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Molecular characterization of the role of Cbl proteins in EGFR endocytosis

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List of abbreviations

3D	Three-dimensional
aa	Aminoacid
AchR	Acetylcholine Receptor
ADAM	A Disintegrin And Metalloproteinase
AML	Acute myeloid leukemia
AP2	Adaptor Protein 2
APP	Amyloid precursor protein
APPL	Adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper-containing
AR	Amphiregulin
Arf6	ADP ribosylation factor 6
ARH	Autosomal recessive hypercholesterolemia
BAR	Bin-amphiphysin-RVS
Bem1	Bud emergence protein 1
Brca1	Breast cancer 1
Brda1	BRCA1-associated RING domain 1
BTC	Betacellulin
C-	Carboxy
c-Cbl	Casitas B-lineage lymphoma
CAP	Cbl-associated protein
CAV1	Caveolin-1
CAV2	Caveolin-2
CAV3	Caveolin-3
Cbl-b	Casitas B-lineage lymphoma b
Cbl-c	Casitas B-lineage lymphoma c
CCP	Clathrin-coated pit
CCV	Clathrin-coated vesicle
Cdc42	Cell division control protein 42 homolog
CHC	Clathrin heavy chain
CIN	c-Cbl- interacting protein
CLASP	Clathrin-associated sorting proteins
Clath	Clathrin
CLC	Clathrin light chain
CLIC	Clathrin-independent carriers
CME	Clathrin mediated endocytosis
CML	Chronic myelogenous leukemia
CMML	Chronic myelomonocytic leukemia
CMS	Cas ligand with multiple SH3 domains
Contr	Control
CtBP1	C-terminal binding protein 1
CTxB	Cholera toxin B
CUE	Cuel-homologues
DAB2	Disabled-2
DAG	Diacylglycerol
DAT	Dopamine transporter
Dkk	Dickkopf

DSL	DELTA, SERRATE, LAG-2
DUB	Deubiquitinating enzymes
Dyn	Dynamin
EAAT2	Glial glutamate transporter
EEA1	Early Endosome Antigen 1
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EH	Eps15 homology
ELISA	Enzyme-Linked ImmunoSorbent Assay
EM	Electron microscopy
EMT	Epithelial-mesenchymal transition
EPR	Epiregulin
Eps15	Epidermal growth factor receptor substrate 15
Eps15L1	Epidermal growth factor receptor substrate 15-like 1
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinase
ESCRT	Endosomal sorting complex required for transport
ET	Endothelin
EV	Empty vector
FACS	Fluorescence-activated cell sorting
FGFR	Fibroblast growth factor receptor
FYVE	Fab 1,YOTB, Vac 1, EEA1
GDP	Guanosine 5'-Diphosphate
GED	GTPase effector domain
GEEC	GPI-AP enriched early endosomal compartment
GGA	Golgi-localized, gamma-ear-containing, Arf-binding protein
GLUE	Gram-like Ub-binding in Eap45
Gpa1	GTP-binding a1 subunit
GPCR	G protein-coupled receptor
GPI	Glycosylphosphatidylinositol
GPI-AP	Glycosyl phosphatidylinositol-anchored proteins
Grb2	Growth factor receptor-bound protein 2
GTP	Guanosine triphosphate
HB-EGF	Heparin-binding EGF-like growth factor
HECT	Homologous to the E6-AP Carboxyl Terminus
HGF	Hepatocyte growth factor
HGFR	Hepatocyte growth factor receptor
hsc	Heat shock cognate
IB	Immunoblotting
IF	Immunofluorescence
IGF-IR	Insulin-like growth factor I receptor
IL-2	Interleukin 2
Ile	Isoleucine
ILV	Intraluminal vesicles
InsP3	Inositol trisphosphate
IP	Immunoprecipitation
JMML	Juvenile myelomonocytic leukemia
KD	Knock-down
kDa	kDalton

KO	Knock-out
LDLR	Low-density lipoprotein receptor
LPA	Lysophosphatidic acid
LRP6	Low-density receptor-related protein 6
lys	Lysine
LZ	Leucine zipper
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MEK	Mitogen activated extracellular signal regulated kinase
MHC	Major histocompatibility complex
min	Minutes
mRNA	Messenger RNA
MURC	Muscle restricted coiled-coil protein
MVB	Multivesicular body
MW	Molecular weight
NCE	Non-clathrin endocytosis
NCID	Notch intracellular domain
Nedd4	Neural precursor cell expressed developmentally downregulated protein-4
NPF	Asparagine-proline-phenylalanine
NRG4	Neuregulin 4
NS	Noonan syndrome
NSCLC	Non small cell lung cancer
NTD	N-terminal domain
NZF	Np14 zinc-finger
o.n.	Overnight
PAZ	Poly-Ub-associated zinc-finger
PDGFR	Platelet-derived growth factor receptor
PDK	Phosphoinositide-dependent kinase
PH	Pleckstrin homology
PI3P	Phosphatidylinositol 3-phosphate
PI3K	Phosphatidylinositol 3-Kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PKC	Protein kinase C
PLCγ	Phospholipase C γ
PM	Plasma membrane
PRD	Proline/arginine-rich domain
PTB	PhosphoTyrosine-binding
PTP	Protein Tyr-specific phosphatases
PTP1B	Protein-Tyr phosphatase 1B
PTRF	Polymerase I and transcript release factor
pY	Phosphotyrosine
qPCR	Quantitative polymerase chain reaction
RING	Really Interesting Gene
RNAi	RNA interference
RNF4	Ring finger protein 4
RTK	Receptor tyrosine kinase
RTPT	Receptor-like PTPs

S	Serine
SARA	Smad anchor for receptor activation
SDPR	Serum deprivation response
SETA	SH3-encoding, expressed in tumorigenic astrocytes
SH2	Src homology 2 domain
SH3	Src homology 3 domain
SHC	Src homology 2 domain containing
siRNA	Small interfering RNA
SNX9	Sorting nexin 9
SOS	Son-of-sevenless
Spry	Sprouty
SRBC	SDR-related gene product that binds to c-kinase
STAMBP	STAM binding protein
STAT	Signal-transducer and activator of transcription
SV40	Simian virus 40
TCR	T-cell receptor
TfR	Transferrin receptor
TGF-βR	Transforming growth factor beta receptor
TGF	Transforming growth factor alpha
TKB	Tyrosine kinase binding
TKI	Tyrosine kinase inhibitors
TRAF2	TNF receptor-associated factor 2
Tyr	Tyrosine
Ub	Ubiquitin
UBA	Ubiquitin binding associated
UBD	Ubiquitin binding domain
UBPY	Ubiquitin isopeptidase Y
UEV	Ubiquitin E2 variant
UIM	Ubiquitin interacting motif
UTR	Untranslated region
VEGFR	Vascular endothelial growth factor receptor
VHS	Vps27, Hrs, STAM
WB	Western blot
WT	Wild type
Y	Tyrosine
YFP	Yellow fluorescent protein

Abstract

c-Cbl is the major E3 ligase involved in ubiquitination of Epidermal Growth Factor Receptor (EGFR). Ubiquitination by c-Cbl plays a critical role in EGFR endocytosis by targeting receptors to lysosomal degradation. Its involvement at early internalization steps is still debated, also due to the fact that multiple internalization pathways were described. Indeed, EGFR ubiquitination is required for non-clathrin mediated endocytosis (NCE), while it is not essential for clathrin endocytosis (CME) (1,2). However, c-Cbl might still play a crucial function also in CME since, in addition to its role as an E3 ligase, it works also as an adaptor, by recruiting several proteins involved in the early phases of this process (3).

Importantly, c-Cbl has been found mutated in different disorders, from myeloproliferative disease to Noonan syndrome and non-small cell lung cancer (NSCLC) (4). Most of these mutations are located within the Ring finger domain and in the regulatory linker region, and are therefore predicted to affect E3 ligase activity. However, some mutations map outside this region, suggesting that they might impinge on the adaptor function without altering E3 ligase activity. None of these mutations was characterized in detail at the mechanistic level. In order to draw a more precise molecular picture of c-Cbl activity in EGFR ubiquitination and endocytosis, we investigated the effects of different set of cancer-relevant mutations, combining two distinct approaches: 1) RNA interference-based functional assays and 2) *in vitro* ubiquitination assays.

1) First, we characterized the effect of the knockdown (KD) of c-Cbl (and its family members) on EGFR ubiquitination and endocytosis in two different cell systems, murine fibroblast and HeLa cells. From our data, we confirmed that c-Cbl is essential for NCE, by ubiquitinating the EGFR; however, it also plays a role in CME. Importantly, reconstitution experiments with RING finger mutants demonstrated that c-Cbl E3 ligase activity is also required for CME. Since EGFR ubiquitination is not essential for CME, we hypothesize

that this activity is exerted not directly on the receptor but on endocytic adaptors. In agreement, Eps15 monoubiquitination is impaired upon c-Cbl KD.

2) We were able to reconstitute the EGFR ubiquitination reaction *in vitro*, and now we can use this tool to study the molecular details of c-Cbl catalysis.

Moreover, in order to dissect c-Cbl adaptor function vs E3 ligase activity, we plan to investigate the phenotype of c-Cbl mutations that map outside the E3 ligase domain in EGFR internalization and ubiquitination, exploiting both *in vivo* analyses (through reconstitution experiments in cell lines) and *in vitro* ubiquitination assay.

Introduction

1 The endocytic process

Endocytosis is a basic cellular process that occurs at the cell surface and involves internalization of the plasma membrane (PM) together with its constituent membrane proteins and lipids. The endocytic process governs almost all cellular relationships with the extracellular environment and intracellular signalling pathways. Over the years, multiple cell entry routes have been described (Figure 1). A rough classification is performed on the basis of the size of the initial membrane invagination. Particles larger than 500 nm, as bacteria or apoptotic cells, are taken up by phagocytosis (5), while fluid uptake occurs by macropinocytosis (6). These processes involve large rearrangements of the plasma membrane, driven by actin cytoskeleton remodelling and RHO-GTPases activity (7). On the contrary, micropinocytosis is characterized by smaller invaginations (<200 nm) and comprises both clathrin mediated endocytosis and non-clathrin endocytosis (8). In this introduction, we will focus on describing micropinocytic entry routes.

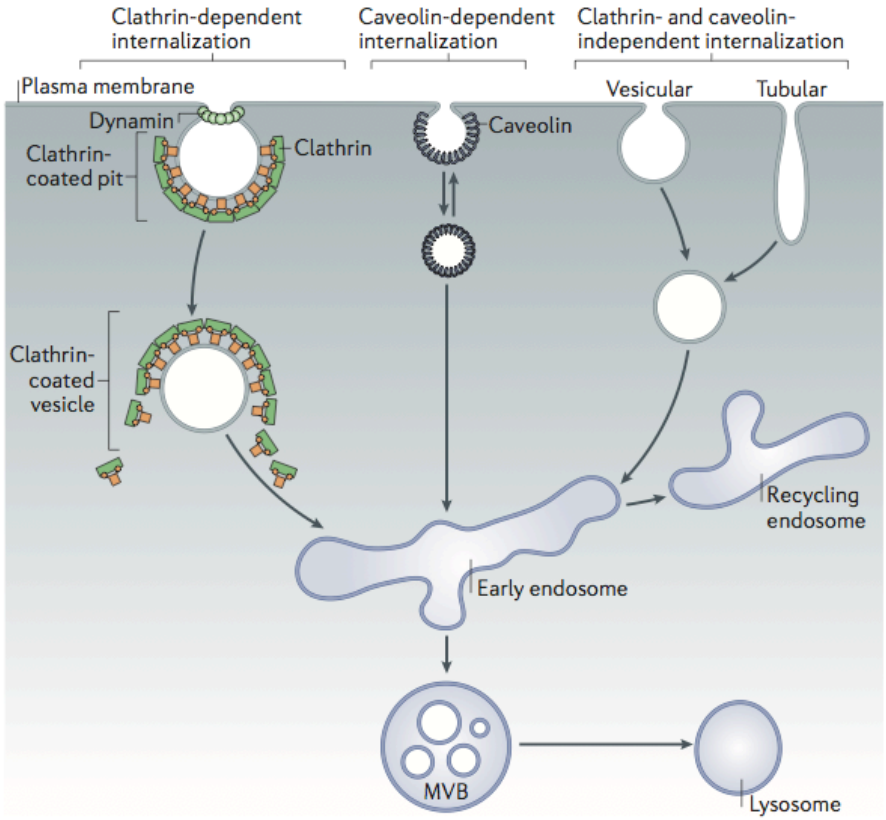

Figure 1 Major micropinocytic entry routes in mammalian cells. Multiple pathways of endocytosis exist, for example, clathrin-dependent, caveolin-dependent and clathrin- and caveolin-independent internalization. Once internalized, cargoes are trafficked into endosomes, where they can be either recycled back to the cell surface or sorted into other compartments [multivesicular bodies (MVB) and lysosomes] for degradation (9).

Pathway	Morphology and Size	Coat or Coat-like	Dynamin Dependence	Small GTPase Involved	Internalized Cargoes	Associated/Regulatory Proteins
Phagocytosis	Cargo-shaped >500 nm	None	No	RAC1/RHOA/CDC42 [depending on type (9, 105, 763)]	Pathogens, apoptotic cells, FcRs (738, 763)	Actin, ARP2/3 (507), Formins (133), PI3K (808), WASP (479), WAVE2 (2), amphiphysin (864), coronin (867), others (763)
Macro-pinocytosis	Ruffled 0.2–10 μm	None	In some cases (575)	RAC1, CDCD42 (560, 837), ARF6 (687), RAB5 (426, 427)	RTKs (575), fluids, bacteria (391, 763)	Actin, ARP2/3, cortactin (413), PI3K (17, 18), SRC (382, 391), PAK1 (164), RAS, CTBP1/BARS (454), others (763)
Clathrin-mediated	Vesicular 150–200 nm	Clathrin	Yes	RAB5 (89)	RTKs (742), GPCR (744), TFR (539), LDLR (539), toxins (681), bacteria (804), viruses (517)	AP-2, epsins, EPS15, intersectin, amphiphysin, ARRs, DAB2, ARH, others (>50) (388, 645, 781); FCHO1/2 (309), ARFGAP1 (36)
Caveolae-mediated	Flask-shaped 50–120 nm	Caveolin 1 and 2	Yes	Not clear	GPI-linked proteins (291), CTxB (681), SV40 (517), TGF-βR (167), IGF-1R (678)	PTRF/cavin1 (290, 546), SDPR/cavin2 (289), SRBC/cavin3 (512), MURC/Cavin4 (49), SRC (702)
CLIC/GEEC	Tubular	None	No	CDC42, ARF1 (416)	Fluids, bulk membrane, GPI-linked proteins (327)	Actin (327), GRAF1 (172), ARHGAP10 (416)
IL-2Rβ	Vesicular 50–100 nm	None	Yes	RHOA, RAC1 (424)	IL-2Rβ (424), γc-cytokine receptor (686)	PAK1 and 2, cortactin, N-WASP (424)
Arf6-dependent	Tubular	None	None as yet	ARF6 (147)	MHC I-II (67, 814), CD59 (551), CD55, GLUT1 (203), AchR (76)	None as yet
Flotillin-dependent	Vesicular	Flotillin 1 and 2	No	None	CTxB, CD59, proteoglycans (230, 258, 591), DAT, EAAT2 (141)	None as yet

Table 1 Internalization pathways. The known pathways of internalization are shown together with the morphology and the size of the internalizing membrane structure, the coat involved, the dependency on dynamin, the involvement of GTPases, the type of internalized cargo and the 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; LDLR, low-density lipoprotein receptor; DAB2, disabled-2; ARH, autosomal recessive hypercholesterolemia; CTxB, cholera toxin B; SV40, simian virus 40; GPI, glycosylphosphatidylinositol; MHC, major histocompatibility complex; TGF-bR, transforming growth factor-beta receptor; IGF-IR, insulin-like growth factor I receptor; PTRF, polymerase I and transcript release factor; SDPR, serum deprivation response; SRBC, SDR-related gene product that binds to

c-kinase; MURC, muscle restricted coiled-coil protein; CtBP1, C-terminal binding protein 1; DAT, dopamine transporter; EAAT2, glial glutamate transporter; AchR, acetylcholine receptor (4).

1.1 Clathrin mediated endocytosis

Among the different cell entry routes, CME has been the most extensively studied (9). CME is ubiquitous to all eukaryotic cells and proceeds through a series of well-defined morphological intermediates: a clathrin-coated pit (CCP) undergoes progressive invagination before scission from the plasma membrane leading to the formation of a clathrin-coated vesicle (CCV) (10). As the name suggests, the main component of the CCV's coat is clathrin, which is assembled as a scaffold on the vesicle surface. The final structure resembles a sandwich with two outer layers: the inner part of the layer contains the cargo while the outer contains clathrin; between these two sheets there is a middle layer consisting of different clathrin-adaptor proteins and other regulatory molecules involved in the CCV assembly (Figure 2).

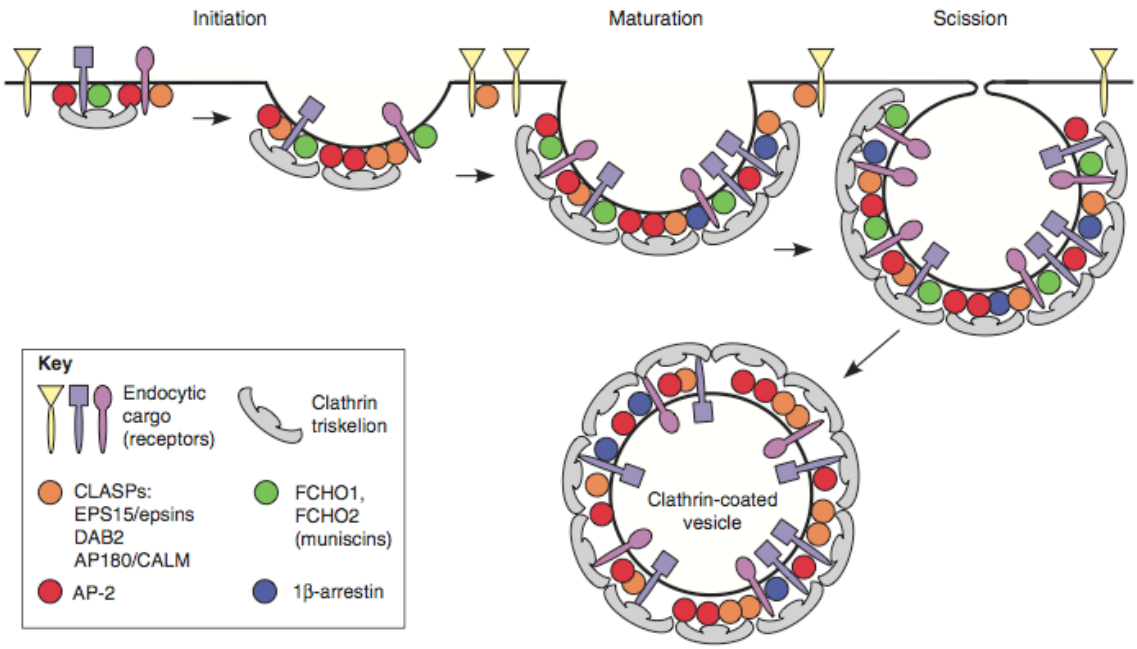

Figure 2 Clathrin-dependent endocytosis. Clathrin-dependent endocytosis starts when adaptor and clathrin complexes associate with cognate cargo, thus initiating the formation of a coated pit. As the pit matures, additional adaptor and scaffold proteins join the pit,

providing a structural platform that regulates interactions between the adaptors and the other endocytic proteins. Increasing membrane deformation eventually leads to vesicle scission, which releases a clathrin-coated vesicle into the cytoplasm. Examples of different cargos are given in different colors. Blue and purple cargos, which contain sorting motifs that bind to clathrin-associated adaptors, can therefore be incorporated into the forming CCP. Yellow cargoes, by contrast, are internalized through an alternative clathrin-independent pathway that might involve a select subset of CLASP (clathrin-associated sorting proteins) adaptors (11).

1.1.1 Clathrin and clathrin coat assembly

A clathrin coat is three-dimensional (3D) array of triskelia. Each triskelion is composed by three clathrin heavy chains (CHCs) with a mass of 170 kDa and three clathrin light chains (CLCs) of 25 kDa: the three CHCs provide the structural backbone of the clathrin lattice, while the three CLCs seem to regulate the formation and disassembly of the clathrin lattice. In vertebrates, two forms of CLCs have been identified, CLCa and CLCb (12). CLCs have been shown not to be essential in the process of vesicle formation since their concomitant ablation does not affect neither Transferrin Receptor (TfR) nor EGFR internalization (13). On the contrary, Ferreira and colleagues demonstrated that CLCa or CLCb KD specifically inhibits the uptake of G protein coupled receptors (GPCRs) (14). Therefore, CLCs seem to play specific roles in receptor uptake; their involvement in this process depends on the receptor being internalized.

The clathrin heavy chain is an invariant polypeptide made of functionally distinct regions (Figure 3). The globular N-terminal domain (NTD) contains the binding sites for adaptor proteins, including Adaptor Protein 2 (AP2) and is connected via the ankle region to a distal and a proximal leg, linked 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 implicated in triskelion formation. *In vitro* experiments show that purified clathrin can spontaneously

assemble into cages, or in the presence of adaptor proteins, into coats. The first 3D map of a clathrin cage, both in presence and in absence of adaptor proteins, was obtained in 1986 using cryo-EM (electron microscopy) (15). In later years, improvements in EM resolution allowed researchers to precisely trace and position the main parts of the triskelion (16).

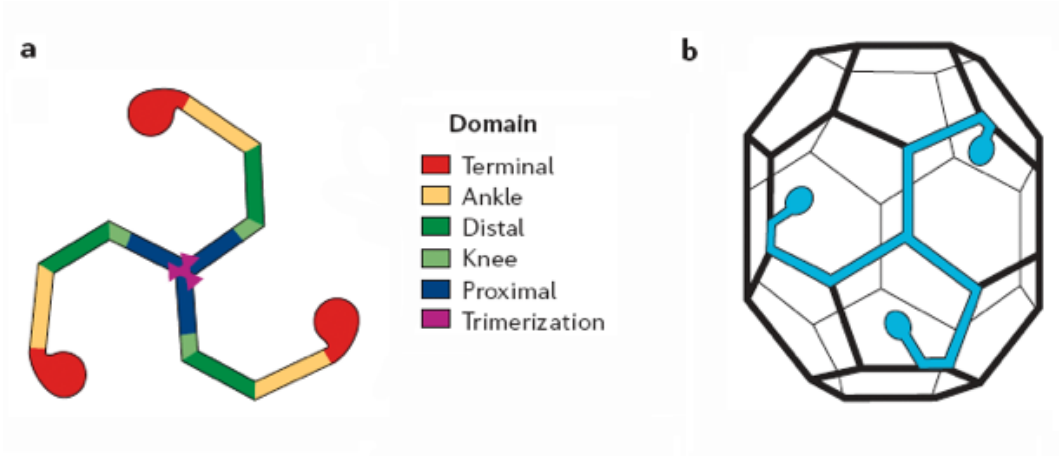

Figure 3 The architecture of clathrin. (A) Schematic representation of a clathrin triskelion, which shows the various domains of CHC with different colours (see the key). **(B)** A clathrin barrel with a single triskelion highlighted in blue (17).

Adaptor and accessory molecules coordinate clathrin nucleation at sites of the plasma membrane that will be internalized (18). The nucleation process promotes the polymerization of clathrin into curved lattices, by stabilizing the deformation of the attached membrane. Clathrin polymerization, together with the concomitant action of other proteins, leads to the formation and constriction of the vesicle neck, bringing the membranes surrounding the neck in close apposition. The GTPase dynamin forms a helical polymer around the constricted neck and mediates the fission of the vesicle from the plasma membrane upon GTP (Guanosine 5'-Triphosphate) hydrolysis (19). This results in an irreversible release of the CCV into the inner part of the cell; subsequently, the clathrin basket is released from the vesicle by the proteins auxilin and hsc70 (heat shock cognate 70 kDa protein).

1.1.2 Clathrin adaptors

A wide range of adaptors and accessory proteins has been implicated in CCP and CCV formation. At least 20 clathrin adaptors have been identified (20). Endocytic adaptor proteins vary greatly in size [about 300-3000 amino acids (aa)] and structure, but possess similar properties. Most of the clathrin adaptors contain regions that interact with some or all the four types of binding partners, namely clathrin, cargo, lipids and accessory proteins (11,20). Endocytic adaptors can be divided into two groups: multimeric adaptor proteins (for instance AP2) and monomeric or non-classic adaptor proteins, also named CLASPs (Figure 4).

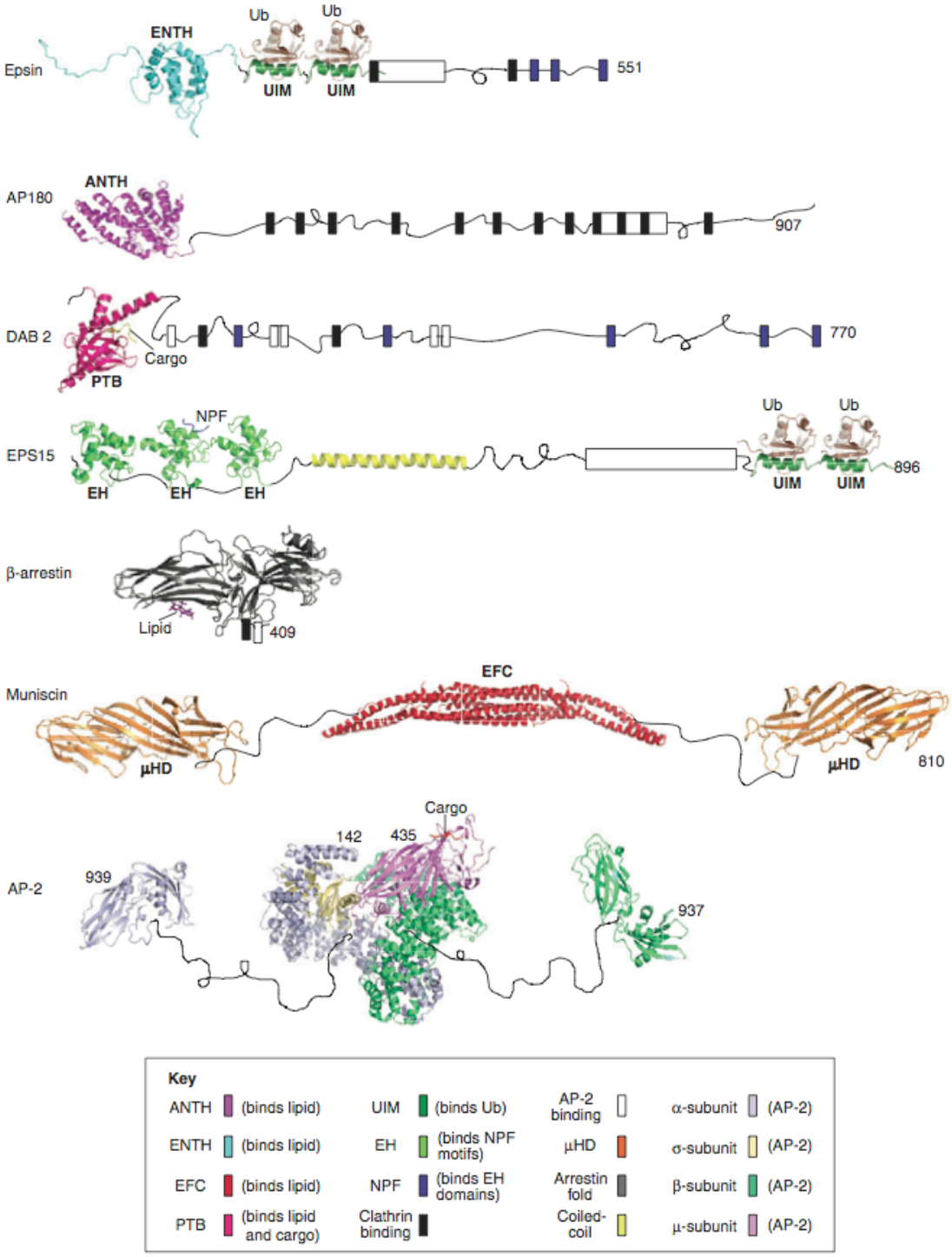

Figure 4 Representation of the major domains and motif organization of human adaptors. Multimeric and monomeric adaptor proteins consist of folded domains (represented by atomic structures), binding motifs (colored boxes) and relatively unstructured regions (represented by thin black curved lines) (11).

The first adaptor to be identified was the cargo-binding and phospholipid-binding heterotetrameric complex AP2. AP2 is the most abundant non-clathrin constituent of

purified endocytic CCVs and remains today the prototypical and best characterized endocytic adaptor. AP2 is a stable complex of four polypeptide chains: the 100 kDa α-subunit, the 100 kDa β2-subunit, the 50 kDa μ2 subunit and the 17 kDa σ2 subunit.

For many years, AP2 was thought to be the only existent cargo-sorting protein. However, recently, the discovery that in cells depleted for the AP2 complex some CCPs were present at the PM and these were sufficient to induce receptor internalization (13,21) challenged this simplicistic view. Indeed, other specific adaptors have been implicated in clathrin-coat assembly (9). Some of them have been demonstrated, or proposed, to work in concert with AP2 or as its substitute, since they can bind both cargoes and clathrin, such as Epsin (22) and β-arrestin (23).

Adaptor proteins are collectively called CLASPs (20) and are able to bind both AP2 and clathrin. CLASPs can be classified on the basis of the motif recognized on the cargo. PhosphoTyrosine-binding (PTB) domain-containing CLASPs recognize the [FY]XNPX[YF] signal, which is not bound by the μ2 subunit of AP2. The PTB domain is able to bind both phosphatidylinositol-4,5-bisphosphate (PIP2) and cargo simultaneously. Examples of CLASPs containing PTB domain are Dab2, ARH (autosomal recessive hypercholesterolemia) and Numb (24). Other CLASPs, like Epsin family proteins, Eps15 (Epidermal growth factor receptor substrate 15) and Eps15L1 (Epidermal growth factor receptor substrate 15-like 1) recognize ubiquitinated cargoes and bind AP2. For these adaptors, cargo selectivity derives from tandemly arrayed ubiquitin-interacting motifs (UIMs). Through its NPF motif, epsins associate with the EH domain of Eps15; moreover, epsins are able to bind to clathrin (25). These properties of epsins allow the assembly of clathrin coat in the absence of AP2.

Accessory proteins can participate in CME during membrane deformation by recruiting other molecules or by performing scaffolding/coordination functions within the endocytic process. N-BAR (Bin-amphiphysin-RVS) and BAR domain-containing proteins, such as SNX9 (sorting nexin 9) and amphiphysin, can generate and stabilize membrane

curvature, bind both clathrin and AP2, and recruit dynamin to the neck of the budding vesicle (26,27). Many accessory proteins have been identified, including NECAP, intersectin, and stonin, that may play important roles during the endocytic process (18).

Due to their overlapping binding abilities, CLASP adaptors often play redundant roles in CME. Indeed, Eps15 and epsins, are redundantly necessary in EGFR clathrin-mediated endocytosis (1). Similarly, Eps15 and intersectin are redundantly involved in the internalization step of both TfR and EGFR (28).

To complicate the picture even more, according to cell context, cargo and signal, the cell may use the same machinery to trigger different intracellular routes. Indeed, although both Eps15 and epsins have traditionally been linked to CME, our lab has recently demonstrated that they are also involved in NCE of the EGFR. This latter function is exerted through binding of adaptor UIM motifs to cargoes [(1) and paragraph 4.2 of this thesis].

1.2 Dynamin in endocytosis

Upon the formation of a coated patch of membrane, the generation of an endocytic vesicle requires fission of the budded membrane from the parental one. Dynamin, a large GTPase, has been implicated in several fission processes in eukaryotic cells (29,30). Dynamin is highly conserved among multicellular organisms: the *Drosophila* and *C. elegans* homologues are 70% and 61% identical to human dynamin, respectively. While both *Drosophila* and *C. elegans* carry only a single *dynamin* gene, mammalian cells express three dynamin isoforms in a tissue-specific manner. Dynamin-1 is neuron-specific, Dynamin-2 is ubiquitously expressed (19) and Dynamin-3 is predominantly expressed in testis and, to lesser extent, in neurons. In addition, numerous splice variants exist, which suggests that, at least in mammals, these different dynamin family members might exert distinct functions, other than receptor-mediated endocytosis.

A great variety of endocytic processes including clathrin-dependent and some clathrin-independent receptor-mediated endocytosis has been shown to require dynamin for the scission process that generates vesicles. Initially, dynamin was identified as homologue of the *Drosophila shibire* gene (31), whose mutations were shown to cause a temperature-sensitive paralysis. The first data were obtained from morphological analysis of synapses in the *shibire*^{ts1} mutant, where a reversible loss of synaptic vesicle at neuromuscular junctions were observed, implicating dynamin in the endocytic process. Moreover, overexpression of a dominant negative mutant of dynamin blocked receptor-mediated endocytosis in different cells, suggesting a further role in clathrin-dependent endocytosis out of the nervous system. A further evidence of the role of dynamin in this process is achieved through its localization to CCPs at the plasma membrane (32).

Dynamin is a large GTPase that contains a Pleckstrin homology (PH) domain, which mediates the binding to PIP2; a GTPase effector domain (GED), which can interact with the same domain of an adjacent dynamin molecule, by causing oligomerization of the proteins and activation of the GTPase activity (33); a C-terminal proline/arginine-rich domain (PRD), which allows interactions with SH3 (Src homology 3)-domain containing proteins. This wide range of partners enables dynamin to recruit and bind proteins involved in coated vesicles formation (Figure 5).

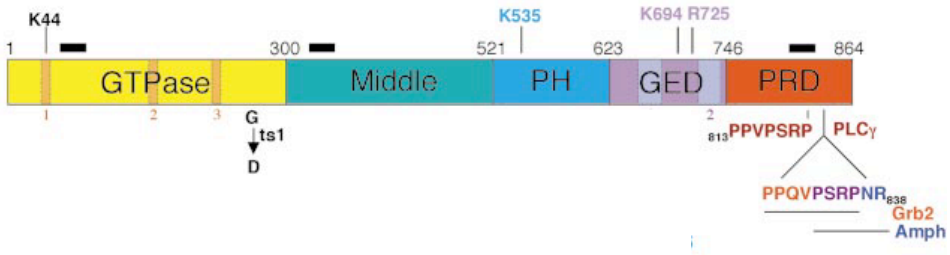

Figure 5 Schematic representation of dynamin domains. Dynamin consists of five domains: the GTPase domain, a middle domain, a PH domain, a GED, and a highly basic C-terminal PRD (34).

Besides acting in the scission machinery for the clathrin-dependent pathway, dynamin is also implicated in vesicle formation in some clathrin-independent pathways,

including caveolar endocytosis (35), Rho-A-dependent IL-2 (Interleukin 2) receptor endocytic route (36), APP (amyloid precursor protein) endocytosis in primary neurons (37) and a class of macropinocytosis (38) (see Table 1 in section 1).

Evidence suggests that dynamin is sufficient to promote constriction and generate tension for vesicle pinching-off; however other factors are required at the neck of the budding vesicle to generate the tension necessary for vesicle budding (39). The mechanism by which dynamin is recruited to the endocytic sites at the plasma membrane is poorly understood; likely, the GTPase is recruited during clathrin pit assembly, a process in which proteins containing N-BAR domain, such as amphiphysin, are involved and can interact with dynamin (40). Indeed, in cells, dynamin recruitment to the plasma membrane is dramatically reduced by concomitant depletion of endophilin and amphiphysin and conversely, depletion of dynamin strongly reduces the recruitment of endophilin (41).

At high concentration, dynamin forms tetrameric structures, possibly through head-to-tail interactions between two dynamin dimers (42). During dynamin-dependent endocytosis, dynamin tetramers oligomerize in a helical polymer at the neck of the rising vesicle. Through its GTPase activity, dynamin then constricts and fuses the two sides of the neck, in order to induce budding of the vesicle from the plasma membrane.

1.3 Non-clathrin mediated endocytosis

Compared to CME, the current understanding of the NCE pathway is much less clear. NCE pathways are a heterogeneous group of internalization routes that share insensitivity to clathrin depletion and dependency on cholesterol-rich plasma membrane microdomains, called rafts. The current classification for non-clathrin mediated pathways rely on the requirement of dynamin for vesicle release; thus, clathrin independent pathways are divided according to the fact that they use a dynamin-mediated scission mechanism (dynamin-dependent) or not (dynamin-independent). A second tier implies the presence of coat-like proteins, like caveolins or flotillins, subdividing the pathways in caveolae-

mediated or flotillin-mediated endocytosis, respectively. A third level of classification is based on the involvement of small GTPases; the terminology Cdc42 (cell division control protein 42 homolog)-, RhoA- or Arf6 (ADP ribosylation factor)-regulated endocytic pathway indicates that modifying the function of these GTPases affects the internalization or trafficking of one set of non-clathrin mediated endocytic markers (43,44).

1.3.1 Caveolae-mediated endocytosis

Caveolae are pits of 60-80 nm diameter formed by the assembly of caveolins (45,46), integral membrane proteins that associate directly to membrane cholesterol. They have been implicated in NCE, signal transduction, redox signalling, cell adhesion, lipid and cholesterol regulation, mechanosensing and even transcription regulation.

The structural and regulatory components of caveolae include two protein families: caveolins and cavins. There are three mammalian caveolin proteins, caveolin-1 (CAV1), caveolin-2 (CAV2) and caveolin-3 (CAV3). Although CAV3 is muscle specific, CAV1 and CAV2 are present widely in nonmuscle cells, with the exception of neurons and leukocytes (47,48). Cells that do not express these proteins lack morphologically evident caveolae (49). CAV1 is necessary for caveolar biogenesis (50); on the contrary, loss of CAV2 has no apparent effect on caveolae formation *in vivo,* which suggests that CAV2 role might be cell specific (51). Recent studies demonstrate that although morphological features of caveolae are widely conserved, the functions of the proteins may appear different (52).

In addition to caveolins, four cavins are also critical for the formation of caveolae at the plasma membrane (53). Cavins form a multiprotein complex that is recruited to the PM by caveolin-1, in a cavin-1 dependent manner (53), where it stabilizes caveolae.

The caveolar pathway is involved in endocytosis of several ligands such as albumin (54), autocrine motile factor (55), tetanus toxin (56), cholera toxin (57), and viruses, like

polyoma (58) and SV40 (59). However, the exact endocytic function of caveolae remains object of debate.

However, several studies have identified common features to all caveolar cell entry mechanisms. Indeed, all caveolar cell entry mechanisms depend on dynamin (35), Src kinase, PKC (protein kinase C) and actin recruitment (59-61).

1.3.2 CLIC/GEEC pathway

Glycosyl phopshatidylinositol-anchored proteins (GPI-APs) are membrane proteins that lack cytosolic extension and are therefore unable to bind directly cytosolic adaptors. The internalization of these proteins is kinetically different from the uptake of transmembrane proteins, since they can bypass Rab-5-positive endosomal compartments. The pathway involved in GPI-AP internalization delivers cargoes to endosomes termed GPI-AP-enriched early endosomal compartments (GEECs), which probably result from fusion of uncoated tubulovesicular CLICs (clathrin-independent carriers) directly derived from the cell surface (62). CLICs/GEECs are selectively enriched for GPI-APs (63), but the determinants at this stage at the plasma membrane are not yet clear. However, this sorting could be based on lipids, since perturbations in cholesterol and sphingolipids levels affect endocytosis through this pathway (61,63).

When the CLIC/GEEC pathway is perturbed, endocytosis of GPI-linked proteins seems to occur via CME (63). The intracellular destination for cargoes internalized through the CLIC/GEEC pathway appears to differ between cell types, including lysosomal and pericentriolar recycling compartments (63,64). There is an increasing number of proteins internalized through routes that resemble to the GEECs, including cholera toxin (62), VacA toxin and SNX9 (65,66).

2 Endocytic sorting

After internalization, cargoes are routed to early endosomes, where distinct trafficking paths lead internalized receptors to their fate: either recycling to the plasma membrane or lysosomal degradation. Since cargoes destined for degradation are separated from those destined for recycling in early endosomes, these structures are considered the first sorting station for internalized receptors.

The specificity of sorting in the early endosome can be defined by: (1) lipid composition (67); (2) specific sorting signals on the receptor (68,69); (3) geometric constraints in which the membrane-bound components are recycled to the PM, while cargoes in the fluid phase are delivered to late endosomes (70).

Recent evidence has drawn a new picture that reveals early endosomes as a morphologically and functionally heterogeneous population, characterized by distinct membrane subdomains, which influence the signalling ability and the fate of receptors within organelle (71-73). For example, an enrichment in phosphatidylinositol 3-phosphate (PI3P) allows the assembly of complexes involving FYVE (Fab1, YOTB, Vac1 and EEA1) domain and PX domain-containing proteins (74).

At the molecular level, two main classes of small GTPases play an essential role for sorting cargoes along the endosomal stations: the Rab (75,76) and the Arf families (44). These proteins act as molecular switches that can shift between a GTP-bound active state and a GDP (Guanosine 5'-Diphosphate)-bound inactive form. The latter is cytosolic, whereas the active form is associated with membranes. Rab5 regulates CCV-mediated transport from the plasma membrane to the early endosome (76), from which cargoes can be recycled back to the PM through either fast Rab4-dependent or a slow Rab8/Rab11-dependent recycling route (77). Arf6 is involved in an additional recycling pathway, which is mainly used by receptors internalized through NCE, such as MHCI (44), although some CME-internalized cargoes can also be recycled through this pathway.

In other cases cargoes are targeted to degradation in lysosomes through late endosomes and multivesicular bodies (MVB) sorting. The transition from early to late endosomes is accompanied by the phenomenon called “Rab conversion”, characterized by the acquisition of Rab7 at the expense of Rab5 (77). A key signal to enter this pathway is cargo ubiquitination, because several proteins that harbour ubiquitin-binding domains (UBD) recognize ubiquitinated cargoes and lead them to the degradative route (see also chapter 4.1). A central role for the sorting of ubiquitinated cargoes is achieved by ESCRT (endosomal sorting complex required for transport) complexes, which act sequentially:

(1) ESCRT-0 acts at the level of the endosome and is composed of two interacting proteins HRS and STAM (Signal Transducing Adaptor Molecule) (78);

(2) ESCRT-I is a heterotetramer composed by Vps23, Vps28, Vps37, Mvb12 (78) and UBAP1 (79) and acts at MVB membrane ;

(3) ESCRT-II is a heterotetramer consisting of one molecule of Vps22, one molecule of Vps36, and two molecules of Vps25. It also acts at MVB membrane (78);

(4) ESCRT-III, unlike other ESCRTs, which are stable complexes, is a dynamic polymer of ESCRT-III proteins whose stoichiometry is not clearly defined. It also acts at the MVB membrane (78).

ESCRT-I, ESCRT-II and ESCRT-III complexes are necessary for the formation of intraluminal vesicles (ILVs) of MVBs, accomplished by involution of the liming membrane. Moreover, they are involved in the recruitment of the enzymes that deubiquitinate receptors before their sorting into ILVs.

3 Endocytosis and signalling

Endocytosis is a major mechanism of long-termed attenuation; however recent evidence has shown that endocytosis has a wider impact on signalling, by enabling cells to adopt several strategies for the regulation of signal propagation and duration. Endocytosis has recently been described as a key player that fine-tunes a diverse array of biological output in response to stimuli, thus defining signal specificity.

3.1 Regulation of signalling at the plasma membrane

Ligand-induced internalization of signalling receptors is an important mechanism able to negatively regulate signalling from the cell surface. Receptor endocytosis can attenuate the duration or strength of signalling from the plasma membrane by physically decreasing the number of cell surface receptors accessible to the ligand (Figure 6). However, in some cases, a reduction in the concentration of surface receptors does not result in attenuation of the maximal signalling response that can be elicited by a ligand, but it shifts the dose response, so that higher concentration of the ligands are required to induce a response of the same magnitude. For instance this mechanism is functional during chemotaxis in response to soluble ligands. Several plasma membrane receptors, such as RTKs and GPCRs, function as motogenic sensors that are able to respond to gradients of chemotactic factors that drive cell migration (80). Besides the directional moving, cells have also to be able to stop at their target sites, where there is the highest concentration of chemotactic factors (81). For example, mammary carcinoma cell lines migrate in response to a gradient of EGF (Epidermal Growth Factor). At the beginning of this process, EGFR is uniformly distributed all over the cell surface; then, EGF binding at the fore end of the cell causes ligand sequestration. The subsequent 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 (82) where the concentration is higher. These kinds of

regulatory mechanisms are very common in nature and are critical to organize migration throughout development.

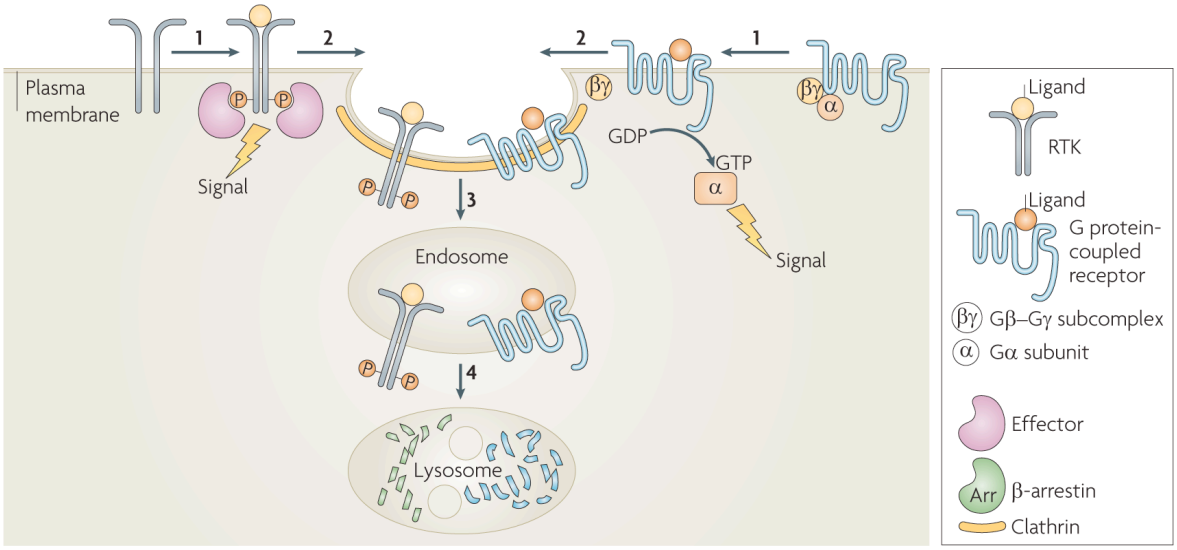

Figure 6 Endocytosis regulates signalling from the plasma membrane. Schematic picture of signal attenuation. Ligand-induced activation of RTKs or GPCRs promotes signalling from the plasma membrane through the receptor-mediated recruitment of signalling effectors to phosphorylated RTKs, or the activation of G proteins associated with GPCRs when the Gα subunit is bound to GTP (step 1). GPCR signalling can be mediated both by the GTP-bound Gα subunit (as depicted) and by the Gβ–Gγ subcomplex. The following receptor recruitment into coated pits (step 2) and clathrin-dependent endocytosis (step 3) attenuate signalling by separating the receptors from plasma membrane-delimited substrates and/or mediators. Some receptors traffic to lysosomes, which results in their downregulation by proteolysis and further signalling attenuation (step 4) (83).

Signalling can also be modulated through the regulation of ligand accessibility to the receptor. An example of this kind of regulation is seen in Notch receptor signalling. The direct binding of DSL ligands (DELTA, SERRATE, LAG-2) – which are anchored at the PM of a signal-sending cell – to the receptor Notch – localized at the membrane of a signal-receiving cell – is necessary to induce Notch signalling. Upon ligand binding, the Notch receptor undergoes two proteolytic cleavage events: the first cleavage is catalyzed by ADAM (A Disintegrin And Metalloproteinase)-family metalloproteases, whereas the

second is mediated by γ-secretase. The second cleavage occurs in the transmembrane region of Notch and releases the soluble Notch intracellular domain (NICD), which then translocates to the nucleus and promotes transcription of target genes (84). In order to activate signalling, 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 γ-secretase cleavage probably occurs in endosomes. Interestingly, Notch activation in the signal receiving-cell also requires endocytosis to occur in the signal-sending cell. At least in the case of Delta ligand, internalization seems to be required to "activate" the ligand (85). Studies from flies and frogs (86,87) identified Delta as a substrate for the E3 ligase Neur. Indeed, Neur localizes to the plasma membrane, physically interacts with Delta, and promotes Delta ubiquitination. The addition of ubiquitin (Ub) moiety to Delta stimulates its removal from the cell surface via endocytosis and correlates with a loss in Delta protein. Endocytosis and recycling of Delta to specific restricted regions of the PM are probably necessary to maintain an enough high local concentration of ligand to induce robust Notch activation, as indicated also by recent evidence in *Drosophila* (88). As found for Neur, Mib mediated ubiquitination promotes ligand endocytosis, although this does not appear to promote ligand degradation as reported for Neur (86,87). These findings are consistent with a role for Mib ubiquitination in generating ligand signalling potential by promoting ligand endocytosis rather than regulating levels of ligand for Notch activation.

Spatial and temporal regulation of signalling can be also achieved through a differential distribution of signalling effectors between the plasma membrane and the endosomal compartment [e.g. signalling molecules, specific phospholipids (4)]. Receptors removal from the plasma membrane, through endocytosis, additionally extinguishes signals that depend specifically on plasma membrane molecules. For example, GPCR signalling through PM-potassium channels requires that receptors and G proteins are present in the same membrane (89); similarly PLCγ1 (phospholipase Cγ1) and PI3K

(phosphoinositide 3-kinase) signalling by EGFR is inhibited by receptor internalization due to the lack of their lipid substrate, PIP2, in endosomes (90).

3.2 Regulation of signalling by internalization routes

The biological output of a specific signal depends not only on internalization of receptor and ligands into endosomal organelles, but also on the endocytic route through which receptors reach the different compartments. Different signalling receptors, including RTKs, GPCRs, TGFβR, Notch and Wnt undergo both CME and NCE and the relative partitioning in the two endocytic routes defines the final output in terms of sustaining or attenuating signalling (Figure 7). TGFβR, for instance, can be internalized both by CME and NCE. Proteins of the TGFβ superfamily signal through the transmembrane Ser-Thr kinase TGFβR type I (TβRI) and type II (TβRII), whose dimerization results in the activation of type I receptor. Consequent phosphorylation of the receptor-regulated Smads causes SARA (SMAD anchor for receptor activation) binding to the receptors. SARA protein contains a FYVE domain, which also binds to membranes through specific interactions with PI3P. Receptors that are internalized through the clathrin pathway sustain signalling; on the contrary, receptors that enter the cell through NCE associate with Smad7 and the E3 Ub ligase SMURF, which results in receptor ubiquitination and subsequent degradation (91). A similar scenario is described for internalization and signalling of EGFR [(92) and see also section 7].

While in the case of TGFβR and EGFR, CME leads to signalling and NCE to degradation, other cargoes, such as Wnt3a-activated low-density receptor-related protein 6 (LRP6), exploit the two internalization pathways in the opposite manner. In presence of Wnt3a, LPR6 is phosphorylated and internalized into a caveolin-positive vesicular compartment, where it stabilizes β-catenin and transduces the signal via the CK1γ kinase. However, when LRP6 binds the Wnt3a antagonist Dkk (Dickkopf), the caveolin pathway

is displaced by the clathrin pathway. In the absence of the kinase, signal transduction does not occur, and this leads to enhanced β-catenin degradation (93).

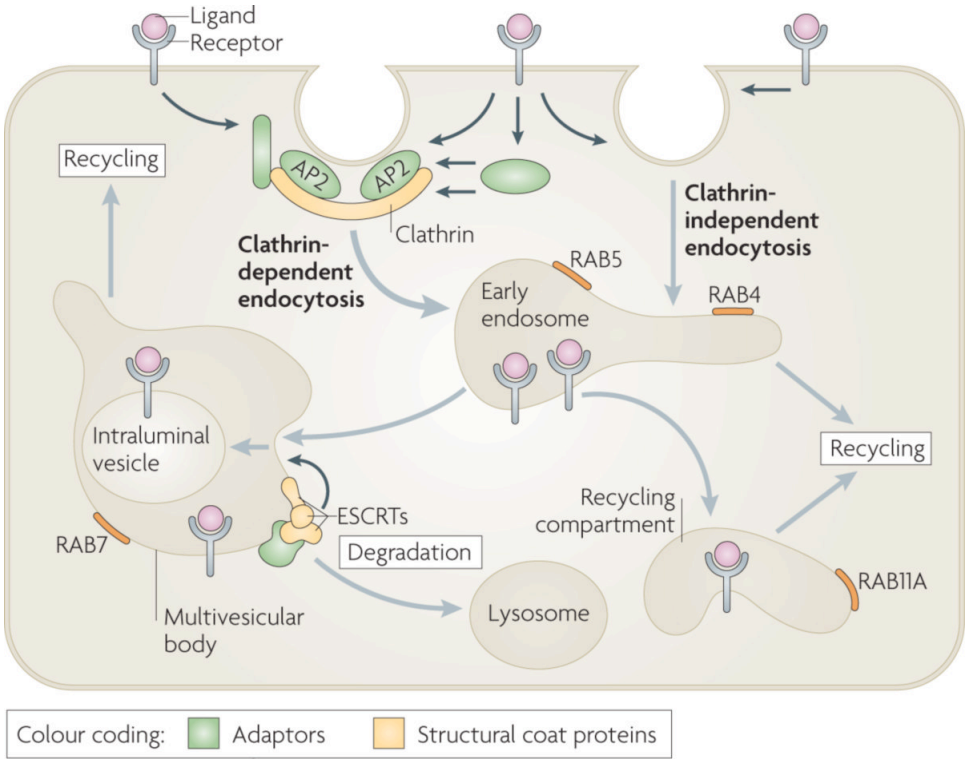

Figure 7 Signalling regulation through different entry routes. Endocytic vesicles derived from both clathrin-dependent and clathrin-independent endocytosis fuse with early endosomes. Endosomal trafficking is controlled by several Rab proteins - small GTP-binding proteins of the Ras superfamily. Each GTP-bound Rab protein resides in a particular type of endosome and functions by recruiting specific effector proteins. Following their internalization into early RAB5-containing endosomes, receptors can rapidly recycle back to the plasma membrane by a RAB4-dependent mechanism, traffic to the recycling compartment that contains RAB11A or remain in endosomes, which mature into MVB and late endosomes. Early-to-late endosome maturation involves the acquisition of RAB7 and the removal of endosomal components that are necessary for recycling. In the MVBs, cargo destined for degradation is incorporated into ILVs. Fusion of late endosomes and MVBs with lysosomes carrying proteolytic enzymes results in cargo degradation (83).

3.3 Regulation of signalling by endosomal compartimentalization

A large body of evidence shows that signalling is not restricted to the plasma membrane. On the contrary, as internalization goes further, activated transmembrane molecules get confined and enriched within endosomal organelles. Endosomes have many unique features that give them the ability to act as signalling platform during signal transduction from various receptors. The fact that endosomes exist in functional states has led to the concept of the "signalling endosome" (83,94). This model has focused on early endosomes in the first place, but recently, new evidence shows that also the other endocytic compartment performs distinct signalling roles. Endosomes are characterized by: (1) a small volume that favors ligand-receptor binding and the maintenance of receptor activity; (2) slow sorting mechanisms, as activated receptors have relatively longer resident times in endosomes, compared to the PM; (3) ability to use microtubular transport to move for long distances and towards the nucleus; (4) enrichment in particular lipids or proteins (such as PI3P and small GTPases), which contribute to the scaffold promoting-microenvironment, by recruiting proteins containing FYVE and PX domain (74); and (5) microenvironment characterized by low pH, especially in late endosomes, which favours specific reactions, such as proteolysis of signalling molecules. Endosomal signalling can mainly occur in two ways: by sustaining signals originating from the plasma membrane, or by assembling specific signal complexes that are not allowed at the PM (94,95).

In systems where activated receptors are rapidly endocytosed, the ability of a receptor to signal after its internalization is crucial to ensure the sufficient duration and intensity of signalling. To this purpose, it is important that receptors remain active in the endosomes. Several RTKs and their ligands, including the complex EGF-EGFR, remain bound and active once internalized in endosomes and throughout the endosomal trafficking (96). Moreover, evidence that EGFR continues to be active after being internalized is given by the fact that all the components of the ERK (extracellular signal regulated kinase) – MAPK (mitogen-activated protein kinase) activation cascade can be detected in

endosomes (96). An additional line of evidence is provided by experiments with siRNAs (small interfering RNAs) targeting proteins involved in internalization, which show that the endocytic process is required for ERK activation by several kinases (92,97). Furthermore, endosomal-specific proteins that are important to sustain signalling 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 (98). A similar mechanism has been described for GPCR signalling, where β-arrestin acts as a specific scaffold stably anchoring ERK1/2 to the endosome. It has also been proposed that β-arrestin promotes signalling towards cytosolic rather than nuclear ERK substates (99).

As previously described, endosomes can support signalling processes that cannot occur, or occur with low efficiency, at the plasma membrane, by acting as obligatory intermediate signalling stations between the PM and the nucleus. An example is provided by the endosomal machinery involved in the propagation of signalling from TGFβ receptor. Internalization of TGFβ receptor allows the interaction between the type I and the FYVE domain containing adaptor SARA in early endosomes (100). The interaction of SARA with SMAD2 allows the phosphorylation of SMAD2 by the receptor in endosomes. Then, SMAD2 dissociates in order to interact with SMAD4 and the complex translocates into the nucleus, by regulating gene transcription. Also the protein endofin contains FYVE domain, potentiating TGFβ signalling from endosomes (101).

Another example of endosome-specific signal is provided by GPCR signalling, in which the activation of Ste2 results in the activation of the trimeric G protein complex through the release of the β and γ subunit from the GTP-binding α1 subunit (Gpa1). Gpa1 translocates to the endosomal compartment and binds Vps15. In these stations, Gpa1 can be subsequently activated by the guanine nucleotide exchange factor Arr4, leading to the activation of the kinase Vps34 that converts phosphatidylinositol to phosphatidylinositol 3-phosphate. Increasing concentration of PI3P promotes the recruitment of Bem1 (Bud

emergence protein 1) and other proteins involved in the potentiation of MAPKs and Cdc42 signalling cascades (83).

A third example that sustains the endosomal hypothesis is provided by RTK signalling. Indeed, it has been proposed that endosomes containing EGFR, Rab5 and the Rab5 effectors APPL1 (adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper-containing 1) and APPL2 can function as signalling platform (72). APPL-containing endosomes are early endosomes that contain Rab5 but not EEA1 (Early Endosome Antigen 1); it seems that APPL proteins compete with EEA1 to bind to the active Rab5 (73). Activation of the kinase VPS34 converts phosphatidylinositol to phosphatidylinositol 3-phosphate, leading to accumulation of EEA1 and concomitant dissociation of APPLs. In HeLa cells, APPL-containing endosomes appear to be necessary for ERK1 and ERK2 activation and Akt signalling to GSK3β and mTOR (73).

4 The Ubiquitin System

The posttranslational modification of signalling receptors by the covalent attachment of one or more ubiquitin moieties has emerged as one of the major mechanism regulating receptor trafficking, sorting and downregulation.

Ubiquitin is a conserved protein of 76 amino acids that is covalently conjugated to several proteins by the formation of an isopeptide bond between its C (carboxy)-terminal glycine carboxy group and the ε-amino group of a lysine (lys) residue on the substrate proteins (102). Ubiquitination is the result of three sequential enzymatic reactions catalyzed by a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3) (102) (Figure 8). The first step catalyzed by the E1 consists of ATP-dependent activation of the carboxyl terminus of ubiquitin and its conjugation to an active site of cysteine residue in the E1. In the second step, ubiquitin is then transferred to a similar cysteine residue in the active site of the E2, also through a thiol-ester linkage. The final step is accomplished by the E3 ligase and results in the formation of an isopeptide bond with the ε-amino group of a lysine residue in the substrate (103). There are two main families of E3 ligases: HECT (Homologous to the E6-AP Carboxyl Terminus)-type and RING (Really Interesting Gene)-type (Figure 8). The HECT family, which includes Nedd4 (neural precursor cell expressed developmentally downregulated protein-4) family ligases, catalyzes the transfer of ubiquitin from the E2 to its catalytic cysteine through the formation of a thiol-ester bond and then to the substrate. Unlike the HECT domain, the RING finger domain does not possess the catalytic cysteine and does not form any catalytic intermediate with ubiquitin. Instead, the RING finger serves as a scaffold that brings E2 and substrate together, and different studies suggest that RING finger domains can also allosterically activate E2s (104). Members of the RING-type family can function as monomers, dimers or multi-subunit complexes. Dimerization generally occurs through the RING finger domain or surrounding regions and can result in homodimers, e.g. RNF4

(ring finger protein 4), and TRAF2 (TNF receptor- associated factor 2) (105,106) or heterodimers, e.g. BRCA1 (breast cancer 1) and BARD1 (BRCA1-associated RING domain 1) (107). Recent studies have demonstrated that dimeric E3 RING finger ligases are not only inert scaffolds that bring E2-loaded ubiquitin and the substrate in close proximity, but they also actively facilitate the transfer of ubiquitin (108).

The ubiquitin modification can be reversed by the action of deubiquitinating enzymes (DUBs) that recycle ubiquitin to the cytoplasmic pool (109). Thus, ubiquitination is a highly reversible process.

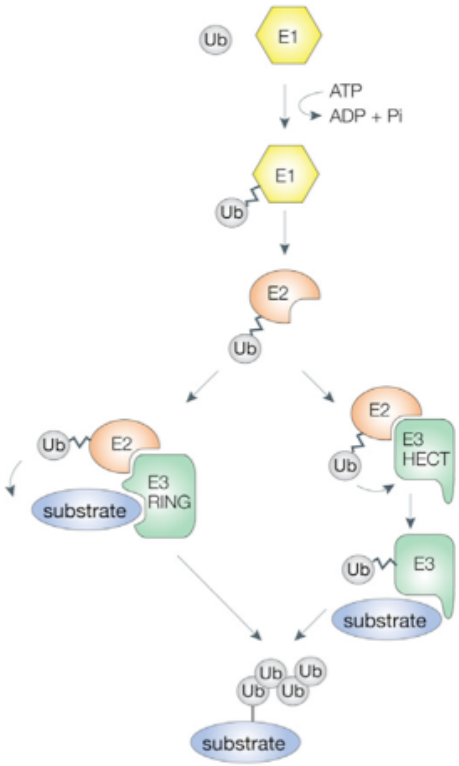

Figure 8 Schematic representation of the ubiquitination process. A hierarchical set of three types of enzyme is required for substrate ubiquitination: ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin-ligase (E3) enzymes. The two major classes of E3 ligases are shown (110).

Ubiquitin can be itself modified on any of its seven lysine residues (lys6, lys11, lys27, lys29, lys33, lys48, lys63), leading to the formation of polyubiquitin chains that show different structures and features depending on how the chains are assembled (111).

Moreover, chains that contain multiple Ub moieties can be formed by either homotypic or heterotypic linkages (111).

The different types of substrate ubiquitination include the conjugation of one ubiquitin moiety (monoubiquitination), several single Ub moieties per substrate (multiple monoubiquitination), or substrate polyubiquitination when ubiquitin chains are linked (Figure 9). Lys48-linked polyubiquitination is composed by a minimal unit of four ubiquitin moieties and targets proteins to proteasomal degradation. In contrast, lys63 chains and monoUb mediate nonproteasomal cellular functions (112): they are implicated in the regulation of endocytosis and targeting for lysosomal degradation, besides to the control of histone activity, DNA repair and virus budding (103,113). In yeast, lys63 chains are required for maximal internalization rates of a subset of nutrient permeases (114,115). A similar behaviour has been observed in mammalian cells. MHC class I molecules (116) and nerve growth factor (NGF) receptor (117) require lys63 chains for internalization, while EGFR is modified by both monoUb and lys63. Lys11-linked chains bind proteasomal receptors and trigger degradation of cell cycle regulators during mitosis (118). In human cells, lys11 linkages accumulate dramatically upon activation of the APC/C, and inhibition of lys11-linked chain formation stabilizes APC/C-substrates and leads to cell cycle arrest (118). Other chain types are involved in proteasomal degradation less frequently. Lys29-linked chains contribute to substrate turnover in the ubiquitin-fusion-degradation pathway (119), and in a few cases, lys63-linked or mixed chains trigger degradation (120). In conclusion, lys11-, lys29-, lys48-, and lys63-linked chains might all mediate proteasomal degradation, a diversity in targeting signals that is reflected by the plasticity in substrate recognition by proteasomal subunits. Much less is known about the precise function of chains that are linked through lys6, lys27 and lys33 (103,121).

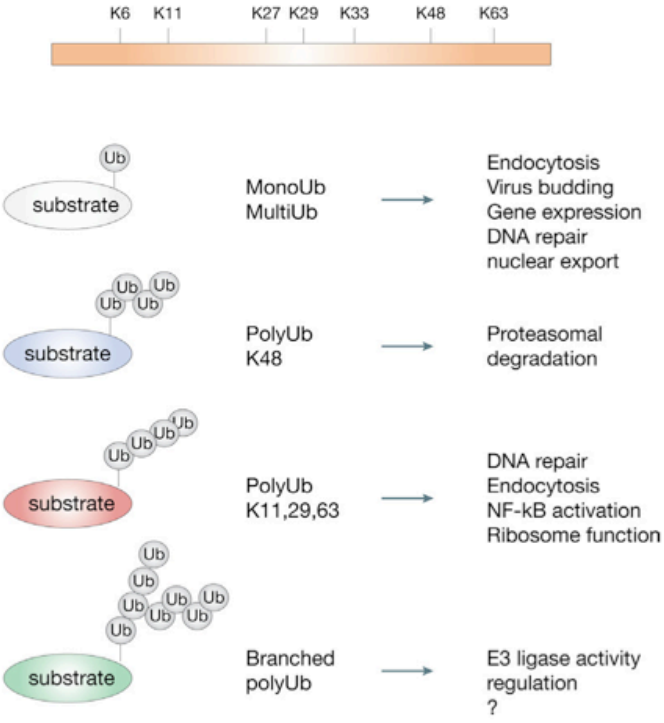

Figure 9 Schematic representation of the different ubiquitin modifications and their functional roles. The question mark indicates that the functions of branched chains are mostly unknown (110).

Ubiquitin modifications on the target proteins are recognized by an array of UBDs that bind noncovalently to ubiquitin. At least nine specialized UBD have been identified so far (122): ubiquitin associated (UBA) domain; UIM; ubiquitin E2 variant (UEV); Cuel-homologues (CUE); poly-Ub-associated zinc-finger (PAZ); Gram-like Ub-binding in Eap45 (GLUE); Np14 zinc-finger (NZF); Vps27, Hrs, STAM (VHS) and GGA (Golgi-localized, gamma-ear-containing, Arf-binding protein) and TOM1 (GAT). These different UBDs might have evolved to allow a great variety of proteins to interact with ubiquitin or ubiquitinated proteins during several cellular processes. The formation of complex ubiquitin-UBD interaction networks controls several cellular processes (123), for instance many endocytic proteins acting at different steps of the endocytic cascade possess UBD, which are crucial to regulate receptor trafficking (see next chapter 4.1).

4.1 Ubiquitin in endocytosis

Ubiquitin-mediated endocytosis was first identified in studies using the yeast *Saccharomyces cerevisiae* that demonstrated that internalization of several PM cargoes, including Ste6 (124) and Ste2 (114,125), required ubiquitin. Indeed, for many yeast cargoes, Ub modifications are both necessary and sufficient for endocytosis (125) although Ub-independent endocytosis of cargoes has also been described (126). The general consensus is that Ub-mediated endocytosis is the preponderant mechanism for internalization of most cargoes studied in yeast. However, in mammalian cells, the role of ubiquitin in endocytosis is somewhat more complicated. In the case of ion channels, ubiquitination is essential for their internalization (83). For many endocytic cargoes in mammalian cells, including RTKs and GPCRs, ubiquitination appears to be sufficient for endocytic uptake (1,127). Importantly, although many of these cargoes exhibit ligand-dependent ubiquitin modification, they also possess Ub-independent endocytosis. Thus, in mammalian cells, ubiquitination is often sufficient, but not required, for internalization. This is indicative of multiple redundant, yet distinct, mechanisms of endocytosis (128).

Despite a clear regulatory role of ubiquitination in targeting cargoes for endocytosis, it has been difficult to establish an exclusive role in this process for several reasons. First, in mammalian cells different kinds of endocytosis exist, which often act in parallel in internalization of specific cargoes. These internalization pathways might have distinct sorting determinants and might depend or not on receptor ubiquitination. One critical example in this sense is represented by the EGFR, as will be illustrated in detail in the section 7. Secondly, the endocytic interactors contain many UBDs, which may act redundantly or serve as avidity sensors to increase the efficiency of sorting for endocytosis. Moreover, some endocytic proteins are ubiquitinated themselves, thus suggesting that some UBDs may mediate interactions not only with the cargo but also with proteins involved in the endocytic network (129).

4.2 Ubiquitination of endocytic adaptors

As previously mentioned, several components of the downstream endocytic machinery, such as Eps15, Eps15L1, epsin1, epsin2, Hrs, Stam and Rabex5, are also modified by monoubiquitination upon RTK activation (22,127,130-132). For many of them, this process takes place following the stimulation of cells with growth factor (22,133). Many of these endocytic proteins, including Cin85, epsins, and eps15 family proteins, are known to undergo coupled monoubiquitination (127), which strictly depends on the ability of the UIM to bind to monoubiquitin.

The precise role of adaptor monoubiquitination in RTK endocytosis is still a matter of debate. Monoubiquitination of adaptors might be responsible for sorting the receptor along the endocytic pathway by allowing binding of ubiquitinated cargoes (through UBDs), thus resulting in signal amplification and progression of ubiquitinated cargoes along the route (134). Alternatively, it has been recently proposed that this modification 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* (132). This mechanism might in turn harbour a series of consequences, for instance, the release of ubiquitinated cargo that would thus become available for the next level of interactions along the endocytic route. A validation of this possibility was shown through an Eps15-Ub chimera, created by the fusion of ubiquitin to the C-terminus of Eps15 to mimic its permanent ubiquitination. This construct fails to localize properly on endocytic vesicles containing internalized EGFR, thereby preventing the interaction between the UIMs contained in Eps15 and EGFR-Ub, and efficient endocytosis and degradation of the receptor (132). Furthermore, an HRS-ubiquitin chimera loses its ability to recognize and sort ubiquitinated cargo (132). However, this evidence is based exclusively on artificial constructs. More studies are required to clarify the role of monoUb in the endocytic process.

4.3 Ubiquitin in endosomal sorting

Ubiquitin plays a critical role at later steps of the endocytic cascade, in the endosomal sorting process. Ligand-induced ubiquitination is indeed required for lysosomal targeting and downregulation of signalling receptors (135). Importantly, there is a continuous process of ubiquitination and deubiquitination of EGFRs all along the endocytic pathway, which is crucial to fine tune and regulate EGFR ubiquitination levels. For instance, in the case of the EGF receptor, the E3 ligase c-Cbl (Casitas B-lineage lymphoma) remains associated with the receptor and promotes receptor ubiquitination all along the endocytic route, therefore ensuring that receptors are sorted to multivesicular endosomes and targeted for lysosomal degradation (127,136-139). There are also different DUBs acting along the pathway at the level of endosomes, multivesicular bodies and lysosomes [e.g. AMSH, UBPY (140)] that may counterbalance the action of c-Cbl. EGFR degradation is regulated by two best studied deubiquitinating enzymes that reside in the MVB, named STAM-binding protein (STAMBP; also known as AMSH) and ubiquitin isopeptidase Y (UBPY, also knowkn as USP8) (141). Both the enzymes interact with ESCRT-0, the complex involved in the initial sorting of receptor for degradation (142). In line with this, conditional inactivation of the gene encoding for UBPY reduces the expression of several RTKs (143).

The ESCRT complexes machinery orchestrates the ubiquitin-directed sorting into MVBs (144) (discussed in section 2.1). This conserved machinery performs three distinct but related functions: first, it recognizes ubiquitinated cargoes and prevents their recycling and retrograde trafficking; second, it bends the endosomal membrane, allowing cargo sorting into endosomal invaginations; third, it catalyzes the final abscission of the invaginations, forming the ILVs that contain the sorted cargo (145). Genetic KD of various subunits of ESCRT -0, -I, -II or –III strongly inhibits EGF-mediated lysosomal degradation of EGFRs (144,146). In addition, an isoform of Eps15 that associates to Hrs, Eps15b, is required for efficient lysosomal degradation of endocytosed EGFRs (147). What is the

exact mechanism of action of this protein in EGFR trafficking is still unknown, but the fact that it contains two C-terminal ubiquitin-interacting motifs suggests that it may contribute to increase the avidity of ESCRT-0 for ubiquitinated receptors.

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 signalling, as observed for mutants in EGFR ubiquitination sites (148). Similarly, RNA or genetic interference with the Ub adaptor Hrs in mammalian cells results in enhanced signalling by various RTKs, including EGFR, Met and VEGFR (vascular endothelial growth factor receptor) (149). Furthermore, genetic disruption of members of the ESCRT complexes, which are required for membrane fission events, including those that lead to endosomal ILV formation, results in sustained EGFR signalling (144). Thus, ubiquitination levels need to be tightly controlled to avoid excess of signalling.

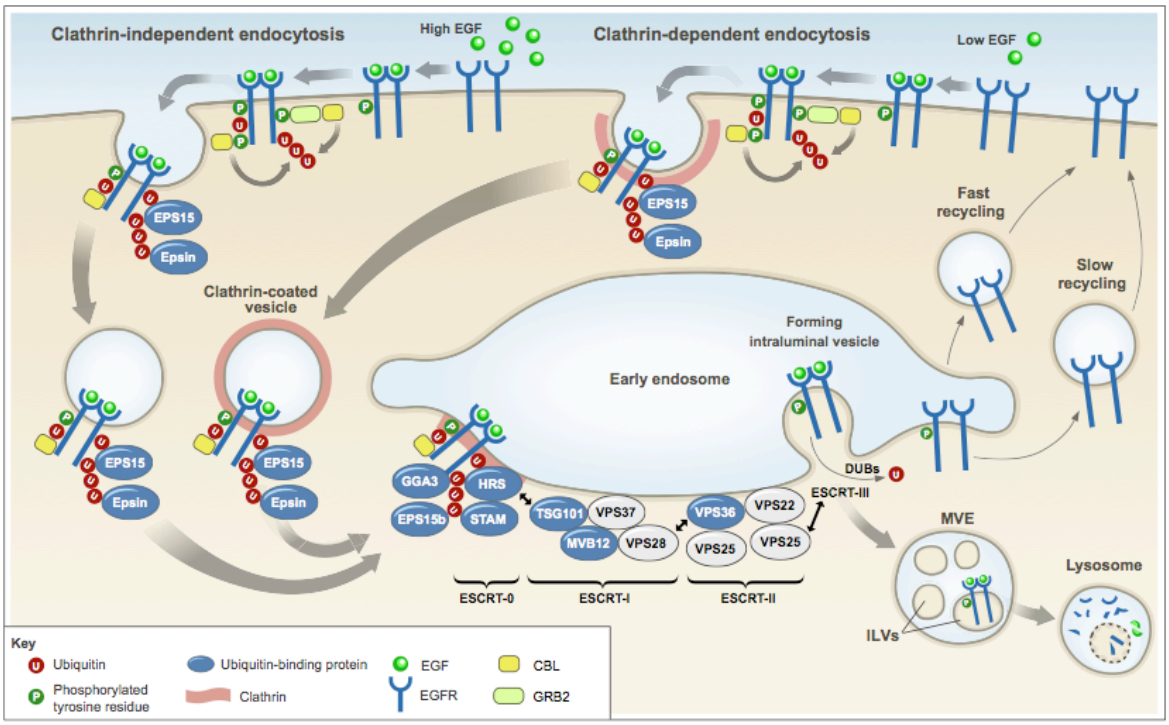

Figure 10 Ubiquitination in receptor endocytosis and endosomal sorting. Following ligand binding, EGFR can undergo clathrin-mediated endocytosis (right) or clathrin-independent endocytosis (left). In both cases, receptors are routed to early endosomes, from where they can be sorted into ILVs of the MVE and subsequently targeted for

lysosomal degradation (ubiquitinated receptors) or recycled to the plasma membrane (non-ubiquitinated receptors). Ubiquitin is an essential signal for endosomal sorting of EGFRs into the ILVs of MVEs. Components of the endocytic machinery, including Eps15, epsins, the ESCRT-0 components HRS and STAM, the ESCRT-I components TSG101 and Mvb12p (in yeast) (and the novel ESCRT-I component UBAP1), the ESCRT-II component VPS36 (EAP45), as well as EPS15b and GGA3 contain ubiquitin-binding domains have been implicated in recognizing and sorting ubiquitinated receptors either at the plasma membrane or at endosomes. The ESCRT-I and -II components assemble in supercomplexes and have been proposed to organize buds at the endosomal membrane. The ESCRT-III complex associates with ESCRT-II and forms polymers that drive membrane scission and ILV biogenesis. DUBs catalyze the removal of ubiquitin from receptors before their translocation into the ILV, without allowing cargo to escape (150).

5 Cbl proteins

The extracellular stimulation of transmembrane receptors leads to the activation of signalling networks that transmit the information throughout the cell. This is achieved through the assembly of protein complexes that convey or inhibit a signal. An important regulation of the signal transduction involves Cbl proteins, a well-conserved family that controls multiple cellular processes, by acting both as ubiquitin ligases and as multiadaptor molecules (3).

The first member of the Cbl family to be discovered was v-Cbl, a retroviral gene encoding a protein of 357 aminoacids, which was found to induce myelogenous leukemia and pre-B-cell lymphoma (151). Subsequently, it was discovered that v-Cbl corresponded to the truncated form of the larger cellular c-Cbl (152). c-Cbl encodes for a 120 kDa cytoplasmic protein that is ubiquitously expressed, in particular in thymus and testis (151).

The mammalian Cbl proteins family comprises other two members, Cbl-b and Cbl-3 (or Cbl-c); the former is mostly found in mature T cells while the latter is expressed at low levels in hematopoietic tissue (153,154). Orthologues in *Drosophila melanogaster* (D-Cbl_L; D-Cbl_S) and *Caenorhabditis elegans* (SLI-1) have also been identified (155,156) (Figure 11).

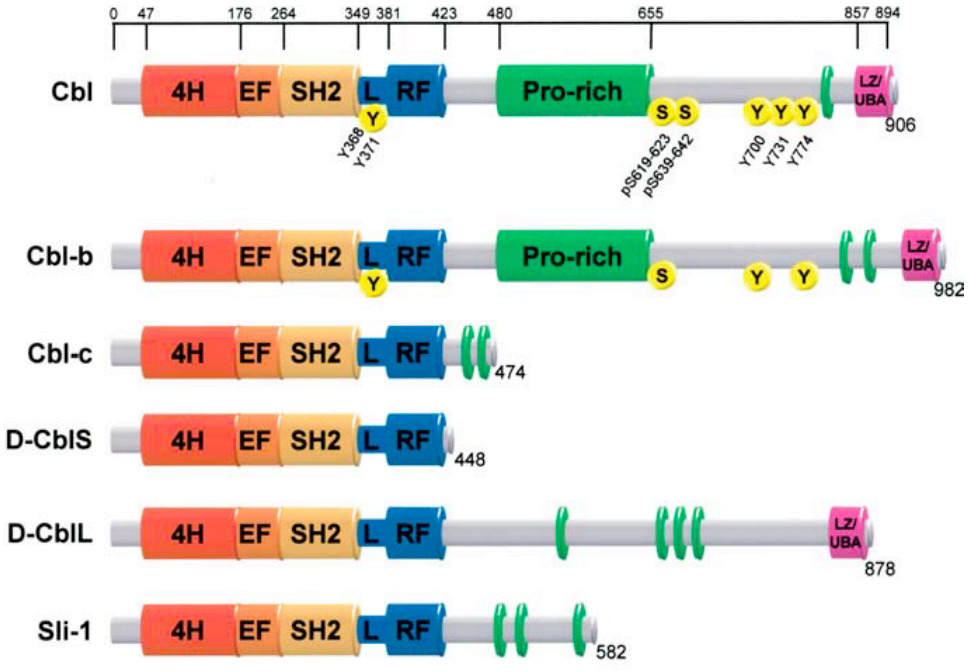

Figure 11 Members of the Cbl proteins family. Domain structure and length of mammalian Cbl, Cbl-b and Cbl-c, Drosophila melanogaster D-Cbl short and long, Caenorhabditis elegans SLI-1 are depicted. The amino-terminal PTB domain is composed of a four-helix boundle (4H), a calcium-binding EF hand and a divergent SH2 domain. The PTB domain, together with the linker region (L) and RING finger (RF), is the most conserved region in all the proteins. The long Cbl homologues have an extended carboxy-terminal part with proline-rich sequences (Pro-rich), several tyrosine and serine phosphorylation sites and a leucine zipper/ubiquitin-associated domain (LZ/UBA). The top scale indicates amino acids positions based on the human c-Cbl (Cbl) sequences (157).

5.1 Cbl proteins structure

All the above-cited Cbl proteins show a high degree of sequence similarity in the N-terminus, which encompasses a phosphotyrosine binding domain, a short helical linker region and a RING finger domain. The phosphotyrosine binding domain, also called TKB (tyrosine kinase binding) domain, contains a four-helix bundle (4H), a calcium-binding EF hand and a modified Src homology 2 domain (SH2) (158). This domain is connected to the RING finger through a linker region, which directs the correct positioning of the TKB domain towards the RING thus allowing the spatial orientation for the substrate and the E2 loading enzyme (159). Another highly conserved region is the zinc-binding C3HC4 RING finger domain, which recruits E2 ubiquitin conjugating enzymes and mediates the transfer of molecules of ubiquitin on the target (160,161).

Among the different members, the most divergent part of the protein is the C-terminal sequence. Indeed, while c-Cbl, Cbl-b and D-CblL (Drosophila-Cbl Long isoform) have extensive proline-rich regions that contain various putative SH3-binding motifs (151,152), Cbl-c and SLI-1 have a very short proline rich region (153,156), which is absent in the short form of D-Cbl. Moreover, the carboxyl-terminal part extends with an acidic box domain with serine/threonine- and tyrosines that can be phosphorylated upon stimulus and ends with a leucine zipper domain that mediates dimerization (162) and a UBA motif that binds to ubiquitin residues (163). Yet, it has been demonstrated that both c-Cbl and

Cbl-b show homo- and hetero-dimerization ability and this property was found to be necessary for efficient c-Cbl tyrosine phosphorylation upon EGF and therefore, its association with the EGFR (164). However, only Cbl-b and not c-Cbl can bind ubiquitin moieties (165).

These multiple binding motifs allow Cbl proteins to interact with a plethora of proteins through distinct mechanisms (Figure 12). For example, c-Cbl interacts with the EGFR directly through its TKB domain and indirectly via an SH3 domain-mediated interaction with Grb2 (Growth factor receptor-bound protein 2) (166). The RING finger domain recruits E2 ubiquitin-conjugating enzymes (159) and also binds its negative regulator Sprouty2 (167). The proline rich region mediates interactions with SH3-containing proteins, such as Sts1-2, Src family PTKs (phospho-tyrosine kinases) and p85 (168-170). Within this proline-rich region a small serine-rich domain provides docking site for 14-3-3 proteins binding (171). At the C-terminus, c-Cbl contains 22 tyrosine residues and those at 700, 731 and 774 are the major phosphorylation sites (172) by Syk and Src-family kinases Fyn, Yes and Lyn (172). Phosphorylation at Y700 and Y774 represents docking site for the SH2 domain of the adaptor protein Crk (173).

This intricate picture suggests that c-Cbl activity needs to be tightly regulated and that c-Cbl can have multiple roles in endocytosis, both as E3 ligase and adaptor; one of the aims of this project was indeed to dissect the two functions.

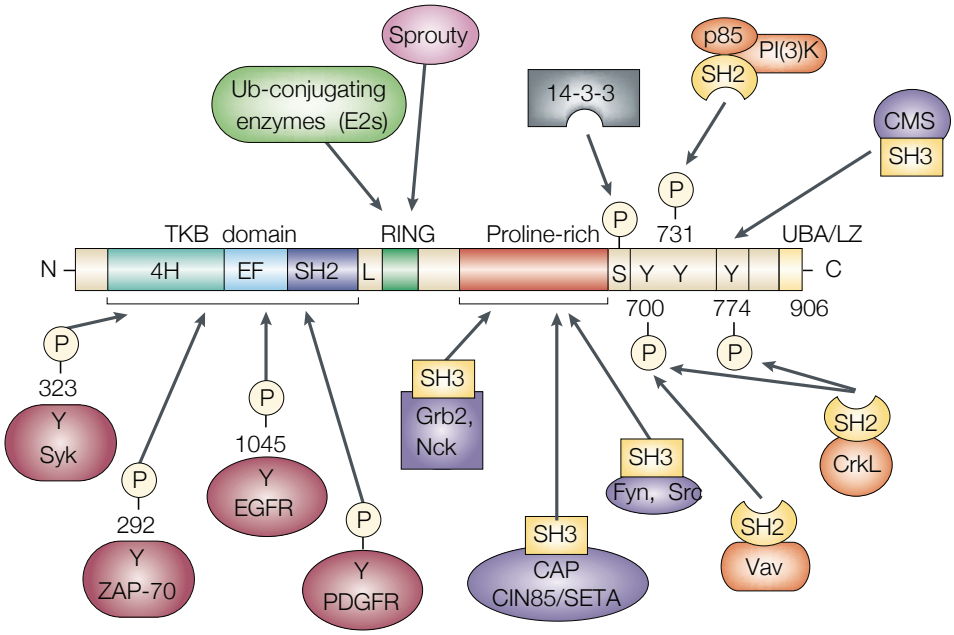

Figure 12 c-Cbl interacts with many signalling proteins. After cell surface stimulation c-Cbl is recruited to the activated receptor and in turn, recruits several other signalling proteins. The TKB domain binds phosphorylated tyrosines on the substrate; the RING finger domain recruits Ub-conjugating enzymes that allow its E3 ligase activity. c-Cbl also becomes phosphorylated on tyrosine (Y) and serine (S) residues, by promoting associations with SH2-domain-containing proteins and 14-3-3 proteins, respectively. Some associations are constitutive, such as those with the numerous SH3-domain-containing proteins that bind one or more of the 15 SH3-binding motifs within the extensive proline-rich regions. (CAP, Cbl-associated protein; CIN c-Cbl- interacting protein; CMS, Cas ligand with multiple SH3 domains; EGFR; PDGFR, platelet-derived growth factor receptor; PI(3)K; SETA, SH3-encoding, expressed in tumorigenic astrocytes; UBA; ZAP-70, z-chain-associated protein kinase 70) (174).

5.2 Cbl proteins as regulators of RTKs

Cbl proteins have been identified as negative regulators of RTKs when genetic experiments in *C. elegans* demonstrated that Sli-1 is a suppressor of EGFR/LET-23 signalling in this organism (156). In agreement with these studies, Levkowitz and colleagues showed that c-Cbl was able to ubiquitinate and downregulate the EGFR (137,161), an ability that requires an intact TKB and RING finger domain (161) (for the role of c-Cbl in EGFR ubiquitination see section 7.1).

Since then, c-Cbl has been shown to ubiquitinate other RTKs, e.g. EGFR (148,175), VEGFR (176), PDGFR (160), FGFR (Fibroblast Growth Factor Receptor) (177) and HGFR (hepatocyte growth factor receptor) (178) (Table 2).

In the case of HGFR (also known as c-Met receptor), the molecular mechanism of its ubiquitination has been widely investigated. Stimulation of HGFR with its ligand, the hepatocyte growth factor (HGF), leads to receptor ubiquitination by c-Cbl and increased degradation (178). This requires a functional c-Cbl TKB domain and the Y1033 located in the juxtamembrane region of the receptor. Indeed, the E3 ligase c-Cbl is recruited to the HGFR directly through its TKB domain that binds the pY1033 and indirectly, via its proline-rich domain, through the adaptor protein Grb2, which binds to the pY1356 on the receptor (178). The substitution of Y1033 with phenylalanine abrogates the recruitment of c-Cbl to the HGFR and its consequent ubiquitination (178). Moreover, an intact indirect binding site for c-Cbl recruitment is not able alone to efficiently induce HGFR ubiquitination (178). Noteworthy, this modality of c-Cbl recruitment could be envisioned for other RTKs [e.g. Ret and EGFR (see section 7)].

RTK	E3-Ligase	Type of Ub-modification	References
EGFR	Cbl	MultimonoUb	Haglund et al. [25]
		PolyUb Lys63	Huang et al. [26]
VEGFR-1	Cbl	?	Kobayashi et al. [87]
VEGFR-2	Cbl	?	Duval et al. [83]
	Nedd4		Murdaca J., JBC 2004
PDGFR	Cbl	MultimonoUb	Joazeiro et al. [86]
		PolyUb	Haglund et al. [25]
FGFR	Cbl	?	Wong et al. [97]
IGF-1R	Nedd4	?	Vecchione et al. [96]
	Mdm2	PolyUb Lys63	Girnita et al. [85]
	Cbl	PolyUb Lys48	Sehat et al. [33]
Met/ HGFR	Cbl	MultimonoUb	Peschard et al. [20]
		PolyUb Lys48	Carter et al. [82]
TrkA	TRAF6	PolyUb Lys63	Geeta et al. [84]
	Nedd4-2	MultimonoUb	Arevalo et al. [38]
ErbB3	NRDP1	PolyUb	Qiu and Goldberg [93]
ErbB4	NRDP1	PolyUb	Omerovic et al. [92]
	AIP1/Itch	MonoUb	Sundvall et al. [94]
c-Kit	Cbl	?	Zeng et al. [98]
			Masson et al. [91]
TGFβ	Nedd4-2	PolyUb	Suzuki et al. [95]
	Smad7/ Smurf2		Komuro et al. [88]
			Kuratomi et al. [89]
	WWP1/ Tiul1		
Ret	Cbl	? MonoUb/PolyUb	Scott et al. [22]
CSF-1R	Cbl	?	Lee et al. [90]

Question mark means "not characterized".

Table 2 Tyrosine kinases receptors regulated through ubiquitination. The RTKs together with the E3 ligase described for their ubiquitination and the type of ubiquitination are shown in the table (135).

5.3 Regulation of c-Cbl functions

Cbl proteins are highly regulated within the cells, both by degradation but also by specific inhibitors, which act through different mechanisms. Several studies have shown that degradation of Cbl proteins is a regulated process that can potentially control RTKs activity. Different mechanisms, comprising both auto-ubiquitination and ubiquitination by other E3 ligases, seem to be involved in the degradation of Cbl proteins (179).

Activated forms of Src kinase induce ubiquitination and proteasomal degradation of c-Cbl (180) thus preventing downregulation of the activated EGFR by c-Cbl (180) (Figure 13). Moreover, c-Cbl colocalizes with activated Src in structures similar to those observed for the activated EGFR, suggesting that Src induces c-Cbl degradation

throughout the endocytic pathway (180). In addition, activated Src enhances the transforming activity of the EGFR, and this might be in part explained by degradation of the Cbl proteins (180).

Another regulatory mechanism involves the HECT E3 ligases Nedd4 and Itch, which bind to and ubiquitinate all the mammalian Cbl proteins, targeting them to proteasomal degradation (181) (Figure 13). Consistent with these observations, Nedd4 prevents c-Cbl-mediated ubiquitination and downregulation of the EGFR, thereby resulting in the persistence of downstream signalling by the EGFR (181).

Recently, two c-Cbl interacting proteins (Sts-1 and Sts-2) have been found to regulate the action of c-Cbl on RTKs signalling (170). Upon EGFR activation, Sts-1 and Sts-2 are recruited to the EGFR and inhibit its ubiquitination by the E3 ligase c-Cbl that leads to receptor downregulation. Sts-1 and Sts-2 can compete with epsin proteins and associate to activated and ubiquitinated EGFR through their UBA domain (170). The mechanism of inhibition is still poorly understood; however, competition with the sorting machinery during receptor endocytosis could be important.

A well-studied example of regulation of c-Cbl functions is achieved through Sprouty2; Cbl proteins bind constitutively but weakly to Sprouty via the RING finger domain (182). Upon growth factor stimulation, Sprouty2 expression is induced and the protein is moved to the plasma membrane where it becomes phosphorylated on Tyr55. c-Cbl binds to this phosphorylated residue through its TKB domain with high affinity (182,183). This interaction results in ubiquitination and proteasomal degradation of Sprouty2 (182,183). Phosphorylated Sprouty2 also competes with the activated EGFR for the binding to the TKB domain of c-Cbl, and this leads to a decrease in Cbl-mediated ubiquitination and downregulation of the receptor (182,183) (Figure 13).

Cdc42 is another example of a protein that competitively inhibits the downregulation of the EGFR by Cbl proteins. Upon EGF stimulation, Cdc42 is activated and binds to the β-Pix protein, which in turn interacts with the proline rich region of c-Cbl

(184). This complex sequesters c-Cbl and prevents its binding to the EGFR, thereby inhibiting ubiquitination and downregulation of the activated EGFR (184) (Figure 13).

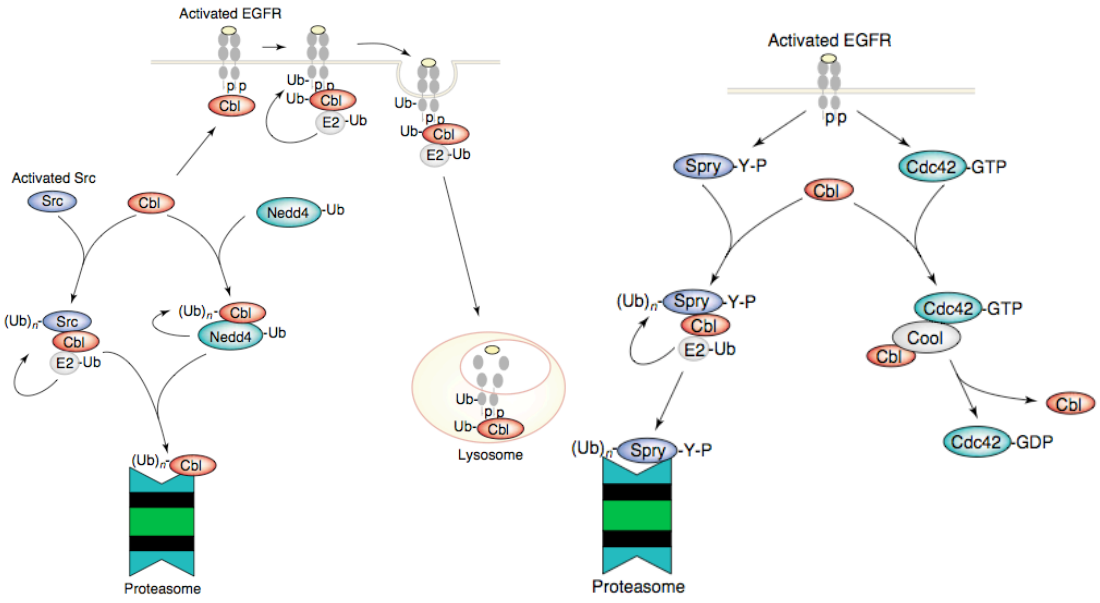

Figure 13 Different mechanisms of c-Cbl regulation. On the left panel regulation with degradation is shown. Cbl proteins are degraded by various mechanisms, including their coordinate degradation along with the EGFR, degradation by Src and degradation by Nedd4. c-Cbl is also degraded along with the EGFR in the lysosome. On the right panel, regulation without c-Cbl degradation is depicted. The two major proteins involved in this mechanism are the inhibitor Sprouty (Spry) proteins the GTPase Cdc42 (179).

All the above-described mechanisms concern negative regulation of c-Cbl activity. But c-Cbl activity is also positively regulated. A crucial mechanism of activation is achieved through c-Cbl phosphorylation by EGFR. Indeed, EGFR can phosphorylate the Tyr371 located in the linker region of c-Cbl. The E3 ligase activity of c-Cbl is stimulated by Tyr371 phosphorylation, and although previous *in vitro* and *in vivo* studies clearly support phosphoregulation of the ligase activity of c-Cbl through this tyrosine (161,185), the detailed mechanisms underlying this positive regulation of c-Cbl activity have not been described yet. However, recently, two structural studies have shed some light on the mechanism of c-Cbl and Cbl-b activation induced by phosphorylation (186,187). In the absence of substrate binding, the TKB and RING domains form a packed structure that masks the E2 binding site. The subsequent binding of the TKB to the substrate induces a

primary rotation of the linker region, which allows phosphorylation of Tyr371 in c-Cbl and Tyr363 in Cbl-b. This phosphorylation event leads to a complete rotation of the linker region that unmasks the RING E2 binding surface, allowing substrate ubiquitination (186,187).

5.4 Functions of Cbl proteins in the immune system

c-Cbl and Cbl-b proteins play crucial roles in controlling several functions of the immune system. Their essential role was demonstrated through the generation of mice deficient for c-Cbl (188) or Cbl-b (189,190). These two proteins display redundant functions, since c-Cbl$^{-/-}$ and Cbl-b$^{-/-}$ double knockout (KO) mice are embryonic lethal (191), while single KO animals are viable and fertile (188-190). The major phenotypes observed in the single KO mice concern the alteration of signalling pathways in thymocytes or mature T cells. In detail, c-Cbl-deficient mice show increased TCR (T cell receptor) signalling in thymocytes involving the upregulation of the activity of the kinase ZAP70 (188). On the contrary, Cbl-b deficient mice display alterations of TCR in mature pheripheral T cells through deregulation of Vav guanine nucleotide exchange factor activity (189,190). c-Cbl$^{-/-}$ mice exhibit increased thymic cellularity and splenomegaly (188), consistent with the ability of c-Cbl to negatively regulate mitogenic signals. On the contrary, no aberrant thymic development has been demonstrated in Cbl-b$^{-/-}$ mice, although they display spontaneous (189) or induced autoimmunity disorders (190). The distinct phenotypes observed between c-Cbl$^{-/-}$ and Cbl-b$^{-/-}$ mice, may reflect differences in the expression of the two proteins between thymocytes and peripheral T cells. Thus, c-Cbl KO mice have raised interesting questions about the abilities of c-Cbl and Cbl-b to negatively regulate different signalling molecules in distinct T-cell populations. Some studies suggest that Cbl-b might act preferentially in the regulation of exchange factors for Rho-family GTPases rather than tyrosine kinases (192).

5.5 c-Cbl mutations from myeloid neoplasm to Noonan syndrome and solid tumors

Over the past 5 years, c-Cbl has been found mutated in almost 5% of a wide variety of myeloid neoplasms, including myelodysplastic syndrome, myelofibrosis, *de novo* and secondary acute myeloid leukemia (respectively AML and sAML), atypical chronic myelogenous leukemia (aCML), juvenile myelomonocytic leukemia (JMML) and chronic myelomonocytic leukemia (CMML) (193-199) (Cosmic. Catalogue of Somatic Mutations in Cancer. 2012. Available from : http://www.sanger.ac.uk/genetics/CGP/cosmic/). The frequency of c-Cbl mutations is spread throughout these diseases; it appears to be higher in JMML (15%), CMML (13%), sAML (10%), and aCML (8%). The majority of these mutations are missense mutations that cluster within the linker region and the RING finger domain (Figure 14) and have been shown to disrupt the E3 ligase activity. These missense mutations are usually homozygous mutations, resulting from copy neutral loss of heterozygosis; on the contrary, the deletions that arise from splicing mutations are more frequently heterozygous (193-199). Nonsense mutations, frame shift mutations and insertions within the linker and RING finger regions have been found as well. Functional studies have demonstrated that the c-Cbl mutants found in myeloid neoplasm loose E3 ligase activity, thus causing increased activity of the receptor tyrosine kinase Flt3, and downstream signalling of PI3K and STAT pathways; indeed they are transforming (193,196,199). One possibility of action of these mutants is that they act as dominant negative proteins on the endogenous c-Cbl (when is in heterozygosis) or on Cbl-b, by uncoupling c-Cbl binding to activated RTKs from their ubiquitination and degradation. In support of this, while deficient mice in c-Cbl, Cbl-b or Cbl-c do not show any evidence of leukemia, mice carrying a RING finger mutant c-Cbl knock-in develop leukemia (200), by suggesting that wild type c-Cbl may act as tumor suppressor, while mutant c-Cbl might function as oncogene.

Mutations in Cbl-b and Cbl-c are much less common in myeloid neoplasm. Only two studies have described frame shift or missense mutations within the RING finger

domain of Cbl-b (194,198). The fact that no other studies have found Cbl-b mutations suggests that the frequency of Cbl-b mutations in myeloid neoplasm is lower compared to c-Cbl (193,196). Moreover, only one report describes polymorphism in the RING finger domain of Cbl-c (198); however, the expression of Cbl-c is restricted to epithelial cells, so the importance and significance of these abnormalities remain to be elucidated (154).

Heterozygous germline mutations in c-Cbl can underlie a phenotype with clinical features fitting or partially overlapping Noonan Syndrome (NS) (201). The Noonan Syndrome is transmitted as an autosomal dominant trait and is genetically heterogenous (202). It is a clinically variable condition characterized by reduced postnatal growth, a wide spectrum of cardiac diseases, facial dysmorphism and ectodermal and skeletal defects (203). c-Cbl mutations associated with this disease are missense mutations that alter evolutionary conserved residues located in the RING finger domain and in the linker region (Figure 14), two known mutational hot spots in myeloid malignancies above described (201). The mutations found in c-Cbl have been shown to affect c-Cbl-mediated receptor ubiquitination and to cause an aberrant signal flux through RAS and the MAPK signalling cascade (201,202).

Somatic mutations of c-Cbl have been found in 8 NSCLC out of 119 samples (204). All but one of the mutations are located outside the RING finger (Figure 14) and are all heterozygous. These mutants have been characterized for their effect on E3 ligase activity, cell viability, cell cycle and cell motility: the E3 ligase activity is maintained, while their overexpression causes increased viability and motility (204). However, the relevance and significance of these mutations still needs to be established.

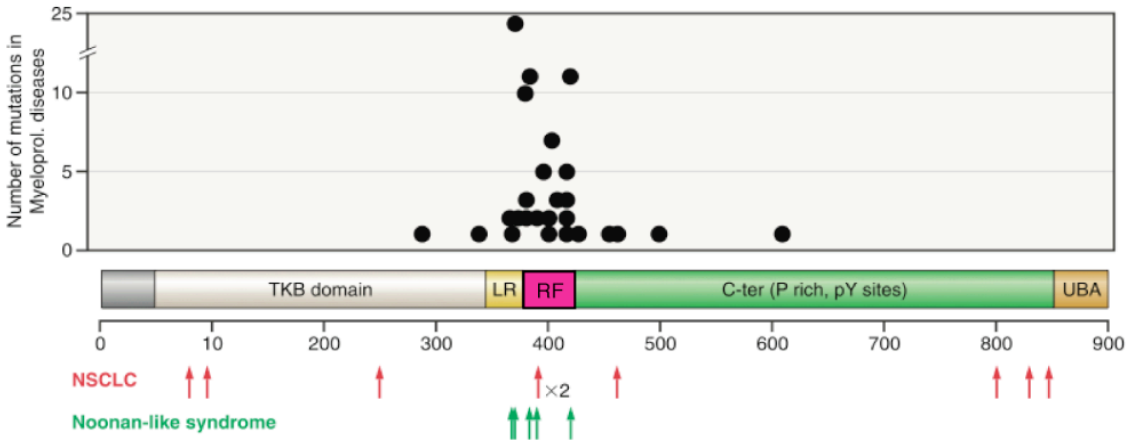

Figure 14 Mutations of c-Cbl in cancer and Mendelian diseases. In the middle of the panel, a schematic of the c-Cbl protein is depicted with its functional domains (LR, linker rregion; RF, RING finger domain). The ruler underneath shows amino acid positions. On the top, the position and the frequency of the mutations detected in myeloproliferative diseases are shown by solid circles, aligned with the amino acid sequence. At the bottom, the position of the mutations detected in NSCLC and in the Noonan-like syndrome is shown by red and green arrows, respectively. In NSCLC, the mutation at position 391 was detected in two tumors (shown as x2). In the Mendelian syndrome, four of five mutations affect the same residues (371, 367, 382, 420) as in myeloproliferative diseases (4).

6 The EGFR system

Receptor tyrosine kinases have been widely described as key regulators of critical cellular processes, such as proliferation, differentiation, cell survival and metabolism, cell migration and cell-cycle control (205). The EGFR is a prototypical RTK (206) with critical roles in physiological and pathological processes in epithelial cells (207). The EGFR, also named ErbB1, belongs to a family that includes three other members: ErbB2/HER-2 (208), ErbB3/HER-3 (208), and ErbB4/HER-4 (209). These receptors share a similar structure that is composed by an extracellular ligand-binding domain, which has four subdomains (I-IV), a short hydrophobic transmembrane helix, and a cytoplasmic region that contains the protein tyrosine kinase domain and an additional carboxy-terminal and juxtamembrane regulatory regions (210). This structure enables signals to be transmitted across the plasma membrane where they activate gene expression and ultimately induce different cellular responses.

A number of distinct ligands activate the receptor by binding to the extracellular domain, such as EGF, transforming growth factor-α (TGFα), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AR), betacellulin (BTC), epiregulin (EPR) and epigen (211-215). All ErbB family ligands are made as type I transmembrane proteins that are inserted into the plasma membrane and then cleaved by cell surface proteases to release mature growth factor that binds the receptor.

Like other transmembrane proteins, EGFR is co-translationally translocated through the endoplasmic reticulum (ER) membrane, transported to the Golgi apparatus, where the extracellular domain becomes N-linked glycosylated. From here, EGFR is delivered to the plasma membrane (216).

Increasing evidence indicates that, in unstimulated cells, EGFR might exist both in a monomeric and in a pre-dimerized state (217). It is now well accepted that binding of EGF (or other agonists) to EGFR shifts a monomer-dimer equilibrium to favour the

dimeric state (218,219). Ligand binding to the pre-dimerized state forms a 2:2 ligand to receptor configuration and induces a rearrangement of each receptor subunit, allowing a rotation of the transmembrane domain (220). The transition to ligand-bound receptor dimers releases inhibitory interactions that maintain the inactive form of the receptor. These conformational changes induce activation of the intracellular tyrosine kinase domain and consequent trans-autophosphorylation of key tyrosine residues within the COOH-terminal portion of EGFR and, as a result, provide specific docking sites for cytoplasmic proteins containing SH2 and phosphotyrosine binding domains. These multiple bindings initiate intracellular signalling via several pathways (210), thereby inducing multiple responses in the cell (Figure 15).

EGFR phosphorylation is counterbalanced by the action of protein Tyr-specific phosphatases (PTPs), which reverse Tyr phosphorylation on EGFR residues. The PTP superfamily includes both cytoplasmic enzymes and transmembrane receptor-like PTPs (RPTPs). Although protein-Tyr phosphatase 1B (PTP1B) resides in the endoplasmic reticulum, it has been implicated in EGFR signalling (221). Additional RTK-PTP interactions take place at the plasma membrane and in endosomes. However the exact impact of phosphateases on EGFR at the plasma membrane is still a matter of investigation.

As we discussed previously, c-Cbl is recruited to specific tyrosines in the EGFR tail upon receptor activation at the plasma membrane and catalyzes ubiquitination of the EGFR (see also next section). Importantly, c-Cbl-mediated ubiquitination starts already at the plasma membrane and continues in the endocytic compartments (222). Indeed, c-Cbl remains associated all along the endocytic route (136,222) counteracting the action of deubiquitinating enzymes at the endosomal stations (141). c-Cbl-mediated ubiquitination is required to target EGFRs to lysosomal degradation, leading to signal extinction (137-139,175). Whether ubiquitination is essential also at the initial steps of EGFR internalization is rather controversial and will be discussed more in details in section 7.1.

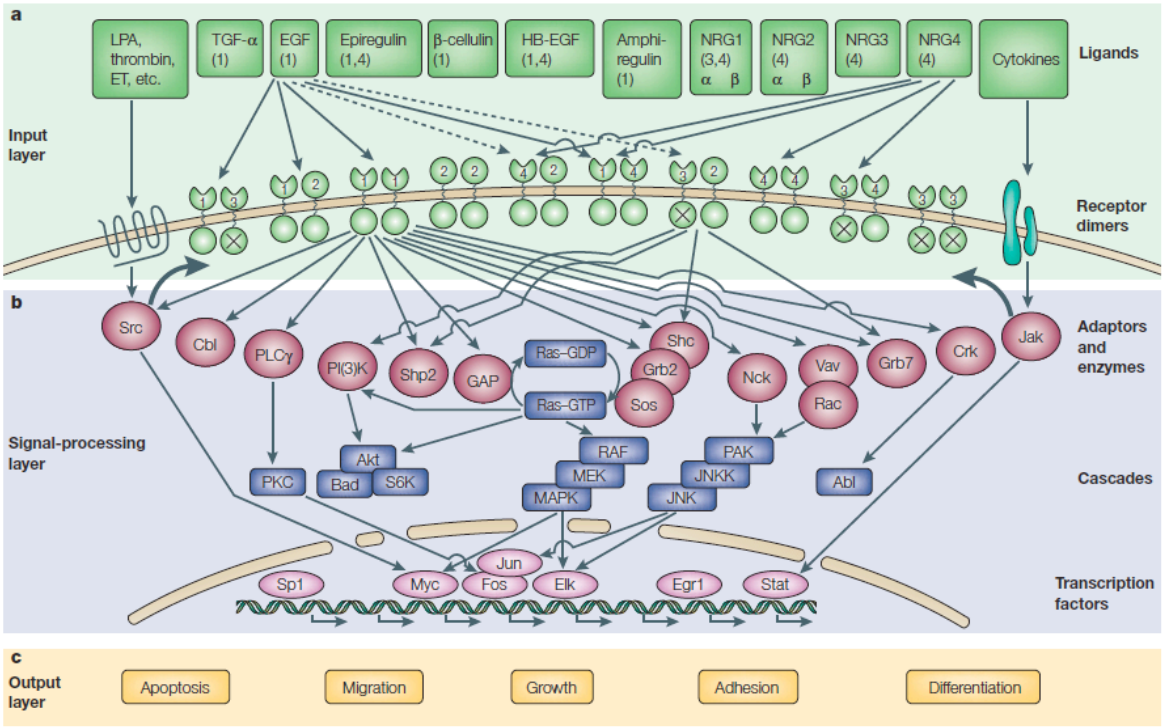

Figure 15 The ErbB signalling network. a Ligands and the ten dimeric receptor combinations comprise the input layer. Numbers in each ligand block indicate the respective high-affinity ErbB receptors. For simplicity, specificities of receptor binding are shown only for EGF and neuregulin 4 (NRG4). ErbB2 binds no ligand with high affinity, and ErbB3 homodimers are catalytically inactive (crossed kinase domains). *Trans*-regulation by G-protein-coupled receptors [such as those for lysophosphatidic acid (LPA), thrombin and endothelin (ET)], and cytokine receptors is shown by wide arrows. **b** Signalling to the adaptor/enzyme layer is shown only for the weakly mitogenic ErbB1 homodimer, and the relatively potent ErbB2–ErbB3 heterodimer. Only some of the pathways and transcription factors are represented in this layer. **c** Translation of the pathways into specific types of output (210).

6.1 Ligand-induced EGFR signal transduction

The ErbB network has been described as a bow-tie structure, where the inputs of multiple growth factors function through eight potential hetero-homodimers receptors and activate common signalling cascades (223). This process results in the specific activation of transcription factors that lead to the selected cell fate. Temporal and spatial control of EGFR signalling dictates its biological outcome possibly by altering the balance among

various signalling pathways (224). As above mentioned, EGFR can be activated by several ligands, each of which is characterized by a consensus sequence consisting of six spatially conserved cysteines residues that create three intermolecular disulfide bonds. This consensus sequence is known as the EGF motif (225).

Upon ligand binding, EGFR triggers several downstream signalling pathways similar to other RTKs. These pathways include:

(1) the activation of PLCγ and its downstream calcium- and PKC-mediated cascades;

(2) the activation of Ras signalling, leading to various MAPKs;

(3) the activation of PI3K, leading to AKT signalling cascade;

(4) the activation of signal transducers and activators of transcription (STAT) pathway;

(5) the activation of Src kinase.

7 EGFR endocytosis

Activation of EGFR through binding of EGF leads to acceleration of receptor endocytosis. EGFR can be internalized through both CME and NCE pathway (1) and the distribution of the receptor into these two entry routes is a highly regulated process, which has a major impact on the final fate of the EGFR (92). CME is characterized by high internalization rates of EGFR and is observed already 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 become active, in different cellular contexts (226). There has been a long controversy in the field regarding the establishment of accepted physiological concentrations of EGF, since there was a historical erroneous perception that only low doses of EGF could be physiological. However, it is known that while plasma EGF concentrations are around 1 ng/ml (227), serum EGF levels are significantly higher (228,229), possibly because of the release of EGF from platelets by degranulation (228) (Table 3). Moreover, different organs seem to regulate their levels of EGF in an independent fashion, further confirming the notion that EGF might act locally rather than systemically as an endocrine factor. Indeed, great differences of EGF concentrations have been observed in various fluids of the body, from low concentration (1–5 ng/ml) in plasma, serum, and saliva (227-229), to medium amount (5–50 ng/ml) in tears, follicular fluid, sperm, and seminal plasma (227,229,230), to high levels (50–500 ng/ml) in bile, urine, milk, and prostate fluid (227,231,232) (Table 3). Therefore, under physiological conditions, EGFR-expressing cells are exposed to a wide range of EGF 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.

Recent studies from our laboratory have shown that ligand concentration can affect the internalization route (1). In particular, at low doses of EGF (<3 ng/ml), receptors undergo exclusively CME (1). On the contrary, higher ligand concentration (10-100 ng/ml) induces a substantial proportion of receptors (~40%) to undergo NCE (1). Moreover, EGFRs internalized through CME are mostly not targeted to lysosomal degradation but rather recycled back to the plasma membrane. This results in a prolonged duration of activated-EGFR signalling pathways (92). By contrast, EGFRs entering cells through clathrin-independent endocytosis are preferentially targeted to degradation, thus leading to signal extinction (92). Interestingly, this dual mechanism seems to cope with the huge variety of physiological EGF concentration present in body fluids. When cells need to maximize the stimulation efficiency (i.e. at low doses of ligand), this mechanism sustains signalling and protects receptors from degradation; when the ligand is present at high concentration, it protects cells from overstimulation by targeting excess of activated

EGFRs to degradation (92). However, not all cells have this dual mechanism, and the presence and significance of EGFR-NCE clearly depends on the cellular context at least in culture (226,233) (Table 4).

Cell Line EGFRs/cell		LOW EGF Ke	HIGH EGF Ke obs	NCE
HeLa Milan 3.0 x 10^5	Control	0.30	0.15	YES
	Cl-KD	0.08	0.08	
	Fil.	0.30	0.08	
	Cl-KD+Fil.	0.08	0.03	
	Dyn-KD	0.07	0.03	
HeLa Milan EGFR KD 0.7 x 10^5	Control	0.24	0.32	YES
	Cl-KD	0.09	0.23	
	Fil.	0.21	0.24	
	Cl-KD+Fil.	0.07	0.04	
HeLa Oslo 0.8 x 10^5	Control	0.30	0.30	NO
	Cl-KD	0.08	0.08	
	Fil.	0.03	0.30	
	Cl-KD+Fil.	0.08	0.08	
NR6-EGFR wt 2.5 x 10^5	Control	0.11	0.11	YES
	Cl-KD	0.03	0.06	
	Fil.	0.11	0.08	
	Cl-KD+Fil.	0.03	0.03	
A431 1.4 x 10^6	Control	0.42	0.05	YES
	Cl-KD	0.07	0.03	
	Dyn-KD	0.08	0.01	
MDA MB-231 0.8 x 10^5	Control	0.24	0.19	YES
	Cl-KD	0.04	0.08	
	Dyn-KD	0.03	0.02	
BT20 4.3 x 10^5	Control	0.10	0.06	YES
	Cl-KD	0.03	0.04	
	Dyn-KD	0.03	0.02	
MCF10A 2.9 x 10^5	Control	0.27	0.19	NO
	Cl-KD	0.04	0.02	
	Dyn-KD	0.02	0.04	
HCT116 0.5 x 10^5	Control	0.23	0.24	NO
	Cl-KD	0.04	0.05	
	Dyn-KD	0.04	0.05	
BT549 0.5 x 10^5	Control	0.23	0.13	NO
	Cl-KD	0.04	0.02	
	Dyn-KD	0.04	0.02	

Table 4 Presence of EGFR-NCE in various cell lines. The presence of an EGFR-NCE pathway in the indicated cell lines has been evaluated by comparing the internalization curves of WT versus clathrin-KD cells or dynamin-KD, or by comparing WT versus

filipin-treated cells, and calculating the endocytic rate constants (Ke or Ke obs, see section 1 , Results) (226).

So how does EGF dosage influence the selection of a particular endocytic pathway? Recent work performed in our lab (226) has shown that the same range of EGF concentration that activates EGFR NCE also causes a sharp increase ("threshold") in EGFR ubiquitination. It is this covalent modification of the EGFR that functions as a "switch" that commits receptors to NCE. The molecular mechanism governing the switch depends on the cooperative recruitment of c-Cbl to the EGFR: as a consequence of EGF binding, the EGFR becomes multiply phosphorylated, a modification that permits the cooperative binding of the E3 ligase c-Cbl, in complex with Grb2, at two specific EGFR phosphorylation sites. A higher concentration of EGF results in a greater probability of EGFR being sufficiently phosphorylated at both critical sites to allow c-Cbl/Grb2 binding. Efficient recruitment of c-Cbl induces massive EGFR ubiquitination and triggers its internalization via NCE (150). Thus, c-Cbl binding to the EGFR allows cells to react to a linear gradient of ligand with a threshold ubiquitination response that, in turn, regulates EGFR internalization via NCE, and ultimately EGFR fate and signalling capacity.

7.1 Role of c-Cbl in EGFR endocytosis

c-Cbl is the major E3 ligase involved in EGFR ubiquitination along the endocytic pathway (136); in addition, through its ligase activity, c-Cbl can ubiquitinate, not only the receptor, but also other proteins involved in the internalization process (3). In addition, as we discussed above, the multi-domain nature of c-Cbl allows the protein to interact with numerous partners. Therefore, beside its role as E3 ligase, c-Cbl can bind and/or regulate around 150 different proteins [reviewed in (3)], making it difficult to separate its E3 ligase activity and adaptor function in EGFR endocytosis. One of the aims of my PhD was indeed to dissect this dual role in EGFR clathrin-mediated endocytosis.

Several experimental data support the role of c-Cbl in EGFR internalization via CME. Firstly, it has been demonstrated EGF-induced translocation of c-Cbl to CCPs (136). Secondly, overexpression of several c-Cbl mutants inhibits EGFR internalization in different cell lines, including HeLa, PAE and NIH3T3 cells (234,235). Thirdly, chimeric proteins composed by the SH2 domain of Grb2 and c-Cbl are able to rescue EGFR endocytosis in Grb2-depleted cells (236). Fourthly, siRNA depletion of both c-Cbl and Cbl-b through RNA interference partially inhibits EGFR clathrin endocytosis (148).

Since c-Cbl ubiquitinates EGFR, it is logical to suppose that c-Cbl-mediated ubiquitination of EGFR is required for receptor internalization. However, the situation is complicated by the fact that distinct internalization pathways show a different requirement for receptor ubiquitination. Indeed, as previously discussed, EGFR ubiquitination is absolutely required for receptor internalization through NCE (1,226). However, its requirement for the initial steps of CME is rather controversial (150). In this sense, several lines of evidence exclude the requirement of c-Cbl-mediated EGFR ubiquitination in CME. The first indication came from an EGFR mutant that lacks Tyr1045 and is weakly ubiquitinated. This mutant undergoes normal internalization via CME (234). Recently, through a mass spectrometry analysis, the ubiquitination sites in EGFR have been mapped in the kinase domain of the receptor and it has been found that the two major modifications are the monoubiquitination (~49% of the modified receptor) and lys63-linked polyubiquitination (~40%) (148), although it is not clear whether they play a distinct role in endocytosis. Mutations of the major ubiquitination sites (16KR) do not affect EGFR CME, arguing that receptor ubiquitination is not essential for this pathway (2,148). Interestingly, the 16KR EGFR mutant still depends on c-Cbl for its internalization via CME. From these results, it emerges that although EGFR ubiquitination is not essential for CME, c-Cbl activity is still needed, possibly because of its adaptor function or because it ubiquitinates other endocytic components of the clathrin machinery. To better understand

the role of c-Cbl in EGFR endocytosis, further studies should focus on additional proteins that can mediate EGFR internalization through c-Cbl-Grb2 complex.

Material ans 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%
Deoxyxholic acid	0.5%
EGTA	5 mM

500X 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 7.4	50 mM
NaCl	150 mM
Glycerol	10%
Triton X-100	1%
EDTA	1 mM
EGTA	1 mM

500X 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, pH6.8	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. ^{125}I-EGF was from Perkin Elmer. Doxycycline hydrocloride was from SIGMA.

3.1 Antibodies

For western-blot, anti-EGFR [epitope: aa 1172-1186 (*Homo sapiens*)], anti-Eps15 [epitope: aa 2-330 (*Mus musculus*)], anti-Eps15 human and anti-GST were produced in-house through the Antibody Facility. Anti-pY(1068)EGFR, anti-pY(1045)EGFR were from Cell Signaling. Anti-CHC, anti-c-Cbl, anti-Grb2, and anti-Shc were from Transduction BD. Anti-HA was from BABCO. Anti-FK2 was from MIOBOL. Anti-pY was from Upstate. Anti-Cbl-b, anti-Eps15, anti-Tubulin, anti-Actin and anti-Ub (P4D1) were from Santa Cruz.

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:

- c-Cbl human (Invitrogen): CCGAUUUGAGAUAGAGGCCUUUAAA

- c-Cbl human (Invitrogen) : AUGAGAAGCUGCCUGGUCUAUUACU

- Cbl-b human (Invitrogen): GAGAGAAGUGUCUCCUCCUCGUGUA

- Cbl-b UTR2: UAAACAAGGUAAAGCAUUUCACAGG

- c-Cbl mouse (Invitrogen): GAGUAUUUCAGGGUGUUCAUGGAAA

- Cbl-b mouse (Invitrogen): GGAGCAGUAUGAACUGUAUUGUGAA

- Grb2 (Dharmacon): CAUGUUUCCCCGCAAUUAU

- Dynamin [Dharmacon (13)]: GACATGATCCTGCAGTTCA;

GAGCGAATCGTCACCACTT

- Clathrin Heavy Chain (Stealth Invitrogen): GAAGAACUCUUUGCCCGGAAAUUUA;

3.3 TaqMAN assays for Q-PCR (Applied Biosystems)

- C-CBL: hs00231981_m1

- CBL-B: hs00969143_m1

- CBL-C: hs00180288_m1

- c-Cbl: mm01343092_m1

- Cbl-b: mm00483069_m1

- Eps15: mm00514740_m1

4 Cloning techniques

4.1 Agarose gel electrophoresis

DNA samples were loaded on 0.8%-1% 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 100 V until desired separation was achieved. DNA bands were visualized under a UV lamp.

4.2 Minipreps

Individual colonies were used to inoculate 3 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 30 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, 900 µl of LB medium was added and the cells were left at 37°C for further 60 minutes before plating them onto agar plates with the appropriate antibiotic. Plates were incubated overnight at 37°C.

5 Constructs and plasmids

N-terminal HA-tagged human c-Cbl WT [kindly provided by Y. Yarden - Weizmann Institute, Israel (137)] and mutants [c-Cbl C381A, c-Cbl I383A and c-Cbl W802* generated by site directed mutagenesis using the Quick Change Mutagenesis Kit (StrataGene), from c-Cbl WT construct] were subcloned into pBABE-puro, pGEX-6P (GE Healthcare) or pSLIK (Invitrogen) vectors, through restriction enzyme digestion (New England Biolabs) and ligation (New England Biolabs), starting from original pcDNA. Human Cbl-b was kindly provided to us by S. Lipkowitz [National Cancer Institute, Maryland, USA (237)] and cloned into pGEX-6P vector. All clones were sequence verified.

EGFR mutant (Y1045/1068/1086F) was generated by site-directed mutagenesis of the human EGFR cDNA and sub-cloned into the pBABE-puro retroviral vector and pSLIK lentiviral vector. All clones were sequence verified.

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.

Human epithelial cervical cancer HeLa-Oslo cells and murine fibroblastic NR6 cells 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.

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. Oligos for c-Cbl and Cbl-b mRNA silencing were designed with BLOCK-iT™ RNAi Designer from Invitrogen. Cells were subjected to a single (in suspension) transfection (in the case of dynamin) or double (in both suspension and adhesion) transfection, treated with 10 nM RNAi oligo (except for clathrin KD: 24 nM RNAi oligo) and analyzed 5 days after transfection (except for dynamin: 2 days after transfection).

- *DNA transfections*

For biochemical purposes (i.e., immunoprecipitation assay), DNA transfections were performed using Lipofectamine reagent from Invitrogen Life Technologies, 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 immunoprecipitation assay.

6.3 Retroviral and lentiviral infection

Stable populations of HeLa cells expressing c-Cbl WT or mutant constructs (c-Cbl C381A and c-Cbl I383A) were generated by infecting HeLa cells 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 "Cell culture media"). 48 hours after infection, selection of infected cells was performed by adding puromycin at a concentration of 1.5 μg/ml. NR6 cells, devoid of endogenous EGFR, were infected with retroviral vectors expressing WT and mutant human EGFR (1) using the same method as described above. NR6 clones stably expressing EGFR WT or mutants were generated by selection with puromycin.

Stable clone of NR6 cells expressing human EGFR WT was infected with lentiviral vectors expressing c-Cbl WT or mutants (c-Cbl C381A and c-Cbl I383A). Lentiviruses were produced by transfecting the 293T helper cell line with 5-10 μg of DNA. 24 hours after transfection, supernatant was concentrated and after 24h supernatant was collected and passed through a 0.45 μm filter. After the addition of 8 μg/ml polybrene, the supernatant was added to NR6 cells plated on 10 cm cell culture dishes. Two cycles of infection were repeated, after which the medium was replaced with NR6 medium added of puromycin. 48 hours after infection, selection of infected cells was performed by adding gentamycin at a concentration of 400 ng /ml.

7 Protein procedures

7.1 Cell lysis

After washing with PBS 1X, cells were lysed in JS or RIPA buffer directly in the cell culture plates using a cell-scraper and clarified by centrifugation at 16,000 *xg* for 20 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**
Acrylamide mix (ml)	2	2.7	3.3
1.5 M Tris HCl, pH 8.8 (ml)	2.5	2.5	2.5
ddH_2O (ml)	5.3	4.6	4
10% SDS (ml)	0.1	0.1	0.1
10% APS (ml)	0.1	0.1	0.1
TEMED (ml)	0.01	0.01	0.01
TOTAL (ml)	10	10	10

Stacking gel mix

Acrylamide mix (ml)	1.68
1 M Tris HCl, pH 6.8 (ml)	1.26

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 or BSA in TBS supplemented with 0.1% Tween (TBS-T). After blocking, filters were incubated with the primary antibody, diluted in TBS-T 5% milk or BSA, for 1 hour at room temperature, followed by three washes of ten 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 (10 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 (10 minutes each) and the bound secondary antibody was revealed using the ECL method (Amersham).

Denaturing solution

Guanidinium 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 (IP)] or in RIPA [for immunoprecipitation (co-IP)] 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 c-Cbl, Shc and Grb2 with EGFR, EGFR was immunoprecipitated using an anti-EGFR antibody produced in-house [epitope: aa 1172-1186 (*Homo sapiens*)]. IP and co-IP assays were performed using 300 μg and 1.5 mg, respectively, of fresh lysate that was subjected to IP (22,238).

8 Protein production and purification

8.1 GST-fusion protein production

Rosetta 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 and cloramphenicol at 34 ug/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, 1 mM IPTG was added and the culture was grown either at 37°C for theree hours or at 18°C overnight. Cells were then pelleted at 4000 x g 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 14000 x g for 30 minutes at 4°C. 600 μl – 1ml of Glutathione Sepharose 4B (GE Healthcare) beads (1:1 slurry), previously washed 3 times with PBS and once with GST-lysis buffer, was added to the supernatants and samples were incubated for either 4 hours or overnight at 4°C with rocking. Beads were washed once 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.

GST-lysis solution

HEPES, pH 7.5	50 mM
NaCl	200 mM
EDTA	1 mM
Glycerol	5%
NP-40	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

List of the GST-proteins used:

- c-Cbl: full length (*Homo sapiens*);
- c-Cbl C381A: full length with mutation C381A (*Homo sapiens*);
- c-Cbl I383A: full length with mutation I383A (*Homo sapiens*);
- c-Cbl W802*: full length with mutation W802* (*Homo sapiens*);
- Cbl-b: full length (*Homo sapiens*);
- Grb2: full length (*Homo sapiens*);
- EGFR: aa 696-end (*Homo sapiens*) from Millipore, cat #14-531

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 incubated either overnight at 4°C or for 4 hours at room temperature. After cleavage the supernatant containing the cleaved protein was collected.

8.3 EGFR *in vitro* ubiquitination assay

A baculovirus-produced GST-EGFR cytoplasmic tail (aa 696-end, Millipore, 250 ng) was

subjected to *in vitro* auto-phosphorylation for 1 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) beads, washed three times 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 ubiquitination buffer (250 mM Tris-HCl pH 7.6, 50 mM $MgCl_2$, 1 M NaCl, 1 mM DTT, 2 mM ATP, plus ATP regeneration system, SIGMA), with the following amount of purified enzymes: 100 ng of E1 (purified from baculovirus), 500 ng of E2 (His-tagged UbcH5c/Ube2D3, purified from bacteria), 500 ng of E3 (either c-Cbl WT, mutants or Cbl-b, purified from bacteria), 1 μg of Ub (SIGMA), with or without Grb2 (purified from bacteria). Beads were then washed four times in YY buffer 0.1% SDS and eluted in Laemmli buffer. Enzymes were purified as described (239).

9 Assays with ^{125}I-EGF

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

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

Low Dose EGF internalization	^{125}I-EGF: 1 ng/ml
High Dose EGF internalization	^{125}I-EGF: 30 ng/ml

After 2, 4, 6 minutes (for HeLa cells) or 4,8, 12 minutes (for NR6 cells) of EGF 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 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. 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 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 Internalized/bound). Internalization rate constants were extrapolated from the internalization curves 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 in binding buffer (MEM or DMEM, BSA 0.1%, Hepes pH 7.4 20 mM) for at least 4 hours and then incubated in the presence of 5 ng/ml of ^{125}I-EGF. 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.

10 Immunofluorescence studies

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 Babco or anti-EGFR in-house-made, or c-Cbl BD), 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 ImageJ software. To detect only surface EGFR (anti-EGFR Ab-1, Calbiochem) permeabilization step was avoided.

11 FACS (Fluorescence-activated cell sorting) analysis

Cells were washed in PBS and resuspended in PBS 0.5 ml and then added formaldehyde 2% 0.5 ml for 20 minutes on ice. They were subsequerntly washed in PBS in presence of 1% BSA and to prevent non-specific binding of the antibodies, resuspended in PBS BSA 5% and incubated for 30 minutes at room temperature. Next, cells were incubated for 1 hour with primary antibody in 1X PBS in presence of 1% BSA (anti-EGFR antibody that recognizes the extracellular domain of the receptor), washed in PBS in presence of 1% BSA and incubated for 1 hour with fluorescently labeled secondary antibodies (Amersham). After washing with PBS, cells were ready to be analysed with FACScanto II (Becton Dickinson).

12 Densitometry and statistical analysis

Quantification of blot was performed with Photoshop. Error bars in the plots represent the standard deviation of the mean. All statistical analyses were performed using Excel.

Aim of the project

The role of ubiquitination in EGFR endocytosis and trafficking has been extensively studied [reviewed in (150)]. The family of E3 ubiquitin ligases involved in EGFR ubiquitination is the family of Cbl proteins (137,161,162,174), which are recruited to the active/phosphorylated receptor leading to its ubiquitination and endocytosis (97,174,222,234,240).

Although ubiquitination mediated by c-Cbl is known to be critical for targeting EGFR to lysosomal degradation, the role played by c-Cbl at the early EGFR internalization steps still needs to be elucidated. This is a very complex question to answer since several factors hinder the study of the role of Cbl proteins in EGFR internalization. First, EGFR can be internalized through different pathways, such as CME and NCE, with different ubiquitin requirements. Indeed, EGFR ubiquitination is not essential for CME (1,2), while it is absolutely required for NCE (1,226). Second, c-Cbl can ubiquitinate other endocytic substrates besides EGFR and this might represent an important regulatory mechanism along the endocytic pathway (3). Third, in addition to its role as E3 ligase, c-Cbl also functions as an adaptor molecule by recruiting several proteins involved in the early phases of CME (3). Having these factors in mind, we developed a multi-approach experimental plan that allowed us to overcome some of them and to further investigate the role of Cbl proteins in EGFR ubiquitination and internalization.

The first aim of this study was, thus, to understand the connection between Cbl proteins and EGFR ubiquitination. To this aim, we analyzed the differential/redundant effect of Cbl proteins in EGFR ubiquitination by performing RNAi-based functional assays in different cellular model systems. In particular, we have: i) ablated c-Cbl and/or Cbl-b (the two Cbl proteins expressed in our cell models) and analyzed their differential or redundant effect on EGFR ubiquitination; ii) performed KD of c-Cbl/Cbl-b with known endocytic players and performed EGFR internalization assays, in order to clarify the role of

Cbl proteins in EGFR CME versus NCE (to gain further insight into the process the requirement of Grb2 in EGFR ubiquitination and endocytosis was also assessed); iii) analyzed the impact of c-Cbl/Cbl-b KD on the ubiquitination of endocytic adaptors.

Our second aim was to study the molecular mechanisms underlying the role of c-Cbl in EGFR internalization. To this end, we undertook a genetic approach and performed reconstitution experiments with c-Cbl WT or mutants defective in E3 ligase activity to clarify whether the role of c-Cbl in EGFR internalization is mediated via its E3 ligase or adaptor function.

Our final aim was to dissect the molecular requirements for the EGFR ubiquitination reaction. To achieve this goal, we set-up an *in vitro* EGFR ubiquitination assay with all purified components, which allowed us to confirm the essential role of Grb2 in c-Cbl-mediated EGFR ubiquitination. In future studies, we expect to employ this *in vitro* assay to better characterize c-Cbl E3 ligase activity in terms of processivity and chain specificity, and to unveil possible differences with Cbl-b.

From this study, we expect results to provide a deeper understanding of possible differential role of c-Cbl and Cbl-b in the endocytic pathway of the EGFR.

Results

1 Experimental set up

We designed an experimental plan that allowed us to investigate the role of Cbl proteins in EGFR endocytosis. We used two cellular model systems: HeLa cells express substantial levels - although within the physiological range - of endogenous EGFR (about 200 000 receptors/cell) and have been extensively characterized by our group and others for EGFR ubiquitination and endocytosis (92,97); in addition we chose another model that does not express endogenous EGFR and was therefore enginereed to express the WT form or mutant of the receptor [NR6 cells, fibroblast derivative of NIH devoid of endogenous EGFR (241)]. The use of these two different cell lines allowed us to highlight a different importance of Cbl proteins according to the cellular context analysed. Importantly, it was crucial to our study to be able to characterize the involvement of Cbl proteins both in EGFR CME and NCE. Our experimental set up was thus based on ^{125}I-EGF internalization assays that were performed in highly controlled conditions that allowed us to study these two mechanisms independently, as described below.

1) Since in our cellular models (HeLa and NR6 cells) EGFR can be internalized either by CME or by NCE (1,226) depending on ligand concentration, we performed experiments using two doses of EGF:

- Non-saturating concentration of EGF (1 ng/ml)

  At a non-saturating dose, only CME - and not NCE - is active. Thus, by using 1 ng/ml of EGF we ensure that any observed effects can be directly and specifically ascribed to the clathrin-mediated internalization pathway. Indeed, at this ligand concentration, silencing of CHC causes, approximately, an 80% inhibition of EGFR internalization (13,92) and see also Figure 19B, Figure 20B left panels). The residual (remaining 20%) EGFR internalization is mediated by an alternative endocytic pathway that is insensitive to clathrin depletion. Indeed, we have

observed that this residual EGFR internalization is also unaffected by filipin treatment (a cholesterol-interfering compound) or dynamin KD. Thus, this residual internalization is not NCE, but rather a third kind of internalization pathway used by EGFR, which we have shown to be mostly kinase independent (226).

- High dose of EGF (30 ng/ml)

  In this experimental condition both CME and NCE pathways are active. In order to activate only NCE, we performed CHC KD, alone or in combination with the gene of interest. For high EGF doses, the kinetic parameter might not measure the actual endocytic rate constant, since at this dose bound counts might not remain constant over time. For this reason, instead of Ke (constant rate), we use the more appropriate term "observed Ke" [Ke obs, see also (242)], when describing the kinetics parameter at high EGF doses.

Internalization assays were performed at 2, 4 and 6 minutes for HeLa cells and 4, 8 and 12 minutes for NR6 cells. From the kinetics, we calculated the Ke.

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 artifacts that could mask or enhance internalization defects.

3) Both HeLa cells and NR6 cells stably expressing EGFR WT or mutants were used as cell model system. NR6 clones expressing EGFR WT or mutants were generated. EGFR levels were assessed through western blot (WB) analysis, immunofluorescence (IF), fluorescence-activated cell sorting (FACS) analysis and iodinated assays in order to select homogeneous EGFR-expressing clones with similar levels to HeLa cells.

4) All KD were performed transiently and at least 2 different oligos against each target were used with similar results. Reconstitution experiments were performed both in HeLa and NR6 cells. HeLa cells were infected with stable retroviral vectors expressing different forms of c-Cbl (WT or mutants) and, due to the heterogeneity of the bulk population,

homogeneous clones were selected, by analyzing c-Cbl expression through IF analysis (Figure 24). NR6 cells expressing either EGFR WT or TripleF/3F (Y1045/1068/1086F) were infected with the doxycycline-inducible vector pSLIK (243) to express homogenously c-Cbl WT or mutants, upon doxycycline treatment (Figure 26).

5) Cell surface EGFR was quantified in KD cells using the ^{125}I-EGF saturation-binding assay. Silencing of Cbl proteins did not alter the cell surface number of EGFR (data not shown). This result allowed us to exclude any involvement of Cbl proteins in EGFR biosynthesis/delivery to the PM, and in basal receptor turnover. Moreover, it also allowed us to exclude that differences in EGFR internalization rates are due to different starting numbers of surface EGFRs in KD cells with respect to control cells.

2 c-Cbl is the major E3 ligase involved in EGFR ubiquitination in HeLa cells

Previous studies have demonstrated that EGFR ubiquitination is essential for targeting the receptor to lysosomal degradation, while it plays a non-essential role at the early internalization steps (1,2,148). In contrast, c-Cbl seems to have a crucial role also at this initial stage (234). The situation is complicated by the existence of multiple entering pathways dependent on cell context and on the experimental conditions used.

In order to depict a clearer picture of the role of Cbl proteins in EGFR ubiquitination and endocytosis (CME vs NCE), we firstly analysed the phenotype of Cbl proteins silencing in HeLa cells. Since the Cbl family consists of three members, c-Cbl, Cbl-b, and Cbl-c (see Introduction), we first determined their expression at the mRNA (messenger RNA) and/or protein level in our cellular model system, HeLa cells (Figure 16A). As depicted in the figure, *CBL-C* expression is undetectable, thus it will be not considered in our further experiments; on the contrary, *C-CBL* is 8-fold greater compared to *CBL-B* mRNA.

We initially investigated the involvement of c-Cbl and Cbl-b in EGFR ubiquitination by ablating the expression of these two proteins, alone or in combination in HeLa cells, using RNA interference technique (Figure 16B). Single and double c-Cbl/Cbl-b KD cells were stimulated with high dose of EGF for 2 minutes and, after EGFR immunoprecipitation, the EGFR ubiquitination levels were analysed. In HeLa cells (Figure 16C), we observed that the KD of c-Cbl dramatically decreased EGFR ubiquitination to less than 10% of residual signal, while Cbl-b KD cells showed only a minor -if any- reduction compared to control cells (Figure 16C). When combined to c-Cbl KD, Cbl-b KD only slightly reduced EGFR ubiquitination. These data suggest that c-Cbl is the major E3

ligase involved in EGFR ubiquitination at early time points in HeLa cells, although Cbl-b might play an additional role in this process.

Since ubiquitination has an established role in targeting EGFR for lysosomal degradation at late stages of endocytosis, we extended our experimental analysis of the effects of c-Cbl/Cbl-b KD on EGFR ubiquitination to later time points. To this aim, we chose to perform an ELISA (Enzyme-Linked ImmunoSorbent Assay)-based assay that allows quantitative measurements of EGFR ubiquitination for a longer period of time. After ablation of c-Cbl and Cbl-b, alone or in combination, HeLa cells were stimulated with high dose of EGF for different periods of time (from 0 to 180 minutes). We observed that, throughout the analysed time points, EGFR ubiquitination was almost completely impaired in the absence of c-Cbl (~90% reduction; Figure 16D). A subsequent 10% reduction in EGFR ubiquitination was further achieved with the KD of Cbl-b. These results suggest that c-Cbl is the major E3 ligase involved in EGFR ubiquitination even at late steps of endocytosis in HeLa cells, and that Cbl-b contributes to this process only to a minor extent.

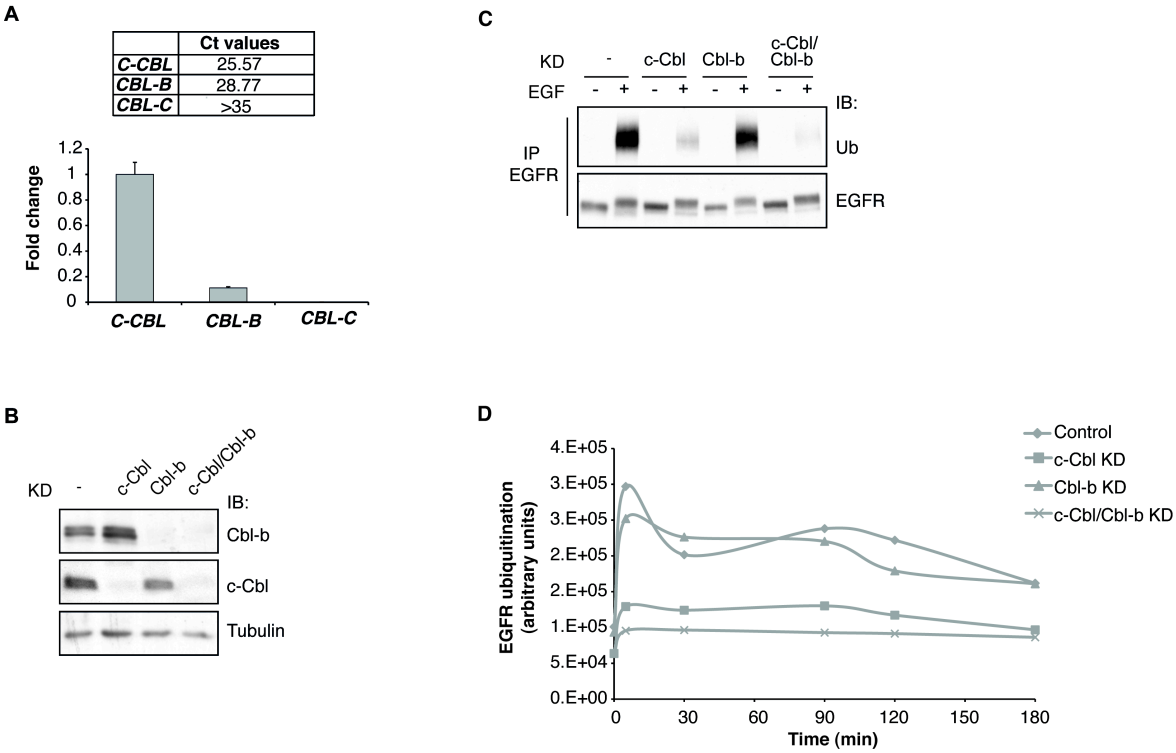

Figure 16 Role of Cbl proteins in EGFR ubiquitination. (A) Expression of *C-CBL*, *CBL-B* and *CBL-C* genes were assessed by qPCR analysis. Ct values and fold change are reported. The mRNA levels of the different genes were normalized to the housekeeping gene 18S and *C-CBL* was used as reference gene. **(B)** HeLa cells were silenced for c-Cbl and Cbl-b. Protein expression was determined by immunoblotting using specific antibodies, as indicated. Tubulin was used as a protein loading control. **(C)** *In vivo* EGFR ubiquitination. HeLa cells, subjected to KD as indicated, were serum starved o.n. (-) and then stimulated (+) with high dose of EGF (100 ng/ml) for 2 minutes. EGFR was immunoprecipitated with anti-EGFR antibodies and ubiquitination was detected through a specific antibody against ubiquitin (P4D1, Santa Cruz). Levels of immunoprecipitated EGFR were determined with anti-EGFR antibodies. Results are representative of more than three experiments. **(D)** Quantitative EGFR ubiquitination analysis at late time points. HeLa cells were silenced for c-Cbl and Cbl-b (single or in combination) and serum starved o.n. At the indicated time points, cells were stimulated with high dose of EGF (100 ng/ml). Samples were subjected to ELISA assay using anti-Ub (FK2) as detecting antibody. Absolute level of EGFR ubiquitination is displayed as arbitrary units.

3 Interplay between c-Cbl and Grb2 in EGFR ubiquitination

The recruitment of c-Cbl to the EGFR follows a cooperative behaviour in which two binding sites are needed: c-Cbl can directly bind to the EGFR through the interaction involving its TKB domain and the phospho-tyrosine (pY)1045 in the cytoplasmic tail of the receptor or it can be recruited to the EGFR via the adaptor protein Grb2 that is composed of one SH2 and two SH3 domains; indeed, the proline rich region in the C-terminus of c-Cbl interacts with SH3 domain of Grb2, which in turn binds to the pY1068/1086 of the receptor through its SH2 domain (Figure 17A).

In order to initially clarify the importance of Grb2 in the system, we silenced Grb2 and analysed the effects in EGFR ubiquitination. As shown in Figure 17B, upon Grb2 KD, EGFR ubiquitination was significantly reduced in stimulated cells, suggesting that Grb2 is a crucial player in this process.

To fully understand the interplay of Grb2/c-Cbl in EGFR ubiquitination and to unveil possible differences in the E3 ligase activity of the two Cbl proteins, we reconstituted the EGFR ubiquitination reaction in an *in vitro* ubiquitination assay. To this aim, we purified EGFR protein from baculovirus and induced the auto-phosphorylation *in vitro* of the intracellular domain of purified EGFR to use it as substrate in the ubiquitination reaction. In this *in vitro* ubiquitination assay, c-Cbl was used as E3 ligase and UbcH5c as E2, in presence or absence of Grb2. As seen in Figure 17C**,** we managed to ubiquitinate EGFR *in vitro* and found that the level of ubiquitination was maximal when Grb2 was present, confirming a strong requirement of Grb2 for efficient catalysis. On the contrary, c-Cbl alone is not very efficient, despite the efficient phosphorylation of its direct binding site. In the same assay, Cbl-b showed a Grb2-dependent ubiquitination activity on EGFR similar to the one observed in c-Cbl, (Figure 17D). These data suggest that the two Cbl proteins ubiquitinate the EGFR via a similar mechanism at least in this *in vitro* assay. However, we cannot exclude that c-Cbl

and Cbl-b proteins have different processivity or specificity towards ubiquitin chains. Further studies are required to better clarify this point.

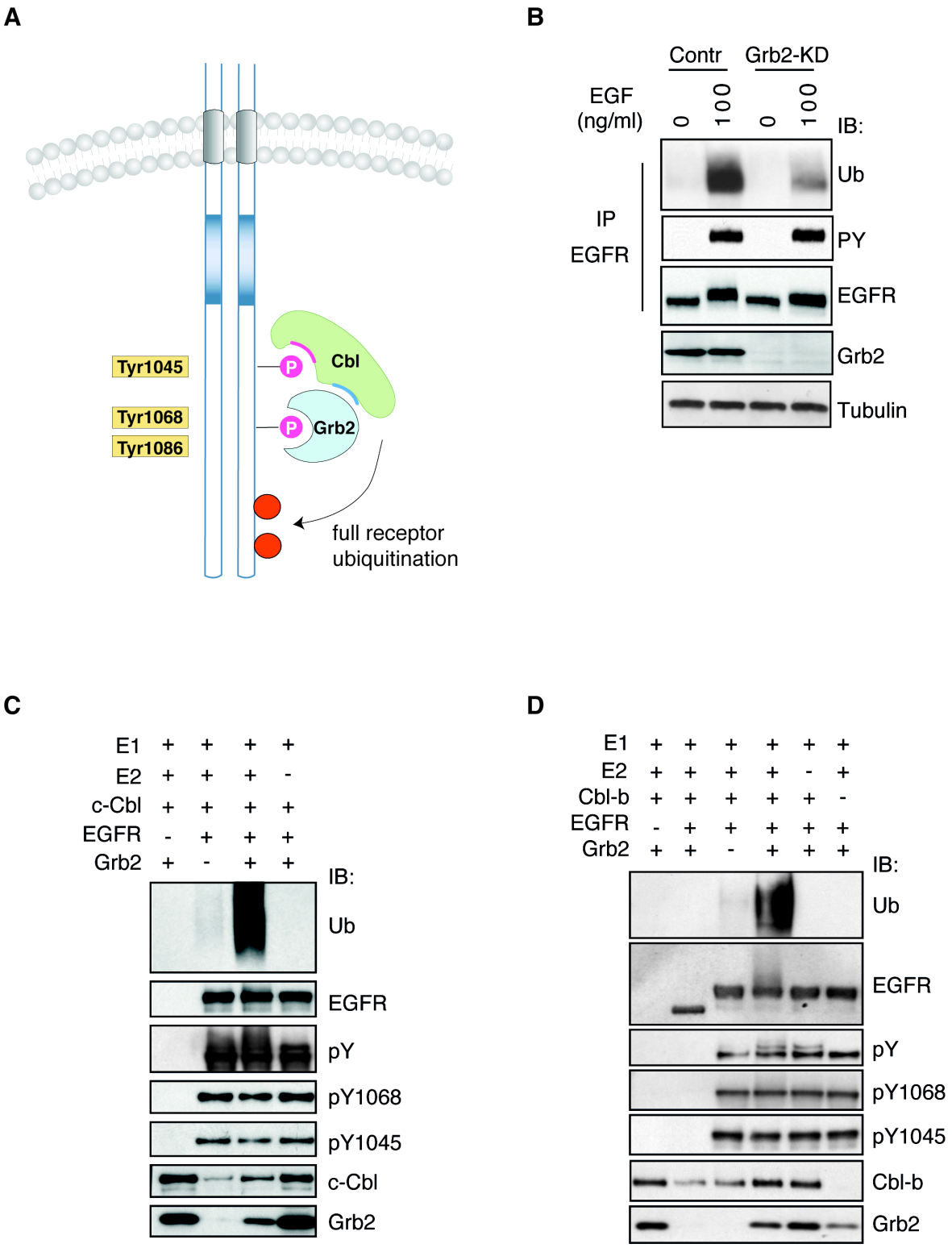

Figure 17 Recruitment of Grb2 for complete EGFR ubiquitination. (A) Modelling of c-Cbl recruitment to the EGFR (schematic representation of EGFR cytoplasmic tail). c-Cbl

is recruited to the activated EGFR directly through pY1045 and indirectly through Grb2 to pY1068/1086. The stable recruitment of c-Cbl via both pY-sites leads to full EGFR ubiquitination. **(B)** HeLa cells were subjected to Grb2 KD or transfection with a scramble oligo. Lysates were stimulated with EGF for two minutes as indicated and subjected to IP/IB as shown. **(C)** *In vitro* ubiquitination assay with c-Cbl as E3 ligase. GST-EGFR cytoplasmic tail was subjected to *in vitro* autophosphorylation reaction. Then, it was subjected to ubiquitination reaction in the presence of ubiquitin, bacterially purified E1, UbcH5c as E2, c-Cbl as E3 and ATP regeneration system, with or without Grb2 (ten times c-Cbl in molarity). Results from control reactions performed without EGFR or without E2 are shown. Immunoblotting was as indicated. Results are representative of more than three experiments. **(D)** *In vitro* ubiquitination assay with Cbl-b as E3 ligase. GST-EGFR cytoplasmic tail was subjected to *in vitro* autophosphorylation and ubiquitination reaction as described in **(C)** with exception of the E3 in the reaction mixture being Cbl-b (500 ng) instead of c-Cbl. Results from control reactions performed with EGFR not phosphorylated (lane 2) or without either E2, E3 or EGFR are shown. Immunoblotting was as indicated. Results are representative of three experiments.

4 Role of Cbl proteins and Grb2 in EGFR CME and NCE

In order to understand a possible specific role of c-Cbl in EGFR internalization, we performed c-Cbl KD in presence or in absence of clathrin and dynamin and we performed internalization assays at low and high doses of EGF. At low EGF doses, a condition where only CME is active, c-Cbl KD cells displayed a decrease of 30% of the EGF internalization rate (Figure 18B, left panel), a result comparable to what other groups have already reported (2). Furthermore, double KDs (c-Cbl/clathrin or dynamin KD) did not result in any worsening of the single clathrin or dynamin KD phenotypes (Figure 18B, left panel). This result showed that, although not being completely essential, c-Cbl plays an important role in CME. At high EGF concentration, where both CME and NCE are active, c-Cbl KD led to an important impairment (about 30-40%) in EGF internalization rate (Figure 18B, right panel). In addition, while in a dynamin KD background c-Cbl KD did not worsen the defect of the individual dynamin KD, double c-Cbl/clathrin KD caused a complete reduction in the EGF internalization rate to dynamin KD-induced levels (Figure 18B, right panel). These data show that c-Cbl is essential for EGFR internalization via NCE, which is compatible with the fact that the EGFR ubiquitination is required for NCE (1). From these data, we can conclude that NCE is completely dependent on c-Cbl, while EGFR CME displays only a partial dependency on this E3 ligase.

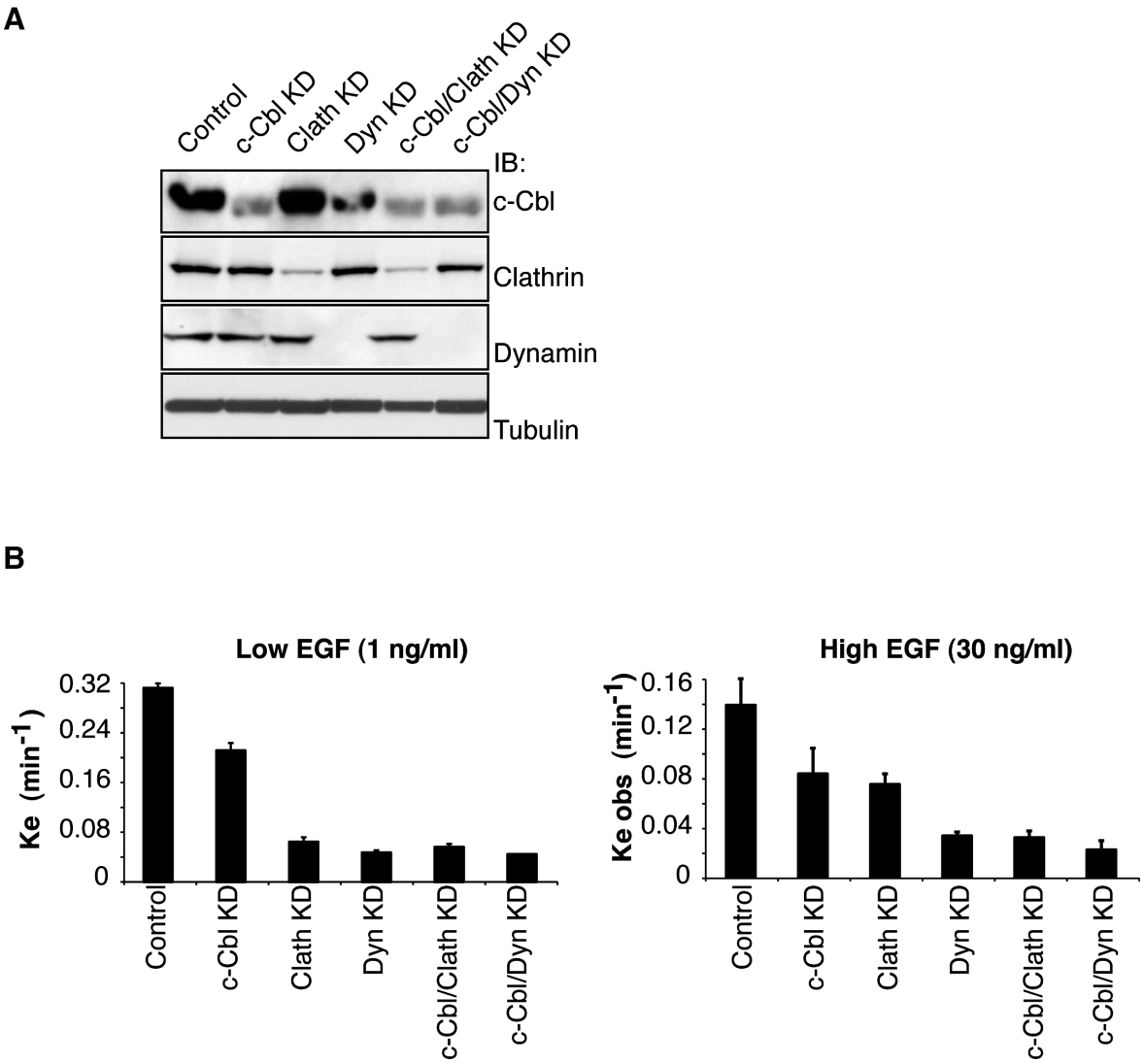

Figure 18 c-Cbl is involved both in CME and in NCE. (A) HeLa cells were silenced for c-Cbl, clathrin and/or dynamin. Protein expression was determined by immunoblotting using specific antibodies, as indicated. Tubulin was used as a protein loading control. Results are representative of more than three experiments. **(B)** ^{125}I-EGF internalization kinetics in control HeLa cells or upon different KD as indicated, at low EGF (1 ng/ml) (left panel) or high dose of EGF (30 ng/ml) (right panel). Results are expressed as internalization rate constants (Ke or Kobs) and are the mean of triplicate experiments.

Grb2 was previously found to be essential for EGFR CME through siRNA depletion of Grb2, which substantially reduces internalization of EGFR in different cell lines (148,244). To confirm the essential role of Grb2 in EGFR CME in our model system, we performed Grb2 KD, alone or in combination with clathrin and dynamin KD (Figure 19A) and performed EGFR internalization assay. We confirmed the essential role of Grb2 in

EGFR clathrin-mediated internalization since we observed a complete impairment of EGFR internalization upon Grb2 KD, comparable to the one observed in clathrin KD cells (Figure 19B, left panel). Importantly, we were also able to establish an essential role of Grb2 in EGFR NCE, since Grb2 KD, alone or in combination with clathrin KD (Figure 19B, right panel) reduced internalization kinetics to the levels observed in dynamin KD cells.

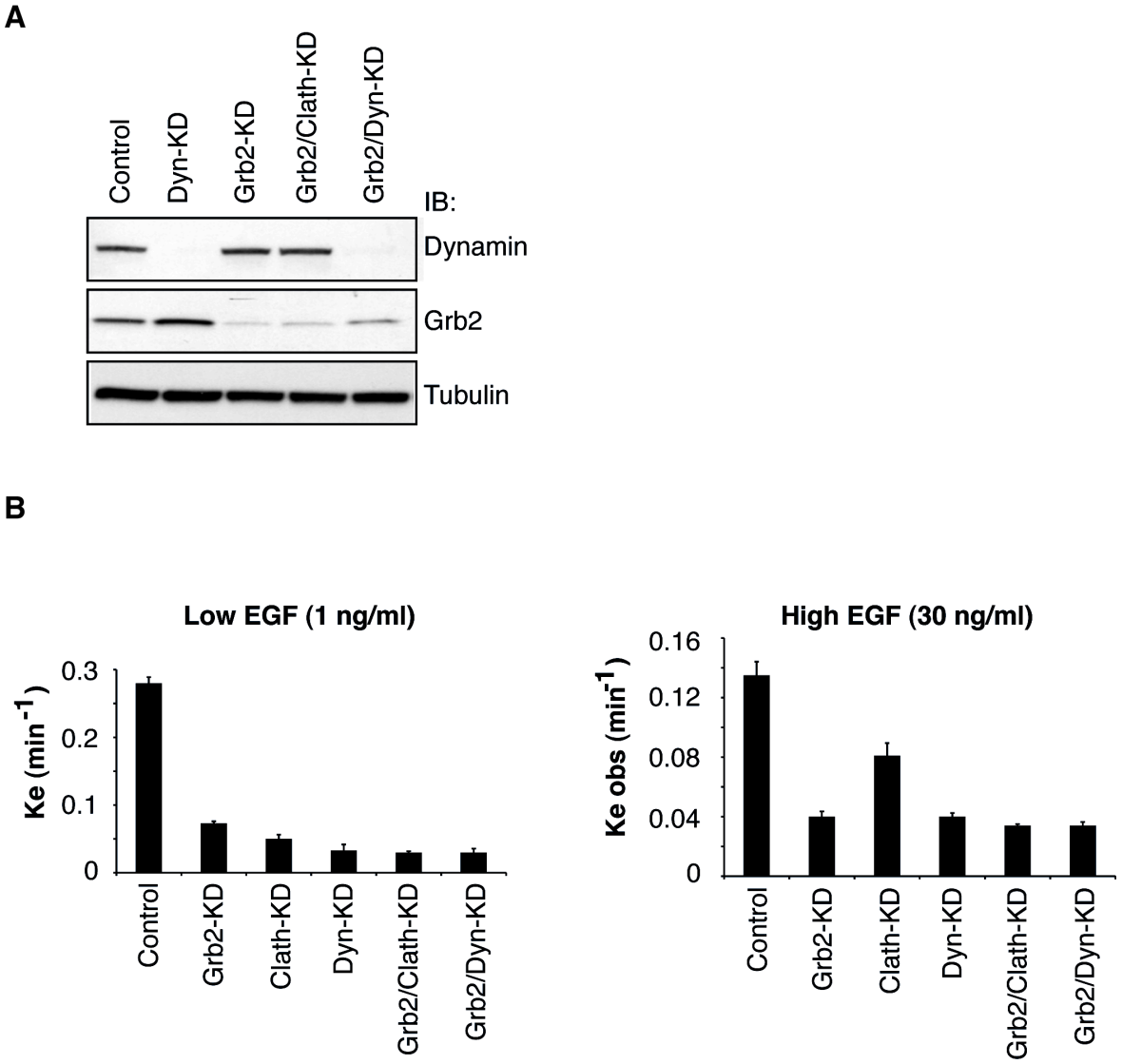

Figure 19 Role of Grb2 in EGFR internalization. (A) HeLa cells were silenced for Grb2, clathrin and/or dynamin. Protein expression was determined by immunoblotting using specific antibodies, as indicated. Tubulin was used as a protein loading control. Results are representative of more than three experiments. **(B)** ^{125}I-EGF internalization kinetics in control HeLa cells or upon different KD as indicated, at low EGF (1 ng/ml) (left

panel) or high dose of EGF (30 ng/ml) (right panel). Results are expressed as internalization rate constants (Ke or Kobs) and are the mean of triplicate experiments.

At this point we wondered whether the two members of Cbl family could act differently in EGFR CME and NCE. Thus, we transiently ablated the two members of Cbl family expressed in HeLa cells in presence or in absence of clathrin (Figure 20A). To this aim, we performed internalization assays upon c-Cbl and/or Cbl-b KD at low and high dose of EGF. As shown in Figure 20B**,** Cbl-b KD did not have any effect in EGFR internalization either at low or high EGF doses. In addition, the combined silencing of c-Cbl and Cbl-b did not further decrease the internalization defect observed in c-Cbl KD cells. These data suggest that, in HeLa cells, c-Cbl plays an important role in EGFR internalization via CME (not essential) and NCE (essential), while Cbl-b seems not involved at this step. Thus, in this cellular system, the two Cbl proteins seem to play a non-redundant role in EGFR internalization.

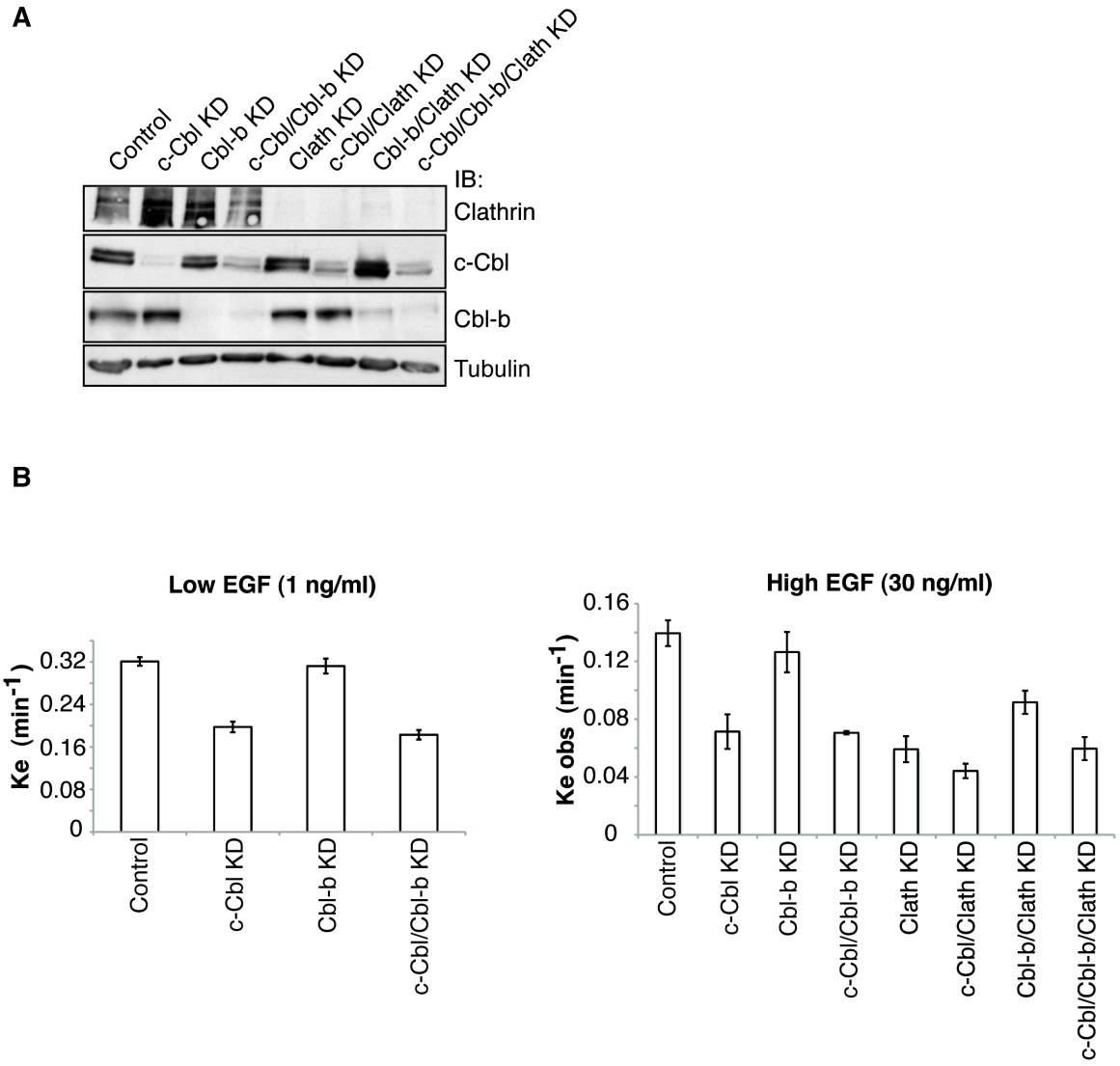

Figure 20 c-Cbl and Cbl-b have different impact on EGFR internalization in HeLa cells. (A) HeLa cells were silenced for c-Cbl, Cbl-b and/or clathrin. Protein expression was determined by immunoblotting using specific antibodies, as indicated. Tubulin was used as a protein loading control. Results are representative of more than three experiments. **(B)** ^{125}I-EGF internalization kinetics in control HeLa cells or upon different KD as indicated, at low EGF (1 ng/ml) (left panel) or high dose of EGF (30 ng/ml) (right panel). Results are expressed as internalization rate constants (Ke or Kobs) and are the mean of triplicate experiments.

5 The role of c-Cbl in CME is independent from EGFR ubiquitination

As we have already discussed, differently from NCE, EGFR ubiquitination seems to be non-essential for clathrin-mediated endocytosis (1,2,148). Thus, we were interested in investigating the role of c-Cbl in CME. To this aim, we took advantage of NR6 cells infected with retroviral vector expressing WT EGFR or a mutant form of EGFR (3F). This mutant carries the three binding sites of c-Cbl (both direct and indirect) mutagenized into phenylalanine (Y1045/1068/1086F) (Figure 21A). Of note, 3F mutant cannot enter the cell via NCE, while it can still be internalized through CME, displaying only minor defects in this clathrin-dependent internalization pathway (226). Using ^{125}I-EGF saturation binding assays and WB analysis (Figure 21B,C), we selected NR6 clones whose expression of EGFR surface levels was comparable to HeLa cells (about 200 000 receptors/cell). Such clones presented also homogenous surface EGFR expression as assessed by IF and FACS analysis (Figure 21D, E).

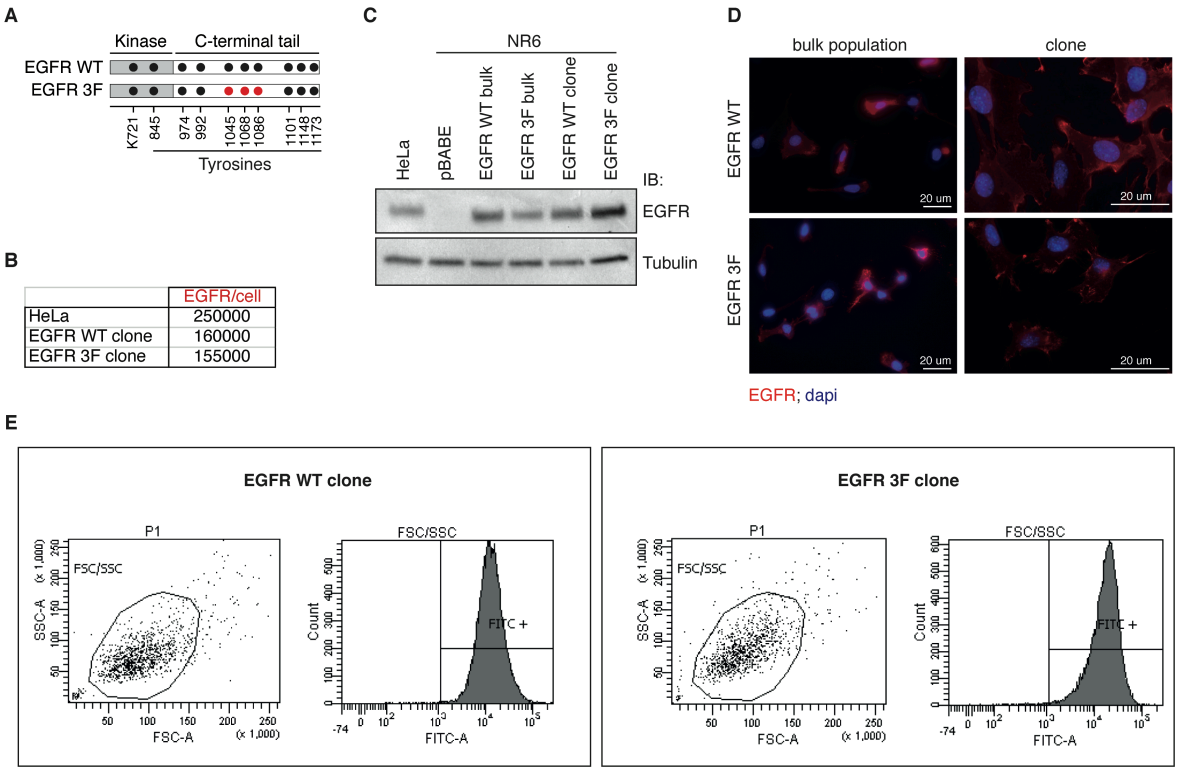

Figure 21 Characterization of NR6 cells expressing either EGFR WT or EGFR 3F. **(A)** Scheme of the Y-to-F mutants used in this study. The intracellular domain comprising the kinase domain and the C-terminal tail of the EGFR is shown with the positions of relevant residues. Mutagenized residues are indicated in red. **(B)** NR6 cells stably expressing EGFR WT or the indicated mutant were analysed by ^{125}I-EGF saturation binding and the number of surface receptors was measured. Data are expressed as surface EGFR/cell. **(C)** EGFR levels of the studied samples were determined by immunoblotting using specific antibodies, as indicated. Tubulin was used as a protein loading control. **(D)** The homogeneity of the selected clones was analyzed through IF; the EGFR was detected through an in-house antibody detecting the extracellular domain of the EGFR. Thus, no permeabilization was performed. The primary antibody was revealed through anti-mouse Cy3 secondary antibody. **(E)** FACS analysis of the selected clones was performed to further validate the homogeneity of the clones. The primary antibody used was the same as the one used for IF and therefore no permeabilization was performed.

We started by determining the expression of c-Cbl and Cbl-b in NR6 cells. As observed in Figure 22A, NR6 cells showed similar levels of *C-CBL* and *CBL-B*, displaying only a lower expression of *CBL-B* compared to *C-CBL* (about 30%). We then characterized the 3F mutant for its ubiquitination level compared to the WT form in the presence or absence of Cbl proteins. We analysed the expression of Cbl proteins upon KD by WB (Figure 22B). Upon EGF stimulation, EGFR WT showed a strong increase in EGFR ubiquitination, which slightly decreased upon c-Cbl and Cbl-b single KD (Figure 22C); only when the two proteins were simultaneously ablated, EGFR ubiquitination dramatically decreased (Figure 22C). Importantly, the mutant EGFR 3F showed a great decrease in EGFR ubiquitination by more than 95% compared to WT receptor and ablation of c-Cbl/Cbl-b in this sample induced only a slight reduction of the already low EGFR ubiquitination levels. Note that the receptor levels are immunoprecipitated less efficiently in the last sample (NR6 EGFR 3F, c-Cbl/Cbl-b KD). From these data we can conclude that EGFR 3F is ubiquitin-defective and could represent a good tool to study the impact of

EGFR ubiquitination in CME. Of note, this mutant has only a minor defect in CME [Figure 22D and (226)], confirming that EGFR ubiquitination is not essential for CME.

To understand whether the internalization of the receptor in the 3F mutant could still depend on Cbl proteins, we performed an internalization assay at low EGF concentration, upon c-Cbl and Cbl-b KD, both singularly and in combination. The combined silencing of c-Cbl and Cbl-b reduced by 50% EGFR internalization, while the single KD did not have any effect, thus suggesting a redundant role of these two proteins in EGFR CME (Figure 22D). The EGFR WT and 3F displayed a similar behaviour since internalization was dependent on c-Cbl/Cbl-b in both cases and to the same extent. This result suggests that c-Cbl/Cbl-b have a role in EGFR internalization independently on receptor ubiquitination.

Since in EGFR 3F the major c-Cbl binding sites (direct and indirect) are mutated, we did not expect c-Cbl to be recruited to the EGFR 3F. Indeed, results from a co-immunoprecipitation experiment showed that while c-Cbl was recruited to the EGFR WT upon EGF stimulus, it did not stably associate with the 3F mutant (Figure 22E). On the contrary, Grb2 binding was only partially affected (Figure 22E), possibly due to the presence of other indirect binding sites. Indeed, Grb2 can interact through its SH3 domain to Shc, which, in turn, binds with its SH2 to the pY1173 on the EGFR. This site might be responsible for the Grb2 recruitment. We cannot exclude that c-Cbl is recruited via Grb2 to this site in an unstable/low affinity interaction (not detected by coIP). Nevertheless, our data show that c-Cbl is required for EGFR internalization even if it is not stably recruited to the receptor. Altogether, these data suggest that the ubiquitination of the receptor itself is not essential for CME and that Cbl proteins are required, either because they ubiquitinate other substrates involved in EGFR internalization or because they can act as endocytic adaptors in CME.

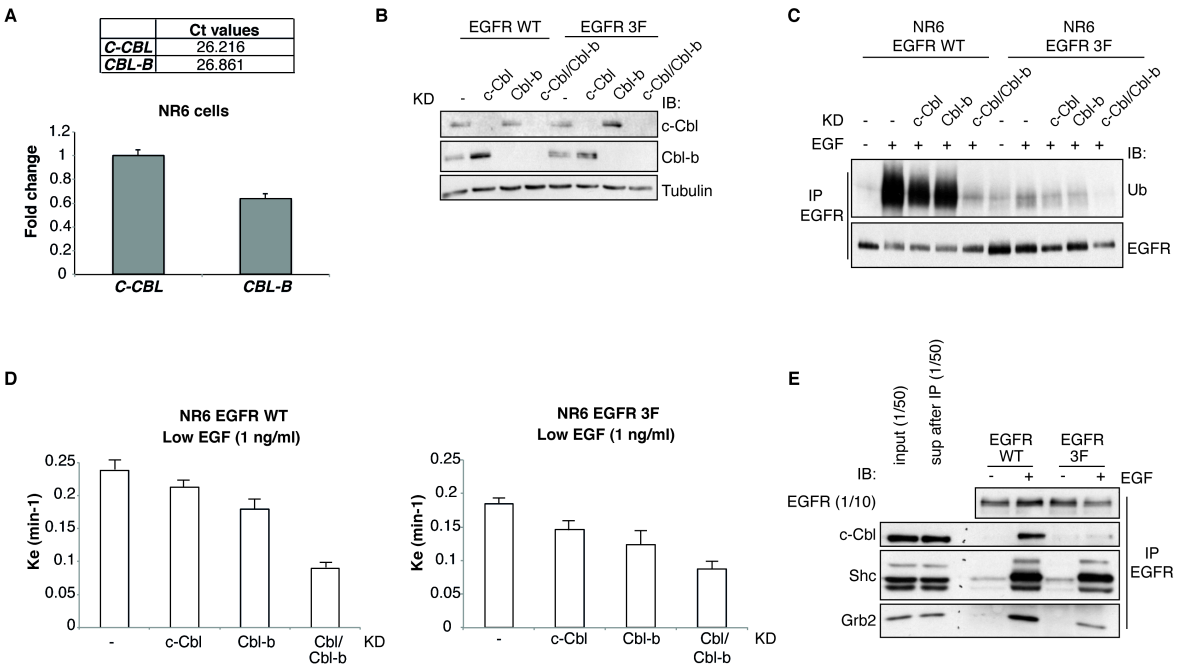

Figure 22 Role of Cbl proteins in EGFR ubiquitination and internalization in NR6 cells. (A) Levels of *C-CBL* and *CBL-B* were assessed by qPCR analysis. Ct values are reported, and the mRNA levels of the different genes were normalized on the housekeeping gene 18S. *C-CBL* was used as reference gene. **(B)** NR6 cells expressing either EGFR WT or EGFR 3F were silenced for c-Cbl and Cbl-b, singularly or in combination. Levels of c-Cbl and Cbl-b expression were determined by immunoblotting using specific antibodies, as indicated. Tubulin was used as a protein loading control. **(C)** *In vivo* EGFR ubiquitination. NR6 cells EGFR WT or 3F, subjected to KD as indicated, were serum starved o.n. (-) and then stimulated (+) with high dose of EGF (100 ng/ml) for 2 minutes. EGFR was immunoprecipitated with anti-EGFR antibodies and ubiquitination was detected through a specific antibody against ubiquitin (P4D1, Santa Cruz). Levels of precipitated EGFR were determined with anti-EGFR antibodies. Results are representative of three experiments. **(D)** ^{125}I-EGF internalization in EGFR WT and EGFR 3F NR6 cells, upon KD of Cbl proteins. EGF internalization was measured in the presence of ^{125}I-EGF 1 ng/ml at initial time points (4, 8 and 12 min). Internalization constants were extrapolated from the internalization curves and correspond to the slopes of the best-fitting curves. Error bars indicate standard deviation calculated on two independent experiments. **(E)** Coimmunoprecipitation of c-Cbl, Shc and Grb2 with EGFR. NR6 cells expressing EGFR WT or EGFR 3F were serum starved o.n. (-) and stimulated with 100 ng/ml of EGF for 2 minutes (+). EGFR was immunoprecipitated with anti-EGFR antibodies and c-Cbl, Shc and Grb2 levels were detected by using specific antibodies, as indicated.

6 RING finger mutations abrogate E3 ligase activity of c-Cbl

Due to its complex structure, c-Cbl has different interactors involved in multiple cellular processes. Indeed, it can work both as E3 ligase and adaptor protein (see introduction section 5.1). To uncover a possible role of c-Cbl as E3 ligase involved in EGFR internalization, we decided to investigate whether c-Cbl mutants in the RING finger domain lacking E3 ligase activity could reconstitute the EGFR internalization defect induced by the ablation of Cbl proteins, both in Hela and NR6 cells.

We generated two c-Cbl mutants: c-Cbl C381A, which carries a mutation previously shown to abolish the E3 ligase activity of c-Cbl and that possibly disrupts the RING finger domain (245); and c-Cbl I383A, carrying a mutation described by Brzovic and colleagues able to impair the E3 ligase activity of Brca1 without disrupting the structure of the RING finger (107) (Figure 23A). In Brca1, the mutation of this aminoacid (Ile26) neither disrupts the overall structure of the RING domain, nor prevents interactions with other protein partners, but the integrity of this residue is critical for E3 ligase activity and can represent a general determinant for E2 recognition and binding (107). Through the *in vitro* ubiquitination assay that we have set up and previously described (Figure 17B), we observed that both mutants were unable to ubiquitinate EGFR, both in absence or presence of the adaptor protein Grb2, even though their binding to the activated EGFR was not affected (Figure 23B).

We first analysed the impact of these two mutants on EGFR ubiquitination in an overexpression system. Indeed, we transiently transfected HeLa cells with vectors carrying either c-Cbl WT or the RING finger mutants or the negative control v-cbl (a truncated viral form of c-Cbl). The constructs were almost equally transfected as shown from IB of total cell lysate in Figure 23C. Cells were serum starved o.n. and stimulated with high dose of EGF for 2 minutes; we immunoprecipitated endogenous EGFR and analysed its ubiquitination by WB. As shown in Figure 23C, as expected, the overexpression of the WT form increased

EGFR ubiquitination, compared to endogenous, while the C381A mutant strongly reduced the endogenous EGFR ubiquitination. Thus, the C381A clearly shows an impaired E3 ligase activity and works also as dominant negative towards the endogenous c-Cbl. Overexpression of the I383A mutant seems to cause an increase (and not a reduction) of EGFR ubiquitination compared to the endogenous, although it does not reach the level of c-Cbl WT. This result was also confirmed using a different cell system that does not express EGFR (phoenix cells) where we cotransfected vector expressing EGFR and the above-cited vectors carrying different forms of c-Cbl (Figure 23D).

This result appears in contrast with the *in vitro* data, where both C381A and I383A mutants behaved equally and appear to be "catalitically dead" (see discussion, section 4).

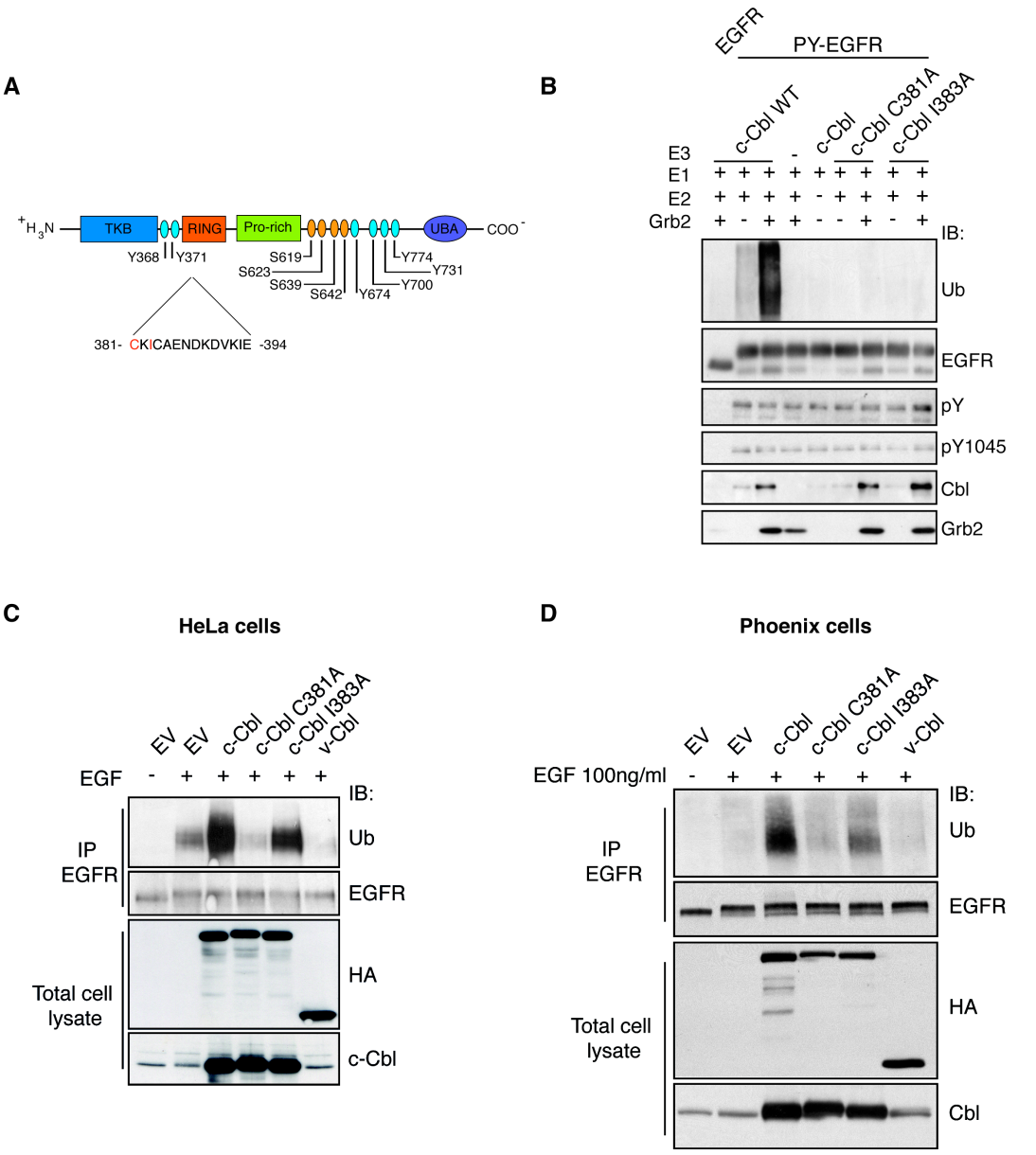

Figure 23 Characterization of c-Cbl E3 ligase mutants. (A) Schematic representation of c-Cbl structure with the domains that compose the protein. In red are indicated the residues within the RING domain that were mutagenized into alanine. **(B)** *In vitro* ubiquitination assay with either c-Cbl WT, C381A or I383A as E3 ligases. GST-EGFR cytoplasmic tail was subjected to *in vitro* autophosphorylation and ubiquitination reaction as described in **Figure 17**B with exception of the E3 in the reaction mixture. The control reactions with EGFR not phosphorylated or without either E2 or E3 or EGFR are shown. Immunoblotting was as indicated. Results are representative of two experiments. **(C)** *In vivo* EGFR ubiquitination. HeLa cells were transiently transfected with either control vector or different forms of c-Cbl (WT, C381A, I383A). Cells were serum starved 8 hours (-) and then stimulated (+) with high dose of EGF (100 ng/ml) for 2 minutes. EGFR was immunoprecipitated with anti-EGFR antibodies and ubiquitination was detected through a specific antibody against ubiquitin (P4D1, Santa Cruz). Levels of precipitated EGFR were

determined with anti-EGFR antibodies. Results are representative of three experiments. Levels of transfection were detected by using specific antibodies as indicated. **(D)** *In vivo* EGFR ubiquitination. Phoenix cells were transiently transfected with vector expressing EGFR and different forms of c-Cbl. Samples were treated as described in **(C)**.

To better understand what is going on in a more physiological situation (i.e. w/o overexpression), we performed reconstitution experiments with E3 ligase mutants upon KD of endogenous c-Cbl. To this aim, we generated stable clones of HeLa cells expressing c-Cbl WT, c-Cbl C381A and c-Cbl I383A at the endogenous level. These clones were selected and characterized, through WB analysis and immunofluorescence for expression level and homogeneity (Figure 24).

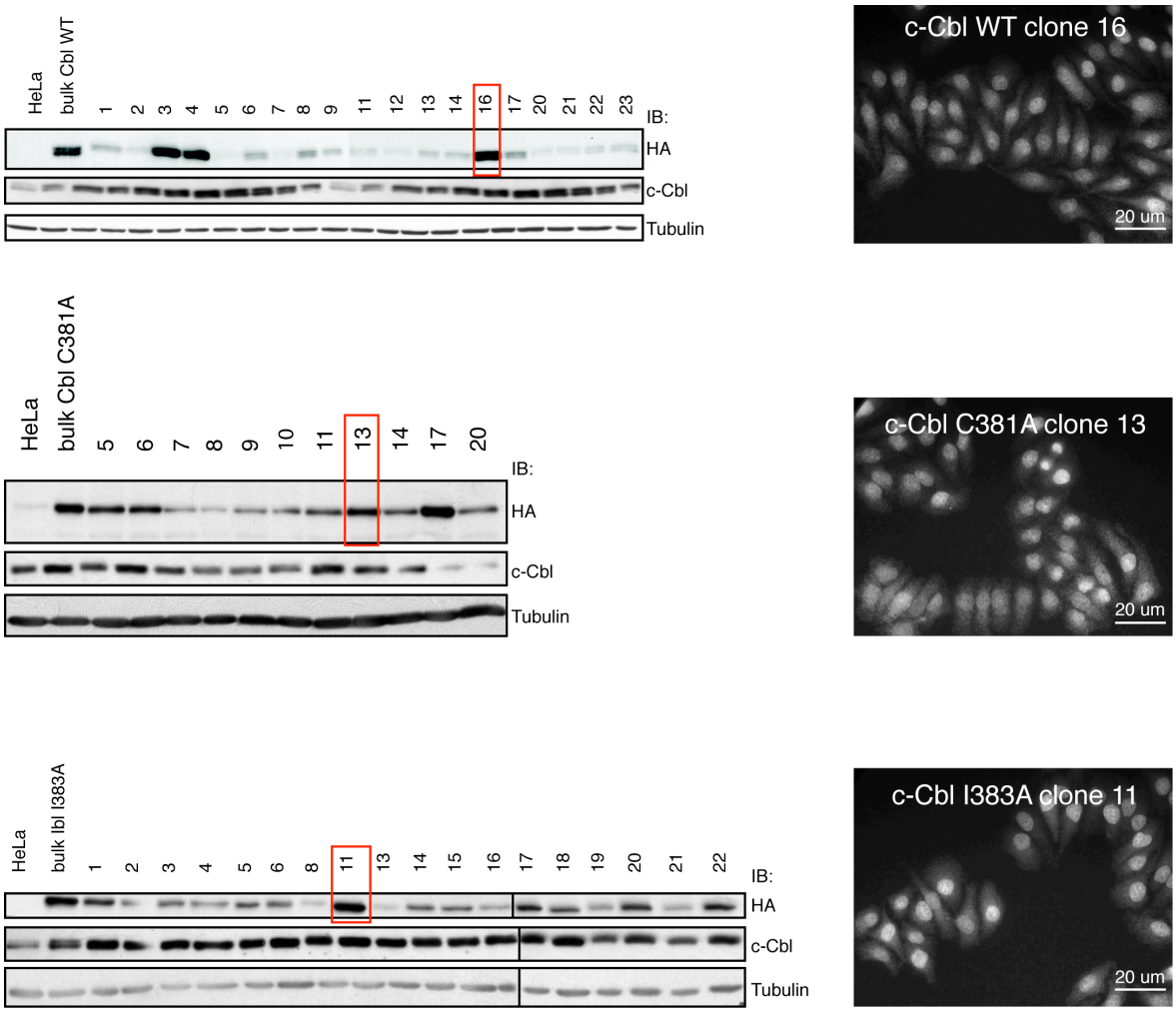

Figure 24 Characterization of HeLa clones expressing c-Cbl WT, C381A and I383A. **(A)** WB analysis of different clones by immunoblotting with specific antibody, as indicated. Tubulin was used as a protein loading control. **(B)** IF analysis of the selected clones (in red rectangle) to validate the homogeneity.

To perform c-Cbl ablation, we used RNAi oligos that target the 3'-UTR (untranslated region) of the mRNA without targeting the exogenous cDNA constructs (transgene). The efficiency of the KD was assessed by qPCR analysis using oligos which recognize the 3'-UTR (Figure 25B), whereas the expression level of each transgene was analysed through WB (Figure 25A). Unfortunately, we noted that the C381A mutant was expressed at lower extent compared to WT; however, the I383A mutant displayed an expression level similar to WT, which allowed us to continue our analysis (Figure 25A, anti-HA WB). To uncover the role of these mutations in EGFR ubiquitination *in vivo*, we analysed EGFR ubiquitination in the different clones by WB upon stimulation with high dose of EGF (100 ng/ml) for 2 min. We observed that EGFR ubiquitination was impaired in the absence of c-Cbl, but expression of the WT protein reconstituted this defect (Figure 25C). On the contrary, both mutants did not rescue the defect of EGFR ubiquitination upon c-Cbl KD. This result shows that both c-Cbl mutants are indeed E3 ligase impaired also *in vivo*, confirming the *in vitro* data.

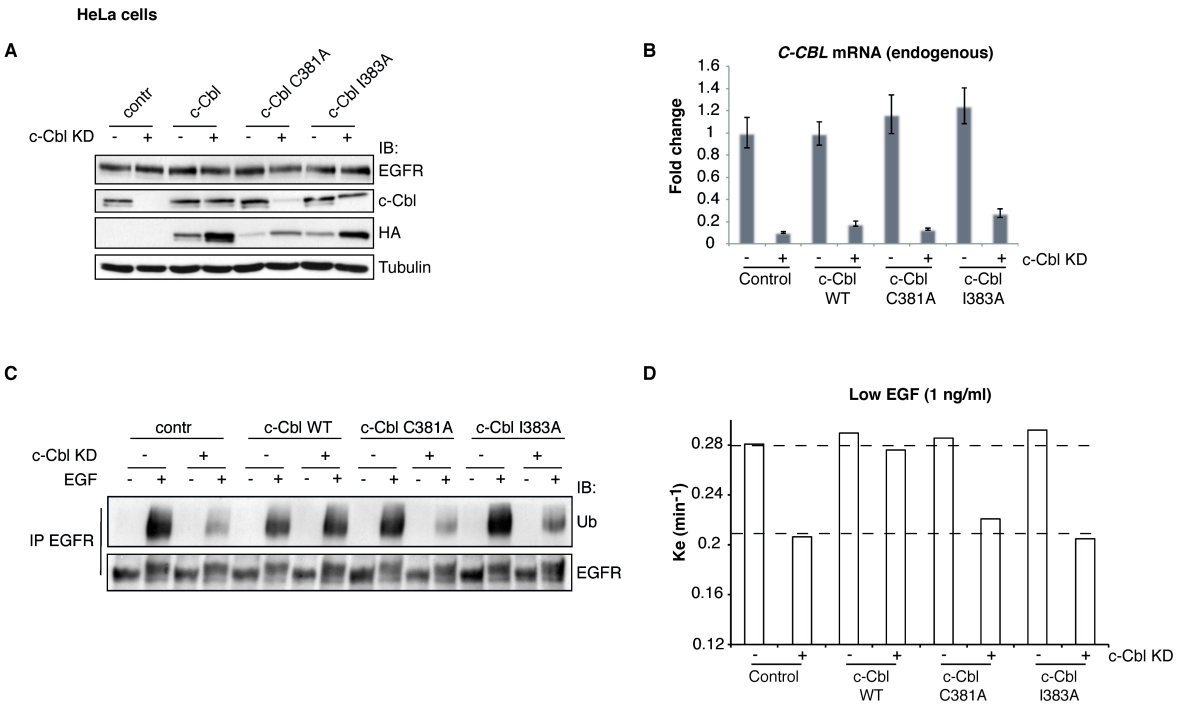

Figure 25 E3 ligase activity of c-Cbl is required for EGFR CME in HeLa cells. (A) HeLa cells stably expressing different c-Cbl constructs were silenced for c-Cbl (+) or transfected with control oligos (-). Levels of c-Cbl (both silenced and overexpressed) were

determined by immunoblotting using specific antibodies, as indicated. Note that with anti-Cbl antibody we are detecting both endogenous c-Cbl and transgene, while with anti-HA we are detecting only the transgene. Tubulin was used as a protein loading control. **(B)** qPCR analysis to detect levels of c-Cbl KD of the sample used in **(A)**. In order to follow only the endogenous *C-CBL* we employed oligos targeting the 3'-UTR. Data were normalized on the housekeeping gene 18S and control sample was used as reference. Fold changes were reported. **(C)** *In vivo* EGFR ubiquitination. HeLa clones used in this study were serum starved o.n. (-) and then stimulated (+) with high dose of EGF (100 ng/ml) for 2 minutes. EGFR was immunoprecipitated with anti-EGFR antibodies and ubiquitination was detected through a specific antibody against ubiquitin (P4D1, Santa Cruz). Levels of precipitated EGFR were determined with anti-EGFR antibodies. Results are representative of two experiments. **(D)** ^{125}I-EGF internalization upon KD of Cbl proteins. EGF internalization was measured in the presence of ^{125}I-EGF 1 ng/ml at initial time points (2, 4 and 6 min). Internalization constants were extrapolated from the internalization curves and correspond to the slopes of the best-fitting curves.

7 The E3 ligase activity of c-Cbl is required for EGFR CME

We then exploited this reconstitution system to investigate whether the E3 ligase activity of c-Cbl is required for EGFR CME by performing ^{125}I-EGF internalization assays in the clones previously described upon c-Cbl KD. As shown in Figure 25D, upon c-Cbl KD, control cells displayed an internalization defect (about 30%) that was rescued in the c-Cbl WT expressing clone. On the contrary, both mutants were not able to reconstitute EGFR internalization defect upon c-Cbl KD. These data show that the E3 ligase activity of c-Cbl is required for EGFR CME.

We further confirmed this hypothesis by taking advantage of NR6 cells expressing EGFR WT. In particular, we infected these cells with lentiviral inducible vectors expressing WT or mutant forms of c-Cbl (C381A and I383A). The homogenous expression of the inducible constructs was verified through WB, qPCR and IF analysis upon doxycycline treatment (Figure 26). All clones expressed c-Cbl WT and mutants at the same level.

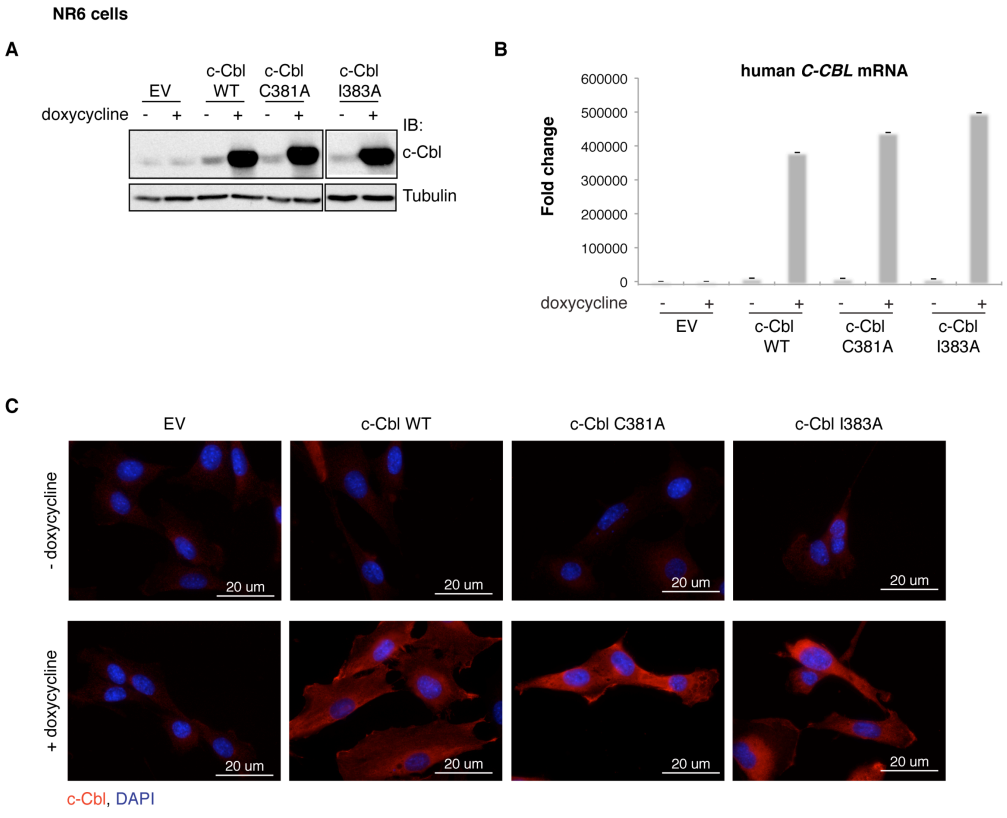

Figure 26 Characterization of NR6 EGFR WT expressing either c-Cbl WT, C381A or I383A. (A) NR6 EGFR WT were infected with lentiviral vectors carrying c-Cbl WT, C381A and I383A and treated (+) with doxycycline 100 ng/ml o.n. Levels of c-Cbl induction were determined by immunoblotting using specific antibody, as indicated. Tubulin was used as a protein loading control. **(B)** qPCR analysis to determine mRNA levels of human *C-CBL* induction upon doxycycline treatment. Data were normalized on the housekeeping gene 18S. Fold changes are reported. **(C)** The homogeneity of the population was analyzed through IF analysis; human c-Cbl was detected through an antibody detecting the C-terminus of the protein. The primary antibody was revealed through anti-mouse Cy3 secondary antibody.

We then performed ^{125}I-ligand internalization assays at low EGF concentration with NR6 cells expressing c-Cbl WT, C381A and I383A, upon c-Cbl/Cbl-b KD. The expression level of each transgene and the ablation of endogenous c-Cbl were analysed through WB and qPCR analysis (Figure 27A, B). Since WB analysis does not allow to discriminate between endogenous and exogenous c-Cbl [the two proteins migrate at the same molecular weight (MW)], we analysed the expression levels of endogenous and exogenous c-Cbl through qPCR, by taking advantage of the fact that the inducible gene is human while the endogenous is mouse. We were able to reach the same KD level in all samples (Figure 27B, middle and lower panel). The overexpression of the different c-Cbl WT and mutants was similar upon doxycycline treatment (Figure 27B, upper panel). We observed that after c-Cbl/Cbl-b KD a slight reduction of transgene expression occurred, which, however, was the same in all samples. We then performed internalization assays at low EGF doses, in control and c-Cbl/Cbl-b KD conditions, both in absence and in presence of doxycycline. The control (EV) was also treated with the doxycycline in order to exclude non-specific effects. As shown in Figure 27C, upon c-Cbl/Cbl-b KD, control cells displayed an internalization defect (about 60%) that was rescued upon induction of c-Cbl WT expression. On the contrary, both mutants were not able to rescue the EGFR internalization defect upon c-Cbl/Cbl-b KD. Of

note, the two mutants also showed a dominant negative effect on EGFR internalization when overexpressed in the presence of the endogenous proteins (+ doxycycline, in absence of c-Cbl/Cbl-b KD, Figure 27C).

Taken together, our data suggest that mutations in the RING finger domain abolish the E3 ligase activity of c-Cbl; this activity is required for the early steps of EGFR CME. Since ubiquitination of the EGFR itself seems not to play a major role in CME, a possibility is that the E3 ligase activity may be required to ubiquitinate endocytic adaptors and not directly the EGFR.

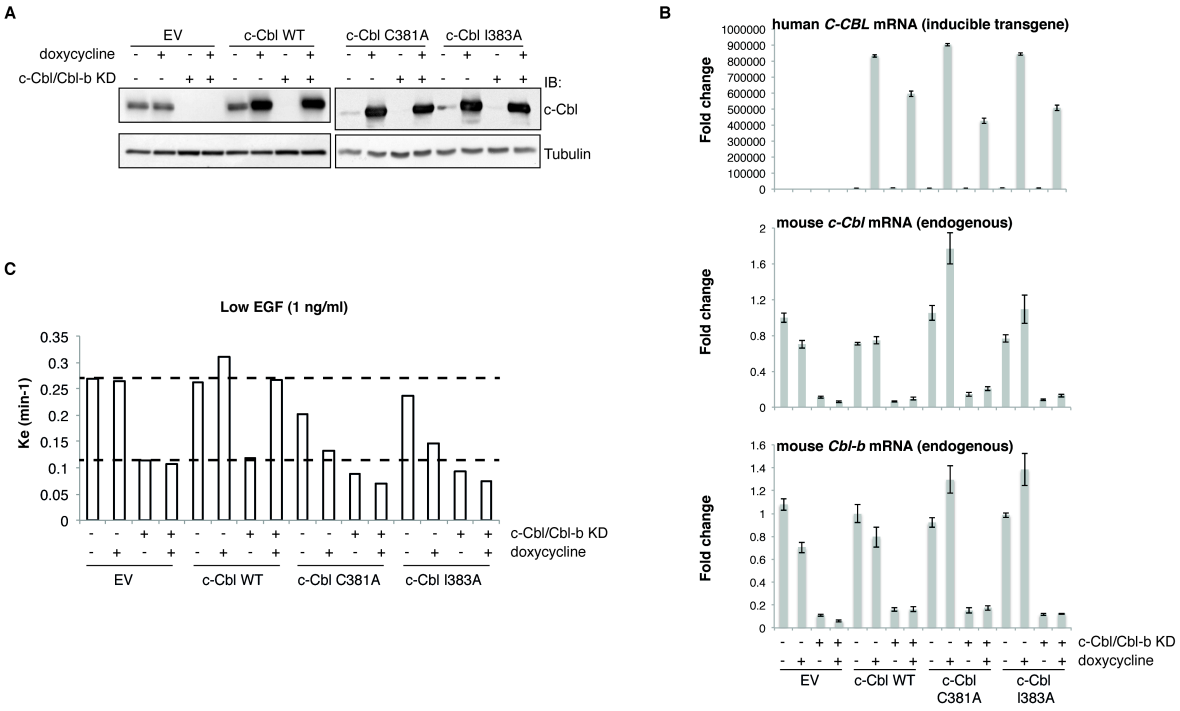

Figure 27 E3 ligase activity of c-Cbl is required for EGFR CME in NR6 EGFR WT cells. (A) NR6 cells infected with inducible constructs encoding for c-Cbl WT, C381A or I383A were silenced for c-Cbl and Cbl-b and treated with 100 ng/ml doxycycline o.n. Levels of c-Cbl were detected by immunoblotting using specific antibody, as indicated. Tubulin was used as a protein loading control. Note that we detected both endogenous and the c-Cbl transgene. **(B)** qPCR analysis to determine human *C-CBL* induction upon doxycycline treatment (upper panel), levels of endogenous *c-Cbl* (middle panel) and *Cbl-b* upon KD (lower panel). Data were normalized on the housekeeping gene 18S and fold change were reported. **(C)** Kinetics of ^{125}I-EGF internalization at low EGF concentrations

(1 ng/ml) upon different KD were analysed at initial time points (4, 8 and 12 min). Internalization constants were extrapolated from the internalization curves and correspond to the slopes of the best-fitting curves.

8 Cbl proteins are involved in ubiquitination of the adaptor protein Eps15

Several proteins are implicated in the initial steps of EGFR internalization and have been suggested to work as clathrin adaptors. Among these, Eps15, Eps15L1 and epsins might represent possible targets of c-Cbl E3 ligase since they are monoubiquitinated upon EGF at early time points (22). Importantly, we know that Eps15 is monoubiquitinated at similar level both at low EGF (only CME is active) and high EGF (CME and NCE are present), suggesting that Eps15 ubiquitination might play a role in EGFR CME (Figure 28A).

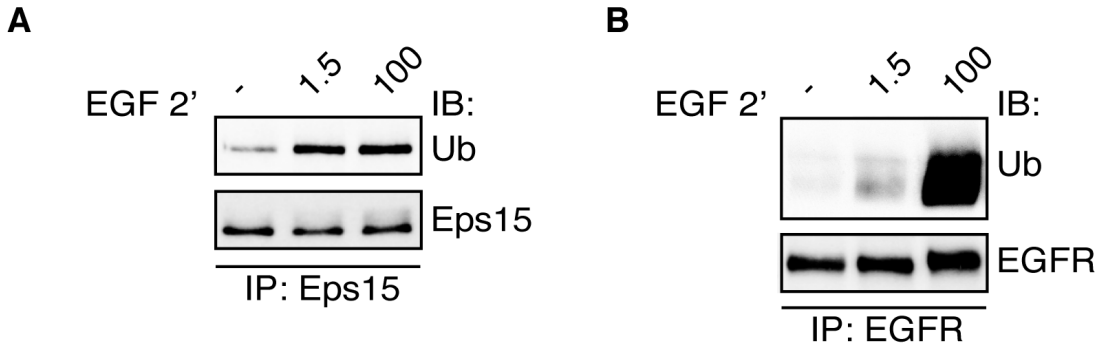

Figure 28 Eps15 ubiquitination follows similar behaviour at low and high doses of EGF. HeLa cells were serum starved o.n. and then stimulated with different concentrations of EGF (1.5 or 100 ng/ml) for 2 minutes. **(A)** Eps15 ubiquitination *in vivo*. Eps15 was immunoprecipitated with specific antibodies and ubiquitination was detected using a specific antibody against ubiquitin (P4D1, Santa Cruz). The levels of Eps15 immunoprecipitated were analysed by using specific antibodies. **(B)** EGFR ubiquitination *in vivo* as a control. EGFR was immunoprecipitated with anti-EGFR antibodies and ubiquitination was detected through a specific antibody against ubiquitin (P4D1, Santa Cruz). Levels of precipitated EGFR were determined with anti-EGFR antibodies.

To test this hypotesis, we analysed Eps15 ubiquitination in c-Cbl KD cells compared to control cells by WB upon EGF stimulation at high dose (100 ng/ml) (Figure 29). At 2

minutes, control cells showed a peak of Eps15 ubiquitination that later decreased; on the other hand, c-Cbl KD cells showed a reduction in Eps15 ubiquitination. These data indicate a possible direct or indirect role for c-Cbl in Eps15 ubiquitination. It will be important to test whether this is true also for other components of the endocytic clathrin machinery.

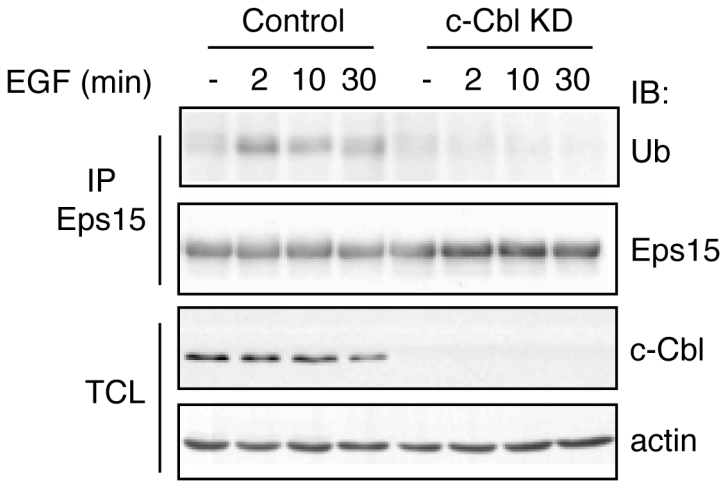

Figure 29 Eps15 as a possible substrate for c-Cbl in HeLa cells. Role of c-Cbl on Eps15 ubiquitination *in vivo*. HeLa cells were transiently KD for c-Cbl or with a scramble as a control. These samples were serum starved o.n. (-) and stimulated with 100 ng/ml EGF at indicated time points. Eps15 was immunoprecipitated with specific antibodies and ubiquitination was detected through a specific antibody against ubiquitin (P4D1, Santa Cruz). The levels of Eps15 immunoprecipitated were analysed by using specific antibodies. Protein expression was determined as indicated. Actin was used as protein loading control.

We further confirmed this result in NR6 EGFR WT cells expressing the inducible c-Cbl WT. To this aim, we assessed whether c-Cbl and Cbl-b might play a role in Eps15 ubiquitination and if c-Cbl WT could be able to rescue a possible ubiquitination defect. We silenced the two proteins simultaneously. c-Cbl expression (both in silencing and inducing conditions) was analysed through WB (Figure 30A) and qPCR analysis (Figure 30B). As

shown in the WB, upon doxycycline treatment, c-Cbl was greatly induced (lanes 2,4). The antibody used for Cbl-b IB recognizes both Cbl-b and c-Cbl (when overexpressed); indeed, as indicated in Figure 30A, the upper band corresponds to Cbl-b, while the lower one represents overexpressed c-Cbl. Importantly, upon c-Cbl/Cbl-b KD, the levels of Eps15, both protein and mRNA, increase (Figure 30A, B upper panel). In these cells, we investigated the Eps15 monoubiquitination by WB, thus we serum starved cells and then stimulated with high dose of EGF for 2 minutes. Lysates were subjected to Eps15 immunoprecipitation and then analysed by WB anti-Eps15; Eps15 ubiquitination levels were inferred from the shift in MW.

As shown in Figure 30C, upon EGF stimulation cells showed a shift in Eps15 MW, corresponding to its ubiquitination, which decreased when Cbl proteins were ablated. Upon exogenous c-Cbl overexpression, we were able to fully rescue the defect of Eps15 ubiquitination, suggesting that Eps15 might be a good candidate on which c-Cbl could exert its E3 ligase activity. c-Cbl might be involved in Eps15 ubiquitination either directly or indirectly (see discussion, section 5).

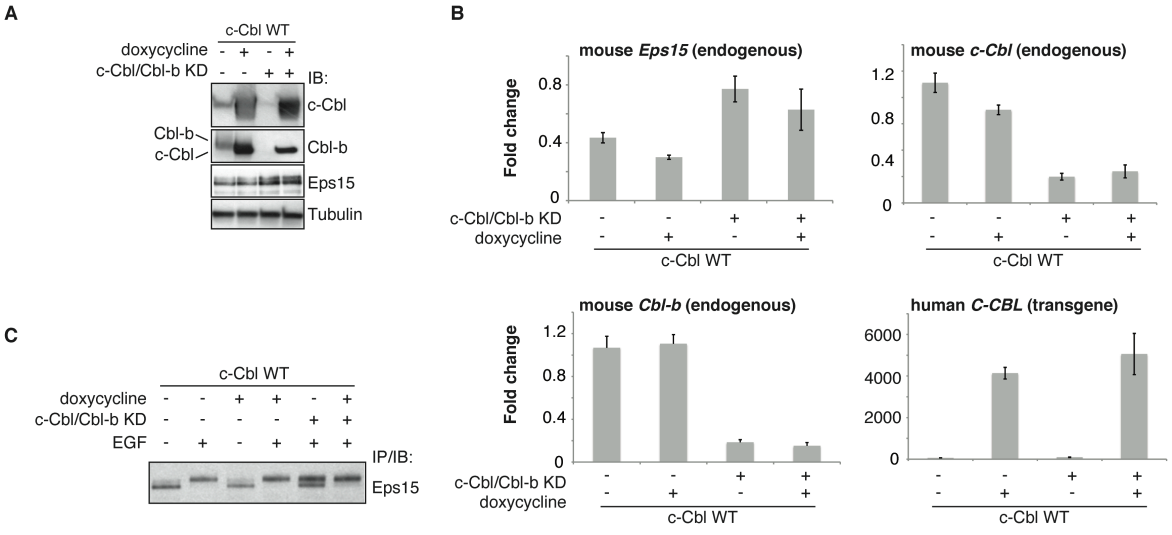

Figure 30 c-Cbl is involved in Eps15 ubiquitination in NR6 cells. NR6 cells infected with lentiviral construct carrying c-Cbl WT were silenced for c-Cbl and Cbl-b and treated with 100 ng/ml doxycycline o.n. **(A)** Protein expression (both silencing and induction) was detected by immunoblotting using specific antibodies, as indicated. Tubulin was used as a

protein loading control. **(B)** qPCR analysis to determine either endogenous *Eps15* levels (upper, left panel), endogenous *c-Cbl* (upper, right panel), endogenous *Cbl-b* (lower, left panel) and the transgene *C-CBL* (lower, right panel) upon c-Cbl/Cbl-b KD. Data were normalized on the housekeeping gene 18S and reported as fold change. **(C)** Eps15 ubiquitination *in vivo*. Cells were serum starved 8 hours (-) and stimulated with 100 ng/ml EGF for two minutes. Eps15 was immunoprecipitated and analysed with specific antibody.

To investigate whether c-Cbl is involved in Eps15 ubiquitination through a direct or indirect mechanism, we performed an *in vitro* ubiquitination assay, by using c-Cbl as E3 ligase and Eps15 as substrate. Since it is known that activation of c-Cbl requires phosphorylation, we used both unphosphorylated and phosphorylated (by purified EGFR kinase) forms of c-Cbl to perform this assay. As shown in Figure 31, only the active form of c-Cbl was able to ubiquitinate the adaptor protein Eps15. This result suggests that active c-Cbl may in principle directly act as E3 ligase on Eps15. However, since *in vitro* assays can be quite unspecific, further experiments are needed to gain a better understanding of c-Cbl E3 ligase activity. In particular, it would be interesting to investigate if Eps15 is a direct target of c-Cbl, whether c-Cbl can ubiquitinate also other endocytic adaptors and if and how ubiquitination of these adaptor proteins is indeed involved in EGFR CME.

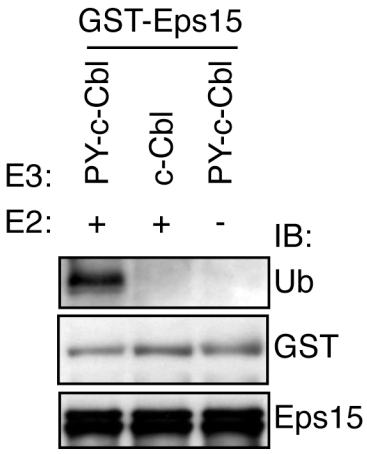

Figure 31 Role of c-Cbl on Eps15 ubiquitination *in vitro*. *In vitro* phosphorylated (pY-c-Cbl) or unphosphorylated c-Cbl (c-Cbl) were used as E3 ligase to perform *in vitro*

ubiquitination reactions that imply incubation of the substrate GST-Eps15 with ubiquitin (1 ug), bacterially purified E1 (100 ng), UbcH5c as E2 (500 ng), E3 (500 ng) and ATP regeneration system. Results from the control reaction without E2 are shown. Immunoblotting was performed with the indicated antibodies. Results are representative of two experiments.

Appendix

1 The contribution of c-Cbl and Cbl-b as EGFR regulators might rely on their relative abundance in the cells

We would like to underline that the role of Cbl proteins as EGFR regulators is cell context-dependent. Indeed, while in NR6 cells, c-Cbl and Cbl-b seem to play redundant roles in the early steps of EGFR CME (Figure 22D), in HeLa cells, c-Cbl is the major player involved in this endocytic pathway (Figure 20B).

To confirm these results, we analysed another cell line, such as another HeLa cell clone (named HeLa-Oslo) exhibiting different expression levels of the two proteins. HeLa-Oslo cells are a clone of HeLa cells, charaterized for the absence of the NCE (226,233). HeLa-Oslo cells show similar levels of the mRNA of the *C-CBL* and *CBL-B* genes, displaying only a lower expression of CBL-B compared to *C-CBL* [about 15% (Figure 32)], as observed in NR6 cells.

	Ct values		
	C-CBL	***CBL-B***	***CBL-C***
HeLa Milan	28.225	31.65	>35
HeLa-Oslo	28.292	28.502	>35

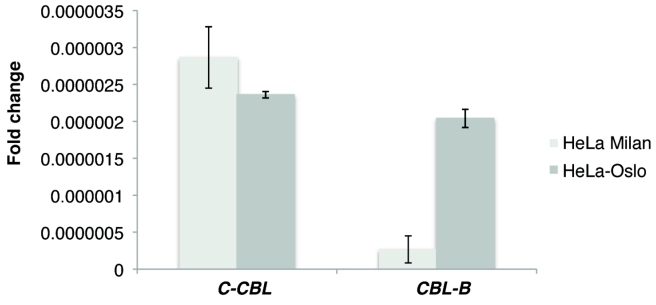

Figure 32 Comparison of *C-CBL* and *CBL-B* expression in HeLa (HeLa-Milan) vs HeLa-Oslo cells. Levels of *C-CBL*, *CBL-B* and *CBL-C* were analysed through qPCR analysis. On the left panel Ct values are reported. The mRNA levels of the different genes were normalized to the housekeeping gene 18S. Fold changes are reported.

We characterized this HeLa-Oslo line for the impact of Cbl proteins in EGFR CME and ubiquitination. As previously described, using an RNAi-based approach [single or

double KD (Figure 33A)], we observed that, like in NR6 cells, EGFR clathrin-mediated internalization is only slightly reduced upon single KD, while it is strongly impaired when both the proteins were silenced (Figure 33B). Also EGFR ubiquitination was affected at the same extent, thus only when the two members were KD (Figure 33C), suggesting a redundant role of c-Cbl and Cbl-b both in EGFR CME and its ubiquitination.

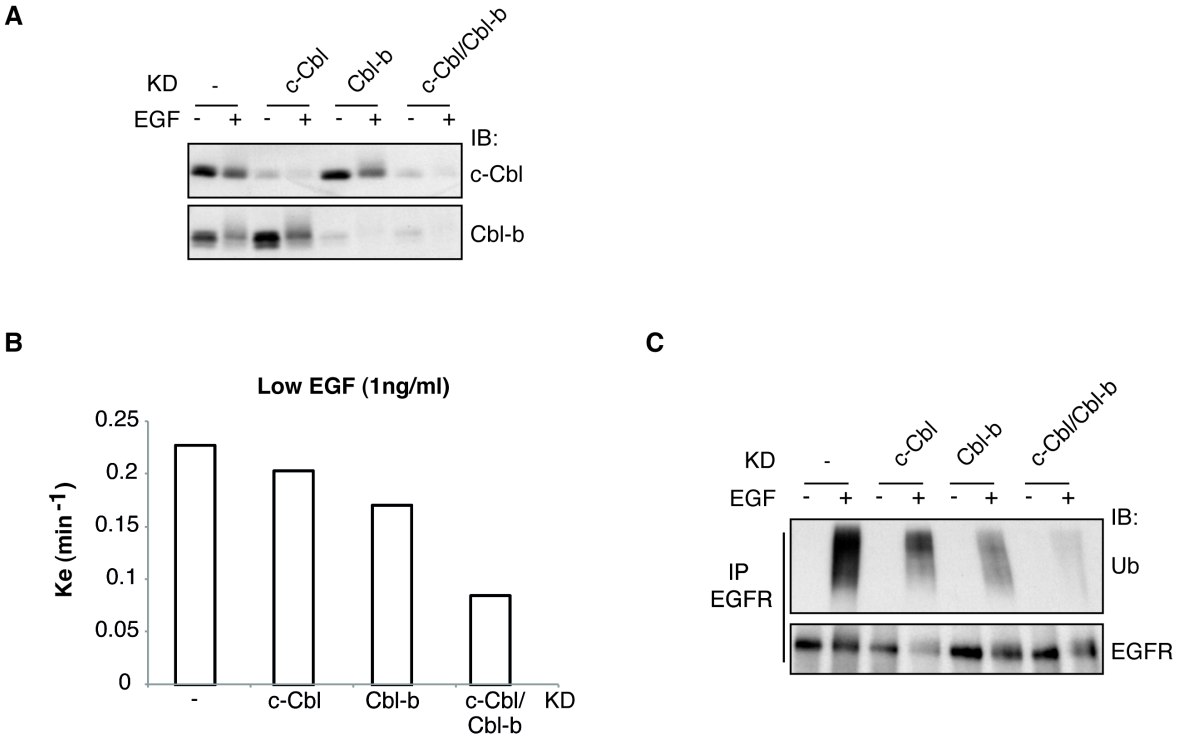

Figure 33 Cbl proteins play redundant roles in EGFR CME and ubiquitination in HeLa-Oslo. (A) HeLa-Oslo cells were silenced for c-Cbl and Cbl-b. Expression levels were determined by immunoblotting using specific antibodies, as indicated. **(B)** ^{125}I-EGF internalization in Hela-Oslo cells, upon KD of Cbl proteins. EGF internalization was measured in the presence of ^{125}I-EGF 1 ng/ml at initial time points (2, 4 and 6 min). Internalization constants were extrapolated from the internalization curves and correspond to the slopes of the best-fitting curves. **(C)** *In vivo* EGFR ubiquitination. HeLa-Oslo cells, subjected to KD as indicated, were serum starved o.n. (-) and then stimulated (+) with high dose of EGF (100 ng/ml) for 2 minutes. EGFR was immunoprecipitated with anti-EGFR antibodies and ubiquitination was detected through a specific antibody against ubiquitin (P4D1, Santa Cruz). Levels of immunoprecipitated EGFR were determined with anti-EGFR antibodies.

One possible explanation for the differential role of c-Cbl/Cbl-b in HeLa (Milan) versus NR6 cells (or HeLa-Oslo) might rely on the different levels of expression observed in HeLa and NR6 cells (or HeLa-Oslo). However, other scenarios might explain this difference. Indeed, it is possible that a differential regulation/localization of the two ligases occurs in the analysed cell lines. We will investigate this possibility in future studies.

2 Role of c-Cbl vs Cbl-b in EGFR degradation

EGFR ubiquitination is known to play an important role in signal extinction, by targeting the receptor to lysosomal degradation. We investigated if the observed effects on EGFR ubiquitination could correlate with a concomitant delay in EGFR degradation and whether c-Cbl and/or Cbl-b could play different roles in EGFR degradation in HeLa cells. To this aim, after stimulation with a high dose of ligand (100 ng/ml of EGF), we assessed the degradation of the receptor by WB (Figure 34B, C). In single KD (c-Cbl and Cbl-b), we did not score any defect in EGFR degradation, while a significant delay was evident in c-Cbl/Cbl-b double KD. This result suggests that Cbl-b might play a specific role at later stages of EGFR endocytosis, redundantly with c-Cbl.

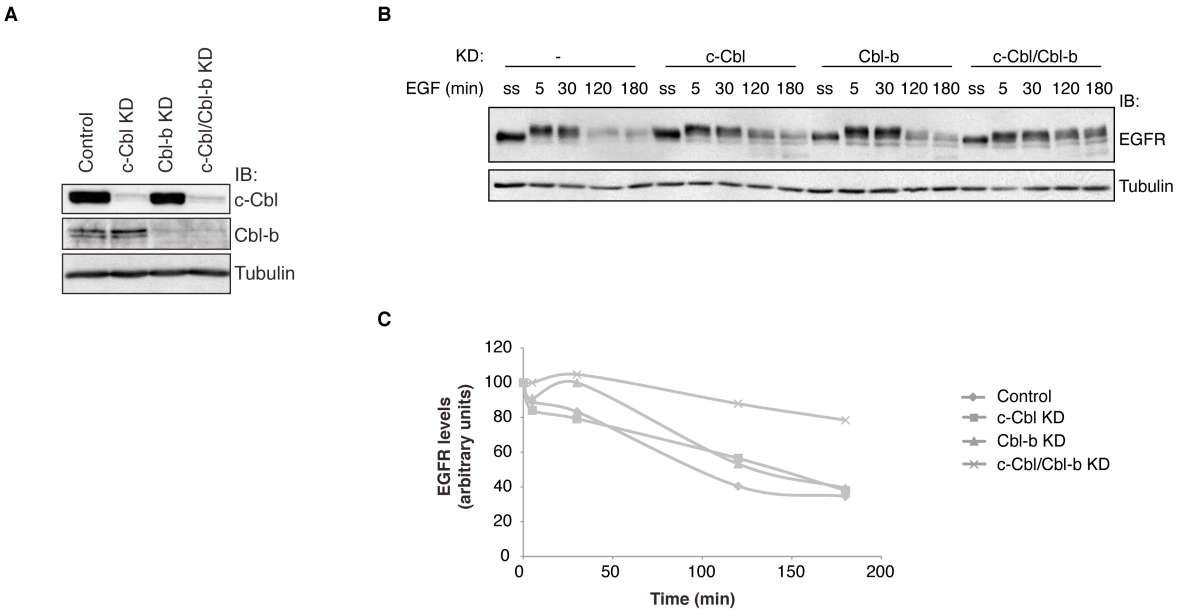

Figure 34 Role of Cbl proteins in EGFR degradation. (A) HeLa cells were silenced for c-Cbl and Cbl-b (single or in combination). Expression levels of Cbl proteins were determined by immunoblotting using specific antibodies, as indicated. Tubulin was used as protein loading control. **(B)** Analysis of EGFR degradation. Upon the indicated KDs, cells were stimulated with high dose of EGF (100 ng/ml) at different time points after an o.n. serum deprivation. EGFR levels were evaluated through WB as indicated. Tubulin was used as a protein loading control. **(C)** Quantification of EGFR levels shown in **(B)** through Photoshop analysis.

An important issue arising from our data is the uncoupling between the major role of c-Cbl in EGFR ubiquitination and its mild effect on EGFR degradation. A first, easy, explanation might be that the residual ubiquitination in c-Cbl KD cells is sufficient to target the receptor to the degradative lysosomal pathway. Only by further reducing ubiquitination through Cbl-b KD were we able to block EGFR degradation. Another possibility might be that c-Cbl and Cbl-b are able to generate different types of ubiquitin signals on the EGFR with distinct role along the endocytic pathway. Indeed, it is known that the EGFR can be modified either by multiple monoubiquitination or by short K63-linked chains in the kinase domain (148), although the role of these two modifications is still unclear. By using EGFR-ERBB4 chimeras, it has been recently demonstrated that c-Cbl is responsible for K48-polyubiquitination upon ligand stimulation (246). In the future, we plan to investigate this issue by performing *in vitro* ubiquitination assays using ubiquitin mutants in which the lysines are mutated into Arg. These mutations do not allow the formation of the corresponding Ub chains and let us discriminate the specificity of the E3 ligase activity.

A more complex hypothesis explaining the observed c-Cbl phenotype would be that another mechanism of EGFR degradation exists and is independent of direct receptor-ubiquitination. For instance, Cbl-b might influence EGFR degradation by ubiquitinating endocytic adaptors involved at a later endocytic stage. Indeed, at this step, different adaptors have been shown to be ubiquitinated, such as Hrs (130), STAM or Eps15b [reviewed in (147)]. However, these are pure speculations and further studies will be required to clarify this point.

Discussion

1 Role of c-Cbl in EGFR CME

Ubiquitination was first implicated in yeast endocytosis. In this model system, ubiquitin modifications are both necessary and sufficient for the internalization of many plasma membrane proteins (114,125). In mammalian cells, the situation is less clear, since for many endocytic cargoes (such as RTKs and GPCRs), ubiquitination appears to be sufficient but not required for internalization (1,2), while for other proteins, such as ion channels, ubiquitination is an essential step of the endocytic trafficking (125). These findings are indicative of the presence of multiple, redundant but distinct mechanisms of endocytosis that may depend on ubiquitination or not (128). In the case of the EGFR, these mechanisms include ubiquitination of Lys residues in the intracellular kinase domain of the receptor, interaction of the receptor with the AP2 complex and with the adaptor protein Grb2, and acetylation of C-terminal Lys residues (128). Likely, the relative contribution of these mechanisms varies depending on the cell type taken into consideration and experimental conditions adopted, that not all mechanisms are simultaneously employed by a single cell and that some mechanisms are preferentially engaged under physiological conditions.

Moreover, we should consider that some studies have shown that EGFR ubiquitination is detectable even at low EGF concentrations, and that ubiquitination can promote the translocation of the receptors to clathrin-coated pits (79). Thus, the contribution of ubiquitination to EGFR CME still remains somewhat elusive and can be influenced by the experimental conditions used (1,2,79,128). Moreover, not only the receptor but also the endocytic adaptors are often ubiquitinated in response to extracellular stimuli (22).

For EGFR, at least two distinct internalization pathways have been described: CME and NCE. The molecular machinery involved in NCE is still poorly defined. Despite this,

we have shown in the past (and confirmed in the present work) that ubiquitination of the EGFR is absolutely essential for EGFR internalization through this pathway. Contrary to NCE, CME is very well characterized at the molecular level. Several groups, including our own, have relied on different tools and approaches to investigate the role of EGFR ubiquitination in CME (1,2). However, if and how EGFR ubiquitination is required at the initial steps of the process is still debated. Thus, one of the goals of this work was to clarify the role of EGFR ubiquitination in CME.

To this aim, we undertook a genetic approach through the generation of an EGFR mutant lacking c-Cbl-binding sites. Importantly, our mutant (3F) was negative for EGFR ubiquitination (as assessed by WB analysis, Figure 22C) and was unable to recruit c-Cbl (Figure 22E). On the contrary, Grb2 binding was only partially impaired (Figure 22E). This observation might be explained by the fact that Grb2 also interacts with the EGFR through the adaptor protein Shc, which upon EGF stimulation binds the pY1173 of the cytoplasmic tail of the receptor and undergoes phosphorylation and activation. Despite this fact, it is clear from our results that either c-Cbl recruitment to the 3F mutant is severely impaired or a labile interaction between c-Cbl and the EGFR is formed. Therefore, this mutant represents a powerful tool to analyse the involvement of c-Cbl in EGFR internalization regardless of its stable recruitment to EGFR. We addressed this issue and observed that internalization of the Ub negative mutant 3F was still dependent on c-Cbl and Cbl-b (Figure 22D). This result suggests that the role of Cbl proteins in EGFR CME is independent of their ability to ubiquitinate the receptor. These results are in agreement with the body of knowledge that indicates that c-Cbl is directly involved in CME through its role as an adaptor or as an E3 ligase for proteins different from the EGFR (3,97). Indeed, we predict that Cbl proteins exert their role in CME possibly through ubiquitination of other substrates required for this process. The ubiquitination of other substrates by c-Cbl may be completely independent on the direct recruitment of c-Cbl to the EGFR or may simply result from a transient interaction of c-Cbl with EGFR, which

would allow the ubiquitin ligase to exert its function on endocytic adaptors. However, it is also possible that c-Cbl works in parallel as adaptor and as E3 ligase, an issue that wants further investigations in the future through the use of mutants, e.g. the mutations that have been mostly shown to be found in tumors (see section 4).

2 Interplay between c-Cbl and Grb2 in EGFR ubiquitination and internalization.

c-Cbl is recruited to the activated EGFR both directly, by binding to the pY1045 and indirectly, through the adaptor protein Grb2, which binds to the pY1068/1086 (Figure 17A). We have demonstrated that the efficient recruitment of c-Cbl to the receptor leads to a massive ubiquitination of the EGFR both *in vitro* (Figure 17B) and *in vivo* (226). Initially, this interplay between c-Cbl and Grb2 was investigated using chimera constructs. Indeed, upon EGF stimulation, c-Cbl-yellow fluorescent protein (YFP) translocated with the EGFR to clathrin-coated pits and endosomes and this localization was dependent on the interaction between the proline-rich region of c-Cbl and the SH3 domain of Grb2 (234). On the contrary, direct binding of c-Cbl to pY1045 of the EGFR was required for its ubiquitination, but was not essential for c-Cbl-YFP localization in EGFR-containing compartments. These data suggest that the binding of c-Cbl to the activated receptor through Grb2 is necessary and sufficient for c-Cbl function during clathrin-mediated endocytosis (234). We confirmed these data, by demonstrating that while c-Cbl KD induces a partial decrease in EGFR CME (Figure 18B, left panel), Grb2 plays an essential role in EGFR clathrin-mediated internalization (Figure 19B, left panel). We were also able to establish that both c-Cbl and Grb2 are critical players in EGFR NCE (Figure 18B and Figure 19B right panel).

3 c-Cbl versus Cbl-b

The role of c-Cbl as adaptor in EGFR endocytosis is well characterized. We wondered whether the other member of the Cbl family expressed in HeLa and NR6 cells - Cbl-b - could have a similar impact on EGFR internalization in our cellular systems. However, we found that these two Cbl proteins behave differently in the two cellular models studies. Indeed, in HeLa cells, Cbl-b does not seem to be involved in the early steps of EGFR internalization, both clathrin-mediated and clathrin-independent (Figure 20B left and right panel, respectively). On the contrary, in NR6 cells, these two proteins seem to play redundant roles in EGFR CME (Figure 22D). Of note, in HeLa-Oslo cells, which show similar expression levels of c-Cbl and Cbl-b as NR6 cells, the redundant behaviour of c-Cbl and Cbl-b in EGFR CME was confirmed (Figure 33B). In addition, our results also show that the roles of c-Cbl and Cbl-b in EGFR ubiquitination are also cell-context dependent. Indeed, while in HeLa cells, c-Cbl is the major E3 ligase involved in EGFR ubiquitination (Figure 16C), in NR6 cells (Figure 22C) and HeLa-Oslo cells (Figure 33C) the two Cbl E3 ligases act redundantly. These discrepant behaviours might be explained by the different expression level of the two genes and consequently of the corresponding proteins (Figure 16A, Figure 22A and Figure 32).

Another difference between c-Cbl and Cbl-b roles in EGFR ubiquitination was observed in HeLa cells. Indeed, we demonstrated that c-Cbl plays the most predominant role in ubiquitinating the EGFR both at the plasma membrane (Figure 16C) and all along the endocytic route (Figure 16D). However, even though c-Cbl has been characterized as the major E3 ligase of the EGFR in HeLa cells, when we analysed the degradation of the receptor, we noticed an uncoupling of the role of c-Cbl in EGFR ubiquitination and degradation. Indeed, when c-Cbl and Cbl-b were singularly KD, no defects in EGFR degradation were observed along the kinetics studied (Figure 34B, C). On the contrary, in the presence of double KD, EGFR degradation was delayed, suggesting that either the minimal

ubiquitination present in c-Cbl KD cells is sufficient to target the receptor to degradation, or that c-Cbl and Cbl-b may generate different types of Ub signals on the EGFR with distinct role along the endocytic pathway, or that the EGFR degradation is independent on its ubiquitination (see appendix, section 2).

A possible explanation for the different impact of c-Cbl and Cbl-b on EGFR internalization and ubiquitination may reside in the structure of these two proteins, namely in the UBA domain at their C-terminus. Importantly, although both c-Cbl and Cbl-b have a UBA domain at their C-terminal, only the UBA domain of Cbl-b has been demonstrated to bind ubiquitin (or ubiquitinated proteins) (165). Indeed, *in vitro*, the UBA domain of Cbl-b, and not of c-Cbl, can interact with ubiquitinated proteins and Ub chains, showing a greater affinity for polyubiquitin chains compared to monoubiquitin. The affinity for ubiquitin resides in differences in several aminoacids between the domains of the two proteins. Thus, this constrasting ability in ubiquitin-binding may reflect distinct regulatory functions of c-Cbl and Cbl-b. Moreover, a study using site-directed mutagenesis has demonstrated that Cbl-b dimerization is regulated by Ub binding and is required for its tyrosine phosphorylation and consequent E3 ligase activity on the substrates (247).

Importantly, another difference between these two proteins can be observed in cancer. Indeed, while several studies have reported c-Cbl mutations located in the linker region and RING finger domain in myeloid neoplasm [see introduction, chapter 5.5 and (226)], only two studies have described frame shift or missense mutations within the RING finger domain of Cbl-b (194,198), suggesting that the frequency of Cbl-b mutations in myeloid neoplasm is lower compared to c-Cbl (193,196). Moreover, c-Cbl$^{-/-}$ and Cbl-b$^{-/-}$ mice display distinct phenotypes, in particular concerning the alteration of signalling pathways in thymocytes or mature T cells (see introduction, chapter 5.4). This may reflect differences in the expression of the two proteins between thymocytes and peripheral T cells.

4 E3 ligase versus adaptor function

It is widely described that c-Cbl can interact with several proteins involved in CME (3). Indeed, by functioning as adaptor, c-Cbl can recruit the adaptor molecules CIN85 and CD2AP (127), which trigger signalling cascades that initiate early phases of receptor endocytosis.

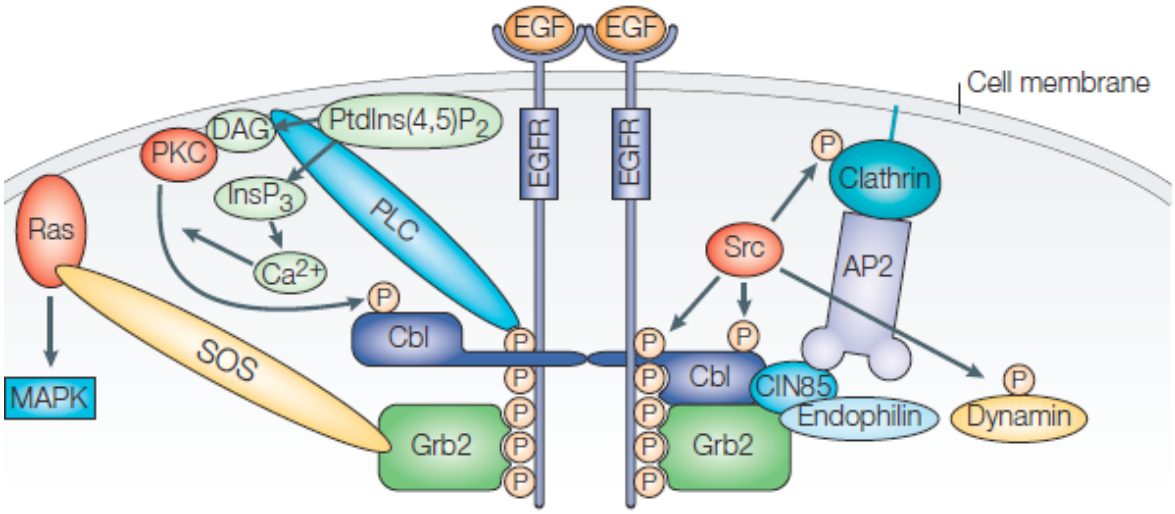

Figure 35 c-Cbl interacts with multiple proteins of CME. Following EGF stimulation, EGFR-associated proteins of the c-Cbl interactome, as c-Cbl itself, clathrin and dynamin, become phosphorylated by Src. This is an essential step for the onset of endocytosis. In parallel, PLC is activated and produces the second-messenger molecules diacylglycerol (DAG) and inositol trisphosphate (InsP3). This results in an increase of intracellular Ca^{2+} concentrations, activation of PKC and initial negative regulation of c-Cbl through phosphorylation of serine residues. At the same time, the Ras pathway is activated through Grb2 and SOS (son-of-sevenless), and this stimulates the MAPK cascade (3).

In order to dissect its role as E3 ligase versus adaptor function in EGFR CME, we generated mutants in the RING finger domain (C381A and I383A) and showed that such mutants display abrogated E3 ligase activity in an *in vitro* ubiquitination assay (Figure 23B). Yet, these two mutants did not display the same behaviour in an overexpression system; indeed, while expression of c-Cbl C381A mutant induced complete EGFR

ubiquitination impairment, expression of c-Cbl I383A mutant affected receptor ubiquitination to a lesser extent (Figure 23C, D). This may be explained by the fact that only the cysteine mutation can completely disrupt the structure of the RING (245). Since c-Cbl can homo- and hetero-dimerize with Cbl-b, when overexpressed this mutant may interact with the endogenous proteins leading to their instability and inhibiting their action. On the contrary, the mutation I383A has been described to prevent the interaction with the E2, leaving intact the RING finger domain. These differences might explain the dominant negative effect exerted by the C381A mutant compared to the I383A. Indeed, in ubiquitination reconstitution experiments, upon c-Cbl and Cbl-b KD, both the RING finger mutants displayed the same ubiquitination defect, as they were not able to rescue the ubiquitination defect, differently from the WT protein (Figure 25C). In this case, upon silencing of the endogenous proteins, we cannot discriminate a dominant negative behaviour of the mutants.

Thus, we demonstrated that these mutations affected E3 ligase activity both *in vivo* and *in vitro* and represented a powerful tool to dissect a possible role of c-Cbl as E3 ligase in EGFR CME. To this aim, we exploited this system in two cellular contexts (HeLa cells and NR6 cells), by performing reconstitution assays of ^{125}I-EGF internalization. In both cases, we demonstrated that c-Cbl E3 ligase activity is required for EGFR CME, since both mutants were not able to reconstitute the defect of EGFR internalization upon Cbl proteins KD (Figure 25D**,** Figure 26C). Moreover, in an overexpression system both C381A and I383A behaved as dominant negative mutants, by decreasing EGFR internalization compared to the untreated samples (Figure 26C). Therefore we can conclude that c-Cbl is involved in EGFR internalization by acting as E3 ligase, an activity that is not exerted on the receptor but possibly on endocytic adaptors involved in CME.

In order to dissect c-Cbl adaptor function vs E3 ligase activity, we additionally undertook a bioinformatic approach, through which we analysed the hotspot mutations present in c-Cbl in the different types of tumors, by taking advantage of the bioinformatic

portal cBio and TCGA (The Cancer Genome Atlas) as source of data. In the Table 5 we represented the most frequent mutations that occur in *C-CBL* gene as well as the frequency of occurency.

gene	original aa	mutated aa	position aa	region	frequency
c-CBL	E	K	276	TKB (SH2 domain)	3.10%
c-CBL	E	K	276	TKB (SH2 domain)	
c-CBL	K	E	287	TKB (SH2 domain)	3.10%
c-CBL	K	R	287	TKB (SH2 domain)	
c-CBL	L	S	338	TKB (SH2 domain)	3.10%
c-CBL	L	S	338	TKB (SH2 domain)	
c-CBL	Q	STOP	367	LINKER	3.10%
c-CBL	Q	L	367	LINKER	
c-CBL	C	R	381	RING	6.25%
c-CBL	C	G	381	RING	
c-CBL	C	Y	381	RING	
c-CBL	C	Y	381	RING	
c-CBL	P	S	395	RING	3.10%
c-CBL	P	L	395	RING	
c-CBL	M	T	400	RING	3.10%
c-CBL	M	T	400	RING	
c-CBL	C	R	401	RING	4.70%
c-CBL	C	R	401	RING	
c-CBL	C	F	401	RING	
c-CBL	R	STOP	420	RING	4.70%
c-CBL	R	STOP	420	RING	
c-CBL	R	Q	420	RING	
c-CBL	W	R	802		3.10%
c-CBL	W	STOP	802		
c-CBL	G	E	868	UBA	3.10%
c-CBL	G	V	868	UBA	

Table 5 Hotspot mutations in c-Cbl. Through a bioinformatic analysis using TCGA as source of data, we found the residues shown in the table, mutated in different types of cancer (breast, lung adenocarcinoma, lung squamous cell carcinoma, rectum and colon adenocarcinoma, uterine corpus endometrioid carcinoma). The original aminoacid, its conversion and the position in the sequence are represented. The position within the structure and the frequency of occurrency considering all the mutations (w/o the silent mutations) are depicted. The color code of the domains is related to the structure of c-Cbl depicted in Figure 14.

From this analysis we importantly scored the presence of several nonsense mutations (STOP codon). We investigated deeper and we found that the nonsense mutations are spread all along the sequence of the protein (Table 6).

gene	original aa	mutated aa	position aa	region
c-CBL	Q	STOP	367	LINKER
c-CBL	R	STOP	420	RING
c-CBL	R	STOP	420	RING
c-CBL	W	STOP	802	
c-CBL	E	STOP	894	UBA

Table 6 Nonsense mutations in c-Cbl. Bioinformatic analysis using TCGA as source of data (same as **Table 5**)

Data from literature have described the mutation c-Cbl W802* by analysing 119 lung cancer patient tumor tissues (204). In an overexpression system, the mutation shows an intact E3 ligase activity on the activated EGFR, results in increased number of viable cells and leads to an increase in cell motility compared to the WT construct. However these studies were performed in presence of the endogenous Cbl proteins, thus investigating for a dominant negative effect of the mutation.

In order to assess whether these mutations could be a useful tool to dissect the E3 ligase activity vs the adaptor function, we firstly investigated whether the E3 ligase activity was impaired in an *in vitro* ubiquitination assay (Figure 36), but for the mutation c-Cbl W802* this was not the case.

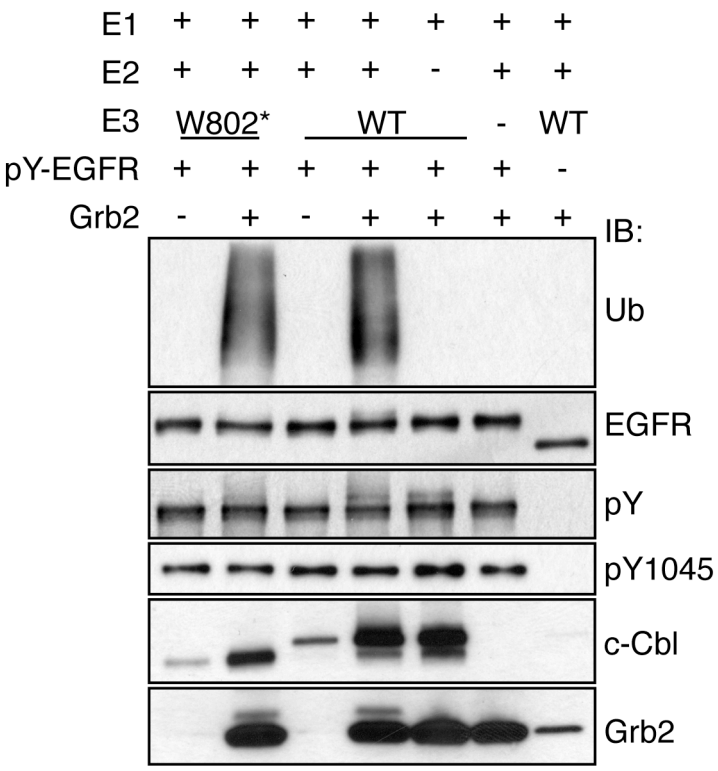

Figure 36 c-Cbl W802* is able to ubiquitinate EGFR *in vitro*. EGFR ubiquitination *in vitro*. GST-EGFR cytoplasmic tail was subjected to *in vitro* autophosphorylation reaction. Then, it was subjected to ubiquitination reaction in the presence of ubiquitin, bacterially purified E1, UbcH5c as E2, c-Cbl WT or c-Cbl W802* as E3, ATP regeneration system, with or without Grb2 (ten times c-Cbl in molarity). The control reactions with EGFR not phosphorylated or without E2 or E3 are shown.

This result goes in the same direction with the findings that only in Cbl-b – and not in c-Cbl – the UBA domain is required for E3 ligase activity on substrates (247).

An in-depth study and characterization of these mutations for EGFR internalization and ubiquitination, exploiting both *in vivo* analyses (reconstitution experiments in cell lines) and *in vitro* ubiquitination assays, will be helpful to possibly dissect the role of c-Cbl as E3 ligase and adaptor.

5 Role of Cbl proteins on Eps15

Ub-mediated internalization or sorting of membrane receptors requires accurate recognition of the ubiquitinated cargo by endocytic Ub receptors, which are proteins that contain UBDs. These endocytic adaptors have the ability to interact with both ubiquitinated cargoes and components of the endocytic machinery. Among these adaptors, Epsins, Eps15 and Eps15L1 are the ones that were shown to recognize ubiquitinated EGFR at the early steps of endocytosis; their cargo selectivity comes from tandemly arrayed Ub-interacting motifs (1). Due to their overlapping binding abilities, these adaptors seem to play redundant roles (1). Indeed, although Eps15, Eps15L1and Epsins have all been traditionally linked to CME (248), they are absolutely required also for clathrin-independent endocytosis of the EGF receptor (1). Importantly, this latter function is exerted through the binding of adaptor UIMs to the ubiquitinated EGFR (1). These proteins are also modified themselves by monoubiquitination upon EGFR activation (22,127,130,131). The presence of a UBD is required for monoubiquitination of the UBD-harbouring adaptor, in a process called "coupled monoubiquitination". This process has been elucidated using Eps15s and Epsins as model systems (22,249). The HECT E3 ligase Nedd4 has been involved in their ubiquitination (249). Moreover, we have also shown here that Eps15 undergoes monoubiquitination at the same extent both at low and high dose of EGF (Figure 28). However, the molecular mechanism by which the upstream signal induced by the activated EGFR causes Nedd4 engagement still needs to be clarified.

This still open scenario led us to better investigate the molecular mechanism underneath the EGFR CME, by focusing on a possible relation c-Cbl/Eps15. Both RNA interference experiments performed *in vivo* [in HeLa and NR6 cells (Figure 29, Figure 30)] and *in vitro* (Figure 31), suggest that c-Cbl may indeed regulate Eps15 monoubiquitination, through a direct or indirect mechanism. In the latter case, since Eps15 can be monoubiquitinated by members of the Nedd4 family of E3 ligases, whose activity is

also regulated by ubiquitination (249), we may hypothesize a circuitry in which Cbl proteins can regulate Nedd4 E3 ligase activity on Eps15. Bidirectional interplay between Nedd4 family and Cbl family have been described in the past. For instance, Nedd4 and Itch bind and ubiquitinate all the mammalian Cbl proteins targeting them to lysosomal degradation. Consistent with this, Nedd4 prevents c-Cbl-mediated EGFR ubiquitination and downregulation and results in a persistent downstream signalling by EGFR (181). Regulation of Nedd4 by Cbl family members are also described: recent studies have differently shed light on the circuitry involving Nedd4 and Cbl proteins in different cell systems (T cells), where loss of Cbl-b enhanced Nedd4 ubiquitination that did not result in its degradation, while the presence of Cbl-b inhibited Nedd4 E3 ligase activity *in vitro* (250). However this type of regulation is apparently in the opposite direction of the simple model that would explain our data, namely that c-Cbl positively regulates Nedd4 activity via ubiquitination.

Another possibility is that c-Cbl directly ubiquitinates Eps15, a scenario that would be compatible with our *in vitro* ubiquitination assay. In this case, a possible scenario would be that the two different E3 ligases (c-Cbl and Nedd4) might play a role at different steps of the endocytic route of the EGFR. Indeed, Eps15 is recruited to the plasma membrane upon EGF stimulation and localizes to the coated pits (251). Recently, it has been demonstrated that Eps15b, an isoform of Eps15, is endosomally localized and directly interacts with Hrs (147). Interestingly, c-Cbl binds to the EGFR at the plasma membrane and remains associated to the ubiquitinated receptor throughout the endocytic route from the plasma membrane to late endosomes (136) and might be involved at early and late steps in combination with other E3 ligases, e.g. Nedd4 family. All these hypotheses will require further investigations in the future.

6 Role of Eps15 and other adaptors monoubiquitination in EGFR internalization

The delivery of membrane receptors to the lysosome requires appropriate recognition of the ubiquitinated cargo by endosomal adaptors and sorting proteins. For the EGFR, for example, Eps15 and epsins act at the initial steps of internalization, serving to recruit the enzymes required for ubiquitination of downstream components of the endocytic pathway. On the contrary, ESCRTs act sequentially at various points of the degradative route, sorting the ubiquitinated cargo at the endosomal membrane for inclusion into the intraluminal vesicle of the MVB. ESCRT-0, composed of the two interacting proteins HRS and STAM, is the first ESCRT complex involved in this process. Three additional complexes, ESCRT-I, ESCRTII and ESCRT-III, then generate the inward vesicle budding, required for MVB maturation (78). These ubiquitin-binding 'route controllers' that ferry the internalized receptor towards a degradative fate into lysosomes are also inducibly ubiquitinated (22,130).

The inducibility of the system illustrates the dynamic nature of EGFR endocytosis regulation based on ubiquitin. Indeed, over the past 15 years, several laboratories have shown a critical role for ubiquitination in receptor down-regulation, a process that is essential for the spatial and temporal resolution of receptor signalling (135). Moreover, we cannot exclude a possible involvement of other endocytic adaptors, such as CIN85, whose interaction with c-Cbl is known to increase upon RTK activation and RTK-induced phosphorylation of c-Cbl (127), even though the mechanism underlying this process is not clear.

But what is the functional role of monoubiquitination of endocytic proteins? Two major hypotheses are most plausible. On the one hand, coupled monoubiquitination would increase the range of intermolecular interactions of the Ub receptor, creating a network of ubiquitin-based interactions, leading to signal amplification and progression of ubiquitinated

cargoes along the endocytic route (110,129). On the other hand, intramolecular interactions between monoUb and UBDs within the endocytic adaptor may lead to an autoinhibitory mechanism, which causes the dissociation of the Ub receptor from the ubiquitinated cargo (e.g., Sts2 and ubiquitinated EGFR) (132). Since these two possibilities are not mutually exclusive, both mechanisms could be involved in the regulation of endocytic processes, possibly by acting at distinct trafficking steps and/or regulating different endocytic adaptors. However, these are pure speculations.

Preliminary data from our lab have demonstrated that an Eps15 mutant in which the six lysines in the UIM are mutated in arginines (6KR mutant) is not able to reconstitute the defect of EGFR internalization upon Eps15/Eps15L1/Epsin1 KD, suggesting the crucial importance of Eps15 monoubiquitination in EGFR endocytosis.

7 c-Cbl in cancer

Interestingly, c-Cbl has been found mutated in human pathologies such NSCLC and myeloproliferative diseases (193); this may lead to dysregulation of RTKs (252). The absence of leukemia in c-Cbl knockout mice and its development in mice with a c-Cbl RING finger mutant knockin are explained by a dominant negative function of the mutant protein. However, the positive functions of Cbl proteins in signalling based on the adaptor function of c-Cbl suggest that the mutations may give rise to both a loss of tumor suppressor function and a gain of oncogene function.

c-Cbl mutations occur throughout all the coding sequence (Figure 37), and are mostly located within the RING finger domain and in the regulatory linker region, therefore are predicted to involve both the ubiquitin ligase activity and protein-protein interactions. However some mutations map outside this region, suggesting that they might impinge on the adaptor function without altering the E3 ligase activity. None of these mutations has been characterized in detail at the mechanistic level.

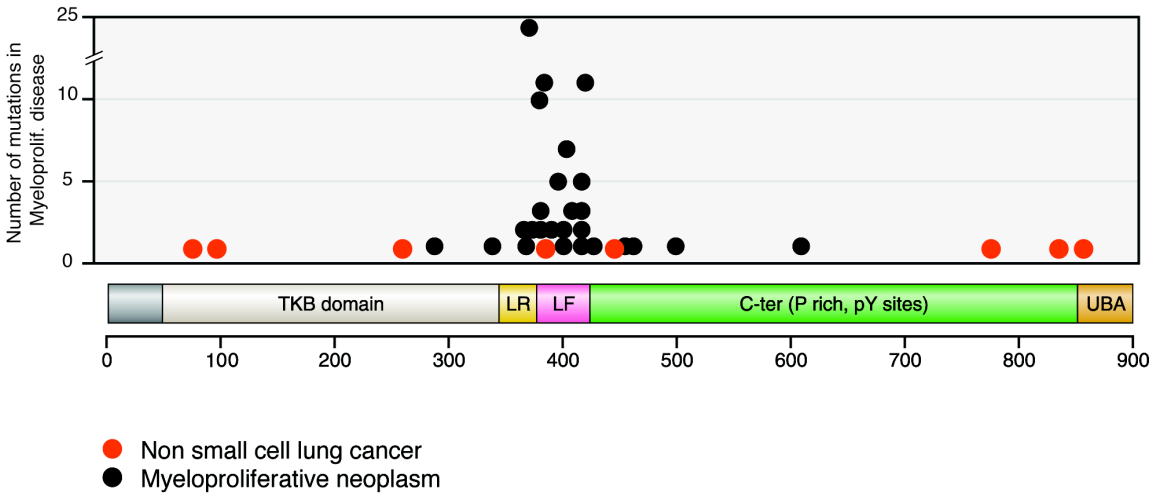

Figure 37 Mutations of c-Cbl in NSCLC and myeloproliferative diseases. At the bottom of the panel, a schematic representation of c-Cbl is shown with its functional domains. The ruler underneath shows aminoacids positions. The position and the frequency of the mutations detected in myeloproliferative diseases are shown by black

circles, while the ones detected in NSCLC are shown by red circles and are aligned with the aminoacid sequence [adapted from (4)].

While c-Cbl WT may work as onco-suppressor, mutants of c-Cbl can function as gain-of-function oncogenes. Since the mutations identified in the different leukemia are located in the RING finger and do not alter the TKB and C-terminal domain, interactions with a variety of signalling proteins are not affected. Thus, it is logical to hypothesize that these E3-ligase impaired proteins will be recruited to activated RTKs and that this will lead to the formation of a signalling complex that lacks the negative regulatory function of the c-Cbl WT. Thus, mutant c-Cbl proteins are likely to function as supramolecular scaffolds to assemble aberrant signalling complexes that can promote hyperactivation of signalling pathways normally attenuated by the WT counterpart. This hypothesis is compatible with analyses of a mutant c-Cbl knock-in mouse model developed by Langdon and colleagues. This model carries a c-Cbl RING finger mutant (C379A, corresponding to C381 in human), which is expressed under the control of the endogenous c-Cbl promoter (253). While homozygous mutant mice show early lethality, heterozygous mutant mice with one WT c-Cbl allele do not show hematopoietic abnormalities. However, mice with one C379A mutant allele in a c-Cbl-null background, but with intact Cbl-b, dispaly myeloid malignancies (200). Interestingly, Akt was constitutively activated in the C379A mutant hematopoietic cells but not in control or Cbl-null mutant cells (200). This went with enhanced phosphorylation of Y737 (corresponding to Y731 in human) of c-Cbl binding site for the p85 regulatory subunit of PI3K.

These findings in mice carrying the RING finger mutant Cbl knock-in (200) suggest that wild type c-Cbl may act as tumor suppressor, while mutant c-Cbl might have oncogene functions.

Further experiments are needed to better elucidate the molecular mechanism underlying the role of c-Cbl mutations in cancer development.

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