Ubiquitin and endocytic internalization in yeast and animal cells.

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Abstract

Endocytosis is involved in a wide variety of cellular processes, and the internalization step of endocytosis has been extensively studied in both lower and higher eukaryotic cells. Studies in mammalian cells have described several endocytic pathways, with the main emphasis on clathrin-dependent endocytosis. Genetic studies in yeast have underlined the critical role of actin and actin-binding proteins, lipid modification, and the ubiquitin conjugation system. The combined results of studies of endocytosis in higher and lower eukaryotic cells reveal an interesting interplay in the two systems, including a crucial role for ubiquitin-associated events. The ubiquitylation of yeast cell-surface proteins clearly acts as a signal triggering their internalization. Mammalian cells display variations on the common theme of ubiquitin-linked endocytosis, according to the cell-surface protein considered. Many plasma membrane channels, transporters and receptors undergo cell-surface ubiquitylation, required for the internalization or later endocytic steps of some cell-surface proteins, whereas for others, internalization involves interaction with the ubiquitin conjugation system or with ancillary proteins, which are themselves ubiquitylated. Epsins and Eps15 (or Eps15 homologs), are commonly involved in the process of endocytosis in all eukaryotes, their critical role in this process stemming from their capacity to bind ubiquitin, and to undergo ubiquitylation.

INTRODUCTION

Endocytosis -the uptake of membrane proteins and lipids, extracellular ligands and soluble molecules from the cell surface- is a hallmark of all eukaryotic cells. Endocytosis is involved in a diverse array of cellular processes, including nutrient uptake, morphogenesis of the neuronal network, synaptic molecule recycling and regulation of the cell-surface expression of signaling receptors, transporters and channels. The original description of endocytosis emerged principally from morphological studies in animal cells. Genetic approches have since been applied to the study of this process in *Drosophila melanogaster*, *Dictyostelium discoideum*, *Caenorhabditis elegans* and *Saccharomyces cerevisiae*. It has become apparent that endocytosis is associated with multiple cellular processes, including actin cytoskeletal dynamics, the addition of ubiquitin and lipid modification. Studies, in yeast in particular, have demonstrated that ubiquitylation occurs at various steps in endocytosis. Ubiquitylation has been shown to be required for the first step of endocytosis: entry into primary endocytic vesicles budding from the plasma membrane. In animal cells, several of the ions channels and signal transduction receptors that undergo regulated internalization are ubiquitylated in response to extracellular signals. Ubiquitylation regulates the endocytosis of these molecules by a cellular machinery including the Nedd4 or Cbl ubiquitin ligases. Whether this triggers their internalization and/or later endocytic steps in still a matter of debate. Moreover, the process seems to differ according to the protein considered. In *S. cerevisiae*, the vast majority of plasma membrane proteins require ubiquitylation of the cytoplasmic domain for internalization. This process involves the ubiquitin ligase Rsp5p (homologous to proteins of the Nedd4 ligase family in higher eukaryotes). Most of the proteins ubiquitylated at the plasma membrane are then targeted for degradation in the lysosome/vacuole (recent reviews [1, 2]). A second step in the endocytic pathway also involves ubiquitin-dependent events. Sorting to internal vesicles that bud inside multivesicular bodies (MVBs) requires cargoes ubiquitylation, and a machinery conserved from yeast to man (class E vps proteins) (recent reviews [3, 4]). This process shares many similarities with ubiquitin-dependent events at the plasma membrane, and even some common actors, including as demonstrated in yeast the ubiquitin ligase Rsp5p [5-8].

E1/E2/E3

Ubiquitin is a 76-amino acid protein, highly conserved throughout evolution, which is found in all eukaryotic organisms and cell types. The C-terminus of ubiquitin is generally linked via an amide isopeptide bond to the ε-amino group of an internal lysine residue of the substrate protein. Ubiquin is conjugated to the protein substrate by means of a three-step cascade mechanism. First, a ubiquitin-activating enzyme, E1, activates ubiquitin in an ATP-dependent reaction. Ubiquitin is then transferred to the active site cysteine of a ubiquitin-conjugating enzyme (E2). Finally, a ubiquitin protein ligase (E3) catalyzes the transfer of ubiquitin from E2 to the substrate. A number of families of E3s or E3 multiprotein complexes have been identified. RING-finger containing E3s catalyze direct transfer of the activated ubiquitin from E2 to the E3-bound substrate. With HECT-(homologous to E6-AP COOH terminus) domain

E3 enzymes, the ubiquitin is transferred from E2 to the active site of E3, generating a high-energy intermediate, and is then transferred to the ligase-bound substrate [9]. E3s play a key role in protein ubiquitylation because they serve as the specific recognition factors of the system. Proteins may be modified by monoubiquitylation: the conjugation of a single ubiquitin to one or several lysines. Monoubiquitylation is involved in histone modification, and in several trafficking events. As ubiquitin itself carries several conserved acceptor lysines — notably Lys29, Lys48 and Lys63 — multiubiquitin chains can be generated, in some cases with the assistance of E4 enzymes. K48-linked (and sometimes K29-linked) multiubiquitin chains, at least four ubiquitin units long, are potent targeting signals leading to the recognition and subsequent degradation of target proteins by the 26S proteasome, a large multisubunit protease complex [10]. K63-linked ubiquitin chains are involved in other functions, including DNA-repair, the activation of translation, the activation of specific kinases, and endocytosis [11, 12]. Ubiquitylation is a dynamic process. Deubiquitylating enzymes (DUBs) cleave ubiquitin from proteins, and disassemble ubiquitin chains [13]. Several such enzymes are involved in endocytosis.

E3 families specifically involved in the internalization step of endocytosis

Ubiquitin ligases are proteins or protein complexes that binds to both E2 and the substrate. Interaction with the substrate may be direct or may involve other proteins. E3s are heterogeneous, but may be classified into two major groups — HECT domain and RING finger-containing E3s — and several minors groups, such as U box-containing proteins, also termed E4, involved in elongating polyubiquitin chains [9], or a subset of the PHD-containing proteins with divergent RING domains [14]. The E3s involved in endocytosis include RING finger E3s, HECT domain proteins, and PHD-containing proteins.

Most of E3 ubiquitin ligases are RING finger-containing enzymes. These E3s serve as a scaffold responsible for optimal positioning of E2 and the substrate for the efficient transfer of activated ubiquitin from E2 to the substrate. The RING finger domain has been defined as a pattern of conserved Cys and His residues forming a cross-brace structure that probably binds two Zn cations. The RING finger family of E3 enzymes is composed of two distinct groups: single and multisubunit proteins. Monomers (or homodimers) contain both the RING finger domain and the substrate-binding/recognition site in the same molecule [9]. This is the case for c-Cbl, a RING finger ligase involved in targeting activated receptor tyrosine kinases [15]. A multisubunit RING E3, SCF^{HOS}, was also described to be involved in endocytosis [16].

The HECT domain superfamily is the only family of E3 enzymes known to catalyze substrate ubiquitylation directly [17, 18]. HECT domain proteins contain a 350 amino acid sequence homologous to the COOH-terminal domain of the prototype member of the E6-AP family (E6-associated protein). This domain contains a conserved Cys residue, to which the activated ubiquitin moiety is transferred from E2 [17, 18]. The NH$_2$-terminal domain, which varies between HECT domain proteins, is probably devoted to specific substrate recognition, as is the case for members of the Nedd4 family. The N-terminus of members of the Nedd4 family harbors a C2 domain, followed by two to four WW domains (Fig. 1) [19]. The C2 domain is a 120-amino acid sequence that has been shown to bind phospholipids and membrane proteins in a Ca^{++}-dependent manner in several proteins, including Nedd4 [20, 21], whereas Rsp5p C2 domain was shown to bind phosphoinositides in a Ca^{++}-independent manner *in vitro* [6]. WW domains

are 40-amino acid protein:protein interaction modules that bind Pro-rich ligands. Members of the Nedd4 family have two to four WW domains, suggesting that they may interact with several proteins simultaneously. Based on their binding specificity, WW domains can be classified into two major and three minor groups [22]. Group I WW domains bind PPXY motifs, whereas Group II WW domains bind PPLP motifs. Two of the three minor groups, Group III and V, bind proline-rich sequences, whereas Group IV WW domains interact with short sequences containing phosphorylated serine and threonine residues followed by proline. The binding of Group IV WW domains to their ligands has been shown to be phosphorylation-dependent [23].

One major function of Nedd4/Rsp5p family members is regulation of the stability of several yeast and animal cell-surface transmembrane proteins by ubiquitylation, which controls subsequent internalization. For example, Nedd4-2 targets the kidney epithelial Na+ channel [24], whereas the degradation of several receptors and transporters in yeast is mediated by the Rsp5p ligase, the only member of this family in *S. cerevisiae* [21].

The third family of E3s involved in the internalization step of endocytosis is that of PHD-containing E3 ligases. The plant homeodomain (PHD) motif encodes a specialized form of Zn finger [25]. This motif consists of seven Cys and one His residue in the order 4 Cys, His, 3 Cys, and is similar in both sequence and structure to RING finger domains. A PHD domain is present in a membrane-bound protein known to be involved in ubiquitin-dependent ER degradation in yeast. This protein was demonstrated to have ubiquitin ligase activity *in vitro* [26]. PHD motifs have more recently been identified in several viral and human proteins playing key roles as E3s in the downregulation of cell surface proteins (reviewed in [14]).

A key issue in the ubiquitin field is the way in which the system achieves its high specificity and selectivity, an E3-dependent property. In the case of cell-surface proteins, ubiquitylation is often specifically triggered in response to extracellular stimuli, such as ligand binding or nutrient modification. For E3s involved in the endocytic pathway, and for other E3s, ubiquitylation may require the involvement of other modifying enzymes and additional proteins. Many substrates are not recognized constitutively and are not recognized directly by E3s. In some instances, E3s must be activated or deactivated by posttranslational modification. In other cases, the substrate undergoes modifications rendering its recognition possible. Thus, in addition to E3s themselves, modifying proteins such as kinases and other proteins also play an important role in the recognition process.

1- Mechanisms underlying the internalization step of endocytosis: insights from yeast and animal cells

Endocytosis is only one branch of multiple intracellular trafficking pathways. The unicellular eukaryote *S. cerevisiae* has been widely used as a model organism for studies of the secretory pathway, a classical example of a process conserved from yeast to humans. The mechanisms underlying trafficking from the Golgi apparatus to the lysosome/vacuole also appeared to have been strongly conserved throughout evolution, and yeast mutants have served as key tools in identification of the genes

involved in this pathway. Endocytosis may be divided in early events, essentially the internalization step, and late steps- delivery to endosomes- followed by either the recycling of internalized cargoes, or their targeting to the lysosome/vacuole for degradation. The late steps of endocytosis were rapidly shown to be conserved from yeast to humans. Interestingly, several years of research initially suggested different requirements for the internalization step of endocytosis in yeast and animal cells, but it now seems that the two processes share many similar requirements (reviewed in [27, 28]).

1-1- Internalization in animal cells: clathrin-dependent and independent endocytosis

1-1-1-Clathrin-dependent endocytosis

Almost forty years ago, Roth and Porter identified clathrin-coated pits as specialized plasma membrane domains responsible for the selective recruitment of cargo molecules [29]. Extensive work then provided critical insight into clathrin-dependent endocytosis, a major pathway responsible for the efficient uptake of various proteins from the cell surface [30]. Clathrin coats at the plasma membrane give rise to endosome-targeted vesicles, by means of a two-step process: membrane budding, and subsequent fission of the resulting vesicle. The major structural components of clathrin coats are two protein complexes, clathrin, and clathrin adaptor proteins. Clathrin consists of three 192-KDa heavy chains, each bound to one light chain of approximately 30 KDa [31]. This complex is called a triskelion and may polymerize *in vitro* to form a polygonal lattice [32]. *In vivo*, triskelia polymerize to form rounded baskets that coat invaginated pits and vesicles. The heterotetrameric adaptors (APs) are responsible both for recognizing signals in the cargoes, and for bridging the clathrin lattices to the membrane [33]. AP2 is involved in internalization at the plasma membrane. Clathrin is also involved in other trafficking steps, together with other APs, such as AP1, in particular, which is involved in Golgi-to-endosome trafficking. Like the other APs, AP2 consists of two large subunits ($\alpha2$ and $\beta2$), one medium ($\mu2$), and one small subunit ($\sigma2$). The $\mu2$ and $\beta2$ subunits interact with sorting signals in the cytoplasmic domains of transmembrane proteins, notably Tyr-based and di-Leu-based motifs, respectively. The $\beta2$ subunit also interacts with clathrin heavy chains *via* a specific motif, the clathrin box, also found in other clathrin partners (e.g. epsins, amphiphysin). The $\alpha2$ and $\beta2$ subunits both contain a 30 kDa "appendage" domain, which is joined to the rest of the protein via a flexible linker. Resolution of the crystal structure of the flexible domain of $\alpha2$ subunit revealed a single binding site for its ligands, which include amphiphysin, Eps15, epsin, and possibly dynamin [34]. A single site for the binding of multiple ligands would facilitate temporal and spatial regulation in the recruitment of components of the endocytic machinery. Eps15 was identified as a partner of AP2, capable of binding the $\alpha2$ subunit *via* the DPF motifs in its C-terminal domain [35]. The N-terminal region of this protein contains three repeated sequences, conserved throughout evolution, which define a family of proteins [36]. These EH (Eps15 homology) domains of Eps15 have been demonstrated to bind several proteins, including epsin [37]. The C-terminal domain of Eps15 also contains also two UIMs (ubiquitin interacting motif). Eps15 has been demonstrated to be crucial for clathrin-dependent endocytosis, probably in the recruitment of AP2 to clathrin-coated pits. It was recently suggested that epsin, a protein that interacts with Eps15 via an NPF motif and may also bind

AP2, plays an important role in internalization. Epsin contains a highly conserved amino-terminal region [38], the ENTH domain (epsin N-terminal homology). This domain binds phosphoinositol-(4, 5)-bisphosphate and it was recently shown that such binding modifies membrane curvature in conjunction with clathrin polymerization [39]. Epsin also contains UIM domains. Following initial invagination of the membrane, the clathrin-pit recruits dynamin, a GTPase which plays a key role in constriction, and endophilin, which may play a direct role in the fission reaction by virtue of its lipid modifying activity [40] before the pinching off of vesicles from the plasma membrane [41]. Another AP2 partner, that also binds clathrin and dynamin, amphiphysin, was demonstrated to induce curvature of lipid bilayers *in vitro*, a feature also displayed by epsin, endophilin and dynamin, indicating that roles in invagination or fission may not be mutually exclusive [42]. AP2 is not the only adaptor molecule involved in clathrin-dependent endocytosis. For some receptors, such as the β-adrenergic receptor, the role of adaptor in clathrin-mediated endocytosis is played by β-arrestins, which link activated receptors to both AP2 and clathrin [43].

Models ordering the successive steps and actors involved in membrane recruitment, invagination, constriction and fission have been raised, in which invaginating clathrin-coated pits were traditionally thought to be covered by AP2. Recent investigations performed in living cells expressing fluorescent-tagged proteins have challenged old models. The use of total internal reflection fluorescence microscopy (TIR-FM) has enabled the tracking of individual clathrin coated pits and vesicles and the direct observation of events occuring within a restricted area adjacent to plasma membrane. Experiments revealing that AP2 is absent from disappearing clathrin spots have suggested that contrary to predictions, AP2 complexes may form stable plateforms from which clathrin coated pits lacking AP2 would laterally emerge [42].

New insights on clathrin-mediated endocytosis were also obtained with the use of small interfering RNA to knock down AP-2 subunits and clathrin heavy chain to undetectable levels. Receptor-mediated endocytosis of several receptors including transferrin receptor (TfR), or Epidermal Growth Factor receptor (EGFR) was severely inhibited in clathrin depleted cells. Strikingly, however, if internalization of TfR was inhibited in AP2-depleted cells, internalization of EGFR was as efficient in these cells as in control cells. AP-2 is thus only one of several endocytic adaptors required for clathrin-dependent the uptake of certain cargo proteins [44].

1-1-2- Endocytosis without clathrin coats

Clathrin-dependent endocytosis is not the only endocytic pathway in animal cells. The use of specific inhibitors of clathrin-dependent endocytosis, dominant negative forms of Eps15 in particular, has made it possible to demonstrate the physiological importance of alternative endocytic pathways. These pathways include caveolae-induced endocytosis. In the electron microscope, caveolae appear as small invaginations with a typical "omega–like" shape. Caveolae have a striated coat, one marker of which is a integral membrane protein, caveolin [45]. Caveolae are directly involved in the internalization of certain plasma membrane components, such as GPI-anchored proteins, some toxins, and several envelope

viruses [46]. The detachment of caveolae from the plasma membrane, like that of clathrin-coated vesicles, is mediated by dynamin [47].

Other, as yet poorly defined, endocytic pathways have been observed following the inhibition of clathrin-dependent endocytosis, in cells devoid of caveolae. This is the case for the endocytosis of the interleukin-2 receptor (IL2-R) [48]. The constitutive endocytosis of IL2-R has been shown to be coupled to the partitioning of IL2-R in lipid rafts (dynamic microdomains of the plasma membrane rich in cholesterol and sphingolipids). Lipid rafts are thought to play a key role in the endocytosis of other receptors, including the high-affinity IgE receptor [49].

1-2- Internalization in yeast: general requirements

Receptor-mediated endocytosis in *S. cerevisiae* has been studied for two G protein serpentine receptors, the **a** and α-factor receptors, both of which display constitutive and ligand-induced endocytosis [27]. The endocytosis of cell-surface transporters has also been thoroughly studied in this organism. These transporters display both constitutive and accelerated endocytosis, specifically regulated by factors such as excess substrate, changes in nutrient availability, and stress conditions [50].

Although yeast cells have no caveolin, and therefore no caveolae-dependent endocytosis, there is still some debate concerning whether clathrin-dependent endocytosis occurs in this organism (reviewed in [51]). Clathrin-coated vesicles have been purified from *S. cerevisiae*. However, attempts to visualize clathrin-coated pits and clathrin-coated vesicles at the yeast plasma membrane have not been successful [51]. Deletions of the genes encoding the clathrin light or heavy chains, or heat-sensitive mutations in the first of these genes, result in only 30-50% inhibition of internalization of the α-factor and **a**-factor receptors [52]**.** A clear difference between yeast and animal cells in the internalization step of endocytosis is the lack of involvement of dynamin, endophilin and AP-type adaptors in yeast. Yeast cells contain genes encoding three AP complexes, known as the AP1, AP2-like and AP3 adaptors. The AP2-like adaptor complex is not only dispensable for endocytosis, but may even fail to associate with clathrin in yeast cells [53, 54].

The other requirements for endocytosis have been investigated by studying several available mutants and screening to identify endocytic mutants. A number of proteins specifically required for the internalization step of endocytosis have been identified in this way (Table I). The correct organization of the actin cytoskeleton is a key requirement for endocytosis in yeast (reviewed in [27]). Many of the first *end* (for endocytosis) mutants identified were found to have mutations in the actin gene itself (*END7*) [55], in genes encoding actin-binding proteins (*END6/RVS161, END5/VRP1*) or in genes encoding proteins required for the correct organization of the actin cytoskeleton (e.g. *END3*, *END4/SLA2*, *ARP2, END9/ARC35*). The precise role of actin in yeast endocytosis is still unclear but, several years after the first study with yeast mutants [56], it was demonstrated using latrunculin A, a drug that sequesters actin monomers that correct actin polymerization is also required for endocytosis in mammals [57]. It has been suggested that, in yeast, actin polymerization may provide, in conjunction with the myosin isoforms Myo3p and Myo5p, the force required for the fission of vesicles from the plasma membrane (reviewed in

[27]). This suggestion was based, in part, on the lack of involvement of dynamin-like proteins in yeast endocytosis. The two homologs of amphiphysin, Rvs161p and Rvs167p (Fig. 1), were identified among the proteins required for endocytosis in yeast. Cells defective in these proteins have impaired actin cytoskeleton organization. As mammalian amphiphysin interacts with dynamin, Rvs167p interacts with actin [58]. Amphiphysin/Rvs proteins are among the actors in endocytosis in both yeast and mammals, with apparent adaptation in their respective roles. Anyhow, the actin cytoskeleton now clearly appears involved in the internalization process in mammalian cells. Several clathrin-coated pits associated proteins, including dynamin, seem to play a role in regulating actin polymerization, and actin accumulation was observed at clathrin spots while they were disappearing. Actin was thus proposed to provide a force during vesicle formation, possibly to push the nascent vesicle away from the plasma membrane [59, 60].

Genetic screening, in addition to identifying mutations in genes related to actin function, also revealed the crucial role of lipids in endocytosis [27], and led to the identification of several clathrin-binding proteins essential for endocytosis. These proteins include Ent1p and Ent2p -the yeast homologs of epsin- and Pan1p, a protein carrying two EH domains. Yeast cells carry several EH domain-containing proteins, including Ede1p, a protein that like Eps15 carries 3 N-terminal EH domains, but has no DPF motifs (Fig. 1). Ede1p has been shown to be required for efficient endocytosis [61]. Extensive genetic studies and two-hybrid analysis led to the proposal that a complex network of interacting proteins linked to the actin cytoskeleton, including End3p (one EH doman), Pan1p and its Ent1/2 partners [62], is involved in endocytosis in yeast [63]. Ede1p may also be part of this complex, given genetic interactions between *EDE1*, *PAN1*, and *END3* [61]. This suggestion was based on the analogy between this complex of EH domain-containing proteins with associated partners and the network of EH domain proteins and their partners involved in endocytosis in mammalian cells, the function of which appears to be more clearly defined [36]. The discovery that some of the corresponding yeast (Ede1p, Ent1/Ent2p), and mammalian proteins (Eps15, Epsins) contain ubiquitin-binding domains (Fig. 1), and improvements in our understanding of the role of ubiquitylation events in yeast and mammalian endocytosis have led to the formulation of new hypotheses concerning the possible function of some of the proteins in these complexes as adaptors, as detailed below.

2- Ubiquitin and the internalization step of endocytosis

A striking difference between endocytosis in mammals and yeast, first described years ago, is the almost complete absence in yeast plasma membrane proteins of the classical Tyr-based, or di-Leu-based internalization signals known to be recognized by AP2 in mammalian cells. This finding is consistent with the lack of involvement of AP2-like adaptors in yeast endocytosis. It is currently thought that, for the vast majority of yeast plasma membrane proteins, endocytic signals result from the posttranslational modification of these proteins by ubiquitin (previously reviewed in [1, 2, 21]. Progress towards understanding the molecular mechanisms underlying ubiquitin-dependent endocytosis in yeast has been made in parallel with the recognition that ubiquitylation events are involved in endocytosis in mammalian cells. The demonstration that ubiquitin has a function in endocytosis was unexpected, given previous

knowledge concerning the requirement of ubiquitin for degradation by the proteasome, and the degradation of proteins in the lysosome/vacuole after endocytosis.

2-1- Ubiquitin-dependent endocytosis in yeast : a general process

2-1-1- The discovery of ubiquitin-dependent endocytosis

The link between ubiquitin and endocytosis in yeast was discovered almost simultaneously in different laboratories, using three approaches: 1) analysis of the downregulation of Ste6p, the ATP-binding cassette (ABC)-transporter for secretion of the pheromone **a**-factor [64], 2) demonstration that the endocytic signal identified in a C-terminal truncated form of Ste2p, the receptor for α-factor, was an ubiquitylation signal [65], and 3) cloning of a gene involved in the downregulation of amino acid permeases, which was found to encode a ubiquitin protein ligase [66].

In a pioneering study, Kölling and coworkers observed the accumulation in plasma membrane fractions prepared from a mutant with impaired endocytic internalization, of ubiquitylated forms of Ste6p [64]. These forms were less abundant in a mutant lacking two ubiquitin-conjugating enzymes, Ubc4p and Ubc5p, and this mutant was protected from Ste6p degradation. This suggested that a ubiquitylation event might precede and be required for Ste6p internalization. Secondly, extensive work was devoted to determining the endocytic signals in the α-factor receptor, Ste2p, by tracing α-factor internalization by truncated or mutated receptors. A non-classical signal, SINNDAKSS, was found to be necessary and sufficient for the ligand-induced endocytosis of a C-terminally truncated Ste2p. Within this sequence, the Lys residue was found to be critical [67]. These observations were clarified with the understanding that this residue is the target for α-factor-induced ubiquitylation of the truncated receptor, which is required for internalization [65]. The third line of evidence for a link between ubiquitin and endocytosis in yeast was obtained by genetic analysis of the ammonium-induced downregulation of amino acid permeases (reviewed in [68]). The discovery that one gene involved in this process, *NPI1,* encodes a ubiquitin-protein ligase of the HECT family, *Rsp5p* (Fig. 1) [66], and that a human homolog of this enzyme, Nedd4, is critical for downregulation of the epithelial sodium channel ENaC, provided major insights into the emerging field of ubiquitin-dependent endocytosis (reviewed in [2, 21, 50]).

Rsp5p was then demonstrated to be involved in constitutive ubiquitylation of the uracil permease, Fur4p [69], and ammonium-induced ubiquitylation of the general amino acid, Gap1p [70]. Impairments affecting Rsp5p, or mutations of the two target lysines in the two permeases inhibit their ubiquitylation, thereby preventing internalization [69-72]. The fusion of ubiquitin in-frame at the N-terminus of a mutant form of uracil permease lacking the two target lysines trapped at the plasma membrane results in the partial restoration of permease internalization [5]. Similarly, the fusion in-frame of ubiquitin restores some internalization of variant forms of α-factor and **a**-factor receptors lacking their own ubiquitylation signals [73, 74]. Moreover, the fusion in-frame of ubiquitin to a stable plasma membrane protein, the [H$^+$] ATPase, leads to the internalization and subsequent vacuolar degradation of this protein [73]. These data, together with the observation that endocytic cargoes accumulate in ubiquitylated forms in mutants defective for the internalization step of endocytosis, demonstrate that ubiquitylation is required for, and

precedes the internalization of many plasma membrane proteins, and that ubiquitin (monoubiquitin) constitutes an internalization signal in itself.

It soon became clear that ubiquitylation is a prerequisite for the internalization of most known plasma membrane substrates of endocytosis, and that Rsp5p, the only HECT ligase of the Nedd4 family in yeast, is the only ubiquitin protein ligase involved in this posttranslational modification [1, 21, 50, 75]. The only exception reported to date in this apparent general requirement for ubiquitin in yeast internalization is the ligand-induced internalization of the **a**-factor receptor, Ste3p. Whereas constitutive Ste3p endocytosis displays typical ubiquitin-dependence [74, 76], Ste3p ligand-dependent internalization and recycling proceeds normally after conservative Lys to Arg mutations at all possible ubiquitin acceptor sites [77]. Ligand-dependent Ste3p endocytosis has been shown to involve a specific signal, NPFSTD, recognized by the protein Sla1p, linked to the actin cytoskeleton, which has been suggested to act as an endocytic adaptor [78]. It should be noted that a di-Leu motif is required for endocytosis of the Gap1p permease [79]. Point mutations in the di-Leu signal, or small deletions in this region, impair endocytosis but not ubiquitylation of the Gap1p permease, suggesting that this region of the protein is involved in endocytosis, at a stage downstream from ubiquitylation [70, 79]. No endocytic adaptor has yet been identified in this case.

2-1-2 Type of ubiquitin modification in cell-surface transmembrane proteins

The efficient recognition of proteins by the proteasome requires polyubiquitin chains at least four subunits long, which in most cases appear to be linked via the Lys48 of ubiquitin [9]. It was suggested that ubiquitylated plasma membrane proteins may escape recognition and subsequent degradation by the proteasome, probably depending on the type of ubiquitin chain they receive. Indeed, some yeast plasma membrane proteins appear to display mono-ubiquitylation, whereas others are modified by the addition of K63-linked di/tri-ubiquitin residues ([1, 2, 21, 50] for review).

The list of membrane proteins which are post-translationaly modify with ubiquitin is continually increasing, and currently comprises thirty three proteins, including transporters, ABC-transporters, (list in [50]), and receptors. Data concerning the ubiquitin profiles of six of these proteins are now available. Ste2p is monoubiquitylated on several lysines (multi-monoubiquitin) [80], as are probably the galactose permease, Gal2p, and the maltose permease [81-83]. Ste3p, Gap1p, the zinc transporter, Ztr1p, and Fur4p have been shown to be modified by small chains of two to three ubiquitins, each attached to one, two or more target lysine residues [71, 74, 84, 85 , 86]. The ubiquitylation pattern of Fur4p and Gap1p has been analyzed in cells lacking the Doa4p ubiquitin isopeptidase (which have lower than normal intracellular free ubiquitin concentrations [87], and which are therefore impaired in ubiquitylation processes) and overproducing either wild-type or variant ubiquitins incompetent for the formation of K29-, K48- or K63-linked ubiquitin chains. Both transporters carry two target Lys residues [71, 72] that can accept up to two or three ubiquitin residues, linked via the Lys63 residue of ubiquitin [84, 86]. For both transporters, the addition of one ubiquitin to the two target lysines (multi-monoubiquitylation) appears to be sufficient for some endocytosis to occur, but the formation of Lys63-linked short ubiquitin chains is required for efficient internalization. Moreover, although the fusion of ubiquitin in-frame at the N-terminus

of the variant of Fur4p lacking the two target lysines restores permease internalization, the rate of uptake is five times higher if ubiquitin is fused in-frame to the wild-type permease also modified by adding a short chain of di-ubiquitin to each of its target lysines [5]. Although mono-ubiquitylation is sufficient for Ste3p endocytosis, there is evidence to suggest that multi-ubiquitylation also increases the rate of internalization [74]. Whether Fur4p and Gap1p, two of the few known ubiquitylated substrates carrying Lys63-linked ubiquitin residues, are representative of a larger class of Rsp5p plasma membrane substrates, remains to be determined. A recent proteomic study of ubiqutylated yeast proteins has shown that Lys63 chains are far more abundant than previously thought [88].

2-1-3- Ubiquitylation motifs on target plasma membrane proteins

Target lysines for ubiquitylation have been identified in a number of cases. Investigations aiming to define the cis signals required for ubiquitylation have revealed that an acidic stretch in the linker region connecting the two halves of the ABC-transporter Ste6p [89], and an N-terminal acidic PEST-like sequence in Fur4p [90] and in the maltose permease Mal61p [81] are essential. Furthermore, many yeast transporters are phosphorylated. It is known at least for soluble proteins that phosphorylation, notably within PEST sequences, is frequently linked to ubiquitylation [9]. The PEST sequence of Fur4p displays serine phosphorylation in its PEST region, a modification required for permease ubiquitylation at nearby lysines [71, 90]. Phosphorylation in this sequence is partly dependent on the redundant Yck1p/Yck2p casein kinase I homologs [71]. Similarly, the unique target Lys in the Zrt1p zinc transporter is 14 amino acids away from a short acidic sequence containing several serines required for Zrt1p ubiquitylation [91]. Two phospho-acceptor residues in the linker region of Ste6p are also important for phosphorylation, efficient ubiquitylation, and internalization [92, 93]. Finally, the acidic, Ser-rich sequence, SINNDAKSS of Ste2p is the target of both phosphorylation partly due to Yck1p/Yck2p, and ubiquitylation [94]. So, when documented, critical Lys residues are generally located within or adjacent to these acidic sequences potentially important for recognition by the ubiquitylation machinery.

The Lys residue of the cis signal SINNDAKSS, is one of the major ubiquitylation sites in the full-length Ste2p receptor [80]. A motif similar to the SINNDAKSS sequence, DAKTI, has been identified in the acidic region of Ste6p required for ubiquitylation of the protein [89], although a Lys-to-Arg mutation within this motif had only a minor effect on Ste6p turnover (suggesting the involvement of additional Lys residues). Two Lys residues included in similar sequences, ERKS and EYKS, have been shown to be essential, together with three other nearby lysines, in the ubiquitylation and turnover of the tryptophan permease, Tat2p [95]. One of the two adjacent lysine residues required for Fur4p ubiquitylation is also included in a similar sequence, EYKSS. We suggest that Lys residues included in D/EXKS/T motifs are probably primary targets for the ubiquitylation of plasma membrane proteins, at least in yeast. In a proteomics approach, attempts have recently been made to identify ubiquitylated proteins in cells with 6His-tagged ubiquitin as their sole ubiquitin [88]. Potentially ubiquitylated proteins (retained on Ni colunms), included 12 plasma membrane transporters. Precise ubiquitylation sites were identified in six transporters including three proteins that have already been shown to be

ubiquitylated. Strikingly, the target Lys identified in these transporters lie in very acidic motifs, often rich in Ser residues. Phosphorylation sites were also identified within nine transporters. For two of these transporters the ubiquitylated target Lys lie very close to the identified phosphorylated amino acids and lie in a D/EXKS/T motif. It remains to define whether the identified target Lys are ubiquitylated at the plasma membrane, as ubiquitylation is also involved in other trafficking steps. The very same physiological condition can trigger both Rsp5p-dependent ubiquitylation and internalization of a given plasma membrane transporter, and ubiquitylation at an intracellular location leading to diversion of neosynthesized transporter for lysosome/vacuolar degradation pathway without passing through the plasma membrane (reviewed in [50]). The target Lys in Gap1p identified in the above study differs from the two target Lys shown to be ubiquitylated at the cell surface following the addition of ammonium to cells cultured in nitrogen-poor medium [72], and the function of this modification remains to be determined. Uracil permease, Fur4p undergoes both substrate-induced plasma membrane internalization, and direct vacuolar routing if synthesized *de novo* in the presence of its substrate [5]. The ubiquitylation required for endosome sorting and vacuolar degradation, also Rsp5p-dependent, did not require prior phosphorylation of the PEST sequence of Fur4p [5], whereas plasma membrane ubiquitylation did [90]. The two target Lys for plasma membrane ubiquitylation [71] were also sites of ubiquitylation for endosome sorting, together with other Lys [5]. Thus, even for the same protein, the sequences required for ubiquitylation by the same enzyme are not the same at different intracellular locations. Obviously, the proteomic approach, performed for cells cultured in standard conditions, should be complemented with data obtained in defined physiological conditions corresponding to known trafficking of a given transporter. If this study requires complementary analyses, it constitutes an important step in determining whether the ubiquitylation sites in plasma membrane transporters display some signature. The data currently available, conforting prior studies, highlight the link between the phosphorylation, D/EXKS/T motifs, and ubiquitylation, possibly at plasma membrane, of some transporters.

2-1-4- Interactions between Rsp5p and its plasma membrane substrates

Although much is already known about the putative ubiquitylation signals in yeast transporters and, to a lesser extent, the role played by Rsp5p domains in this process (see below), we still have no clear overall picture of the way in which Rsp5p interacts with transporters.

Rsp5 is the only member of the Nedd4 family of HECT-E3 proteins in yeast and is currently the only ubiquitin ligase shown to be involved in the posttranslational modification of yeast cell-surface proteins [21]. The three WW domains of Rsp5p have been shown to be important for Ste2p ubiquitylation [96], whereas only the second and moreover the third WW are required for the efficient ubiquitylation of Fur4p ([97] and Marchal and Urban-Grimal, unpublished data). These data suggest that the Rsp5p WW domains, or a subset of these domains, are involved, directly or indirectly, in substrate recognition. However, Rsp5p-WW domains are Group I WW that bind PPXY motifs [98] and yeast cell-surface proteins include generally no obvious PPXY motifs that could accommodate the direct binding of Rsp5p-WWs. Thus, there may be other, currently unknown motifs involved in the interaction of Rsp5p *via* its WW domains, or Rsp5p may interact with its plasma membrane substrates *via* a novel type of interaction, or

with the assistance of adaptors. The Bul proteins are possible candidate adaptor proteins. Bul1p interacts physically with Rsp5p via its PPXY motif, and Bul2p is a homolog [99, 100] . Both Bul proteins contain a PPXY motif and a mutant Bul1p protein with an altered PY-motif has been shown to be defective in Rsp5p binding [100]. Bul1p has been shown to interact with Rsp5p functionally, biochemically, and genetically [99, 100]. Bul1p has been shown to assist Rsp5p in several of its many functions in the cell [72, 101-104]. Both proteins are conjugated with ubiquitin, the role of this modification being unknown [88]. Deletion of *BUL1* and *BUL2* impairs cell-surface ubiquitylation and downregulation of the cell-surface transporters Gap1p [72] and Fui1p (Volland, personal communication) but has no effect on Fur4p (Castillon and Urban-Grimal, personal communication). Thus, the Bul proteins may be involved in the recognition of only a subset of of Rsp5p plasma membrane substrates.

A last possibility would be that productive Rsp5p-membrane proteins interaction at the cell surface could be only transient and weak as already postulated for Nedd4-Eps15 interaction [105]. No physical interaction has been found between Rsp5p and the cytoplasmic part of Fur4p containing the PEST signal for ubiquitylation (even if a PEST variant efficiently constitutively ubiquitylated *in vivo* after changing all Ser residues to Glu, which mimics phosphorylation, was used) (Castillon and Urban-Grimal, unpublished data). Rsp5p is peripherally associated with membranes and has been shown to function as part of a multimeric protein complex at the plasma membrane [96, 106]. One possiblity would be that at this location Rsp5p could be able to recognize any of its target lysines provided that they are uncovered following a change in the conformation of the substrate protein due to phosphorylation at a nearby site.

Whatever the mode of interaction between Rsp5p and its plasma membrane substrates, little is known about the regulation of this interaction. Ste2p displays ligand-induced hyperphosphorylation, which is required for subsequent ubiquitylation, and the downregulation of many transporters is also highly controlled. The cell-surface ubiquitylation of Gap1p, Zrt1p, the Mg^{++} transporter, sugar transporters and Fur4p is induced and/or accelerated in the presence of ammonium, zinc, magnesium, glucose, and uracil, respectively. Other plasma membrane transporters have been reported to undergo controlled downregulation, but it is not yet defined whether this downregulation depends on ubiquitylation events (reviewed in [50]). It is unclear whether such regulation depends on phosphorylation, or whether these transporters display specific conformational changes, exposing critical lysine residues. The possibility that Rsp5p activity is specifically regulated also remains to be addressed, by analogy with data reported in mammals (see below).

2-1-5- Other roles of Rsp5p in the internalization process

Several observations have suggested that, in addition to its function in the ubiquitylation of plasma membrane cargoes, Rsp5p may have other functions in internalization. WW1 and WW3 domains appear to be important in fluid-phase endocytosis [96, 97]. Strikingly, mutations in the WW1 domain strongly inhibit fluid-phase endocytosis, but have no effect on Fur4p ubiquitylation and internalization ([97], and Marchal and Urban-Grimal, unpublished observations). Hence, the WW1 domain of Rsp5p may have a substrate/partner that is essential for fluid phase endocytosis. The C2 domain of Rsp5p appears to be the critical element controlling the location of the protein in both the plasma membrane and endosomal compartments [106, 107]. Although deletion of the C2 domain has no effect on α-factor

internalization it has been reported to inhibit fluid phase endocytosis [96], and to lead to a marked decrease in the rates of internalization of Fur4p [106] and Gap1p [108], with no apparent effect on the ubiquitylation of these proteins. These findings indicate that, in addition to its role in the ubiquitylation of these plasma membrane proteins, Rsp5p is involved in their internalization, *via* a process dependent on the C2 domain. This led to the suggestion that the Rsp5p-dependent ubiquitylation of a *trans*-acting protein might be required for the internalization step of endocytosis [109]. Support for this hypothesis was provided by the observation that temperature-sensitive *rsp5* mutant cells are defective in the internalization of α-factor by a Ste2p-ubiquitin chimera, a receptor that does not require posttranslational ubiquitylation for internalization. Similarly, a modified version of Ste2p bearing a NPFXD linear peptide sequence as its only internalization signal (ubiquitin-independent) was not internalized in *rsp5* cells. The internalization of these variant receptors and fluid-phase endocytosis were found to be dependent on the catalytic cysteine residue of Rsp5p [109].

The substrate(s) of Rsp5p critical for efficient internalization remains to be identified. Proteins playing an important role in organization of the actin cytoskeleton are potential candidates. Synthetic lethality was observed between mutations in *RSP5*, and mutations in several genes encoding proteins important for cytoskeleton organization, including Vrp1p/End5p, Pan1p and End3p [110, 111] or proteins colocalized with the actin cytoskeleton, such as Ede1p, the homolog of Eps15 [61]. Mutants with impaired actin cytoskeleton organization have been shown to display dislocalization of Rsp5p [106, 111]. Moreover, point mutations in the WW1 domain of Rsp5p result in resistance to latrunculin, a drug that sequesters actin monomers [111]. The link between Rsp5p and the actin cytoskeleton was further underlined by systematic genomic approaches. Large-scale analysis of protein complexes has revealed that Rsp5p interacts with actin, and with Las17p (Bee1p), a member of the Wiskott-Aldrich Syndrome protein (WASP) family of actin-assembly proteins. Affinity precipitation and two hybrid analysis, respectively, have shown an interaction between Rsp5p and two Las17p partners, *Lsb1*, and *Lsb7p/Bzz1p* required for the recruitment of actin polymerization machinery [112]. Affinity precipitation also evidenced an interaction between Rsp5p and the two amphiphysin homologs, Rvs161p and Rvs167p. Interestingly, **Lsb1p** and Rvs167p both display PPXY motif potentially recognized by Rsp5p WW domains. The interaction between Rsp5p and the amphiphysin homolog Rvs167p was indeed documented by two hybrid and biochemical technics [107]. Furthermore, Rvs167p was demonstrated to undergo Rsp5p-dependent monoubiquitylation on a target Lys (Lys481) within Rvs167p SH3 domain. However, mutation of this Lys to Arg did not impair fluid phase endocytosis nor α-factor internalization [107]. Although these data provide the first identification of an Rsp5p substrate among proteins required for endocytosis and actin cytoskeleton organization, the potential role of Rsp5p on the endocytic machinery and/or actin cytoskeleton organization remains to be defined. Whether the mammalian amphiphysin also undergo ubiquitylation also remains an open question.

2-2- Ubiquitylation events and endocytosis in animal cells: a variety of situations

Early reports described the ubiquitylation of several cell-surface receptors in mammalian cells (first published example [113], other cases reviewed in [2, 114], and Table II). In parallel to the study of ubiquitin-dependent endocytosis in yeast, a link was also established between ubiquitin and endocytosis in mammalian cells, although only some plasma membrane proteins undergo cell-surface ubiquitylation. A variety of situations were identified, involving different ubiquitin ligases for different classes of cell-surface proteins. The direct ubiquitylation of very few endocytic substrates seems to be required for their internalization. In other cases, recruitment of the ubiquitylation machinery to a receptor appears to be the critical step. Some receptors appear to undergo ligand-induced ubiquitylation at the cell surface, but this process is not required for the internalization of these receptors. Moreover, the ubiquitylation of some of the proteins of the endocytic machinery seems to accompany or to be required for internalization. The link between ubiquitylation and internalization thus appears to be more complex in mammalian cells than in yeast.

2-2-1- A role for ubiquitin ligases of the Nedd4/Rsp5 family in the downregulation of channels, transporters and receptors

In parallel to the discovery that Rsp5p is crucial for the downregulation of several yeast plasma membrane proteins [66, 69], the crucial role played by one isoform of Nedd4s, namely Nedd4-2 [24] in downregulation of the sodium channel ENaC was demonstrated [115-117]. ENaC plays an essential role in renal sodium management. This channel consists of three subnits (α, β, γ), each of which contains a conserved PXY motif at its C-terminus. Deletion or mutation of the PXY motifs of the ß or γ subnit of ENaC results in Liddle's syndrome, a hereditary form of arterial hypertension in which ENaC activity is abnormally high. The PXY motifs of ENaC serve as binding sites for the WW domains of Nedd4, and mutations in these PY motifs abolishes the binding of this enzyme. Consistent with its ability to associate with Nedd4, ENaC is regulated by ubiquitylation, which takes place primarily on a cluster of lysine residues in the γ subnit. Mutation of these lysines leads to the impairment of channel ubiquitylation and an increase in channel density at the cell surface. Moreover, in a *Xenopus* oocyte system, it has been shown that the overproduction of wild-type Nedd4, but not of catalytically inactive Nedd4, leads to the inhibition of channel activity in a PXY-dependent manner, providing conclusive proof that Nedd4 is responsible for the ubiquitylation and downregulation of ENaC (reviewed in [21]). Two other channels also appear to display ubiquitin-dependent downregulation controlled by E3s of the Nedd4 family (Table II). The cardiac voltage-gated Na+ channel (rh1), which contains PXY motifs, is also negatively regulated by Nedd4 when produced in *Xenopus* oocytes [118]. The C-terminus of the chloride channel ClC5 has a PXY motif that is critical for its downregulation in response to interaction with an E3 of the Nedd4 family, possibly WWP2 [119].

A few receptors belonging to different families were also demonstrated to undergo downregulation mediated by ubiquitin ligases of the Nedd4 family (Table II). Insulin-like growth factor I receptor (IGF-IR), previously known to interact with the adapter Grb10, was demonstrated to undergo Nedd4-dependent ubiquitylation. Surprisingly, in this case, interaction of Nedd4 with its substrate was not mediated by Nedd4 WW domain, but by Grb10, acting as an adapter linked to Nedd4 C2 domain, as first evidenced by Nedd4/Grb10 two hybrid interaction [120]. AIP4/Itch was found to promote agonist-induced

ubiquitylation of the chemokine receptor CXCR4, a representative of the superfamily of G protein-coupled receptors (GPCRs) [121], that plays a critical role in HIV infection [122]. CXCR4 is ubiquitylated (likely monoubiquitylated) at the plasma membrane, as inhibition of internalisation by expression of dominant negative form of dynamin leads to an accumulation of ubiquitylated CXCR4 [123]. In this case, ubiquitylation seems to be required for late steps of receptor endocytosis rather than internalization [123].

Ubiquitin ligases of the Nedd4 protein family are not always constitutively active on their plasma membrane substrates. It has long been known that ENaC activity is subject to complex regulation by a number of hormones, including aldosterone. This regulation seems to involve Sgk1 kinase (serum and glucocorticoid regulated kinase), a member of the Akt family of Ser/Thr kinases, which is induced by aldosterone, and stimulates ENaC. Based on the observation that Sgk1 has a PXY motif, and that Nedd4-2 includes two consensus sites for phosphorylation by Sgk1, Staub and coworkers showed that Sgk1 phosphorylates Nedd4-2 in a PXY-dependent manner in *Xenopus* oocytes, and that this phosphorylation reduces the interaction between Nedd4-2 and ENaC, leading to high levels of ENaC at the cell surface [124]. Extensive use of the Xenopus oocyte system injected with cRNA encoding active or inactive forms of Nedd4-2 and Sgk1 has revealed similar opposite role of these two enzymes in the downregulation of several transporters, the astrocyte aminoacid transporter SN1 [125], the glial glutamine transporter EAAT1 [126], and the intestinal phosphate transporter NaPi [127], supporting the idea that Sgk-dependent regulation of the interaction of Nedd4-2 with its substrates might be a general process.

A further level of complexity in the role of the ubiquitin system in the endocytic pathway was also illustrated in several cases with the demonstration that ubiquitylation of a given substrate sometimes involves several E3s. Ubiquitylation and downregulation of the epidermal growth factor receptor (EGFR) appear, in some cases, dependent on both the HECT ligase AIP4, and the RING finger protein Cbl3 that were described to interact [128]. Similarly, the receptor Notch, a protein involved in cell fate decision in many mammalian cell types, was described to interact with the mouse Nedd4-like Itch [129] and the RING cCbl [130], and to undergo ubiquitylation (polyubiquitylation ?) by both enzymes. The precise function of ubiquitylation in Notch signalling was recently documented, and is quite unsual. Binding of Notch ligands (such as Delta) was known to trigger successively a proteolytic cleavage in the extracellular domain of the receptor due to a protease of the ADAM family, TACE [131], followed by a cleavage in the transmembrane domain of the remaining protein by γ-secretase. This leads to release of the intracellular (ICv) domain, which then translocates to the nucleus. It was recently demonstrated that γ-secretase cleavage requires prior monoubiquitylation and subsequent endocytosis of Notch [132].The E3 responsible for this precise modification of Notch remains to be identified.

In this rapid overview of the involvement of proteins of the Nedd4 family in the endocytic pathway of various plasma membrane substrate, it seems that in most cases, ubiquitylation is a plasma membrane event leading to cargo internalization (channels, transporters, some receptors). In at least one case (CXCR4), ubiquitylation, although occuring at plasma membrane, appears required for later endocytic steps. In addition, it seems that Nedd4 might also display a dual regulation of some receptors at two trafficking steps, in scenarios similar to the downregulation of yeast transporters by Rsp5p-dependent ubiquitylation at two intracellular levels. Drosophila Nedd4 has been shown to control the Roundabout (Robo) receptor in axon guidance at the central nervous system (CNS) midline. It was previously defined

by genetic studies that the transmembrane protein Comm is a negative regulator of Robo. Myat *et al.* showed that the internalization of Comm and of Comm/Robo from the cell surface is a DNedd4-mediated, ubiquitin-dependent event. They demonstrated that DNedd4 binds and ubiquitylates Comm in a PPxY-dependent way. These data suggested that DNedd4 activity is necessary for the internalization of Comm and that Comm acts as an adaptor-like protein that can cotarget Robo for internalization [133]. Data reported by Keleman *et al.* showed that transient production of Com prevents Robo from reaching the cell surface by binding to this receptor and targeting it directly to endosomes [134]. The two processes might also work together for optimal downregulationof Robo, by similarity to Rsp5p-dependent trafficking of yeast transporters [50].

2-2-2: Role of known and unknown E3s in ubiquitin-dependent downregulation of cytokine receptors

The historical example of ubiquitin-dependent downregulation of GHR differs considerably from the ubiquitin-dependent endocytosis of ENaC, or of most other studied receptors. GHR is a member of the cytokine receptor superfamily. In reponse to growth hormone (GH), two receptor polypeptides dimerize, turning on a cascade of events leading to signal transduction and degradation of the receptor [135]. In 1987, it was observed that the growth hormone receptor (GHR) was ubiquitylated [135]. Nine years later, Strous and coworkers made the link between ubiquitin and endocytosis of the GHR: GHR was not degraded upon ligand binding at restrictive temperature in Chinese hamster ovary cells, which present a temperature-sensitive defect in ubiquitin conjugation [136]. Thus, the ubiquitin system is required for ligand-induced GHR endocytosis. The amino acid sequence $DSWVEF_{337}IELD$ was shown to be required for the ubiquitin-dependent internalization of GHR, and was designated the UbE motif [137]. However mutation of lysines in the cytoplasmic tail of a truncated version of the GHR (GHR 1-399), the internalization of which depends on the UbE motif and the integrity of the ubiquitin conjugation system [138], did not impair internalization of the truncated GHR. Thus, internalization requires recruitment of the ubiquitin conjugation system to the GHR UbE motif, rather than the conjugation of ubiquitin to the GHR [137]. These data suggest that ancillary proteins of the endocytic machinery may be ubiquitylated, or that factors of the ubiquitin conjugation system itself may act as adaptors for the endocytosis machinery (reviewed in [139]). Strikingly, the UbE motif has been demonstrated to be required for recruitment of the GHR to clathrin-coated pits [140]. The use of a glutathione S-transferase (GST)-pulldown assay indeed allowed identification of a protein binding the UbE (small glutamine-rich tetratricopeptide repeat (TPR)-containing protein), but in an ubiquitin-independent way [141], suggesting that other UbE partners have yet to be indentified. Although GHR ubiquitylation does not appear to be required for endocytosis, GHR nonetheless undergoes ubiquitylation during endocytosis. The use of two different approaches to inhibit internalization, β-methyl cyclodextrin treatment, which inhibits endocytosis at the stage of coated vesicle formation, and overproduction of a dominant negative mutant form of dynamin, which prevents the detachment of clathrin-coated vesicles from plasma membrane, made it clear that GHR is ubiquitylated at the plasma membrane before endocytosis [142].

The β and γ chains of the interleukin-2 receptor (IL2R) also belong to the cytokine receptor superfamily, whereas the IL2R chain does not. The IL2R is internalized following the binding of

interleukin-2 (IL2). After endocytosis, the three subunits are sorted differently: the α chain is recycled, whereas, the β and γ chains are targeted to late endocytic compartments [143]. The β subunit of this receptor is monoubiquitylated. Neither this monoubiquitylation, nor an intact ubiquitin conjugation system, is required for the internalization step of endocytosis. However, ubiquitylation seems to be a signal involved in sorting from the early/recycling endosome to late endocytic compartments [144]. Hence, ubiquitylation events are associated with the endocytosis of several receptors of the cytokine receptor superfamily, some of which are internalized by clathrin-coated vesicles (GHR), and some of which are not (IL2R) [48]. It should be noted that, neither for IL2R nor for GHR has the E3 involved in ubiquitylation been identified.

The type I interferon (IFN) receptor, another cytokine receptor consisting of IFNAR1 and IFNAR2 subunits, is still todate, to our knowledge, the unique example of a receptor ubiquitylated by a RING finger enzyme of the SCF (Skp1/Cullin/F-box) family of E3. In these multisubunits E3s, the substrates are recognized by specific F-box proteins. IFNAR1 was shown to interact with the Homolog of Slimb (HOS) F-box protein, an interaction promoted by interferon alpha (IFNα) that triggers IFNARI phosphorylation. SCF^{HOS} expression and activity is required for IFNalpha-stimulated ubiquitylation and downregulation of IFNAR1, probably associated with IFNAR2 [16]. In contrast to the case of IL2R, SCF^{HOS}- dependent ubiquitylation of IFNAR1 appears required for receptor internalization.

2-2-3- Ubiquitin-dependent downregulation of cell surface proteins by PHD ubiquitin ligases

Ubiquitylation events have also been reported to play a key role in the downregulation of cell surface proteins during host immunity system evasion following infection with Kaposi's sarcoma-associated herpes virus (KSHV). KHSV is a recently identified herpes virus responsible for Kaposi's sarcoma, a neoplasm common in individuals suffering from AIDS. After viral infection, major histocompatibility complex (MHC) class I molecules and other molecules (B7-2 and ICAM-1) involved in immune recognition are rapidly downregulated. These molecules are efficiently synthesized and targeted to the plasma membrane, but then rapidly undergo endocytosis followed by degradation in the lysosomes. The viral genome contains two genes, K3 and K5, encoding transmembrane proteins (now named modulator of immune recognition MIR1 and MIR2) with cytoplasmic PHD domains. MIR1 and MIR2 were reported to promote the PHD-dependent cell-surface ubiquitylation of MHC Class I, B7-1 and ICAM-1 [145, 146]. Mutation of the two lysine residues in the cytosolic tail of MHC class I molecules blocks both MHC class I ubiquitylation and downregulation [145, 146]. The transmembrane segment of MIR2 plays a critical role in target recognition, and the transmembrane and juxtamembrane regions of the target MHC class I molecule make a key contribution to recognition by the MIR proteins [145].

Analysis of the sequences of other viral genomes has indicated that transmembrane PHD-containing proteins are present in a considerable number of DNA viruses of the herpesvirus and poxvirus families, and it was predicted that some of these proteins could act as E3 involved in trafficking events [14]. Indeed, M153R, a PHD-containing protein of poxviruses, homolog to K3 and K5 downregulates the T-cell coreceptor CD4 and MHC-I in an ubiquitin-dependent way [147]. The discovery of transmembrane human proteins homolog to K3/K5, membrane-associated RING-CH (MARCH) proteins, suggested that

they might play similar roles, an hypothesis which was tested with known substrates of the viral K3 family. Two closely related proteins, MARCH-IV and MARCH-IX, reduced surface expression of MHC-I molecules whereas MHC-I molecules lacking lysines in their cytoplasmic tail were resistant to downregulation [148]. The functional similarity of the MARCH family and the K3 family suggests that the viral immune evasion proteins were derived from MARCH proteins, a novel family of transmembrane ubiquitin ligases.

2-2-3- Cbl and its role in the ubiquitylation and endocytosis of tyrosine kinase receptors

Cbl was first identified as the cellular homolog of v-Cbl, a protein expressed by murine retroviruses that potently induces B-cells lymphomas [149]. In mammals, the Cbl family comprises three members, c- Cbl, Cbl -b and Cbl -c (also named Cbl3), of which c- Cbl is the most studied. c- Cbl, Cbl -b are ubiquitous proteins which exhibit enhanced expression in hematopoïetic tissues, whereas Cbl-c is mostly expressed in epithelial cells. The N-terminus of Cbl proteins contains a TKB (Tyrosine Kinase Binding) domain consisting of a four-helical domain followed by a EF-Hand domain (which link Ca++) and a non classical Src homology 2 (SH2) domain. The variable C-terminal half of Cbl carries a long proline-rich domain, several phosphorylation sites, and a UBA (ubiquitin associated) domain except for Cbl-c. A centrally located RING finger domain separates these two parts of the protein (Fig. 1). Extensive studies in the past years have highlighted the key role of Cbl family members in downregulation of several tyrosine-kinase receptors (RTK).

(RTKs) are downregulated following ligand binding to surface receptors. As RTKs play a determinant role as regulators of cell growth, proliferation and differentiation, extensive investigations have been carried out by many groups with a view to elucidating the process of ligand-induced RTKs downregulation. One prototype RTK is the epidermal growth factor receptor, EGFR. Upon ligand binding, monomeric receptors rapidly dimerize and catalyse auto-phosphorylation on several C-terminal Tyr residues that recruit signaling molecules at the cell surface. Efficient internalization then occurs by means of clathrin-coated pits that invaginate to form coated vesicles. The first clue to the process underlying the internalization of EGFR came from studies of vulva development in worms. Genetic screening in *C. elegans* identified the SLI-1 protein as an inhibitor of the EGFR-induced differentiation of vulva precursor cells [150]. SLI-1 is a worm ortholog of the mammalian proto-oncogene Cbl [151].

Extensive mutagenesis in the genes encoding both EGFR andc-Cbl, as well as the possibility to reproduce EGFR ubiquitylation *in vitro* showed the sequence of events to be as follows. Binding of EGF to EGFR which stimulates the EGFR tyrosine kinase domain, results in an increase in the phosphorylation of the C-terminal Tyr residues, including a specific Tyr that then serves as a docking site for the TKB domain of Cbl. This interaction triggers the phosphorylation of c-Cbl, resulting in recruitment of an E2 and the activation of c-Cbl, which promotes EGFR ubiquitylation [15]. Thus, c-Cbl acts as an E3, and its RING finger domain is required for this activity [152]. Cbl mutants lacking a functional RING finger domain are unable to mediate receptor ubiquitylation and downregulation. Such mutants include oncogenic variants of Cbl, such as N-Cbl [153].

It was initially suggested that ubiquitylation of EGFR occured in endosomes [154]. However, it was then reported that polyubiquitylated forms of the EGFR receptor accumulated upon addition of EGF

to HeLa cell overproducing a mutant form of dynamin that blocks the internalization step of endocytosis. Thus, conjugaison with ubiquitin must occur at the plasma membrane before recruitment to clathrin-coated pits [155, 156]. It was further shown that Cbl and EGFR associate at the plasma membrane, and remain associated throughout the endocytic pathway [155]. Whether cell surface EGFR ubiquitylation is the key element of receptor internalization, or merely required for later steps of endocytosis is still a matter of controversy in the literature. Overproduction of Cbl accelerates degradation of EGFR, while overproduction of v-Cbl, a dominant negative form of Cbl inhibiting ubiquitylation does not prevent internalization, but favors recycling of internalized EGFR at the cell surface [15, 154]. This led to the suggestion that EGFR ubiquitylation, despite its occurrence at the plasma membrane, is probably required for late events in the endocytic pathway, such as sorting to the internal vesicles of multivesicular bodies (MVB), an ubiquitin-dependent process [3]. This conclusion was further supported by experiments performed with a cell line carrying a thermosensitive E1 enzyme, which inhibited receptor degradation and not internalization [157]. Contrary to initial conclusions that growth factor receptor are polyubiquitylated, experiments performed with antibodies specific against mono- or polyubiquitin, and overproduction of mutant forms of ubiquitin unable to form polyubiquitin chains demonstrated that EGFR is monoubiquitylated on multiples cytoplasmic tail Lys residues (multi-monoubiquitylation) [158, 159]. Fusion of ubiquitin to a truncated form of EGFR lacking its cytoplasmic tail triggers the ability to internalize radio-labeled EGF in constitutive way, but with lower rate than that observed with wild type EGFR [158]. This observation was interpreted as an additional argument in favor of ubiquitylation acting as an internalization signal, and underlined the requirement of multiple monoubiquitins for efficient internalization. A possible way to reconcile the various data and models would be to consider that EGFR can be internalized by redundant internalization pathways [160, 161], one of these being the CIN85/endophilin pathway. In addition to its role in the ubiquitylation of EGFR and various other RTKs (Table II), c-Cbl was indeed reported to play a critical role in the internalization of RTKs by recruiting various components of the endocytic machinery ([162, 163]. Soubeyran and coworkers searched for partners of c-Cbl; they identified CIN85, an adaptor containing three Src-homology 3 (SH3) domains and a proline-rich region (Fig. 1). The binding of CIN85 to Cbl is increased by EGF stimulation. Furthermore, CIN85 constitutively associates with endophilins via its proline-rich domain and thus recruits endophilins to the complex with activated EGFR receptors [162]. In EGF-stimulated cells, EGFR internalization requires association with an intact Cbl-CIN85-endophilin complex. These data convincingly demonstrate the dual role of c-Cbl in the internalization step of endocytosis. In addition to its action as an E3, c-Cbl recruits endophilin *via* CIN85, possibly facilitating the invagination of membrane pits [164]. Furthermore, the Cbl-CIN85-endophilin complex remains associated with EGFR along the endocytic pathway.

3- Role of ubiquitin as an internalization signal, and/or in activating or regulating the endocytic machinery

The mechanism by which ubiquitin directs cell-surface protein internalization is still unknown. Receptors and transporters in yeast lacking sites for posttranslational ubiquitylation in their cytoplasmic

domains are internalized by the fusion in-frame of a ubiquitin molecule lacking lysine residues (monoubiquitylation) [5, 73, 74], and a similar situation was reported for a EGFR-ubiquitin chimera [158]. Ubiquitin does not carry a functional Tyr- or di-Leu-based internalization signal. However, the single fused ubiquitin carries, within its three-dimensional structure, all the information necessary to promote the internalization of cell-surface proteins. Scanning alanine mutagenesis revealed that the signal involved consists of two hydrophobic patches on the surface of the folded ubiquitin protein. Two surface residues, Ile44 and Phe4, are absolutely required for internalization in yeast [73]. Ile44 is also important for ubiquitin-dependent internalization in mammalian cells, but the function of Phe4 is unknown [165]. Interestingly, whereas Ile44 and the surrounding hydrophobic residues are required for both proteasome recognition and endocytosis, a distinct surface region of ubiquitin surrounding Phe4 is required only for endocytosis [166].

3-1. Ubiquitin binding domaisn in endocytic proteins

The question raised by ubiquitin acting as an internalization signal immediately suggested the existence of ubiquitin receptors playing a key role in internalization [167]. This hypothesis appeared strongly conforted by the identification of a number of ubiquitin binding domains in proteins involved in endocytosis. Two ubiquitin-binding motifs, the ubiquitin-associated (UBA) domain and the ubiquitin-interacting (UIM) motif have been identified in the past years, by bioinformatics approaches [168, 169]. The UBA domain consists of about 40 residues and was initially identified in E2s, E3s and other proteins associated with ubiquitylation [168]. It was subsequently demonstrated that several UBA-containing proteins bind proteins modified by ubiquitin, or bind ubiquitin chains (reviewed in [170]). The UIM motif is a stretch of about 20 amino acid residues that probably forms an α-helix. It was originally identified in the S5a/Rpn10 subunit of the proteasome, where it was shown to function as a receptor for ubiquitin chains [171]. A search for sequence similarities revealed the presence of UIMs, often in tandem, in a variety of proteins involved in ubiquitylation and, in trafficking [169]. Several of these UIM- or UBA-containing proteins, including Eps15, Ede1p and epsins, are involved in the endocytic pathway in yeast and mammalian cells [38, 61, 172]. The ubiquitin ligase c-Cbl also possesses a UBA domain [168], as does the yeast Swa2p, a protein required for clathrin assembly/disassembly *in vivo* [173]. Other ubiquitin binding domains more recently identified include the CUE, NZF and GAT domains. A number of publications have summarized our present knowledge of the biochemical and structural properties of all these domains in the presence or absence of linked ubiquitin, and the present understanding of their effect on intracellular trafficking (reviewed in [174]). We will focus below on some points relative to the role of UBA and UIM-containing proteins, a number of which are more specifically involved in the internalization process. But strikingly, very similar observations were made for ubiquitin-binding proteins involved in sorting of ubiquitylated cargoes into MVBs [3].

3-2. Ubiquitylation of ubiquitin-binding proteins

Experiments carried out by van Delft *et al.* showed that, upon stimulation of cells with EGF or transforming growth factor alpha, Eps15 is transiently phosphorylated on tyrosine residues and is modified by monoubiquitylation [175]. It was recently reported that two UIM motifs at the extreme C-

terminus of Eps15 are essential for the monoubiquitylation of this molecule [105], and for the binding of Eps15 to ubiquitin-containing proteins, with a preference for polyubiquitin chains. Mutations in the first UIM motif abolish the monoubiquitylation of Eps15 but do not affect the ability of the protein to bind to ubiquitin-binding proteins, whereas mutations in the distal motif did both [105, 176]. Finally, Eps15 UIM domains are required for ubiquitylation but are not the sites of ubiquitylation [105, 176]. Eps15 ubiquitylation appears to be Nedd4-dependent [105]. Initial attemps at understanding the function of Eps15 ubiquitylation by construction of various mutant forms of Eps15 with deletions of the UIM-containing region, or point mutations that abolish ubiquitylation have not revealed clear effects, as the mutant proteins were correctly targeted to clathrin-coated pits, and internalization of certain receptors was not inhibited [176, 177].

Very similar results were obtained for mammalian epsins, known Eps15. Interestingly, epsin possesses two UIMs (Fig. 1). It was demonstrated that epsin is predominantly monoubiquitylated and that these UIM motifs are necessary for epsin ubiquitylation but that they are not the site of ubiquitylation [178]. Epsin is possibly ubiquitylated in its ENTH domain. This domain interacts with phosphoinositides. This interaction leads to conformational changes, suggesting that it may initiate membrane curvature (reviewed in [179]). Epsin ubiquitylation may thus regulate these events. The observation that mammalian epsin is ubiquitylated confirms earlier genetic reports on *liquid facets*, the *Drosophila* epsin. Mutations in *liquid facets* were found to increase endocytic defects associated with mutations in the gene encoding the de-ubiquitylating enzyme *Fat facets* [180]. Formal prove that Fam, the mammalian homolog of *Fat facet* is indeed the specific ubiquitin isopeptidase responsible for epsin deubiquitylation was recently obtained in experiments where si-RNA-mediated suppression of Fam inhibits *in vivo* epsin deubiquitylation triggered by Ca2+ influx into synaptosomes [181]. These overall data indicate that epsin deubiquitylation plays a key role in the endocytic process that remains to be determined.

Isolated UIMs from both mammalian epsins and Eps15 are sufficient to promote the ubiquitylation of a chimeric glutathione-S transferase (GST)-UIM fusion protein, suggesting that UIMs may serve as a general signal for ubiquitylation [178]. Thus, the same motif in several endocytic proteins may be responsible for ubiquitin recognition and monoubiquitylation, a notion that extends to UIM-containing proteins involved in MVB sorting [3], and to a subset of proteins containing other ubiquitin binding domains. In the case of UIM-containing proteins, a general rule seems to emerge. Ubiquitin attachment occurs outside the UIM domain, is limited to monoubiquitylation, and catalyzed by HECT E3, that can also catalyze polyubiquitylation of different types. One possible interpretation of these observations is that the E3/UIM recognition is mediated by the ubiquitin present in the thiol-ester intermediate of the E3. Once ubiquitin is transferred to a UIM-containg protein, the E3 would dissociate, yielding a monoubiquitylated substrate.

Eps15 and epsins have yeast homologs that have been shown to be involved in endocytosis [38, 61], Deletion of the *EDE1* gene, or of one of the two *ENT* genes, together with a heat-sensitive mutation in the second gene (the deletion of both *ENT* genes is lethal) leads to defective fluid-phase endocytosis and to the defective internalization of Fur4p, Ste6p, and Ste2p, respectively [38, 61, 182]. The yeast epsins, Ent1p and Ent2p, each display two UIMs, and Ede1p, a yeast homolog of the mammalian Eps15, has a C-terminal UBA domain (Fig. 1). The yeast epsins and Ede1p were shown to bind monoubiquitin *in*

vitro, in a way dependent of several conserved UIM residues [182], and the two UIMs cooperate for the interaction [183]. The two UIMs of epsins may display functional redundancy in endocytosis with Ede1p, possibly due to the ubiquitin-binding properties of the UBA domain of Ede1p [182]. Data reported by Aguilar and Wendland slightly modify the view of Ede1p and Ent1/2 properties. These authors showed that Ede1p and Ent1p bind one another, like their mammalian counterpart. The UBA Domain of Ede1p binds yeast membranes in a ubiquitin-dependent way, and the ENTH domain of Ent1p cooperates with the UIMs for membrane recruitment [183]. These data emphasize that the properties of these ubiquitin-binding proteins are intimately linked to their interactions with lipids. Whereas these data are compatible with the attractive hypothesis of Ent/epsin acting as adaptors for ubiquitylated plasma membrane proteins, the formal proof of an interaction *in situ* with a precise ubiquitylated cargo is still lacking, as are informations relative to the affinity of UBA/UIM domains of these proteins for monoubiquitin versus ubiquitin chains (UbK63-based). It will also be important to determine whether Ent1/2p and Ede1p are ubiquitylated, like mammalian epsins and Eps15.

3-3. Other ubiquitylated endocytic proteins

In addition to ubiquitylation of UIM-containing endocytic proteins, a number of other proteins of the endocytic machinery were described to undergo ubiquitylation. The list of examples of substrates/E3 pairs is extending, but clear ideas about the corresponding functions of this modification is often lacking. Examples include once more proteins invi-olved in the internalization step or in later endocytic steps. We already described the link between Rsp5p and endocytic proteins important for the organization of the actin cytoskeleton, and the function still undefined of Rvs167/amphiphysin ubiquitylation [107]. Amphiphysin displays domains (SH3, BAR domain) and properties similar to that of endophilin. A specific endophilin isoform, endophilin A1, was described to undergo ubiquitylation by the Nedd4-like protein Itch, probably at endosomes [184].

Agonist-dependent internalization of G protein-coupled receptors *via* clathrin-coated pits is dependent on the adaptor protein β-arrestin, which interacts with elements of the endocytic machinery such as AP2 and clathrin. For the β2-adrenergic receptor (β2-AR) this requires ubiquitylation of β-arrestin by monomeric RING E3, Mdm2. Although the receptor is ubiquitylated, it is β-arrestin ubiquitylation which is critical for its internalization. Two distinct patterns of β-arrestin trafficking within the cell have been observed for different classes of GPCRs. For class A (Ex : β2AR), β-arrestin interacts transiently with the receptor at the cell surface, and for class B (Ex : vasopressin receptor V2R), β-arrestin displays simultaneous trafficking with the receptors from the cell membrane to endocytic vesicles. This differential behavior was shown to rely on different ubiquitylation/deubiquitylation patterns of β-arrestin during trafficking of these two class of receptors. β2AR stimulation leads to only transient β-arrestin ubiquiitylation, whereas V2R stimulation leads to stable β-arrestin ubiquitylation Expression of arrestin-ubiquitin chimera transforms a class A receptor to class B with respect to intracellular trafficking. One possible interpretation could be that persistently ubiquitylated β-arrestin in complex with internalized receptors somehow leads to their MVB sorting, instead of recycling [185]. Mechanisms underlying regulation of β-arrestin deubiquitylation have still to be defined.

Similar features prevail in the case of EGFR internalization. In the CIN85-endophilin-Cbl complex required for EGFR endocytosis, CIN85 itself is massively monoubiquitylated by Cbl after EGF treatment [186]. Both Cbl and CIN85 are then targeted to MVBs and degraded together with EGFR. Strikingly, Cbl-directed CIN85 monoubiquitylation is not specific of a particular Lys residue. Dominant negative forms of CIN85 which delay EGFR degradation display impaired monoubiquitylation, indicating that this modification plays a critical role in EGFR endocytic pathway, likely for both internalization, and MVB sorting. These data are rather similar to that reported for β2-AR and β-arrestin, since in both case there is a ligand stimulated ubiquitylation simultaneously for the endocytic cargoes, and for a corresponding adaptor. In addition,

Conclusion and future perspectives

The ubiquitylation of yeast cell-surface proteins clearly acts as a signal triggering their internalization. Todate, however, the adaptors recognizing ubiquitin or ubiquitin chains in endocytic cargoes had not been formerly identified, even if the Ent/epsins are likely candidates. In mammalian cells, the ubiquitylation of plasma membrane proteins appears to play a distinct role in the endocytic pathway. Although ubiquitylation often takes place at the plasma membrane, it may (ENaC, MHC class I, various transporters), or may not (GHR, IL2R, GPCRs) be required for internalization, and some cases are still discussed (EGFR). Thus, mammalian cells may have developed a more complex and sophisticated ubiquitin-dependent internalization system than that of yeast. The internalization of some receptors seems to require not their ubiquitylation, but interaction with the ubiquitin conjugation system (GHR), or with another protein. The ubiquitylation of these ancillary proteins triggers the internalization of the receptor. Interaction of these proteins with the receptors may be direct (ß-arrestin) or indirect (CIN85, which recruits Cbl). Hence, mammalian cells display variations on the common theme of ubiquitin-linked internalization according to the cell-surface protein considered. For both yeast and mammalian cells, strikingly, several of the E3s involved in endocytosis display multiple protein and lipid interacting domains, and appear to have a dual role both as ubiquitylating enzymes, and as adapters recruiting elements of the endocytic machinery. For several of these enzymes, understanding the mechanisms underlying substrate recognition is not yet achieved, and a major unanswered question concerns how the decision is made to as to whether mono- multi- or polyubiquitylate a substrate.

Current investigations into the role of UIM and UBA domains in endocytosis in yeast and mammalian endocytosis emphasize the close link between ubiquitin-binding domains and endocytosis, but a common overview is still lacking. Informations obtained on various organisms, together with findings concerning the crystal structure of the ENTH domain, and its role in membrane curvature strongly suggested that epsins may act as endocytic adaptors: they bind clathrin, possibly endocytic ubiquitylated cargoes, and phospholipids likely in a way regulated by their own ubiquitylation [187]. In addition, they also bind other protein components of the endocytic machinery. The prevailing model could be that epsins may interact, *via* their UIMs, with ubiquitylated endocytic cargoes. This interaction may result in the attraction of these cargoes to regions of membrane curvature, ultimately stimulating the formation of clathrin-coated

vesicles in these regions. This model is especially attractive in yeast, in which endocytic adaptors are not yet formerly identified. In mammalian cells, epsins may also correspond to the missing adaptor for EGFR, linking either ubiquitylated receptor, or ubiquitylated EGFR partners. Rather than delineating two endocytic pathways in yeast and higher eukaryotic cells, this model highlights the possibility that very similar processes may be involved in some endocytic pathways in all eukaryotes, together with the crucial role played by ubiquitin-dependent interactions. In spite of the multiple recent progress in dissecting ubiquitin-depedent endocytosis, the above appealing model is far from demonstrated. We already have many informations relative to interactions of proteins involved in endocytosis, and the role of ubiquitylation processes leading to possible changes in activity, location, or interactions of the various partners. But the assembly of the endocytic machinery is, by necessity, a cooperative process, given the low affinity of interactions between individual binding partners. The main challenge for the future will be to determine the order of assembly of the components of the endocytic machinery, and the regulation of these associations.

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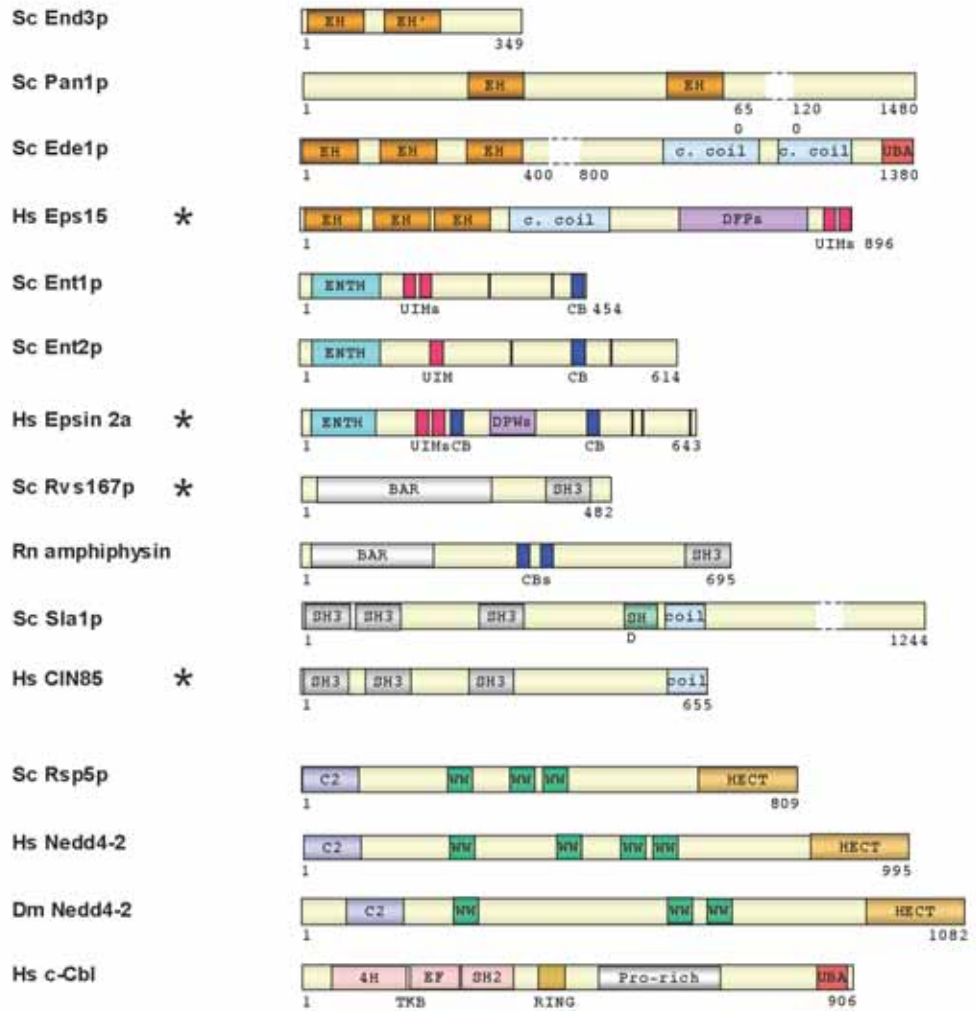
Legends of Figure and Tables

Fig. 1: Schematic representation of proteins involved in endocytic internalization and ubiquitylation processes drawn to scale with their main domains and motifs.
BAR: domain with unknown function. CB: clathrin-binding domain. TKB: Tyrosine Kinase Binding domain. 4H: four helix. EF: EF-hand domain. SH2: Src homology 2 domain. DPF/DPW: Asp-Pro-Phe/Asp-Pro-Trp sequences that bind AP2. EH: Eps15 homology domain, pair of EF hand motifs that recognise proteins containing Asn-Pro-Fhe (NPF) sequences. ENTH: Epsin N-terminal homology domain. RING: RING-finger domain that exhibits binding activity towards E2 enzyme. SH3: Src homology 3 domain, sequence that binds Pro-rich sequences containing a PXXP core-conserved binding motif. UIM: ubiquitin interaction motif. UBA: ubiquitin-associated domain. Black vertical line: Asn-Pro-Phe (NPF) sequences. Asterisk : the protein is ubiquitylated. For the various domains of Rsp5p/Nedd4 and c-Cbl: see text. Dm: *D. melanogaster*, Hs: *H. sapiens*; Rn: *Rattus novegicus* Sc: *S. cerevisiae*. Domains indicated are those designated by Pfam (available on the WWW at pfam.cgb.ki.se) or SMART (available on the WWW at smart.embl-heidelberg.de).

Table I: Yeast proteins required for the endocytic internalization of receptors and transporters. (I) Proteins in connection with the cortical actin cytoskeleton; (II) Proteins involved in lipid biosynthesis; (III) Proteins involved in ubiquitylation; (IV) Proteins with no obvious connection with actin cytoskeleton, or lipid biosynthesis, or ubiquitylation.

Table II: Ubiquitylated plasma membrane proteins in higher eukaryotic cells.
RTKs: Tyr-kinase receptors; R-S/TKs: Ser-Thr kinase receptors; RK-associated: receptors associated with kinases; GPCRs: G-protein-coupled receptors. *E3s of the Nedd4 family, °RING finger E3s, "PHD-containing E3s.

	Yeast proteins	Mammalian homologs	comments	References
I	Act1p/End7p	actin	-	[55, 56]
	Arp2	Arp2	actin-related protein	[188]
	Arp3	Arp3	actin-related protein	Marchal and Urban-Grimal, unpublished
	Arc35p/End9p	Arc35	Arp2/Arp3 coomplex	[189, 190]
	Cmd1p	calmodulin	-	[191]
	Myo3p and Myo5p	Type I myosin	-	[192]
	Sac6p	Fimbrin	-	[56]
	End3p	-	EH domain	[193, 194]
	Pan1p/Dim2p	-	EH domain	[195, 196]
	Ede1p	Eps15	EH and UBA domains for Ede1 UIM domain for Eps15	[61]
	Ent1p and Ent2p	Epsin1 and 2	ENTH and UIMs domains	[37, 38]
	Sla1	CIN85	-	[107]
	Sla2p/End4p/Mop2p	HIP1, HIPR	-	[193, 197]
	Rvs161p/End6p	amphiphysin	BAR domain	[55]
	Rvs167p	amphiphysin	BAR domain	[55, 107]
	Sjl1p, Sjl2p, Sjl3p	synaptojanin	-	[198]
	Las17p/Bee1p	WASP	-	[199, 200]
	Vrp1p/End5p	WIP/verprolin	-	[55]
	Akr1p and Akr2p	ankyrin	Ankyrin repeat	[201]
	Ark1p	-	kinase	[202]
	Prk1p	-	kinase	[203]
	Ypk1p and Ypk2p	SGK1	kinase	[204]
II	Lcb1p/End8p	-	ceramide biosynthesis	[189]
	Erg2p/End11p	-	ergosterol biosynthesis	[189]
III	Rsp5p	Nedd4 family	E3 HECT enzyme	[66]
	Ubc1p, Ubc4p, Ubc5p	Ubch…	E2 enzyme	[21]
	Yck1p and Yck2p	-	kinase	[71, 94, 202]
	Bul1p and Bul2p	-	PxY motif	[72]
IV	Chc1p	Clathrin heavy chain	-	[52, 205]
	Clc1p	Clathrin light chain	-	[206]

Proteins	E3	References
<u>Channels</u>		
ENaC	Nedd4-2*	[24, 117, 207]
rH1	Nedd4*	[118]
ClC5	Nedd4*	[119]
<u>Ligand-gated ion channel</u>		
GlyR		[208]
GluR		[209]
<u>Transporters</u>		
SN1	Nedd4*	[125]
EAAT1	Nedd4*	[126]
NaPi	Nedd4*	[127]
<u>Tyr-kinase receptors</u>		
c-Met	Cbl°	[163, 210]
PDGFRß	Cbl°	[211, 212]
EGFR	Cbl°/ Cbl-c° + AIP4/Itch*	[15, 128, 154, 213]
CSF-1R	Cbl°	[214, 215]
Notch	Cbl° + Itch*	[129, 132, 216]
c-kit		[217]
<u>Ser/Thr-kinase receptors</u>		
TGFß	Smurf2* (+Smad7)	[218]
<u>Cytokine receptors</u>		
GHR		[135-137, 142]
IFNAR1 / IFNAR2	SCF^HOS	[16]
IL-2R		[144]
Prolactine R		[219]
<u>GPCRs</u>		
Rhodopsin		[220]
V2		[221]
CXCR4	AIP4*	[121, 123]
ß2-AR	Mdm2°	[222]
<u>Other proteins</u>		
Comm	DNedd4*	[133]
E-cadherin	Hakaï°	[223]
<u>Immune recognition</u>		
MHC Class I	KK3/MIR1" / KK5/MIR2" MARCH-IV"/MARCH-IX" M153R"	[145, 146] [148] [147]
B7.2	MIR1/MIR2"	[145]
ICAM-1	MIR1/MIR2"	[145]
T-cell coreceptor CD4	M153R"	[147]
TCR	Cbl°	[224, 225]
TCR ζchain		[226]
FcεRI		[227, 228]