ErbB2 has a tethered structure which makes ligand binding difficult (reviewed in Garrett et al. 2003). It therefore seems that ErbB2 functions primarily as a co-receptor, and it is considered the preferred dimerization partner for the other ErbB proteins, as the tethered structure causes the dimerization loop to be constitutively exposed (Garrett et al. 2003).

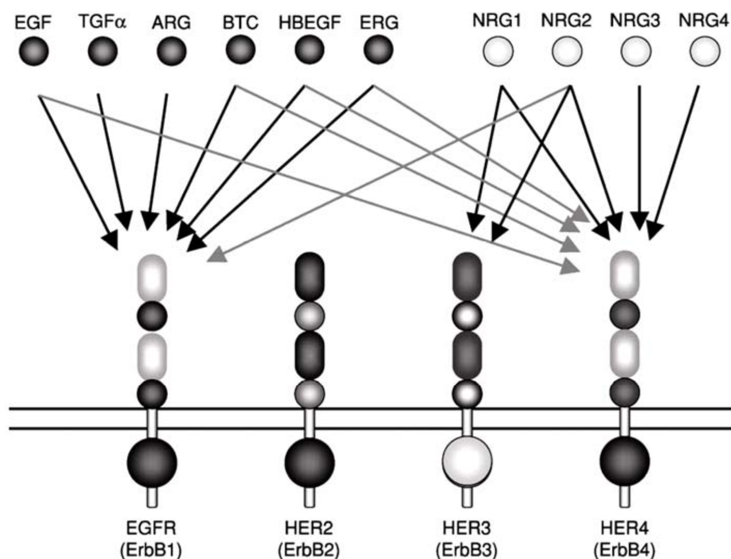

Figure 1. Members of the ErbB protein family and their ligands. EGFR, ErbB3 and ErbB4 bind a distinct set of ligands, including EGF, Transforming Growth Factor α (TGF), Amphiregulin (ARG), Betacellulin (BTC), Heparin-binding EGF (HBEGF), Epiregulin (ERG) and Neuregulins (NRG1-4). ErbB2 does not bind any ligand. Reprinted from Advances in Protein Chemistry, Vol 68, Daniel J. Leahy, Structure and Function of the Epidermal Growth Factor (EGF/ErbB) Family of Receptors, 1-27, ©2004, with permission from Elsevier. The figure legend is modified.

1.1.1 EGFR

EGFR is the most extensively studied ErbB protein, and it was also the first member of this family to be discovered (Carpenter et al. 1978). The EGFR is a 175-kDa protein, and like the other ErbB-proteins, it is highly glycosylated with 9 out of 11 potential glycosylation sites utilized (Zhen et al. 2003). The glycosylation seems to be essential for translocation of EGFR to the cell surface (Slieker et al. 1986). The EGFR ligands bind to the extracellular domain which is comprised of four subdomains (I-IV). These subdomains are arranged as tandem repeats of two different types of domains. Subdomains I and III (also named L1 and L2) are two large homologous domains shown to be responsible for ligand binding, while subdomains II and IV are cysteine-rich subdomains responsible for dimerization (reviewed in Burgess et al. 2003). One single α-helix comprises the transmembrane domain (Rigby et al. 1998). The cytoplasmic domain can be divided in three regions: the juxtamembrane domain, the tyrosin kinase domain and the C-terminal regulatory region (see Figure 2 for an overview). The juxtamembrane domain is considered to have a regulatory role in receptor down-regulation (Fuller et al. 2008). The kinase domain of EGFR is structurally similar to other tyrosine kinases, and it catalyzes the transfer of phosphate from ATP to acceptor tyrosines in other substrates as well as in the C-terminal tail of the receptor itself (a process termed autophosphorylation) (Honegger et al. 1989; Honegger et al. 1990). The C-terminal regulatory region contains several tyrosine, serine and threonine residues which can be phosphorylated and thereby play important roles in signal transduction (Olayioye et al. 2000; Yarden and Sliwkowski 2001).

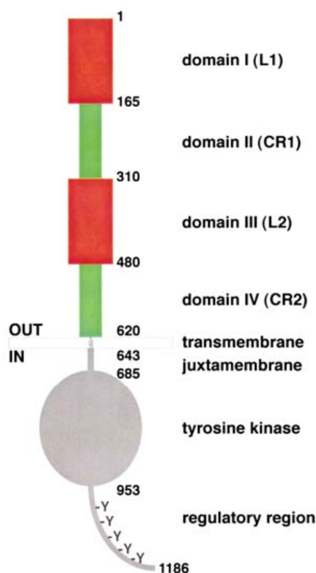

Figure 2. Schematic overview of the domain organization in EGFR. the domains are referred to using the I, II, III, IV nomenclature. An alternative nomenclature using domain names L1, CR1, L2, CR2 is also used in the literature. Numbers refer to the amino acid residues in EGFR. Reprinted from Molecular Cell, Vol 12, Burgess et al., An Open and Shut Case? Recent Insight into the Activation of EGF/ErbB Receptors, 541-52, ©2003 with permission from Elsevier. The figure legend is modified.

1.1.2 EGFR activation and signal transduction

In the absence of ligand, EGFR proteins exist in both monomeric and dimeric forms. However, upon ligand binding, the EGFR is stabilized in an active state with the dimerization loop exposed, thus increasing the formation of and stability of EGFR dimers (reviewed in Jorissen et al. 2003). Binding of ligand and receptor dimerization triggers the tyrosine kinase activity of EGFR, leading to phosphorylation of tyrosine residues in the receptor itself, in the dimerization partner, and in other effector molecules. The C-terminal tail of the receptor contains five tyrosines that upon phosphorylation serve as docking sites for src homology (SH) 2 or phosphotyrosine binding (PTB) domains of intracellular signal transducers or adaptor proteins (reviewed in Singh and Harris 2005). This recruitment leads to activation of different signaling pathways (see Figure 3 for an overview).

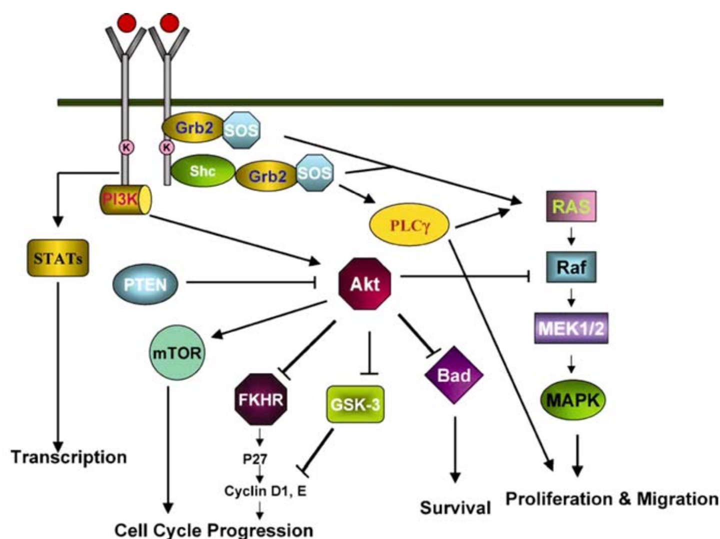

Figure 3. The diversity of the signaling network activated by ligand-dependent activation of the EGFR. Ligand binding induces receptor homo- or hetero-dimerization resulting in tyrosine phosphorylation of the cytoplasmic tail. The selective activation of well characterized signaling transduction pathways depends on the various arrangements of ligand-receptor engagement, tyrosine phosphorylation, and subsequent receptor dimerization combinations as well the stage and context of cell type and growth. Reprinted from Cellular Signalling, Vol 17, Sing, A.B. and Harris, R.C., Autocrine, paracrine and juxtacrine signalling by EGFR ligands, 1183-93, ©2005, with permission from Elsevier.

The signaling network originating from EGFR is complex and involves protein-protein interactions, protein-lipid interactions, phosphorylation, dephosphorylation, ubiquitination, and second messengers. This signaling network comprises several different signaling

pathways which involves Phospholipase C γ (PLC-γ), Ras and Mitogen-activated protein kinases (ras-MAPKs), Multiple signal transducer and activator of transcription (STAT) isoforms, heterotrimeric G-proteins, Phosphatidylinositol 3-kinase (PI3K), Phospholipase D (PLD) and the proto-oncogene cytoplasmic tyrosine kinase Src (reviewed in Singh and Harris 2005). The biological outcome of EGFR signaling is highly diverse, ranging from cell division, migration, adhesion, and differentiation to apoptosis (reviewed in Yarden and Sliwkowski 2001), and it is determined both by the identity of the ligand and by the EGFR dimerization partner (Olayioye et al. 2000).

1.1.3 Ubiquitination of EGFR

As described above, the EGFR is covalently modified by phosphorylation upon activation with EGF. Another post-translational modification of EGFR induced by binding of EGF is ubiquitination. Ubiquitination refers to the covalent attachment of ubiquitin to a lysine residue in a target protein (reviewed in Pickart 2001). Ubiquitin is a highly conserved small polypeptide consisting of 76 amino acids which is ubiquitously expressed in all eukaryotes (thus, giving rise to its name). Ubiquitination of proteins can be induced by a broad range of stimuli and signaling events. This highly regulated mechanism involves three enzymes; a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin ligase (E3) (see Figure 4 and figure text). If one single ubiquitin molecule is conjugated to the target protein, the modification is called monoubiquitination. Alternatively, several ubiquitin molecules can each be conjugated to different lysine residues within the target protein, resulting in multiple monoubiquitination. Ubiquitin itself contains several lysine residues and can thus also be substrate for ubiquitination, forming polyubiquitin chains, a process denoted polyubiquitination (reviewed in Haglund and Dikic 2005). In the case of polyubiquitination, different linkages between the ubiquitin monomers exist, defined by which lysine residue in the ubiquitin monomer that is the substrate for further ubiquitination. Lysine-48 linkage is mainly a signal for targeting proteins to the proteasome for degradation, whereas lysine-63 linkage is involved in DNA-repair, inflammatory responses, ribosomal protein synthesis and in the endocytic pathway (which will be discussed in more detail later) (reviewed in Mukhopadhyay and Riezman 2007).

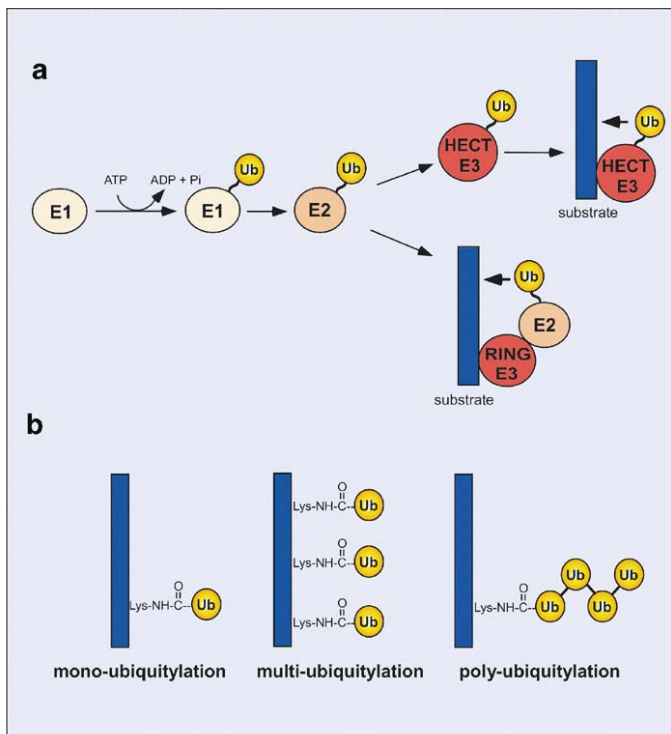

Figure 4. Protein ubiquitination. (a) The ubiquitination reaction consists of three steps: the single ubiquitin activating enzyme or E1 mediates the ATP-dependent activation of the C-terminal glycine of ubiquitin, which is conjugated to an active site cysteine through a thiol-ester linkage. Ubiquitin is then transferred to a cysteine in the active site of one of several ubiquitin-conjugating enzymes or E2s. Although some E2s can ubiquitinate substrates directly, in most cases an E3 ubiquitin ligase is required, and they provide the specificity of the ubiquitination reaction by binding substrates. There are multiple E3 ubiquitin ligases, with two major types that mediate ubiquitination in a distinct manner: HECT domain-containing E3s, such as Nedd4, form a thiol–ester linkage with a ubiquitin transferred from the E2, which is subsequently conjugated to the substrate. RING finger E3s, including Cbl, function as adaptors that bind both E2 enzymes and substrates to coordinate substrate ubiquitination. Both reactions result in the formation of an isopeptide bond between the C-terminal glycine of ubiquitin and the ε-amino group of a lysine residue within the substrate. Several types of substrate modification by ubiquitin can ensue **(b)** conjugation with one ubiquitin moiety (monoubiquitination), conjugation with multiple ubiquitin monomers (multiubiquitination) or the formation of a ubiquitin chain branched at internal lysines within ubiquitin (polyubiquitination). Reprinted by permission from Macmillan Publishers Ltd: Oncogene, 2004; 23:2057-70, ©2008.

In 1995, Galcheva-Gargova and coworkers discovered that EGFR from cells incubated with EGF migrated as a smear of differently increased molecular weight after SDS Polyacrylamide gel electrophoresis, and that this smear represented ubiquitinated EGFR (Galcheva-Gargova et al. 1995). The EGF-induced ubiquitination was additionally found to be dependent on the activity of EGFR's kinase domain (Galcheva-Gargova et al. 1995). It is well established that the EGF-induced ubiquitination of EGFR is mediated by the ubiquitin

ligase Cbl (Joazeiro et al. 1999; Levkowitz et al. 1999; Lill et al. 2000; Thien and Langdon 2001). Cbl binds to EGFR upon ligand-induced phosphorylation of EGFR, either directly via its PTB domain to phosphotyrosine 1045 or indirectly through Grb2 (Levkowitz et al. 1999; Waterman et al. 2002). Both direct and indirect association of Cbl with EGFR is reported to result in ubiquitination of EGFR (Levkowitz et al. 1999; Waterman et al. 2002). Initially, EGFR was reported to be polyubiquitinated (Galcheva-Gargova et al. 1995). However, a few years later, it was reported that EGFR was multiply monoubiquitinated rather than polyubiquitinated (Haglund et al. 2003; Mosesson et al. 2003). More recently, mass spectrometry studies revealed that upon EGFR activation, the receptor was in fact polyubiquitinated as well as monoubiquitinated, and that the majority of the ubiquitin moieties were attached to lysine residues within the kinase domain (Huang et al. 2006). Ubiquitination of EGFR mediates sorting to and degradation in lysosomes (reviewed in Miranda and Sorkin 2007). It was initially proposed that the ubiquitination of EGFR takes place in endosomes, after EGFR internalization from the plasma membrane (Levkowitz et al. 1998). It was however later demonstrated by our group and others that ubiquitination of EGFR also occurs in cells where the internalization step is blocked in different ways (Stang et al. 2000; de Melker et al. 2001; Longva et al. 2002). Additionally, it is clear that the ubiquitin ligase Cbl is recruited to EGFR localized at the plasma membrane (de Melker et al. 2001; Longva et al. 2002). Taken together, these results reveal that ligand-induced ubiquitination of EGFR is a process taking place at the plasma membrane prior to internalization.

1.2 ENDOCYTOSIS

Macromolecules are taken into cells by the process called endocytosis. Endocytosis is the internalization of extracellular materials together with a part of the plasma membrane. Endocytosis can be divided into two broad categories: phagocytosis and pinocytosis (also termed “cell eating” and “cell drinking”, respectively). Phagocytosis is the uptake of large particles, e.g. bacteria, and it is mostly performed by specialized cells, like macrophages, monocytes and neutrophils. Pinocytosis refers to fluid-phase uptake, and can further be divided into macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis and caveolae- and clathrin-independent endocytosis (Figure 5) (reviewed in Conner and Schmid 2003b). Different classifications exist, defined by requirements for different factors in endocytosis. In this thesis, the classification from Conner and Schmid is

used (Conner and Schmid 2003b). At present, the best characterized endocytic pathway is the one mediated by clathrin.

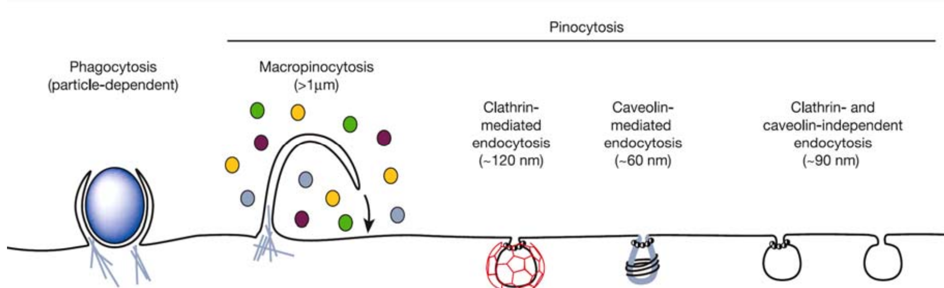

Figure 5. Multiple portals of entry into the cell. Large particles are taken up by phagocytosis, while fluids and smaller particles are taken up by pinocytosis. This can occur either through macropinocytosis or by formation of vesicles by invagination of the plasma membrane. The major route of endocytosis is clathrin-mediated endocytosis, where a clathrin-coat encloses the forming vesicle. Endocytosis can also occur independently of clathrin. In caveolin-mediated endocytosis the uptake is via vesicles enriched in caveolin. There is also evidence for other routes of internalization independent of both clathrin and caveolin. At least two different pathways have been described, one dependent on dynamin, and one independent on dynamin. Reprinted by permission from Macmillan Publishers Ltd: Nature, 2003; 422:37-44, ©2008. The figure legend is modified.

1.2.1 Clathrin-mediated endocytosis

Clathrin-mediated endocytosis occurs in all nucleated cells, either constitutively or in response to certain stimuli, and is an important mechanism for entry of nutrients and membrane-bound receptors and their ligands into cells. Examples of such receptors and ligands are transferrin (Tf) and the Tf receptor (TfR) and low-density lipoprotein (LDL) and its receptor, the LDLR (reviewed in Conner and Schmid 2003b). Clathrin-mediated endocytosis is characterized by the formation of a protein coat mainly consisting of clathrin on the cytosolic side of the plasma membrane. Invagination of the plasma membrane in the clathrin-coated area generates clathrin-coated pits. Moreover, clathrin-mediated endocytosis involves the concentration of transmembrane receptors and their ligands in clathrin-coated pits, further invagination of the plasma membrane, and eventually pinching off from the membrane to form clathrin-coated vesicles inside the cell (Conner and Schmid 2003b). The endocytic pathway is named after the definitive requirement for clathrin, although a number of different proteins are also reported to be involved and required in the process. Such accessory endocytic proteins include among others the clathrin adaptor complex 2 (AP-2) and dynamin (Figure 6), which will be described in more detail below. These proteins, and others, are recruited to areas of the plasma membrane enriched in phosphatidylinositol 4,5-

P_2 (PIP_2), followed by concentration of the endocytic cargo in clathrin-coated pits (Ehrlich et al. 2004).

1.2.1.1 Clathrin

As mentioned, clathrin is the major component of the endocytic clathrin coat. In the clathrin coat, it exists as a trimeric protein complex consisting of three heavy chains and three light chains. The three heavy chains comprise a three-legged structure, a triskelion. The triskelion in turn oligomerizes into a cage-like lattice at the plasma membrane, thereby forming the protein coat (reviewed in Kirchhausen 2000; Conner and Schmid 2003b). It has been reported that clathrin is essential for the invagination of coated pits (Hinrichsen et al. 2006). Clathrin has no identified lipid-binding motifs, and is thus dependent on other proteins, often referred to as adaptor proteins, to associate with the lipid bilayer in the plasma membrane (reviewed in Kirchhausen 2000; Sorkin 2004). It is well documented that the clathrin adaptor complex AP-2 has such a function (reviewed in Robinson and Bonifacino 2001; Conner and Schmid 2003b; Traub 2005).

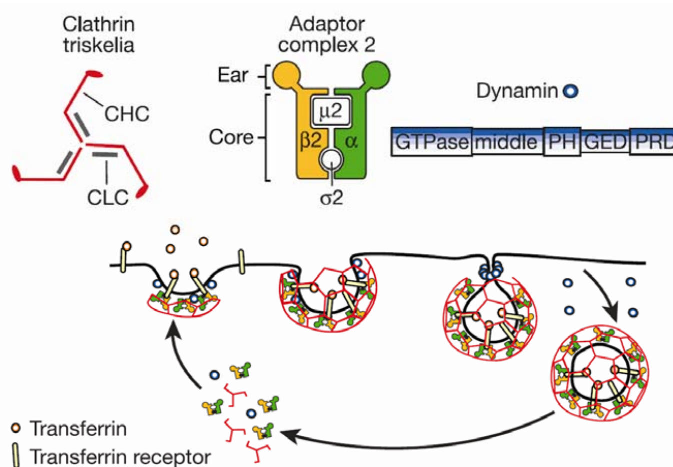

Figure 6. Core components of clathrin-coated pits. Clathrin triskelions, composed of three clathrin heavy chains (CHC) and three tightly associated light chains (CLC), assemble into a polygonal lattice, which helps to deform the overlying plasma membrane into a coated pit. Heterotetrameric AP-2 complexes are targeted to the plasma membrane by the α-adaptin subunits, where they mediate clathrin assembly through the β2-subunit, and interact directly with sorting motifs on cargo molecules through their μ2 subunits. Dynamin is a multidomain GTPase that is recruited to clathrin-coated pits, where it can assemble into a spiral or 'collar' to mediate or monitor membrane fission and the release of clathrin-coated vesicles. Reprinted by permission from Macmillan Publishers Ltd: Nature, 2003; 422:37-44, ©2008. The figure legend is modified.

1.2.1.2 AP-2

Four structurally related adaptor complexes (AP-1 to AP-4) have been described, and they are reported to be involved in coat assembly at different subcellular sites (reviewed in Robinson and Bonifacino 2001). Of these four, only AP-2 is involved in clathrin-coat assembly at the plasma membrane. AP-2 is a heterotetrameric complex consisting of two large core subunits, the α- and β2-adaptins, a medium subunit, the μ2, and a small subunit, the σ2 (see Figure 6 for an overview) (Conner and Schmid 2003b). The core of the α-subunit binds to PIP_2 in the plasma membrane (Beck and Keen 1991), and the α-appendage of the α-subunit is thought to serve as an interacting hub for accessory endocytic proteins (Praefcke et al. 2004). The β2-subunit binds to clathrin, and the μ2-subunit is considered to be the cargo selection unit of the complex, binding to sorting motifs in cargo molecules at the plasma membrane. Finally, the σ2-subunit seems to have a role in stabilizing the AP-2 complex (Conner and Schmid 2003b). The AP-2 complex is thought to be one of the key components of clathrin-mediated endocytosis. Consistent with this, it is demonstrated that depleting cells of AP-2 disrupts endocytosis of the TfR (Hinrichsen et al. 2003; Motley et al. 2003). However, endocytosis of EGFR is reported to be less affected by AP-2 depletion (Hinrichsen et al. 2003; Motley et al. 2003; Huang et al. 2004; Johannessen et al. 2006), suggesting that AP-2 is not absolutely critical for clathrin-mediated endocytosis.

1.2.1.3 Dynamin

Dynamin is a large GTPase belonging to the dynamin superfamily of proteins. This family of proteins includes the classical dynamins, dynamin-related proteins and the guanylate-binding proteins, all of which are classified according to their domain structure. The classical dynamins comprise dynamin1, which is mainly expressed in neuronal-tissue, dynamin2 which is ubiquitously expressed, and the brain- and testis-enriched dynamin3. All dynamins have identical domain architecture. They have a GTPase domain, a middle domain with no known function, a pleckstrin homology (PH) domain binding to PIP_2, and a GTPase effector domain which functions as an internal GTPase activating protein domain. The C-terminal part of the proteins encloses a proline rich domain (PRD) that interacts with proteins containing SH3 domains (Figure 6) (reviewed in Praefcke and McMahon 2004; Kruchten and McNiven 2006). Dynamin was reported to be involved in endocytosis as early as 1991 by van der Bliek and Meyerowitz (van der Bliek and Meyerowitz 1991). It is proposed that the GTPase activity of dynamin is necessary for its function in endocytosis (Damke et al. 1994; Hill et al. 2001), but the exact mechanism of action is still debated.

Dynamin is proposed to function either directly as a "pinchcase" pinching off membrane invaginations, or as a regulator of other mechanochemical enzymes (reviewed in Conner and Schmid 2003b; Praefcke and McMahon 2004). Dynamin is not exclusively involved in clathrin-mediated endocytosis, it is also a required factor in several of the clathrin-independent endocytic pathways.

1.2.2 Clathrin-independent endocytosis

Endocytosis can also occur clathrin-independently. clathrin-mediated endocytosis has for several years been considered as the main internalization route for plasma membrane localized proteins; however, the knowledge of clathrin-independent endocytic pathways is emerging, and a number of 'new' internalization routes have now been described.

1.2.2.1 Macropinocytosis

Macropinocytosis is a triggered process where the cells internalize large amounts of fluid and membrane. It is often induced by growth factors, and it is characterized by actin-driven membrane ruffling, formation of protrusions that collapse onto the plasma membrane, and formation of large endocytic vesicles called macropinosomes (Swanson and Watts 1995; Pelkmans and Helenius 2003).

1.2.2.2 Caveolae-mediated endocytosis

Caveolae are small flask-shaped invaginations of the plasma membrane present in many cell types, but enriched in endothelial cells. Caveolae lack a cytoplasmic coat but are characterized by the presence of the protein caveolin, which is inserted into the cytoplasmic leaflet of plasma membrane and stabilizes the membrane curvature (reviewed in Gong et al. 2007). Caveolae are one type of rafts, which are specialized lipid microdomains of the plasma membrane enriched in sphingolipids, cholesterol and glycosyl phosphatidyl (GPI) - anchored proteins (reviewed in Conner and Schmid 2003b; Mayor and Pagano 2007). Caveolae were initially reported to be relatively immobile structures of the plasma membrane (Thomsen et al. 2002), but it was later demonstrated that caveolae can internalize slowly to form caveosomes and that this process is dependent on dynamin and actin assembly (reviewed in Parton and Simons 2007).

1.2.2.3 Caveolae- and clathrin-independent endocytosis

Several endocytic pathways independent of both clathrin and caveolin are also described. Some of these internalization routes are based on lipid microdomains, exemplified by

internalization of interleukin-2 receptor and γc cytokine receptor (Lamaze et al. 2001; Kirkham and Parton 2005; Sauvonnet et al. 2005). A flotillin-dependent endocytic pathway has recently been described (Glebov et al. 2006), and it has been proposed that flotillin displays structural functions during endocytosis similar to the role caveolin has in caveolae-mediated endocytosis (Bauer and Pelkmans 2006; Gong et al. 2007). The requirement for dynamin varies among the clathrin and caveolin-independent endocytic pathways; receptor for interleukin-2 and the γc cytokine receptor both are dependent on dynamin for internalization, whereas cholera toxin B and some GPI-anchored proteins can be internalized from lipid rafts independently of dynamin (reviewed in Kirkham and Parton 2005; Gong et al. 2007). A new classification of clathrin-independent endocytosis was recently proposed, defined by the requirement for dynamin and further subdivided into pathways characterized by the GTPase involved (Mayor and Pagano 2007). It seems that cargo can utilize different endocytic pathways depending on cell line and accessible adaptor proteins (Sandvig and van Deurs 2002). The number of clathrin and caveolin-independent endocytic pathways and the molecules involved thus remain unclear at present.

1.2.3 Ubiquitination as endocytic signal

Ubiquitination of proteins was initially discovered to be a signal for proteasomal degradation of cytosolic proteins, but has later been implicated in a number of other cellular processes, including endocytosis. In 1996, Linda Hicke and coworkers identified ubiquitination as a signal for endocytosis in yeast, and demonstrated that one single ubiquitin molecule could be sufficient for mediating endocytosis (Hicke and Riezman 1996). The requirement for ubiquitination has been demonstrated in endocytosis of certain plasma membrane proteins in yeast, for example Ste2p, a G-protein coupled receptor (Hicke and Riezman 1996), and the Fur4-encoded uracil permease (Galan et al. 1996; Galan and Haguenauer-Tsapis 1997).

In mammalian cells, tyrosine-based and di-leucine motifs are well described signals for directing endocytosis of plasma membrane localized proteins (reviewed in Bonifacino and Traub 2003). These motifs serve as recognition signals for cargo selecting adaptor proteins (i.e. AP-2 and disabled 2) during endocytosis. The TfR and LDLR are both internalized constitutively via clathrin-mediated endocytosis, and both harbor tyrosine based motifs that are recognized by AP-2 (Chen et al. 1990; Jing et al. 1990; Boll et al. 2002; reviewed in

Bonifacino and Traub 2003). In addition to these internalization motifs, ubiquitination has later been identified as an endocytic signal in higher eukaryotes, as ubiquitination of certain plasma membrane proteins appears to be sufficient for internalization. After fusing one single ubiquitin molecule to the cytoplasmic part of a truncated form of EGFR, the EGFR-ubiquitin chimera was constitutively endocytosed (Haglund et al. 2003). It has also been shown that fusing ubiquitin moieties to the plasma membrane localized protein CD4, induces its endocytosis (Barriere et al. 2006). Furthermore, endocytosis of the growth hormone (GH) receptor (GHR) has been shown to depend on an intact ubiquitination machinery, although the direct requirement for ubiquitination of the receptor itself could not be demonstrated (Strous et al. 1996; Sachse et al. 2001; van Kerkhof et al. 2007). In contrast, it has been demonstrated that ubiquitination of the receptors for nerve growth factor, for leptin and for dopamine is instrumental in initial steps of endocytosis of these receptors (Geetha et al. 2005; Belouzard and Rouille 2006; Miranda et al. 2007). In order to function as endocytic signal in clathrin-mediated endocytosis, ubiquitin has to be recognized by proteins associated with the clathrin-coated pit. Interestingly, several proteins involved in clathrin-mediated endocytosis (e.g. epsin and eps15, which will be discussed later) indeed possess ubiquitin interacting motifs (UIMs) (Hofmann and Falquet 2001). Ubiquitination can also direct proteins from the *trans*-Golgi network to endosomes (Piper and Luzio 2007) and can additionally serve as signal for sorting of proteins at late endosomes.

1.2.4 Endocytic down-regulation of EGFR

After ligand binding and activation, EGFR is rapidly internalized. Endocytosis of EGFR is a common model system for studying receptor-mediated endocytosis from clathrin-coated pits. The requirement for different adaptor proteins and accessory endocytic proteins are, however, still disputed, and the exact mechanism for endocytic down-regulation remains unclear. There is general agreement that EGFR can be internalized through clathrin-coated pits after ligand binding (Carpentier et al. 1982; Hanover et al. 1984). It has also been suggested that endocytosis of EGFR can occur independently of clathrin (Hinrichsen et al. 2003) and that EGFR is enriched in and can be internalized from caveolae at high concentrations of ligand (Sigismund et al. 2005). More recently, it was, however, demonstrated that the main internalization route for EGFR is clathrin-mediated endocytosis, both at high and low concentrations of EGF and that EGF did not induce mobilization of caveolae (Kazazic et al. 2006). In addition to clathrin, the internalization of EGFR is

controlled by interaction of EGFR with several different proteins, such as Grb2, Cbl, Eps15, and epsin.

1.2.4.1 Grb2

Grb2 consists of an SH2 domain flanked by two SH3 domains. It binds via its SH2 domain to phosphotyrosines 1086 or 1068 of EGFR upon activation of EGFR. It is well established that Grb2 is important in signaling from EGFR by recruiting important proteins involved in activation of signaling cascades via its SH3 domains. One example of such a protein is Son of sevenless (Sos) (Jorissen et al. 2003). Additionally, Grb2 has been shown to be critical for endocytosis of EGFR, since mutation of the two Grb2-binding sites in EGFR as well as depletion of Grb2 by RNA interference (RNAi) effectively hinder EGFR internalization (Sorkina et al. 2002; Jiang et al. 2003). In addition, it has been proposed that Grb2 plays an important role in recruiting the activated EGFR to clathrin-coated pits (Jiang et al. 2003). Furthermore, Grb2 has been suggested to recruit the ubiquitin ligase Cbl to EGFR upon activation. The Grb2 SH3 domain associates with proline rich sequences in Cbl (Meisner et al. 1995) and thereby guides Cbl to EGFR (Huang and Sorkin 2005). Consistently, a mutant form of Grb2 not able to bind Cbl was demonstrated to inhibit the internalization of EGFR (Jiang et al. 2003).

1.2.4.2 Cbl

Cbl is a protein which functions as a negative regulator of signaling from tyrosine kinases, and it is involved in endocytic down-regulation of EGFR. Three different mammalian forms of Cbl have been identified; c-Cbl, Cbl-b and Cbl-3 (also called Cbl-c). All these three forms are reported to interact with EGFR in an EGF-dependent manner (Thien and Langdon 2001; Thien and Langdon 2005; Swaminathan and Tsygankov 2006). The Cbl proteins have several highly conserved domains including the N-terminal PTB domain recognizing phosphorylated tyrosines on RTKs and the centrally located RING-finger domain recruiting ubiquitin conjugating enzymes. Additionally, c-Cbl and Cbl-b contain several tyrosine phosphorylation sites as well as proline rich sequences that can bind to SH3-domain containing proteins (Ryan et al. 2006). These characteristics define the Cbl-proteins as multifunctional proteins capable of acting both as ubiquitin ligases and as adaptor proteins. As previously mentioned, Cbl interacts with activated EGFR in two ways; directly via the PTB domain and indirectly through Grb2 (Figure 7). Both direct and indirect association of Cbl with EGFR is known to mediate ubiquitination of EGFR (Waterman et al. 2002). It is,

however, reported that both direct and indirect association of Cbl with EGFR are required for efficient EGFR ubiquitination (Lill et al. 2000). It has furthermore been proposed that the direct EGFR-Cbl interaction is necessary for degradation of EGFR in lysosomes (Jiang et al. 2003; Grovdal et al. 2004), while the indirect binding of Cbl through Grb2 mediates recruitment of activated EGFR to clathrin-coated pits prior to internalization (Huang and Sorkin 2005). Although it seems clear that Cbl positively regulates endocytosis of EGFR, it is currently an open question whether Cbl regulates endocytosis of EGFR as a ubiquitin ligase or as an adaptor protein.

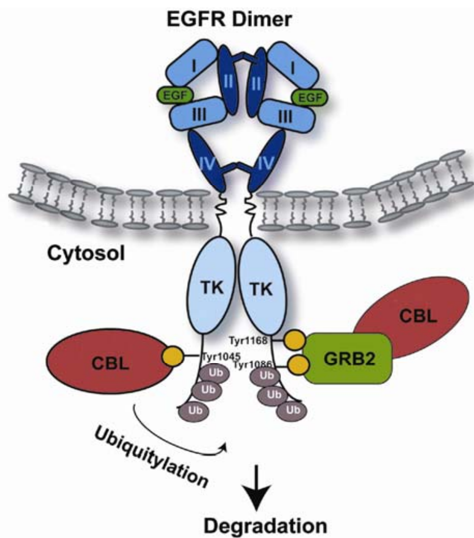

Figure 7. Binding of c-Cbl to EGFR. Upon ligand binding, c-Cbl is recruited to the plasma membrane where it associates with, and ubiquitinates, the activated EGFR prior to receptor internalization. c-Cbl binds to the activated EGFR both directly and indirectly. Direct binding is mediated through phosphorylated tyrosine residue 1045 on the EGFR whereas indirect binding is mediated through the adapter protein Grb2, which binds to phosphorylated tyrosine residues 1086 and 1068 on the receptor. Reprinted from Cellular Signalling, Vol 19, Zandi et al, Mechanisms for oncogenic activation of the epidermal growth factor receptor, 2013-23, ©2007, with permission from Elsevier. The figure legend is modified.

1.2.4.3 Eps15

Eps15 was originally discovered as a substrate for the EGFR kinase, but has later been implicated in clathrin-mediated endocytosis, primarily by binding to AP-2 (Tebar et al. 1996). In the N-terminal part, Eps15 harbors three Eps15 homology (EH) domains, which are domains often found in proteins associated with endocytosis and vesicle transport. Eps15 also contains a coiled-coil domain, DPF repeats and UIMs (Salcini et al. 1999). Upon EGFR activation, Eps15 is recruited to clathrin-coated pits at the plasma membrane (Torrisi et al. 1999; Stang et al. 2004), and the EH-domains of Eps15 were demonstrated to be required for this relocalization (Benmerah et al. 1999). The relocalization of Eps15 to clathrin-coated pits upon activation of EGFR might indicate that Eps15 is involved in endocytosis of EGFR, but this has to date not been confirmed. Some research groups have

been able to demonstrate that RNAi-mediated knock down of Eps15 negatively impacts on internalization of EGFR (Huang et al. 2004; Fallon et al. 2006), whereas others have reported no effect on EGFR endocytosis upon Eps15 knock down (Sigismund et al. 2005). The involvement of Eps15 in endocytic down-regulation of EGFR is thus still unclear. Concerning that a) Eps15 harbors two UIMs which binds to ubiquitinated proteins, b) ubiquitination is suggested to be involved in recruiting activated EGFR to clathrin-coated pits (Stang et al. 2004), c) EGFR is ubiquitinated at the plasma membrane in an EGF-dependent manner, and d) Eps15 localizes to clathrin-coated pits in response to EGF, one could speculate that Eps15 might function as an adaptor protein for sorting of ubiquitinated cargo, such as EGFR, to clathrin-coated pits prior to endocytosis.

1.2.4.4 Epsin

Another UIM-containing protein suggested to be involved in endocytosis of EGFR is epsin. At present, five human epsin homologs have been described (hEpsin, hEpsin1, hEpsin2a, hEpsin2b and hEpsin3) (Sugiyama et al. 2005). The N-terminal part of the protein is highly conserved among the different epsin family members, and it is denoted the ENTH-domain (epsin N-terminal homology domain). The ENTH-domain targets epsin to the plasma membrane by binding to PIP_2, and this interaction has been demonstrated to be necessary for EGFR endocytosis (Itoh et al. 2001). Following the ENTH-domain, the epsins contain tandem UIMs that can interact with ubiquitinated proteins (Chen and De Camilli 2005; Hawryluk et al. 2006). The central and C-terminal parts of epsins contain motifs that bind to clathrin, AP-2 and EH-domain-containing proteins such as Eps15 (Horvath et al. 2007). Epsin has been shown to be involved in formation or stabilization of membrane curvature (Ford et al. 2002; Ritter and McPherson 2006) and is also thought to serve as an adaptor protein in clathrin-mediated endocytosis (Horvath et al. 2007). Additionally, interaction of epsin with EGFR has been reported (Sigismund et al. 2005). This interaction has been characterized in paper III.

1.2.4.5 Intracellular sorting of EGFR

Clathrin-coated pits containing activated EGFR bud into the cell and form clathrin-coated vesicles, a process involving dynamin and the actin cytoskeleton. The clathrin-coated vesicles are then uncoated. The uncoated vesicles and their content mature into – or fuse with – early endosomes. Membrane fusion generating early endosomes is mediated by Rab5, early endosome antigen 1 (EEA1) and SNAREs. Moreover, the *Trans*-Golgi network

(TGN) contributes to the maturation of endosomes by providing lysosomal hydrolases (for review see Maxfield and McGraw 2004). In early endosomes, also termed sorting endosomes, the internalized receptors are sorted to distinct microdomains, for recycling back to the plasma membrane, for transport to the TGN or for lysosomal degradation. In early endosomes the environment is acidic due to proton pumps in the membrane. The low pH causes some of the receptor-bound ligands to dissociate from their receptor. This is the case if Transforming growth factor alpha (TGFα) is bound to EGFR. TGFα is released from EGFR, and the EGFR then enters early endosomal tubules and is recycled back to the plasma membrane. In contrast, the EGFR-EGF complex remains stable also at the low endosomal pH, and EGFR and EGF are sorted as a complex to intraluminal vesicles by inward budding of the limiting membrane of the endosome (Longva et al. 2002) (Figure 8). Formation of inner vesicles in endosomes generates the endosomal structures referred to as multivesicular bodies (MVBs). MVBs finally fuse with lysosomes, where both EGFR and EGF are degraded by acid hydrolases (Dunn et al. 1986; Futter et al. 1996; Raiborg et al. 2003).

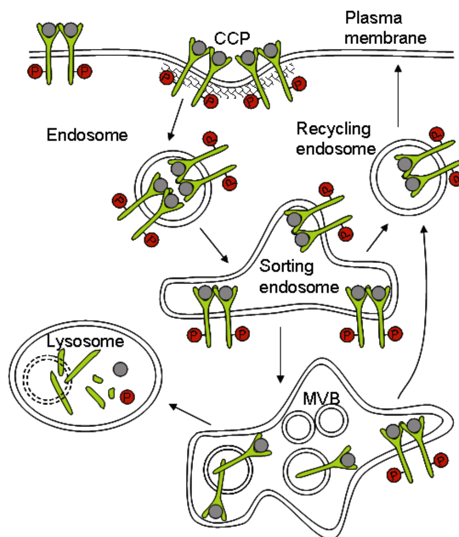

Figure 8. Trafficking of EGFR. The EGFR is internalized from clathrin-coated pits. The forming vesicles fuse with -or mature into- endosomes. In the sorting endosome, the receptors that are to be recycled are transferred to recycling endosomes while the receptors that are to be degraded are sorted to inner vesicles of a multi-vesicular body (MVB), and are eventually degraded in lysosomes. (The figure is from Breen 2008, reprinted with permission).

Ubiquitination is the best characterized signal for sorting receptors into MVBs. For EGFR, it is well established that Cbl-mediated ubiquitination is a prerequisite for translocation to inner vesicles of MVBs. Moreover, it is the direct binding of Cbl to phosphotyrosine 1045 of EGFR that is required for sorting to MVBs and thus receptor degradation (Grovdal et al.

2004). EGFR is sorted to inner vesicles of MVBs by sequential interaction with the four sorting protein complexes named ESCRT-0 to ESCRT-III (Endosomal Sorting Complexes Required for Transport). ESCRT-0 is a protein complex consisting of two proteins possessing one UIM each, the Hepathocyte growth factor regulated tyrosine kinase substrate (Hrs) and the Signal-transducing adaptor molecule (STAM). The ESCRT-0 protein complex seems to be essential for the initial sorting of ubiquitinated cargo at the endosomal membrane (Williams and Urbe 2007). Eps15 is not included in the ESCRT-0 complex. However, a fraction of Eps15 is also localized to endosomes and is reported to bind to Hrs. Interestingly, Eps15b, a novel isoform of Eps15, was recently described to be involved in sorting of EGFR for degradation (Roxrud et al. 2008). Downstream of binding ESCRT-0, ubiquitinated cargo is handed over to ESCRT-I, -II and III, respectively, before transport to inner vesicles of the MVBs and degradation (Raiborg et al. 2003; Piper and Luzio 2007; Williams and Urbe 2007). In the case of EGFR, it is reported that depletion of key components of all the four ESCRT-complexes by RNAi each strongly inhibits degradation of EGFR (Raiborg et al. 2008).

1.3 EGFR IN CANCER

Cell division and reduced apoptosis are among the biological outputs of signaling from EGFR. Attenuation of EGFR signaling by endocytic down-regulation is important in controlling these processes. Thus, dysregulation of endocytosis and intracellular trafficking can lead to increased and uncontrolled cell proliferation and lower rate of apoptosis, both characteristics associated with cancer progression. EGFR was the first cell surface receptor reported to be directly linked to cancer (Todaro et al. 1976). Since then, the involvement of EGFR, as well as the other ErbB proteins in cancer, has been extensively studied (reviewed in Gschwind et al. 2004; Ciardiello and Tortora 2008). Overexpression of both EGFR and its ligands was demonstrated in various cancer forms, including brain malignancies and non-small cell lung cancers (reviewed in Gullick 1991) and is often associated with poor prognosis (Holbro et al. 2003; Zandi et al. 2007). A number of mutations in the EGFR gene have been observed in various tumors, most common are deletions in the extracellular domain. These deletions are often associated with reduced ligand-induced down-regulation of EGFR, leading to sustained signaling (reviewed in Zandi et al. 2007). The EGFRvIII is the best characterized mutant form of EGFR. The EGFRvIII has a large extracellular deletion, rendering it incapable of ligand binding, but having a constitutively active kinase

domain (reviewed in Kuan et al. 2001). It has recently been demonstrated that the constitutively active EGFRvIII is less effectively ubiquitinated, and because of impaired internalization and increased recycling, this receptor escapes down-regulation (Grandal et al. 2007).

Because of its role in tumor progression, the EGFR has been extensively studied. The EGFR is targeted in cancer treatment by two major strategies, by using antibodies binding to the extracellular domain of EGFR and/or inhibitors of EGFR's kinase activity (Zandi et al. 2007). It could also be of future interest to target EGFR in cancer treatment by altering its endocytic down-regulation.

2 AIMS OF THE STUDY

The main focus of the work presented in this thesis has been to gain more insight into the molecular mechanisms controlling the initial steps of endocytic down-regulation of the EGFR. The importance of ubiquitination in endocytosis of EGFR has been a matter of debate, but the fact that the ubiquitin ligase Cbl is instrumental in endocytosis of EGFR is now well documented. It is still discussed whether Cbl functions as a ubiquitin ligase or as an adaptor protein in this process. A main aim has therefore been to investigate how Cbl-mediated ubiquitination affects endocytosis of EGFR, with different subgoals:

- In Paper I we wanted to use the Cbl-binding protein TULA (T-cell ubiquitin ligand) as a tool to inhibit EGF-induced ubiquitination of EGFR, and further to investigate how this would affect endocytosis of EGFR

- In Paper II the aim was to study the role of Grb2 and Cbl-mediated ubiquitination in endocytosis and EGF-induced recruitment of EGFR to coated-pits

- In Paper III we wanted to characterize the described interaction of EGFR and epsin 1, and to investigate whether epsin 1 could function as an endocytic adaptor protein recruiting ubiquitinated EGFR to clathrin-coated pits

- One of the aims in Paper IV was to characterize the role of the ubiquitin ligase Cbl in endocytosis of EGFR and in recruitment of activated EGFR into clathrin-coated pits. Another goal was to find out whether ubiquitination as such could function as a signal for EGFR endocytosis.

3 SUMMARY OF PAPERS

3.1 PAPER I

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

In this paper we studied how overexpression of TULA, a Cbl- and ubiquitin binding protein, affected endocytosis in HeLa cells. We confirmed that overexpression of TULA inhibited both ubiquitination and endocytosis of EGFR, as previously reported. Surprisingly, the endocytosis of Tf receptor, LDL receptor, Major histocompatibility complex class I (MHC-I), and CD59, all endocytosed dynamin-dependently, was also strongly inhibited upon overexpression of TULA. In contrast, endocytosis of the plant toxin ricin, which uses dynamin-independent endocytic pathways for internalization, was not affected by TULA overexpression. By confocal microscopy and co-immunoprecipiation experiments, we found that TULA colocalized and interacted with dynamin. We therefore proposed that the SH3-domain of TULA can interact with proline rich sequences in dynamin, and that overexpression of TULA inhibited dynamin-dependent endocytic pathways by functionally sequestering dynamin. Consistent with this, we showed that the TULA-induced inhibition of endocytosis could be counteracted by raising the cellular level of dynamin, or by disrupting the TULA-dynamin interaction. This supported the idea that TULA inhibited these dynamin-dependent endocytic pathways by sequestering dynamin. However, overexpression of dynamin or mutation of the SH3-domain of TULA did not counteract the inhibitory effect TULA had on ubiquitination of EGFR, and consistently nor on endocytosis of EGF. We concluded that TULA inhibited clathrin-dependent and -independent endocytic pathways by functionally sequestering dynamin, and that there was a correlation between inhibition of ubiquitination and endocytosis of the EGFR.

3.2 PAPER II

Cbl-dependent ubiquitination is required for progression of EGF Receptors into clathrin-coated pits. *Espen Stang, Frøydis D. Blystad, Maja Kazazic, **Vibeke Bertelsen**, Tonje Brodahl, Camilla Raiborg, Harald Stenmark, and Inger Helene Madshus*

In this paper we studied the relevance of Grb2 and Cbl-induced ubiquitination in recruitment of activated EGFR into clathrin-coated pits. When the EGFR at the plasma membrane is activated by binding EGF, it also becomes ubiquitinated by the ubiquitin ligase Cbl. Immuno electron microscopy (Immuno-EM) studies demonstrated that when the EGFR was activated by EGF on ice, it translocated from smooth areas to clathrin coated areas at the plasma membrane. When ubiquitination of the EGFR was inhibited due to overexpression of a mutant form of Grb2 or overexpression of the Cbl-interacting protein hSpry2, the recruitment of EGFR into central parts of clathrin-coated pits was inhibited. Under such conditions, the EGFR localized predominantly to the rim of clathrin-coated pits. Additionally, Grb2, Cbl and hSpry2 were all found to localize to the rim of clathrin coated pits after incubation with EGF on ice, indicating that these proteins cooperate in recruitment of activated EGFR into the clathrin-coated pits. In addition to the correlation of inhibited ubiquitination and inhibited recruitment into clathrin-coated pits, we found that overexpression of a conjugation-defect ubiquitin similarly inhibited the ligand-induced recruitment of EGFR into clathrin-coated pits as well as the endocytosis of EGF. Although we cannot from these data conclude that ubiquitination of EGFR itself is a prerequisite for recruitment of EGFR into clathrin-coated pits, our data led us to propose that Cbl-induced ubiquitination is required to recruit EGFR into interior parts of clathrin-coated pits.

3.3 PAPER III

Epsin recruits ubiquitinated EGF receptors into clathrin-coated pits. *Maja Kazazic, **Vibeke Bertelsen**, Ketil Winther Pedersen, Tram Thu Vuong, Michael Vibo Grandal, Marianne Skeie Rødland, Linton M. Traub, Espen Stang, and Inger Helene Madshus*

In this paper we studied the role of epsin as a ubiquitin-binding adaptor protein in endocytosis of EGFR. Epsin is a UIM-containing protein which is demonstrated to localize to clathrin-coated pits at the plasma membrane and also to associate with the EGFR. In this

work we showed by co-immunoprecipitation experiments that epsin interacted with ubiquitinated EGFR in a UIM-dependent manner. In cells where EGFR was less effectively ubiquitinated or when the UIMs of epsin 1 were mutated, the formation of an EGFR-epsin complex was reduced. Knocking down epsin 1 by RNAi resulted in a reduced internalization ratio of EGF, both at high and low EGF concentrations, whereas the internalization of Tf was unaffected. This indicated that epsin specifically was involved in endocytosis of EGFR and not in clathrin-mediated endocytosis in general. Immuno-EM studies confirmed the role of epsin 1 in clathrin-dependent endocytosis of EGFR; RNAi-mediated knock down of epsin 1 interfered with the EGF-induced recruitment of the EGFR into clathrin-coated pits. When the cells were depleted of epsin 1, the recruitment of EGFR into central parts of clathrin-coated pits was strongly inhibited compared to in control cells. We therefore concluded that epsin 1 acts as an adaptor protein that recruits ubiquitinated EGFR into clathrin-coated pits prior to endocytosis.

3.4 PAPER IV

Cbl and ubiquitination promote translocation of EGF Receptor into clathrin-coated pits.

Vibeke Bertelsen*, Malgorzata M. Sak, Kamilla Breen, Espen Stang, and Inger Helene Madshus.*

In paper IV we further studied the relevance of Cbl and Cbl-induced ubiquitination for the recruitment of activated EGFR into clathrin-coated pits as well as whether ubiquitin in isolation could induce endocytosis of EGFR. By siRNA-mediated knock down of c-Cbl and Cbl-b, we found that the EGF-induced ubiquitination and endocytosis of EGFR were both inhibited. Moreover, we also found by using immuno-EM that by depleting the cells of c-Cbl and Cbl-b, activated EGFR was less efficiently recruited to clathrin coated pits. We additionally found that the distribution of EGFR within coated pits was affected. In control cells, most of the clathrin-coated pit-associated EGFRs were found in interior parts of the pits. Contrastingly, in cells where c-Cbl and Cbl-b had been knocked down, clathrin-coated pit associated EGFRs predominantly localized to the rim of coated pits. We therefore suggested that consistent with our previous findings, Cbl-mediated ubiquitination facilitates endocytosis of EGFR by guiding EGFR into clathrin-coated pits prior to internalization. To address whether ubiquitin could function as an endocytosis signal for EGFR, we constructed an EGFR-ubiquitin chimera with 4 ubiquitins fused to the C-terminal tail of full-length

EGFR via a flexible linker (EGFR-Ub_4). By confocal microscopy, we observed this chimeric protein in early endosomes in the absence of added EGF. The EGFR-Ub_4 was internalized from the plasma membrane in a clathrin-dependent manner without requirement for EGF or EGFR kinase activity and also without recruiting c-Cbl. This strongly indicates that ubiquitination of EGFR *per se* can mediate clathrin-dependent endocytosis of EGFR. We thus concluded that Cbl-mediated ubiquitination of EGFR facilitates clathrin-dependent endocytosis of the EGFR by guiding ubiquitinated EGFR into clathrin-coated pits.

4 METHODOLOGICAL CONSIDERATIONS

Detailed descriptions of the methods used in this work are given in the papers. In general, all experiments included have been performed three times or more, and when statistics has been needed, standard deviation (SD) has been calculated. The following section will discuss the advantages and limitations of some of the methods used.

4.1 CELL LINES/EXPERIMENTAL CELL SYSTEMS

Different cell lines are used throughout this work: HeLa cells, Hep2-cells and Porcine Aortic Endothelial (PAE) cells. Most of the experiments are performed in the human cervical carcinoma cell line, HeLa. We have mainly used HeLa cells because they are easy to work with, they are easy to transfect, they express relatively high levels of EGFR and they are well suited for confocal microscopy studies due to the flat morphology and the high cytosol/nucleus ratio. In some of the microscopy studies (mainly immuno-EM), human laryngeal carcinoma cells line Hep2 was chosen since these cells express higher levels of EGFR at the plasma membrane than do the HeLa cells. Higher EGFR level is an advantage due to the highly limited volumes/areas contained within an immuno-EM section compared to volumes examined by fluorescence microscopy. When studying the Y1045F-EGFR mutant (paper III) or the EGFR-Ub$_4$ chimera (paper IV), PAE cells were used, either stably or transiently expressing the recombinant EGFR. PAE cells were used since they do not express endogenous EGFR and thereby enable investigation of the effect of an introduced alteration in the EGFR. As PAE cells do not express EGFR endogenously, it may be argued that the cells can exploit different mechanisms for endocytic down-regulation of the EGFR. However, the PAE cell line stably transfected with wild type EGFR is well described and was reported to internalize EGFR at a level comparable to that of human cells endogenously expressing EGFR (Carter and Sorkin 1998).This cell line has since then been widely used as a model system to study endocytic down-regulation of different mutant forms of EGFR as well as of other ErbB proteins (Jiang et al. 2003; Haslekas et al. 2005; Huang et al. 2006; Huang et al. 2007; Pedersen et al. 2008).

One of the advantages of using immortalized cells is that these cells can be maintained in culture for a long time, thereby allowing several repeated experiments. On the other hand, genotypic or phenotypic alterations may occur after long times in culture, and the cells were therefore kept in culture for a limited time period. PAE cells stably transfected with wt- or Y1045F-EGFR were routinely tested for EGFR expression level and were not kept for more than 20 passages.

4.2 TRANSIENT TRANSFECTION WITH PLASMIDS AND OVEREXPRESSION OF PROTEINS

Transfection of cells is a widely used and powerful tool for studying the importance of mutants or domains of a protein, or for detecting protein-protein interactions. Nevertheless, one should be aware of the pitfalls in this method. Transient transfection can result in artificially high levels of the encoded protein (overexpression) and may therefore cause cellular responses that have no physiological relevance. For example, altered stoichiometry may cause sequestration of endogenously expressed proteins. This was the case when epsin 1 was overexpressed in paper III (supplementary information). High levels of epsin 1, which possesses clathrin- and AP-2-binding motifs, disrupted clathrin-coated pits by sequestering AP-2 and clathrin away from the plasma membrane and thus inhibiting all endocytosis from clathrin-coated pits. In other cases, the effect of protein overexpression is useful. Overexpression of proteins, such as hSpry2 (used in paper II and III) or TULA (paper I), disrupts the interaction between Cbl and EGFR, thereby making it a useful tool to study the relevance of Cbl and/or ubiquitination in endocytosis of EGFR. One problem is that the transfection efficiency most often is low and that only a minority of the cells express the protein of interest. Thus, when performing biochemical analyses on a transiently transfected population of cells, the effect can easily be underestimated or even difficult to detect.

4.3 RNA INTERFERENCE (RNAI)

RNAi is a tool to suppress mRNA levels and thereby the expression level of a protein of interest. The technique is based on the discovery that double-stranded small interfering RNAs (siRNAs) are a part of the post translational gene silencing in plants (Hamilton and Baulcombe 1999) as well as the finding that siRNAs also induce RNAi

in cultured mammalian cells (Elbashir et al. 2001). However, a pitfall in this technique is that RNAi may generate off-target effects, where the siRNAs, due to sequence similarity, reduce expression of non-targeted genes. To be sure that the observed phenotype is due to specific RNAi, it is useful to verify results by using siRNA sequences complementary to different regions of the mRNA-sequence transcribed from the same target gene. One should additionally be aware of the fact that introducing high amounts of siRNA to the cells may create an interferon response resulting in activation of intracellular signaling cascades (Sledz et al. 2003) which in turn may lead to misinterpretation of results. It is therefore important to transfect control cells with control siRNAs with similar length and identical concentration. In paper III where epsin I was knocked down using siRNAs, the control cells were treated with the transfection reagent only. However, we confirmed that under our experimental conditions, transfection of siRNA specific for a non-relevant gene (green fluorescent protein (GFP)) did not display the phenotype observed with the epsin 1 specific siRNAs (paper III, supplementary information). One should also be aware of the fact that it can be difficult to distinguish a direct effect of protein depletion from its indirect effects. For example, inhibiting endocytosis by siRNA-mediated down-regulation of clathrin may prevent import of essential cells, thereby generating an indirect effect which in turn may cause an altered phenotype (Roth 2008). Despite the limitations and pitfalls using RNAi, this method is a powerful technique to study the function of proteins.

4.4 IMMUNOLOGICAL METHODS

In this work, several immunological techniques have been used. The prerequisite for all immunological methods is the specific interaction between an antibody and its antigenic epitope. Thus, it is important to include controls which ensure that the interaction is specific without immunoreactivity against irrelevant molecules.

Western blotting was used to study protein level, formation of protein complexes and protein modifications, such as ubiquitination and phosphorylation. In this technique, the result exclusively depends on antibody-antigen interaction. Antibodies used in Western blotting were tested for specificity prior to use. Only antibodies recognizing proteins with the expected molecular weight were used. In addition, when it was

possible, antibody specificity was tested in cells overexpressing the protein of interest. This was assayed as increased intensity of the band detected by the antibody.

Immunoprecipitation experiments were performed to purify proteins prior to analysis of protein modification and to study protein complex formation. In this method, antibodies are coupled to protein A or protein G which is pre-coupled to magnetic beads by the manufacturer. Proteins recognized by the antibody are then precipitated and isolated. It was tested whether the precipitation was specific for the protein of interest. This included a sample with protein A- or protein G-beads without antibody ruling out that the protein of interest was precipitated by unspecific interactions with the beads.

Immunostaining of fixed cells was used to study intracellular localization of proteins and to detect protein expression levels upon transfection of cells with a plasmid encoding the protein of interest. The specificity of the antibodies used was also carefully tested by comparing the localization of the target protein with previous reports regarding the localization of the encoded protein as well as by including cells either not expressing or overexpressing the protein, whenever it was possible. All secondary antibodies used in this work were characterized in a control experiment using only secondary antibodies in order to investigate the level of background staining and to ensure that there was minimal unspecific binding. Prior to use in double or triple labeling experiments, different combinations of secondary and primary antibodies were tested for possible cross-reactions.

4.5 MICROSCOPY

A major part of the research covered by this thesis is based on results obtained with confocal and electron microscopy. Confocal microscopy was used to study internalization of fluorescent ligands or antibodies, to study subcellular localization of proteins and to detect cells overexpressing proteins of interest. A major advantage of microscopical analysis is its possibility to study single cells. This is especially of interest in experiments involving transient transfection with plasmids, when not all cells express the protein of interest. However, results from single cells may display variations, and it is thus important to include several cells in the study to correct for

individual variations. It is thus useful to quantify cells displaying a certain phenotype and thereby obtain a more objective result. Such quantification was for example included in paper I, in which confocal microscopy was the only method used to analyze the effect of a mutant form of dynamin or TULA on internalization of different ligands and plasma membrane proteins.

Immunofluorescent staining of proteins at the same subcellular localization was considered colocalization of these proteins. Our confocal microscopy analyses, however, only investigated localization in the x and y planes thereby allowing the possibility of superimposing in the z plane. We can therefore not be absolutely certain that the proteins reside in the same subcellular compartment based on these analyses, even though the images obtained strongly suggested colocalization. It should be pointed out that when cells were labeled with more than one antibody, the pictures were taken sequentially to avoid bleed-through from the distinct fluorochromes used.

Since light microscopy has a relatively low resolution, immuno-EM was used to investigate the localization of proteins more precisely. In particular, the plasma membrane localization of EGFR before and after EGF-treatment under different experimental conditions was studied. One major advantage of immuno-EM is the high resolution achieved by this technique. Another important factor is that electron-dense protein coats at the plasma membrane can be detected without using specific markers. This has been useful in this kind of studies when localization of EGFR to clathrin-coated areas at the plasma membrane has been a main focus. That these coats contain clathrin was confirmed by labeling for clathrin. A general problem in immuno-EM studies, however, is a limited labeling efficiency using EGFR antibodies combined with the small volumes contained within an EM-section (Griffiths 1993). Due to this, we often detect only one gold particle, i.e. one EGFR molecule in a single coated pit. Quantification of the results obtained using immuno-EM has thus been important, and in paper III we also included cell lines with higher expression of EGFR to confirm the results. Quantification was performed by counting gold particles localized to the plasma membrane followed by calculation of the percentage of the total labeling for EGFR at the plasma membrane that was associated with coated areas.

4.6 INTERNALIZATION OF EGF

Different approaches to study internalization of EGF were used in this work. Internalization of EGF was used to indirectly study internalization of EGFR, since EGF is internalized when bound to its receptor. To measure the internalization ratio, cells were incubated with 1 ng/ml ^{125}I-EGF. The amount of surface-labeled and internalized ^{125}I-EGF at different time periods was measured, and the internalization ratio was calculated. This method is very sensitive, allowing small amounts of intracellularly localized EGF to be detected. It is thus particularly useful when studying internalization of EGFR at low EGF concentrations. To detect fluorescent EGF by confocal microscopy, however, higher concentrations of EGF were required. It could possibly be due to the low sensitivity of our system suggesting that multiple fluorescent EGF molecules have to coexist in endosomes to allow detection of the fluorescence signal above background level. In confocal microscopy studies included in this work, 10 ng/ml or 15 ng/ml of fluorescent labeled EGF were used, both of which are within the range of physiological concentrations of EGF (Sigismund et al. 2005, supplementary Material). One exception though is in paper II where we used 100 ng/ml EGF in order to study effects on macropinocytosis, which can be induced by high concentrations of EGF (Roepstorff et al. 2008). Detection of EGF using immuno-EM required high EGF concentration. This is probably due to a combination of the small volumes retained within an immuno-EM section and a limited labeling efficiency. We therefore incubated the cells with 60 ng/ml EGF prior to immuno-EM analyses. Moreover, 60 ng/ml EGF was routinely used in experiments where a cellular response to EGF-treatment (i.e. recruitment of proteins, posttranslational modifications and translocation of EGFR at the plasma membrane) was analyzed, in order to obtain a maximal response.

5 GENERAL DISCUSSION

It is generally accepted that EGFR undergoes rapid clathrin-mediated endocytosis upon binding EGF. Despite the extensive investigation of EGFR endocytosis, the exact molecular mechanisms involved in this process are, however, not fully elucidated. There are conflicting reports regarding different posttranslational modifications required for EGFR endocytosis. Furthermore, there is also to some degree inconsistency in the literature concerning requirements for adaptor proteins and accessory endocytic proteins in translocation of activated EGFRs into clathrin-coated pits.

5.1 SIGNALS FOR EGFR ENDOCYTOSIS

Receptors constitutively internalized from clathrin coated pits, such as TfR, are sorted to clathrin-coated pits via linear sequences such as tyrosine-based and di-leucine sorting signals selected by AP-2 (reviewed in Bonifacino and Traub 2003). EGFR also possesses linear sorting signals, both di-leucine and tyrosine-based. The tyrosine-based signal is demonstrated to interact with AP-2 (Nesterov et al. 1995; Sorkin et al. 1996), and the di-leucine sequences have in a recent paper been reported to be essential for EGFR endocytosis (Wang et al. 2007). This is, however, in conflict with another report arguing that AP-2 is not strictly required as a cargo-selecting adaptor protein for EGFR, but that AP-2 is rather required as a clathrin-coat component having a more structural function (Johannessen et al. 2006).

In contrast to constitutively internalized receptors, EGFR is rapidly internalized only in response to ligand binding, and its endocytic signal is therefore most likely exposed or switched on by ligand-induced events, such as receptor dimerization, kinase activation, phosphorylation, and/or ubiquitination.

5.1.1 Is EGFR kinase activity required for EGFR endocytosis?

A recent paper argues that the EGFR kinase is dispensable for its internalization and moreover that receptor dimerization is sufficient to mediate endocytosis of EGFR (Wang et

al. 2005). This is, however, in conflict with several other reports demonstrating that endocytosis of EGFR is strictly dependent on the activity of the EGFR kinase. These reports are based on experiments using a kinase deficient mutant of EGFR or on the use of EGFR kinase inhibitors (Lamaze and Schmid 1995; Sorkina et al. 2002). Taking into consideration that overexpression of a dominant negative mutant of Grb2 efficiently inhibited EGF internalization (paper II) and that Grb2 is recruited to autophosphorylation sites on EGFR (Batzer et al. 1994), it is logical that kinase activity is required for EGFR endocytosis. This is supported by a previous paper from our group, where it was demonstrated that incubation with EGF induced formation of clathrin-coated pits, and that this was dependent on EGFR kinase activity (Johannessen et al. 2006). The role of Grb2 in EGFR endocytosis has been thoroughly investigated by the group of Alexander Sorkin. A recent paper revealed that the SH2-domain of Grb2 coupled to the RING-finger domain of Cbl was sufficient for restoring EGFR endocytosis in cells where endogenous Grb2 had been knocked down by RNAi, suggesting that an important function of Grb2 in EGFR endocytosis is to recruit Cbl to EGFR (Huang and Sorkin 2005).

5.1.2 Is ubiquitination of EGFR necessary for its endocytosis?

Upon EGF-binding, the EGFR is activated and becomes ubiquitinated by the ubiquitin ligase Cbl while still present at the plasma membrane (see Introduction, Section 1.1.3). However, whereas it is well established that ubiquitination of EGFR is a prerequisite for sorting EGFR to inner vesicles of MVBs, it is controversial whether ubiquitination is required for the initial steps of EGFR endocytosis (reviewed in Miranda and Sorkin 2007). In the work included in this thesis, there is consistently a correlation between inhibition of EGFR ubiquitination and inhibition of EGFR endocytosis. Moreover, a correlation between inhibited ubiquitination and inefficient translocation of EGFR into clathrin-coated pits has been observed. Overall, these data point in the direction of ubiquitination as a prerequisite also for initial steps of endocytic down-regulation of EGFR.

In paper I, we used the Cbl-interacting protein TULA as a tool to investigate how impaired ubiquitination affected endocytosis of EGFR. It had previously been reported that ubiquitination and endocytic down-regulation of EGFR were both reduced upon overexpression of TULA (Feshchenko et al. 2004; Kowanetz et al. 2004). In paper I we additionally demonstrated that overexpression of TULA sequestered dynamin and thereby inhibited all dynamin-dependent endocytosis. It was therefore difficult to study how TULA

and its Cbl-interaction specifically affected endocytosis of EGFR. Since it further has been reported that TULA binds to Cbl via its SH3-domain and that the negative regulation of EGFR endocytosis is dependent on this interaction (Feshchenko et al. 2004; Kowanetz et al. 2004), we mutated the SH3-domain of TULA. However, in conflict with the previously reported data, we found that the negative effect of TULA on ubiquitination and endocytosis of EGFR was independent of a functional SH3-domain (paper I). The reason for this discrepancy is unclear, but our data suggest either that Cbl can interact with TULA independently of the SH3-domain, or that TULA negatively impacts ubiquitination and endocytosis of EGFR regardless of Cbl binding.

Correlation between inhibited ubiquitination and inhibited endocytosis of EGFR was also described in paper II, both by overexpression of the Cbl-interacting protein hSpry2 and overexpression of a dominant negative mutant of Grb2 not binding Cbl. In paper IV we further observed that inhibited endocytosis of EGF coincided with inhibited ubiquitination of EGFR in cells where c-Cbl and Cbl-b were knocked down by RNAi. This repeatedly observed correlation argues that ubiquitination and endocytosis of EGFR are linked. However, a correlation between ubiquitination and endocytosis is not direct evidence that ubiquitination of EGFR itself is necessary for its endocytosis. Also a correlation between ubiquitination of GHR and clathrin-mediated endocytosis of this receptor has been reported (Govers et al. 1997; Strous et al. 1997). Initially it was suggested that ubiquitination of the GHR was required for its endocytosis (Govers et al. 1997). However, it was later demonstrated that a domain of GHR designated ubiquitin-dependent endocytosis motif, rather than ubiquitination of GHR itself, was critical for its endocytosis (Govers et al. 1999). It was further suggested that this motif in GHR interacts with an E3 ubiquitin ligase which in turn mediates ubiquitination of another factor in the GHR-ubiquitination complex leading to GHR endocytosis (Govers et al. 1997; van Kerkhof et al. 2007).

That the multidomain-containing protein and ubiquitin ligase Cbl is important in endocytic down-regulation of EGFR seems to be generally accepted (reviewed in Thien and Langdon 2001). The question remains, however, whether it is the ubiquitin ligase activity or the domains involved in protein-protein interactions that is essential for its function in EGFR endocytosis. The finding of the RING-finger domain of Cbl being instrumental in EGFR endocytosis (Huang and Sorkin 2005) indicates that the ubiquitin ligase activity is essential for mediating EGFR endocytosis. However, it has yet not been clarified whether efficient

endocytosis of EGFR required Cbl-induced ubiquitination of the receptor itself or of an accessory endocytic protein. In paper II we made use of a ubiquitin mutant which could not be conjugated to substrates, but which could potentially bind to proteins harboring ubiquitin binding domains thereby disturbing their binding to ubiquitinated proteins. When this ubiquitin mutant was overexpressed, the EGF-induced endocytosis of EGFR was strongly inhibited (paper II). Again, this is not direct evidence that ubiquitination of EGFR itself is required for its endocytosis, but it strongly argues that the ubiquitin system is required for effective endocytosis of EGFR. If one or more endocytic adaptor proteins need to be ubiquitinated for endocytosis of EGFR, such candidates could potentially be Eps15 or epsin, both of which are demonstrated to be ubiquitinated in response to EGFR activation (van Delft et al. 1997; Polo et al. 2002). However, ubiquitination of these proteins is known to be mediated by ubiquitin ligases other that Cbl (Polo et al. 2002; Timsit et al. 2005), thereby making it unlikely that Cbl-mediated ubiquitination of either of these proteins is critical for EGFR endocytosis. However, we cannot rule out that ubiquitination of other, yet unidentified proteins, has a role in EGFR endocytosis.

It was recently reported that ubiquitination of EGFR is not necessary for its endocytosis. This conclusion was based on the finding that an EGFR with multiple lysine mutations (designated 15KR), rendering it only marginally ubiquitinated, was internalized at a rate comparable to the rate of wild type EGFR (Huang et al. 2007). Moreover, internalization of the 15KR mutant of EGFR was sensitive to expression of both clathrin and Cbl, since depletion of clathrin heavy chain or of Cbl resulted in a reduced rate of internalization. From these findings, it was argued that Cbl is required to ubiquitinate another protein than EGFR itself. It should, however, be noted that the mutant EGFR used in this reported work contained residual lysine residues that could possibly be targets for ubiquitination and further that complete absence of ubiquitination of the EGFR mutant could not be achieved. A residual ubiquitination of approximately 1 % was reported (Huang et al. 2007). A problem when investigating ubiquitination level of proteins by Western blotting is that marginal ubiquitination can be difficult to detect. It was recently reported that an antibody to polyubiquitin did not show linear reactivity since the quantitative Western blotting did not correspond to the amount of ubiquitinated proteins added to the gel (Umebayashi et al. 2008). To what extent this is the case for other anti-ubiquitin antibodies as well is unknown, but this could potentially explain some of the discrepancy in the literature regarding the level of ubiquitination of EGFR versus the rate of endocytosis.

Ubiquitination has been demonstrated to be a prerequisite for internalization of several cell surface receptors in both yeast and mammalian cells (Galan et al. 1996; Hicke and Riezman 1996; Galan and Haguenauer-Tsapis 1997; Geetha et al. 2005; Belouzard and Rouille 2006; Miranda et al. 2007). Since our results suggested that ubiquitination is important for EGFR endocytosis, we wanted to investigate whether ubiquitin could induce endocytosis of the EGFR in the absence of added ligand or of the EGFR kinase activity (paper IV). Based on experiments with the EGFR-Ub_4 chimeric protein in which 4 ubiquitin moieties were recombinantly fused to the C-terminus of EGFR via a flexible linker, we could indeed demonstrate that ubiquitin *per se* promoted clathrin-dependent endocytosis of EGFR. Chimeric proteins of EGFR and ubiquitin have been described earlier (Haglund et al. 2003; Mosesson et al. 2003; Sigismund et al. 2005). A chimeric protein consisting of one single ubiquitin fused to a truncated EGFR consisting of the transmembrane and extracellular parts was reported to be internalized in a clathrin-independent, but caveolin-dependent, manner. The view of ubiquitination as signal for caveolae-mediated endocytosis has later been argued against by data from Barriere et al. (Barriere et al. 2006). By RNAi they demonstrated that endocytosis of ubiquitinated cargo was clathrin-dependent and caveolin-independent. This is in accordance with our finding that endocytosis of the EGFR-Ub_4 depended on clathrin. The different conclusions in these studies could possibly be explained by the differences in the chimeric fusion proteins used. Sigismund et al studied a truncated version of EGFR with one single ubiquitin as the intracellular signaling unit (Sigismund et al. 2005). In contrast, we used a full length EGFR. Moreover, since the endocytic adaptors containing UIMs, epsin 1 and eps15, preferably interact with polyubiquitin chains (Hawryluk et al. 2006), we fused 4 ubiquitins to the EGFR. Interestingly, the observation that 4 ubiquitins is sufficient to mediate clathrin-dependent endocytosis of EGFR argues that the EGFR does not necessarily need to be heavily ubiquitinated to bind endocytic adaptor proteins via ubiquitin binding domains. This challenges the conclusion that EGFR ubiquitination is not necessary for its endocytosis based on the finding that the 15KR EGFR mutant was endocytosed as effectively as was the wild type EGFR (Huang et al. 2007).

Altogether, our data suggest that ubiquitination plays an important role in endocytosis of EGFR. However, how the ubiquitinated EGFR interacts with adaptor proteins and which adaptor proteins are required are still not fully resolved.

5.2 TRANSLOCATION OF EGFR INTO CLATHRIN-COATED PITS

As described, EGFR is mainly endocytosed from clathrin-coated pits. The molecular mechanisms and the adaptor proteins involved in the translocation of EGFR into clathrin-coated pits are not fully elucidated. In paper II we demonstrated that a fraction of the plasma membrane localized EGFR was translocated from smooth areas to clathrin-coated areas at the plasma membrane upon incubation of cells with EGF. Since the EGF-induced endocytosis of EGFR is a rapid process at 37 °C, we performed experiments on ice as it was previously reported that maximal accumulation of EGFR in clathrin coated pits was observed at 4 °C (Sorkina et al. 2002). This allowed us to study the involvement of specific proteins in recruitment of activated EGFR into clathrin-coated pits. Upon EGF-treatment, we detected a relocalization of Cbl, Grb2 and Eps15 from the cytosol to the plasma membrane. By immuno-EM studies, we could demonstrate in more detail that Cbl and Grb2 both localized to the rim of clathrin-coated pits upon EGF-treatment, suggesting that these proteins participate in the recruitment of activated EGFR to clathrin-coated pits. The finding that the nonconjugable ubiquitin mutant inhibited recruitment of activated EGFR to clathrin-coated pits further suggested that interaction between ubiquitinated proteins and proteins with ubiquitin binding domains is important in this process. Consistently, when EGF-induced ubiquitination of EGFR was inhibited by overexpression of the Cbl-binding protein hSpry2, the translocation of EGFR into clathrin-coated pits was inhibited, and the activated EGFR was retained at the rim of clathrin-coated pits. This observation led us to speculate that ubiquitination mediates translocation of EGFR from the rim to inner parts of clathrin-coated pits. In paper IV this hypothesis was further investigated. When c-Cbl and Cbl-b were knocked down by RNAi and the ubiquitination of EGFR was strongly inhibited, less EGFR was associated with clathrin-coated pits. Moreover, the distribution of EGFR within clathrin-coated pits was affected by knockdown of c-Cbl and Cbl-b. Coat-associated EGFR from cells depleted of c-Cbl and Cbl-b localized predominantly to the rim of the coats, whereas the coat-associated EGFR mainly was found in the interior of the coated pits in control cells (paper IV).

The adaptor complex AP-2 is a key component in clathrin-coated pits due to its clathrin-binding and its direct binding to several cargo proteins, as well as to its ability to recruit endocytic accessory proteins (Conner and Schmid 2003b). However, the involvement of AP-2 in endocytosis of EGFR has been disputed, and different requirements for this adaptor

complex in EGFR and TfR endocytosis have been reported (Conner and Schmid 2003a; Motley et al. 2003; Johannessen et al. 2006). If EGFR needs to be ubiquitinated to be effectively translocated from the rim to interior parts of clathrin-coated pits, this translocation probably requires one or more adaptor proteins that contain ubiquitin binding domains. Two such candidate proteins harboring UIMs are Eps15 and epsin (Hofmann and Falquet 2001). Eps15 has previously been shown to localize to the rim of clathrin-coated pits (Tebar et al. 1996; Stang et al. 2000). It is thus unlikely that this protein sequesters ubiquitinated EGFR in the interior parts of the coated pits. In contrast, epsin 1 was found to localize all along the clathrin-coats, both in flat and in invaginated coated areas (paper II). Moreover, siRNA-mediated depletion of epsin 1 inhibited both recruitment of activated EGFR into interior parts of clathrin-coated pits and endocytosis of EGF to the same extent (paper III). We additionally demonstrated that epsin 1 and EGFR interacted in a ubiquitin- and UIM-dependent manner. In paper III, we therefore proposed a model in which Eps15 and epsin 1 cooperate in packaging of ubiquitinated EGFR into the invaginated section of clathrin-coated pits. Our model is that ubiquitinated EGFR binds transiently to Eps15 and is subsequently handed over to epsin 1 which is localized inside the edge of the clathrin-coat. This resembles the described cooperation of GGAs (Golgi-associated, γ-adaptin homologues) and AP-1 in packaging of Mannose-6-Phosphate receptors at the TGN (Doray et al. 2002). It should be noted that we in paper II reported that not all EGF-positive clathrin-coated pits were epsin 1-positive, which could suggest that also other ubiquitin binding proteins could function as adaptors for ubiquitinated EGFR. However, a later study has shown that epsin 1 is present in all clathrin-coated pits (Hawryluk et al. 2006). Our conclusion may thus be a misinterpretation explained by limited labeling efficiency for epsin 1 as discussed in Section 4.5. Our data demonstrating that epsin 1 depletion inhibited recruitment of ubiquitinated EGFR into the interior of clathrin-coated pits are consistent with previous reports suggesting that epsin 1 is involved in endocytosis of ubiquitinated cargo (Barriere et al. 2006) and that epsin 1 is involved in clathrin-mediated endocytosis (Hawryluk et al. 2006). It has, however, been argued against epsin as adaptor protein for ubiquitinated cargo in clathrin-mediated endocytosis based on data suggesting that the ubiquitin binding and clathrin binding capacity of epsin 1 were mutually exclusive (Chen and De Camilli 2005) and that epsin 1 was involved in caveolae-mediated endocytosis (Sigismund et al. 2005). However, the work presented by Hawryluk et al. demonstrating that epsin 1 colocalized with AP-2 and not with caveolin argues against this conclusion (Hawryluk et al. 2006).

Upon RNAi-mediated epsin 1 knock down, we observed a reduced EGF internalization ratio of approximately 30 % compared to in control cells (paper III). The relatively low effect of epsin 1 depletion can possibly be explained by incomplete epsin 1 knock down, or it can be argued that epsin 1 is not strictly required for EGFR endocytosis. We can thus not exclude the possibility that other unidentified UIM-containing proteins or epsin 2 can mediate translocation of ubiquitinated EGFR into clathrin-coated pits (as suggested in paper II). If epsin 1 is not strictly required for EGFR endocytosis, it could, however, possibly act by facilitating or speeding up endocytosis of the ubiquitinated EGFR.

5.3 CONCLUDING REMARKS

This work has shown that:

- Grb2 and c-Cbl localize to the rim of clathrin-coated pits upon activation of EGFR and these proteins are both involved in endocytosis of EGFR
- there is correlation between inhibited ubiquitination and inhibited endocytosis of EGFR
- when ubiquitination of EGFR was inhibited by different means, activated EGFR was less efficiently recruited into interior parts of clathrin-coated pits
- ubiquitin recombinantly added to EGFR promoted internalization of EGFR from clathrin-coated pits
- there is a positive correlation between expression of epsin 1 and localization of EGFR into interior parts of clathrin-coated pits

Based on these data, I suggest the following model: upon incubation of cells with EGF, the EGFR dimerizes and the EGFR kinase is activated leading to recruitment of Grb2 and Cbl to EGFR and the EGFR is translocated to clathrin-coated areas at the plasma membrane. Cbl-mediated ubiquitination of EGFR, which occurs at the plasma membrane, facilitates the recruitment of EGFR into interior parts of clathrin-coated pits by ubiquitinated EGFR interacting sequentially with Eps15, localized at the rim of clathrin-coated pits, and epsin 1 which sequesters ubiquitinated EGFR in interior parts of clathrin-coated pits, from where EGFR is endocytosed.

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