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Molecular characterization of clathrin-dependent EGFR endocytosis unveils distinct internalization mechanisms that couple with different signaling outputs

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LIST OF ABBREVIATIONS

ANTH	AP180 N-terminal homology
AP2	Adaptor protein 2
ARH	Autosomal Recessive Hypercholesterolemia
BAR	Bin-Amphiphysin-Rvs
CCP	Clathrin coated pit
CCV	Clathrin coated vesicle
CHC	Clathrin heavy chain
CLASP	Clathrin associated sorting protein
CLC	Clathrin light chain
CLIC	Clathrin-independent carriers
CME	Clathrin-mediated endocytosis
CTxB	Cholera toxin B
CtBP1	C-terminal binding protein 1
DAB2	Disabled homolog 2
DAG	1,2-diacylglycerol
DEG	Delayed early gene
EE	Early endosome
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
EPS15	Epidermal growth factor receptor substrate 15
EPSIN	Eps15-interacting protein
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinase
ESCRT	Endosomal sorting complex required for transport
FAK	Focal adhesion kinase
FCHo	FCH domain only
FCH	F-BAR domain-containing Fer/Cip4 homology
GAK	Cyclin G-associated kinase
GAP	GTPase activating protein
GEEC	GPI-AP enriched early endosomal compartment
GPCR	G protein-coupled receptor
GPI	Glycosylphosphatidylinositol
GRB2	Growth factor receptor-bound protein 2
HB-EGF	Heparin-binding EGF-like growth factor
HSC70	Heat Shock Cognate 70
ID-miR	Immediately downregulated microRNA
IEG	Immediate early gene
IL2Rβ	Interleukin 2 receptor type β
ILV	Intraluminal vesicle
IP_3	Inositol 1,3,5-triphosphate
JNK	c-Jun NH_2-terminal kinase
KD	Knock down
LDLR	Low-density lipoprotein receptor
LE	Late endosome
LRP6	Wnt3a-activated low-density receptor-related protein 6
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MVB	Multivesicular body
NCE	Non-clathrin endocytosis
NGFR	Nerve growth factor receptor
NICD	Notch intracellular domain
NTD	N-terminal domain

PAR1	Protease-activated receptor-1
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PKC	Protein kinase C
PLCγ1	Phospholipase Cγ1
PLZF	Promyelocytic leukemia zinc finger protein
PM	Plasma membrane
PDGFR	Platelet-derived growth factor receptor
PTB	Phosphotyrosine-binding
PTRF	Polymerase I and transcript release factor
RTK	Receptor tyrosine kinase
SDPR	Serum deprivation response
SARA	Smad anchor for receptor activation
SH2	Src homology 2
SH3	Src homology 3
SILAC	Stable isotope labelling with amino acids in cell culture
siRNA	Small interfering RNA
shRNA	Short hairpin RNA
SNX9	Sorting Nexin 9
SRBC	SDR-related gene product that binds to c-kinase
SRG	Secondary response gene
STAT	Signal transducer and activation of transcription
SV40	Simian virus 40
TfR	Transferrin receptor
TGFβR	Tumor growth factor β receptor
TGN	Trans-Golgi network
TKB	Tyrosine kinase-binding
TNFR	Tumor necrosis factor receptor
TIRFM	Total internal reflection fluorescence microscopy
Ub	Ubiquitin
UIM	Ubiquitin-interacting motif
Vps27	Vacuolar protein sorting 27
WT	Wild type
β2AR	β2 adrenergic receptor
μHD	μ-Homology domain

ABSTRACT

The epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor that induces cell differentiation, proliferation and migration upon ligand activation. EGFR endocytosis is critical for the regulation of its signalling. Despite the enormous amount of work on this topic, the endocytic mechanisms that regulate EGFR signalling have not been completely elucidated.

The EGFR can be endocytosed through either clathrin-mediated endocytosis (CME) or non-clathrin endocytosis (NCE), depending on ligand concentration. NCE of the EGFR is mainly associated with receptor degradation; CME, on the other hand, primarily leads to EGFR recycling and sustained signaling, although a minority of receptors is also delivered to degradation through this pathway.

Recent proteomic and imaging studies have revealed a complex molecular portrait of CME with more than 30 proteins involved in the assembly and maturation of clathrin-coated pits. The involvement of such a wealth of proteins in CME – together with the large variety of endocytic sorting signals – raises the possibility of the existence of distinct types of clathrin-coated pits, specialized in terms of cargo-selection and intracellular fate.

To investigate this possibility, we carried out a complete characterization of ligand-induced EGFR internalization and signaling, upon RNA interference of different endocytic adaptor proteins. Molecular genetics and biochemical assays were employed in this study allowing a comprehensive analysis of EGFR endocytosis. Our results indicate the existence of specialized clathrin adaptors involved in EGFR internalization, namely eps15, eps15R and epsin1, which function in parallel with AP2, previously considered to be the exclusive clathrin adaptor. Importantly, our data show that this molecular heterogeneity of EGFR clathrin vesicles ultimately impacts on EGF-dependent signaling and biological response. On the mechanistic level, we found that monoubiquitination of these adaptors is absolutely required for AP2-independent EGFR internalization through CME. Importantly, this is the first demonstration of a positive role of monoubiquitination in early EGFR internalization events.

INTRODUCTION

1. At the cell's gates: entry portals and endocytic routes

Endocytosis is the process of internalization of plasma membrane (PM) proteins and extracellular materials. It is tightly linked with almost all aspects of cellular signaling. This led to the notion that endocytosis is actually the master organizer of cellular signaling, providing the cell with understandable messages, resolved in space and time. In essence, endocytosis provides the communications and supply routes (the logistics) of the cell [**Figure 1**; (Le Roy and Wrana, 2005; Platta and Stenmark, 2011; Sigismund et al., 2012)].

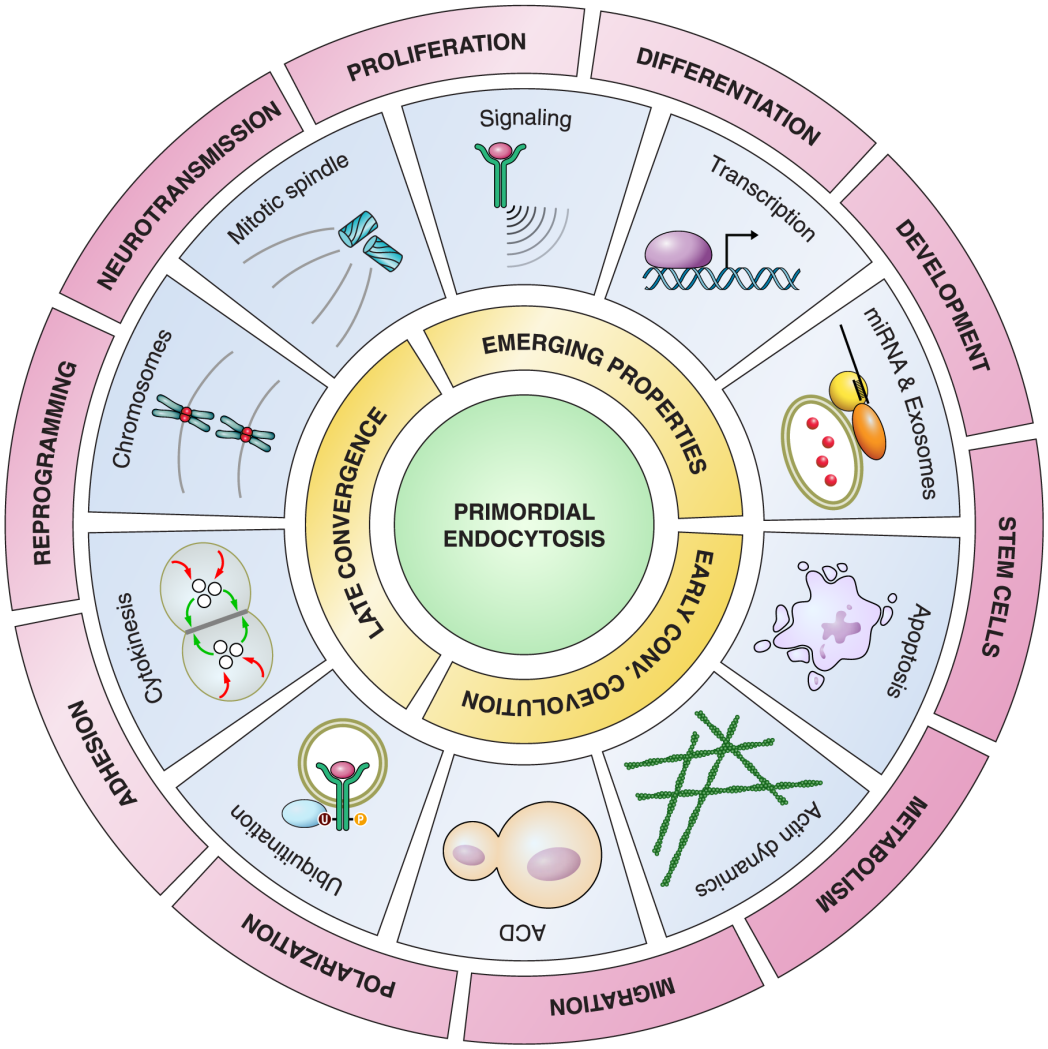


Figure 1: The endocytic matrix.
A conceptual drawing is displayed. Starting from the primordial functions of endocytosis, connected with competition for food, a series of additional functions became associated with the endomembrane system during evolution [taken from (Sigismund et al., 2012)].

The vastness of the impact of endocytosis on cellular homeostasis is revealed through the intricacy of entry portals and the subsequent modalities of cargo sorting in intracellular membranous compartments. Cellular and molecular biologists are still trying to understand the mechanisms that regulate endocytosis. This is a very difficult task to achieve because of: (i) a lack of generally accepted markers/inhibitors to elucidate the pathways, (ii) technical limitations such as imaging resolution and toxicity/cell homeostasis perturbation, and (iii) cross-talk between the different pathways.

In **Figure 2**, the main endocytic mechanisms are depicted together with the intra-cellular fate of internalized materials. All of these mechanisms generally share four fundamental steps: (i) specific binding event at the cell surface; (ii) PM budding and pinching off; (iii) tethering of the resulting trafficking vesicle and finally (iv) trafficking of the vesicle to a specific subcellular organelle.

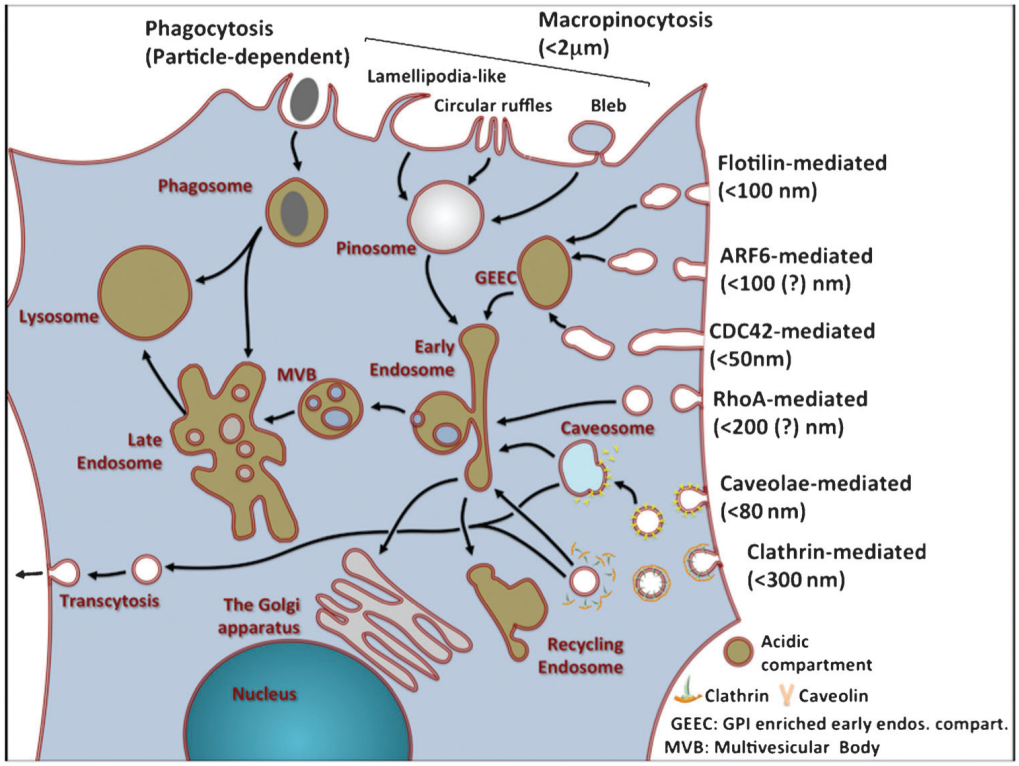


Figure 2: **Mechanisms of extracellular uptake by endocytosis in a typical eukaryotic cell.**
The known pathways of endocytosis are shown together with their intracellular trafficking route [taken from (Canton and Battaglia, 2012)].

1.1 Different entry routes

Because endocytosis is important to many cellular functions, a variety of routes have evolved to accomplish these tasks (Howes et al., 2010). The complexity of the system kicks off at the PM where multiple entry portals have been described (**Table 1**). The traditional classification divides uptaken cargoes by size. Large particles (about 500 nm) are taken up by phagocytosis, as is generally the case for bacteria or for apoptotic cells. This type of endocytosis is typical of only few specialized cells. Fluid uptake occurs by macropinocytosis and is ubiquitous to almost all eukaryotic cells. Both processes involve large rearrangements of the PM guided by actin cytoskeleton remodeling, and coordinated by Rho-GTPases (Swanson, 2008). Micropinocytic events are instead characterized by smaller invaginations (200 nm) and include clathrin-mediated endocytosis (CME) and non-clathrin endocytosis (NCE). The two following chapters of this thesis' introduction are entirely dedicated to CME. CME is the best described endocytic pathway. Compared with CME, the current picture of NCE is at a lower level of resolution. The term NCE is used to refer to a heterogeneous group of pathways that share the common property of being insensitive to clathrin depletion, but that frequently depend on cholesterol-rich PM microdomains, thus being sensitive to pharmacological cholesterol depletion. While knowledge of the molecular machinery of NCE(s) is still somewhat limited, attempts to classify these pathways rely on three major criteria (**Table 1**): 1) dependency on dynamin for vesicle release; 2) presence of "coatlike" proteins involved in membrane curvature and stabilization, such as caveolins or flotillins, in the case of caveolae-mediated or flotillin-mediated internalization, respectively; and 3) dependency on small GTPases, which control the entry of specific cargoes such as interleukin (IL)-2Rβ (dependent on RHO-A), major histocompatibility complex (MHC)-I (dependent on ARF6), or fluid-phase markers, which enter through the so-called CLIC/GEEC pathway (CDC42 and GRAF-1 dependent).

Pathway	Morphology and size	Coat	Dynamin dependence	Small GTPase involved	Internalized cargoes	Associated/regulatory proteins
Phagocytosis	Cargo shaped > 500 nm	None	No	RAC1/RHOA/ CDC42	Pathogens, apoptotic cells, FcRs	Actin, ARP2/3; Formins; PI3K; WASP; WAVE2; amphiphysin; coronin; others
Macro-pinocytosis	Ruffled 0.2-10 μm	None	In some cases	RAC1, CDCD42, ARF6, RAB5	RTKs; fluids, some bacteria	Actin, ARP2/3, cortactin; PI3K; SRC; PAK1; RAS, CTBP1/BARS; others
Clathrin-mediated	Vesicular 150-200 nm	Clathrin	Yes	RAB5	RTKs; GPCR; TfR; some toxins; bacteria; viruses	AP-2, EPSINS, EPS15, intersectin, amphiphysin (plus many others, >50)
Caveolae-mediated	Flask-shaped 50-120 nm	Caveolin 1 and 2	Yes	Not clear	GPI-linked proteins; CTxB; SV40	PTRF/cavin; SRC, SDPR; SRBC
CLIC/GEEC	Tubular	None	No	CDC42, ARF1	Fluids, bulk membrane, GPI-linked proteins	Actin; GRAF1; ARHGAP10
IL2Rβ	Vesicular 50-100 nm	None	Yes	RHOA, RAC1	IL2Rβ; γc-cytokine receptor	PAK1 and 2, cortactin, N-WASP
Arf6-dependent	Tubular	None	None as yet	ARF6	MHC I; MHC II; CD59; CD55, GLUT1	None as yet
Flotillin-dependent	Vesicular	Flotillin 1 and 2	No	None	CTxB, CD59, proteoglycans	None as yet

Table 1: Internalization pathways.
The known pathways of endocytosis are shown together with morphology and the size of the internalizing membrane structure, the coat involved, the dependence of the pathway on dynamin, the involvement of GTPases, the type of cargo internalized, and the known associated molecular machinery (CLIC, clathrin-independent carriers; GEEC, GPI-AP enriched early endosomal compartment; RTK, receptor tyrosine kinase; GPCR, G protein-coupled receptor; TfR, transferrin receptor; CTxB, cholera toxin B; SV40, simian virus 40; GPI, glycosylphosphatidylinositol; MHC, major histocompatibility complex; PTRF, polymerase I and transcript release factor; SDPR, serum deprivation response; SRBC, SDR-related gene product that binds to c-kinase; CtBP1, C-terminal binding protein 1).

1.2 Endocytic compartments

Following the complete detachment from the PM, the resulting vesicle, referred to as pinosome for macropinocytosis, phagosome for phagocytosis, and trafficking vesicle for the other mechanisms, delivers its cargo to other subcellular compartments. The endocytic pathway is a spatiotemporal succession of different compartments, which continuously interchange their content while undergoing structural transformation and functional makeover (**Figure 3**). How this is achieved is still a matter of debate and different models have been proposed. Most probably, the progressive maturation, with acquisition/loss of different markers, combined to vesicular transport and fusion between compartments (“kiss and run” theory) is the real scenario, in a succession and synergy yet to be discovered.

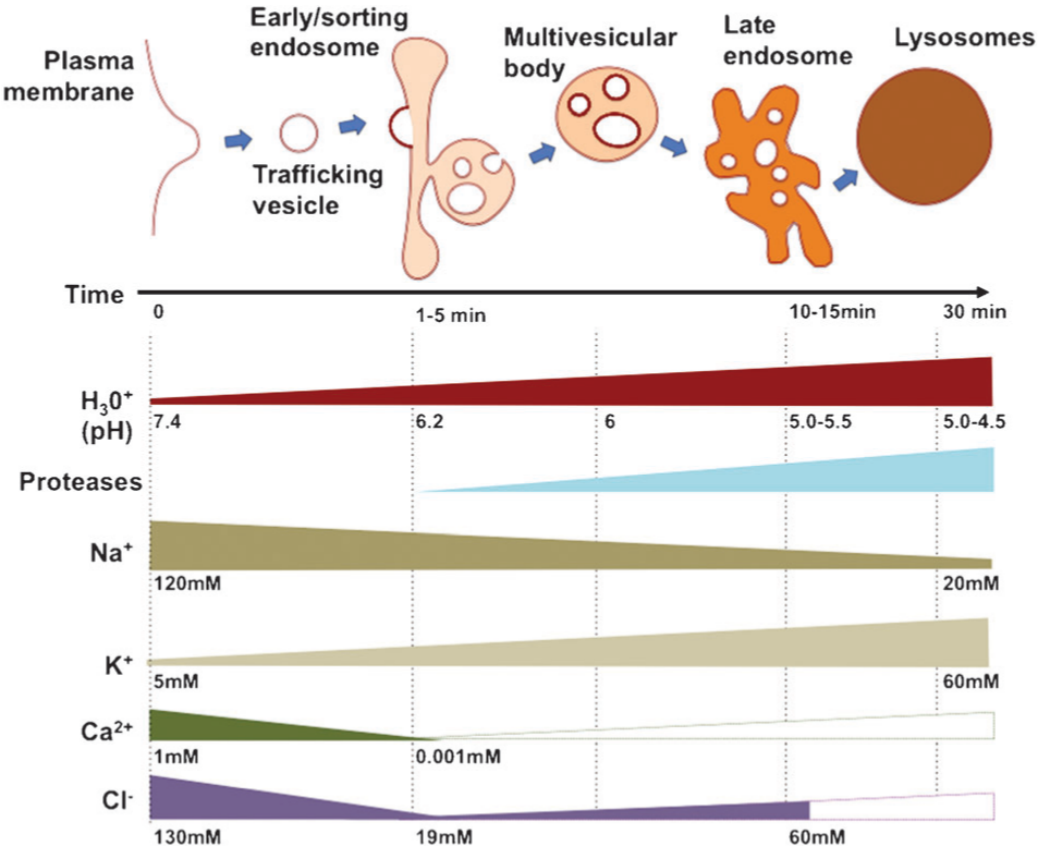


Figure 3: Endocytic compartments.
Schematic representation of the different endocytic compartments involved in the delivery of material from the plasma membrane to lysosomes, i.e., trafficking vesicles, early endosomes, multivesicular bodies, late endosomes and finally lysosomes. The time that the endocytosed material takes, from its entry into the cell, to reach each organelle is plotted alongside the organelle's internal pH, protease content and concentration of several ions [taken from (Canton and Battaglia, 2012)].

Each entry mechanism is related to a specific cellular function. For example, material that is internalized by phagocytosis involves large volumes of membrane and requires prompt and effective processing. Thus, the resulting phagosomes are directly fused with lysosomes to accelerate the degradative process (Gagnon et al., 2002; Jutras and Desjardins, 2005). Most of the other entry mechanisms regulate cargo degradation as well as receptor recycling. The internalized material is therefore transported to coordinating stations (the early endosomes, EEs), where it is sorted for degradation or recycling back to the PM.

The EEs are responsible for: (i) ensuring that housekeeping receptors are recycled back to the PM (directly or indirectly via recycling endosomes); (ii) sorting material towards the trans-Golgi network (TGN); and (iii) shuttling receptors and internalized

materials, which require downregulation/degradation, to the late endosomes (LEs). The EE structure is directly correlated to its function. The tubules regulate communication with the TGN and the recycling endosomes, as well as the fusion with the trafficking vesicles. Instead, the multivesicular parts bud off to form multivesicular bodies (MVBs). Endosomal membrane rearrangements are regulated by the ‘‘endosomal-sorting complex required for transport’’ (ESCRT). These complexes, on the one hand, drive membrane fission from the cytosolic side of the EE membrane stabilizing the resulting vesicle bud necks, and on the other hand, recognize the ubiquitinated cargoes and target them for degradation [see below; (Henne et al., 2011; Raiborg and Stenmark, 2009)].

The EE membrane starts to bud off towards the inner side forming intraluminal vesicles (ILVs) containing factors and proteins that need to be degraded in lysosomes (Bache et al., 2003; Raiborg et al., 2006). These ILVs are believed to evolve into globular MVBs (Hurley et al., 2010). MVBs rapidly acidify their lumen to approximately pH 5.5. LEs are more acidic than EEs (pH 5.5–5.0) and they also receive newly synthesized endo-lysosomal proteins from the TGN en route to lysosomes. LEs are characterized by low levels of endocytosed recycling proteins and high levels of endocytosed proteins destined for degradation. LEs have also a pleomorphic architecture that comprises tubular and multivesicular areas rich in ILVs. Each of these regions has a different protein and lipid composition, which allows further sorting of the internalized material (Russell et al., 2006). Finally, after approximately 30 minutes from its entry into the cell, the internalized material reaches the terminal station of the endocytic pathway: the lysosome.

At the molecular level, small GTPases play an essential role for sorting cargos along the endosomal stations. Two main classes of molecules are involved: the Rab (Behnia and Munro, 2005; Galvez et al., 2012; Miaczynska and Zerial, 2002; Stenmark, 2012) and the Arf families of small GTPases (D'Souza-Schorey and Chavrier, 2006). These proteins act as molecular switches that can alternate between a GTP-bound active form and a GDP-bound inactive form. The latter is cytosolic, whereas the active form is associated with membranes. Rab5 regulates clathrin-coated vesicle (CCV)-mediated transport from the PM to the EE (Behnia and Munro, 2005), from which cargos can be

recycled back to the PM through either a fast Rab4-dependent or a slow Rab8/Rab11-dependent recycling route (Stenmark, 2009). In other cases, cargos are destined to degradation in lysosomes via sorting through MVBs and LEs. This route depends on Rab7 (Stenmark, 2009).

Cargo ubiquitination is the key signal to enter this latter pathway, because several proteins harboring ubiquitin (Ub)-binding domains recognize ubiquitinated cargos and lead them along the degradative route. These are ESCRT complexes that act sequentially (Hurley and Hanson, 2010; Raiborg and Stenmark, 2009):

(1) ESCRT-0 acts at the level of the EEs and is composed of two interacting proteins Hrs and STAM;

(2) ESCRT-I is a heterotetramer of Vps23, Vps28, Vps37 and Mvb12 and acts at the MVB membrane;

(3) ESCRT-II is a heterotetramer composed of one molecule of Vps22, one molecule of Vps36, and two molecules of Vps25. It also acts at the MVB membrane;

(4) ESCRT-III, unlike other ESCRTs, which are stable complexes, is a dynamic polymer of ESCRT-III proteins that does not have a clearly defined or unique composition. It also acts at the MVB membrane.

ESCRT-I, ESCRT-II and ESCRT-III complexes are required for the formation of ILVs in MVBs, achieved by involution of the inner membrane. They are also involved in the recruitment of enzymes that deubiquitylate receptors before they are packaged into ILVs.

2. Clathrin-mediated endocytosis

Among the different forms of endocytosis, the clathrin-mediated pathway has been most extensively studied (McMahon and Boucrot, 2011). CME is ubiquitous to all eukaryotic cells and involves the internalization of PM proteins into the cell via CCVs. Although CCVs can also be formed from other membranous compartments (McGough and Cullen, 2011; Radulescu et al., 2007), the term CME is used to refer only to intake through vesicles formed from the PM.

CCVs participate in the endocytosis of a wide variety of molecules including nutrient-receptor complexes, membrane transporters, adhesion molecules, and signaling receptors. CCVs are also involved in the recycling of synaptic vesicles. Cell-surface receptors, including the transferrin receptor [TfR; (Hopkins, 1983; Hopkins and Trowbridge, 1983)], the low-density lipoprotein receptor [LDLR; (Anderson et al., 1976)], α2 macroglobulin (Willingham et al., 1979), the epidermal growth factor receptor [EGFR; (Gorden et al., 1978)], the insulin receptor (Fan et al., 1982), immunoglobulin G (Rodewald, 1973), G-protein coupled receptors [GPCRs; (Chuang et al., 1986; Silva et al., 1986)] and the Met receptor (Naka et al., 1993) are some examples of cargos concentrated in CCVs. When cargo has been taken up, it is sorted in endosomes and either sent back to the cell surface through recycling vesicles or targeted to degradative compartments, such as MVBs and lysosomes (Grant and Donaldson, 2009; Huotari and Helenius, 2011; Luzio et al., 2009).

In keeping with cargo versatility, CME performs a range of different functions. These include: regulating the surface expression of proteins; sampling extracellular environment for growth and guidance cues; bringing nutrients into cells; controlling the activation of signaling pathways; retrieving proteins deposited after vesicle fusion and turning over membrane components by sending these components for degradation in lysosomes. Thus, defects in the formation of CCVs are implicated in diseases such as familial hypercholesterolemia, hereditary hemochromatosis, leukemia, and muscle defects (Conner and Schmid, 2003; McMahon and Boucrot, 2011). Moreover, CME is also exploited by pathogens, such as toxins, bacteria and viruses to enter the cell (McMahon and Boucrot, 2011).

2.1 The clathrin coat

CCVs were first detected in 1964 by Roth and Porter (Roth and Porter, 1964) when they observed that deposition of protein yolk in mosquito oocytes correlated with a 15-fold increase in PM depressions with a coat on their cytoplasmic side. These depressions, later named clathrin-coated pits (CCPs), are the precursors of CCVs. About a decade

later Barbara Pearse developed a subcellular fractionation procedure to isolate CCVs from pig brain revealing a protein of 180 KDa as the major protein component, which she named clathrin (Pearse, 1976). Subsequent studies demonstrated that clathrin isolated from the brain is composed of three copies of the clathrin heavy chain (CHC) assembled into a three-legged, curled pinwheel structure, referred to as a clathrin triskelion, which is the assembly subunit of a clathrin coat [**Figure 4** (Kirchhausen and Harrison, 1981; Ungewickell and Branton, 1981)].

Nowadays, we know that each triskelion is composed of three CHCs with a mass of 180 kDa and three clathrin light chains (CLCs) with a mass of 25 kDa: the three CHCs provide the structural backbone of the clathrin lattice, while the three CLCs are thought to regulate the formation and disassembly of the clathrin lattice. Two kinds of CLCs have been identified in vertebrates, CLCa and CLCb (Brodsky et al., 2001). They have both been shown to be not essential in the process of vesicle formation since upon their concomitant double depletion neither TfR, nor EGFR internalization are affected (Huang et al., 2004). Very recently, however, small interfering RNA (siRNA)-mediated knockdown (KD) of either CLCa or CLCb have been shown to specifically inhibit the uptake of GPCRs (Ferreira, Foley et al. 2012). Moreover, it has been demonstrated that phosphorylation of CLCb is required for efficient endocytosis of a subset of GPCRs (Ferreira et al., 2012).

The CHC is an invariant polypeptide with functionally distinct regions (**Figure 4**). The globular N-terminal domain (NTD) contains the binding sites for endocytic adaptors (see below). This NTD is linked via the ankle region to a distal and a proximal leg, connected by a region behaving like a knee. The proximal leg portion contains the binding site for the light chain. The hub region consists of the three C-terminal segments of the heavy chains, belonging to the three monomers that participate in triskelion formation (Fotin et al., 2004b).

Figure 4: The clathrin coat.
A. Schematic representation of a clathrin triskelion, which highlights the various domains of the clathrin heavy chain in different colors (see key) and the position of the clathrin light chains inside the triskelion. **B.** Clathrin-cage reconstructions using cryo-EM. Three single triskelions are highlighted in different colors [adapted from (Edeling *et al.*, 2006)].

2.2 Life, death and miracles of the clathrin-coated vesicle

CCV formation starts as a shallow invagination called a CCP. The magnitude of the CME process can be estimated by weighting against the amount of cell membrane occupied by the CCPs, approximately 2% (Goldstein et al., 1979; Griffiths et al., 1989). The rate of PM internalization via CME ranges between 1-5% per minute (Bretscher, 1984).

CCV formation proceeds through five stages that correspond to ultrastructural and cell biological observations: nucleation, cargo selection, coat assembly, scission and uncoating (**Figure 5**).

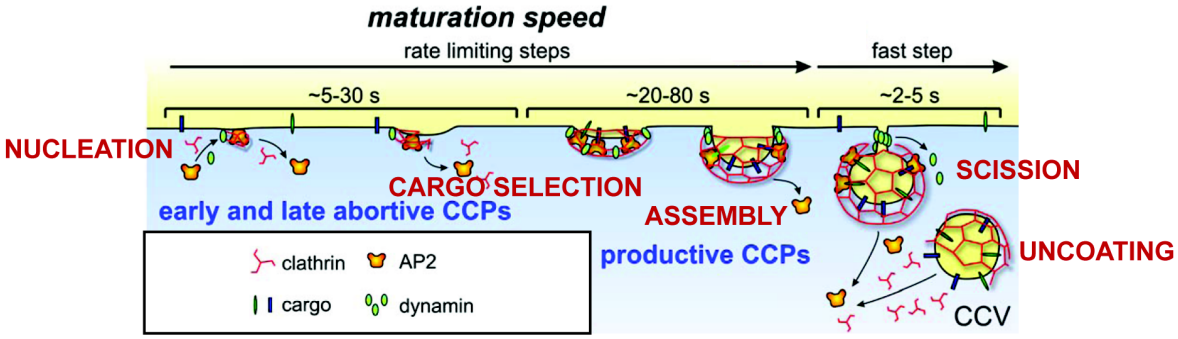


Figure 5: Schematic representation of clathrin-coated vesicle formation.
Clathrin-coated vesicles (CCVs) are formed from productive clathrin-coated pits (CCPs) that have undergone the maturation process, which involves nucleation, cargo selection, clathrin-coat assembly, vesicle scission, and vesicle uncoating (see main text for details). An endocytosis checkpoint, dependent on the concentration of cargo, AP2 adaptors, and likely other factors, controls progress through the maturation process. CCPs that do not progress beyond this restriction point abort by sequential disassembly of AP2 and clathrin [adapted from (Loerke et al., 2009)].

Each of these steps has been extensively characterized in terms of dynamics and biochemical actors [**Figure 6** (Mettlen et al., 2009a; Taylor et al., 2011; Traub, 2011)]. Indeed, this maturation process involves a complex hierarchy of molecular events (i.e., spatial/temporal cues and cargo-dependent accessory factors) and is governed by a checkpoint system that determines pit fate between progressive and abortive states.

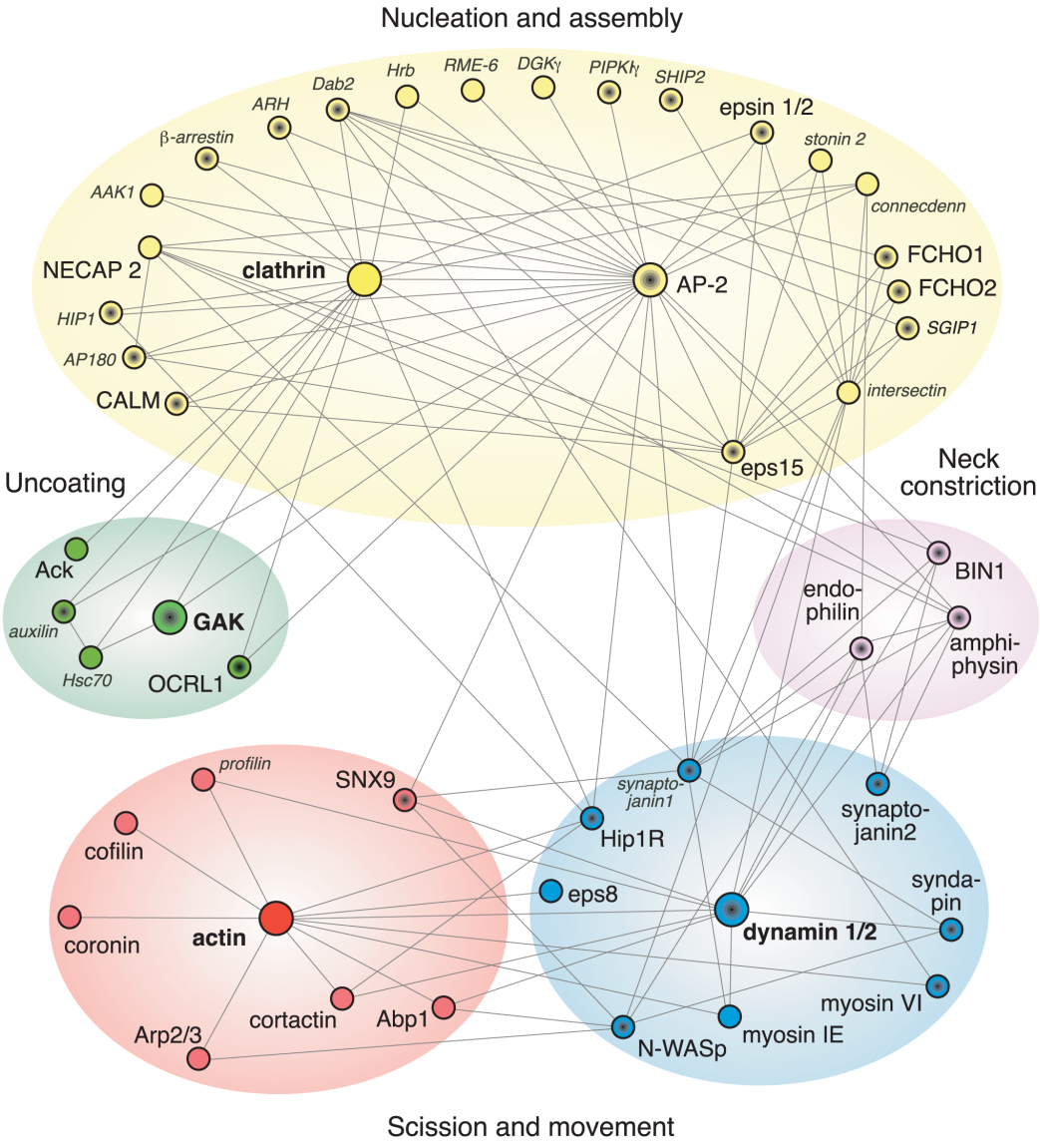


Figure 6: The endocytic clathrin-coat interaction network.
Hub-and-spoke depiction of a selected subset of the known proteins participating in clathrin-mediated endocytosis. Established interactions are indicated by the spokes. The symbols with black centers indicate proteins that bind to phosphatidylinositol 4,5-bisphosphate, a lipid marker of the PM [taken from (Traub, 2011)].

Following cargo selection and initiation of pit formation, soluble clathrin triskelia polymerize into hexagons and pentagons, like a soccer ball (**Figure 4B**), the relative ratio of which accommodates a wide range of membrane curvatures. Clathrin does not bind directly to the membrane or to cargo receptors and thus relies on adaptor proteins and accessory proteins to be recruited to the PM (see dedicated chapter “Clathrin adaptors”). Similarly to clathrin, the adaptor/accessory proteins are cytoplasmic proteins that are recruited to sites of vesicle budding. Following vesicle formation, these proteins together with clathrin are released back into the cytoplasm for reuse in another cycle of endocytosis, as depicted by adaptor protein 2 (AP2) in **Figure 5**.

2.2.1 Nucleation

Morphologically, the first stage of vesicle budding involves the formation of a membrane invagination called a pit. The nucleation of productive CCPs at the PM appears to be a stochastic process (Banerjee et al., 2012; Ehrlich et al., 2004). CCP initiation was traditionally thought to be triggered by the recruitment of the highly conserved adaptor protein AP2 to the PM (**Figure 5**). AP2 recruitment is mediated by its binding to endocytic motifs present in cytoplasmic tails of receptors (Ohno et al., 1995) and to the PM-specific lipid, phosphatidylinositol-4,5-bisphosphate [PIP2; (Ohno et al., 1995)].

Recent studies in yeast and mammalian cells, however, indicate that the initiation stage may involve the formation of a putative nucleation module that defines the sites on the PM where clathrin will be recruited and vesicles will bud (Henne et al., 2010; Stimpson et al., 2009). This putative nucleation module is thought to assemble only at the PM because of a preference for PIP2 and includes FCH (F-BAR domain-containing Fer/Cip4 homology) domain only (FCHo) proteins, EGFR pathway substrate 15 (eps15) and intersectins. The nucleation module is thought to be required for CCP formation, as depletion of either FCHo proteins or eps15 and intersectins has been shown to inhibit clathrin coat recruitment (Stimpson, Toret et al. 2009; Henne, Boucrot et al. 2010). The F-BAR domain of FCHo proteins can bind to very low curvatures, and its membrane-

bending activity is required for progression of the CCPs, suggesting a need for membrane curvature generation even before clathrin recruitment.

The involvement of the nucleation module in the CCP formation, however, appears to apply mostly to constitutive endocytosis (see below "Cargo selection"), as it has recently been shown that FCHo is dispensable for ligand-induced CME, for example of the EGFR [(Uezu et al., 2011), see also Results section "1.4 Differential requirement of Grb2, FCHo 1/2 and intersectin 1/2 in EGFR and TfR internalization"]. This result could be explained if other factors can substitute FCHo in ligand-induced CME (Merrifield, 2012; Umasankar et al., 2012), or if the nucleation step is not always necessary for CME (see below "Preexisting and *de novo* pits"). Moreover, a more recent work showed that FCHo1/2 are not essential for CCVs initiation; instead, they are required for sustained CCVs growth (Cocucci et al., 2012).

Starting from nucleation and all along the process of vesicle formation, proteins that act as membrane curvature sensors and generators play a master role (Shen et al., 2012). Proteins that initiate curvature by inserting a wedge in the bilayer [for example, amphipathic helices (e.g., epsin and endophilin) and hydrophobic loops (e.g., synaptotagmin 1)] will bind preferentially to a precurved bilayer, where bending has created a gap in lipid packing. Likewise, proteins that function primarily as curved scaffolds (e.g., amphiphysin, FBP17/TOCA1/CIP4 and FCHo) generally assemble into polymers that propagate curvature, and their binding to the bilayer and their polymerization are both facilitated by a precurved surface (Shen et al., 2012). Protein-protein crowding has also been showed to function in membrane bending (Stachowiak et al., 2012).

2.2.2 Cargo selection

Several receptor types are endocytosed constitutively (i.e., with or without ligands bound) via CME from the PM to endosomes, and a major portion of these is recycled back to the PM within minutes. TfR is the classic example of such a receptor (Hopkins et al., 1985). LDLR is also reported to traffic constitutively through CME (Anderson et al., 1977).

Indeed, a significant amount of data collected on the mechanism of CCV formation refers to constitutive endocytosis (Taylor et al., 2011). Many receptors, however, including receptor tyrosine kinases (RTKs, such as EGFR) and GPCRs (e.g., β2 adrenergic receptor), undergo stimulated, or ligand-induced, endocytosis. Internalization via ligand-induced CME depends on the unmasking of signals in the receptors or on posttranslational modifications. Indeed, binding of the cognate ligands induces dimerization (in the case of RTKs) or a change in conformation (in the case of GPCRs) of the receptors, as well as posttranslational modifications, which are necessary for their recruitment by adaptors to CCPs.

Cargo capture plays a fundamental role in CCV maturation. Indeed, cargo recruitment acts as a sort of checkpoint in allowing the progression of vesicle formation (**Figure 5**). In the absence of cargo, the process stops, giving rise to the so-called abortive pits, which last not more than 20-30 seconds and can represent up to 50% of total clathrin events, depending on the cell line (Ehrlich et al., 2004; Mettlen et al., 2009). If cargo does not enter the pit, the local concentration of membrane PIP2 becomes insufficient to stabilize the growing network of clathrin and other adaptor/accessory proteins, leading to the dissolution of the coated pit prior to an endocytic event (Ehrlich et al., 2004; Loerke et al., 2009). This allows the cell to save energy.

Although it is known that different cargos can be transported via the same CCP, it has been proposed that specialized CCPs exist, specific for particular cargos (Keyel et al., 2006; Lakadamyali et al., 2006; Leonard et al., 2008; Mundell et al., 2006; Puthenveedu and von Zastrow, 2006). Indeed, the pathway is versatile, as many different cargoes can be packaged using a range of adaptor proteins. This issue will be discussed in depth in the section “Clathrin adaptors”.

2.2.3 Clathrin coat assembly

As cargo is selected and bound by adaptor proteins, the clathrin coat has to be assembled (**Figure 5**). Clathrin triskelia are recruited directly from the cytosol to sites of adaptor concentration on the membrane. Polymerization of clathrin results in stabilization of membrane curvature and displacement of cargo, accessory/adaptor proteins (e.g.,

eps15 and epsin) and curvature effectors, to the edge of the forming vesicle (Saffarian et al., 2009; Tebar et al., 1996). It had been assumed that clathrin polymerization could mediate membrane bending as the coated pit invaginates (Dannhauser and Ungewickell, 2012; Hinrichsen et al., 2006). Accordingly, in clathrin-deficient fibroblasts PM areas coated with AP2 and other endocytic proteins are visible, but do not appear to be invaginated (Hinrichsen et al., 2006). However, because clathrin binds to the flexible region of most adaptor proteins, the potential force generated by polymerization would be inefficiently transmitted to the PM, and thus insufficient to deform the membrane. Instead, direct membrane interactions of curvature effectors are now thought to sculpt the vesicle.

In some cell types, a substantial pool of clathrin can also be found as flat lattices, in which triskelia are arranged as hexagons only (Heuser, 1980). These lattices, also referred to as clathrin plaques, are mostly found on the cytoplasmic side of the adherent membrane surface of a subset of cells and have a slower turnover than CCPs (Saffarian et al., 2009). Their exact function is still under investigation.

2.2.4 Vesicle scission

The scission of a fully invaginated CCP to form a CCV is mediated by the 100-kDa GTPase, dynamin [(Kosaka and Ikeda, 1983) **Figure 5**]. Three dynamin isoforms have been found in mammalian cells: dynamin-1, dynamin-2 and dynamin-3. Dynamin-1 is neuron-specific (Ferguson et al., 2007), dynamin-2 is ubiquitously expressed (Ferguson et al., 2007; Praefcke and McMahon, 2004) and dynamin-3 is predominantly expressed in testis and, to lesser extent, in neurons (Cao et al., 1998; Ferguson and De Camilli, 2012; Raimondi et al., 2011).

Dynamin is recruited by N-BAR (Bin-Amphiphysin-Rvs) domain-containing proteins (Ferguson et al., 2009; Sundborger et al., 2011), which have a preference for the curvature of the vesicle neck and likely assist in its formation. Examples of such N-BAR domain-containing proteins are amphiphysin, endophilin and sorting nexin 9 (SNX9), which have Src homology 3 (SH3) domains that bind the proline-rich domain of dynamin.

Polymerization of dynamin around the neck of the nascent vesicle favors GTP

hydrolysis and consequently membrane fission (Hinshaw and Schmid, 1995; Sweitzer and Hinshaw, 1998). The precise scission mechanism is still not completely clear, although it is known that dynamin undergoes a GTP hydrolysis-dependent conformational change that likely helps to mediate scission (Bashkirov et al., 2008; Roux et al., 2006). Inhibition of dynamin recruitment or activity, arrests vesicle formation at the stage of clathrin coat assembly or vesicle scission (Macia et al., 2006; van der Bliek et al., 1993). Dynamin is also found in many other vesicle-budding pathways, in which it is recruited by a different subset of interaction partners. However, the essential activity of dynamin in CME has recently been challenged (Boucrot et al., 2012). Boucrot and colleagues investigated membrane fission by shallow hydrophobic insertions and provided evidence that membrane insertion of the ENTH domain of epsin leads to liposome vesiculation, and that epsin is required for CCV budding in cells. Nevertheless, while the existence of this alternative scission mechanism has been clearly demonstrated *in vitro*, its relevance *in vivo* still needs to be elucidated.

2.2.5 Uncoating and clathrin component recycling

Once the newly formed CCV is detached from the membrane, the clathrin coat is disassembled by the ATPase heat-shock cognate 70 protein (HSC70) and its cofactor, auxilin [or cyclin G-associated kinase (GAK) in non-neuronal tissues; (Schlossman et al., 1984; Ungewickell et al., 1995)], allowing the detached and uncoated vesicle to travel to and fuse with its target endosome. Auxilin is recruited after CCV budding (Massol et al., 2006; Taylor et al., 2011) by binding to the terminal domains and ankles of clathrin triskelia (Fotin et al., 2004a). Notably, when CCV scission takes place, it is unlikely that the clathrin cage is completed across the zone where the neck was attached, thus leaving a defect in the clathrin cage that allows the uncoating apparatus to start the uncoating process with ease. This is why uncoating takes place only after vesicle scission, as this is the only point when a defect becomes apparent. Changes in the phosphoinositide composition of CCVs mediated by the phosphatase synaptojanin are required for uncoating (Cremona et al., 1999), but whether synaptojanin acts by facilitating auxilin

recruitment (Massol et al., 2006) or via another mechanism is unclear. Uncoating releases the clathrin machinery back into the cytoplasm to be recruited and reused in another round of CCV formation (**Figure 5**).

2.2.6 Actin involvement

CCV formation is associated with actin polymerization (Merrifield et al., 2002). Disruption of actin polymerization inhibits the formation of CCPs, internalization of CCVs, and further restricts the limited lateral mobility of CCPs (Yarar et al., 2005). The clathrin coat contains actin-binding proteins; however, CCV preparations do not contain actin or proteins that promote actin polymerization. Although none of the steps underlying CCV formation (nucleation, assembly, and scission) requires actin, sustained CCV formation grinds to a halt without an actin cytoskeleton. It is likely that the forced localization of clathrin structures due to the restricted mobility imposed by the cortical actin network aids vesicle formation by ensuring the association of all the participants necessary for vesicle formation (Boucrot et al., 2006). However, interference with actin polymerization does not seem to preclude CME. Drugs that affect actin polymerization have varying effects on endocytosis and, in some cases, have no effect [reviewed in (Engqvist-Goldstein and Drubin, 2003)]. These apparently contradictory results suggest that although endocytosis is possible without actin, efficient CME requires the presence of actin, but this is a quite controversial issue.

Taylor and colleagues have recently investigated the coordination and interdependencies between the recruitment of dynamin, the actin cytoskeleton, and N-BAR proteins to individual clathrin-mediated endocytic scission events. These measurements revealed that a feedback loop exists between dynamin and actin at sites of membrane scission. The kinetics of dynamin, actin and N-BAR protein recruitment were modulated by the GTPase activity of dynamin. Conversely, acute ablation of actin dynamics led to a ~50% decrease in the incidence of scission and in the amplitude of dynamin recruitment, and abolished actin and N-BAR recruitment to scission events.

Collectively these data suggest that dynamin, actin, and N-BAR proteins work cooperatively to efficiently catalyze membrane scission (Taylor et al., 2012).

2.2.7 Clathrin-coated vesicle heterogeneity

The regulation of clathrin-coat assembly and vesicle size *in vivo* is a matter of debate. The precise mechanism is poorly understood, but is thought to involve a complex interplay between PIP2 levels, cargo capture and sequestration, and signaling.

CCPs from different species appear to differ widely in size. The size of CCVs depends on the size of its cargo (Ehrlich et al., 2004), with an observed upper limit of ~200 nm exterior diameter comprising the coat, as in the case of virus uptake (Cureton et al., 2009). Yeasts (e.g., *Saccharomyces cerevisiae*) and plants (e.g., *Arabidopsis thaliana*) have very small coated pits of ~35-60 nm in exterior diameter (Dhonukshe et al., 2007; Smaczynska-de et al., 2010), which is considerably smaller than those found in mouse fibroblasts (Bretscher et al., 1980) or chicken oocytes (Perry and Gilbert, 1979), which are ~150 nm in diameter. This difference in size may be because plant cells and yeasts have rigid walls, thus, the need to counteract the internal pressure to deform the PM might limit the size of CCPs. The size of CCPs also varies between different cell types within the same species. For example, CCPs in rat and mouse brains are ~70-90 nm in exterior diameter, which is comparable to the pit size in lamprey synapses (Cheng et al., 2007). However, they are significantly smaller than those generally observed in mouse or human epithelial cells, which are ~120-150 nm in diameter (Cheng et al., 2007). This difference in size may be because the brain vesicles do not internalize large extracellular cargo, but simply retrieve the synaptic vesicle components.

Within the same cell, Ehrlich *et al.* observed that the size of coated pits and subsequent vesicles were dependent on the amount of loaded cargo, with coated pits persisting longer and becoming larger upon higher levels of cargo incorporation (Ehrlich et al., 2004). It was also observed that the checkpoint at which coated pits were either aborted or committed to vesiculation was largely standardized, strengthening the notion that cargo binding controls the biochemical checkpoint controlling CCP maturation. The

authors propose that the bending rigidity of the underlying membrane determines coat size by opposing curvature, and that cargo binding stabilizes the coat and allows further membrane bending.

CCVs also differ in maturation time. Expression of fluorescently tagged clathrin molecules, coupled with total internal reflection fluorescence (TIRF) microscopy, has allowed the measurement of CCV lifetime, starting from the time of appearance at the PM and ending with the uncoating and consequent disappearance of the clathrin signal. HeLa, BSC1 and COS7 cells displayed wide variation in the time between nucleation and scission (35-65 s), with a mean CCV lifetime of ~50 s. Swiss 3T3 cells displayed an even broader distribution and increased mean lifetime [~55±30 s; (Ehrlich et al., 2004; Saffarian et al., 2009)]. More in-depth analyses in BSC1 cells report a longer average CCV lifetime of ~90 s (Loerke et al., 2009; Taylor et al., 2011). This discrepancy can be explained by the existence of two CCP subpopulations, the first being represented by short-lived abortive CCPs, the second being comprised of CCVs that effectively reach maturation. Substantial lifetime heterogeneity is still present in this last subpopulation of vesicles.

Real-time imaging of the sorting of different sized cargos (LDL, Tf and rheovirus) undergoing constitutive endocytosis has indicated that the time required to assemble a CCV was proportional to the size of the cargo (Ehrlich et al., 2004). This might be an explanation for the ample range of dynamics observed. Moreover, it is known that cargo regulates CCP dynamics. For example, β-arrestin-mediated internalization of GPCRs is delayed when compared with constitutively endocytosed TfR. The prolonged surface retention of GPCRs has been attributed to receptor association with scaffolding proteins (Puthenveedu and von Zastrow, 2006). Other activated signaling receptors might display similar properties, dependent on the individual signal transduction cascades they elicit.

Another contributing factor to the broad differences in internalization kinetics may be the spatial variations in the underlying actin cortex (Liu et al., 2009). Actin has also been shown to regulate formation of so-called hot spots, selected regions of the PM where more frequent and continuous assembly and disassembly of CCVs has been recorded (Gaidarov et al., 1999).

2.2.8 Preexisting *vs. de novo* clathrin-coated pits

By analogy with the recruitment of signaling molecules to activated growth factor receptors, it has been suggested that receptor activation could also recruit the molecular machinery for *de novo* CCP assembly and their own internalization. An alternative scenario is that activated receptors are targeted to CCPs, either already formed or in the process of assembly before receptor activation (preexisting CCPs).

These two models, which remain a matter of debate, involve major differences in CCP behavior. *De novo* CCP assembly implies that receptor activation regulates the assembly machinery and that newly formed CCPs are probably restricted to the stimulated receptor, possibly bypassing the nucleation process. The preexisting model implies that receptor activation controls cargo recruitment to CCPs, but not CCP assembly, and suggests that activated receptors share CCPs with cargos taken up by constitutive endocytosis. However, also in this latter model cargo could influence subsequent CCP assembly by modifying adaptor recruitment and vesicle retention (Puthenveedu and von Zastrow, 2006).

Most of the support in favor of the *de novo* model of CCP formation comes from studies on EGFR endocytosis. An initial study by the group of Brodsky reported that at high EGF doses, rapid tyrosine phosphorylation of the CHC occurred in a Src-dependent manner, resulting in the massive recruitment of clathrin to the PM (Wilde et al., 1999). This hypothesis was further confirmed by an electron microscopy analysis, which showed that the number of CCPs doubled in cells stimulated by EGF (Johannessen et al., 2006; Puri et al., 2005).

A few other receptors can also induce assembly of their own endocytic structures. One example is the nerve growth factor receptor (NGFR). Early studies indicated that treatment with NGF increased the total number of CCPs present at the PM (Connolly et al., 1981, 1984). However, in contrast to what has been observed for EGF, NGF treatment also stimulates TfR internalization (Beattie et al., 2000) suggesting that the NGF-induced CCPs do not differ from the CCPs involved in constitutive endocytosis. An increase in clathrin recruitment at the PM and/or in tyrosine phosphorylation of the CHC has also

been described for the insulin receptor (Corvera, 1990; Corvera and Capocasale, 1990) and for the B-cell and T-cell receptors (Crotzer et al., 2004; Stoddart et al., 2002).

Although studies in fixed cells indicate that ligands may promote recruitment of clathrin, live-cell imaging is needed to determine if there is indeed an increased formation of endocytic clathrin-coated structures or just an accumulation of CCPs/altered dynamics due to altered signaling of the preexisting CCPs. Indeed, live-cell imaging has recently been applied to Shiga Toxin, revealing that it increases the formation of CCPs (Utskarpen et al., 2010).

3. Clathrin adaptors

The variety of transmembrane cargos concentrated into CCVs at the PM requires the use of diverse sorting signals (Traub, 2009), and diverse adaptors to recognize them. This variety prevents competition for entry and allows plasticity in the selection of cargo for internalization.

The term endocytic adaptor was coined in 1981 (Pearse and Bretscher, 1981) to describe a yet-to-be-identified group of proteins that mediate the interactions between 'address tickets' on cargo proteins and clathrin, as clathrin cannot bind directly to cargo or membranes. At the time, it was hypothesized that these adaptors have three characteristics: i) they interact with clathrin; ii) they recognize cargo 'address tickets'; iii) possibly contain signals that direct them towards the desired organelle. Nowadays, we know that endocytic adaptor proteins vary greatly in size (~300-3000 amino acids) and structure, but possess similar properties. Most of the clathrin adaptor proteins contain regions that interact with some or all of the four types of binding partner: lipids, cargo, clathrin and accessory proteins [**Figure 7** and **Table 2;** (Owen et al., 2004; Reider and Wendland, 2011)]. Cooperation between these interactions is required for efficient recruitment of adaptors to the PM and is crucial for progression of the internalization process.

Endocytic adaptors are divided into two main groups: multimeric adaptor proteins (e.g., AP2) and monomeric or non-classic adaptor proteins – the clathrin-associated

sorting proteins (CLASPs; **Table 2**). The characterization of adaptor proteins began with the purification and identification of HA-2 (now known as AP2) from CCVs in 1984 (Pearse and Robinson, 1984).

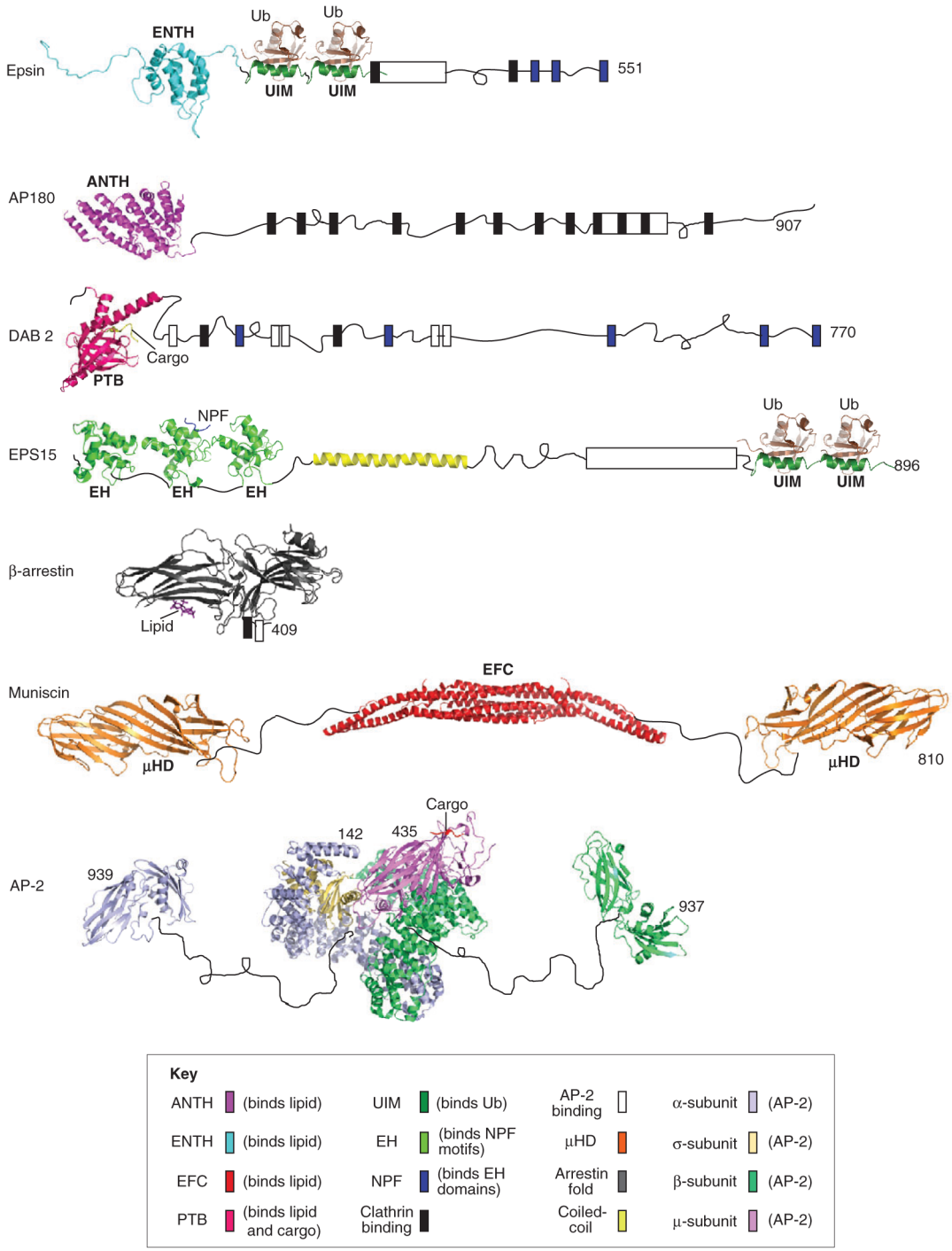


Figure 7: Representation of the overall domain and motif organization of human adaptor proteins.
Multimeric and monomeric adaptor proteins consist of folded domains (represented by atomic structures), binding motifs (colored boxes), and relatively unstructured regions [represented by thin black curved lines; taken from (Reider and Wendland, 2011)].

Adaptor protein	Lipid binding	Cargo binding	Clathrin binding	Accessory protein binding	Adaptor type	References
Mammalian adaptor proteins						
AP-2	+	+	+	+	Heterotetramer	(Jackson et al., 2010; Owen et al., 2004)
Epsin1, epsin2	+	+	+	+	CLASP	(Chen et al., 1998; Itoh et al., 2001; Overstreet et al., 2004)
ARH, NUMB, DAB2	+	+	+	+	CLASP	(Gallagher et al., 2004; Keyel et al., 2006; Mishra et al., 2002a; Mishra et al., 2002b)
β-arrestin	+	+	+	+	CLASP	(Gurevich and Gurevich, 2006)
AP180/CALM	+		+	+	CLASP	(Ford et al., 2001)
Stonin2		+		+	CLASP	(Martina et al., 2001; Walther et al., 2004)
Eps15		+		+	CLASP	(Benmerah et al., 1996)

Table 2: Endocytic adaptor proteins and their interactions.
The table shows with + the ability of the indicated adaptors to bind lipid, cargo, clathrin and accessory protein. Adaptor type is also highlighted [CLASP: clathrin-associated sorting proteins; adapted from (Reider and Wendland, 2011)].

3.1 Adaptor protein 2 (AP2)

AP2 is the most abundant non-clathrin component of endocytic vesicles purified from brain and is, consequently, the longest-studied and best-understood adaptor (Boehm and Bonifacino, 2001; Keen et al., 1979). AP2 was named “adaptor protein” because of its intrinsic ability to stimulate the assembly of clathrin triskelions *in vitro* (Zaremba and Keen, 1983). Indeed, *in vivo* AP2 plays a major role in CME as demonstrated by the recent observation that knocking down its expression using siRNAs strongly decreased (by >90%) the number of CCPs at the PM in basal condition (Hinrichsen et al., 2003; Motley et al., 2003).

The AP2 complex consists of two large subunits (α, β2), one medium subunit (μ2) and one small subunit (σ2; **Figure 7**). The large subunits can be subdivided into a trunk domain (70–75 kDa) and an appendage domain (~30 kDa), which are connected by an extended, proteolytically sensitive, flexible linker (Kirchhausen et al., 1989; Zaremba and Keen, 1985). The large subunit trunk domains plus the medium and small subunits represent the biochemically stable AP-2 ‘core’ domain. The large subunits are involved in membrane targeting, clathrin binding (clathrin-binding motif: LF[D/E]F[D/E]) and the recruitment of accessory proteins, through binding to Dx[F/W] motifs (Owen et al., 1999; Traub et al., 1999). The σ2 subunit stabilizes the complex and binds to the cargo motif [D/E]xxxL[L/I/M] together with the α subunit (Doray et al., 2007; Kelly et al., 2008). The μ2 subunit binds well-characterized lipid-binding and cargo-binding tyrosine-based motifs, such as NPxY and Yxxf, which were first identified in the LDLR and the TfR, respectively

(Owen et al., 2004), while the β2 subunit binds to dileucine motifs that are mostly found in the cytoplasmic tails of immune receptors. Of note, the EGFR contains both tyrosine-based and dileucine motifs (Goh et al., 2010). These motifs can target membrane cargo for constitutive CME, as in the case of TfR, or function upon activation of signaling receptors, such as in the case of the EGFR. Interestingly, the tyrosine-based and dileucine endocytic signals do not compete with each other for internalization (Craig et al., 1998; Marks et al., 1996) or for binding to purified AP2 complexes (Honing et al., 2005), suggesting that they are involved in distinct sorting mechanisms in CCPs, despite the fact that they are both bound by AP2. These results indicate that individual AP2 complexes can independently and simultaneously sort several different subsets of cargo through interactions involving different subunits or subunit subdomains. Through protein crystallography and other biophysical techniques, it has been demonstrated that endocytic cargo binding of AP2 is driven by a conformational change induced by its interaction with PIP2-containing membranes (Jackson et al., 2010).

It was initially thought that AP2 was the only existent cargo-sorting, clathrin endocytic adaptor protein. This simplistic view was recently challenged by the discovery that cells depleted of functional AP2 complex still contained a few CCPs present at the PM and that these AP2-independent CCPs were sufficient to internalize certain receptors (Huang et al., 2004; Johannessen et al., 2006; Maurer and Cooper, 2006; Motley et al., 2003). Indeed, novel cargo-specific adaptors have been characterized. Some of these have been demonstrated, or proposed, to work as substitute adaptors for AP2, since they can bind both cargo and clathrin (see paragraph “3.2.1 Cargo-specific adaptors”). Despite these recent findings, the existence of alternative clathrin adaptor proteins is still a matter of debate and conservative thinking about the essential role of AP2 still persists within the endocytic community (Boucrot et al., 2010; Henne et al., 2010).

3.2 CLASP adaptor proteins

In addition to the tetrameric AP2 complex, eukaryotic cells also contain a plethora of additional adaptor proteins that participate in CME. These CLASP adaptors are monomeric and vary in structure and binding properties (**Table 2** and **Figure 7**).

3.2.1 Cargo-specific adaptors

CLASP adaptors drive efficient sorting of specific cargoes into CCPs and are involved in the formation and maturation of CCVs. CLASPs are often specific for a single transmembrane receptor or a small family of receptors. Like AP2, CLASPs recognize short peptide motifs within select cargo that function as endocytic signals and in some cases are found in combination with conventional endocytic motifs.

Numb, ARH (autosomal recessive hypercholesterolemia) and DAB2 (disabled homolog 2) all contain an N-terminal phosphotyrosine-binding (PTB) domain that preferentially binds to non-phosphorylated tyrosines within [F/Y]xNPxY cargo-sorting motifs (Uhlik et al., 2005), followed by several hundred amino acids that are predicted to lack a defined secondary structure. Numb is involved in Notch internalization, at least in *Drosophila Melanogaster* (Berdnik et al., 2002; Guo et al., 1996), while cargo recognition sites in ARH and DAB2 are essential for clathrin-dependent internalization of megalin and members of the LDLR family (Gallagher et al., 2004; Keyel et al., 2006; Mishra et al., 2002).

Two recent studies investigated the respective roles of AP2, ARH and DAB2 in the CME of LDLR (Keyel et al., 2006; Maurer and Cooper, 2006). Both studies confirmed that AP2 is dispensable for the clathrin-dependent internalization of LDLR. ARH-mediated internalization was dependent on AP2, whereas DAB2-mediated, clathrin-dependent internalization of LDLR was independent of AP2. These results suggest that DAB2 can assemble a subset of AP2-independent CCPs at the PM, consistent with its ability to interact with membrane phospholipids and to polymerize clathrin *in vitro* (Mishra et al., 2002). However, endogenous DAB2, like ARH, was found to colocalize strongly with AP2 in various cell types (Keyel et al., 2006; Morris and Cooper, 2001), suggesting that, under

normal conditions (i.e., in the presence of AP2), DAB2 acts as a specific LDLR adaptor targeted to AP2-containing CCPs. In the absence of AP2, DAB2 is likely involved in the assembly of and/or is recruited to the few (5-10%) CCPs that remain in AP2-depleted cells (Hinrichsen et al., 2003; Motley et al., 2003). It is unclear, however, how these AP2-depleted CCPs contribute to the normal rate of LDL uptake. It may be that alternative adaptors, which are normally evenly distributed among CCPs (Keyel et al., 2006), become more concentrated in the AP2-depleted CCPs, thereby increasing the concentration of the cargo in fewer structures. Although these issues are crucial to our understanding of events in cells lacking AP2, they remain to be directly investigated.

Stonin 2 was identified as a CLASP for the internalization of presynaptic vesicles via association with synaptotagmin, and is able to function independently of AP2 (Jung et al., 2007; Willox and Royle, 2012). Arrestins, on the other hand, play a role in the endocytosis of GPCRs. Upon binding to GPCR, β-arrestin undergoes a conformational change, thereby exposing a C-terminal region that contains motifs for AP2 and clathrin binding; this, in turn, allows for efficient CME of the receptor (Edeling et al., 2006; Gurevich and Gurevich, 2003).

Eps15, eps15R and epsins are thought to specifically mediate the recruitment of ubiquitinated cargos. For these adaptors, cargo selectivity is mediated by tandemly arrayed Ub-interacting motifs (UIMs). The internalization of fusion proteins comprised of CD4 and Ub fused to their cytoplasmic domains, was shown to be inhibited by the depletion of clathrin, epsin or eps15/eps15R, but not by the depletion of AP2 (Barriere et al., 2006). Interestingly, the depletion of epsin and clathrin, but not of AP2, has also been reported to inhibit the Ub-dependent internalization of MHC-I molecules in cells expressing the K63 Ub ligase of the Kaposi's sarcoma-associated herpes virus (Duncan et al., 2006), suggesting that an AP2-independent, clathrin- and epsin-dependent pathway may be involved in the internalization of physiological cargos. Indeed, modulation of epsin function alone or together with eps15/eps15R function, by siRNA techniques or the overexpression of mutant forms, has been reported to affect the internalization of ubiquitinated receptors, including the dopamine receptor (Sorkina et al., 2006) and the

epithelial sodium channel (Wang et al., 2006). Meloty-Kapella identified a molecularly distinct mode of CME requiring epsin, but not AP2, and actin for Notch-ligand expressing-cells to pull on Notch and activate its signaling (Meloty-Kapella et al., 2012). Chen and coworkers instead reported an essential cooperation between AP2 and epsin for the internalization of protease-activated receptor-1 (PAR1), a GPCR for thrombin (Chen et al., 2011). Of note, epsin was found to colocalize with clathrin-positive CCPs observed in AP2-depleted cells (Hinrichsen et al., 2003; Motley et al., 2003). Epsin has also been shown to be sufficient to drive the *in vitro* assembly of clathrin on phospholipid monolayers (Ford et al., 2002), suggesting that epsin may replace AP2 in CCP assembly.

Importantly, at least some cargo proteins appear to concentrate in discrete CCPs and display different internalization kinetics suggesting specialization of the CME pathway (Keyel et al., 2006; Lakadamyali et al., 2006; Leonard et al., 2008; Mundell et al., 2006; Puthenveedu and von Zastrow, 2006). Clearly, CME of receptors is complex and every receptor seems to use and arrange the endocytic apparatus in different ways. For these reasons, generalizations are not possible and specific studies focused on the different receptor systems are required.

3.2.2 Lipid-binding adaptors that lack cargo selectivity

Whereas some adaptors only recognize one receptor family, it appears that other adaptors do not recognize cargo at all. Although neuronal AP180 and its ubiquitous counterpart CALM (officially known as PICALM) are referred to as adaptor proteins, so far there is no evidence to suggest that they directly bind to cargo. Therefore, it seems that the sole purpose of these proteins is to link clathrin to membrane phospholipids. These proteins possess an AP180 N-terminal homology (ANTH) domain that binds PIP2 (Norris et al., 1995; Ye and Lafer, 1995). This structured domain is followed by a long unstructured region that contains NPF motifs, a series of DLL and DLF variant clathrin-binding motifs and, in non-yeast species, Dx[F/W] AP2-binding motifs (Morgan et al., 2000; Zhuo et al., 2010).

Numerous studies support a requirement for AP180 and its homologs in the internalization of the v-SNARE, VAMP (also known as synaptobrevin), suggesting a cargo-specific role for these proteins (Bao et al., 2005; Dittman and Kaplan, 2006; Harel et al., 2008). However, to date, no direct physical interaction has been demonstrated between v-SNAREs and AP180s. Thus, AP180 proteins might associate with an undetermined accessory protein or with other adaptor proteins that bind cargo directly.

3.2.3 Cargo-binding adaptors that lack lipid- and clathrin-binding selectivity

There are a few proteins that have been classified as adaptor proteins on the basis of their ability to bind cargo, but that do not contain any known clathrin or lipid-binding motifs. These proteins include eps15, so far known to select ubiquitinated cargo, and stonins that bind to and sort synaptotagmin family cargos (Maritzen et al., 2010).

The N-terminal domain of stonins binds AP2 (Walther et al., 2004) and is followed by a conserved region of unknown function (the stonin homology domain). The C-terminus contains a μ-homology domain (μHD) that is homologous to the cargo-binding C-terminus of the μ2 subunit of AP2 and is essential for selecting synaptotagmin cargo. Interestingly, Stonin 2, the best-studied stonin, also contains two NPF motifs that serve as potential binding partners for EH-domain proteins, such as eps15 and intersectin (Martina et al., 2001).

Given the centrality of eps15s and epsins in this thesis, I have dedicated the following two sections to them.

3.3 Eps15s

Eps15 was originally identified as a substrate for EGFR (Fazioli et al., 1993) and was subsequently implicated in endocytosis (Carbone et al., 1997). It has a modular structure, which endows it with multiple binding activities (**Figure 8**). The N-terminal region contains three EH protein-protein interaction domains (Salcini et al., 1997), which allow eps15 to establish interactions with several other endocytic/sorting proteins, including epsin (Chen et al., 1998), Numb (Salcini et al., 1997), and Hrb (Doria et al., 1999). The central region

of eps15 mediates homodimerization and heterodimerization with its homolog eps15R, intersectin (Sengar et al., 1999), and Hrs, a protein implicated in endosomal function (Bean et al., 2000). The C-terminal region of eps15 contains several DPF motifs that mediate the interaction with AP2 (Benmerah et al., 1996; Iannolo et al., 1997; Owen et al., 2000), a proline-rich region involved in CRK and Grb2 binding (Parachoniak and Park, 2009) and two UIMs (Klapisz et al., 2002; Polo et al., 2002).

Eps15 forms dimers and tetramers with distinct shapes. The eps15 dimer is an elongated molecule in which two subunits are arranged parallel to each other. The eps15 tetramer has a "dumbbell" shape and is formed by the anti-parallel association of two eps15 dimers. This "dumbbell" shape has been shown to have profound implications on the role of eps15 as an endocytic adaptor (Cupers et al., 1997; Tebar et al., 1997).

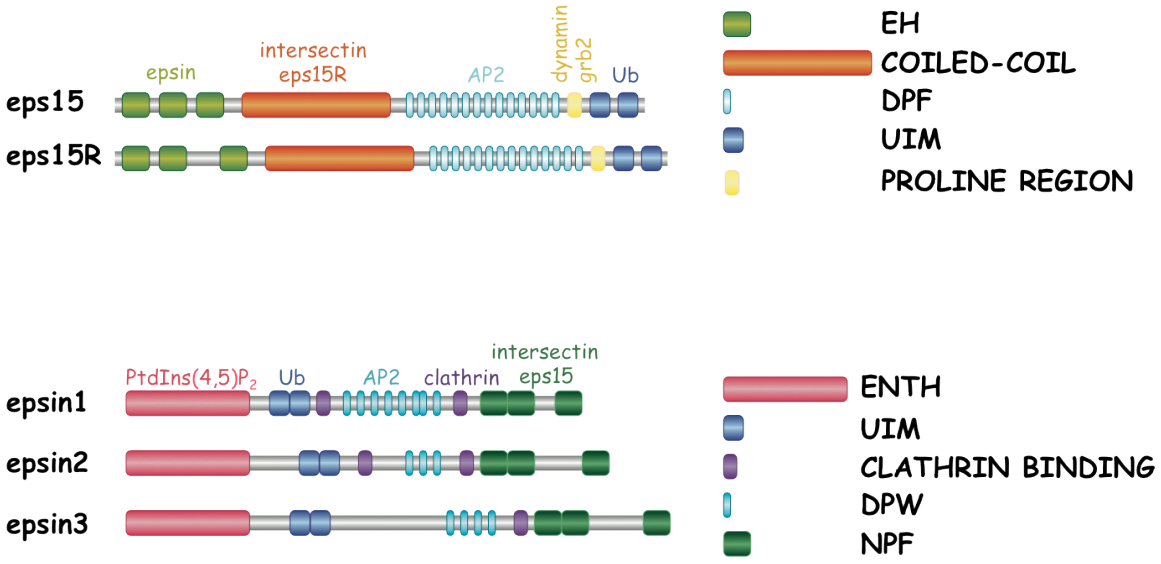


Figure 8: Representation of the overall domain and motif organization of human eps15/R and epsin1/2/3.
A selection of binding partners is written above the single domains/motifs.

The targeting of eps15 to CCPs has long been described to be AP2 dependent (Benmerah et al., 2000), however recent studies have established an inverted order of recruitment at the PM: eps15 appears to be recruited before AP2 and clathrin (Henne et al., 2010; Taylor et al., 2011). By binding multiple AP2 molecules simultaneously, eps15 can generate a sufficient local concentration to promote clathrin polymerization. Since clathrin association with AP2 disrupts the eps15 binding site, eps15 is continually pushed

to the rim of the growing CCP, where it can effectively recruit AP2 to increase the size of the clathrin coat (Praefcke et al., 2004; Tebar et al., 1996). In addition to recruiting additional AP2, eps15 interacts with dynamin, by complexing with intersectin, and recruits it to the membrane, to permit deep invagination into a budding vesicle (Koh et al., 2007; Sengar et al., 1999). For most classes of endocytic cargo, eps15 is not incorporated into budding CCVs (van Delft et al., 1997).

Upon EGFR activation, eps15 is strongly recruited to the PM and localizes to CCPs (Tebar et al., 1996; Torrisi et al., 1999). Eps15 is tyrosine phosphorylated upon EGF stimulation and this event is essential for EGFR internalization, but not for constitutive uptake of the TfR (Confalonieri et al., 2000). Phosphopeptide experiments demonstrate that phosphorylated tyrosines in eps15 likely serve as binding sites for PTB-domain containing proteins (Confalonieri et al., 2000). Since unphosphorylated eps15 does not remain within CCPs, it is likely that the PTB-domain containing protein in question serves to anchor the eps15/EGFR complex within the coated pits. This suggests that eps15 might be involved in the differential regulation of specific endocytic pathways in response to proliferation signals. Indicative of a broader role in cellular physiology, eps15 and eps15R are transported into the nucleus and might have a role in nucleocytoplasmic transport or transcriptional regulation (Doria et al., 1999).

Eps15 is also monoubiquitinated upon EGF stimulation (van Delft et al., 1997) via a mechanism called “coupled monoubiquitination” (Polo et al., 2002), where monoubiquitination is dependent on a functional UIM domain. The role of monoubiquitination and its regulation in cells is still unclear and will be discussed in more detail in the section “5.2 Adaptors ubiquitination”.

3.4 Epsins

Epsin was initially discovered in 1998 by virtue of its binding to eps15, hence its name the eps15 interacting protein (Chen et al., 1998). The epsin family includes 3 members: epsin 1 and 2 are housekeeping proteins broadly expressed in all tissues from early stages of development (Chen et al., 2009), while epsin 3 expression is highly restricted to migratory

keratinocytes, gastric parietal cells and aggressive carcinomas (Coon et al., 2011; Ko et al., 2010; Spradling et al., 2001). All three family members contain a N-terminal ENTH domain that is followed by UIMs and by central and C-terminal regions containing binding sites for clathrin, AP2 and EH-domain-containing proteins, such as eps15 and intersectin (**Figure 8**).

Most of that we know about epsin function comes from studies on epsin 1. It has been demonstrated that:

i) epsin, through its ENTH-domain, has a role in mediating membrane curvature in endocytic pits. It binds membrane PIP2 and inserts an amphipathic helix into the membrane, producing a curvature-inducing deformation (Capraro et al., 2010; Yoon et al., 2010). Related to this ability, a critical role of epsins has recently been proposed in fission-mediated membrane scission (Boucrot et al., 2012).

ii) epsin, similarly to eps15, is involved in the recruitment of AP2 to the PM (Taylor et al., 2011).

iii) epsin is an integral component of CCVs that internalizes along with clathrin. Although previous biochemical studies were unable to resolve whether epsin is an integral component of CCVs (Blondeau et al., 2004; Chen et al., 1998; Nossal and Zimmerberg, 2002), using live-cell TIRFM it has been possible to unambiguously demonstrate that epsin internalizes along with clathrin (Rappoport et al., 2006; Zoncu et al., 2007).

iv) epsin regulates actin polymerization at CCPs, either through interaction with GTPase activating proteins (GAPs) interacting with Cdc42, such as RalBP1/RLIP76 (Aguilar et al., 2006), or by membrane recruitment and phosphorylation of Hip1r (Brady et al., 2010).

v) epsin, likewise eps15, may have a nuclear function. Indeed, the ENTH domain of epsin interacts with the transcription factor, promyelocytic leukemia zinc finger protein (PLZF), suggesting that, epsin may function as a transcriptional regulator (Hyman et al., 2000).

vi) epsin, like eps15, is also monoubiquitinated upon EGF stimulation (Polo et al., 2002).

4. Endocytosis and signaling

Endocytosis is a major mechanism for the control of signal attenuation, through the removal and degradation of signaling receptors from the cell surface. Recent evidence, however, has demonstrated that endocytosis has a much wider impact on signaling. Indeed, several molecules have been shown to play a role in both endocytosis and signaling. The emerging picture portrays endocytosis as a key factor in fine-tuning signal propagation in space and time. Moreover, not only does endocytosis regulate signaling, but also signaling controls endocytosis. This latter concept has already been touched on in the section “2.2.8 Pre-existing *vs. de novo* clathrin-coated pits” and for the purposes of this thesis will not be further described.

4.1 Regulation of signaling at the PM

Ligand-induced internalization of signaling receptors is an important mechanism for the negative regulation of signaling from the cell surface. Indeed, receptor endocytosis can control the magnitude and duration of many PM-regulated signaling processes by physically reducing the number of cell surface receptors accessible to the ligands.

In some cases a reduction in the number of surface receptors does not necessarily lead to a reduction of maximal signaling response that can be elicited by a ligand. Rather, it causes a dose-response shift, so that a higher concentration of ligand is required to trigger a response of the same magnitude. This regulatory mechanism is of physiological importance, for instance, during chemotaxis in response to soluble ligands. Migrating cells should be able not only to move directionally, but also to stop when they reach their target sites, where the concentration of the chemoattractant is the highest. The continuous internalization and degradation of the ligand-receptor complex renders the cells progressively less sensitive to the chemotactic stimulus, until they stop at their target sites (Bailly et al., 2000; Raz, 2004). This kind of regulatory mechanism plays a critical role throughout development.

Signaling can be modulated also by the regulation of ligand accessibility through endocytosis, as exemplified by the Notch receptor system. Both Notch receptor and its

ligands, Delta, Serrate and Lag2, are transmembrane proteins; therefore, signal activation occurs through direct engagement of a signal-sending cell, carrying the ligand, and a signal-receiving cell harbouring the receptor. Ligand binding promotes two proteolytic cleavage events in the Notch receptor. The second cleavage releases the Notch intracellular domain (NICD), which then translocates to the nucleus and promotes transcription of target genes (D'Souza et al., 2008; Fortini, 2009; Fortini and Bilder, 2009). In order to activate signaling, endocytosis seems to be necessary both in the signal-receiving and in the signal-sending cell. In the signal-receiving cell, endocytosis of Notch is required for its activation, since the second cleavage probably occurs in endosomes. In the signal-sending cell instead, one model contemplates a mechano-transduction mechanism in which pulling forces exerted by the internalizing ligand “strip” the extracellular domain of Notch from the intracellular membrane-anchored moiety, thereby allowing proteolytic cleavage of Notch (Meloty-Kapella et al., 2012; Rajan et al., 2009). In addition, at least in the case of Delta, internalization seems to be required to “activate” the ligand [**Figure 9Aa**; (Windler and Bilder, 2010)]. Endocytosis and recycling of Delta to specific restricted regions of the PM are probably necessary to maintain a high enough local concentration of ligand to induce robust Notch activation, as indicated also by recent evidence in *Drosophila melanogaster* (Rajan et al., 2009).

Receptor endocytosis can also regulate signaling by physically separating the receptors from substrates or mediators located at the PM. As an example, GPCR signaling through PM potassium channels requires that receptors and G proteins are present in the same membrane [**Figure 9Ab**; (Mathie, 2007)]. Similarly PLCγ1 (phospholipase Cγ1) and PI3K (phosphoinositide 3-kinase) signaling by EGFR are inhibited by receptor internalization owing to the lack of their lipid substrate, PIP2, in endosomes [**Figure 9Ac**; (Haugh and Meyer, 2002)].

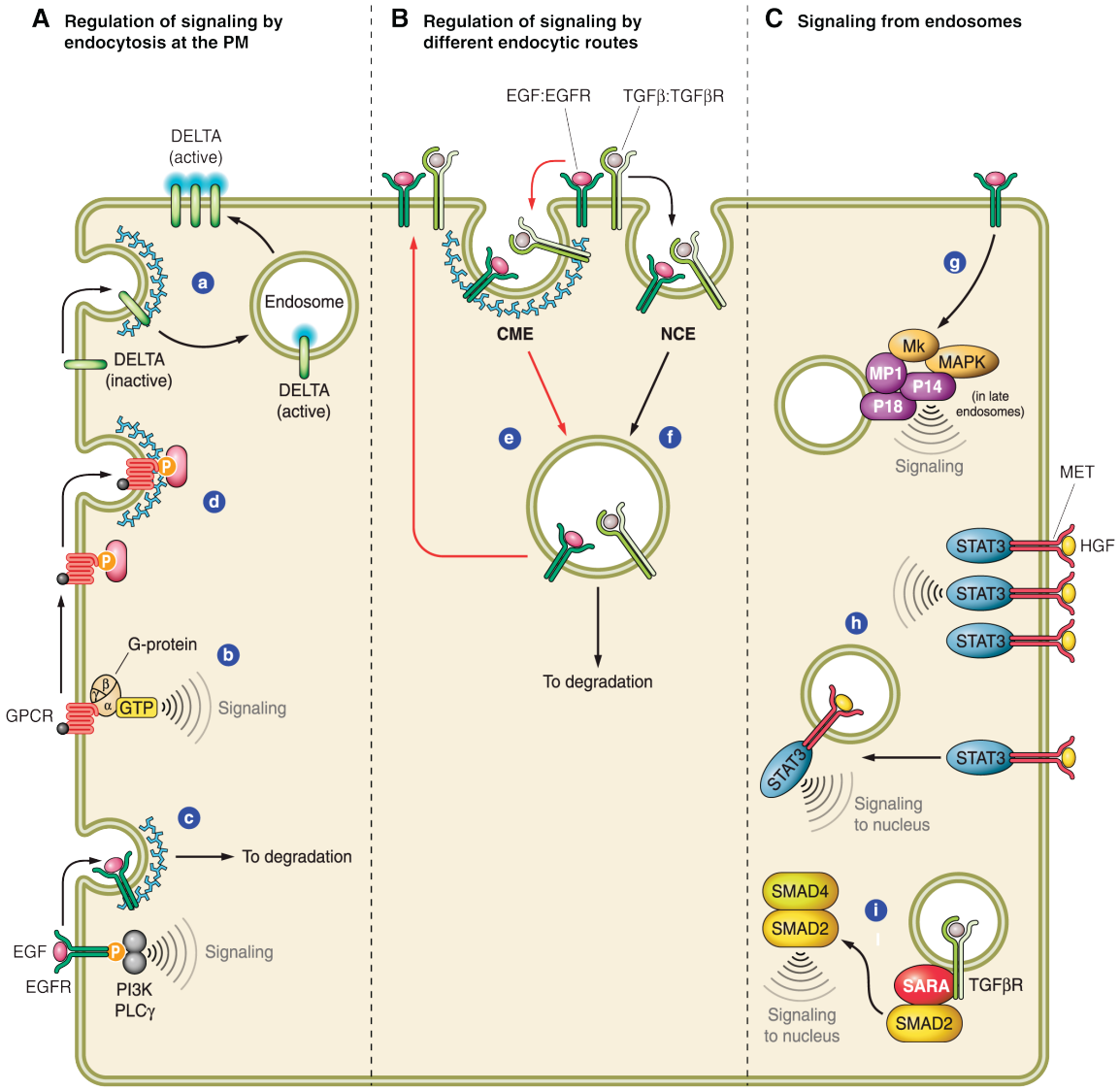


Figure 9: Endocytic control of signaling.
[see text for details, adapted from (Sigismund et al., 2012)].

Finally, retention time inside endocytic structures at the PM has been proposed to influence signaling activation. This is the case of GPCRs, whose regulated endocytosis occurs preferentially through a specialized subset of CCPs. These specialized CCPs have an increased surface residence time, with respect to the other CCPs, which is regulated locally by GPCR cargo via PDZ-dependent linkage to the actin cytoskeleton. This increased surface residence time has been proposed, yet not demonstrated, to be required for proper signal regulation [**Figure 9Ad**; (Puthenveedu and von Zastrow, 2006)].

4.2 The integration of different endocytic routes controls the net biological output

The biological output of a specific signal depends also on the endocytic route through which receptors reach the different compartments. Several signaling receptors, including RTKs, GPCRs, transforming growth factor β receptor (TGFβR), Notch and Wnt, undergo both CME and NCE, and the relative partitioning in the two endocytic routes establishes the final output in terms of sustaining or attenuating signalling [**Figure 9Be-f**]. In the case of TGFβR, ligand binding induces initiation of Smad signaling through the Smad adaptor protein SARA (Smad anchor for receptor activation). Receptor internalization through CME is essential for signaling and SARA has been found in EEA1-positive endosomes that are downstream of this route. Conversely, receptors that enter cells through NCE are associated with Smad7 and the E3 Ub ligase SMURF. This results in their ubiquitination and subsequent degradation (Di Guglielmo et al., 2003). A similar scenario also operates during internalization and signaling of EGFR [(Sigismund et al., 2008), see also Introduction section "6.2 EGFR endocytosis and trafficking"].

The use of CME for signaling and NCE for degradation, although common for TGFβR and EGFR, is not a rule. Other cargos exploit the two internalization pathways in the opposite manner, as exemplified by LRP6 (Wnt3a-activated low-density receptor-related protein 6). Wnt3a signaling by its receptor LRP6 involves the raft (non-clathrin) route, while degradation requires CME (Yamamoto et al., 2008). In the presence of Wnt3a, LRP6 is phosphorylated and internalized into a caveolin-positive vesicular compartment, where it can stabilize β-catenin and transduce the signal via the CK1γ kinase. When LRP6 binds the Wnt3a antagonist Dkk (Dickkopf) instead, it is diverted away from the caveolin pathway towards the clathrin pathway where signal transduction cannot occur, because of the absence of the kinase.

4.3 Endosomes are signaling stations

A large body of evidence shows that signaling is not restricted to PM. On the contrary, as internalization advances, activated transmembrane molecules become confined and

enriched within endosomal organelles. This was first realized in neurons, where the signal given by neurotrophins must be transported from distal axons to the cell body of the neuron, named soma, to activate the transcription of anti-apoptotic genes (Howe and Mobley, 2004). Passive diffusion of signaling effectors from the synapse to the soma is too slow to explain retrograde signaling. The current model of retrograde signaling suggests a dynein-mediated microtubular transport of signaling endosomes containing activated receptors to the soma (Watson, Heerssen et al. 2001; Wu, Ramirez et al. 2007). Endosomes impact on signaling mainly in two ways: by sustaining signals originating from the PM, or by assembling specific signaling complexes that are prohibited at the PM (Gould and Lippincott-Schwartz, 2009; Sadowski et al., 2008; Scita and Di Fiore, 2010).

In systems where active receptors are quickly internalized, the ability of a receptor to signal after internalization is critical to ensure sufficient duration and magnitude of signals. Several RTKs and their ligands, including EGF-EGFR, remain bound and active after internalization in endosomes. Moreover, all the components of the ERK (extracellular signal regulated kinase) - MAPK (mitogen-activated protein kinase) activation cascade can be detected in endosomes (Sorkin and Von Zastrow, 2002), providing evidence that EGFR continues to signal following endocytosis. Additional evidence has been provided by experiments with dominant negative mutants and siRNAs targeting proteins involved in internalization, which show that endocytosis is required for ERK activation (Sorkin and von Zastrow, 2009; Vieira et al., 1996). Furthermore, endosomal-specific proteins that are important to sustain signaling have been identified: for instance, P18 works as an anchor for an ERK-activating scaffold and is required for the maximal amplitude of ERK1/2 phosphorylation [**Figure 9Cg**; (Nada et al., 2009)]. An analogous mechanism occurs in the case of GPCR signaling, where β-arrestin, similarly to P18, acts as a specific scaffold stably anchoring ERK1/2 to the endosome. This seems to promote signaling on the one hand, and, on the other, to shift signaling towards cytosolic rather than nuclear ERK substrates (DeWire et al., 2007).

Importantly, EEs are a morphologically and functionally heterogeneous population, characterized by the presence of biochemically distinct membrane subdomains, which

ultimately impact on receptor signaling and fate (Lakadamyali et al., 2006; Miaczynska et al., 2004; Sonnichsen et al., 2000; Zoncu et al., 2009). Recently, a specific intermediate station before EEs, called APPL endosomes, has been described. APPL endosomes participate in growth factor receptor trafficking and signaling, and represent an early endocytic intermediate common to a subset of clathrin-derived endocytic vesicles and macropinosomes. Due to their lipid composition, APPL endosomes are the only intracellular compartments competent for AKT activation, thus, allowing enhancement of the PM signal (Zoncu et al., 2009).

In a few cases it has been demonstrated that endocytic structures can operate as obligatory intermediate signaling stations between the PM and the nucleus. An example is provided by the endosomal machinery involved in the propagation of signaling from the RTK Met to the activation of the transcription factor STAT3 (signal transducer and activation of transcription 3). Upon ligand stimulation of Met, STAT3 is recruited and probably activated by the receptor at the PM. STAT3 then travels, via endocytic organelles, to the nucleus, where it regulates the transcription of its target genes [**Figure 9Ch**]. Recent evidence has shown that the magnitude of the signaling response is dependent on trafficking of the receptor and downstream signaling components (Kermorgant and Parker, 2008). Moreover, it appears that endosomal trafficking of STAT3 may also protect weakly activated STAT3 from cytosolic phosphatases (Kermorgant and Parker, 2008).

The second way in which endosomes can function as signaling organelles is by supporting signaling processes that cannot happen, or that happen with low efficiency, at the PM. The ability of endosomes to recruit proteins that bind PI3P, which is particularly enriched in endosomes compared to the PM, is used during TGFβR signaling. As previously described, the activated receptor interacts with the adaptor protein SARA in EEs. SARA is associated with the receptor target SMAD2, and this allows the efficient phosphorylation of SMAD2 by TGFβR in endosomes [**Figure 9Ci**; (Chen et al., 2007; Hayes et al., 2002; Tsukazaki et al., 1998)].

Another example of endosome-specific signaling is provided by the TNFR (tumor

necrosis factor receptor) signaling cascade. The components that promote the pro-apoptotic signaling pathway induced by TNFR, are recruited to the ligand-bound TNFR at the PM. For apoptosis to occur, the cysteine protease caspase-8 has to be activated. Interestingly, it has been demonstrated that both recruitment and activation of caspase-8 only occurs in endosomes. The mechanisms that prevent caspase-8 recruitment and activation at the PM are not yet known (Schneider-Brachert et al., 2004).

4.4 Regulation of signaling by endosome sorting

Endosome sorting plays an essential role in the spatial restriction of signals, which prevents signals from becoming uniformly distributed throughout the cell, and consequently uninformative. It is necessary for the execution of a number of polarized cellular functions, including directed cell migration, cell-fate decisions, epithelial-cell polarization, growth cone movement, tissue morphogenesis during development, and cell invasion into the surrounding tissues of metastatic cells. Indeed, during the chemotactic migration of cells, the cells must reorient themselves in the direction of travel through the polarization of sensors that are present in the PM. Moreover, the cells must coordinate the intracellular trafficking of molecules, the adhesion of the cell to the substrate and the remodeling of the actin cytoskeleton to generate the propulsive forces. In *Drosophila melanogaster* border cells, when endocytic pathways that depend on the small GTPase Rab5 are disrupted, cells migrate aberrantly in response to stimulation (Jekely et al., 2005). There are also similar circuitries in mammalian cells: recycling of Rac proteins (small GTPases that relay signals from cell surface receptors to the actin cytoskeleton) to specific regions of the PM is Rab5-dependent and is required for the transduction of motion-inducing stimuli [**Figure 10-1**; (Palamidessi et al., 2008)]. Endosomal sorting also controls the trafficking of integrins, which are the best-characterized cell surface adhesion receptors and have a crucial role in cell migration. It has been suggested that the continual internalization and recycling of integrins between the PM and the endosomal compartment is essential for controling cell locomotion (Disanza et al., 2009). Consistent with this view, it has been found that inhibiting Rab25, which associates with α5β1-

integrins in endosomes, blocks the recycling of these integrins to the surface and impairs the formation of cellular protrusions, thus preventing the cell from migrating in three dimensions [**Figure 10-2**; (Caswell et al., 2007)].

Endosome sorting also controls signaling fate. Transfer of activated receptors to LEs/MVBs terminates signaling, either by sequestering receptors in ILVs, thus preventing their interaction with effectors, or by promoting their lysosomal degradation. On the other hand, recycling of internalized receptors to the PM replenishes the cell surface with ligand-free receptors and also restores receptor sensitivity to extracellular ligands, as is the case for GPCRs. The coupling of β2 adrenergic receptor (β2AR) to trimeric G proteins upon ligand binding is rapidly inhibited by receptor phosphorylation at the PM (Pitcher et al., 1992). However, sorting of internalized arrestin-bound receptor into a rapid recycling pathway promotes receptor dephosphorylation by an endosome-associated PP2A protein phosphatase, thus ensuring the return of intact receptor for successive rounds of signaling [resensitization (Pitcher et al., 1995)].

A functionally similar process also occurs during EGFR signaling. Both TGFα and EGF elicit rapid internalization of EGFR. EGF binding to EGFR is relatively stable at the pH of endosomes, so EGFR remains active in these organelles, before being ubiquitinated and transported to lysosomes for degradation. In contrast, TGFα rapidly dissociates from the receptor when exposed to the acidic endosomal environment. As a consequence, the receptor becomes deactivated and is recycled back to the PM (French et al., 1995; Longva et al., 2002). This differential trafficking fate is crucial to the duration of receptor signaling. Indeed, following receptor recycling, the cell is immediately able to undergo an additional round of signaling activation. In accordance with this, TGFα is a more potent mitogen than EGF (Waterman et al., 1998).

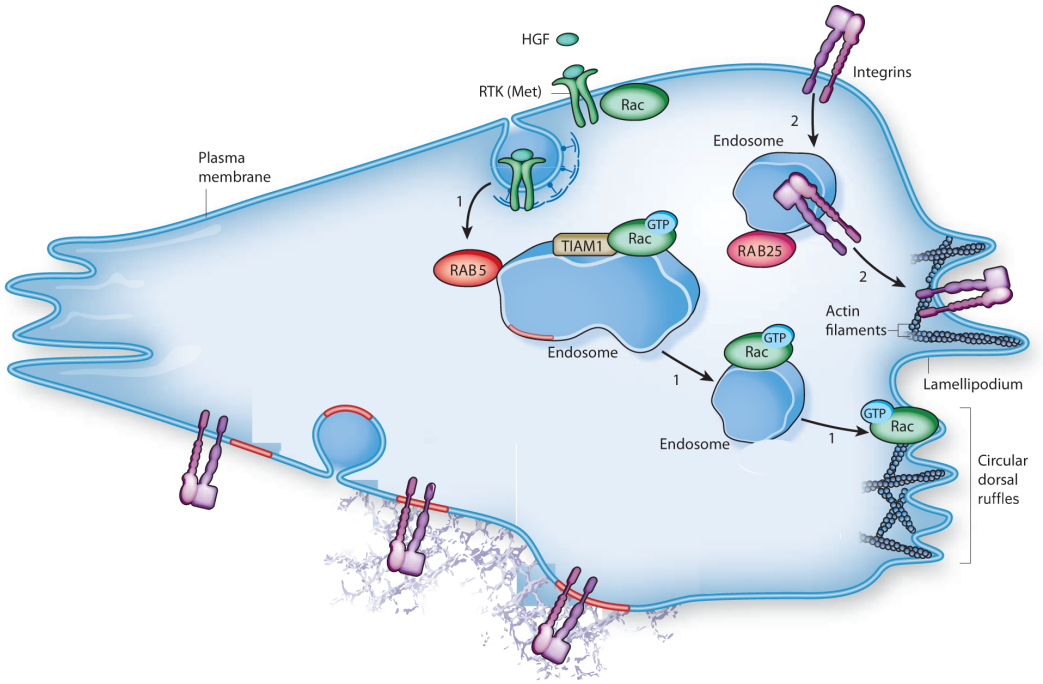


Figure 10: Regulation of signaling by endosome sorting.
[see text for details, adapted from (Scita and Di Fiore, 2010)].

5. Multiple role of ubiquitin in endocytosis

Posttranslational modification of signaling receptors by the covalent attachment of one, or often more, Ub moieties has emerged as the major regulatory mechanism responsible for receptor downregulation. Ubiquitination is a complex process executed by a cascade of enzymes, whose final effectors, the Ub ligases, or E3 enzymes, catalyze the addition of a Ub moiety or of a Ub chain to their substrates. E3 substrates can therefore be monoubiquitinated (when a single Ub is appended), multiple monoubiquitinated (when single Ubs are appended to multiple sites), or polyubiquitinated (when substrates are conjugated to a Ub chain). In addition, Ub chains display different topologies, according to the linkages joining the various Ub moieties in the chain (Macgurn et al., 2012). Ub moieties can be recognized by Ub-binding domains (UBDs) found in many proteins all along the endocytic route (Hurley et al., 2006).

5.1 Cargo ubiquitination

Ub-mediated endocytosis first came into focus with studies in yeast demonstrating a Ub requirement for endocytosis of various PM cargoes, including Ste6 (Kolling and

Hollenberg, 1994) and Ste2 (Hicke and Riezman, 1996). Indeed, for many yeast cargoes, Ub modifications are both necessary and sufficient for endocytosis (Shih et al., 2000), although Ub-independent endocytosis of cargoes has also been described (Chen and Davis, 2002). The general consensus is that Ub-mediated endocytosis is the dominant mechanism for internalization of most cargoes studied in yeast. However, in mammalian cells, the role of Ub in endocytosis is somewhat more complicated. In the case of ion channels, ubiquitination is essential for the internalization. For many endocytic cargoes in mammalian cells, including RTKs and GPCRs, Ub modification appears to be sufficient for endocytic uptake (Haglund et al., 2003; Sigismund et al., 2005). Strikingly, although many of these cargoes exhibit ligand-dependent Ub modification, they also exhibit Ub-independent endocytosis. Thus, in mammalian cells, ubiquitination is often sufficient, but not required, for internalization by endocytosis. This is indicative of multiple redundant, yet distinct, mechanisms of endocytosis (Goh et al., 2010).

The fact that receptor ubiquitination is not indispensable for the internalization step, in the mentioned cases, does not imply that it has no role at all. At least in some cases, the Ub modification might selectively couple the same receptor with different entry portals. In the case of the EGFR, molecular genetic evidence obtained with receptors mutated in the E3 ligase-binding sites or in the Ub-acceptor lysines showed that direct EGFR ubiquitination is not essential to promote CME (Huang et al., 2007; Huang et al., 2006; Jiang and Sorkin, 2003; Sigismund et al., 2005), while it is essential for NCE (Sigismund et al., 2005b). Also in the case of TGFβR, receptor ubiquitination is associated with the caveolar endocytic pathway, but not with CME, although it is not clear whether receptor ubiquitination is the signal that triggers caveolar endocytosis (Di Guglielmo et al., 2003). In addition, different types of ubiquitination might direct the cargo to distinct endocytic routes. In the case of the IGF-1R, the E3 ligase Mdm2 catalyzes the formation of Lys63-linked Ub chains and targets receptors to CME, while the E3 ligase Cbl preferentially utilizes Lys48 and, under these conditions, the internalization of IGF-1R seems to proceed via caveolae (Sehat et al., 2008).

Importantly, interaction of the EGFR with Cbl is required for Ub-independent endocytosis of the EGFR (Huang et al., 2007), suggesting that Cbl-mediated EGFR internalization may not be related to receptor ubiquitination. Such a function may be related to Cbl-mediated recruitment of additional factors to active RTK signaling complexes, including Cin85 and endophilin, which are part of the endocytic protein interaction network. Furthermore, many such endocytic proteins, including Cin85, epsins, and eps15 family proteins, are known to undergo coupled monoubiquitination (Bezsonova et al., 2008; Polo et al., 2002) and as such may be modified by Cbl. Thus, it is tempting to speculate that while EGFR ubiquitination is not required in CME, a Ub signal could still be required, maybe in the form of ubiquitinated adaptors.

5.2 Adaptor ubiquitination

Similarly to direct receptor ubiquitination, the ubiquitination of endocytic adaptors plays a critical role in endocytosis, as exemplified by the arrestin family proteins, which direct internalization of the GPCR cargo (Shenoy et al., 2009). Signaling from activated GPCRs is terminated when GPCRs are phosphorylated, leading to the recruitment of arrestins that binds to AP2 and clathrin, causing the whole complex to be internalized. Agonist-stimulated ubiquitination of arrestin mediated by the E3 Ub ligase Mdm2 is critical for rapid receptor internalization (Shenoy et al., 2009). Mdm2-arrestin binding occurs constitutively and does not persist after receptor activation, suggesting that Ub modification might cause a conformational change in arrestin that is required to promote internalization. GPCRs themselves can also be ubiquitinated, an event required for cargo degradation, but not internalization (Shenoy et al., 2008).

Arrestin is not the sole example of an endocytic adaptor subjected to Ub modification. Several components of the downstream endocytic machinery, such as eps15, epsin and Hrs, are also modified by monoubiquitination upon RTK activation (Haglund et al., 2002; Katz et al., 2002; Polo et al., 2002; Shih et al., 2002).

The precise role of adaptor monoubiquitination in RTK endocytosis is still a matter of debate. Monoubiquitination of adaptors might permit the formation of several tiers of

ubiquitination-dependent interactions in the endosome, by allowing binding of ubiquitinated cargo (through UBDs) and the recruitment of another layer of Ub receptors through a monoubiquitination signal. The result would be signal amplification and progression of ubiquitinated cargoes along the endocytic pathway. Alternatively, it has been proposed that ubiquitination (in particular coupled monoubiquitination) could represent a signal to "switch off" the binding activity of the adaptor (or of other endocytic proteins that undergo the same process), by allowing intramolecular interactions between the UBD and the Ub moiety present in cis (Hoeller et al., 2006). This mechanism might in turn harbor a series of consequences, for instance, the release of ubiquitinated cargo that would thus become available for the next tier of interactions along the endocytic route. In favor of this possibility, it was shown that an eps15-Ub chimera fails to localize properly on endocytic vesicles containing internalized EGFR, thereby preventing the interaction between the UIMs contained in eps15 and EGFR-Ub, an event associated to delayed internalization and degradation of the receptor (Fallon et al., 2006; Hoeller et al., 2006). It is to be noted, however, that in many cases endocytic proteins are ubiquitinated at a rather low stoichiometry: an occurrence not immediately compatible with a "switch off" function of ubiquitination, unless the process is tightly regulated locally (i.e., it occurs and it is relevant only on a minor fraction of the endocytic protein in a particular location). In addition, it was recently reported that monoubiquitination of vacuolar protein sorting 27 (Vps27; the yeast homologue of Hrs), a component of ESCRT-0, is not required for cargo sorting along the degradative endocytic route (Stringer and Piper, 2011).

In conclusion, while the relevance of the ubiquitination of endocytic adaptors is clear in some cases, it remains obscure in others. One possibility is that the simple idea of a general mechanism should be abandoned and that the role of ubiquitination in endocytosis should be established on a case-by-case basis.

5.3 Ubiquitin in endosomal sorting

Beside the role of Ub at the PM, Ub plays a critical role at later steps of endosomal sorting. Ligand-induced ubiquitination is indeed required for lysosomal targeting and

downregulation of signaling receptors. ESCRT complexes orchestrate the Ub-directed sorting into MVBs [see also Introduction section “1.2 Endocytic compartments”; (Babst et al., 2002a; Babst et al., 2002b; Bache et al., 2003)]. This conserved machinery performs three distinct but connected functions: first, it recognizes ubiquitinated cargos and prevents their recycling and retrograde trafficking; second, it bends the endosomal membrane, allowing cargo to be sorted into endosomal invaginations; third, it catalyzes the final abscission of the invaginations, forming the ILVs that contain the sorted cargo [for an exhaustive review, see (Stuffers et al., 2009)].

Since the rate of receptor downregulation and MVB targeting typically correlates with the extent of receptor ubiquitination in endosomes, interference with this posttranslational processing enhances signaling, such as for mutants in EGFR ubiquitination sites (Huang et al., 2006). Similarly, RNA or genetic interference with the Ub adaptor Hrs in mammalian cells or in *Drosophila melanogaster* results in enhanced signaling by various RTKs, including EGFR, Met and VEGFR (Hammond et al., 2003; Lloyd et al., 2002). Furthermore, genetic disruption of members of the ESCRT complexes, which are required for membrane fission events, including those that lead to endosomal ILV formation, leads to sustained EGFR signaling (Bache et al., 2006; Malerod et al., 2007), and, in *Drosophila melanogaster*, to Notch hyperactivation and neoplastic transformation (Vaccari et al., 2008).

6. The EGFR system

EGFR and its ligands are cell signaling molecules involved in many distinct cellular functions, including tissue development, cell proliferation, motility, differentiation, and survival. EGFR, also called ErbB1, belongs to the ErbB family of RTKs (Holbro and Hynes, 2004) that includes ErbB2/HER2/Neu (Citri et al., 2003), ErbB3/HER3 (Citri et al., 2003), and ErbB4/HER4 (Carpenter, 2003). As other transmembrane proteins, ErbBs are co-translationally translocated through the endoplasmic reticulum (ER) membrane, transported to the Golgi apparatus, where the extracellular domain acquires N-linked

glycosylation, and from where the receptors are finally delivered to the PM (Cummings et al., 1985; Todderud and Carpenter, 1989).

The ErbB family of receptors is characterized by a large extracellular ligand-binding region, a single membrane spanning α-helix and an intracellular region that contains a juxtamembrane portion, followed by a tyrosine kinase domain and a C-terminal regulatory sequence.

A series of EGFR crystal structures are now available, which have helped to better understand mechanisms of EGFR activation (Lemmon, 2009). It is now well accepted that binding of EGF (or other agonists) to EGFR shifts a monomer-dimer equilibrium to favor the dimeric state (Lemmon and Schlessinger, 2010). This induces activation of the intracellular tyrosine kinase domain and consequent trans-autophosphorylation in the C-terminal tail, which promotes the recruitment of downstream signaling proteins and the endocytic machinery (Yarden and Sliwkowski, 2001).

6.1 Ligand-induced EGFR signal transduction

EGFR can be activated by several ligands, including EGF, TGFα, HB-EGF (heparin-binding EGF-like growth factor), amphiregulin, betacellulin, epiregulin and epigen. Upon ligand binding, EGFR triggers several downstream signaling pathways similar to other RTKs (**Figure 11**). The activation of these signaling pathways elicits multiple responses in the cell. Temporal and spatial control of EGFR signaling dictates the biological outcome possibly by altering the balance among various signaling pathways (Oda et al., 2005).

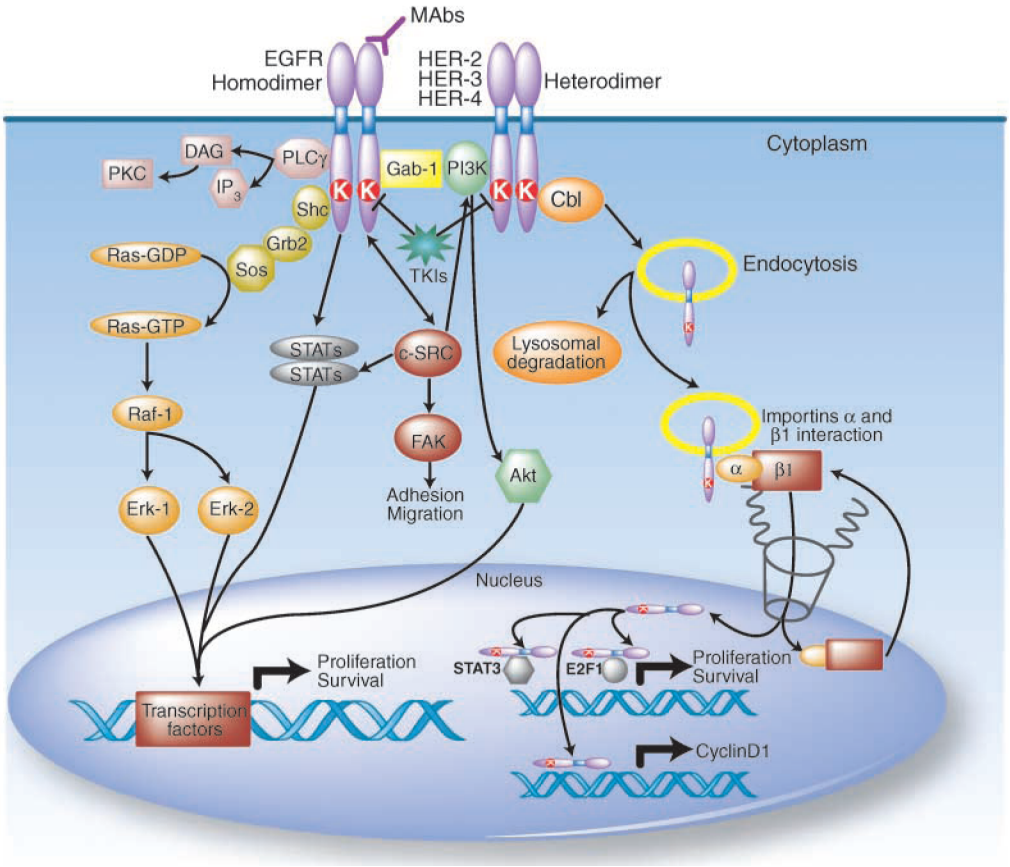


Figure 11: EGFR signaling pathways.
Activation of EGFR leads to its homodimerization/heterodimerization, which triggers phosphorylation of specific tyrosine residues in its intracellular domain and subsequent recruitment of several signaling proteins. PLCγ (pink) and STAT transcription factors (blue) bind directly to the receptor, whereas Ras (orange) and PI3K (green) require specific adaptor molecules (yellow) to interact with the receptor. Concomitantly, the activated receptors undergo endocytosis [see text for details; taken from (Scaltriti and Baselga, 2006)].

6.1.1 Ras/Raf/mitogen-activated protein kinase pathway

The Ras/Raf/MAPK pathway is a critically important route that regulates cell proliferation and survival. Following EGFR phosphorylation, the complex formed by the adaptor proteins Grb2 and Sos binds directly, or through association with the adaptor molecule Shc, to specific docking sites on the receptor [**Figure 11;** (Batzer et al., 1994; Lowenstein et al., 1992)]. This interaction leads to a conformational modification of Sos, which is now able to recruit Ras-GDP, resulting in Ras activation (Ras-GTP). Ras-GTP activates Raf-1 that, through intermediate steps, phosphorylates the MAPKs, ERK1 and ERK2 (Hallberg et al., 1994; Liebmann, 2001). Activated MAPKs are imported into the nucleus where they

phosphorylate specific transcription factors involved in cell proliferation (Gaestel, 2006; Hill and Treisman, 1995).

6.1.2 Phosphatidylinositol 3-kinase/Akt pathway

The PI3K/Akt pathway is involved in cell growth, apoptosis resistance, invasion, and migration. PI3K is a dimeric enzyme composed of a regulatory p85 subunit, responsible of the anchorage to ErbB receptor-specific docking sites, and a catalytic p110 subunit that generates the second messenger phosphatidylinositol 3,4,5-trisphosphate, which is responsible for phosphorylation and activation of the serine/threonine kinase Akt [**Figure 11;** (Vivanco and Sawyers, 2002)]. The principal mechanism that drives EGFR-dependent PI3K activation is the dimerization of the receptor with HER-3. Indeed, docking sites for p85 are absent on EGFR, whereas, on the contrary, docking sites for p85 are abundant on HER-3 (Carpenter et al., 1993; Yarden and Sliwkowski, 2001). Alternatively, the p85 subunit can interact with EGFR through the docking protein Gab-1 [**Figure 11**; (Mattoon et al., 2004)].

6.1.3 Phospholipase Cγ

PLCγ interacts directly with activated EGFR and hydrolyses PIP2 to give inositol 1,3,5-triphosphate (IP_3), which is important for intracellular calcium release, and 1,2-diacylglycerol (DAG), a cofactor in protein kinase C (PKC) activation [**Figure 11**; (Chattopadhyay et al., 1999; Patterson et al., 2005)]. PKC activation can, in turn, result in MAPK and c-Jun NH_2-terminal kinase (JNK) activation (McClellan et al., 1999; Schonwasser et al., 1998).

6.1.4 Signal transducers and activators of transcription pathway

STAT proteins interact with phosphotyrosine residues via their Src homology 2 (SH2) domains and, on dimerization, translocate to the nucleus and drive the expression of specific target genes [**Figure 11**; (Haura et al., 2005)]. Constitutive activation of STAT proteins and especially STAT3 has been observed in numerous primary cancers and

tumor-derived cell lines. Augmented activity of membrane-associated tyrosine kinases, such as EGFR, HER-2, and platelet-derived growth factor receptor (PDGFR), promotes STAT3 persistent activation, which contributes to oncogenesis or tumor progression (Bromberg, 2002).

6.1.5 Src kinase pathway

Src is the archetypal member of a nine-gene family of non-receptor tyrosine kinases that has a critical role in the regulation of cell proliferation, migration, adhesion, angiogenesis, and immune function. Src, which is located in the cytosol, activates a series of substrates, including focal adhesion kinase (FAK), PI3K, and STAT proteins [**Figure 11**; (Summy and Gallick, 2006; Yeatman, 2004)]. Although Src can function independently, it also cooperates with other RTKs. The interaction between EGFR and Src is complex. On one hand, Src serves as a signal transducer and an enhancer of EGFR activation (Jorissen et al., 2003; Leu and Maa, 2003). On the other, Src may be involved in resistance to EGFR therapies via independent activation or association with other receptors.

6.1.6 Regulatory loops downstream of EGFR

The early phase of EGF signaling largely engages pre-existing components involved in phosphorylation events. It also involves the immediate turnover of a group of microRNAs [immediately downregulated microRNAs (ID-miRs)], which allows the onset of later responses [**Figure 12**, (Avraham and Yarden, 2011)]. Newly EGF-induced RNAs and proteins drive the late regulatory mechanisms. The transcriptional response to EGF can be divided into three temporal phases. The initial wave, up to 45 minutes from stimulation, consists of a limited set of genes, called immediate early genes (IEGs), which include transcription factors and cell cycle regulators, like FOS, JUN and EGR1 (Herschman, 1991). The second wave is activated 45-120 minutes after the stimulation and comprises both positively and negatively acting components (Amit et al., 2007). These are called delayed early genes (DEGs) and include many newly induced phosphatases, as well as DNA- and RNA-binding proteins. DEGs act by strongly shutting down IEGs either by inhibiting upstream signal transduction pathways or by promoting transcriptional

attenuation. The late, secondary response genes (SRGs) are activated after 120 minutes and confer stable phenotypes, which are crucial for fate determination. One example of this late response is epithelial-mesenchymal transition (EMT) (Yilmaz and Christofori, 2009). Growth factors such as EGF and TGFβ enhance EMT by inducing several transcription factors, by upregulating the synthesis and secretion of specific matrix metalloproteinases and by downregulating protease inhibitors.

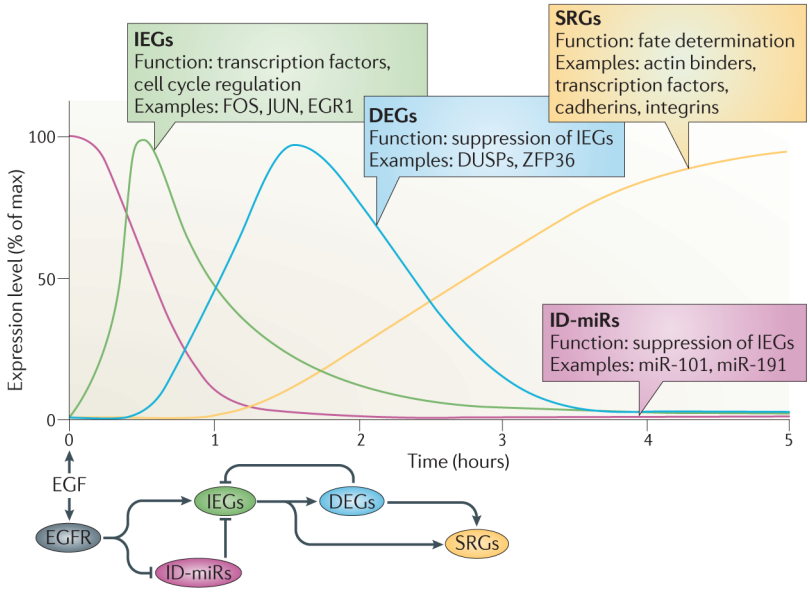


Figure 12: Wave-like regulation of mRNAs and microRNAs by EGF.
Cyclic transitions between a resting cellular state and an active state involve several binary switches that are able to control the kinetic profile of immediate early genes (IEGs). The onset of the cycle is induced by an extracellular stimulus, such as epidermal growth factor (EGF). The earliest event involves the immediate turnover of a group of microRNAs [immediately downregulated microRNAs (ID-miRs)], which allows the onset of IEG induction. Subsequently, IEGs induce the transcription of delayed early genes (DEGs), which shut down the activity of IEGs. The kinetic profiles of the IEGs appear to define the specificity of downstream transcription programmes, including the identity of the secondary response genes (SRGs), which define the cellular outcome. The temporal relationships (graph), as well as the topological interactions (schematic) among the groups of gene products are shown [taken from (Avraham and Yarden, 2011)].

6.2 EGFR endocytosis and trafficking

Binding of EGF to EGFR results in acceleration of receptor endocytosis. EGFR can be internalized through both CME and NCE routes (Sigismund et al, 2005, Orth et al, 2006, Lund et al, 1990; West et al, 1989) and the distribution of the receptor into the two pathways is a highly regulated process. High internalization rates of EGFR, that are

characteristic of CME, are observed only when EGF is used at low concentrations (≤1-2 ng/ml), whereas the apparent rate of EGF uptake decreases with increasing EGF concentrations, as clathrin-independent pathways are activated. Indeed, NCE of the EGFR was first observed more than 20 years ago upon stimulation with high EGF doses (Lund et al, 1990; West et al, 1989). This initial observation has recently been confirmed and extended by studies in our laboratory, where we have shown that EGFR internalization through CME and NCE is tightly controlled by ligand concentration; at low EGF doses (1 ng/ml), receptors are exclusively internalized by CME, while at high, physiologically-relevant, EGF doses (20-100 ng/ml), a substantial proportion of EGFR is internalized by NCE (Sigismund et al., 2005). Ubiquitination appears to be required for clathrin-independent endocytosis while it is dispensable for clathrin-dependent internalization (Huang et al., 2007; Sigismund et al., 2005). However, it is important to note that the presence and the significance of EGFR-NCE clearly depend on the cellular context (Kazazic et al, 2006; Madshus and Stang, 2009; Orth et al, 2006; Rappoport and Simon, 2009; Sigismund et al, 2005).

It has been a huge diatribe in the field regarding the establishment of accepted physiological concentrations of EGF, since historically the erroneous perception has been that only low doses of EGF are physiological. However, whereas plasma concentrations of EGF are around 1 ng/ml (Grau et al., 1994; Hayashi and Sakamoto, 1988), serum levels of EGF are 5-fold greater (Oka and Orth, 1983; Westergaard et al., 1990), and in some zones can be even higher due to the release of EGF from platelets by degranulation [**Table 3**, (Oka and Orth, 1983)]. Moreover, various organs appear to regulate their levels of EGF independently, further confirming the notion that EGF might act locally rather than systemically as a "true" endocrine factor. Thus, huge differences of EGF concentrations can be found in various bodily fluids, from low levels (1-5 ng/ml) in plasma, serum, and saliva (Grau et al., 1994; Hayashi and Sakamoto, 1988; Oka and Orth, 1983; Westergaard et al., 1990), to medium levels (5-50 ng/ml) in tears, follicular fluid, sperm, and seminal plasma (D'Cruz and Haas, 1989; Hayashi and Sakamoto, 1988; Westergaard et al., 1990), to high levels (50-500 ng/ml) in bile, urine, milk, and prostate fluid [**Table 3**,

(Beardmore and Richards, 1983; Gann et al., 1997; Grau et al., 1994; Hayashi and Sakamoto, 1988)]. Finally, EGF is produced as a transmembrane precursor, which is not always processed, but might still act (especially in the kidney) as a juxtacrine stimulator (Carpenter and Cohen, 1990). Thus, under physiological conditions, EGFR-expressing cells are exposed to a wide range of EGF (and EGF-like ligand) concentrations, ranging from a few to a few hundred ng/ml.

	TISSUE/ BODILY FLUIDS	CONCENTRATION
LOW	Plasma	~1 ng/ml
	Serum	~5 ng/ml
	Saliva	1-3 ng/ml
MEDIUM	Tears	10-30 ng/ml
	Follicular fluid	3-30 ng/ml
	Sperm	20-40 ng/ml
	Seminal plasma	~50 ng/ml
HIGH	Bile	~150 ng/ml
	Urine	~100 ng/ml
	Milk	~400 ng/ml
	Prostate fluid	150 ng/ml

Table 3: Concentration of EGF in human tissues and bodily fluids.
The concentration of EGF is locally regulated and varies in different tissues and bodily fluids.

After internalization, EGF and EGFR are efficiently degraded, which results in the dramatic decrease in the half-life ($t_{1/2}$) of the EGFR protein (Stoscheck and Carpenter, 1984). Accelerated internalization and degradation of activated EGFR lead to the decreased number of receptors at the cell surface, a phenomenon referred to as EGF-induced downregulation of EGFR. Thus, the process of EGFR downregulation and degradation is the major negative feedback regulatory mechanism that controls the intensity and duration of receptor signaling (Wells et al., 1990). On the other hand, EGF-receptor complexes remain active in endosomes and continue to signal after

internalization. Therefore, endocytosis has both “positive” and “negative” effects on the signaling network.

One way this could be achieved is through coupling with different entry portals. Indeed, we have shown that in HeLa cells NCE and CME are associated with distinct intracellular fates: CME is mainly involved in recycling and is required to sustained signaling, while the NCE pathway preferentially commits the receptor to degradation [of note, one third of the EGFR that is internalized via clathrin is also delivered to degradation; (Sigismund et al., 2008)]. Interestingly, this dual mechanism seems to be designed to cope with the vast variety of physiological EGF concentrations in bodily fluids; it sustains signaling and protects the receptor from degradation when the cell needs to maximize the stimulation efficiency (*i.e.,* at low EGF doses), and it protects from overstimulation when the ligand is present at high concentrations (Sigismund et al., 2008).

6.3 Everything you ever wanted to know but never dared to ask about EGFR endocytosis via clathrin-coated vesicles

CME appears to be the most efficient pathway for internalizing EGFR, active already at low EGF doses. Two important issues need to be addressed when considering CME of the EGFR: (i) what are the molecular determinants in the EGFR that are responsible for its rapid CME upon receptor activation and (ii) what are the components of the endocytic machinery that mediate EGFR internalization. Studies attempting to address these questions over the last 20 years have produced numerous observations, which allow us to reconstruct many steps in the process. However, some observations are difficult to reconcile with each other, possibly due to differences in the cell lines used and the diverse experimental settings. Here, I have summarized the evidence, highlighting common observations, as well as contrasting data, and have attempted to provide a reconciled view of CME of the EGFR.

One of the first endocytic signals characterized in the EGFR cytoplasmic tail was the AP2-binding site. Indeed, the C-terminus of EGFR (downstream of the kinase domain) contains several sequence motifs that are capable of interaction with AP2; however,

several studies have failed to establish an essential role of the EGFR-AP2 interaction in EGFR internalization. EGFR was found to directly interact with the μ2 subunit of AP2 through the Y^{974}RAL motif, although mutations in this motif did not affect EGFR internalization (Nesterov et al., 1995; Sorkin and Carpenter, 1993; Sorkin et al., 1996; Sorkin et al., 1995). Consistently, mutations in the binding interface for the YRAL motif in the μ2 protein did not affect EGFR internalization (Nesterov et al., 1999), suggesting that YRAL interaction with AP2 is not essential for EGFR internalization. Similarly, mutations of the NPxY motifs in the EGFR, which also mediate interaction with the μ2 subunit, did not reduce EGFR internalization (Chang et al., 1993). The dileucine motif (residues Leu1010/1011) was shown to be involved in the tyrosine phosphorylation of the β2 subunit of AP2, indicative of its possible role in the receptor interaction with AP2. However, again this LL motif was not essential for the internalization of the full-length EGFR (Huang et al., 2003). Double mutation of the YRAL and dileucine motifs did not impair EGFR internalization, ruling out a redundant role of these endocytic signals (Goh et al., 2010). Finally, depletion of AP2 by siRNA did not block EGFR internalization, although there is disagreement among different reports regarding the effect of AP2 depletion on EGFR endocytosis due to differential experimental conditions (Huang et al., 2004; Johannessen et al., 2006; Motley et al., 2003). In summary, while EGFR is capable of interaction with AP2, the role of this interaction remains unknown. Recent work suggests that the binding of EGFR with AP2 might be just one of the possible redundant mechanisms mediating EGFR internalization (Goh et al., 2010).

Upon ligand binding, the EGFR undergoes phosphorylation on multiple tyrosines, which represents docking sites for both signaling molecules and endocytic adaptors. Mutations of several major tyrosine phosphorylation sites in the EGFR partially reduced internalization when these EGFR mutants were expressed in fibroblasts (Chang et al., 1993; Sorkin et al., 1991). Surprisingly, mutation of the major binding sites of the Grb2 adaptor protein (Tyr1068 and Tyr1086) strongly inhibited EGF internalization in porcine aortic endothelial (PAE) cells (Jiang et al., 2003). Interestingly, EGFR mutants lacking Grb2 binding sites due to large deletions of the carboxyl-terminus were rapidly internalized

in mouse fibroblasts (Chang et al., 1993), but internalized very slowly in PAE cells (Jiang et al., 2003). It is possible that C-terminal truncations (involving residues 1022-1023) uncover cryptic internalization motifs leading to Tyr1068/1086-independent endocytosis of truncated EGFR mutants in some cells. It is also possible that Grb2 can bind to truncated mutants by the means other than pTyr1068/1086 in fibroblasts, but not in PAE cells. The key evidence for the endocytic function of Grb2 was obtained in experiments where siRNA depletion of Grb2 substantially and specifically reduced internalization of EGFR in PAE and HeLa cells (Jiang et al., 2003). Furthermore, Grb2-EGFR complexes were found in coated pits, and Grb2 was shown to be necessary for the EGFR recruitment into coated pits (Jiang et al., 2003; Johannessen et al., 2006; Stang et al., 2004). Thus, Grb2, in addition to being a fundamental signaling molecule, also has a central role in EGFR endocytosis.

One of the major Grb2-interacting proteins, Cbl, has been implicated in the regulation of EGFR internalization and degradation (Levkowitz et al., 1998). Cbl is a RING finger containing E3 Ub ligase that mediates ubiquitination of the EGFR via recruitment of E2 Ub conjugating enzymes (Levkowitz et al., 1999; Umebayashi et al., 2008). Cbl possesses a tyrosine kinase-binding (TKB) domain that can directly bind to phosphorylated Tyr1045 of EGFR (Levkowitz et al., 1999). In addition, it can also be recruited to the activated receptor through Grb2 (via its proline-rich region). Several sets of the experimental data support the role of Cbl in the CME of EGFR. Firstly, EGF-induced translocation of Cbl to CCPs has been demonstrated (de Melker et al., 2001). Secondly, overexpression of several Cbl mutants inhibited EGFR internalization in HeLa, PAE and NIH 3T3 cells (Jiang and Sorkin, 2003; Thien et al., 2001). Thirdly, chimeric proteins consisting of the Grb2 SH2 domain and Cbl could rescue EGFR endocytosis in Grb2-depleted cells, confirming the function of Cbl downstream of Grb2 (Huang and Sorkin, 2005). Fourthly, knockdown of Cbl using siRNAs resulted in partial inhibition of EGFR internalization (Huang et al., 2006). Interestingly, direct Cbl binding to pTyr1045 appears to play a minor, if any, role in the CME of EGFR, whereas Grb2-mediated interaction with EGFR is critical (Jiang and Sorkin, 2003).

In line with this latter finding, several lines of evidence exclude the requirement of Cbl-mediated EGFR ubiquitination for CME, and suggest that Cbl could play an additional role in CME, besides ubiquitinating the EGFR. Indeed, normal internalization of an EGFR mutant that lacks Tyr1045 and is weakly ubiquitinated has been observed (Jiang and Sorkin, 2003). Recently, the ubiquitination sites in EGFR were mapped in the kinase domain of the receptor (Huang et al., 2006). Mutation of these sites did not affect EGFR internalization, confirming that EGFR ubiquitination is not essential for internalization. Interestingly, add-back of two major ubiquitination sites to the multi-lysine EGFR mutant (16KR) that displayed partial inhibition of its kinase activity and, therefore, partial inhibition of internalization, restored its internalization, suggesting that Ub moieties are potentially capable of mediating EGFR internalization (Huang et al., 2007; Huang et al., 2006). This is in agreement with initial studies based on the use of a chimeric protein composed of EGFR and an Ub mutant that could not be extended by polyubiquitination. These studies showed that a single Ub was sufficient to drive internalization, although at a lower rate compared to wild-type receptor (Haglund et al., 2003). To better understand the role of Cbl in EGFR endocytosis future studies should focus on the search of additional proteins that can mediate internalization of the EGFR-Grb2-Cbl complex. The RING domain of Cbl could be necessary for ubiquitination of another protein or for the interaction with a protein other than E2 enzymes. In addition, Cbl has other domains through which it can bind proteins involved in endocytosis (Schmidt and Dikic, 2005), suggesting that it might possess an endocytic adaptor function, besides its role as E3 ligase. An example of a potential candidate that might mediate Cbl function in endocytosis is intersectin, a protein that is found in CCPs, capable of interaction with Cbl, and which is necessary for internalization and/or degradation of EGFR (Frosi et al., 2010; Martin et al., 2006).

Other proteins have been implicated in the initial step of CME of the EGFR. Among these epsin1, eps15 and eps15R have been shown to have a major role, in a redundant manner (Sigismund et al., 2005). These endocytic adaptors are associated with EGFR-containing CCPs and have been found to interact with clathrin components (Rosenthal et al., 1999; van Delft et al., 1997). Indeed, they possess domains involved in

binding to AP2 or clathrin (only in the case epsin1) or other clathrin components (e.g., intersectin). In addition, it has been proposed that they can recruit the ubiquitinated EGFR to CCPs via their UIM (Bertelsen et al., 2011; Hawryluk et al., 2006; Stang et al., 2004). However, this hypothesis is clearly incompatible with the non-essential role of EGFR ubiquitination in CME. Indeed the silencing of UIM-containing adaptors significantly affects EGFR CME (Sigismund et al., 2005), while the absence of EGFR ubiquitination has no effect. One of the aims of my PhD was also to understand the precise roles of epsin1, eps15 and eps15R in CME. Additional information regarding the relationship between these adaptors and EGFR can be found in the Introduction sections “3.3 Eps15s”, “3.4 Epsins” and “5.2 Adaptors ubiquitination”.

MATERIALS AND METHODS

1. Solutions

1.1 Phosphate-buffered saline

NaCl	137 mM
KCl	2.7 mM
Na_2HPO_4	10 mM
KH_2PO_4	2 mM

8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 were dissolved in 800 ml of distilled water. The pH was adjusted to 7.4 with HCl and the volume was brought to 1 litre with distilled H_2O.

1.2 Tris-HCl (1 M)

121.1 g of Tris base were dissolved in 800 ml distilled H_2O. The pH was adjusted to 7.4, 7.6 or 8.0 with HCl, and distilled H_2O was added to bring the volume up to 1 litre.

1.3 Tris-buffered saline (TBS)

NaCl	137 mM
KCl	2.7 mM
Tris HCl pH 7.4	25 mM

8 g of NaCl, 0.2 g of KCl, and 3 g of Tris base were dissolved in 800 ml of distilled H_2O. The pH was adjusted to 7.4 with HCl and distilled H_2O was added to bring the volume up to 1 litre.

1.4 10X SDS-PAGE running buffer

Glycine	192 mM
Tris HCl, pH 8.3	250 mM
SDS	1%

1.5 10X Western transfer buffer

Glycine	192 mM
Tris HCl, pH 8.3	250 mM

For 1X western transfer buffer, the 10X stock was diluted 1:10 with ddH_2O and 20% v/v methanol or ethanol.

1.6 50X TAE (Tris-Acetate-EDTA)

Tris base	2 M
Acetic acid	1 M
EDTA, pH 8	10 mM

The pH was adjusted to 8.5 with HCl and distilled H_2O was added to bring the volume up to 1 litre.

2. Protein buffers

2.1 1X JS buffer

HEPES, pH 7.4	50 mM
NaCl	150 mM
Glycerol	10%
Triton X-100	1%
$MgCl_2$	1.5 mM
EGTA	5 mM

2.2 1X RIPA buffer

Tris HCl, pH 7.6	50 mM
NaCl	150 mM
NP-40	1%
SDS	0.1%
Deoxycholic acid	0.5%
EGTA	5 mM

200X Protease inhibitor cocktail from Calbiochem, sodium pyrophosphate pH 7.5 20mM, sodium fluoride 250 mM, PMSF 2 mM, and sodium orthovanadate 10 mM were added to the buffer just before use.

2.3 1X YY buffer

Hepes, pH 7.5	50 mM
NaCl	150 mM
Glycerol	10%
Triton X-100	1%
EDTA	1 mM
EGTA	1 mM

200X Protease inhibitor cocktail from Calbiochem and PMSF 2 mM were added to the buffer just before use.

2.4 1X Laemmli buffer

SDS	2%
Tris HCl, pH 6.8	62.5 mM
Glycerol	10 %
Bromophenol blue	0.1 %
β-Mercaptoethanol	5 % (v/v)

SDS-PAGE sample buffer was prepared as a 5X stock solution and stored at -20°C, protected from light.

3. Reagents

Human recombinant EGF was from INALCO. Human transferrin was from SIGMA. Rhodamine-Tf was from Invitrogen. ^{125}I-EGF and ^{125}I-Tf were from Perkin Elmer.

3.1 Antibodies

For western-blot, anti-EGFR [epitope: aa 1196-2010 (*Homo sapiens*)], anti-eps15 [epitope: aa 2-330 (*Mus musculus*)], anti-eps15R [epitope: aa 216-266 (Mus musculus)], anti-epsin1/2 [(epitope: aa 249-401 of epsin1 (*Homo sapiens*)], intersectin1 [epitope: aa 583-888 (*Homo sapiens*)] and intersectin2 [epitope: aa 628-885 (*Homo sapiens*)] were produced in-house through the Antibody Facility. Anti-pY(1068)EGFR, anti-pERK1/2, anti-pAKT and anti-total-AKT were from Cell Signaling. Anti-AP2μ and anti-CHC were from Transduction BD. Anti-AP2α, anti-total-ERK1/2, anti-tubulin and anti-vinculin were from Sigma. Anti-HA was from BABCO. Anti-APPL1 was a kind gift of Pietro De Camilli (Zoncu et al., 2009). For immunoprecipitation and immunofluorescence, see below.

3.2 RNAi oligos

- Negative control siRNA

The negative control siRNA used in our assays was All Stars from Qiagen.

- Specific RNAi oligos

Oligos used in experiments reported in the figures in the Results section:

- Epsin1 (Dharmacon): GGAAGACGCCGGAGUCAUU (Huang et al., 2004b);

- Epsin2 (Stealth, Invitrogen): AAGAAAGCCGAAGGGACACAGUUAA;

- AP2, μ subunit #1 (Dharmacon): AAGUGGAUGCCUUUCGGGUCA (Motley et al., 2003b);

- AP2, μ subunit #2 (Dharmacon): UCAAGCGCAUGGCAGGCAU (Sigismund et al., 2008a);

- AP2, α subunit (Dharmacon): AAGAGCAUGUGCACGCUGGCCA (Motley et al., 2003b);

- AP2, σ subunit (Stealth, Invitrogen): CCGAGUAACCGUGCCGUUGUCGUGU;

- Grb2 (Dharmacon): CAUGUUUCCCCGCAAUUAU (Huang et al., 2004b);

- FCHo1 #1 (Stealth, Invitrogen): HSS118255;

- FCHo2 #1 (Stealth, Invitrogen): HSS151016;

- FCHo1 #2 (Stealth, Invitrogen): CCACAGCCUUCACAGAGUAUGUCCA;

- FCHo2 #2 (Stealth, Invitrogen): UGGUGGCACCUAGUGUGCUUUCCAA;

- Clathrin Heavy Chain (Stealth Invitrogen): GAAGAACUCUUUGCCCGGAAAUUUA;

- Intersectin1 (Stealth, Invitrogen): HSS109703;

- Intersectin2 (Stealth, Invitrogen): GGCAGCCUAUUCAGUUGGAGAAGAA (Henne et al., 2010);

- APPL1 (Stealth, Invitrogen): GACAAGGUCUUUACUAGGUGUAUUU (Zoncu et al., 2009).

Alternative oligos used to confirm data (data not shown):

- Epsin1 (Stealth, Invitrogen): CACAACUACUCAGAGGCGGAGAUCA;

- Epsin2 (Stealth, Invitrogen): UCUGGGUCCUUUGAGCUCUUCAGUA;

- AP2, α subunit (Dharmacon): CCCCACCUUCAUGUGCCUG (Sigismund et al., 2008a);

- Grb2 (Stealth, Invitrogen): CGUCCAGGCCCUCUUUGACUUUGAU;

- Clathrin Heavy Chain (Dharmacon): CCUGCGGUCUGGAGUCAAC (Hinrichsen et al., 2003);

3.3 TaqMAN assays for Q-PCR (Applied Biosystems)

The TaqMAN assays for Q-PCR (Applied Biosystems) of FCHo1 and FCHo2 were respectively Hs00322606_m1* and Hs00385279_m1*.

4. Cloning techniques

4.1 Agarose gel electrophoresis

DNA samples were loaded on 0.8%-2% agarose gels along with DNA markers (1 kb DNA Ladder, NEB). Gels were made in TAE buffer containing Gel Red (Biotium), according to manufacturer's instructions, and run at 80 V until desired separation was achieved. DNA bands were visualized under a UV lamp.

4.2 Minipreps

Individual colonies were used to inoculate 2 ml LB (containing the appropriate antibiotic) and grown overnight at 37°C. Bacteria were transferred to Eppendorf tubes and centrifuged for 5 minutes at 16,000*xg* using a 5415 R centrifuge. Minipreps were performed with the Wizard Plus SV Minipreps Kit (Promega) following manufacturer's instructions. The plasmids were eluted in 50 µl nuclease free H_2O.

4.3 Diagnostic DNA restriction

Between 0.5 and 5 µg DNA were digested for 2 hours at 37°C with 10-20 units of restriction enzyme (New England Biolabs). For digestion, the volume was made up to 20-50 µl with the appropriate buffer and distilled H_2O.

4.4 Large scale plasmid preparation

Cells containing transfected DNA were expanded into 250 ml cultures overnight. Plasmid DNA was isolated from these cells using the Qiagen Maxi-prep kit according to manufacturer's instructions.

4.5 Transformation of competent cells

An aliquot of competent cells TOP10 (Invitrogen) were thawed on ice for approximately 10 minutes prior to the addition of plasmid DNA. Cells were incubated with DNA on ice for 30 minutes and then subjected to a heat shock for 45 seconds at 42°C. Cells were returned to ice for an additional 5 minutes. Then, 300 µl of SOC was added and the cells were left

at 37°C for a further 60 minutes before plating them onto agar plates with the appropriate antibiotic. Two plates for each reaction were used, one plated with 100 µl of the transformed bacterial cells and the other one with the rest. Plates were incubated overnight at 37°C.

5. Constructs and plasmids

N-terminal HA-tagged mouse eps15 WT, eps15 L883A [previously described in (Woelk et al., 2006b)], eps15 6KR (aminoacidic mutations: 803, 818, 820, 837, 861, 891) and eps15 P771A were cloned into pbabe vectors, through restriction enzyme digestion (New England Biolabs) and ligation (New England Biolabs), starting from original pcDNA. The 6KR construct was generated and kindly provided by Prof. Simona Polo (IFOM, Milan, Italy). All eps15 constructs were mutagenized using the Quick Change Mutagenesis Kit (StrataGene), in order to generate shRNA resistant constructs.

6. Cell culture

6.1 Cell culture media

Human epithelial cervical cancer HeLa cells were grown in GlutaMAX™-Minimum Essential Medium (MEM, Gibco Invitrogen), supplemented with 10% Fetal Bovine Serum South American (FBS SA, Invitrogen), sodium pyruvate 1 mM (Euroclone), non-essential aminoacids (Euroclone), and 2 mM glutamine. Eps15 and/or eps15R stable KD HeLa clones were previously generated by transfecting pSUPER vectors coding for the specific targeting sequences (Sigismund et al., 2005a).

Human epithelial cervical cancer HeLa-Oslo cells, murine fibroblastic NR6 cells and kidney epithelial BSC1 cells of monkey origin were grown in Dulbecco's Modified Eagle's Medium (DMEM, Lonza), supplemented with 10% Fetal Bovine Serum South American (FBS SA, Invitrogen) and 2 mM glutamine.

Non-tumorigenic epithelial breast MCF10A cells were grown in GlutaMAX™-DMEM/F-12 (Gibco Invitrogen), supplemented with 5% Horse Serum (Invitrogen), 20 ng/ml EGF, 50 ng/ml cholera toxin, 0.01 mg/ml insulin, and 500 ng/ml hydrocortisone.

Phoenix helper cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum South American (FBS SA, Invitrogen) and 2 mM glutamine.

6.2 Transfections

- RNAi transfections

RNAi transfections were performed using LipofectAMINE RNAi MAX reagent from Invitrogen, according to manufacturer's instructions. Cells were subjected to a single reverse transfection, treated with 8 nM RNAi oligo (except for clathrin KD: 24 nM RNAi oligo) and analyzed 4 days after transfection (except for clathrin, AP2 and FCHO KD: 5 days after transfection).

- DNA transfections

For biochemical purposes (i.e., GST-protein pull-down assay), DNA transfections were performed using Lipofectamine reagent from Roche, according to manufacturer's instructions. Briefly, cells were plated at 80% confluency on 10 cm cell culture dishes. The day after cells were transfected with 5 µg DNA and 20 µl Lipofectamine. 24 hours after transfection cells were lysed and subjected to pull-down assay.

For live imaging experiments (see below "11.1 Live imaging"), DNA transfections were performed using FuGENE 6 reagent from Roche, according to manufacturer's instructions. Briefly, cells were plated at 50% confluency in 10 cm cell culture dishes. The day after cells were transfected with 5 µg DNA and 30 µl of FUGENE 6. The following day cells were plated on 28 mm coverslips and 48 hours after transfection cells were analyzed by live imaging.

6.3 Retroviral infection

Stable populations of HeLa cells expressing eps15 WT or mutant constructs (eps15 L883A and 6KR were generated by infecting the eps15/R stable KD HeLa clone using a retroviral vector. Retroviruses were produced by transfecting the Phoenix helper cell line

with 5-10 μg of DNA. 48 hours after transfection, supernatant was collected and passed through a 0.45 μm filter. After the addition of 8 μg/ml polybrene (Hexadimethrine bromide, Sigma), the supernatant was added to HeLa cells plated on 10 cm cell culture dishes. Two cycles of infection were repeated, after which the medium was replaced with standard HeLa medium (see above "6.1 Cell culture media"). 48 hours after infection, selection of infected cells was performed by adding puromycin at a concentration of 1.5 μg/ml.

7. Protein procedures

7.1 Cell lysis

After washing with PBS 1X, cells were lysed in JS or RIPA directly in the cell culture plates using a cell-scraper and clarified by centrifugation at 16,000 *xg* for 10 min at 4ºC using a 5415 R centrifuge. Protein concentration was measured by the Bradford assay (Biorad) following manufacturer's instructions.

7.2 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Gels for resolution of proteins were made from a 30%, 37,5:1 mix of acrylamide: bisacrylamide (Sigma). As polymerization catalysts, 10% ammonium persulphate (APS) and TEMED were used.

Separating gel mix

	Gel %			
	6	**8**	**10**	**15**
Acrylamide mix (ml)	2	2.7	3.3	5
1.5M Tris HCl pH 8.8 (ml)	2.5	2.5	2.5	2.5
ddH_2O (ml)	5.3	4.6	4	2.3
10% SDS (ml)	0.1	0.1	0.1	0.1
10% APS (ml)	0.1	0.1	0.1	0.1
TEMED	0.01	0.01	0.01	0.01
TOTAL (ml)	10	10	10	10

Stacking gel mix

Acrylamide mix (ml)	1.68
1M Tris HCl pH 6.8 (ml)	1.36
ddH_2O (ml)	6.8
10% SDS (ml)	0.1
10% APS (ml)	0.1
TEMED (ml)	0.01
TOTAL (ml)	10

7.3 Western Blot (WB)

Desired amounts of proteins were loaded onto 1-1.5 mm thick SDS-PAGE gels for electrophoresis (Biorad). Proteins were transferred in western transfer tanks (Biorad) to nitrocellulose (Schleicher and Schnell) in 1X Western transfer buffer (supplemented with 20% methanol or ethanol) at 30 V overnight or 100 V for 1 hour for small gels and at 30 V overnight or 0.8 A for 2 hours for large gels. Ponceau staining was used to determine the efficiency protein transfer onto the filters. Filters were blocked for 1 hour (or overnight) in 5% milk in TBS supplemented with 0.1% Tween (TBS-T). After blocking, filters were incubated with the primary antibody, diluted in TBS-T 5% milk, for 1 hour at room temperature, followed by three washes of five minutes each in TBS-T. Filters were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody diluted in TBS-T for 30 min. After the incubation with the secondary antibody, the filter was washed 3 times in TBS-T (5 minutes each) and the bound secondary antibody was revealed using the ECL method (Amersham).

7.4 Anti-Ub western blot

After SDS-PAGE, proteins were transferred on a PVDF (polyvinylidene fluoride) membrane (Immobilion P, Millipore), previously activated by incubation in 100% MeOH for 5 minutes at room temperature. Ponceau staining was avoided since it might interfere with antibody recognition. After transfer, filters were subjected to a denaturing treatment in a dedicated solution for 30 minutes at 4ºC. This treatment denatures Ub and facilitates the recognition of latent Ub epitopes by anti-Ub antibody resulting in intensification of the anti-

Ub signal. After extensive washing in TBS-T buffer, filters were blocked overnight at 4ºC in 5% BSA (dissolved in TBS-T). After blocking, filters were incubated with the antibodies against Ub, diluted in TBS-T 5% BSA, for 1 hour at room temperature, followed by 3 washes of 10 minutes each in TBS-T. Filters were then incubated with the anti-mouse horseradish peroxidase-conjugated secondary antibody, diluted in TBS-T 3% BSA, for 30 minutes at room temperature. After incubation with the secondary antibody, the filter was washed 3 times in TBS-T (5 minutes each) and the bound secondary antibody was revealed using the ECL method (Amersham).

Denaturing solution

Guanidium Chloride	6 M
TRIS, pH 7.4	20 mM
PMSF (freshly added)	1 mM
β-Mercaptoethanol (freshly added)	5 mM

7.5 Immmunoprecipitation

Lysates prepared in JS (for coimmunoprecipitiations) or in RIPA (for immunoprecipitation) buffer were incubated in the presence of specific antibodies (about 1-2 μg/mg of lysates) for 2 hours at 4ºC with rocking. Protein G Sepharose beads (Zymed) were then added, and samples were left for an additional hour at 4ºC, rocking. Immunoprecipitates were then washed 4 times in JS buffer or in RIPA buffer. To detect coimmunoprecipitation between eps15 and EGFR, EGFR was immunoprecipitated using an anti-EGFR antibody produced in-house [epitope: aa 1196-2010 (*Homo sapiens*)]. To detect coimmunoprecipitation between eps15 and AP2, HA-eps15 was immunoprecipitated using an anti-HA antibody (BABCO).

8. Protein production and purification

8.1 GST-fusion protein production

BL21 cells transformed with the indicated GST-fusion construct were picked from individual colonies and, used to inoculate 50 ml LB (containing ampicillin at 25 μg/ml). Cultures were grown overnight at 37°C. The 50 ml overnight culture was diluted in 1 litre of LB and was grown at 37°C until it reached an OD of approximately 0.6. Then, 0.5-1 mM IPTG was added and the culture was grown at 18°C overnight. Cells were then pelleted at 4000 rpm for 10 minutes at 4°C and pellets were resuspended in GST-lysis solution (20 ml/liter of bacteria). Samples were sonicated 5 times for 20 seconds each on ice and were pelleted at 14,000 rpm for 30 minutes at 4°C. 1 ml of glutathione-sepharose beads (Amersham; 1:1 slurry), previously washed 3 times with GST-lysis buffer, was added to the supernatants and samples were incubated for 3-4 hour at 4°C with rocking. Beads were washed 3 times in PBS containing 1% triton, followed by 2 times in PBS alone. Beads were finally resuspended in 1:1 volume of GST-maintenance solution and kept at -80°C.

List of the GST-proteins used:

- AP2 α ear: aa 706-938 (*Mus musculus*);
- Grb2: full length (*Homo sapiens*);
- Cbl: full length (*Homo sapiens*);
- eps15: full length (*Mus musculus*);
- eps15: EH, aa 2-330 (*Mus musculus*);
- eps15: COIL, aa 321-520 (*Mus musculus*);
- eps15: DPF, aa 501-897 (*Mus musculus*);
- eps15R: full length isoform2, aa 1-784 (*Mus musculus*);
- eps15R: DPF, aa 1-907 (*Mus musculus*);
- epsin1: full length (*Homo sapiens*);
- Crk: full length (*Homo sapiens*);
- Ub: full length, 3 in tandem (*Homo sapiens*).

GST-lysis solution

Hepes pH 7.5	50 mM
NaCl	200 mM
EDTA	1 mM
Glycerol	5%
NP40	0.1%
Protease Inhibitors (Calbiochem)	1:500

GST-maintenance solution

Tris pH 7.4	50 mM
NaCl	100 mM
EDTA	1 mM
Glycerol	10%
DTT	1 mM
Protease Inhibitors (Calbiochem)	1:500

8.2 Cleavage of GST-fusion proteins

GST-fusion proteins were cleaved with PreScission Protease (provided by the IFOM Biochemistry Unit). 1 unit of enzyme for 100 μg of fusion protein was added to the beads in the presence of GST-maintenance solution and either incubated overnight at 4°C or for 4 hours at room temperature. After cleavage the supernatant containing the cleaved protein was collected and beads were washed twice to allow the complete recovery of the cleaved protein.

8.3 GST pull-down

GST-protein were incubated either with free protein (i.e. cleaved from GST) in JS buffer (unless differently specified) or with JS cell lysates for 2h at 4ºC. Beads were washed 4 times with JS buffer plus proteases and/or phosphatases inhibitors.

8.4 EGFR in vitro ubiquitination assay

A baculovirus-produced GST-EGFR cytoplasmic tail (aa 696-end, Millipore, 1 μg) was subjected to *in vitro* auto-phosphorylation for 1.5 h at 30 ºC in kinase buffer [2 mM ATP, 10 mM $MnCl_2$, 0.8 M $(NH_4)_2SO_4$]. Phosphorylated GST-EGFR tail was then bound to Glutathione Sepharose 4B (GE Healthcare), washed thrice in YY buffer (50 mM Hepes pH 7.5, 10% glycerol, 150 mM NaCl, 1% Triton, 1 mM EDTA, 1 mM EGTA plus protease/phosphatase inhibitors) and subjected to an *in vitro* ubiquitination reaction for 1 h at 30 ºC in Ub buffer (250 mM Tris-HCl pH 7.6, 50 mM MgCl2, 1 M NaCl, 1 mM DTT, 2 mM ATP, plus ATP regeneration system, SIGMA), with the following amount of purified enzymes: 400 ng of E1 (purified from baculovirus), 1 μg of E2 (His-tagged UbcH5c/Ube2D3, purified from bacteria), 2 μg of Cbl as E3 (purified from bacteria), 4 μg of Ub (SIGMA), with or without Grb2 (purified from bacteria). Beads were then washed four times in YY buffer and eluted in Laemmli buffer. Enzymes were purified as described (Maspero *et al*, 2011). Finally, these samples were subjected to pull-down with free eps15 (equimolar to EGFR).

9. Assays with ^{125}I-EGF and ^{125}I-Tf

9.1 Receptor internalization assays with ^{125}I-EGF and ^{125}I-Tf

Cells were serum starved for at least 4 hours and then incubated at 37°C in the presence of ^{125}I-EGF or ^{125}I-Tf in binding buffer (MEM, BSA 0.1%, Hepes pH 7.4 20 mM). The concentration of radiolabelled EGF/Tf used in the assays was the following:

LOW DOSE EGF internalization	^{125}I-EGF: 1 ng/ml
Tf internalization	^{125}I-Tf: 1 μg/ml

After 2, 4, 6 minutes of EGF/Tf treatment, cells were washed 3 times in PBS, and then incubated for 5 minutes at 4°C in 300 μl of acid wash solution, pH 2.5 (acetic acid 0.2 M, NaCl 0.5 M). The solution was then removed from the cells and the radioactivity present in it was measured. This sample represents the amount of ^{125}I-EGF/Tf bound to the receptor on the cell surface. Cells were then lysed with 300 μl of a solution containing 1 M NaOH.

This sample represents the amount of internalized ^{125}I-EGF/Tf. The unspecific binding was measured at each time point in the presence of an excess of non-radioactive EGF/Tf (300X). After correction for non-specific binding, the ratio between internalized and surface-bound radioactivity was determined for each time point. This data was used to obtain the internalization curves (x-axis time in min y-axis ^{125}I-EGF (or Tf) Internalized/bound). Internalization rate constants (K_e) were extrapolated from the internalization curves (at early time points: 2, 4 and 6 min) and correspond to slopes of the best-fitting curves.

9.2 Measurement of the number of EGFRs at the cell surface by saturation binding with ^{125}I-EGF

Cells were serum starved for at least 4 hours and then incubated in the presence of 5 ng/ml of ^{125}I-EGF in binding buffer (MEM, BSA 0.1%, Hepes pH 7.4 20 mM). To reach the final concentration of 100 ng/ml, unlabeled EGF was added to the mix. Samples were cooled on ice for 30 minutes and incubated at 4°C with mix containing ^{125}I-EGF. After 6 hours, cells were washed 3 times in PBS, and then were lysed with 300 µl of a solution containing 1 M NaOH. This sample represents the amount of ^{125}I-EGF bound at equilibrium, which is dependent on the number of EGFRs on the cell surface. The unspecific binding was measured at each time point in the presence of an excess of non-radioactive EGF (300X). After correction for non-specific binding, the assay provides the quantitative measurement of the number of EGFRs for each well. By counting the number of cells plated in each well, this assay allows the determination of the number of surface EGFRs/cell.

9.3 EGFR degradation assay with ^{125}I-EGF

Cells were plated at 60% confluency in triplicate in 12-wells plates for each time point, plus an additional well to assess non-specific binding. Cells were serum-starved for at least 4 hours in binding buffer and incubated for 6 minutes at 37°C with 1.5 ng/ml of ^{125}I-EGF. Cells were put on ice, washed twice with cold PBS and incubated with mild acid/salt

wash buffer (pH 4.5) for 5 minutes (to remove remaining surface-bound ^{125}I-EGF) and then washed 2 times with cold binding buffer. These cells (^{125}I-EGF-loaded cells) contained a large pool of intracellular ^{125}I-EGF and a minimal (less than 5%) surface pool of ^{125}I-EGF. The ^{125}I-EGF-loaded cells were further incubated in binding medium at 37°C for a chase time (0', 30', 60', 90'), and surface labeled ligand was determined at each time point, as well as the amount of degraded and intact ^{125}I-EGF in the medium and in the cytoplasm, distinguished by TCA precipitation. The degraded EGF was calculated as the sum of the TCA soluble ^{125}I-EGF recovered in the medium and in the cytoplasm at each time point.

10. EGFR signaling

HeLa cells (800˙000) or MCF10A cells (500˙000) were plated on five 10 cm cell culture dishes. The following day cells were serum starved for 16 hours and stimulated for the indicated time points with either low dose (1.5 ng/ml) or high dose (100 ng/ml) EGF. At least 30 µg of total cell lysate were loaded on a 10% SDS polyacrylamide gel. Western blots were performed as described in section 7.4.

11. Fluorescence studies

11.1 Immunofluorescence

Cells were plated on glass coverslips pre-incubated with 0.1% gelatin in PBS at 37°C for 30 minutes. Cells were fixed in 4% paraformaldehyde (in 1X PBS) for 10 minutes, washed with PBS and permeabilized in 0.1% Triton X-100, BSA 0.2% in 1X PBS for 8 minutes at room temperature. To prevent non-specific binding of the antibodies, cells were then incubated with 1X PBS in presence of 2% BSA for 30 minutes at room temperature. Next, cells were incubated for 1 hour with primary antibody in 1X PBS in presence of 0.2% BSA (anti-HA 1:1,000 Babco), washed 3 times with 1X PBS and incubated for 30 minutes with fluorescently labeled secondary antibodies (Amersham). After 3 washes with PBS, nuclei were DAPI-stained for 5 minutes and washed again 3 times with 1X PBS. Coverslips were immediately mounted with moviol and examined under a wide-field fluorescence

microscope (Olympus). Images were further processed with the Adobe Photoshop software (Adobe). To detect only surface EGFR (anti-EGFR 1:50, Ab-1, Calbiochem) permeabilization step was avoided. When indicated, cells were stimulated with fluorescently labeled Tf (Rhodamine-Tf 1:100, Invitrogen) for the indicated times.

11.2 Live imaging

TIRFM was performed on HeLa cells transiently co-expressing rat brain mRFP-LCa (kindly provided by Dr. K. Rottner, University of Bonn) and the AP2 rat brain σ2-adaptin fused to EGFP (kindly provided by Dr. T. Kirchhausen, Harvard Medical School, see above "6.2 Transfections – DNA transfection") using a 63X 1.47 NA objective (Leica) and a iXon 885 camera. Cells were kept under the microscope at 37°C in Dulbecco's Modified Eagle's Medium (DMEM, Lonza) w/o phenol red, supplemented with 3% Fetal Bovine Serum South American (FBS SA, Invitrogen), Hepes pH 7.4 20 mM and 2 mM glutamine. Time frame of acquisition was 1.5 sec for 10 min and the exposure time was 150 msec for each channel.

12. Cell migration assay

Cell migration assays were performed using a BD Boyden Chamber (BD Biosciences) with 8 µm pores. Both chambers were filled with medium. The lower chamber contained serum-starved medium, EGF (1.5 ng/ml) or complete medium. Serum-starved cells (4 x 10^4 cells/well) were seeded into the upper chamber of the transwell and allowed to migrate overnight at 37°C. Three replicates for each condition were performed. After the incubation period, cells remaining in the upper chamber were washed away with PBS and removed by a cotton swab. Fixed cells were then stained with DAPI. Cells were counted in three randomly chosen fields using an inverted fluorescence microscope (10X magnification).

13. Densitometry and statistical analysis

Quantification of blots was performed with ImageJ. Average results, calculated from at least three independent experiments, are shown. Error bars in the plots represent the standard deviation of the mean. All statistical analyses were performed using Excel. The statistical significance was obtained applying *t*-test.

AIM OF THE PROJECT

CME is a well-characterized and complex process involving multiple proteins and endocytic signals, which function to internalize cell surface cargoes and deliver them to early endosomes. Most studies examining the molecular mechanisms of CME are focused on constitutive endocytosis, as exemplified by TfR, and often conclusions have been extended to other types of cargoes. However, it is emerging that distinct receptors display different molecular requirements to undergo CME and, thus, it is not possible to derive a generalized CME mechanism valid for all cargoes. This is particularly true for ligand-dependent cargoes, such as the EGFR. For this type of cargo, a reciprocal regulation exists between the endocytic and signaling machineries: on the one hand, signaling receptors regulate their own endocytosis, while on the other hand, endocytosis controls the type and duration of the downstream signaling response, and ultimately the biological output (Sigismund et al., 2012). Despite the importance of endocytosis in regulating signaling, the mechanisms controlling EGFR internalization, and the precise impact of endocytosis on EGFR signaling, are not fully understood.

The overall aim of this thesis was to address this knowledge gap by characterizing novel mechanisms of CME of the EGFR. In particular, we were interested in identifying alternative endocytic adaptors that function independently of AP2 in CME of the EGFR. AP2 is the principal non-clathrin constituent of purified CCVs and has long been considered as the major endocytic adaptor for CME (Conner and Schmid, 2003). However, while this holds true for TfR, it is emerging that a different scenario exists for other types of cargoes (Maurer and Cooper, 2006; Motley et al., 2003). Indeed, knockdown (KD) experiments have shown that CME of EGFR can still occur in absence of detectable AP2 at the PM (Motley et al., 2003; Huang et al., 2004; Johannessen et al., 2006), suggesting the existence of an AP2-independent pathway of CME of the EGFR. However, the molecular nature of this alternative CME pathway has not yet been characterized.

Thus, we aimed to characterize the AP2-independent clathrin adaptors involved in EGFR internalization and to elucidate the mechanisms of their action. To this end, we

employed a systematic approach, based on the combinatorial ablation of candidate endocytic adaptor proteins, to identify the molecular players involved in CME of the EGFR following ligand-induced activation (see Results Section 1). Moreover, we employed adaptor mutants to investigate the involvement of adaptor UIM/monoUb in CME of the EGFR (see Results Section 3). Importantly, since EGFR is a signaling cargo, we investigated whether any observed endocytic phenotypes were associated with specific receptor fates/signaling activation and biological readouts, in order to gain a better understanding of how the endocytic machinery, and in particular the different endocytic adaptors, affect cellular response (see Results Section 2).

RESULTS

1. Molecular heterogeneity of EGFR containing CCPs: the genetic approach

1.1 Experimental set up

To investigate the involvement of different endocytic adaptors specifically in CME of the EGFR, we analyzed the impact of their knockdown (KD) on EGFR internalization in HeLa cells by performing ^{125}I-EGF internalization assays, using the following experimental conditions:

1) Non-saturating concentration of ligand (1 ng/ml). At this dose, only CME, but not NCE, is active, thus ensuring that any observed effects can be directly ascribed specifically to the clathrin pathway. Indeed, silencing of the CHC causes approximately 80% inhibition of EGFR internalization at this ligand concentration [(Huang et al., 2004; Motley et al., 2003; Sigismund et al., 2008) and see also **Figure 14A,C**]. We obtained similar results using pitstop (**Figure 13A**, on the left), a recently discovered pharmacological tool that specifically inhibits CME (von Kleist et al., 2011). Considering that TfR internalization is impaired by up to 95% both in clathrin KD cells [(Huang et al., 2004; Motley et al., 2003; Sigismund et al., 2008) and see also **Figure 14B-C**] and in pitstop treated cells (**Figure 13A**, on the right), it is likely that the residual 20% of EGFR internalization observed in these cells is mediated by an alternative endocytic pathway that is insensitive to clathrin depletion. This residual 20% of EGFR internalization is also unaffected by filipin treatment (a cholesterol-interfering compound), highlighting that this clathrin-independent pathway is not NCE (Sigismund et al., 2008), but rather a third kind of internalization pathway used by EGFR.

2) The ligand was applied to the cells *in continuum*, without any ligand pre-binding on ice. This represents a more physiological situation and prevents molecular rearrangements that could mask internalization defects.

3) To evaluate the specificity of adaptors in terms of cargo-selectivity, we measured clathrin-dependent constitutive internalization of TfR in the KD cells using ^{125}I-Tf, in parallel

to ligand-induced EGFR internalization. The endocytic rate constant [K_e; (Wiley and Cunningham, 1982)] was measured as described in the section Materials and Methods.

4) HeLa cells were used as a cell model system; however, the most critical results were also confirmed in other cell lines. Eps15 and/or eps15R have been stably knocked down using pSUPER vectors coding for the specific short hairpin RNA [shRNA; (Sigismund et al., 2005)], while all the other proteins have been silenced transiently. The use of HeLa cells (together with the development of more efficient transfection conditions) allowed us to easily obtain multiple KD combinations, silencing up to 5 proteins contemporarily, using low concentrations of siRNA oligos, thereby avoiding toxic effects. This allowed us to evaluate redundancy and/or genetic interactions among the endocytic adaptors. At least 2 different targeting oligos against each adaptor was used with similar results.

5) As control we compared initially HeLa cells and a HeLa cells clone stably transfected with pSUPER empy vector, both treated with control oligo (see Materials and Methods). They behave exactly the same (**Figure 13B**), and in successive experiments we used HeLa cells treated with control oligo, referred in the following figures as “control”.

6) Cell surface EGFR number was measured in the KD cells using the ^{125}I-EGF saturation binding assay. In HeLa cells, the number of surface EGFRs was calculated to be ~250,000 receptors/cell, and this number was unchanged when endocytic adaptors were silenced (except for FCHo1 #1 and FCHo2 #1, see Figure 21 C-D). This result allowed us to exclude an involvement of the adaptors in EGFR biosynthesis/delivery to the PM, and in basal receptor turnover. Moreover, this control permitted us to exclude that phenotypes relating to internalization, signaling and trafficking were due to different starting numbers of surface EGFRs in KD cells with respect to control cells. As an example, **Figure 13C** shows number of surface EGFRs upon the indicated proteins silencing condition.

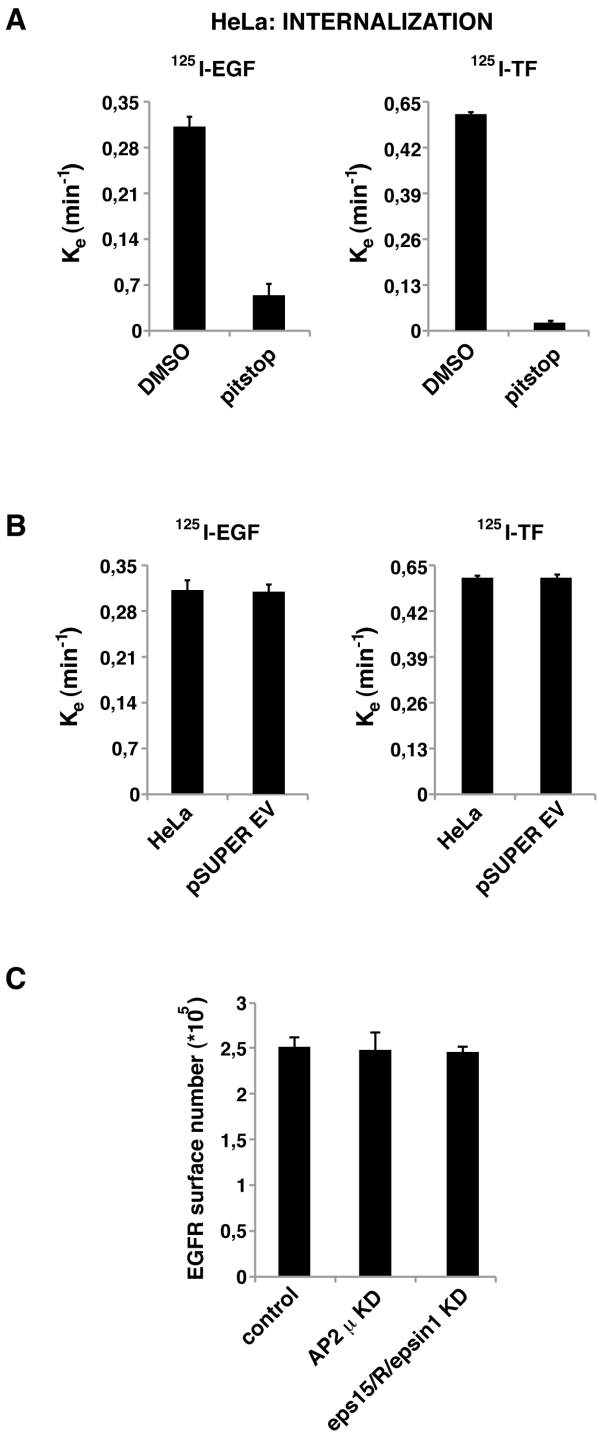


Figure 13: Experimental set up.
A. Analysis of ^{125}I-EGF and ^{125}I-Tf internalization upon pitstop treatment. Kinetics of ^{125}I-EGF (1 ng/ml) and ^{125}I-Tf (1 μg/ml) internalization were followed at early time points (0-6 min) following 15 minutes of preincubation with either DMSO, as control, or pitstop (90 μM) in HeLa cells. **B. Analysis of ^{125}I-EGF and ^{125}I-Tf internalization in control oligo treated HeLa cells and in control oligo treated pSUPER empy vector HeLa cells clone.** Kinetics of ^{125}I-EGF (1 ng/ml) and ^{125}I-Tf (1 μg/ml) internalization were followed at early time points (0-6 min) in HeLa cells and in a HeLa cells clone stably transfected with pSUPER empy vector (pSUPER EV), both treated with control oligo. **A-B.** Internalization constants (K_e) were extrapolated from the internalization curves and correspond to the slopes of the best-fitting curves (see Materials and Methods). Results are MEAN+/-SDEV of three independent experiments. **C. Surface EGFR numbers upon AP2 KD and eps15/R/epsin1 KD in HeLa cells.** The number of EGFRs on the cell surface was measured by saturation binding upon AP2 μ KD and eps15/R/epsin1 KD in HeLa cells. Results are MEAN+/-SDEV of at least six independent experiments.

1.2 AP2 is dispensable for EGFR, but essential for TfR, internalization

Several studies investigating the impact of AP2 KD on EGFR internalization have produced varying results, possibly due to the different experimental settings [(Hinrichsen et al., 2003; Huang et al., 2004; Motley et al., 2003); see also Introduction paragraph **"**6.3 Everything you ever wanted to know but never dared to ask about EGFR endocytosis via clathrin-coated vesicles**"**]. Therefore, we first determined the effect of AP2 KD on EGFR clathrin internalization in our experimental setting using different siRNA oligos directed against two different AP2 subunits (α and μ). Note that silencing of α subunit causes reduction of μ subunit protein level, as previously reported (Motley et al., 2003). In all conditions tested, we scored a reduction in the EGFR internalization rate constant of about 50%, which was less than that observed upon clathrin ablation (~80% reduction; **Figures 14A,C**). In contrast, ^{125}I-Tf internalization is blocked at the same degree in AP2 and clathrin KD cells, indicating that TfR internalization is entirely clathrin- and AP2-dependent (**Figures 14B-C**). Importantly, these results have been confirmed with another set of siRNA oligos targeting both clathrin and AP2 (see Materials and Methods).

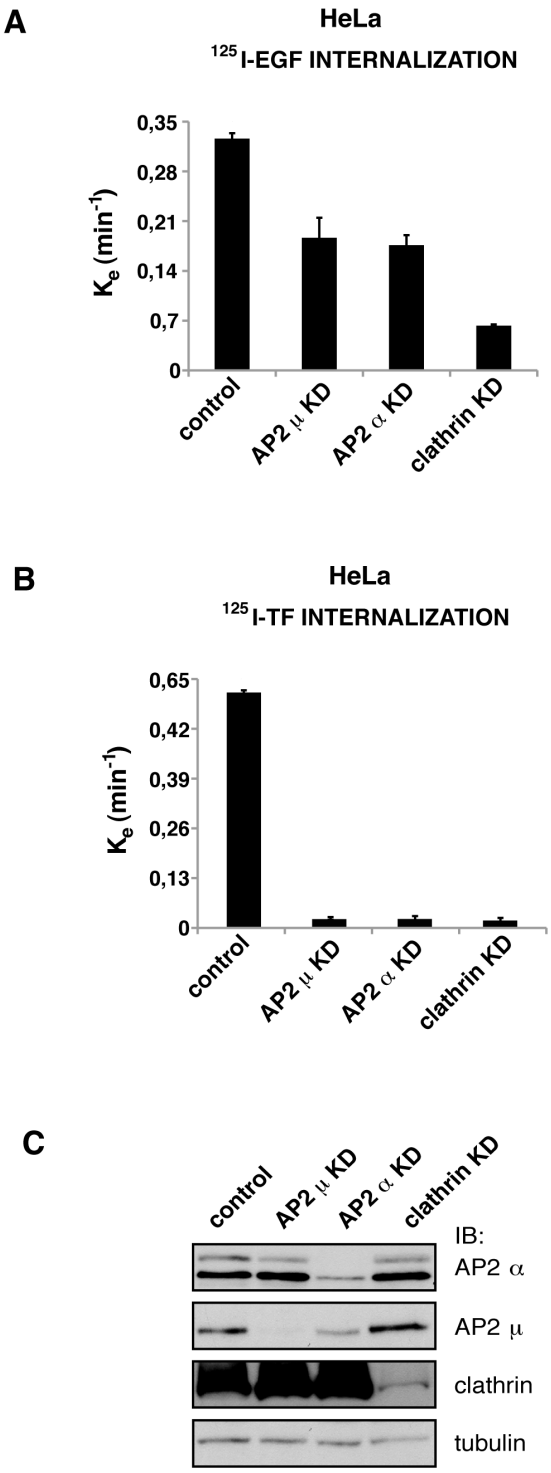


Figure 14: Assessment of AP2 role in CME of EGFR.
A. Analysis of ^{125}I-EGF internalization upon AP2 and clathrin KD. Kinetics of ^{125}I-EGF internalization were followed at early time points (0-6 min) using low EGF concentrations (1 ng/ml). **B. Analysis of ^{125}I-Tf internalization upon AP2 and clathrin KD.** Kinetics of ^{125}I-Tf (1 μg/ml) internalization were followed at early time points (0-6 min). **A-B.** Internalization constants (K_e) were extrapolated from the internalization curves and correspond to the slopes of the best-fitting curves (see Materials and Methods). Results are MEAN+/-SDEV of at least five independent experiments. **C. Levels of AP2 and clathrin KD.** The efficiency of KD was determined by immunoblotting using specific antibodies, as indicated (immunoblots are representative of at least five independent experiments). Clathrin immunoblotting was performed with an anti-CHC antibody. Tubulin was used as protein loading control. We calculated a KD level of at least 95% by densitometry analysis in all samples.

Considering the difference in EGFR internalization between AP2 KD and clathrin KD cells (~30%), we hypothesized that a clathrin-dependent, AP2-independent, internalization pathway exists that is specific for EGFR, but not for TfR, as has been previously suggested (Motley et al., 2003; Sigismund et al., 2008). This idea, however, has been a point of controversy in the endocytic field [see Introduction paragraph **"**3.1 Adaptor protein 2 (AP2)**"**].

To test further our hypothesis, we extended our analysis to other cell lines, i.e. another HeLa cell clone (HeLa-Oslo) and a normal mouse fibroblast cell line [NR6 cells reconstituted with physiological EGFR levels, (Sigismund et al., 2005)]. HeLa-Oslo cells are a clone of HeLa that, contrarily to our HeLa cells (referred to here simply as "HeLa"), were found to lack the NCE pathway (Kazazic et al., 2006). Despite this difference, we observed the same quantitative effect of AP2 KD on CME of the EGFR as in our HeLa strain (**Figures 15A-B**). This result was also confirmed in NR6 cells (**Figure 15C-D**), which displayed ~30% clathrin-dependent internalization upon AP2 KD, despite the good KD level. To further substantiate this data we are planning to use cells derived from AP2 conditional knockout mice in collaboration with Volker Haucke (Freie Universität, Berlin).

These data confirm the existence of clathrin-dependent AP2-independent pathways of EGFR internalization both in cancer and normal cells, and suggest an involvement of alternative endocytic adaptors in EGFR CME.

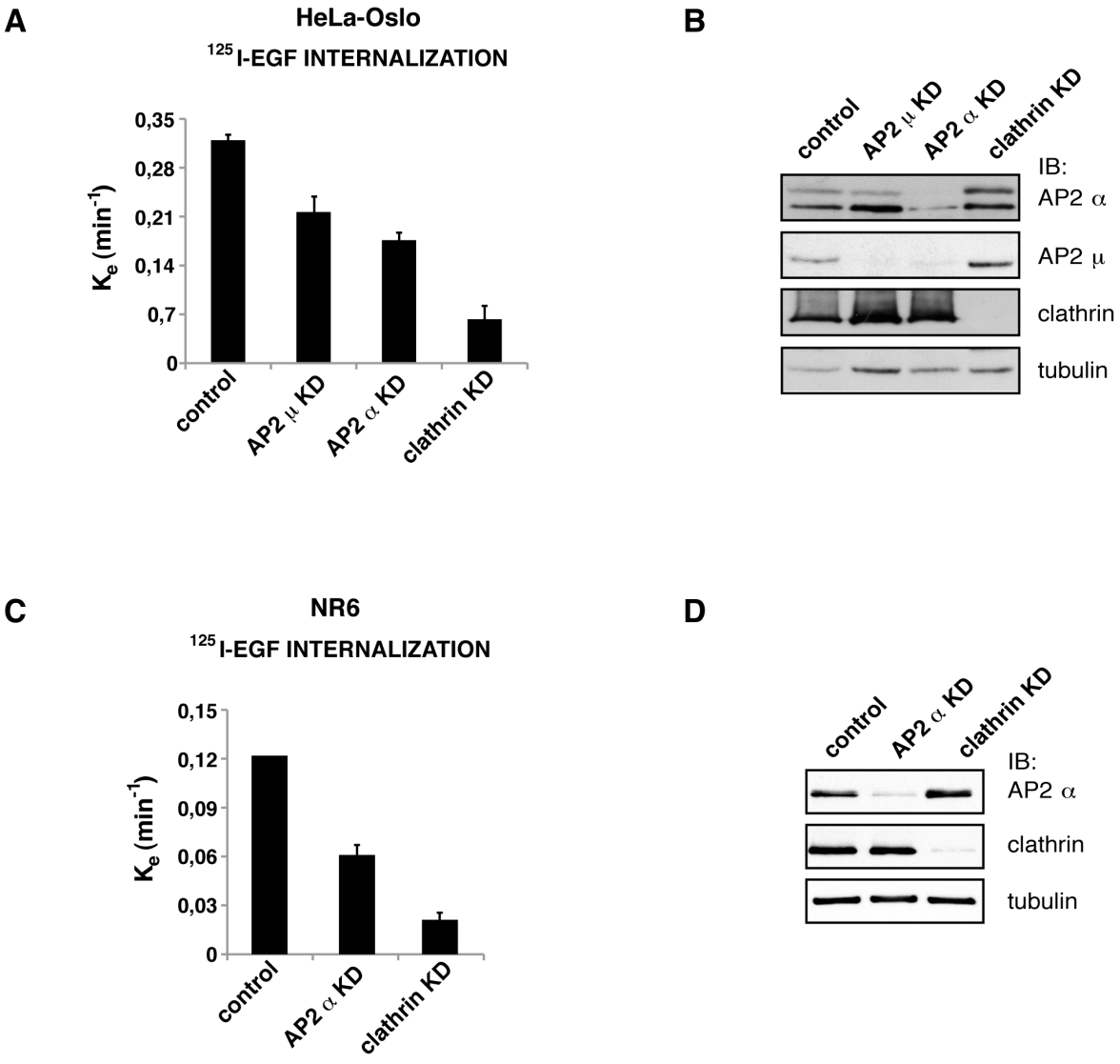


Figure 15: Confirmation of AP2 role in CME of EGFR in various cell lines.
A, C. Analysis of ^{125}I-EGF internalization upon AP2 and clathrin KD in (A) HeLa-Oslo and (C) NR6 cells. Kinetics of ^{125}I-EGF internalization were followed at early time points (0-6 min) using low concentrations of EGF (1 ng/ml). Internalization constants (K_e) were extrapolated from the internalization curves and correspond to the slopes of the best-fitting curves (see Materials and Methods). Results are MEAN+/-SDEV of three independent experiments. **B, D. Levels of AP2 and clathrin KD in (B) HeLa-Oslo and (D) NR6 cells.** The efficiency of AP2 and clathrin KD was determined by immunoblotting using specific antibodies, as indicated (immunoblots are representative of three independent experiments). Clathrin immunoblotting was performed with an anti-CHC antibody. Tubulin was used as protein loading control. We calculated a KD level of at least 95% by densitometry analysis in all samples.

1.3 Eps15, eps15R, epsin1 and epsin2 are involved in CME of EGFR

In seeking for alternative adaptors to AP2 working in EGFR clathrin-dependent internalization, the most obvious candidates were epsins and eps15s. Indeed, we have previously shown that the simultaneous KD of eps15, eps15R and epsin1 reduces the EGFR internalization rate constant by ~50% (Sigismund et al., 2005). Since the epsin family consists of 3 members, epsin1 and 2 that are broadly expressed, and epsin3 whose expression is restricted to a few cell types (see Introduction "3.4 Epsins"), we first determined their expression at the mRNA and/or protein level in our cell model systems, HeLa and HeLa-Oslo. To extend our analysis, and to have positive controls for epsin3 expression, we also tested their expression in normal and tumor breast epithelial cells, MCF10A and BT474 cells, respectively. Epsin1 and 2 were expressed in all cells tested; epsin2 was expressed at lower mRNA levels compared with epsin1 (**Figures 16A-B**). In contrast, epsin3 was not expressed in HeLa and HeLa-Oslo cells, but was expressed at low levels in normal breast epithelial cells MCF10A and at high levels in BT474 epithelial breast tumor cells as expected (**Figures 16A-B**).

Based on this data, we investigated the involvement of epsins and eps15s in EGFR clathrin-dependent internalization by systematically knocking down the expression of eps15, eps15R, epsin1 and epsin2, alone and in different combinations among them and with AP2.

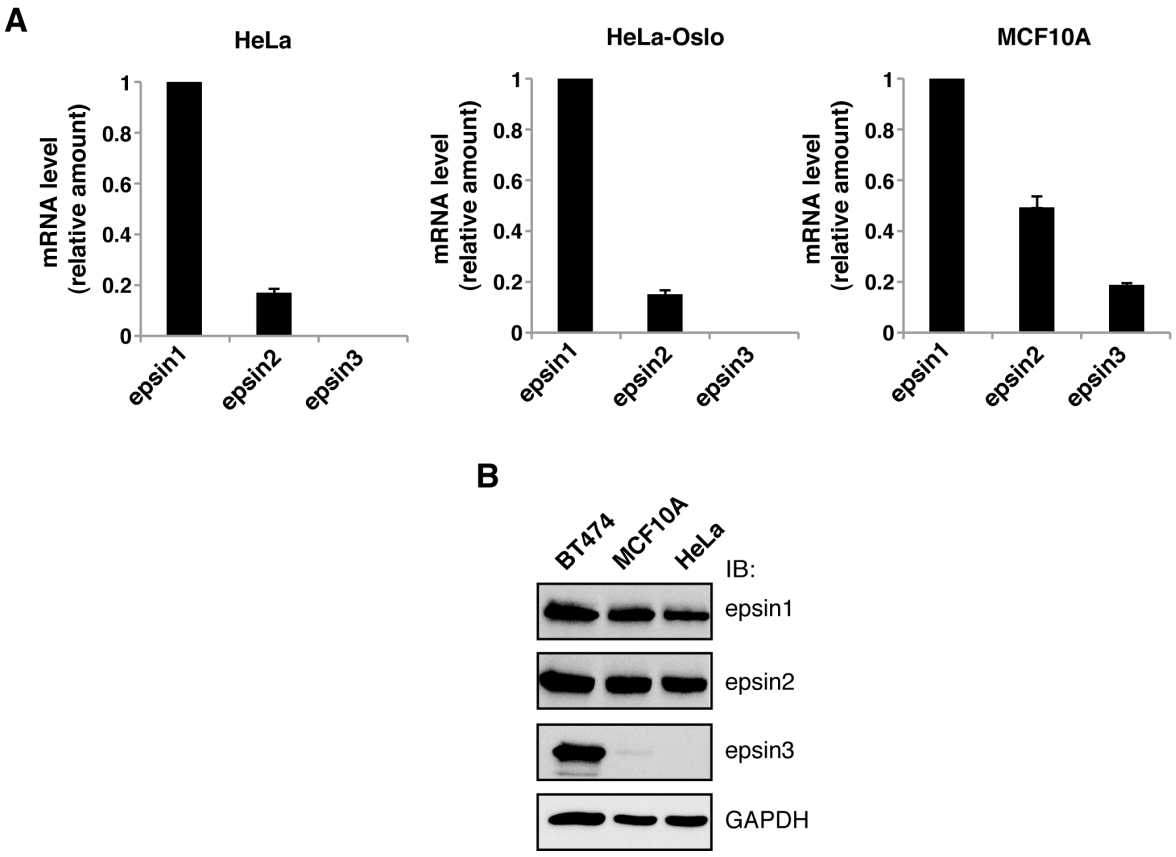


Figure 16: Analysis of epsin family member expression in different cell lines.
A. mRNA levels of epsin1, 2 and 3 were measured by qPCR in HeLa, HeLa-Oslo and MCF10A cells. Values were expressed as relative amount with respect to the most abundant epsin, epsin1. Note that mRNA of epsin3 is undetectable in HeLa and HeLa-Oslo cells. Results are MEAN+/-SDEV of three independent experiments. **B.** Protein levels of epsin1, 2 and 3 were assessed by immunoblotting with specific antibodies, as indicated, in BT474, MCF10A and HeLa cells. GAPDH was used as protein loading control (immunoblots are representative of three independent experiments).

To obtain the different KD cells, we took advantage of previously generated HeLa clones in which eps15 and/or eps15R have been stably knocked down using pSUPER vectors coding for the specific short hairpin RNA [shRNA; (Sigismund et al., 2005)]. These stable clones present impaired EGFR internalization at levels comparable to those reported in literature upon transient siRNA transfection: ~10% reduction in the single KDs and ~20% reduction in the double KD [**Figures 17A-B** (Huang et al., 2004)]. These clones allowed us to carry out experiments in a more reproducible setting and represent a good starting point to obtain multiple KDs.

Of note, three different eps15 isoforms have recently been described: a, b and S (Chi et al., 2011; Fazioli et al., 1993; Roxrud et al., 2008). Isoform ‘a’ represents the full-

length variant, which contains three N-terminal EH domains and is known to be localized at the PM. Isoform 'b' lacks the three EH domains while contains a short unique N-terminal region and localizes to Hrs-positive microdomains on endosomes. Isoform 'S' lacks the 111 C-terminal amino acids present in the full-length protein, but contains the three N-terminal EH domains, and localizes to the Rab11-positive recycling endosomes. By western blot analysis, we only detected a single 140 KDa band in HeLa cells (**Figure 17B**), which corresponds to the full-length isoform 'a'. However, we cannot exclude a low, but biologically significant level of expression of the other two isoforms. Since we employed shRNAs that target the EH domain which is present only in isoforms 'a' and 'S', we can conclude that the experiments described here are pertinent only to these isoforms and not to isoform 'b'. This is important since it means that any effects observed on EGFR trafficking and signaling can be associated with a role of eps15 in internalization and not in endosomal sorting.

Transient KD of epsin1 or 2 by siRNA in HeLa cells caused a slight reduction in EGFR internalization kinetics (~20%), while the double KD had an additive effect compared with the single KDs (~40%, **Figures 17C-D**).

These results indicate that eps15, eps15R, epsin1 and epsin2 are working in CME of EGFR. The defects observed can either be AP2-related or AP2-independent.

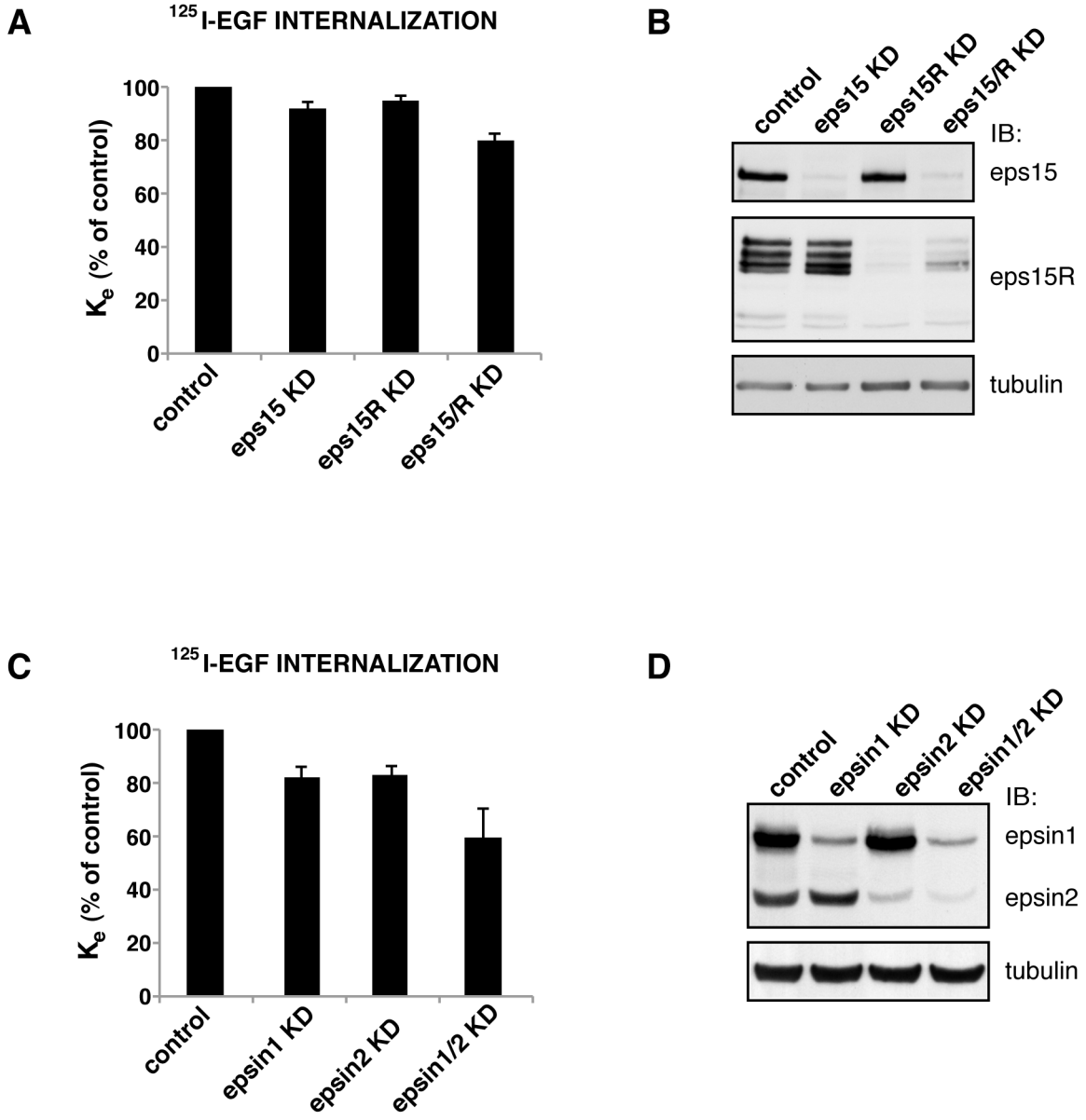


Figure 17: Assessment of the involvement of eps15, eps15R, epsin1 and epsin2 in CME of EGFR.

A, C. Analysis of ^{125}I-EGF internalization following KD of different endocytic adaptors in HeLa cells. Kinetics of ^{125}I-EGF internalization were analyzed at early time points (0-6 min) using low concentrations of EGF (1 ng/ml) in HeLa cells (**A**) stably silenced for eps15 ad eps15R alone or together (eps15/R KD) or (**C**) transiently silenced for epsin1 and epsin2, alone or together (epsin1/2 KD). Internalization constants (K_e) were extrapolated from the internalization curves and correspond to the slopes of the best-fitting curves (see Materials and Methods). Results are MEAN+/-SDEV of at least four independent experiments and expressed as % of the control oligo treated HeLa cells. **B, D. Efficiency of adaptor KD in stably (B) and transiently (D) silenced HeLa cells.** The efficiency of KD of the indicated adaptors was determined by immunoblotting using specific antibodies, as indicated. Tubulin was used as protein loading control (these are representative of at least four independent experiments). We calculated a KD level of at least 95% by densitometry analysis in all the samples.

We followed on adding transient silencing of epsin1 in the stable single and/or double eps15/eps15R KD HeLa cell clones. Addition of epsin1 interference to the single eps15 or eps15R stable KDs, do not cause any further reduction respect to single epsin1 alone (~20% reduction in EGFR internalization rate), possibly indicating that we are affecting the same process of EGFR internalization, as when silencing epsin1 alone. On the contrary, the transient KD of epsin1 in the double eps15/eps15R (hereafter referred to as eps15/R) stable KD HeLa clone resulted in a ~55% decrease in EGFR internalization rate, confirming previously published data [**Figures 18A,C**; (Sigismund et al., 2005)]. Of note, the defect observed in eps15/R/epsin1 KD was higher than the sum of the defects observed in single epsin1 KD and in the double eps15/R KD, and this difference was statistically significant (see asterisk in **Figure 18A**). This result might indicate that the three proteins are playing a synergistic function. Again, the defects observed can either be AP2-related or AP2-independent.

In contrast, transient KD of epsin2 in the double eps15/R KD cells had little effect on EGFR internalization rate, with triple eps15/R/epsin2 KD cells displaying a similar decrease in K_e as double eps15/R KD cells (**Figures 18A,C**). Finally, ablation of epsin2 in eps15/R/epsin1 triple KD cells caused slight further reduction of the phenotype (~65%; **Figures 18A,C**). These results point to a differential involvement of epsin1 and epsin2 in EGFR internalization and strengthen the hypothesis of redundancy between epsin1, eps15 and eps15R.

To assess the specificity of this phenotype, we performed in parallel ^{125}I-Tf internalization assays. In this case, the triple eps15/R/epsin1 KD had a less pronounced and additive effect (**Figures 18B-C**), indicating that the redundant action of these adaptors is specific for EGFR. Since internalization of TfR is totally AP2-dependent and eps15/R/epsin1 KD perturb only partially this AP2-related pathway, while in the case of EGFR internalization AP2 KD and eps15/R/epsin1 KD showed a comparable extent of defect, it is possible to speculate that the EGFR internalization defect observed upon eps15/R/epsin1 KD could be partially AP2-dependent and -independent.

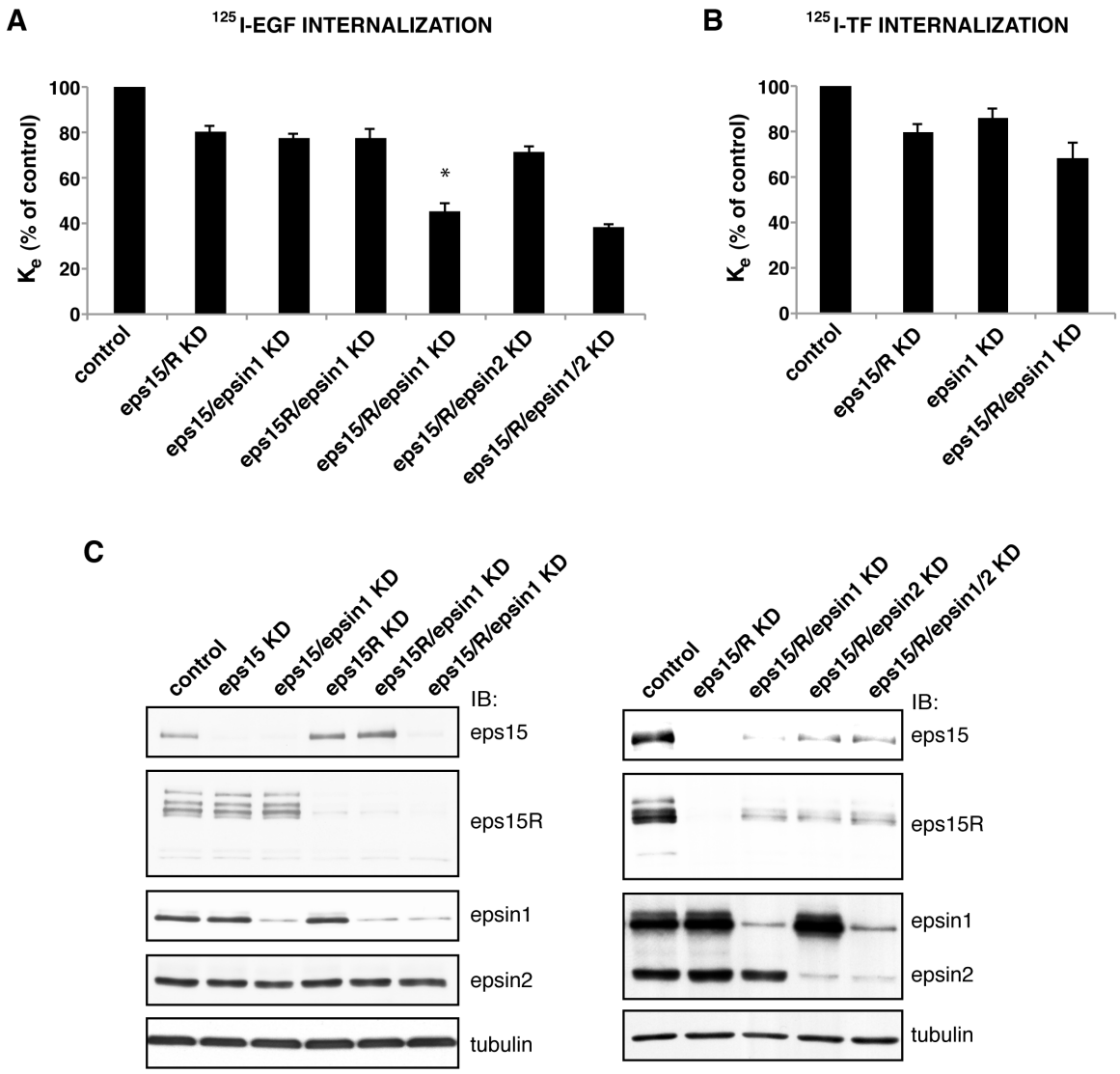


Figure 18: Assessment of the effect of the combinatorial interference among eps15, eps15R, epsin1 and epsin2 on CME of EGFR.
A, B. Analysis of ^{125}I-EGF (A) and ^{125}I-Tf (B) internalization upon KD of different adaptors in HeLa cells. Kinetics of (**A**) ^{125}I-EGF (1 ng/ml) and (**B**) ^{125}I-Tf (1 μg/ml) internalization were followed at initial time points (0-6 min) upon silencing of the indicated adaptors in HeLa cells: eps15/R were silenced stably, while epsin1/2 were silenced transiently. Internalization constants (K_e) were extrapolated from the internalization curves and correspond to the slopes of the best-fitting curves (see Materials and Methods). Results are MEAN+/-SDEV of at least three independent experiment and expressed as % of the control oligo treated HeLa cells. * pvalue < 0,01. **C. Efficiency of adaptor KD.** The KD level of the different adaptors was determined by immunoblotting using specific antibodies, as indicated. Tubulin was used as protein loading control (these are representative of at least three independent experiments). We calculated a KD level of at least 95% by densitometry analysis in all the samples.

1.4 Eps15, eps15R and epsin1 work redundantly in CME of EGFR in a pathway independent from AP2

Considering that AP2 KD and the triple eps15/R/epsin1 KD both resulted in a ~50% decrease in EGFR internalization rate, it is possible that these adaptors could function in the same or distinct endocytic pathways. To investigate this issue, we analyzed the effect of knocking down AP2 in combination with the other adaptors. In this set of experiments, we used siRNA oligos targeting the AP2 μ subunit because they gave the best level of AP2 KD.

The combination of AP2 KD with single (epsin1/AP2, epsin2/AP2, eps15/AP2 and eps15R/AP2) or double KDs (epsin1/2/AP2 and eps15/R/AP2) gave exactly the same effect as the AP2 KD alone (**Figures 19A-B**), possibly meaning that partial defects showed in Figure 17 are due to impact on the same pathway where AP2 is operating. However, we observed that the simultaneous silencing of the four adaptors eps15/R/epsin1/AP2 reduced EGFR internalization rate to clathrin KD levels (**Figures 19A-B**), indicating that eps15/R/epsin1 and AP2 can also function in distinct pathways. The addition of epsin2 KD to the eps15/R/epsin1/AP2 KD did not cause any worsening of the phenotype (**Figures 19A-B**). Indeed, eps15/R/epsin1 could be responsible of 30% residual AP2-independent CME of EGFR. The higher defect observed in eps15/R/epsin1 KD cells (i.e., ~55%) is possibly due to the contemporary partial impairment of the AP2-dependent pathway (as also highlighted by partial defects in single and double combinations of eps15, eps15R and epsin1; Figures 17-18). These results support the idea of a functional overlap between the three adaptors, meaning that any one of them (either epsin1, eps15 or eps15R) is sufficient to sustain AP2-independent EGFR clathrin internalization.

However, from the vesicle formation point of view, these results can be subjected to different interpretations: AP2 and eps15/R/epsin1 are cooperating - through different mechanisms - in building the same EGFR-containing vesicles and their alternative depletion simply slows down the same process; AP2 and epsin1/eps15/R are involved in the formation of different types of clathrin vesicles, meaning that their alternative depletion

selectively blocks the formation of one type of vesicle. It is even plausible that the real scenario is a mixed situation.

Together, these data prompted us to test the hypothesis that eps15, eps15R and epsin1 can act as specialized clathrin adaptors in EGFR internalization process, in a redundant manner and via an AP2-independent mechanism. Importantly, these adaptors do not appear to have the same function in TfR endocytosis, pointing to specific mechanisms of internalization between distinct cargoes.

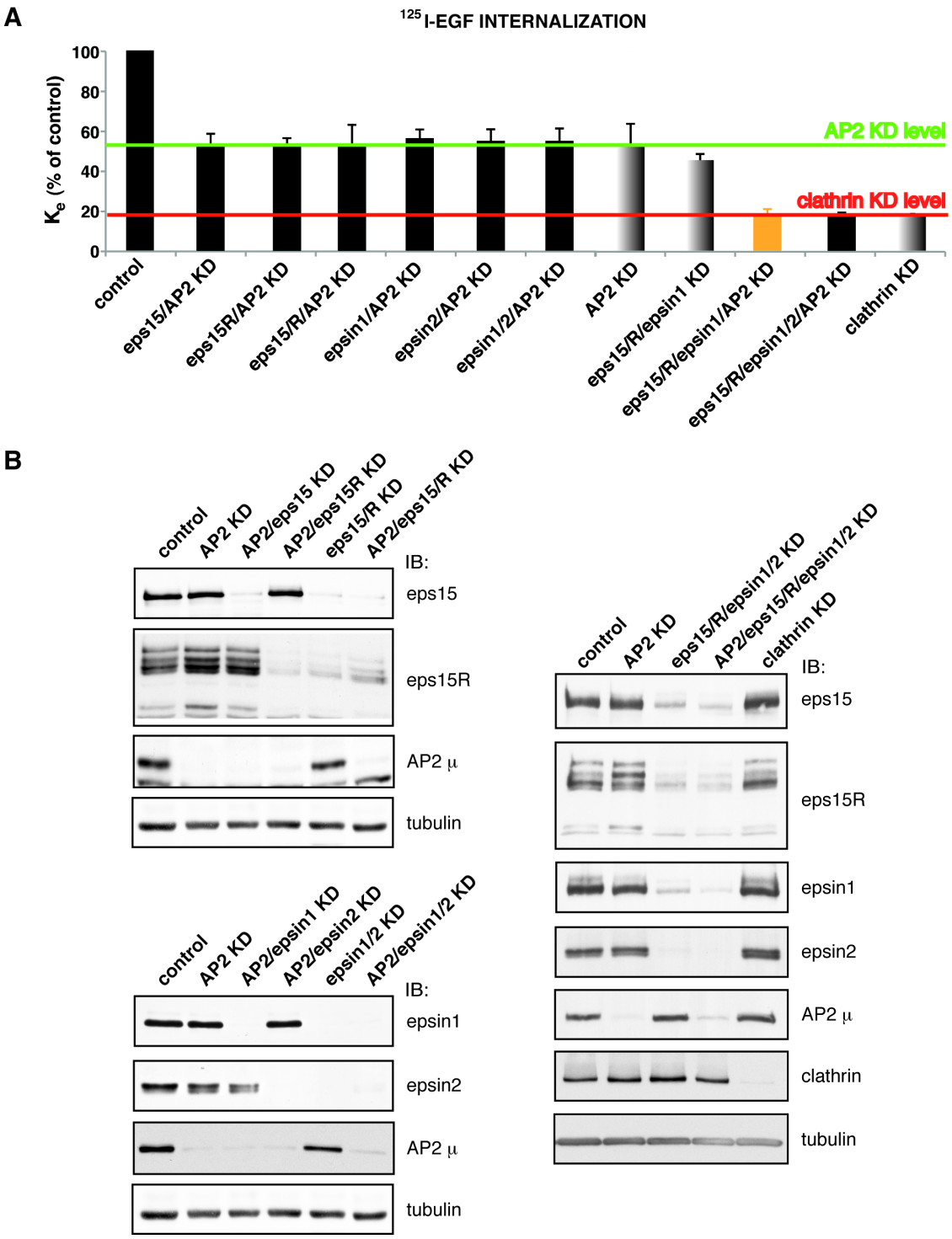


Figure 19: Assessment of the effect of the combinatorial interference among eps15, eps15R, epsin1, epsin2 and AP2 on CME of EGFR.
A. Analysis of ^{125}I-EGF internalization upon KD of different adaptors in HeLa cells. Kinetics of ^{125}I-EGF internalization were followed at early time points (0-6 min) upon silencing of the indicated adaptors using low concentrations of EGF (1 ng/ml) in HeLa cells: eps15/R were silenced stably, while epsin1/2 and AP2 were silenced transiently. Internalization constants (K_e) were extrapolated from the internalization curves and correspond to the slopes of the best-fitting curves (see Materials and Methods). Results are MEAN+/-SDEV of three independent experiments and expressed as % of the control oligo treated HeLa cells. **B. Efficient of adaptor KD.** The KD level of the different adaptors was determined by immunoblotting using specific antibodies, as indicated. Tubulin was used as protein loading control (these are representative of three independent experiments). We calculated a KD level of at least 95% by densitometry analysis in all the samples.

1.5 Differential requirement of Grb2, FCHo 1/2 and intersectin 1/2 in EGFR and TfR internalization

As largely illustrated in the introduction, the issue of cargo-specific internalization machinery via CCPs is debated. Henne and colleagues recently proposed a unique mechanism of CCP nucleation, regardless of the cargo being endocytosed (Henne et al., 2010). In their model, these authors described FCHo proteins as the fundamental membrane-bending proteins that are responsible for the initiation of the vesicle formation process. Through single-cell analysis based on imaging experiments, Henne *et al.* demonstrated that the mechanism of CCP formation is the same for the TfR, LDLR and EGFR. More recently, using TfR as a model cargo, Taylor and colleagues confirmed the early involvement of FCHo proteins in CCP formation (Taylor et al., 2011). We, therefore, investigated the role of FCHo proteins in EGFR and TfR endocytosis in our experimental setting.

Using the same oligos at those used in Henne's study, we confirmed using the quantitative ^{125}I-ligand internalization assay, the essential role for FCHo1/2 in TfR internalization (FCHo1/2 #1, **Figures 20A,C**). Moreover, our results indicate that FCHo2, in contrast to FCHo1, has a major role in this process (**Figures 20A,C**). Since qPCR analysis showed the same level of expression between FCHo1 and 2, it is likely that the differential role of the two proteins in TfR internalization is due to a real functional difference rather than differences in expression levels. On the contrary, we found that neither the single, nor the double FCHo1/2 KD had any impact on EGFR endocytosis (FCHo1/2 #1, **Figures 20B-C**). These results were confirmed with an additional pair of oligos (FCHo1/2 #2, **Figures 20A-C**).

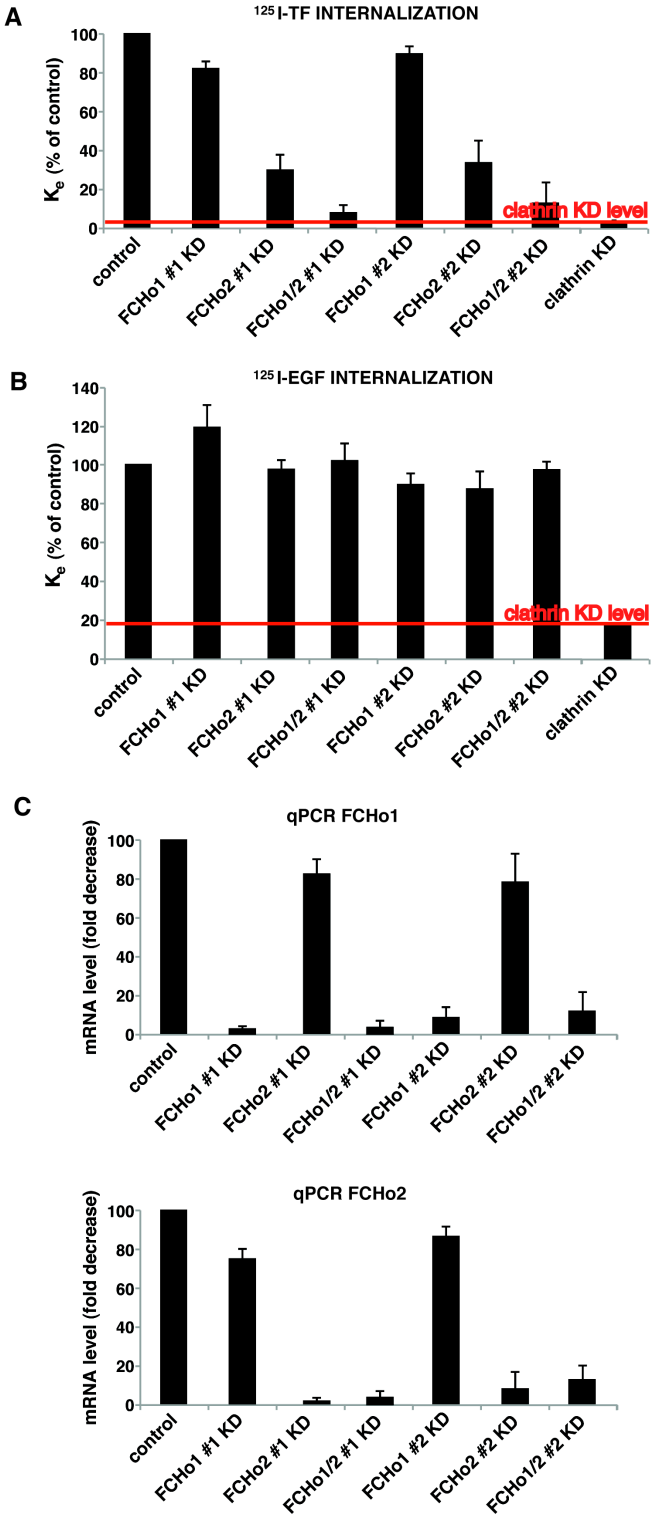


Figure 20: Assessment of the involvement of FCHo1/2 in CME of EGFR.
A-B. Effect of FCHo1/2 KD on ^{125}I-Tf (A) and ^{125}I-EGF (B) internalization in HeLa cells. Kinetics of (A) ^{125}I-Tf (1 μg/ml) and (B) ^{125}I-EGF (1 ng/ml) internalization were followed at early time points (0-6 min) upon transient silencing of FCHo1/2 as indicated. Two different pairs of siRNA oligos (FCHo1/2 #1 and FCHo1/2 #2) were used, as indicated. Internalization constants (K_e) were extrapolated from the internalization curves and correspond to the slopes of the best-fitting curves (see Materials and Methods). Results are MEAN+/-SDEV of three independent experiments and expressed as % of the control oligo treated HeLa cells. **C. Efficiency of FCHo1/2 KD.** The efficiency of FCHo1/2 KD was determined by qPCR. FCHo1/2 mRNA levels were normalized to mRNA levels of the housekeeping gene GAPDH. Results are expressed as fold decrease in mRNA levels relative to the control and are MEAN+/-SDEV of three independent experiments.

A possible explanation for the discrepancy between our results on EGFR and those of Henne *et al.* could be the use of different HeLa cell populations used in the two studies, although we could recapitulate the non-essential role of FCHo1/2 in EGFR endocytosis also in HeLa-Oslo cells (**Figures 21A-B**). Another explanation might lie in the oligos used in the Henne's study. Indeed, these oligos cause a reduction in the levels of surface EGFR (FCHo1/2 #1, **Figure 21C**), which is also visible as a decrease in total EGFR in western blots (**Figure 21D**). This effect of EGFR levels appear to be an off-target effect specific to this set of oligos, as the other pair of oligos (FCHo1/2 #2) does not show this phenotype, despite having the same KD efficiency (Figure 20C). This reduction in the starting level of surface EGFR would translate in a reduction in the intracellular EGFR signal (when revealed by single-cell imaging) that could erroneously be interpreted as a defect in internalization. More recently, our observations have been reproduced in another study, in which the authors showed that in their HeLa cells only FCHo2 is expressed, and that its ablation blocks TfR, but not EGFR, endocytosis (Uezu et al., 2011).

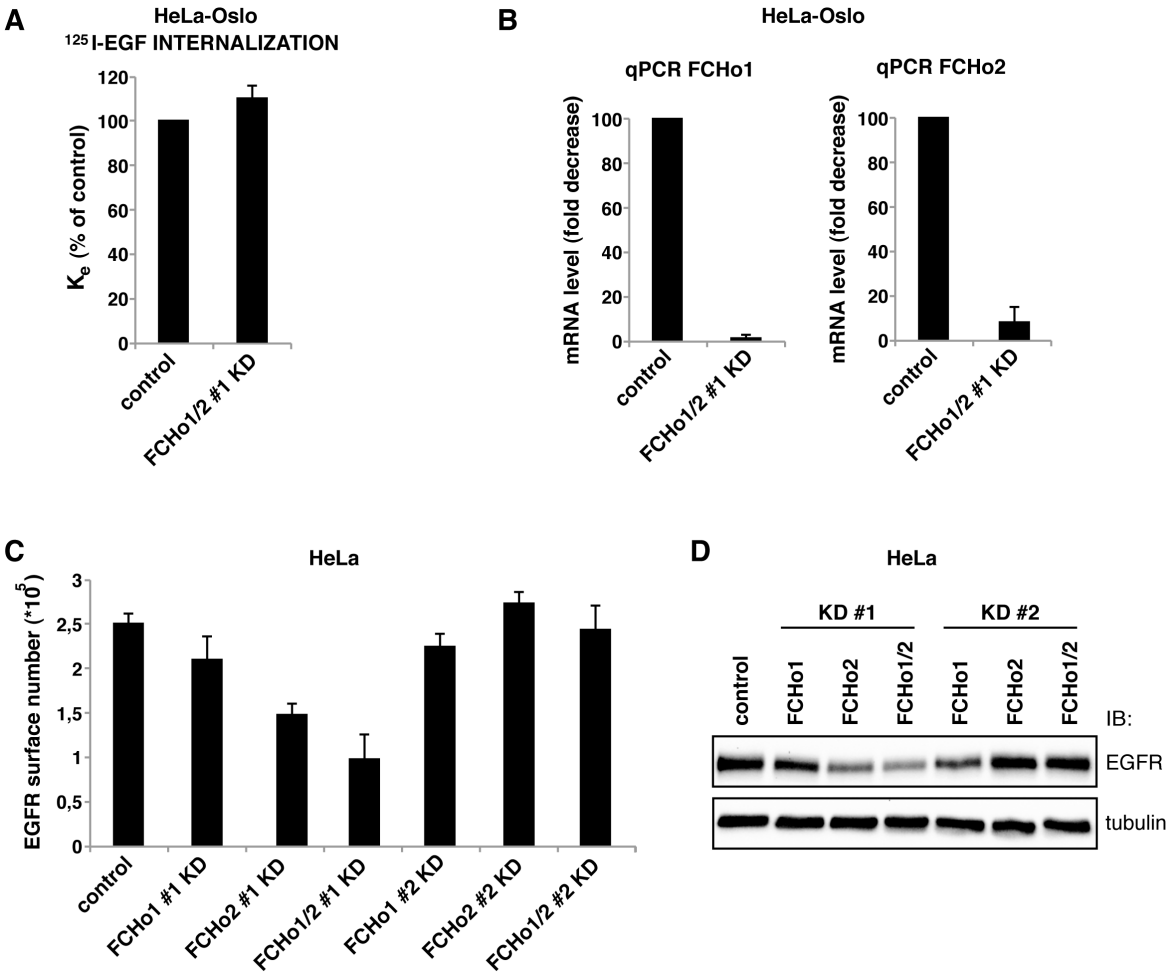


Figure 21: Confirmation of FCHo1/2 non-involvement in CME of EGFR.
A. Analysis of ^{125}I-EGF internalization upon FCHo1/2 KD in HeLa-Oslo cells. Kinetics of ^{125}I-EGF internalization (1 ng/ml) were followed at early time points (0-6 min) upon transient FCHo 1/2 KD. Internalization constants (K_e) were extrapolated from the internalization curves and correspond to the slopes of the best-fitting curves (see Materials and Methods). Results are MEAN+/-SDEV of three independent experiments and expressed as % of the control oligo treated HeLa-Oslo cells. **B. Efficiency of FCHo1/2 KD.** The efficiency of FCHo1/2 KD was determined by qPCR. FCHo1/2 mRNA levels were normalized to mRNA levels of the housekeeping gene GAPDH. Results are expressed as fold decrease in mRNA levels relative to the control and are MEAN+/-SDEV of three independent experiments. **C. Surface EGFR numbers upon FCHo1/2 KD in HeLa cells.** The number of EGFRs on the cell surface was measured by saturation binding upon FCHo1/2 KD. Two different pairs of siRNAs (FCHo1/2 #1 and FCHo1/2 #2) were used, as indicated. Results are MEAN+/-SDEV of three independent experiments. **D. Total EGFR protein level upon FCHo1/2 KD in HeLa cells.** The total level of EGFR was determined by immunoblotting using a specific antibody, as indicated. Tubulin was used as protein loading control (results are representative of three independent experiments).

We also analyzed the role of Grb2 in receptor endocytosis in our experimental setting. Grb2 has been reported to be involved in EGFR internalization, but not constitutive endocytosis of TfR (Huang et al., 2004), indicating that Grb2 is a cargo-specific adaptor. We confirmed these results showing that Grb2 KD had a significant impact on EGFR internalization (75% reduction; **Figures 22A,C**), comparable to clathrin KD, but had no effect on TfR internalization (**Figures 22B-C**). Given the striking effect of Grb2 KD on EGFR internalization, we wondered whether Crk, a structurally similar adaptor protein that has previously been shown to bind to eps15 (Schumacher et al., 1995) could also have a role in EGFR internalization. However, we found that Crk KD, alone or in combination with Grb2 KD, did not have any effect (**Figures 22A-C**).

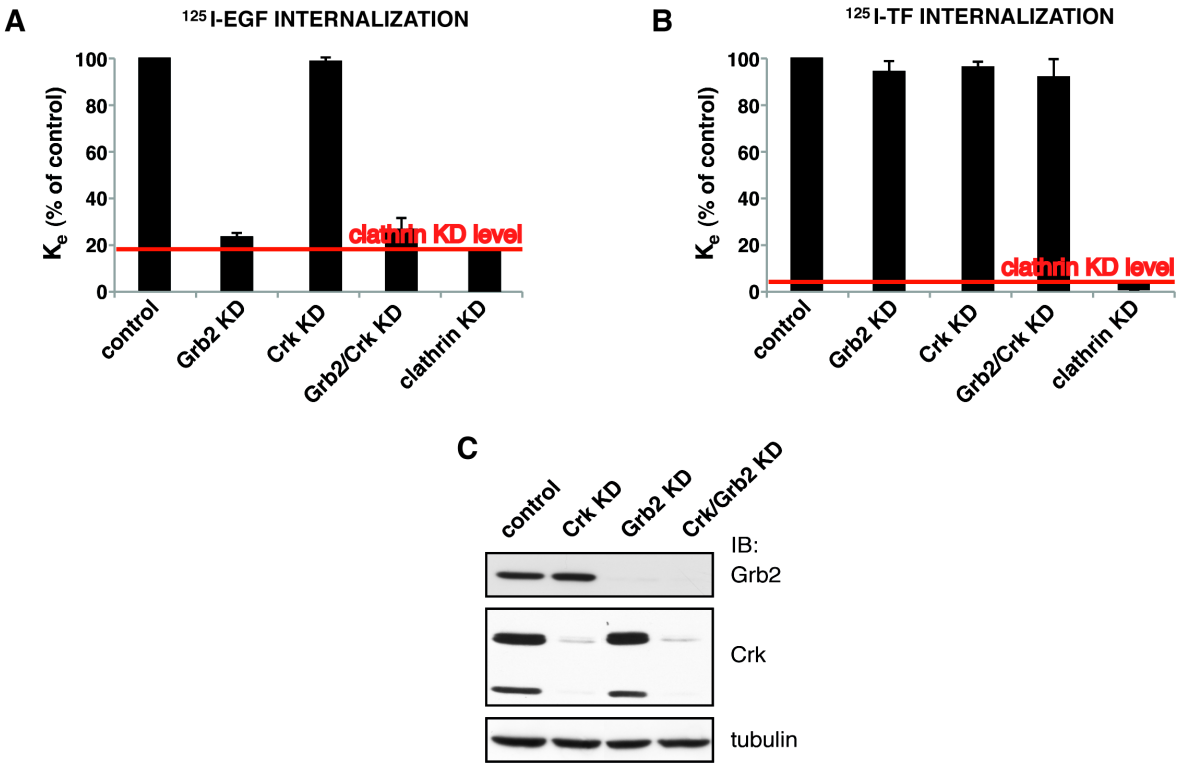


Figure 22: Assessment of Grb2 and Crk role in CME of EGFR.
A-B. Analysis of ^{125}I-EGF (A) and ^{125}I-Tf (B) internalization upon transient silencing of Grb2 and/or Crk in HeLa cells. Kinetics of (**A**) ^{125}I-EGF (1 ng/ml) and (**B**) ^{125}I-Tf (1 μg/ml) internalization were followed at early time points (0-6 min) upon transient silencing of the indicated proteins in HeLa cells. Internalization constants (K_e) were extrapolated from the internalization curves and correspond to the slopes of the best-fitting curves (see Materials and Methods). Results are MEAN+/-SDEV of three independent experiments and expressed as % of the control oligo treated HeLa cells. **C. Efficiency of Grb2 and Crk KD.** The efficiency of Grb2 and Crk KD was determined by immunoblotting using specific antibodies, as indicated. Tubulin was used as protein loading control (these are representative of three independent experiments). We calculated a KD level of at least 95% by densitometry analysis in all the samples.

In the cascade proposed by Henne *et al.* FCHo1/2 interacts with either eps15/R or intersectin1/2, which all function in recruiting AP2 to PM (Henne et al., 2010). Therefore, we also investigated the role of intersectins in our system, which have also been recently described to be involved in clathrin-dependent EGFR internalization (Frosi et al., 2010). We observed a slight decrease in EGFR internalization following KD of intersectin 1 and 2, either singly or in combination: ~10% reduction upon single KD and ~20% reduction in the double KD (**Figures 23A-B**). The simultaneous interference of eps15/R/intersectin1/2 reduces internalization to the same extent as single AP2 KD. Moreover, the addition of AP2 KD did not cause any ulterior impairment, suggesting that the four proteins work together upstream of AP2, in agreement with Henne's work (Henne et al., 2010). This latter result also allows us to exclude an unspecific effect caused by the simultaneous KD of 5 proteins. Importantly, the addition of epsin1 KD to the eps15/R/intersectin1/2 KD, completely blocked clathrin-dependent EGFR internalization (**Figures 23A-B**), in line with our previous finding that eps15/R/epsin1 function independently of AP2.

Our data highlight a different molecular requirement between TfR, a constitutively internalized cargo, and EGFR, whose internalization is ligand-induced.

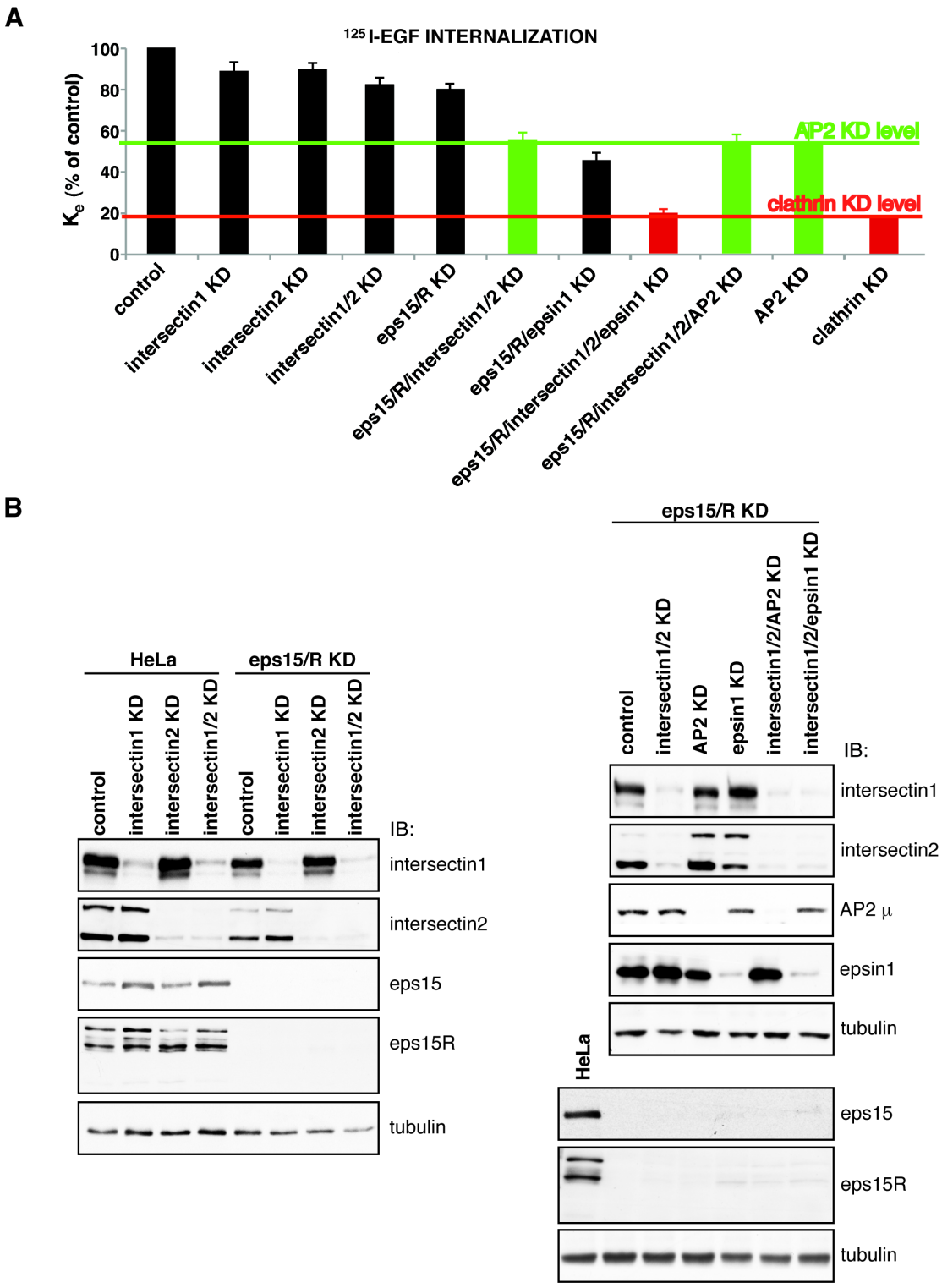


Figure 23: Assessment of the role of intersectin1/2 and their combinatorial interference with eps15, eps15R, epsin1 and AP2 in CME of EGFR.
A. Analysis of ^{125}I-EGF internalization upon multiple KD in HeLa cells. Kinetics of ^{125}I-EGF internalization (1 ng/ml) were followed at early time points (0-6 min) upon KD of the indicated proteins in HeLa cells: eps15/R were silenced stably, while intersectin1/2, epsin1 and AP2 were silenced transiently. Internalization constants (K_e) were extrapolated from the internalization curves and correspond to the slopes of the best-fitting curves (see Materials and Methods). Results are MEAN+/-SDEV of three independent experiments and expressed as % of the control oligo treated HeLa cells. **B. Efficiency of KD.** The KD of the indicated proteins was determined by immunoblotting using specific antibodies, as indicated. Tubulin was used as protein loading control (these are representative of three independent experiments). We calculated a KD level of at least 95% by densitometry analysis in all the samples.

1.6 Setting up of live TIRFM to analyze EGF-induced clathrin endocytosis of the EGFR

Our data suggest that EGFR-specialized CCVs might exist characterized by a specific repertoire of adaptor proteins. A potential problem of the KD approach utilized in the above experiments is that it might cause cellular rearrangement and compensation phenomena, which cannot be easily predicted, and could lead to erroneous interpretation of the results. For this reason, we plan to parallel our genetic studies with live-imaging techniques, in order to follow the recruitment, in time and space, of different clathrin endocytic adaptors to the EGFR. This approach will provide a snapshot of the situation, upon minimal manipulation of the system. Besides giving molecular details, live imaging can also provide information about the dynamics of the events. For this part of the project we established a collaboration with Gaudenz Danuser and Francois Aguet (Harvard Medical School, Boston), who are leaders in data analysis. The ideal technique is total internal reflection fluorescence microscopy (TIRFM), which allows visualization of events within 200 nm of the basal PM. Our final aim is to perform 4-color movies in order to follow simultaneously labeled-EGF, fluorescent clathrin (to distinguish CME from NCE) and fluorescent adaptors (both AP2 and eps15 or epsin1), to understand if different populations of CCVs exist, containing distinct subsets of adaptor proteins.

To this end, we are setting up the system as follows:

1) we selected some well-characterized constructs in the literature (**Table 4**) and set up transient co-transfection conditions. Moreover, we confirmed functionality of EGFP-AP2 σ subunit (Ehrlich et al., 2004) in rescuing fluorescent-Tf internalization upon depletion of endogenous AP2 (oligo targeting 3'UTR has been used; **Figure 24A**);

Clathrin light chain **(N-terminal tag)**	(Gaidarov et al., 1999) (Liu et al., 2010)
AP2 σ subunit **(C-terminal tag)**	(Ehrlich et al., 2004) (Loerke et al., 2011)
Epsin1 **(C-terminal tag)**	(Chen and Zhuang, 2008)
Eps15 **(N-terminal tag)**	(Zhang et al., 2010)

Table 4: **List of the constructs selected from literature**

2) to follow the EGFR we are employing EGF-Alexa647. Since the fluorophore is quite big and might affect activity of the ligand, we tested its ability to compete with ^{125}I-EGF in saturation binding conditions at equilibrium. In parallel, we also tested unlabeled EGF and found that both the labeled and unlabeled EGF were comparable in their ability to compete with ^{125}I-EGF (**Figure 24B**). As a future plan, we also want to test the ability of EGF-Alexa647 to induce EGFR signaling;
3) our HeLa cells present clathrin plaques on the basal membrane (Saffarian et al., 2009). These are more intense structures than CCVs, and they might disturb the analysis of CCVs. Thus, we checked a different model system, commonly used in this type of analysis, BSC1 cells (kidney epithelial cells of monkey origin). These cells showed a very low level of EGFR by WB analysis (**Figure 24C**). Moreover, IF analysis revealed heterogeneous EGFR expression between cells (**Figure 24D**). Therefore, we are currently considering sorting EGFR-expressing BSC1 cells to obtain a homogeneous population.

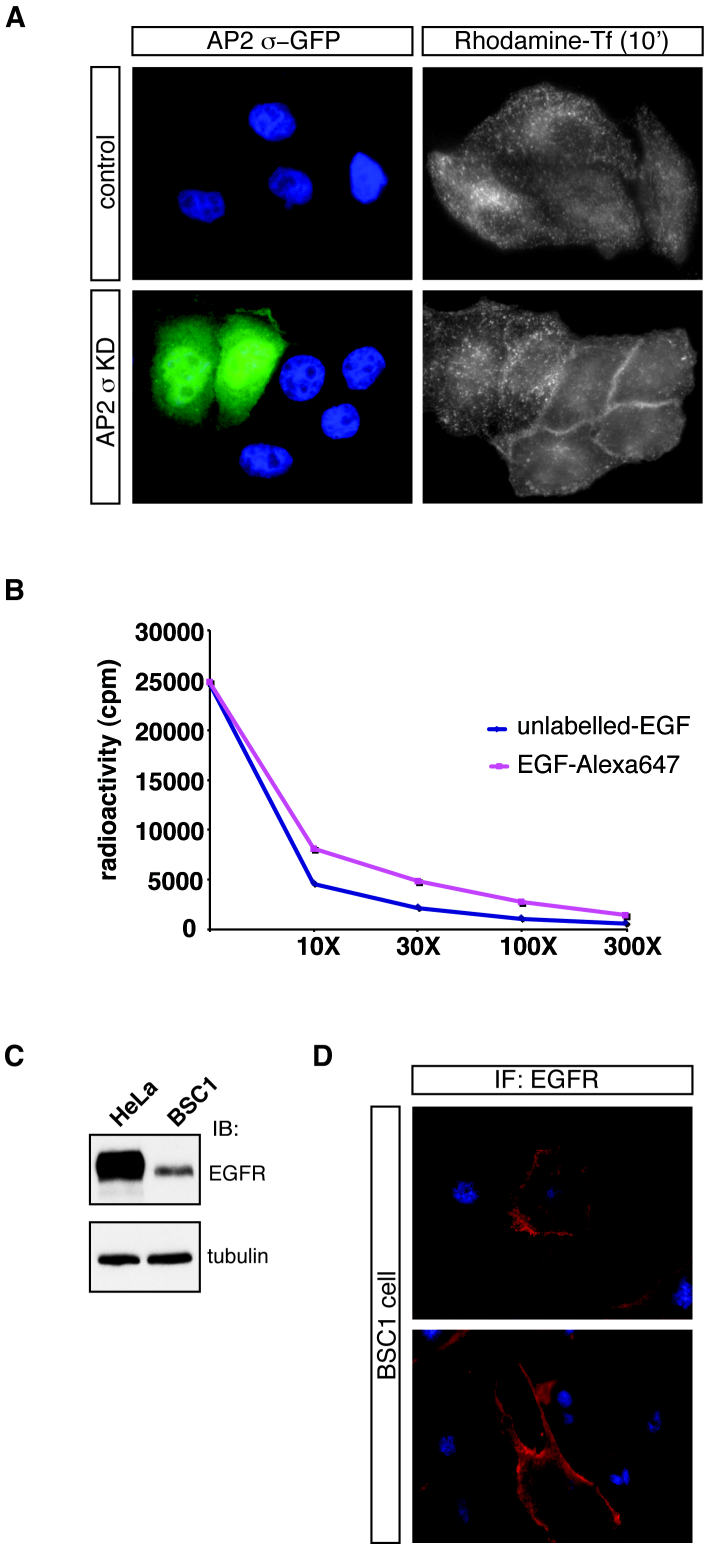


Figure 24: Setting up of live TIRFM.
A. Test of functionality of AP2 σ-GFP. HeLa cells depleted of endogenous AP2 have been transfected with AP2 σ-GFP. Rhodamine-Tf internalization assays were performed at 37°C for 10 minutes. Untreated cells have been used as controls. **B. Competition assay with EGF-Alexa647.** Saturation binding assay has been performed with ^{125}I-EGF**.** Competition with unlabeled-EGF, as control, and EGF-Alexa647 was performed using increasing concentrations, as indicated, up to 300X with respect to ^{125}I-EGF. **C. Levels of EGFR expression in BSC1 cells.** The level of EGFR expression was determined by immunoblotting using a specific antibody, as indicated. Tubulin was used as protein loading control. **D. Levels of surface EGFR expression in BSC1 cells.** EGFR expression was determined by immunofluorescence (w/o membrane permeabilization to detect only surface EGFR) using a specific antibody, as indicated.

2. Different adaptors, different fate

Considering the importance of endocytosis in signaling regulation, we investigated the impact of adaptor KD on EGFR signal propagation and attenuation, using the same experimental settings employed in the abovementioned internalization assays in HeLa cells. Our hypothesis is that different adaptors (AP2 *vs.* eps15/R/epsin1, in this case) might generate distinct populations of CCVs that differ not only at the molecular level, but also from a functional point of view, in that they direct the EGFR to different intracellular fates and signaling outputs.

2.1 AP2 and eps15/eps15R/epsin1 differentially regulate EGFR signaling

In order to investigate the impact of the different adaptors on EGFR signaling, we followed MAPK and AKT phosphorylation in HeLa cells stimulated with a low dose of EGF (1.5 ng/ml) following KD of different adaptors (AP2 *vs.* eps15/R/epsin1). Interestingly, AP2 KD caused a decrease in both the peak phase and the sustained phase of AKT phosphorylation (**Figures 25A-B**). A minor reduction in MAPK signaling was also visible (**Figures 25A-B**). These data are in agreement with previously reported results obtained upon stimulation with a high dose of EGF after AP2 KD (Sigismund et al., 2008). Since AP2 does not act in NCE, this effect was attributed to its role in CME. Strikingly, eps15/R/epsin1 KD showed the opposite phenotype compared to AP2 KD, displaying enhanced and sustained AKT signaling at later time points, while MAPK signaling was unchanged (**Figures 25A-B**). Of note, the single epsin1 KD and double eps15/R KD had no effect, supporting the notion of functional redundancy between eps15, eps15R and epsin1 (**Figure 25C**). Together these data support a role of AP2 in sustaining signaling through the clathrin pathway, as previously suggested (Sigismund et al., 2008), and indicate an opposite function (signal downregulation) for epsin1, eps15 and eps15R, at least regarding AKT activation.

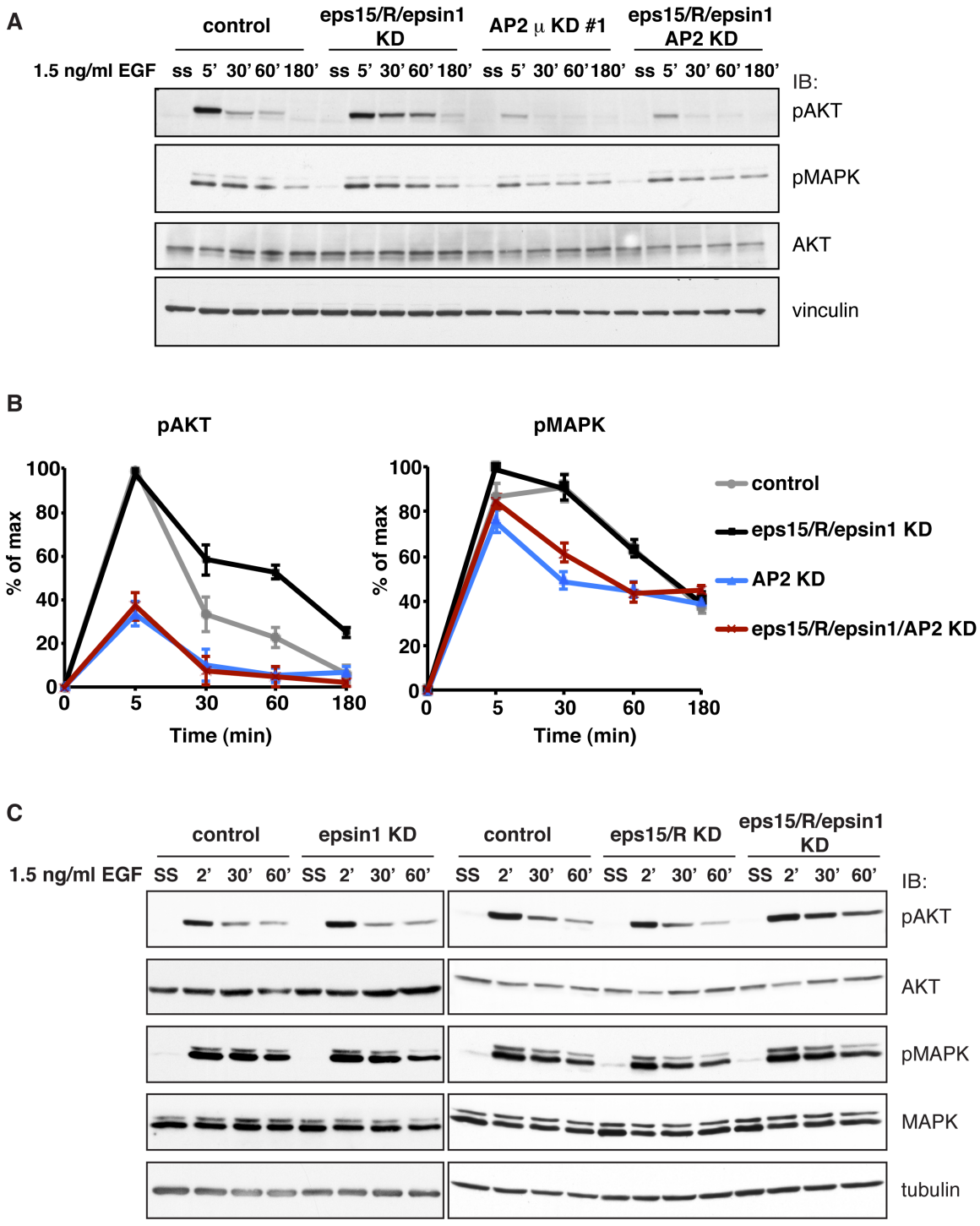


Figure 25: Assessment of the impact of AP2 KD and single/multiple eps15/R/epsin1 KD on EGFR signaling.
A. The effect of AP2 and eps15/R/epsin1 KD on EGFR signaling following stimulation with a low dose of EGF. HeLa cells were stimulated with 1.5 ng/ml EGF for the indicated times after overnight serum deprivation. Eps15/R were silenced stably, while epsin1 and AP2 were silenced transiently. Control oligo treated HeLa cells were used as control. The level of AKT and MAPK activation was determined by immunoblotting using antibodies that recognize the phosphorylated forms (pMAPK and pAKT). Tubulin was used as a protein loading control. Results are representative of at least three independent repeats. **B**. Quantification by densitometry analysis of pAKT (on the left) and pMAPK (on the right) activation. Results are MEAN+/-SDEV of three independent experiments. **C. The effect of epsin1 and eps15/R KD on EGFR signaling following stimulation with a low dose of EGF.** Experiments were performed as described in **A**.

However, AKT phosphorylation is highly sensitive to cellular stress and therefore needs careful verification. In the case of AP2 KD, we validated the phenotype using two additional siRNA oligos in HeLa cells (**Figure 26A**). Moreover, we confirmed this phenotype also in the normal breast epithelial cell line, MCF10A. In these cells, EGFR is internalized only through CME (our unpublished results), thus clathrin-dependent EGFR signaling can be assessed at any EGF concentration. Upon 100 ng/ml EGF we observed that AKT and MAPK activation were reduced in AP2 KD MCF10A cells compared with control (**Figure 26B**), thus confirming in this cell line what we have shown in HeLa cells.

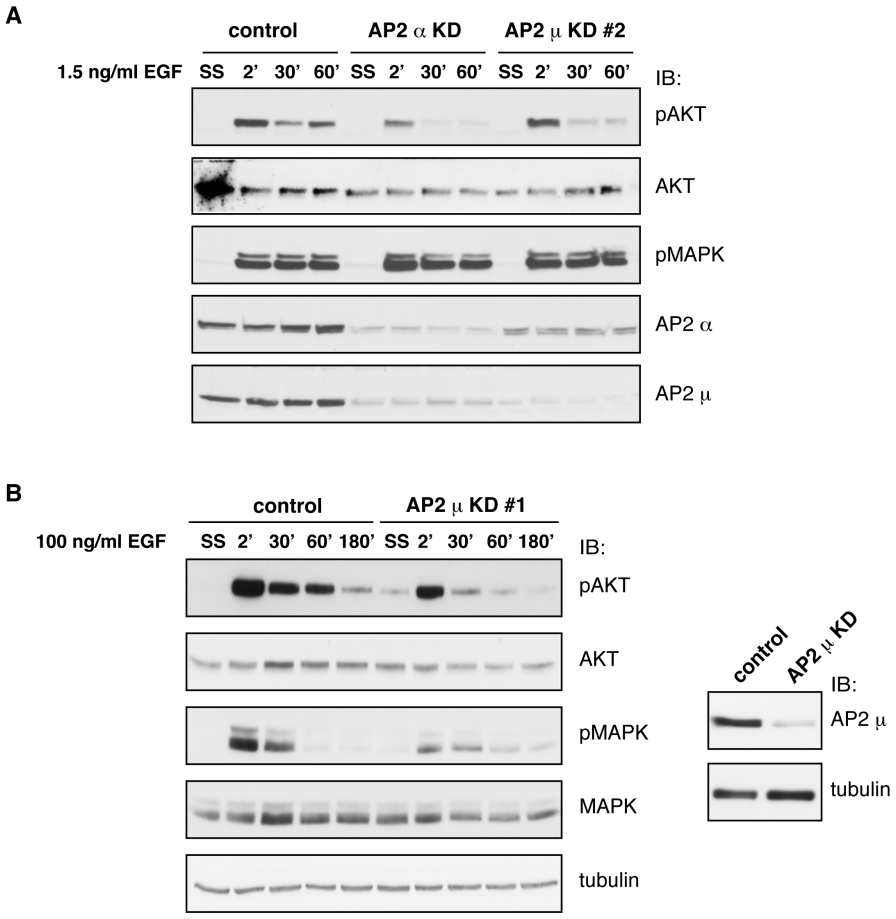


Figure 26: Confirmation of AP2 KD effect on EGFR signaling.
A. Effect of AP2 KD on EGFR signaling upon stimulation with low EGF dose in HeLa cells. HeLa cells were stimulated with 1.5 ng/ml EGF for the indicated time points after overnight serum deprivation, upon transient silencing of different subunit of AP2 with the indicated oligos. Control oligo treated HeLa cells were used as control. The level of AKT and MAPK activation was determined by immunoblotting using antibodies that recognize the phosphorylated forms (pMAPK and pAKT). Tubulin was used as a protein loading control. Results are representative of three independent repeats. **B. Effect of AP2 KD on EGFR signaling in MCF10A cells.** MFC10A cells were stimulated with 100 ng/ml EGF for the indicated times after overnight serum deprivation, upon transient silencing of μ subunit of AP2 with the indicated oligo. Control oligo treated MCF10A cells were used as control. The level of AKT and MAPK activation was determined as described in **A**. Level of AP2 KD (μ subunit) is shown on the right. Results are representative of three independent repeats.

In the case of eps15/R/epsin1 KD, we attempted to rescue the phenotype. We stably reintroduced the murine version of wild-type (WT) eps15 in the stable eps15/R KD HeLa clone. We chose to reintroduce the mouse protein because it is highly similar to human eps15, and mutated versions of murine eps15 were already available in the lab and could be exploited for structure-function analysis (see Result section 3). A stable eps15/R KD HeLa cell clone expressing WT murine eps15 at levels comparable to the endogenous protein was selected (referred to as "clone eps15 WT #1", see also Figures 31A-B; note that exogenous mouse eps15 displays a higher apparent molecular weight in immunoblot with respect to the human copy, due to a higher content in acidic amino acids; **Figure 27A**). To select this clone by WB we took adavantage of anti-eps15 antibodies that recognize mouse and human eps15 with the same efficiency (**Figure 27B;** 3T was used in **Figure 27A**). We then silenced epsin1 by transient transfection of siRNA oligos in the clone eps15 WT #1. We observed that re-expression of the eps15 transgene in the triple KD cells was able to revert sustained AKT activation back to control levels (**Figure 27C**). This result corroborates the signaling phenotype and constitutes a proof of the redundancy amongst the three proteins.

Together these data support the notion that AP2 and eps15/eps15R/epsin1 could give rise to separate pools of EGFR-loaded CCVs that have opposing effects on EGFR signaling, with AP2-positive CCVs sustaining signaling, while eps15/eps15R/epsin1-positive CCVs attenuate signaling.

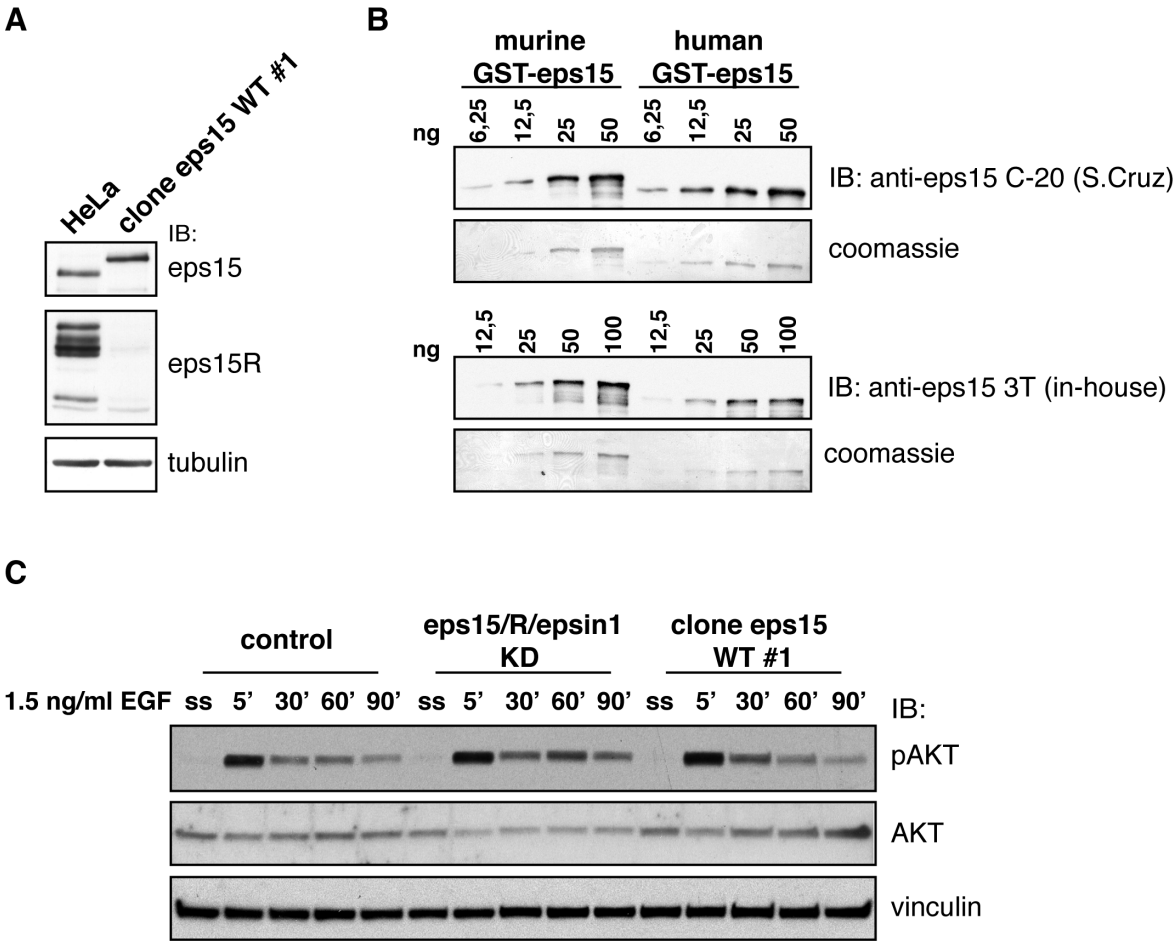


Figure 27: Rescue of eps15/R/epsin1 KD effect on EGFR signaling by eps15 WT.
A. Selection of a HeLa cell clone stably re-expressing WT eps15. The stable eps15/R KD HeLa cell clone was infected with a construct stably expressing murine eps15. A clone expressing eps15 at levels comparable to the endogenous protein was selected. The level of eps15 expression was determined by immunoblotting using a specific antibody, as indicated (anti-eps15 3T was used here, see pannel **B**). Tubulin was used as protein loading control. **B. Test of eps15 antibodies.** Increasing amounts of murine and human GST-eps15 was loaded and immunoblotted with two different anti-eps15 antibodies, as indicated. Coomassie staining was used to control protein loading. **C. Effect of re-expression of WT eps15 in eps15/R/epsin1 KD HeLa cells on EGFR signaling upon stimulation with low EGF dose.** HeLa cells were stimulated with 1.5 ng/ml EGF for the indicated time points after overnight serum deprivation. Eps15/R were silenced stably, while epsin1 was silenced transiently. Control oligo treated HeLa cells were used as control. The level of AKT activation was determined by immunoblotting using antibodies that recognize the phosphorylated forms (pAKT). Vinculin was used as a protein loading control. Results are representative of three independent repeats.

2.2 AP2 antagonizes EGFR degradation

We next asked how distinct CCVs could have differential effects on EGFR signaling. The obvious explanation is that the different CCVs are associated with diverse intracellular trafficking routes. Considering the above data, we hypothesized that EGFRs internalized through AP2 are targeted for recycling, while those internalized through eps15/R/epsin1 might be subjected to CME-dependent degradation, as previously suggested (Lakadamyali et al., 2006; Sigismund et al., 2008). In line with this notion, it is known that AKT activation occurs mainly at the PM and that sustained AKT activation depends on continuous recycling of activated EGFR from the cytoplasm to the PM. To test this hypothesis, we analyzed the impact of silencing AP2 and eps15/R/epsin1 on receptor degradation.

Since at low EGF concentrations only a small fraction of receptors is engaged, it was not possible to follow ligand-induced EGFR degradation by WB analysis. We therefore employed a degradation assay based on iodinated ligand that was previously set up in the laboratory [(Sigismund et al., 2008) and Materials and Methods]. A caveat of this assay is that we are following the ligand and not the receptor, however, in unperturbed conditions the two molecules follow the same trafficking route, i.e., either recycling or degradative. Indeed, the receptor-ligand complex does not dissociate at the endosomal pH, but only at the level of the MVBs when they are already committed to degradation.

Using the iodinated ligand degradation assay, we observed an increase in ^{125}I-EGF degradation in AP2 KD cells compared with control (**Figure 28A**) compatible with the previously reported decrease in EGFR recycling (Sigismund et al., 2008), and with the reduced EGFR signaling (Figures 25A-B), in these cells. However, no difference in EGFR degradation was visible in eps15/R/epsin1 KD cells with respect to control cells (**Figure 28A**). Moreover, also in the normal breast epithelial cell line MCF10A (where EGFR only enters through CME), upon 100 ng/ml EGF, we observed by WB that EGFR degradation was increased in AP2 KD cells compared with control (**Figure 28B**). These data suggest that AP2 has a role in preventing EGFR degradation, while eps15/R/epsin1 are not

involved in the degradative route, as we had hypothesized. It remains, therefore, to be determined how silencing of these alternative adaptors results in augmented EGFR signaling.

One intriguing possibility is that distinct adaptors differentially regulate CCP retention at the PM and vesicle dynamics, thereby, differentially controlling signaling duration at the cell surface, as demonstrated for GPCR (Puthenveedu and von Zastrow, 2006). Alternatively, distinct adaptors might target EGFR to distinct endosomal compartments with specific signaling abilities: AP2 to a signaling-competent compartment while eps15/R/epsin1 to signaling-incompetent endosomes. Colocalization studies with different endosomal markers (e.g., EEA1, APPL1/2, Hrs and others) and with markers of slow and fast recycling (e.g., Rab11 and Rab4, respectively) in the different KD cells will help to clarify this issue.

We have investigated the involvement of APPL endosome. This peculiar organelle, which precedes Rab5 early endosomes, has recently been shown to be an intracellular station for AKT activation. Very interestingly, it has been reported that only about half of the EGFR-containing CCVs reaches this site, while the remaining EGFR-loaded CCVs go directly to Rab5 endosomes, bypassing the intermediate APPL endosomes (Zoncu et al., 2009). Since AP2 KD resulted in reduced AKT activation, we hypothesized that AP2-dependent EGFR-containing CCVs might traffic to APPL endosomes. To test this hypothesis, we checked whether APPL1 KD could phenocopy AP2 KD. APPL1 KD did not affect AKT activation (**Figure 28C**). From this data, we inferred that the AKT phenotype is not linked to AKT activation in APPL endosomes. Thus, it is possible that AP2 impacts on AKT activation by mediating EGFR recycling and PM activation of AKT. Whether AP2-reliant CCVs are targeted to APPL endosomes in our experimental setting remains to be addressed by immunofluorescence.

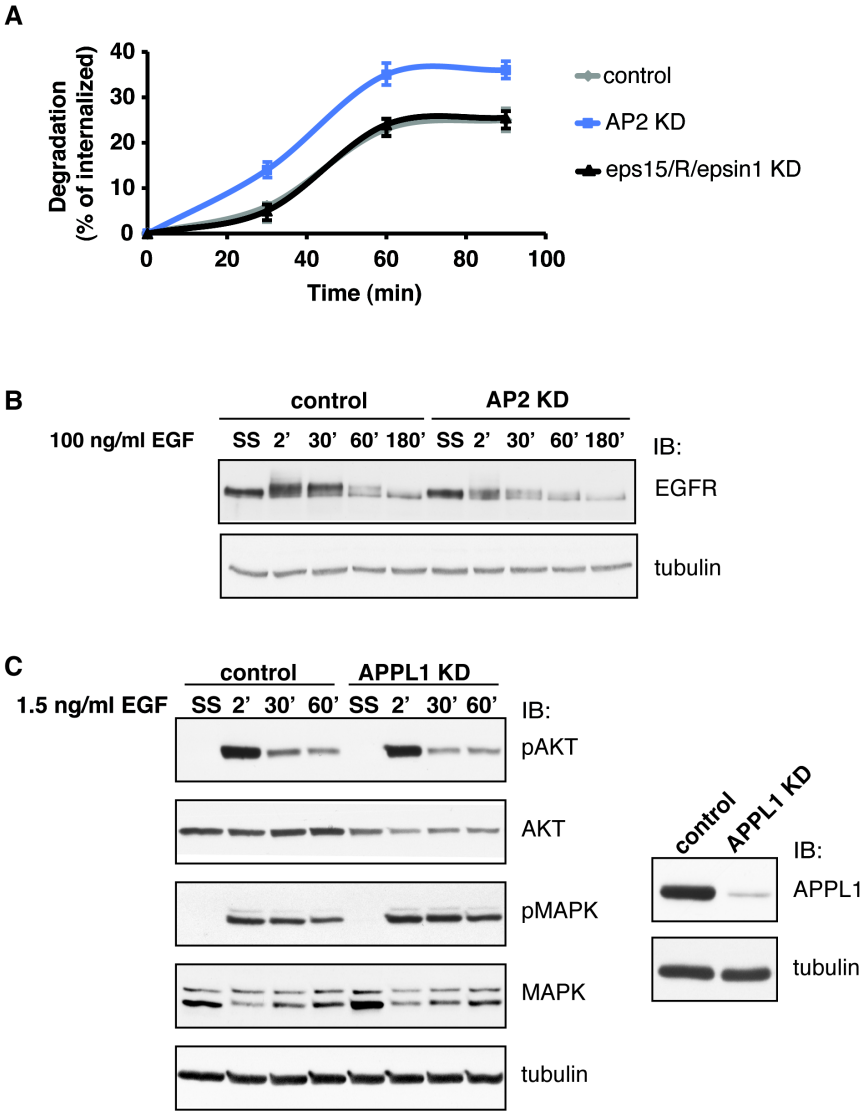


Figure 28: Assessment of AP2 role in EGFR trafficking.
A. ^{125}I-EGF low dose degradation assay upon AP2 and eps15/R/epsin1 KD in HeLa cells. HeLa cells were incubated with 1.5 ng/ml of ^{125}I-EGF at 37°C for 6 minutes, then put on ice, incubated with mild acid wash (pH 4.5) for 5 minutes and shifted to 37°C again for 30, 60 and 90 minutes to follow ^{125}I-EGF fate. Internalized ligand and free ligand in the supernatant were recovered and subjected to TCA precipitation to separate intact from degraded ligand. Rebound ligand at the plasma membrane was also recovered by strong acid wash (pH 2.5). Results are expressed as the % degraded EGF (TCA soluble, medium+internalized) with respect to the internalized EGF at time zero. Eps15/R were silenced stably, while epsin1 and AP2 (μ subunit, oligo #1) was silenced transiently. Control oligo treated HeLa cells were used as control. Results are MEAN+/-SDEV of three independent experiments. **B. Effect of AP2 KD on EGFR degradation in MCF10A cells.** MFC10A cells were stimulated with 100 ng/ml EGF for the indicated times after overnight serum deprivation upon transient silencing of μ subunit of AP2 with oligo #1. Control oligo treated MCF10A cells were used as control. The level of EGFR was determined by immunoblotting using a specific antibody, as indicated. Tubulin was used as a protein loading control. Results are representative of three independent repeats. **C. Analysis of signaling at low dose of EGF upon APPL1 KD in HeLa cells.** HeLa cells were stimulated with low dose of EGF (1.5 ng/ml) at different time points after overnight serum deprivation, upon transient silencing of APPL1. Control oligo treated HeLa cells were used as control. The level of AKT and MAPK activation was determined by immunoblotting using antibodies that recognize the phosphorylated forms (pMAPK and pAKT). On the right, the level of APPL1 KD was assessed by immunoblotting with a specific antibody, as indicated. Tubulin was used as a protein loading control. Results are representative of two independent repeats.

2.3 AP2 specifically promotes EGF-induced migration

We next addressed the impact of altered AKT activation upon AP2 KD on the final biological outcome. Since AKT activation is known to promote migration (Dillon and Muller, 2010), we investigated the impact of AP2 KD on migration using Boyden chamber assays. We observed that reduced AKT activation in AP2 KD HeLa cells correlated with a statistically significant impairment in EGF-induced migration at low dose of ligand. This defect is slighter and not statistically significant upon serum stimulation, highlighting that this phenotype is specific to the EGFR system (**Figures 29A-B**). In contrast, eps15/R/epsin1 KD HeLa cells displayed no differences in migration with respect to control cells (**Figures 29A-B**). A possible explanation for the lack of effect in these cells, in which AKT activation is prolonged, is that the migration assay is saturated. However, it is also possible that sustained AKT does not translate into a migratory phenotype or that it might be involved in other types of EGF-induced cellular responses.

Thus, we can conclude that AP2 acts as a molecular determinant that protects EGFR from degradation and, on the basis of previous work (Sigismund et al., 2008), directs it to recycling. This process might determine continuous cycles of AKT activation, which ultimately lead to cell migration.

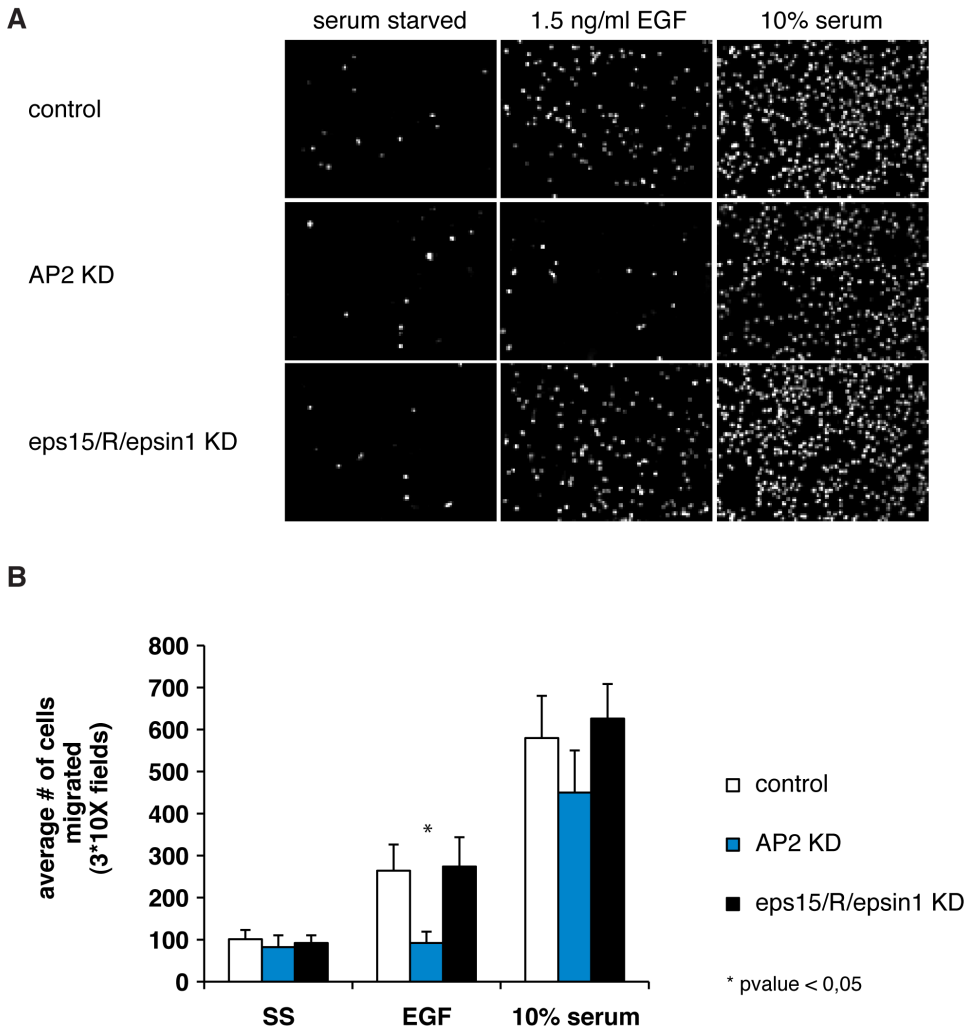


Figure 29: Analysis of migration at low dose of EGF upon AP2 and eps15/R/epsin1 KD HeLa cells.
A. HeLa cells were serum starved for 24 hours and then subjected to Boyden chamber assay in the presence of the indicated amounts of EGF or serum. Eps15/R were silenced stably, while epsin1 and AP2 (μ subunit, oligo #1) was silenced transiently. Control oligo treated HeLa cells were used as control. Migrating cells attached to the membrane were subjected to DAPI staining and analyzed by fluorescence microscopy. Quantitation (MEAN+/-SDEV) of three independent experiments is shown in **B**.

I reported in **Table 5** a summary of the results obtained in AP2 KD and eps15/R/epsin1 KD HeLa cells.

	AP2 KD	**eps15/R/epsin1 KD**
AKT signaling	strong decrease	increase
MAPK signaling	decrease	no change
EGFR degradation	increase	no change
EGF-induced migration	no induction	no change

Table 5: Summary of phenotypes observed in AP2 KD and eps15/R/epsin1 KD HeLa cells.

3. Characterization of the molecular mechanisms underlying eps15, eps15R and epsin1 function

Based on the above genetic data, a novel scenario can be envisioned where the EGFR is recruited to CCPs via an AP2-independent mechanism, which requires eps15/R/epsin1 as alternative adaptors. The AP2-binding site on the EGFR has been mapped and extensively characterized (Sorkin et al., 1996). This site is exposed upon conformational changes induced by ligand binding and receptor dimerization (Nesterov et al., 1995). In contrast, the mechanism through which eps15, eps15R and epsin1 are recruited to the EGFR in an AP2-independent fashion, and the basis for their functional redundancy in CME of the EGFR, are unknown.

From the structure/function point of view, the three proteins share several characteristics:

a) the ability to bind to AP2;

b) the ability to interact with intersectins;

c) the ability to bind to Ub and to be monoubiquitinated, owing to the presence of UIMs.

Since we have demonstrated that eps15, eps15R and epsin1 can function independently of AP2 in CME of the EGFR (Figure 19), their functional redundancy cannot be explained by their shared ability to bind AP2. Moreover, functional redundancy cannot be explained by their ability to bind intersectins, since if this were the case double KD of intersectin1 and 2 would phenocopy the eps15/R/epsin1 KD in ^{125}I-EGF internalization assays. However, we demonstrated that this is not the case (Figure 23). Since eps15, eps15R and epsin1 all possess Ub-binding domains and undergo EGF-dependent monoubiquitination, we hypothesized that these adaptors might be recruited to the EGFR through an Ub-based mechanism. This mechanism should not involve receptor ubiquitination (model A, **Figure 30**), which has been extensively demonstrated to have only a minor impact -if any- at early steps of CME (Huang et al., 2007; Sigismund et al., 2005). The mechanism could involve, however, the binding of eps15, eps15R and epsin1 to ubiquitinated intermediate adaptors (model B, **Figure 30**), rather than direct binding to the receptor. Additionally,

since all the three proteins are monoubiquitinated upon stimulation with low dose EGF (our unpublished data), then monoubiquitination might be necessary for the recruitment of essential components of this internalization pathway (model C, **Figure 30**).

To investigate whether the mechanism of eps15/R/epsin1 recruitment in CME is Ub/UIM-dependent, we used two mutants (**Figure 30** on the top): 1) a lysine-less eps15 (eps15 6KR; point mutations are indicated in Materials and Methods), which cannot be monoubiquitinated (monoUb-defective), but can bind Ub; 2) eps15 L883A mutant, which is defective in Ub-binding (Woelk et al., 2006). Unfortunately, the L883A mutation not only affects Ub-binding, but also impairs the monoubiquitination of the protein (Polo et al., 2002; Woelk et al., 2006). To completely uncouple the two functions, a mutant that abrogates Ub-binding without affecting monoubiquitination is required.

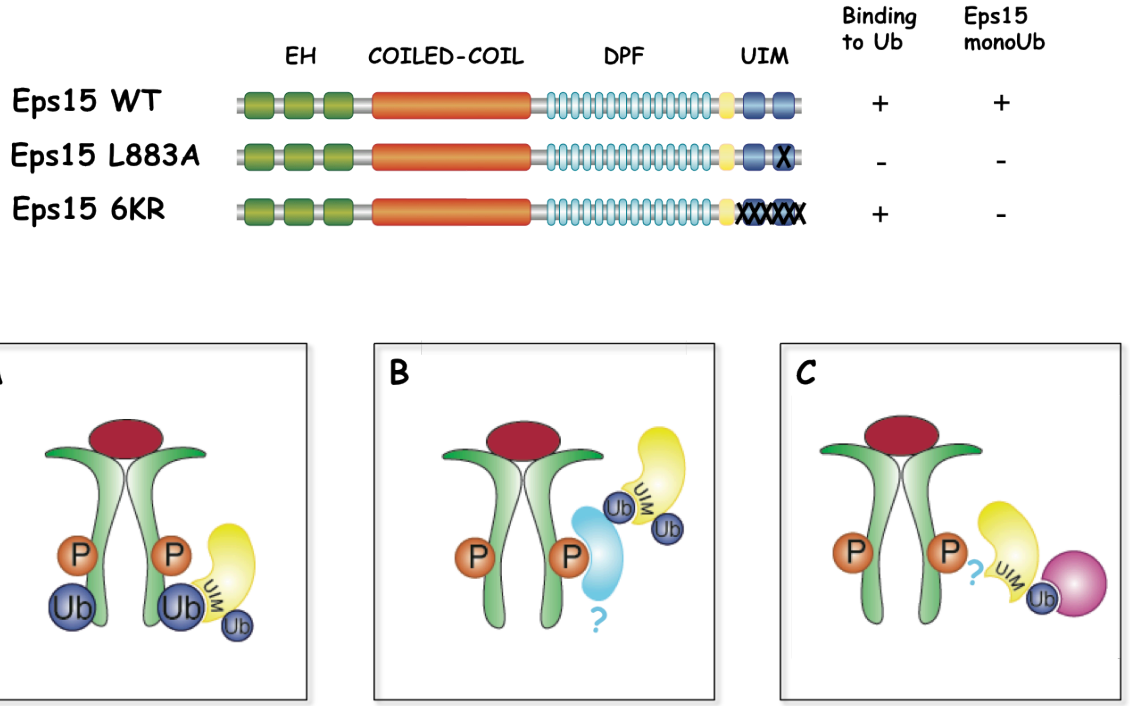


Figure 30: Experimental strategy and hypothesis.
On the top: Schematic view of the properties of eps15 WT and mutants re-expressed in eps15/R stably depleted HeLa cells clone. X indicates point mutation (see Materials and Methods for details). On the bottom: Illustration of three hypothetical mechanisms.

3.1 Eps15 is able to rescue the eps15/R/epsin1 KD endocytic defect

Eps15 WT, 6KR and L883A mutants were stably expressed as N-terminal HA-tagged versions in the stable eps15/R KD HeLa cell clone, and clones expressing the different constructs at endogenous levels were selected (**Figures 31A-B**).

Initially, we assessed the ability of eps15 WT to rescue the EGFR internalization defect in KD cells. We chose two eps15 WT clones (#1 and #2) that express the transgene at endogenous levels in a homogeneous manner (**Figures 31A-B**) and measured EGFR internalization in these cells using the ^{125}I-EGF internalization assay. Re-expression of eps15 WT *per se* had no significant effect. Re-expression of eps15 WT was however able to rescue the internalization defect resulting from transient KD of epsin1 in the stable eps15/R KD cells (**Figures 31C-D**). This result further supports functional redundancy between eps15, eps15R and epsin1 in clathrin-dependent internalization of EGFR, and allowed us to conclude that our experimental model was a good setting to analyze the effects of re-expression of the eps15 mutants.

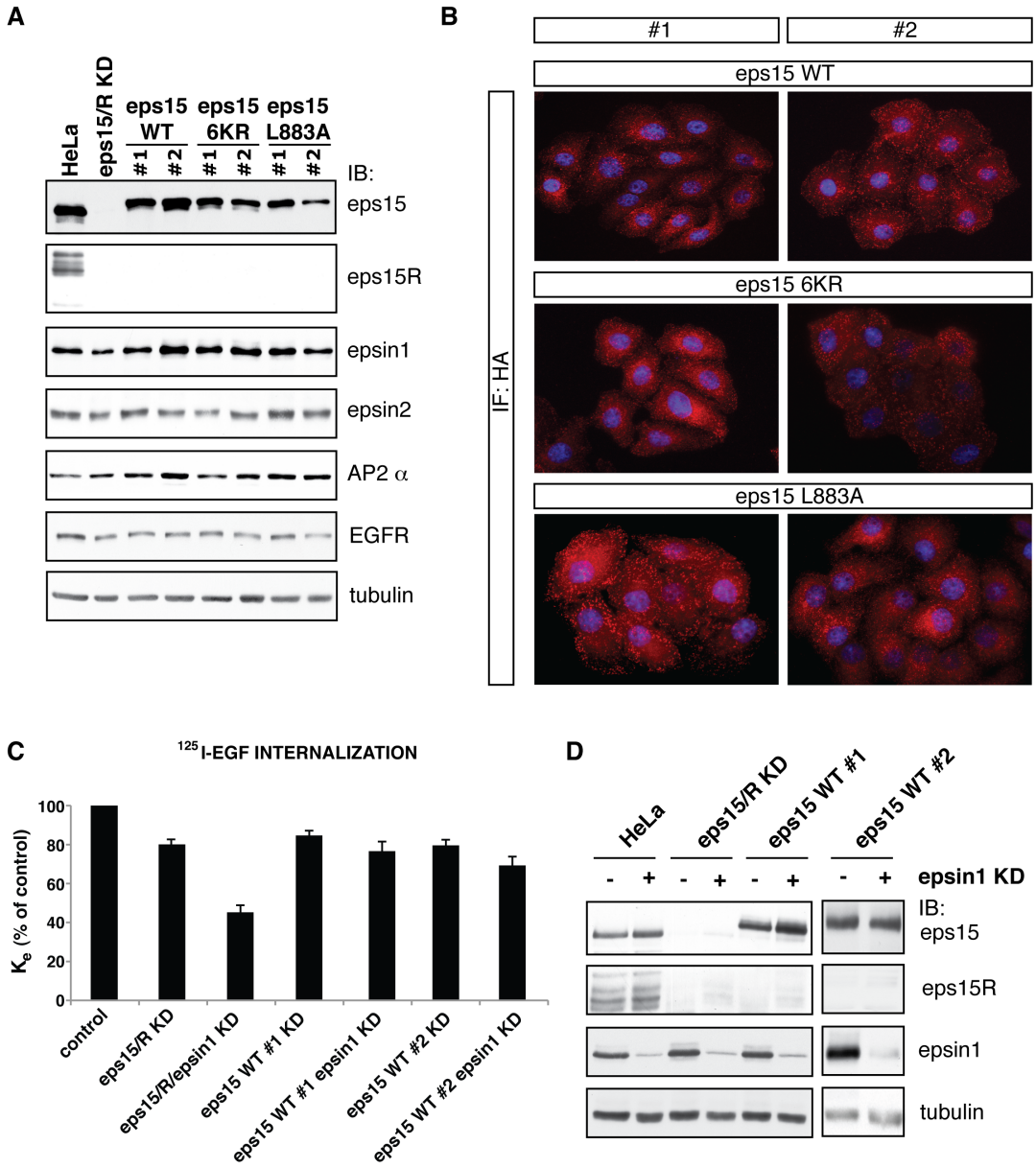


Figure 31: Rescue of eps15/R/epsin1 KD defect in EGFR internalization by eps15 WT.

A-B. Characterization of eps15/R KD HeLa cell clones stably re-expressing WT and mutant eps15. Stable eps15/R KD HeLa cells clone were infected with retroviral constructs expressing WT and mutated (6KR, L883A) eps15. Expression levels of some relevant proteins were determined by immunoblotting using specific antibodies, as indicated. Tubulin was used as protein loading control. Clones expressing WT or mutant eps15 at levels comparable to the endogenous protein in control HeLa cells were selected. In **B**, homogeneity of eps15 expression within the clones was assessed by immunofluorescence using anti-HA antibody. **C. Effect of eps15 WT re-expression on ^{125}I-EGF internalization in cells silenced for different adaptors in HeLa cells.** Kinetics of ^{125}I-EGF internalization were measured at initial time points (0-6 min) after stimulation with 1 ng/ml EGF in stable eps15/R KD HeLa cells clone stably re-expressing eps15 WT (eps15 WT #1 and #2) and/or transiently silenced for epsin1. Control oligo treated HeLa cells were used as control. Internalization constants (K_e) were extrapolated from the internalization curves and correspond to the slopes of the best-fitting curves (see Materials and Methods). Results are expressed as % of the control and are expressed as MEAN+/-SDEV of three independent experiments. **D. Efficiency of epsin1 KD.** The level of epsin1 transient KD in the indicated stable HeLa cell clones was determined by immunoblotting using specific antibodies, as indicated (these are representative experiments of three independent experiments). Tubulin was used as protein loading control.

3.2 Characterization of eps15 mutants

We next characterized the ability of the eps15 mutants, 6KR and L883A, to bind to Ub and to be monoubiquitinated upon EGF stimulation.

We first checked whether amino acid changes in the eps15 6KR mutant affects binding to Ub. Since both monoubiquitination and Ub-binding sites reside in the C-terminal portion of the protein, we used N-terminally FLAG-tagged versions of eps15 WT and the 6KR mutant. Constructs expressing these proteins were transiently transfected into WT HeLa cells and total cell lysate was subjected to a Ub pull-down assay using GST-3Ub produced in bacteria. As shown in **Figure 32A**, eps15 6KR did not present any impairment in binding to Ub.

We also assessed the binding of WT and mutant eps15 to the ubiquitinated-EGFR. (**Figure 32B**). In contrast, eps15 L883A displayed a strongly reduced interaction with EGFR (**Figure 32B**), as previously shown (Woelk et al., 2006).

Finally, we confirmed previous results showing that both eps15 L883A and 6KR display an impaired monoubiquitination upon EGF stimulation [**Figure 32C**; (Woelk et al., 2006)].

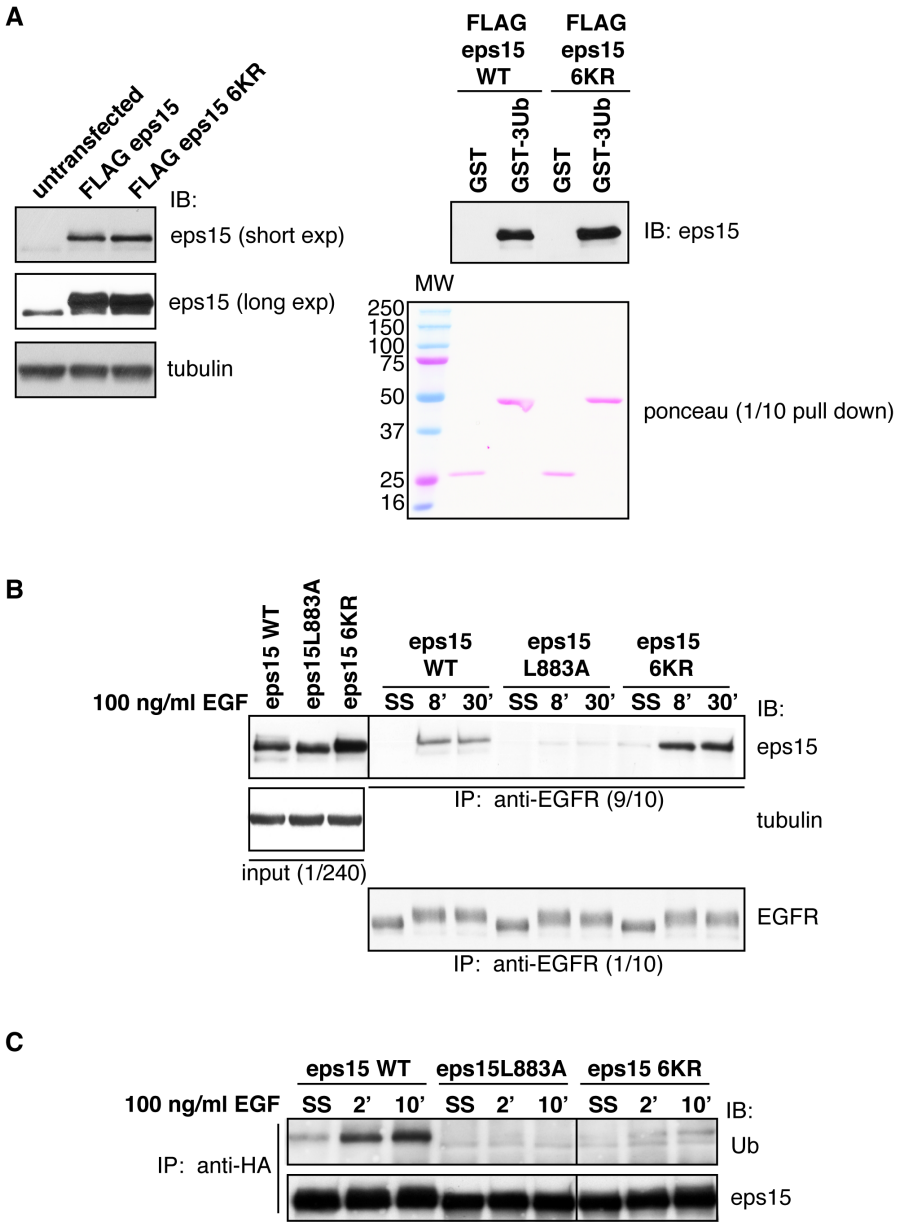


Figure 32: Characterization of eps15 L883A and 6KR mutants.
A. Ub-binding ability of N-terminally FLAG-tagged eps15 WT and 6KR. An N-terminally FLAG-tagged version of eps15 WT and eps15 6KR were transiently transfected into HeLa cells and total cell lysate was subjected to a Ub pull-down assay using GST-3Ub produced in bacteria. Incubation with GST was used to assess pull-down specificity. Left: efficiency of transfection was evaluated by immunoblotting using a specific anti-eps15 antibody**.** Right: amount of eps15 protein pulled-down was evaluated by immunoblotting using a specific anti-eps15 antibody**.** Results are representative of two independent repeats. **B. Ability of WT and mutant eps15 to interact with ligand-activated EGFR.** Eps15/R KD HeLa clones stably re-expressing eps15 constructs (WT, L883A, 6KR #1 clones - see Figure 31A-B) were stimulated with a high dose of EGF (100 ng/ml) for the indicated lengths of time after an overnight serum deprivation. Fresh lysates were subjected to anti-EGFR immunoprecipitation. The extent of co-immunoprecipitation with eps15 was evaluated through WB analysis. 1/10 of IPs was blotted for EGFR as a protein loading control. As input, amount of serum starved lysates corresponding to 1/240 of IP were loaded. Results are representative of three independent repeats. **C. Ability of WT and mutant eps15 to undergo EGF-dependent monoubiquitination.** Cells described in (**B**) were stimulated with EGF as in (**B**). Lysates were subjected to anti-HA immunoprecipitation. Eps15 monoubiquitination status was evaluated through WB analysis using anti-Ub antibody. Anti-eps15 was used as a protein loading control. Results are representative of two independent repeats.

3.3 Eps15 binding to AP2 and its ability to be monoubiquitinated are inversely correlated

Eps15 mutants were then controlled for their ability to bind AP2 in co-immunoprecipitation (co-IP) experiments. Interestingly, the two mutants not only retained binding to AP2, but also displayed a stronger interaction with respect to WT protein (**Figure 33**). This effect was independent of EGF stimulation.

Therefore, there appears to be an inverse correlation between eps15 monoubiquitination and AP2 binding.

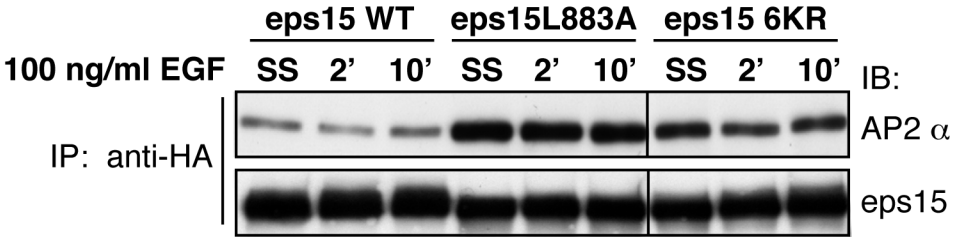


Figure 33: The ability of WT and mutant eps15 to bind to AP2 α.
Eps15/R KD HeLa clones stably re-expressing eps15 constructs (WT, L883A, 6KR #1 clones - see Figure 31A-B) were stimulated with a high dose of EGF (100 ng/ml) for the indicated lengths of time after an overnight serum deprivation. Fresh lysates were subjected to anti-HA immunoprecipitation. The extent of co-immunoprecipitation of eps15 with AP2 α was evaluated through WB analysis. Anti-eps15 was used as a protein loading control. Results are representative of two independent repeats.

3.4 Monoubiquitination of eps15 is required in clathrin-dependent internalization of EGFR

Finally, we assessed the impact of expression of eps15 mutants on clathrin-dependent internalization of EGFR using the clones (#1 and #2) shown in Figure 31A-B. Expression of the eps15 mutants in the eps15/R KD HeLa clone did not have any effect on the rate of EGFR internalization (**Figure 34**), as observed with WT eps15 (Figure 31C). Following transient epsin1 KD, however, the eps15 mutants were unable to rescue the impaired clathrin-dependent internalization of EGFR (**Figure 34**), at variance with WT eps15 (Figures 31C-D). This result indicates a requirement for eps15 monoubiquitination in CME of the EGFR, although the exact role remains to be determined. Since both eps15 mutants were equally unable to rescue CME of the EGFR, we could not make any

conclusions about a possible involvement of eps15 Ub-binding in CME. To resolve this issue, we are currently expressing a chimeric protein comprised of the eps15 L883A mutant fused to Ub, however, this is a difficult strategy which is subject to potential artefacts that will need to be carefully controlled. Indeed, this construct might not be able to mimic properly physiological monoubiquitination, either because Ub is not bound in the correct position or because during EGFR internalization continuous cycles of eps15 ubiquitination and deubiquitination are necessary to regulate the entire process.

In order to clarify the dependency on eps15 monoubiquitination, we plan to identify, via a proteomic approach, EGF-induced eps15 WT and 6KR interactors. In particular, we are interested in interactors that can bind WT but not eps15 6KR. In fact, monoubiquitin may be the signal for the recruitment of eps15 to the activated receptor (through an unknown protein containing a Ub-binding domain) or it may be required to recruit the clathrin machinery via Ub-mediated interactions. The same mechanism might apply to epsin1 and eps15R, since they are also monoubiquitinated upon EGF stimulation. Thus, monoubiquitination could embody the basis of redundancy between eps15, eps15R and epsin1 in CME of EGFR. As a matter of fact, at variance with EGFR polyubiquitination, monoubiquitination of the adaptors occurs to the same extent upon low and high dose EGF treatment (our unpublished results), underlying its importance in CME.

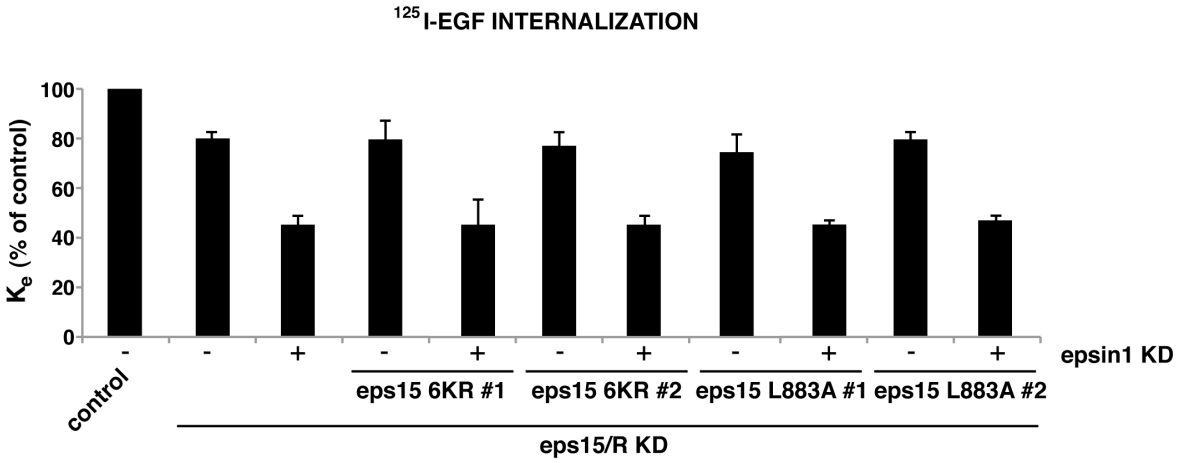


Figure 34: Effect of eps15 6KR and L883A re-expression on CME of EGFR in HeLa cells silenced for eps15/R/epsin1.
Kinetics of ^{125}I-EGF internalization were measured at initial time points (0-6 min) after stimulation with 1 ng/ml EGF in stable eps15/R KD HeLa cells clone stably re-expressing eps15 6KR or L883A (eps15 6KR #1 and #2 and eps15 L883A #1 and #2) and/or transiently silenced for epsin1. Control oligo treated HeLa cells were used as control. Internalization constants (K_e) were extrapolated from the internalization curves and correspond to the slopes of the best-fitting curves (see Materials and Methods). Results are expressed as % of the control and are expressed as MEAN+/-SDEV of three independent experiments.

3.5 Eps15-Grb2 binding is not essential in clathrin-dependent endocytosis of the EGFR

Parachoniak and colleagues recently demonstrated that direct binding of the eps15 coiled-coil domain to the Met receptor is involved in its internalization. They also uncovered a role of an eps15-Grb2 interaction in the same process (Parachoniak and Park, 2009). Since we, and others, have demonstrated that Grb2 exerts a specific role in CME of the EGFR [Figure 22; (Grandal et al., 2011; Huang et al., 2004)], we investigated whether an eps15-Grb2 interaction mediates recruitment of eps15 to EGFR. Parachoniak and colleagues mapped the site of the eps15-Grb2 interaction to residue P769 in human eps15, through co-IP experiments from cells overexpressing both Grb2 and eps15 WT and mutants (Parachoniak and Park, 2009). Therefore, it is likely that the eps15-Grb2 interaction is mediated by the interaction of a proline rich region in eps15 and an SH3 domain in Grb2. By *in vitro* pull-down assay with purified proteins, we confirmed a direct binding between Grb2 and eps15, which is dependent on the eps15 proline residue at the C-terminus, as demonstrated using a murine eps15 P771A mutant (note that we employed murine eps15 in which P771 corresponds to human P679; **Figure 35B**, lanes

7,8). Interestingly, free eps15 (free means that it has been cleaved from GST) is able to bind to GST-AP2 α-ear [(i.e., the portion of α subunit known to bind to eps15; (Benmerah et al., 1996)] and GST-Grb2 with comparable affinity in a purified system at 600 nM concentration, even using a buffer with 0.1% SDS (**Figure 35A**). Of note, the region around P771 of murine eps15 is highly conserved in murine eps15R (**Figure 35C**), which is also able to bind Grb2 *in vitro* [**Figure 35B**; eps15R isoform2, that lacks the C-terminus, doesn't bind to Grb2 (lane 9), while eps15R DPF domain, that corresponds to the C-terminus, indeed binds to Grb2 (lane 10)]. The same region is also conserved in the human counterparts (**Figure 35C**). The obvious question arising from these results is whether epsin1 can also bind to Grb2. Pull-down experiments showed that epsin1 could also bind to Grb2 *in vitro* (**Figure 34D**).

We also investigated whether we could obtain *in vitro* a tricomplex formed by GST-EGFR intracellular domain (GST-EGFR ID; commercially available – see Materials and Methods), free Grb2 and free eps15. We set up a protocol to induce autophosphorylation and Cbl-induced ubiquitination of the GST-EGFR ID, in a pure *in vitro*-reconstituted system. We incubated GST-EGFR ID either unmodified, phosphorylated only, or phosphorylated and ubiquitinated, with increasing amounts of Grb2 and fixed amounts of eps15 (**Figure 35E**). GST was used as negative control. Note that, Grb2 is known to bind only to phosphorylated EGFR, at specific residues: pY1068 and pY1086. We observed that eps15 is unable to bind to unphosphorylated and phosphorylated GST-EGFR ID in the absence of Grb2 (**Figure 35E**, lanes 1,4-6). This result indicates that eps15 cannot be directly recruited to nonubiquitinated, pY-EGFR ID. In contrast, binding of eps15 to the pY-EGFR ID occurs in the presence of increasing amounts of Grb2 (**Figure 35E**, lanes 7,8) and there is a further increment in binding when the receptor is also ubiquitinated (**Figure 35E**, lanes 9-11). Based on these data, we hypothesized that Grb2 could function as a bridge between activated EGFR and eps15.

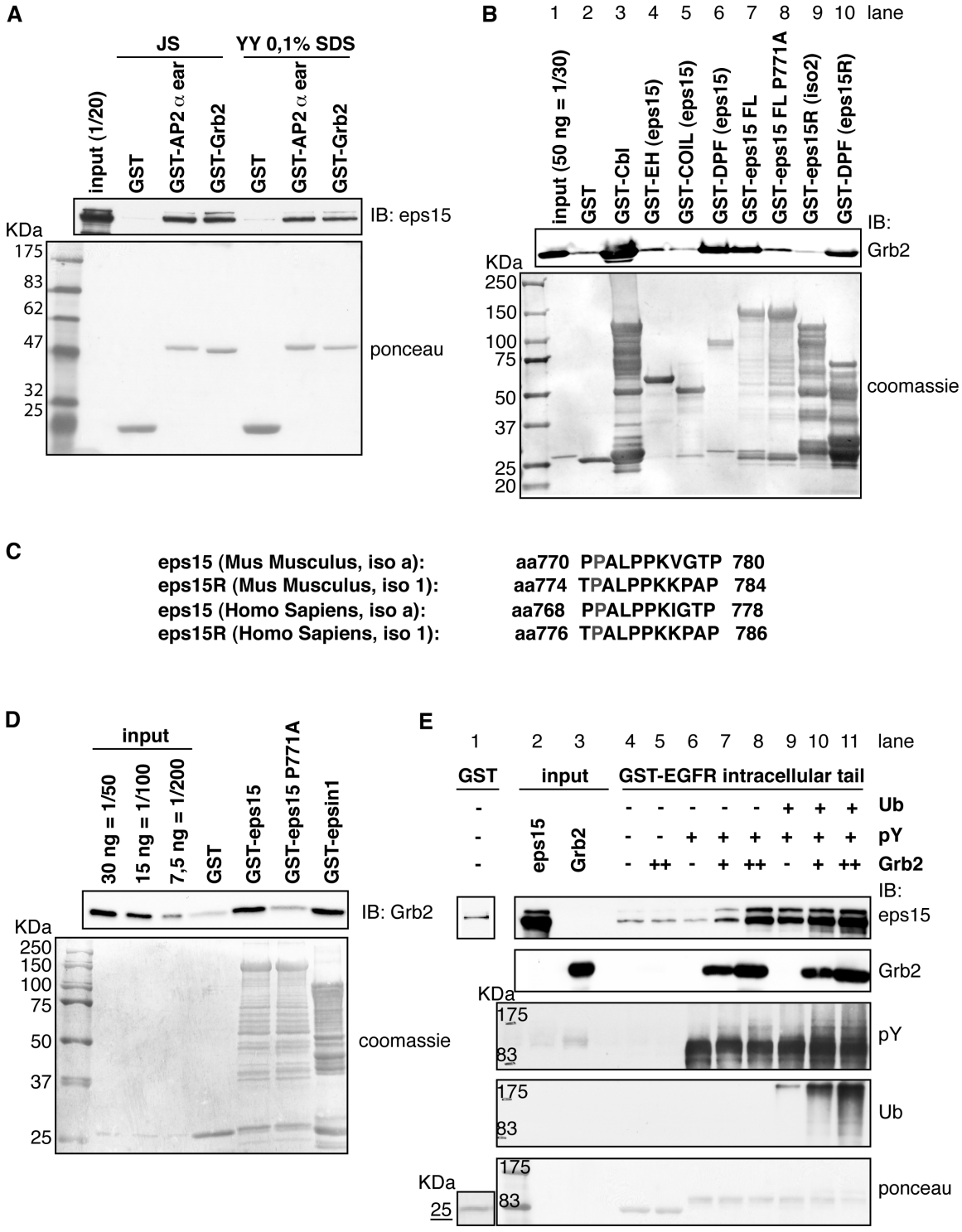


Figure 35: Analysis of Grb2 interaction with eps15, eps15R and epsin1 *in vitro*.
A. Eps15 interacts with Grb2 *in vitro*. The indicated GST-tagged proteins were incubated with equimolar amounts of free eps15 (600 nM) for 2 hrs at 4°C, in two different buffers, as indicated. GST alone was used as a negative control. The extent of interaction with eps15 was evaluated through WB analysis, using a specific anti-eps15 antibody, as indicated. Ponceau staining was used as GST-protein loading control. Results are representative of three independent repeats. **B. Eps15 interacts with Grb2 through its P771 residue *in vitro*.** The indicated GST-tagged proteins were incubated with equimolar amount of free Grb2 (600 nM) for 2 hrs at 4°C. GST alone was used as a negative control. The extent of interaction with Grb2 was evaluated through WB analysis, using a specific anti-Grb2 antibody, as indicated. Coomassie staining was used as GST-protein loading control. For constructs details see Materials and Methods. Results are representative of

three independent repeats. **C. Amino acid sequence conservation between murine eps15 and eps15R. D. Epsin1 interacts with Grb2 *in vitro*.** The indicated GST-tagged proteins were incubated with equimolar amounts of free Grb2 (600 nM) for 2 hrs at 4°C. GST alone was used as a negative control. The extent of interaction with Grb2 was evaluated through WB analysis, using a specific anti-Grb2 antibody, as indicated. Coomassie staining was used as GST-protein loading control. Results are representative of three independent repeats. **E: Eps15 binds to phosphorylated GST-EGFR intracellular tail through Grb2.** GST-EGFR intracellular tail has been either phosphorylated and phosporylated/ubiquitinated or not and then subjected to pull-down with equimolar amounts of free eps15 (600 nM) in presence or absence of increasing amounts of free Grb2 (1X and 20X) for 2 hrs at 4°C. GST was used as negative control. The extent of interaction with Grb2 and eps15 was evaluated through WB analysis, using specific antibodies, as indicated. Phosporylation and ubiquitination status of GST-EGFR intracellular tail has been assessed by immunoblotting with specific antibodies (respectively anti-pY and anti-Ub), as indicated. Ponceau staining was used as GST-protein loading control. Results are representative of three independent repeats.

We next investigated the relevance of the Grb2-mediated EGFR-eps15 interaction in cells by performing pull-down assays with purified GST-tagged proteins and HeLa cell lysate (we used GST-α ear and GST-Crk as positive controls; **Figure 36A**) and co-IP assays in HeLa cells (**Figure 36B**). With both assays, we observed a very low stoichiometry of interaction between eps15 and Grb2. One possibility is that the interaction could be too transient and labile to be detected in these assays. Thus, we performed rescue experiments to investigate the involvement of the eps15-Grb2 interaction in CME of EGFR. We stably expressed murine eps15 P771A in eps15/R KD HeLa cells, and selected two clones (#1 and #2) that expressed this mutant eps15 at endogenous levels (**Figures 36C-D**). We observed that expression of the eps15 P771A mutant in eps15/R KD HeLa cells had no effect on EGFR internalization (**Figure 36E**). However, similarly to WT eps15 (Figure 31C), and at variance with eps15 K6R and L883A (Figure 34), eps15 P771A could rescue the internalization defect observed in the triple eps15/R/epsin1 KD cells (**Figure 36E**). These data indicate that eps15-Grb2 binding is not essential for eps15/R/epsin1-dependent EGFR internalization and that the shared ability of eps15/R/epsin1 to bind Grb2 (demonstrated *in vitro*) cannot explain the functional redundancy between these adaptors. These rescue data are in agreement with the low level of binding observed in cells.

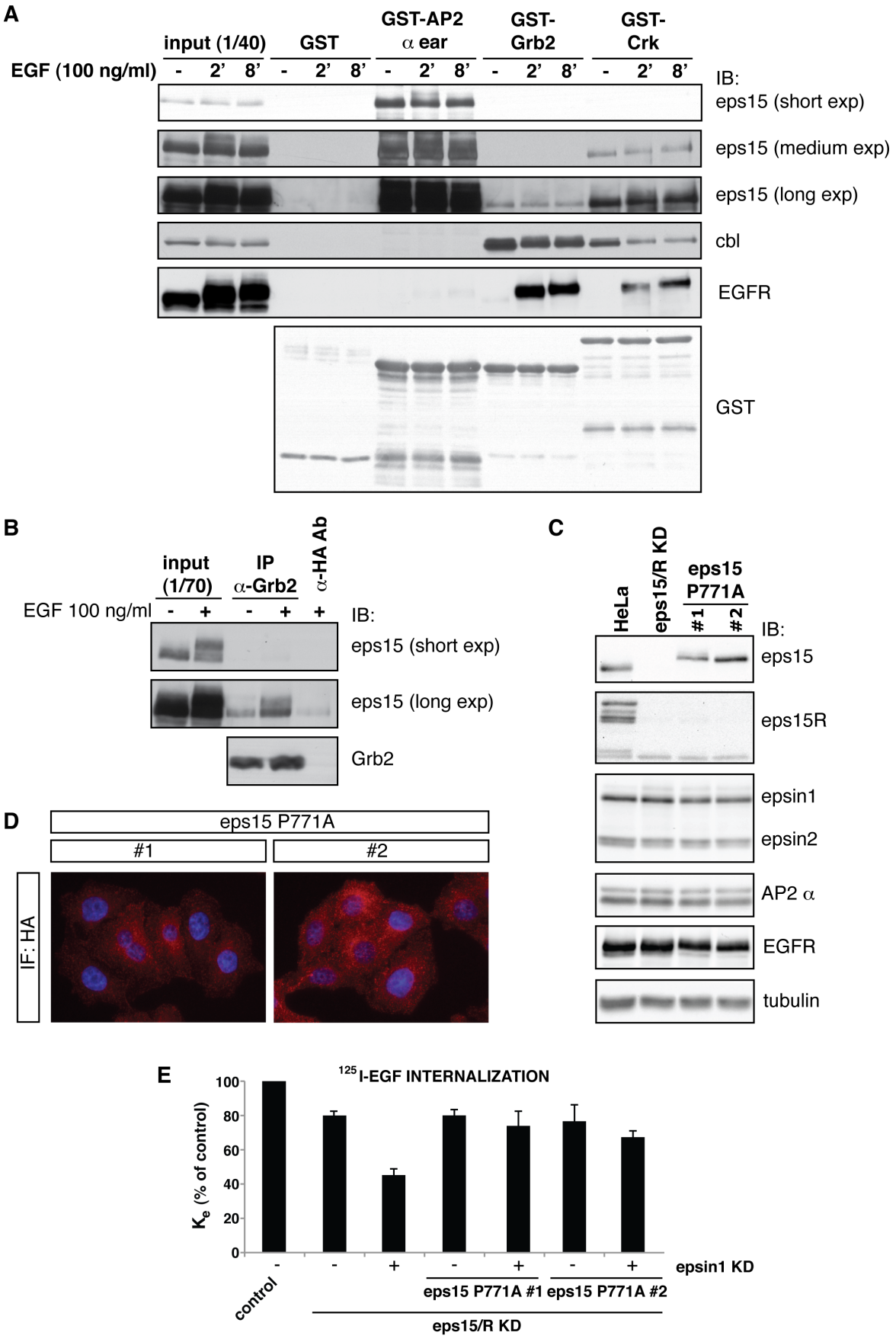


Figure 36: Evaluation of the role of the Grb2-eps15 interaction in CME of EGFR.
A. Analysis of the interaction between GST-Eps15 and Grb2 in cellular lysate. The indicated GST-tagged proteins were incubated with Hela cells lysate for 2 hrs at 4°C. GST alone was used as a negative control. Hela cells were stimulated with high dose of EGF

(100 ng/ml) at indicated time points after an overnight serum deprivation (high dose was used in order to try to increase eventual EGF-dependent binding). The amount of proteins pulled-down was evaluated through immunoblotting, with the indicated antibodies. Anti-GST was used as a protein loading control. As input, amount of lysate corresponding to 1/40 of total amount used in the pull down (1mg) were loaded. Results are representative of three independent repeats. **B. Analysis of the interaction between eps15 and Grb2 in cellular lysate.** HeLa-Oslo cells were stimulated with high dose of EGF (100 ng/ml; high dose was used in HeLa-Oslo where EGFR enters only through CME, in order to try to increase eventual EGF-dependent clathrin-related eps15-Grb2 binding) at indicated time points after an overnight serum deprivation. Fresh lysates (1mg) were subjected to anti-Grb2 immunoprecipitation (and to anti-HA immunoprecipitation as a negative control). The amount of eps15 co-immunoprecipitating with Grb2 was evaluated through immunoblotting. Anti-Grb2 was used as a protein loading control. Results are representative of three independent repeats. **C-D. Characterization of eps15/R KD HeLa clones stably expressing eps15 P771A.** Eps15/R KD HeLa cells were infected with constructs expressing murine eps15 P771A. Clones expressing eps15 P771A at levels comparable to the endogenous eps15 in WT HeLa cells were selected. The levels of other relevant proteins were determined in the eps15 P771A clones by immunoblotting using specific antibodies, as indicated. Tubulin was used as protein loading control. In **D**, homogeneity of eps15 expression within the clones was assessed by immunofluorescence using anti-HA antibody. **E. Effect of eps15 P771A re-expression on ^{125}I-EGF internalization in cells silenced for different adaptors.** Kinetics of ^{125}I-EGF internalization were measured at initial time points (0-6 min) after stimulation with 1 ng/ml EGF in eps15/R KD HeLa cells stably re-expressing eps15 P771A (eps15 P771A #1 and #2) and/or transiently silenced for epsin1. Control oligo treated HeLa cells were used as control. Internalization constants (K_e) were extrapolated from the internalization curves and correspond to the slopes of the best-fitting curves (see Materials and Methods). Results are expressed as % of the control and are expressed as MEAN+/-SDEV of three independent experiments.

DISCUSSION

1. Molecular heterogeneity in CME of EGFR

1.1 AP2-dependent and -independent routes of CME

CME is the most studied and best characterized pathway of EGFR internalization. Despite this, during the last 20 years numerous observations have been produced, which are sometimes difficult to reconcile with each other and which highlight gaps in our understanding of the process. In particular, the roles of AP2 and EGFR ubiquitination in CME of the EGFR are highly debated.

AP2 is the principal non-clathrin constituent of purified endocytic CCVs and has long been considered to be the major adaptor for CME. Recently, however, novel clathrin adaptors have been characterized, which have been demonstrated, or proposed, to work as substitute adaptors for AP2 in CME, since they can bind to both cargo and clathrin. Nevertheless, some scientists in the endocytic field still believe in the essential role of AP2 in CCV formation (Boucrot et al., 2010; Henne et al., 2010).

Indeed, silencing of the different AP2 subunits in HeLa cells completely blocks CME of the TfR, while CME of the EGFR can still occur in the absence of AP2 (Huang et al., 2004; Johannessen et al., 2006; Motley et al., 2003). Since there is disagreement in the literature about the effect of AP2 depletion on EGFR endocytosis, likely due to different experimental conditions (Huang et al., 2004; Johannessen et al., 2006; Motley et al., 2003), we initially assessed the effect of AP2 silencing on EGFR internalization in different cellular systems. We used an internalization assay that more faithfully represents the physiological situation, since ligand was applied to the cells *in continuum*, without any ligand prebinding on ice, which could mask internalization defects (Huang et al., 2004). In three different cell lines, using different siRNAs against AP2, we scored a 50% decrease of EGFR internalization rate (Figures 14-15). These data confirm the existence of AP2-dependent and -independent pathways of EGFR internalization, in both cancer and normal cell lines, and suggest that alternative endocytic adaptors might be involved in CME of the EGFR. However, an alternative explanation for the AP2 KD phenotype has been put forward: since EGFR is a signaling receptor, it might be able to induce the

recruitment of the few AP2 molecules left in the KD cells more efficiently than cargo that internalizes constitutively and has no signaling ability, such as TfR. Indeed, EGF is able to induce formation of new CCPs in cells depleted of AP2 (Johannessen et al., 2006). These CCPs contained EGF, EGFR and Grb2, but not the TfR (Johannessen et al., 2006). Nevertheless, it has never been directly investigated whether these new CCPs also contain residual AP2. Moreover, EGFR mutated in AP2-binding sites is still internalized in a clathrin-dependent manner, supporting the existence of an AP2-independent route of CME of the EGFR (Huang et al., 2003; Nesterov et al., 1999; Sorkin et al., 1996).

To further substantiate our data and finally resolve this issue, we are planning to perform ^{125}I-EGF internalization assays in cells derived from AP2 conditional knockout mice in collaboration with Volker Haucke (Freie Universität, Berlin). In these cells there should not be any AP2 left that can be used by activated EGFR. Thus, in the experimental condition in which only CME is active, if in these cells EGF internalization won't be block, this will be an irrefutable evidence of the existence of an AP2-independent EGFR clathrin internalization pathway.

1.2 Role of eps15, eps15R and epsin1 as redundant and alternative-to-AP2 adaptors

Thanks to the genetic approach we followed, we were able to identify alternative adaptors to AP2 involved in CME of EGFR. In contrast to previous studies, we performed multiple combinations of adaptor KD, which permitted us to identify eps15, eps15R and epsin1 as alternative EGFR-specific adaptors that function in a redundant manner.

1.2.1 Redundancy among eps15, eps15R and epsin1

Several experimental evidences support the fact that eps15, eps15R and epsin1 have a redundant function in CME of EGFR. First, single and double KD of eps15, eps15R and epsin1 did not cause major defects in EGFR internalization (Figure 17), while contemporary silencing of all the three proteins resulted in a 55% decrease in the EGFR internalization rate (Figure 18). The defect observed in eps15/R/epsin1 KD was higher

than the sum of the defects observed in single epsin1 KD and in the double eps15/R KD, and this difference was calculated to be statistically significant (Figure 18). This was the first suggestion of a putative redundancy among epsin1, eps15 and eps15R.

The second indication of the just mentioned redundancy comes from the comparison with the milder effect observed in triple eps15/R/epsin2 KD (Figure 18). Indeed, while single epsin1 and epsin2 KD behave similarly and display an additive effect when silenced together (Figure 17C-D), they showed a clear difference when combined with eps15/R KD (Figure 18). Indeed the defect found in eps15/R/epsin2 KD is even less than the sum of the partial defect, possibly indicating that the three proteins are working in the same pathway. This highlights a functional difference between epsin1 and epsin2 in the CME of the EGFR. However, from the amino acid alignment (**Figure 37**), the two proteins show high structural similarity. Future proteomic and structure-function studies should allow us to unveil the reason of this functional difference.

The confirmation of the redundancy among eps15, eps15R and epsin1 comes from rescue experiment. In fact, re-expression of eps15 WT reverted the EGFR internalization defect of eps15/R/epsin1 KD (Figure 31). Moreover, as discussed below, functional redundancy was not only observed in internalization experiments, but also had an impact in EGF-induced signaling (Figure 25). Also in the case of signaling, re-introduction of eps15 rescued the eps15/R/epsin1 KD phenotype back to control level (Figure 27).

1.2.2 Eps15, eps15R and epsin1 are redundant AP2-independent adaptors

The EGFR internalization defects observed upon single and multiple depletion of eps15, eps15R, epsin1 and epsin2 could be due to partial or total impairment of the same pathway where AP2 is functioning. To investigate this issue, we analyzed the effect of knocking down AP2 in combination with the other adaptors.

Thus, the combination of AP2 KD with single (epsin1/AP2, epsin2/AP2, eps15/AP2 and eps15R/AP2) or double KDs (epsin1/2/AP2 and eps15/R/AP2) gave exactly the same effect as the AP2 KD alone (Figures 19A,B). These results suggest that defects observed

in single and double eps15/R and epsin1/2 (Figure 17) are upstream to AP2. Indeed, all these adaptors can bind AP2 (Benmerah et al., 1998; Chen et al., 1998) and it is known that these adaptors can work with an optimizing function in AP2-containing CCVs (Ford et al., 2002; Henne et al., 2010), as also indicated by the fact that their depletion also affect TfR internalization (Figure 18), which is entirely AP2-dependent. However, we observed that the simultaneous silencing of the four adaptors epsin1/eps15/R/AP2 reduced EGFR internalization rate to clathrin KD levels (Figure 19), indicating that eps15/R/epsin1 and AP2 can also function in distinct pathways.

We reasoned that eps15/R/epsin1 could be responsible of 30% residual AP2-independent CME of EGFR. We explained the higher defect observed in eps15/R/epsin1 KD cells (i.e., ~55%) with the contemporary partial impairment of the AP2-dependent pathway. This represents the fourth result in support of the idea of a functional overlap between the three adaptors, meaning that any one of them (either epsin1, eps15 or eps15R) is sufficient to sustain AP2-independent EGFR clathrin internalization.

Notably, epsin1 has already been proposed to function as an endocytic adaptor independently of AP2, since it is able to bind both clathrin and cargo. Indeed, epsin was found to colocalize with CCPs in AP2-depleted cells (Hinrichsen et al., 2003; Motley et al., 2003). In the case of eps15/R, the results described in this thesis represent the first evidence supporting a role of these proteins as endocytic adaptors in CME that function independently of AP2. A logical extension of these data is that eps15/R - possibly through indirect interactions - should be able to recruit clathrin and promote CCV formation.

Consistent with our findings, a recent paper describes the requirement of epsin1 and eps15 for the formation of clathrin pedestal structures generated during enteropathogenic *Escherichia Coli* infection (Darkoh and DuPont, 2011). Epsin1 and eps15 recruit clathrin in these structures in an AP2-independent manner. However, differently from our findings related to the EGFR, epsin1 and eps15 are not redundant in this system, since depletion of one is sufficient to block pedestal formation. Therefore, given also our results on TfR internalization, we can speculate that redundancy between

epsin1, eps15 and eps15R is specific to CME of the EGFR. The structural basis of this redundancy and its function will be discussed later.

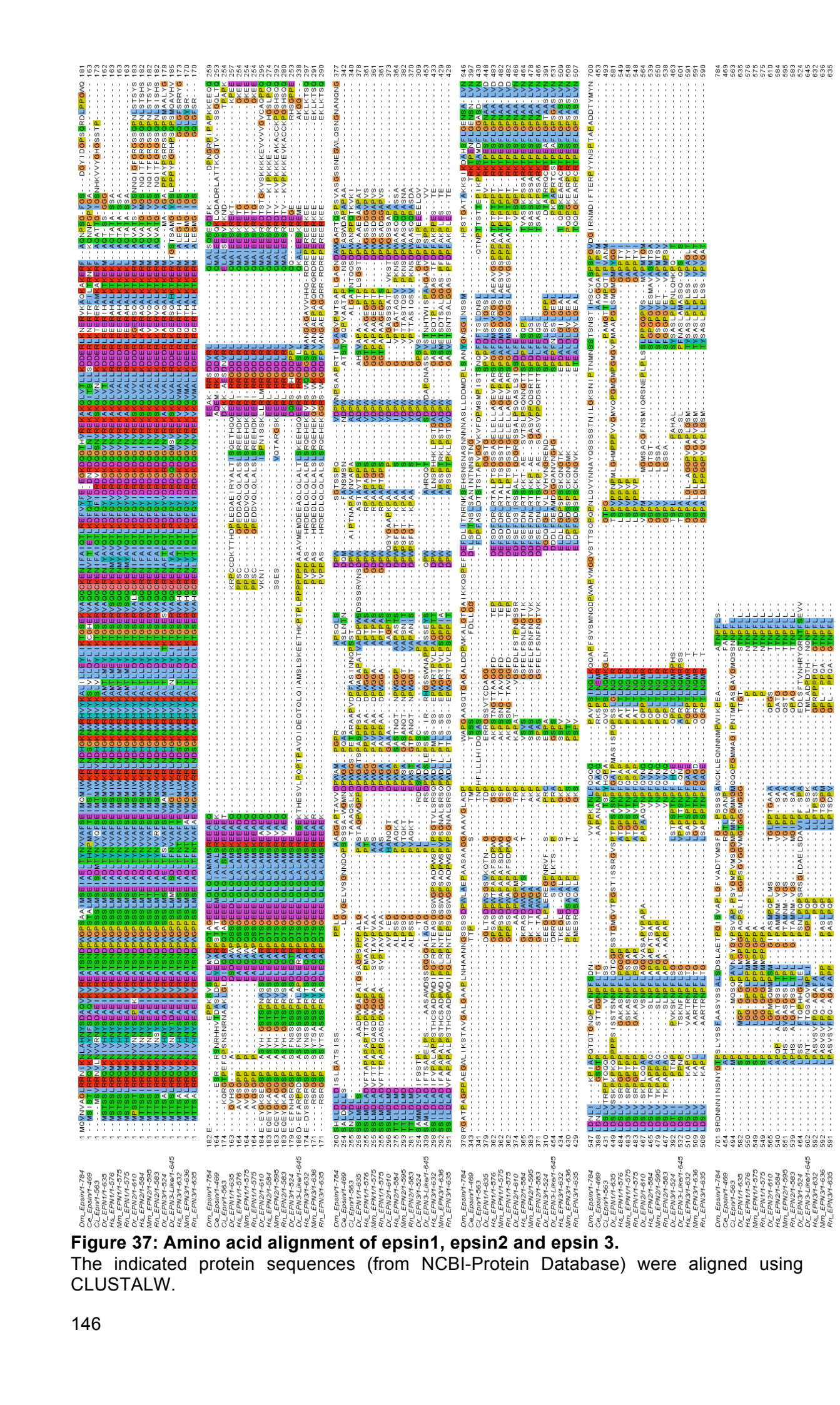


Figure 37: Amino acid alignment of epsin1, epsin2 and epsin 3.
The indicated protein sequences (from NCBI-Protein Database) were aligned using CLUSTALW.

1.3 Cargo-specific mechanisms are involved in CME

In the present thesis, we have demonstrated, through a quantitative analysis, that the different endocytic adaptors are capable of differentially regulating clathrin endocytosis of the EGFR *vs.* TfR. Indeed, KD of eps15, eps15R and epsin1, singularly or in combination, had a less marked effect on TfR, respect to EGFR internalization. Of note, in the case of TfR, silencing of eps15/R/epsin1 KD cause a delay of a process that is entirely AP2 dependent, while we showed that they could work also independently of AP2 in EGFR internalization (Figure 19). This result points to specific mechanisms of internalization between distinct cargoes. We also confirmed in our system that Grb2 specifically affects EGFR internalization without delaying TfR endocytosis [Figure 22; (Huang et al., 2004)]. Notably, Crk, which is structurally very similar to Grb2 and can bind to eps15 (Figure 36), is not involved in either EGFR or TfR internalization. In contrast to Grb2, FCHo proteins are essential for TfR internalization, while dispensable for EGFR internalization (Figure 20). Finally, we have highlighted an involvement of intersectin1/2 in CME of the EGFR, accounting for ~20% of internalization (Figure 23). We have confirmed their function upstream of AP2 and also that intersectin1/2 share this function with eps15/R, in line with Henne's work [Figure 23; (Henne et al., 2010)].

These findings, pointing to distinct, cargo-specific, CME pathways, are in agreement with the emerging concept that distinct receptors display different molecular requirements during their internalization and that internalization could proceed through specific subsets of CCVs (Maurer and Cooper, 2006; Puthenveedu and von Zastrow, 2006). This specialization in the CME pathway could be necessary to avoid competition between different receptors for internalization. Indeed, a series of studies have addressed this issue in the past. Specifically, in the case of EGFR and TfR, pioneering studies revealed that saturation of the TfR endocytic pathway does not influence endocytosis of the EGFR (Warren et al., 1997). Later, EM analysis described poor co-trafficking of EGFR and TfR into the same CCV (Stang et al., 2004; Tosoni et al., 2005). Recently, TIRFM live imaging studies revealed that there was very little overlap between the EGF and Tf signals at the PM (Leonard et al., 2008). Moreover, these studies showed that EGF and Tf

bind to their receptor in very different patterns: the Tf signal comprised both diffuse and concentrated patches distributed over the entire adherent cell surface. In contrast, EGF was mostly observed in smaller spots that tended to concentrate around the periphery of the cell. Finally, live imaging studies showed that a different Rab5 GTPase content between Tf and EGF-containing vesicles is responsible for cargo sorting at the endosomes (Leonard et al., 2008).

A more intriguing hypothesis to explain the existence of distinct pools of CCVs, containing different repertoires of adaptors, is that they are generated to target receptors to distinct intracellular fates. Indeed, we can envisage a system where a decision taken at the PM (i.e., one or the other vesicle) determines the fate of the cargo once internalized (Lakadamyali et al., 2006). This might be crucial in the case of signaling receptors, such as EGFR or GPCRs, since it will have an impact on downstream signaling cascades and biological responses. Indeed, our data on EGF-dependent signaling support such a scenario (see below "2. Functional heterogeneity in CME of EGFR" for further discussion on this point).

1.4 CCPs heterogeneity: reality or consequence of genetic perturbations?

The main problem of the approach utilized above is that knocking down expression of a protein (and in many cases more than one protein) might cause cellular rearrangement and compensation phenomena that give rise to indirect effects that cannot be easily predicted, and which could lead to erroneous interpretation of the results. For this reason, we need to validate our genetic studies with live-imaging techniques, which will allow us to monitor in time and space the recruitment of different clathrin endocytic adaptors to EGFR upon EGF stimulation. The challenge will be to perform 4-color movies to follow contemporarily labeled-EGF, fluorescent clathrin and fluorescent adaptors (both AP2 and eps15 or epsin1). This approach will provide a snapshot of the real situation in cells, with minimal manipulation of the system.

Indeed, from the vesicle formation point of view, our results can be subjected to different interpretations: i) AP2 and eps15/R/epsin1 are cooperating, through different mechanisms, in the formation of the same EGFR-containing vesicles, as has recently been proposed (Goh et al., 2010); thus, their alternative depletion simply slows down the same process. ii) AP2 and epsin1/eps15/R are involved in the formation of different types of CCVs, hence, their alternative depletion selectively blocks the formation of one type of CCV (**Figure 38**). Indeed, from an evolutionary perspective, these two possibilities are equally reasonable. In fact, in mammalian cells, important cellular processes usually have redundant and/or cooperative mechanisms, thus ensuring that cellular homeostasis is maintained. Alternatively, we could speculate that the existence of specialized pools of vesicles, associated with distinct functions, could be a way to diversify functions. It is also plausible that a mixed situation exists, in which the enrichment of different adaptors varies among the vesicles. Live imaging will be instrumental in addressing these crucial biological questions.

The existence of multiple types of EGFR containing CCVs might imply the existence of differential sorting signals in the EGFR cytoplasmic tail upon EGF, which, however, has not been addressed in this thesis. Another possibility is that local concentration and distribution of the EGFR and its adaptors can regulate vesicle formation. In other words, intrinsic properties of PM (and surrounding) microdomains might determine the molecular composition of vesicles.

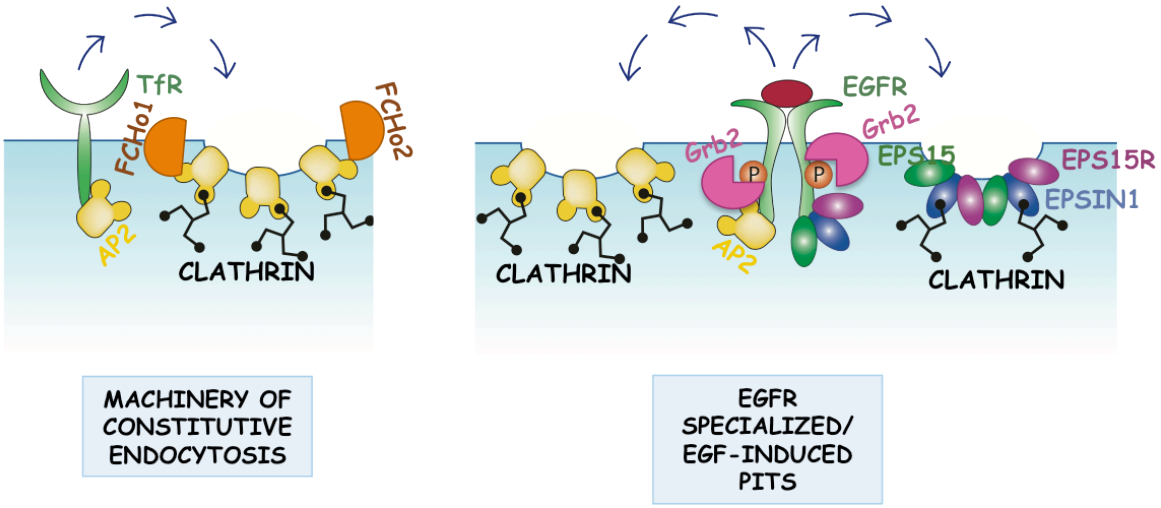


Figure 38: Differential molecular requirements for CME of EGFR and TfR.
Various endocytic adaptors are involved in CME of the EGFR, possibly functioning in distinct pathways, leading to the generation of specialized CCPs (depicted on the right). The picture represents a simplified model, just to underline the different master players in the two CCPs. Our data indicates the presence of eps15, eps15R, epsin1, epsin2, intersectin1 and intersectin2 as well in AP2-containing CCP. In contrast, the majority of TfR is internalized through a single CME route involving AP2 and FCHo proteins (depicted on the left). Eps15, eps15R and epsin1 might play an optimizing function in the formation of vesicles internalizing TfR.

A debated issue is whether EGFR is internalized through so-called preformed pits or whether it induces formation of "*de novo*" CCPs. In the endocytosis field, there is a general consensus that a mixed scenario exists, although different studies have reported varying proportions of internalizing EGFR in these different types of pits, possibly attributable to the different cell lines and techniques used (Puri et al., 2005; Rappoport and Simon, 2009). Based on the observation that the TfR, which is a constitutively recycled cargo, is internalized via preformed CCPs (Mayle et al., 2012), and that EGFR recycling is dependent on AP2 (Sigismund et al., 2008), we can envisage that the AP2-dependent EGFR pool is internalized via preformed CCPs. On the other hand, epsin1, eps15 and eps15R could be responsible for "*de novo*" pit formation, via a mechanism that is AP2-independent and that requires receptor activation. Further investigations are required to test these hypotheses and to resolve the issue of preformed *vs.* "*de novo*" CCPs.

2. Functional heterogeneity in CME of the EGFR

2.1 Distinct pools of vesicles, distinct signaling outcomes

Mounting evidences indicates that endocytosis controls the type and duration of downstream signaling response (Sigismund et al., 2012). Several reports in the literature have suggested that the different mechanisms of EGFR clathrin-dependent internalization are molecularly redundant pathways, but have not deeply investigated whether there are any biological implications of the different pathways (Goh et al., 2010).

In this present thesis, by investigating the functional significance of the distinct molecular mechanisms of CME, we have found that they have strikingly different impacts on EGFR signaling and biological outputs. Indeed, AP2 KD showed a decrease both in the peak phase and in the sustaining phase of AKT phosphorylation; a minor reduction in MAPK signaling was also detected (Figures 25A,B). This result confirms our previous data with high dose of EGF in AP2 KD HeLa cells (Sigismund et al., 2008). Since AP2 does not act in NCE, the effect on EGFR signaling was attributed to its role in CME (Sigismund et al., 2008). Strikingly, eps15/R/epsin1KD showed exactly the opposite phenotype compared to AP2, displaying enhanced and sustained AKT signaling at later time points, while MAPK signaling was unchanged (Figures 25A-B). Of note, the single epsin1 KD and double eps15/R KD had no effect, supporting the notion of functional redundancy between eps15, eps15R and epsin1 (Figure 25C).

These data are consistent with a scenario in which AP2 and eps15/R/epsin1 could give rise to distinct pools of vesicles with opposing signaling functions (**Figure 39**). Thus, different CME pathways could be specialized, not only in terms of cargo, but also in terms of vesicles containing the same cargo. In this sense, internalization at the PM appears to be the first sorting step for EGFR intracellular trafficking. In this case, evolution would have favored the diversification, rather than the preservation, of function.

The notion that internalization of the EGFR into specialized CCVs could represent an initial sorting step that influences signaling outcome is supported by the fact that the contribution of eps15 to the signaling phenotype is directly linked to its internalization role rather than its role at later sorting step. This reasoning is based on the fact that we

employed shRNAs that target the EH domain present in the full-length protein, but not the shorter Hrs-endosome-specific eps15 isoform b, which mediates EGFR degradation (Roxrud et al., 2008). Moreover, the signaling phenotype was rescued by the reintroduction of the full-length PM isoform of eps15 (Figure 27). Nevertheless, this data does not exclude the possibility that multiple levels of regulation exist, acting at different stations along the endocytic route, which renders the system more robust.

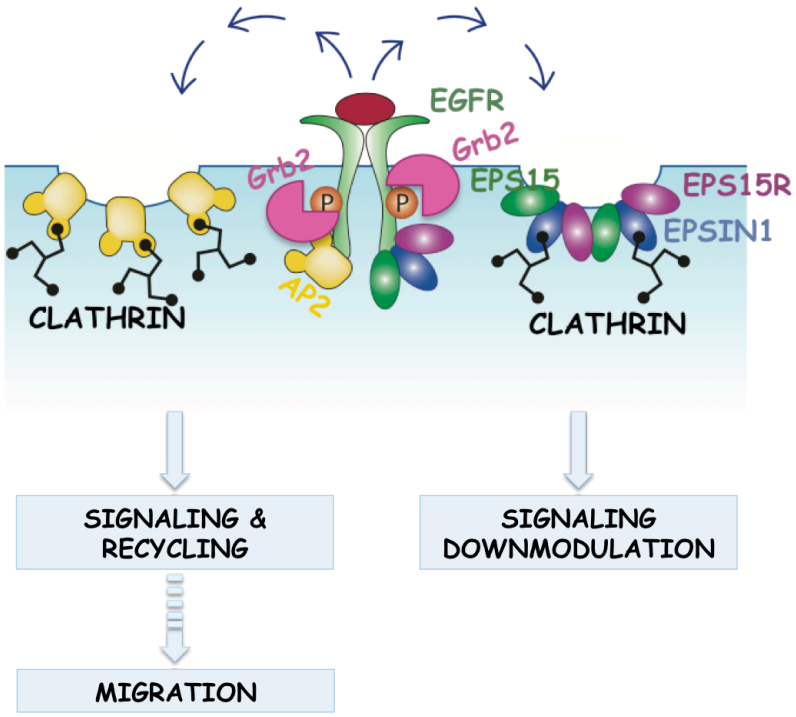


Figure 39: Different pools of EGFR-containing CCVs are linked to different EGFR fates and signaling outcomes.
EGFR that enters through AP2-dependent CCVs is preserved from degradation and destined for recycling to PM. This might contribute to sustaining of signaling, which, together with polarized recycling, can ultimately lead to cell migration. Conversely, formation of EGFR-containing CCVs that rely redundantly on eps15/R/epsin1, determines EGFR signaling downmodulation, through a yet to be defined mechanism.

2.2 How do endocytic adaptors impact on the final signaling response?

How is differential signaling regulation by distinct pools of CCV achieved? One obvious possibility is that the presence of distinct pools of adaptors, directly or indirectly, impacts on the subsequent sorting step at the endosomal level. Based on our data, it appears that AP2 functions as a molecular determinant able to direct EGFR trafficking, sustaining its recycling (Sigismund et al., 2008) and preventing its degradation (Figures 28A-B, **39**). Strikingly, we also clearly established a positive correlation between AP2 and EGF-

induced cell migration, a process that requires polarized recycling [(Palamidessi et al., 2008); Figures 29, **39**]. This finding could have profound implications on our understanding of cancer pathogenesis and metastasis, in which deregulation of signaling causes aberrant proliferation and migration. Since AP2 usually detaches from vesicles after uncoating, before reaching the endosomal compartment (Loerke et al., 2011), we can envisage that AP2-containing vesicles are already committed to recycling. This notion is supported by the fact that TfR, which is constitutively recycled, is internalized via an AP2-dependent default pathway (Mayle et al., 2012).

While the function of AP2 in CME of the EGFR is well defined, the exact roles of eps15, eps15R and epsin1 are less clearly understood. Although depletion of these alternative adaptors caused sustained AKT activation, which was reverted by re-expression of eps15 WT (Figure 27), no effects on EGFR trafficking or migration were observed (Figures 28A, 29). It is possible, therefore, that impaired EGFR endocytosis caused prolonged signaling in eps15/R/epsin1 KD cells, but that this aberrant signaling might be uniformly localized at the cell surface, leading to loss of the spatial information necessary to induce directed cell migration upon EGF stimulation. This possibility could be addressed through immunofluorescence experiments. Alternatively, AKT sustainment might be involved in other types of EGF-induced cellular responses, such as proliferation. We plan to test this latter possibility.

Instead of influencing intracellular sorting steps, endocytic adaptors might influence signaling activation by impacting on cargo retention time inside endocytic structures at the PM. Indeed, this appears to be the case for GPCRs, the endocytosis of which occurs preferentially through a specialized subset of CCPs. These CCPs display an increased surface residence time compared to the other CCPs, which is regulated locally by GPCR cargo via PDZ-dependent linkage to the actin cytoskeleton (Puthenveedu and von Zastrow, 2006). This retention of GPCR-CCPs at the PM has been proposed, yet not demonstrated, to be required for proper regulation of GPCR signaling (Puthenveedu and von Zastrow, 2006). Live imaging analysis of clathrin-dependent events upon EGF stimulation will be instrumental to examine whether the different adaptors (AP2 *vs.*

eps15/R/epsin1) differentially regulate CCPs retention at the PM and vesicles dynamics, thereby differentially regulating signaling activation.

3. Mechanism of AP2-independent CCV formation: role of adaptor ubiquitination

One critical issue that remains unsolved is the mechanism of recruitment of eps15/R and epsin1 to the activated EGFR, independently of AP2. The involvement of these alternative adaptors in CME of the EGFR is not specific to the EGFR, since they are also involved in clathrin-dependent internalization of other cargos, such as influenza virus and Notch (Chen and Zhuang, 2008; Darkoh and DuPont, 2011; Meloty-Kapella et al., 2012). However, what is specific to CME of EGFR, is their functional redundancy in this process.

Eps15/R and epsin1 have been shown to be associated with EGFR-containing CCPs (Benmerah et al., 2000; Chen et al., 1998). Since these adaptors possess the UIM, they have been proposed to mediate CME of the EGFR by binding to the ubiquitinated EGFR (Bertelsen et al., 2011; Hawryluk et al., 2006; Stang et al., 2004). However, several lines of evidence argue against a direct binding of eps15/R/epsin1 to ubiquitinated EGFR. Firstly, the simultaneous silencing of eps15/R/epsin1 significantly affects EGFR internalization at low dose of ligand when EGFR ubiquitination is negligible (Sigismund et al., 2005). Secondly, using different cell lines and experimental approaches it has been demonstrated that defects in EGFR ubiquitination do not have an impact on CME (Huang et al., 2007; Sigismund et al., 2005). Therefore, receptor ubiquitination appears not to be a major mechanism of recruitment of the EGFR to CCPs. Indeed, an EGFR mutant that lacks Tyr1045 (Cbl binding site) and that is weakly ubiquitinated, is internalized normally in PAE (Porcine Aortic Endothelial) cells (Jiang and Sorkin, 2003). Moreover, mutation of the ubiquitination sites in the EGFR kinase domain did not affect its internalization (Huang et al., 2007; Huang et al., 2006). Collectively, these data indirectly suggest that epsin1, eps15 and eps15R are not recruited to the EGFR in CCPs via mechanism dependent on receptor ubiquitination. This does not imply that the Ub signal *per se* is not involved. Indeed, all three proteins undergo UIM-dependent monoubiquitination (Polo et al., 2002).

Importantly, monoubiquitination of eps15 is already induced at low EGF doses (our unpublished data) and might be involved in generating a network of protein-protein interactions involved in CME.

To investigate whether the mechanism of adaptor recruitment in CME is Ub/UIM-dependent, we performed a structure-function investigation taking advantage of two types of eps15 mutants: 1) a lysine-less eps15 6KR, which is monoubiquitination-defective, but still able to bind to Ub; 2) a eps15 L883A mutant, which is defective both in Ub-binding and in monoubiquitination (Woelk et al., 2006). We assessed the ability of these eps15 mutants, as well as of eps15 WT, to restore EGFR internalization rate to control levels in eps15/R/epsin1 KD HeLa cells. We observed that:

1) eps15 WT re-expression was able to restore normal EGFR internalization rates (Figure 31). This result corroborates the notion of functional redundancy among eps15, eps15R and epsin1 in EGFR clathrin internalization;

2) monoubiquitination of eps15 is essential to its ability to rescue internalization (Figure 34). Monoubiquitination may be the signal for the indirect recruitment of eps15 to the activated receptor through an unknown protein containing a Ub-binding domain, or it may be required to recruit the clathrin machinery via Ub-mediated interactions. In order to define the exact role of eps15 monoubiquitination, we will employ a proteomics approach to identify candidate proteins that interact with monoubiquitinated eps15 upon EGF stimulation. Likely candidates will be those that interact with eps15 WT but not the eps15 6KR mutant (see below “4. Future directions”).

Although the exact role of eps15 monoubiquitination in CME of EGFR is unknown, our results highlight, for the first time, a crucial involvement of this post-translational modification in CME. The requirement of adaptor monoubiquitination in clathrin internalization has already been demonstrated for GPCR (Shenoy et al., 2009). In this case, ubiquitination of the adaptor arrestin appears to cause a conformational change that renders arrestin free to bind receptor and to induce its internalization (Shenoy et al., 2009). In the case of EGFR, the role of adaptor ubiquitination has been addressed through the use of a chimera made by the fusion of eps15 and Ub, which should mimic

monoubiquitinated eps15 (Hoeller et al., 2006). This eps15-Ub chimera fails to localize properly to endocytic vesicles containing internalized EGFR, and this has been associated with delayed internalization and degradation of the receptor (Fallon et al., 2006; Hoeller et al., 2006). A possible interpretation of this result is that the chimera forms an intramolecular interaction between the UIM and Ub, which confers a closed conformation to eps15 resulting in its detachment from the activated EGFR. Therefore, monoubiquitination was proposed to negatively regulate eps15 function (Hoeller et al., 2006). However, the eps15-Ub chimera might not represent a physiological situation. In addition, it is possible that cycles of ubiquitination/deubiquitination are required for normal eps15 function, which would be altered in the chimera. Our results, instead, indicate that monoubiquitination might represent a positive signal required for CME, possibly involved in recruiting adaptors. However, the situation could be more complex and a mixed scenario is also possible.

Furthermore, we observed an inverse correlation between eps15 monoubiquitination and AP2 binding (Figure 33). With respect to AP2 binding by eps15, eps15 monoubiquitination appears to be a negative regulator. Such an effect might be exerted through a conformational closure, as previously hypothesized (Hoeller et al., 2006), or through the masking of AP2-binding sites by Ub given the close proximity of the Ub- and AP2-binding sites in eps15 (van Bergen En Henegouwen, 2009). Whatever the case, the fact that AP2-binding and monoubiquitination appear to be mutually exclusive is in line with our idea about the two distinct functions of eps15 in CME of the EGFR: one function dependent on AP2, and the other one independent from AP2, but dependent on its monoubiquitination and redundant with eps15R and epsin1. In the future, it will be important to address the molecular mechanism underlying the regulation of AP2-eps15 binding possibly through eps15 monoubiquitination and its functional implications (see below “4. Future directions”).

Based on our data, it is not possible to draw conclusions about the requirement of Ub-binding by eps15 in CME of the EGFR, since the L883A mutant, which is Ub-binding deficient is also monoubiquitination-impaired. To uncouple these two functions, a mutant

displaying impaired Ub-binding, but normal monoubiquitination, is required. However, since EGFR ubiquitination does not appear to be essential for CME (see above), we could speculate that eps15 Ub-binding might be important to bind ubiquitinated intermediate adaptors, rather than for direct binding to the receptor. One candidate intermediate adaptor is Grb2, which others and we have demonstrated to be a master player in EGFR clathrin internalization [Figure 22, (Huang et al., 2004)]. Moreover, our preliminary data indicates that Grb2 is monoubiquitinated upon EGF stimulation (Appendix Figure 2). In addition, it has recently also been demonstrated that eps15 is able to interact with Grb2 (Parachoniak and Park, 2009). The site of interaction in eps15 has been mapped to a proline-rich region, which is also conserved in eps15R (Figure 35A-C). Our data showed that epsin1 could contain the same functional domain (Figure 35D). Despite this conservation of the proline-rich region, an eps15 mutant that is defective in binding to Grb2 was able to rescue EGFR internalization rate when expressed in the triple eps15/R/epsin1 KD cells (Figure 36E). Thus, the eps15-Grb2 interaction does not appear to be essential for CME of the EGFR. However, we cannot exclude that *in vivo* the proline-rich region in eps15, together with Ub-mediated binding of eps15 to monoubiquitinated Grb2 could be involved in EGFR internalization. Since the interaction between UIM and monoubiquitin is labile, the involvement of such interactions in CME is difficult to prove biochemically.

Another candidate intermediate adaptor is CIN85, which has already been implicated in EGFR endocytosis (Soubeyran et al., 2002). CIN85 is also able to bind to Ub and can be monoubiquitinated (Bezsonova et al., 2008; Haglund et al., 2002). Moreover, CIN85 is an adaptor protein linking Cbl to the clathrin-machinery, through endophilin (Jozic et al., 2005; Soubeyran et al., 2002).

A more complicated scenario could be that epsin1 and eps15/R exert functional redundancy through distinct molecular strategies and specific binding partners that are concurrently recruited to the activated EGFR. More experiments are required to distinguish between these possibilities.

4. Future directions

One of our priorities is to directly visualize EGFR internalization and its associated clathrin adaptors by live imaging. As anticipated in the above paragraph "1.4 CCPs heterogeneity: reality or consequence of genetic perturbations?", this analysis will be instrumental to complement our ^{125}I-EGF internalization data obtained upon interference of the different adaptors and to allow appropriate interpretation of the data. This study will be performed using constructs already characterized in literature (Table 4). EGFR internalization events will be detected by following EGF-Alexa647 that we have shown to be functional (Figure 24B).

As an initial step, we checked our ability to reproduce data reported in the literature in terms of vesicle lifetime. We started transiently co-transfecting cells with AP2σ-GFP and mRFP-CHC and recording events of constitutive endocytosis in growing (unstimulated) conditions using TIRFM. To run the analysis, we utilized the free tracking software ICY (de Chaumont et al., 2012), which has already been employed for the purpose of endocytic studies (Genovesio et al., 2006). We were able to reproduce data reported in literature, in terms of vesicle lifetime distribution, using the same experimental conditions (Loerke et al., 2009), with productive events (i.e., CCPs that reaches maturation into CCVs) having a mean lifetime in the range of 60 sec (**Figures 40A-B**). Interestingly, we noticed that the number of clathrin tracks was greater than the number of AP2 tracks (**Figure 40C**), consistent with published data (Boucrot et al., 2010; Loerke et al., 2011). An accurate analysis revealed that the excess of clathrin tracks was due to the presence of high number of short-lived events, lasting not more than 10 seconds (**Figure 40C**). These short-lived events might correspond to intracellular structures that contain clathrin, but not AP2, and which transiently pass close to the PM (Loerke et al., 2011). Alternatively, they could represent abortive pits with undetectable or null AP2 content, or simply noise due to the use of diverse fluorophores (this latter point will be investigated by inverting fluorophores).

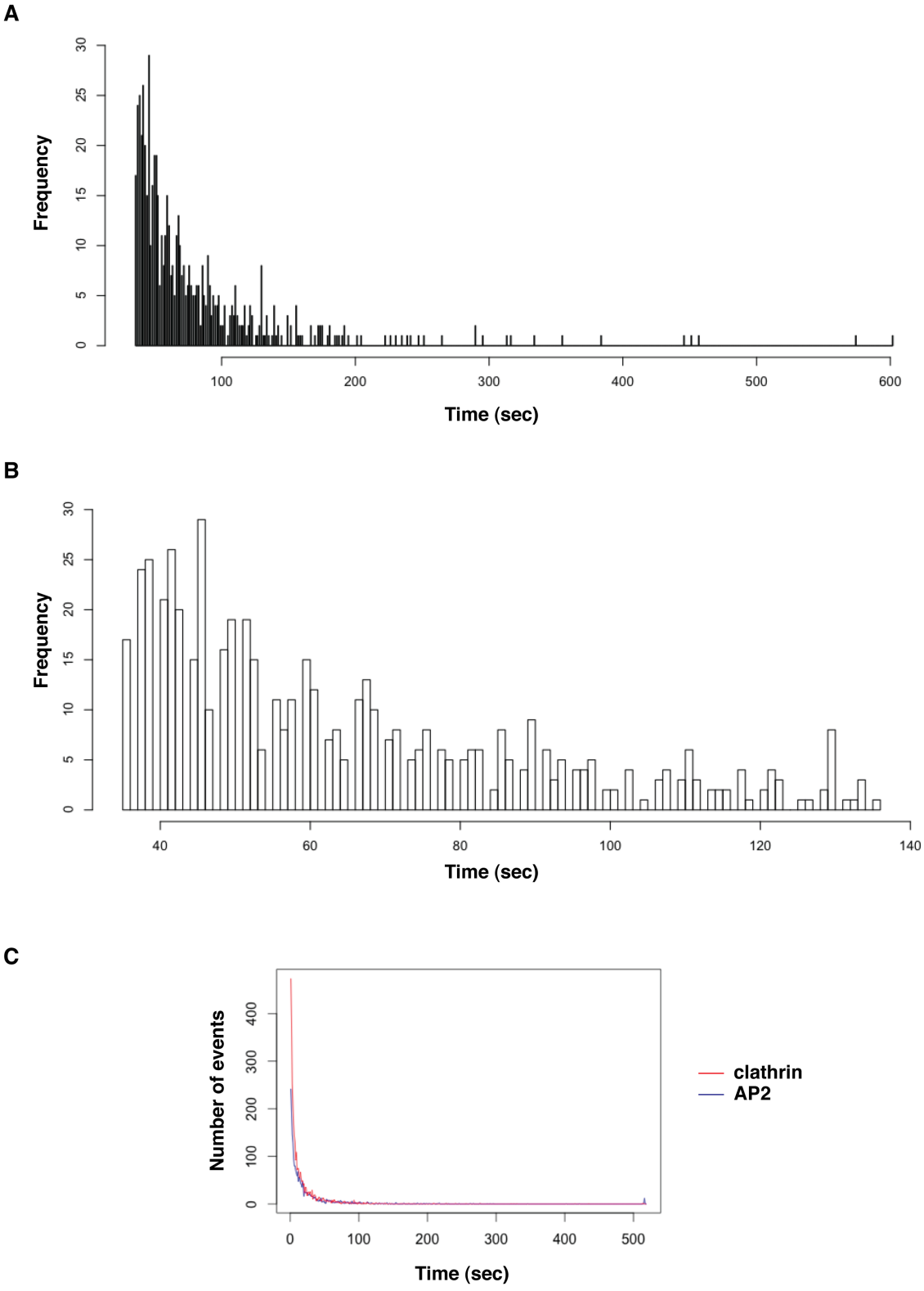


Figure 40: Live imaging analysis of constitutive CCVs.
A-C. Distribution of CCVs lifetimes followed by TIRFM in HeLa cells transiently expressing mRFP-CHC and AP2σ-GFP. HeLa cells were co-trasfected with vectors encoding mRFP-CHC and AP2σ-GFP. Images were acquired every 1.5 sec for 10 min using TIRFM. Reconstruction of clathrin (**A**, **C**) and AP2 (**B**-**C**) tracks in the time was done by particle tracking software ICY. Data in **A** and **B** are represented as histograms showing time (sec; it indicates the lenght of a single track, that is the lifetime of a CCV) on the x-axis and frequency on the y-axis. In **C** graph shows time (sec; it indicates the lenght of a single track, that is the lifetime of a CCV) on the x-axis and number of events on the y-axis (event means a single track, that is the lifetime of a CCV). The graphs in **A**, **B** and **C** are relative to the same single HeLa cell.

Having established the reliability of our system and of the analysis software, we will now stimulate cells with EGF to investigate how ligand stimulation affects vesicle lifetime and whether EGF can induce the formation of CCVs lacking AP2. It will be interesting also to analyze whether EGF can influence the number of short tracks of clathrin detected under unstimulated conditions.

An interesting feature that emerged from this thesis is the requirement of adaptor monoubiquitination in EGFR internalization. Moreover, we observed an inverse correlation between eps15 monoubiquitination and binding to AP2 (Figure 33). This result is in accordance with an AP2-independent function of eps15 in clathrin-dependent EGFR internalization. To investigate further this issue, as a simple initial experiment to complement those employing eps15 mutants, we plan to analyze the impact of AP2 KD on eps15 monoubiquitination, in the presence and absence of EGF. To investigate directly EGF-dependent regulation of AP2-eps15 binding (possibly through eps15 monoubiquitination), live-imaging analysis, as described above, will allow us to determine the extent of eps15/AP2 colocalization in CCPs, and to determine whether this colocalization is influenced by EGF. In parallel, we will address changes in the localization between AP2 and eps15 upon EGF treatment in fixed samples by TIRFM. In these experiment we will utilize eps15 mutants defective in AP2-binding (eps15 ΔAP2 should be impaired in colocalization with AP2) or in monoubiquitination (the extent of colocalization should increase). We also plan to address this point biochemically, through co-immunoprecipitation experiments. However, since AP2 and eps15 are constitutively associated and EGF induces monoubiquitination of only a minor fraction of total eps15, we expect that variations in eps15/AP2 binding will be less-easily detected biochemically.

To directly address the role of EGF-induced eps15 monoubiquitination in CME of the EGFR, we plan to perform comparative proteomics, after immunoprecipitating eps15 in cells expressing eps15 WT or eps15 6KR upon EGF treatment, using stable isotope labelling with amino acids in cell culture [SILAC; (Ong et al., 2002; Ong and Mann, 2006)]. SILAC is a simple and straightforward approach for *in vivo* incorporation of a label into proteins for quantitative proteomics. SILAC relies on metabolic incorporation of a given

'light' or 'heavy' form of an amino acid into proteins, in which the difference between light and heavy amino acids is the substituted stable isotopic nuclei (^{13}C instead of ^{12}C and ^{15}N instead of ^{14}N). Thus, in a SILAC experiment, two cell populations are grown in almost identical culture media, the only difference being that one medium contains the 'light' version of a particular amino acid, while the other contains the 'heavy' version (e.g., ^{12}C and ^{13}C labeled Lys respectively). When the labeled analogue of an amino acid is supplied to cells in culture instead of the natural amino acid, it is incorporated into all newly synthesized proteins. After a number of cell divisions, the natural version of a particular amino acid will be replaced by its isotope-labeled analogue. Incorporation of the heavy amino acid into a peptide leads to a known mass shift compared with the peptide that contains the light version of the same amino acid, without any other chemical changes. The advantage of this technique is that the two samples can be treated as a unique sample, starting from the immunoprecipitation step and throughout the mass spectrometry analysis, thus minimizing experimental variations between the two samples. This procedure will allow us to identify specific and low-stoichiometry interactors of the monoubiquitinated form of eps15, which will be present in higher amounts in WT *vs.* mutant sample, and to eliminate contaminants or common binders, not regulated by Ub, which should be found in equal amounts in the two samples. In addition, we expect also to identify interactors that bind more strongly to the 6KR mutant compared to WT eps15, as in the case of AP2, where monoubiquitination appears to negatively regulate the interaction. As previously mentioned, only a minor fraction of eps15 is monoubiquitinated upon EGF treatment and thus we do not expect a high enrichment of eps15-Ub specific interactors. Thus, it is essential to analyze the two samples with a comparative and quantitative proteomic technique, such as SILAC.

Our structure-function analysis of eps15 did not allow us to draw conclusions about the requirement of eps15 binding to Ub, since the Ub-binding defective L883A mutant is also monoubiquitination impaired. Therefore, to dissect the two functions, a mutant that abrogates Ub-binding without affecting monoubiquitination is required. However, the UIM is absolutely required for monoubiquitination. Thus, we plan to use an

artificial eps15 construct, similar to the one already described by Hoeller *et al.* (Hoeller et al., 2006): i.e., a chimeric protein comprised of eps15-L883A and Ub, which would represent a Ub-binding defective eps15 that is constantly ubiquitinated. With this construct, we plan to perform rescue experiments in our cell model system. However, this construct might not mimic the physiological ubiquitination of eps15 and, therefore, will be only informative in the case of rescue of the phenotype.

Finally, to validate eps15 AP2-independent function, we also plan to assess the capability of a ΔAP2-eps15 mutant to rescue the internalization defect of the triple KD cells. Eps15 possess multiple DPF motifs that have the potential to bind AP2, however 4 major DPF motifs have been demonstrated to be the minimal essential AP2-binding unit (Iannolo et al., 1997). Thus, we plan to mutate this minimal AP2-binding unit, thereby avoiding the use of a large deletion mutant, which might present perturbation in the protein structure. This ΔAP2-eps15 mutant, together with the eps15 L883A-Ub chimeric protein, will be also used to investigate the potential regulation of eps15 monoubiquitination by AP2.

APPENDIX

1. EGFR activation influences TfR internalization

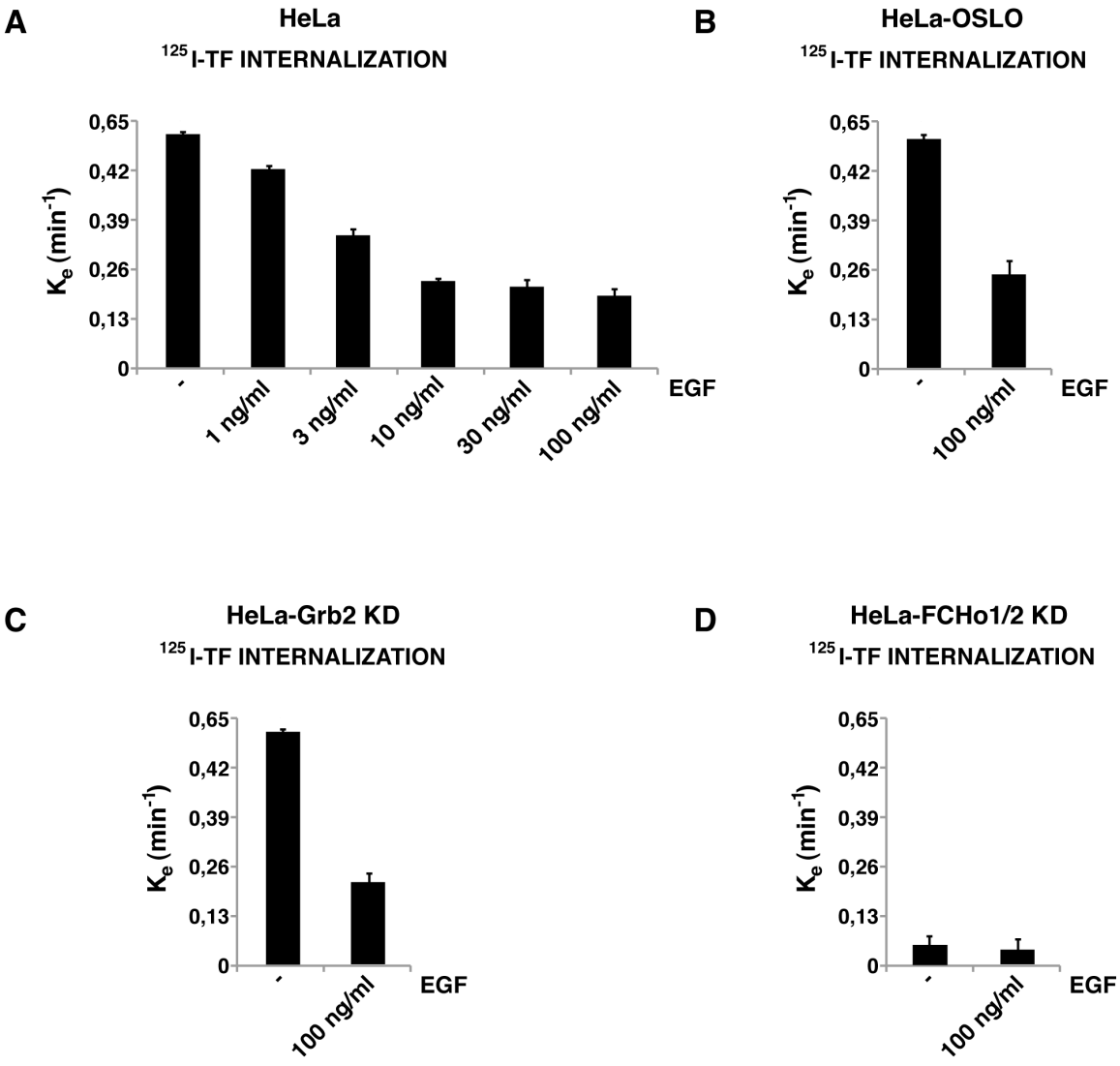
Our data suggest that there are common players, but also specific factors involved in clathrin-mediated internalization of EGFR and TfR. This finding prompts several questions: are different cargoes internalized in the same vesicles or are they endocytosed through separate/specialized vesicles? Do different cargoes compete for common endocytic machinery? Different studies have touched on this latter point over the past years, specifically with regards to the EGFR and TfR, as described in the Discussion section "1.3 Cargo-specific mechanisms are involved in CME". However, a number of outstanding issues remain to be resolved, such as how EGFR activation and internalization impact on TfR internalization kinetics.

In order to investigate further the interplay between TfR and EGFR clathrin-dependent internalization, we performed ^{125}I-Tf internalization assays upon stimulation with increasing doses of EGF. Since the NCE pathway, in addition to the CME pathway, is activated in our HeLa cells at high EGF dose, we performed, in parallel, the same experiment in HeLa-Oslo cells, in which EGFR is internalized solely through CME even at high EGF dose. We observed in both HeLa and HeLa-Oslo cells a decrease in the TfR internalization rate, already at non-saturating doses of EGF, which became more evident with increasing doses of EGF, becoming less than half the initial rate upon maximum EGF dose (**Appendix Figures 1A-B**).

Importantly, this reduction in TfR internalization rate, induced by EGF, was still observed even in Grb2 KD cells, in which EGFR endocytosis and recruitment to CCPs is specifically inhibited (**Appendix Figure 1C**). This result suggests that EGFR is not subtracting a saturable component of the endocytic machinery that is required for TfR endocytosis, in agreement with previous reports (Warren, Green et al. 1997). A possible explanation of the results could therefore be that upon EGF stimulation, the EGFR recruits a certain amount of TfRs in a subpopulation of EGFR-specific CCPs, which display a slower internalization rate. However, KD of FCHo, which is specifically involved in TfR endocytosis (Figure 20), blocked TfR internalization to the same extent in presence or

absence of EGF, indicating that active EGFR is not able to divert TfRs from their canonical internalization pathway to an EGF-specific pathway (**Appendix Figure 1D**).

Together these results suggest that EGF is influencing TfR internalization possibly through its signaling ability and not by directly cotrafficking with it. This is in line with published data that indicates minimal cotrafficking of the EGFR and TfR (Tosoni, Puri et al. 2005; Leonard, Hayakawa et al. 2008). It is possible that an indirect crosstalk between the EGFR and TfR internalization machinery exists, mediated by the EGFR signaling cascade (Wilde et al., 1999). Moreover, since the TfR appears to be ubiquitinated upon EGF stimulation, it is possible that ubiquitination-dependent mechanisms are responsible for this crosstalk (Argenzio, Bange et al. 2011). It will be interesting to determine whether EGF stimulation can influence the internalization of other constitutive cargoes.



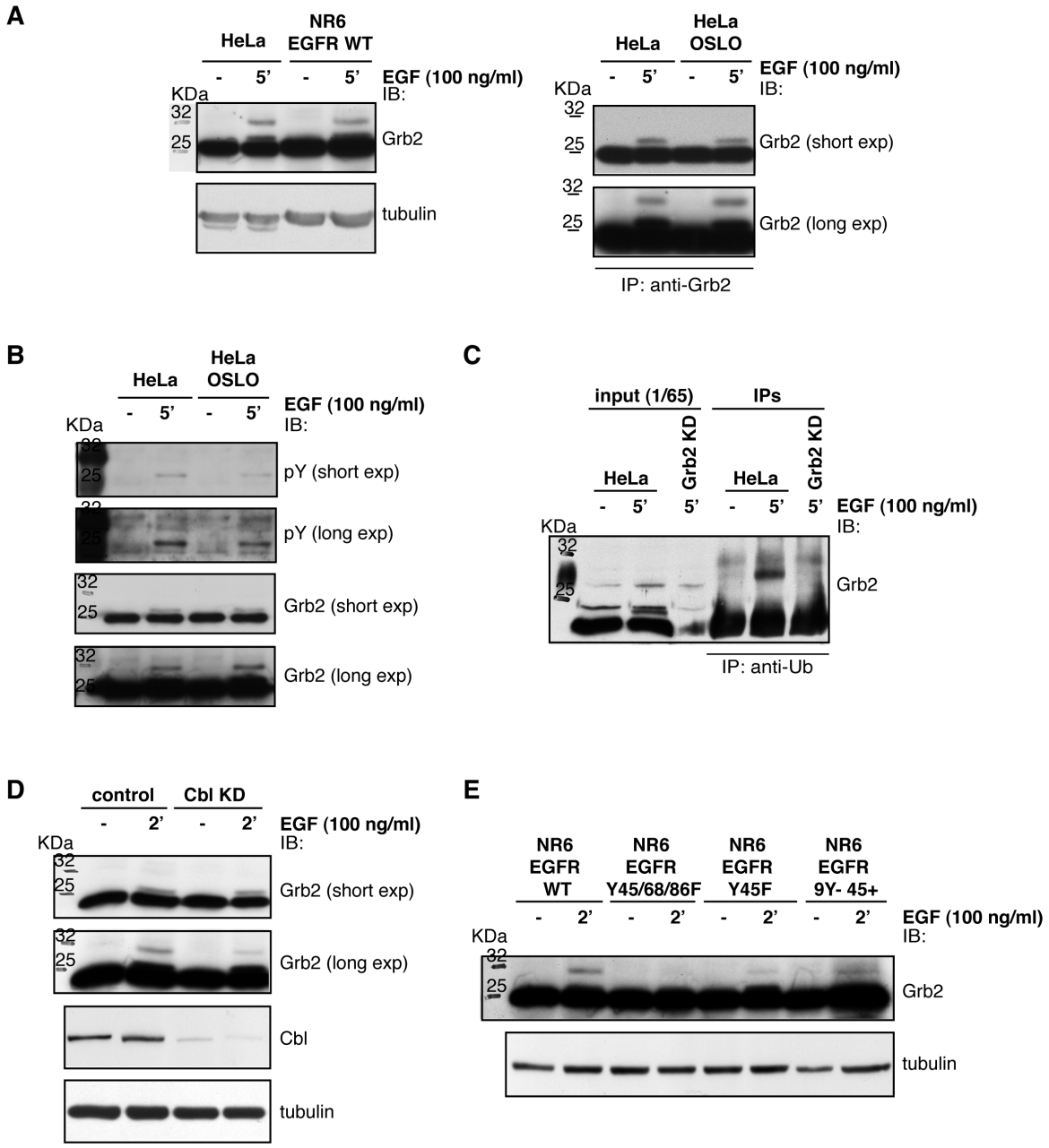
Appendix Figure 1: EGFR activation influences TfR internalization
A-D. Effect of EGF treatment on TfR internalization under different experimental conditions. Kinetics of ^{125}I-Tf (1 μg/ml) internalization were followed at at early time points (0-6 min) in the absence or presence of the indicated amounts of EGF, in different cell lines and upon transient KD of the indicated proteins. Internalization constants (K_e) were extrapolated from the internalization curves and correspond to the slopes of the best-fitting curves (see Materials and Methods). Results are MEAN+/-SDEV of two independent experiments.

2. Grb2 is tyrosine phosphorylated and monoubiquitinated upon EGF stimulation

We observed in anti-Grb2 immunoblots the appearance of two higher molecular weight bands in HeLa, HeLa Oslo and NR6 cells, following stimulation with a high dose of EGF (**Appendix Figure 2A**). The lower of the two bands is compatible with protein phosphorylation. Indeed, it has been recently proposed that upon stimulation, FGFR2 phosphorylates tyrosine residues on Grb2, promoting dissociation from the receptor and allowing full activation of downstream signaling (Lin et al., 2012). The upper band displays a shift of around 8 KDa compatible with monoubiquitination. The stoichiometry of both these modifications is, however, low. To directly assess whether these modifications could represent phosphorylation and ubiquitination, we immunoprecipitated Grb2 from both HeLa and HeLa Oslo cells, under conditions of serum starvation and stimulation with high EGF dose for 5 min. Anti-phosphotyrosine immunoblotting revealed that indeed the lower band corresponded to tyrosine phosphorylation (**Appendix Figure 2B**). However, we were unable to detect any signal in the anti-ubiquitin immunoblots. Thus, we performed the reverse experiment: we immunoprecipitated ubiquitinated proteins with an anti-Ub antibody under partial denaturing conditions and immunoblotted with anti-Grb2 antibody. As a negative control, we used cell lysate from Grb2 KD HeLa cells. Using this approach, we detected a band at the expected molecular weight only in cells treated with EGF, indicating that Grb2 is indeed monoubiquitinated upon EGF stimulation (**Appendix Figure 2C**). A further indication that Grb2 might indeed be directly ubiquitinated upon EGF treatment was the observation that the intensity of the upper band in Grb2 immunoblots decreased upon Cbl KD (**Appendix Figure 2D**). This result indicates that Cbl might be involved in EGF-induced Grb2 ubiquitination. Moreover, in NR6 cells, mutation of the Cbl binding site (Y1045F) in EGFR, also reduced the intensity of the Grb2 upper band. This effect was reversed however upon restoration of the Cbl binding site (**Appendix Figure 2E**). Thus, direct binding of Cbl to the EGFR appears to be required for EGF-induced Grb2 monoubiquitination. Finally, upon mutation of direct Grb2 binding sites in the EGFR (Y1068F and Y1086F), both the lower (phosphorylated) and upper (monoubiquitinated)

Grb2 bands disappeared (**Appendix Figure 2E**), indicating that EGF-induced posttranslational modification of Grb2 requires direct binding of Grb2 to the EGFR.

We have not yet assessed whether these modifications occurs upon low dose of EGF in HeLa. The fact that we see the same molecular bands in HeLa Oslo cells, in which only CME (and not NCE) is functional, suggests that these modifications could have a role in CME. The exact functional roles of these modifications in the EGFR CME internalization mechanism are yet to be explored. The identification, and subsequent mutation, of the Grb2 phosphorylation and ubiquitination sites will be instrumental for elucidating the functional significance of these modifications.



Appendix Figure 2: Grb2 is tyrosine phosphorylated and monoubiquitinated upon EGF stimulation.

A. Molecular weight shifts of Grb2 upon EGF stimulation. Left: HeLa cells or NR6 cells expressing WT EGFR were stimulated with 100 ng/ml EGF for 5 min, or left untreated, after overnight serum deprivation. Immunoblotting using a specific anti-Grb2 antibody was performed on total cell lysates. Tubulin was used as a protein loading control. Right: HeLa or HeLa-Oslo cells were stimulated with 100 ng/ml EGF for 5 min, or left untreated, after overnight serum deprivation. Grb2 was immunoprecipitated from total cell lysates using an anti-Grb2 antibody and visualized by immunoblotting. This experiment is representative of three independent experiments. **B: Grb2 is tyrosine phosphorylated upon EGF stimulation.** HeLa or HeLa-Oslo cells were treated with EGF as described in (A). Immunoprecipitation with a specific anti-Grb2 antibody was performed on total cell lysates, and immunoprecipitates were subjected to anti-phosphotyrosine immunoblotting. Grb2 was used as a protein loading control. This experiment is representative of three independent experiments. **C. Grb2 is monoubiquitinated upon EGF stimulation.** WT and Grb2 KD HeLa cells were treated with EGF as described in (A). Immunoprecipitation using a specific anti-Ub antibody was performed on total cell lysates, and immunoprecipitates were subjected to anti-Grb2 immunoblotting. This

experiment has been done just once. **D. Cbl is involved in Grb2 monoubiquitination.** Control oligo treated and Cbl KD HeLa cells were stimulated with 100 ng/ml EGF for 2 min, or left untreated, after overnight serum deprivation. Immunoblotting using a specific anti-Grb2 antibody was performed on total cell lysates. Level of Cbl KD was assessed by immunoblotting using a specific anti-Cbl antibody. Tubulin was used as a protein loading control. This experiment has been done just once. **E. Grb2 posttranslational modifications require binding of Grb2/Cbl to the EGFR.** NR6 cell lines expressing human EGFR WT, mutant EGFR Y1045F (EGFR Y45F), mutant EGFR Y1045/1068/1086F (EGFR Y45/68/86F) and mutant EGFR that possess only Y45 as autophosporylation site (EGFR 9Y- Y45+) were stimulated with EGF as described in (D). Immunoblotting using a specific anti-Grb2 antibody was performed on total cell lysates. Tubulin was used as a protein loading control. This experiment has been done just once.

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