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*Topical Review*

# Ubiquitination and Endocytosis of Plasma Membrane Proteins: Role of Nedd4/Rsp5p Family of Ubiquitin-Protein Ligases

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**Abstract.** In addition to its well-known role in recognition by the proteasome, ubiquitin-conjugation is also involved in downregulation of membrane receptors, transporters and channels. In most cases, ubiquitination of these plasma membrane proteins leads to their internalization followed by targeting to the lysosome/vacuole for degradation. A crucial role in ubiquitination of many plasma membrane proteins appears to be played by ubiquitin-protein ligases of the Nedd4/Rsp5p family. All family members carry an N-terminal $Ca^{2+}$-dependent lipid/protein binding (C2) domain, two to four WW domains and a C-terminal catalytic Hect-domain. Nedd4 is involved in downregulation of the epithelial $Na^+$ channel, by binding of its WW domains to specific PY motifs of the channel. Rsp5p, the unique family member in *S. cerevisiae,* is involved in ubiquitin-dependent endocytosis of a great number of yeast plasma membrane proteins. These proteins lack apparent PY motifs, but carry acidic sequences, and/or phosphorylated-based sequences that might be important, directly or indirectly, for their recognition by Rsp5p. In contrast to polyubiquitination leading to proteasomal recognition, a number of Rsp5p targets carry few ubiquitins per protein, and moreover with a different ubiquitin linkage. Accumulating evidence suggests that, at least in yeast, ubiquitin itself may constitute an internalization signal, recognized by a hypothetical receptor. Recent data also suggest that Nedd4/Rsp5p might play a role in the endocytic process possibly involving its C2 domain, in addition to its role in ubiquitinating endocytosed proteins.

**Key words:** Ubiquitin — Endocytosis — Ubiquitin protein ligase — Nedd4/Rsp5p — Plasma membrane proteins

## Introduction

Ubiquitination (or ubiquitylation) of proteins is a post-translational modification in which a 76 amino acid polypeptide, ubiquitin, or a multiubiquitin chain, is attached onto proteins and tags them for degradation (reviewed in [22]). The process is carried out by the sequential action of E1, E2 and E3 activities. The E1 (ubiquitin-activating) enzyme first activates ubiquitin in an ATP-dependent reaction by forming a thioester bond at its active-site cysteine with the COOH-terminus of ubiquitin. Ubiquitin is then transferred to the active site cysteine of a ubiquitin-conjugating (carrying) enzyme (E2), and then sometimes to the active site cysteine of a ubiquitin-protein ligase (E3). In a last reaction, catalyzed either by an E2 with the help of an E3, or directly by an E3, an isopeptide bond is formed between the C-terminal Gly of ubiquitin, and the ϵ-amino group of a Lys residue on the target protein [22]. The generation of multiubiquitin chains is often (but not always) mediated by the repeated attachment of ubiquitin onto Lys48 of another ubiquitin or of a growing ubiquitin chain. The E3 enzymes therefore carry out the important task of target recognition. The best known E3 classes include (i) the RING-finger

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containing proteins or complexes, such as the anaphase promoting complex (APC or cyclosomes), the SCF complex, the VCB-like complex (reviewed in [173]) and c-Cbl [180], and (ii) the Hect domain family of E3s [71]. The Hect domain E3s include the Nedd4/Rsp5p family of ubiquitin-protein ligases (Rsp5p is also known as Npi1p or Mdp1p), which are the main focus of this review. In some cases, an E4 enzyme is involved in polyubiquitination of proteins already carrying several (up to 3) ubiquitin moieties [79]. Multiubiquitination of numerous cytosolic and ER proteins leads to their degradation by the 26S proteasome, a large multisubunit protease complex composed of a 20S core and two 19S regulatory subunits flanking it [43]. Degradation of a large number of cellular proteins is carried out by the proteasome, including numerous cell cycle proteins (in which time-dependent regulation of the amount of protein in the cell is critical), transcription factors, signal transduction proteins, enzymes of metabolic pathways and MHC-I antigen complexes [58]. Furthermore, many aberrantly expressed or misfolded proteins in the ER are also degraded by the proteasome following their exit from the ER into the cytosol [156]. In addition to the E1–E4 enzymes, de-ubiquitination enzymes (UBPs and UCHs) play an important role both in the release of ubiquitin from lysine residues preceding protein degradation, thus replenishing the cellular pool of free ubiquitin, and in ubiquitin biosynthesis and processing [22, 184].

In recent years, it has become apparent that in addition to cytosolic and ER proteins, numerous transmembrane proteins are also ubiquitinated, and that their ubiquitination plays a pivotal role in their endocytosis and subsequent degradation, often in the lysosome (vacuoles in yeast) [13, 59, 60, 166]. The nature of this ubiquitination, how it signals endocytosis of these cell surface proteins, and how it is regulated by the Nedd4/Rsp5p family of ubiquitin-protein ligases both in yeast and mammalian cells, is the focus of this review.

## Nedd4/Rsp5 Family of Ubiquitin-Protein Ligases

The Nedd4/Rsp5p family belongs to the Hect-domain superfamily of E3 enzymes, the only known E3 that form ubiquitin-thioester intermediates and directly catalyze substrate ubiquitination. All family members are comprised of a variable N terminus, a C2 domain, 2 to 4 WW domains and a C terminal Hect domain (Fig. 1) [53].

The C2 domain, first identified in $Ca^{2+}$-responsive isoforms of Protein Kinase C (PKC), is a ~120 amino acid long domain demonstrated to bind phospholipids and membranes in a $Ca^{2+}$-dependent manner in several proteins (e.g., synaptotagmin, cytosolic Phospholipase A2, Phospholipase C, rasGAP) (reviewed in [111]), including Nedd4 [126], and hence believed to play a role in membrane targeting, intracellular localization and trafficking of proteins. The domain is composed of 2 four-stranded β sheets [132], with conserved aspartates, located in connecting loops, which are the main coordinators of $Ca^{2+}$ ions binding [132]. In addition to phospholipids, several C2 domains, including that of Nedd4 (*see below*), have been reported to bind proteins in a $Ca^{2+}$-dependent fashion. For example, the second C2 domain of synaptotagmin I binds clathrin AP-2 [190] and rasGAP-C2 domain binds annexin VI [24].

The WW (or WWP) domain is a small (~40 aa) protein:protein interaction module found in diverse signaling and other proteins [4, 14, 63, 124]. It is composed essentially of 3 β strands (antiparallel β sheet) with a hydrophobic binding surface [96, 127], which usually binds proline-rich sequences. So far, 4 types of WW binding motifs have been described: (i) the PY motif (xPPxY, P = Pro, Y = Tyr, x = any amino acid) which binds the WW domains of YAP [21], Nedd4 ([164], *see below*) and other proteins, (ii) the PPLP motif which binds several Formin Binding Proteins (FBPs) [8], FE65 and others, (iii) the PGM motif found in FBP21 [9], and (iv) phosphorylated Ser and Thr in specific sequence motif(s) [93, 107], (J. Noel, *personal communication*). As seen in Fig. 1, the number of WW domains in the Nedd4/Rsp5 or Nedd4-like proteins varies between 2 to 4, suggesting that they can form multiple interactions and complexes with various proteins simultaneously.

The Hect (homologous to E6-AP carboxy terminal) domain, located at the C terminus of all proteins that harbor it (including Nedd4/Rsp5p), is a 350 amino acid long ubiquitin-protein ligase domain first identified in E6-AP which targets p53 for ubiquitination and degradation in the presence of the E6 protein encoded by oncogenic human papilloma viruses (HPVs) [71, 72]. The domain contains an invariant Cys that forms a thioester bond with ubiquitin upon its transfer from E2 enzymes [71, 144]. The Hect domain has at least four known properties: (i) direct binding to E2, (ii) formation of a thioester bond via its active Cys with the ubiquitin moiety transferred from the E2, (iii) transfer of ubiquitin to the ϵ amino group of Lys on the substrate protein and (iv) transfer of additional ubiquitins onto the growing end of the multiubiquitin chain [69]. The E2 best able to bind to and transfer ubiquitin to the Hect domains of Rsp5p/Nedd4 are Ubc4p/Ubc5p (*S. cerevisiae*), and UbcH5 (human) [54, 115], although other E2s (e.g., UbcH6, UbcH7) have been reported to carry out this function, albeit less effectively [84, 147]. The tertiary structure of the E6-AP Hect domain in complex with UbcH7 (the preferred E2 for E6-AP) was recently solved [69]. The crystal structure of the domain reveals a bilobal structure (N and C lobes) with a broad catalytic cleft, which includes the invariant Cys (located at the interface between the two lobes) necessary for transfer of ubiqui-

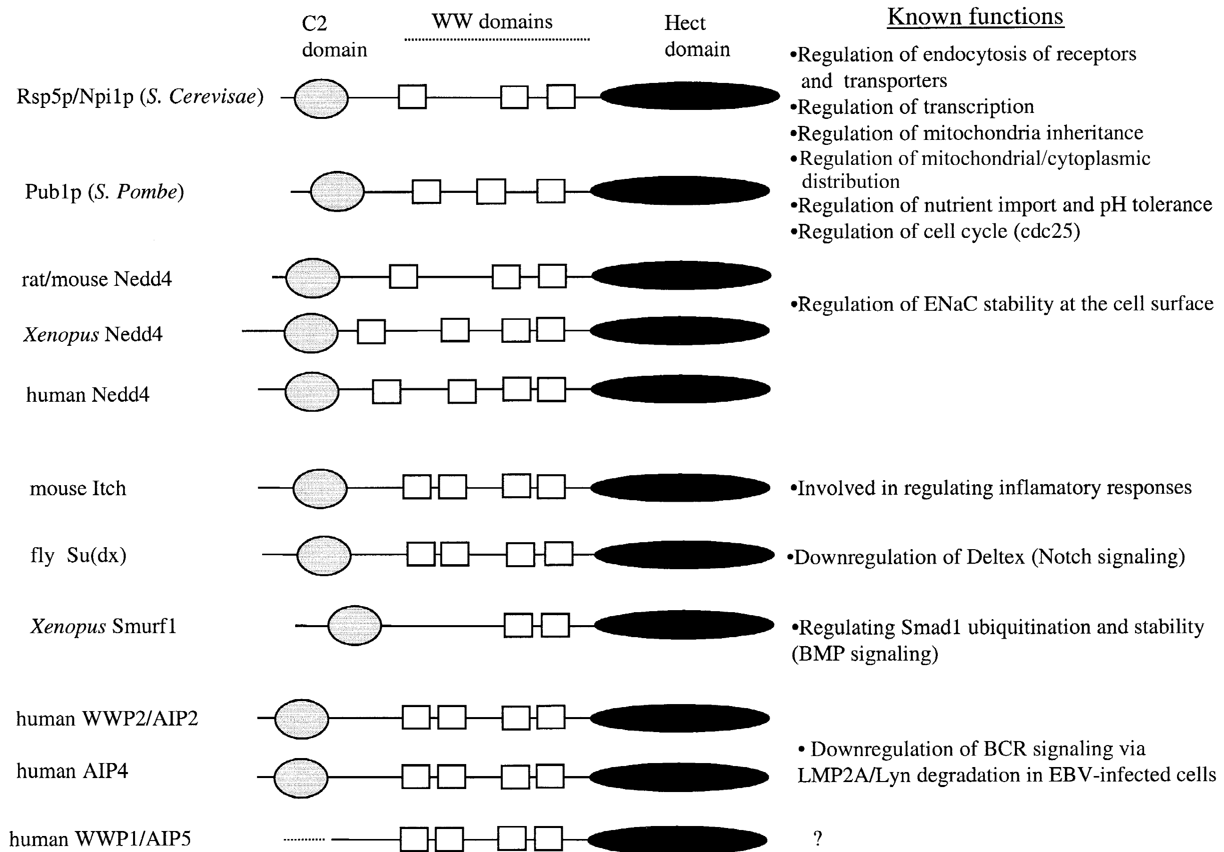


**Fig. 1.** Examples of Nedd4/Rsp5 and Nedd4-like family members.

tin from the E2. The E2 binds to the N lobe, and a Phe conserved in the L1 loop of Hect-specific E2s plays a key role in this binding, hence explaining the specificity of these E2s towards Hect type E3s. Indeed, mutation of the equivalent Phe to Asn in UbcH5 abolished its ability to transfer ubiquitin to Rsp5p [114]. The specificity towards different Hect-E3s (e.g., UbcH7 binds E6-AP whereas UbcH5 binds Rsp5p) may be conferred by the more variable L2 loop of the E2 [69]. The mechanism(s) by which ubiquitin is transferred from the E2 active Cys to the E3 (Hect) active Cys, located quite far apart from each other, is not yet known, and may require extensive conformational changes in the complex [69].

A major function of Nedd4/Rsp5p family members, detailed in this review, is to regulate the stability of several yeast and mammalian transmembrane proteins by ubiquitination, which controls subsequent endocytosis of these cell surface proteins. However, other functions have been ascribed to these proteins, and to several Nedd4-like proteins (Fig 1); these are mentioned here only briefly.

The large subunit of RNA polymerase II (Rpb1) in *S. cerevisiae* was found to associate with Rsp5p [70], an interaction requiring WW2 and 3 of Rsp5p and the C terminus of Rpb1, which contains numerous YSPTSPS motifs [179]; it is not known, however, whether these repeats can bind Rsp5p-WW domains directly, although they can bind the Pin1-WW domain when phosphorylated on Ser residues (J. Noel, *personal communication*). Nonetheless, the interaction (direct or indirect) leads to ubiquitination and degradation of Rpb1 by the proteasome and is enhanced following DNA damage [6], suggesting a regulatory mechanism to impede transcription of damaged DNA, by destroying Rpb1. Rsp5p was also demonstrated to be involved in degradation of the single stranded DNA binding protein Rfa1 in yeast [30], and, independently, to be involved in glucose activation of the plasma membrane $H^+$-ATPase [26]. It also regulates mitochondrial/cytoplasmic distribution of proteins, and mitochondrial inheritance in *S. cerevisiae* [33, 192], an effect dependent on intact Hect domain of Rsp5p. The mechanisms for the involvement of Rsp5p and ubiquitination in mitochondrial inheritance is not known, nor are its targets, but may involve a PY motif-containing protein, Bul1, which binds Rsp5p and stimulates its activity [33, 186, 187]. In *S. pombe,* the Rsp5p homologue Pub1 has been shown to regulate stability not only of nutrient permeases (described below), but also of the tyrosine phos-

phatase cdc25 [112], a key player in cell cycle regulation which dephosphorylates and activates cdc2, thus initiating mitosis [140].

Several Nedd4-like proteins have been identified in recent years [53, 124] (Fig. 1). Examples include Itch, whose disruption in mice causes immunological disorders [121], *Suppressor of Deltex* involved in regulating *Drosophila* Notch signaling [23], and Smurf1, which ubiquitinates Smad1, a PY motif-containing protein mediating signaling via the Bone Morphogenetic Proteins (BMPs) pathway (TGFβ-like) [191], and AIP2 (WWP2) and AIP4, which both bind the PY motifs of the Epstein-Barr virus latent protein 2A (LMP2A), leading to enhanced degradation of LMP2A and its associated tyrosine kinase Lyn, resulting in inhibition of B cell receptor signaling [73].

### Ubiquitin-dependent Downregulation of Yeast Plasma Membrane Proteins: A Central Role for Rsp5p

The yeast endocytic pathway was found to be rather similar to the mammalian endocytic pathway, with internalization, followed by transport to the vacuole/lysosome via early and late endosomes [131]. Studies on endocytosis in yeast have been performed on two types of plasma membrane proteins. One class includes two G protein-coupled receptors for pheromones, comprised of seven transmembrane-spanning segments, extracellular N-terminus and a cytoplasmic C-terminus: the α-factor receptor (Ste2p) and the a-factor receptor (Ste3p). Both undergo constitutive endocytosis and ligand-induced endocytosis. Internalization of the pheromones bound to their receptors initiates a signal transduction cascade that leads to changes in the yeast cells, that are required for mating [130]. The second class of yeast endocytic substrates includes, in addition to two transporters belonging to the ABC-transporter family (ATP Binding Cassette), a number of transporters of the MFS (Multi Facilitators Superfamily), i.e., proteins that have a central hydrophobic core of 10–12 predicted membrane-spanning segments, flanked by hydrophilic domains which presumably face the cytoplasm [3, 113]. The plasma membrane transporters of *S. cerevisiae* play a critical role in growth rate control. Most of them exhibit both constitutive and accelerated endocytosis specifically regulated in each case by factors such as excess substrate, changes in nutrient availability, or stress conditions. The tight regulation of their turnover rate is a key feature in the capacity of this unicellular organism to adapt rapidly to fluctuating extracellular signals and changing nutrient availability [65]. The only case reported so far of a plasma membrane protein whose turnover does not result from endocytosis is the copper-transport protein (Ctr1p), a three-transmembrane spanning protein which undergoes copper-induced proteolysis at the plasma membrane [116].

The first hint of a link between ubiquitination and endocytosis came from studies with Ste6p, a member of the ABC transporter family responsible for the secretion of the yeast-mating pheromone a-factor. It was reported that ubiquitin-conjugated forms of this transporter accumulate at the plasma membrane in endocytosis-deficient mutants. Consistent with a role of ubiquitination in the turnover of the protein, Ste6p was observed to be partly stabilized in cells lacking the ubiquitin-conjugating enzymes Ubc4p and Ubc5p [80].

Yeast genetics then brought a key observation regarding ubiquitin-dependent endocytosis. Multiple earlier studies had been devoted to the analysis of "catabolite inactivation" of many sugar and amino acid transporters, induced in the first case by the addition to the medium of glucose, the preferred carbon source for yeast (reviewed in [65, 85]), and in the second case by the addition of ammonium, preferred over amino acids as a source of nitrogen (reviewed in [48]). In the latter, mutants were isolated that were deficient in this apparent "inactivation", hence their name, *npi1* and *npi2* (Nitrogen Permease Inactivator). Cloning of the *NPI1* gene, essential for viability, revealed that it encodes the ubiquitin-protein ligase Rsp5p [57]. The corresponding report established that ammonium triggered internalization and subsequent degradation of the general amino acid permease, Gap1p, and that the internalization was prevented in *npi1* cells. The same cells were also deficient in stress-induced internalization and subsequent vacuolar degradation of another transporter, the Fur4p uracil permease, a process independent of ammonium. It was shown later on that both Gap1p and Fur4p indeed undergo Rsp5p-dependent ubiquitination, a plasma membrane event required for their internalization [36, 160]. In vivo ubiquitination of Gap1p requires the invariant cysteine of the Rsp5p Hect-domain [161], that was demonstrated to form thioester bond with ubiquitin [70].

Similar data arguing that ubiquitination acts as a signal for internalization were obtained for an expanding list of plasma membrane proteins. Deficiency in either *UBC1/4/5, RSP5,* or both, also resulted in impaired ubiquitination and/or endocytosis of Ste2p and Ste3p, sugar transporters Mal11p, Mal61p and Hxt6/7p, the amino acid permease Tat2p, or the Zinc transporter Zrt1p [7, 41, 61, 82, 94, 101, 137] (Table 1). Likewise, depletion in the intracellular ubiquitin pool resulting from either repression of ubiquitin biosynthesis [137], or from a point mutation or deletion of a single ubiquitin-isopeptidase, Ubp4p/Doa4p, resulted in impaired ubiquitination and internalization of several of these proteins [7, 35, 82, 94, 162, 172]. Interestingly, mutants in *UBP4/DOA4* were identified years ago as "*npi2*" mutants, impaired in am-

**Table 1.** Plasma membrane proteins that undergo ubiquitin dependent endocytosis

A. Yeast (*S. cerevisiae*)

| | Proteins | Ub′n[i] | E2 involved | E3 involved | Mole of Ub′n[e] | Mode of degradation | References |
|---|---|---|---|---|---|---|---|
| receptors | Ste2p (α-factor) | + | *UBC1–4*[a] | *RSP*[a] | Mono (several K) | Vacuole | [61] [172], R. Dunn & L. Hicke (*personal communication*) |
| | Ste3p (a-factor) | + | *UBC1–4*[a] | | Mono+di+tri (3K) (truncated receptor) | Vacuole[f] | [25], [136], [137] |
| ABC transporters | Ste6p (a-factor) | | *UBC1–4*[a] | | Poly | Vacuole + proteasome? | [80], [91] |
| | Pdr5p (multidrug transporter) | + | | | Poly | Vacuole | [28], [29] |
| MFS transporters | Gap1p (general amino acid permease) | + | | *RSP5*[a] | Mono+di+tri (2K)(K63 linked) | Vacuole | [57], [159], [160], [162] |
| | Put4p (proline permease) | | | *RSP5*[c] | | | [48] |
| | Dal5p (allantoate permease) | | | *RSP5*[c] | | | [48] |
| | Gnp1p (glutamine permease) | | | *RSP5*[c] | | | [48] |
| | Tat2p (tryptophan permease) | + | | *RSP5*[a] | Poly | Vacuole[f] | [7] |
| | Hxt6p/Hxt7p (glucose transporters) | | | *RSP5*[b] | | Vacuole | [82] |
| | Gal2p (galactose transporter) | + | | | Poly | Vacuole | [66] |
| | Mal11p (maltose permease) | | | *RSP5*[b] | | Vacuole | [94], [129] |
| | Mal61p (maltose permease) | + | *UBC1–4*[b] | *RSP5*[a] | Mono | Vacuole | [100], [101] |
| | Fur4p (uracil permease) | + | | *RSP5*[a] | Mono+di+tri (2K)(K63 linked) | Vacuole | [35], [36], [57], [177] |
| | Fui1p (uridine permease) | + | | *RSP5*[a] | | | M. Blondel & C. Volland (*personal communication*) |
| | Zrt1p (zinc transporter) | + | | *RSP5*[a] | Mono+di (1K) | Vacuole[f] | [41], [42] |
| | Itr1p (inositol permease) | | *UBC1–4*[bd] | | | Vacuole[f] | [86], [133] |
| | Can1p (arginine permease of *C. albicans* expressed in *S. cerevisiae*) | | | *RSP5*[b] | | | [99] |

monium-induced downregulation of the Gap1p permease [48, 49].

Most significant regarding the link between ubiquitination and endocytosis, sequences were identified in a number of these proteins, whose modification or deletion impaired simultaneously ubiquitination and internalization (Table 2), as detailed below. Additional evidence for a positive role of ubiquitination in the internalization process came from the observation that ubiquitination signals in Ste3p and Ste6p behaved as autonomous degradation signals, that could promote cell-surface ubiquitination and accelerated endocytosis of the stable plasma membrane $H^+$-ATPase Pma1p [81, 138]. As described in the early report on Ste6p ubiquitination, a common

**Table 1** (*continued*). Plasma membrane proteins that undergo ubiquitin dependent endocytosis

B Mammaliam[g]

| Proteins | Ub′n | E2 involved | E3 involved | Mode of Ub′n | Mode of degradation | References |
|---|---|---|---|---|---|---|
| ENac | + | UbcH5 (?) | Nedd4 (Hect) | Poly (state of ub′n at the cell surface not known) | Lysosome + Proteasome? | [164], [165] |
| PDGFRβ<br>EGFR<br>CSF-1R | +<br>(h) | Ubc4<br>(?) | c-Cbl<br>(ring finger) | Poly | Lysosome + Proteasome | [38], [89], [103, 104], [180] |
| c-Met | + (h) | | | Poly | Proteasome | [76] |
| GHR | + (h) | | | Poly | Lysosome + Proteasome | [166;167] [174] |
| TCR | + (h) | | | Poly+mono | Lysosome | [19] |

[a] Effect on ubiquitination and endocytosis/degradation reported.
[b] Only effect on endocytosis/degradation reported.
[c] Only effect on ammonium-induced catabolite inactivation reported.
[d] Restricted to the case of endocytosis in exponentional growth.
[e] Indicates in each case the probable number of ubiquitin moieties per target lysine "poly" describes the observation of high MW ubiquitin-conjugates.
[f] Absence of proteasomal degradation not yet reported.
[g] Partial list only.
[h] Ligand induced.
[i] Ubiquitination reported.

feature of most of these receptors and transporters is their accumulation in ubiquitinated forms at a restrictive temperature in various internalization-defective mutants, indicating that the ubiquitination event precedes the internalization step of endocytosis.

Given the common degradation of ubiquitinated proteins by the proteasome, it was crucial to determine the proteolytic system involved in the degradation of all these plasma membrane proteins. This was investigated for many of them with similar experimental approaches. The α-factor receptor, the ABC transporter Pdr5p, or the MFS tranporters Gal2p, Fur4p, or Mal11p display normal constitutive and induced degradation in mutants affected in catalytic or regulatory proteasome subunits (respectively *pre1pre2,* and *yta5* or *cim3cim5*) [29, 36, 61, 66, 82, 129][1], but strongly reduced degradation in mutants defective in vacuolar proteases activities (*pep4, pep4prb, or pra1pra2*) [7, 25, 29, 36, 61, 66, 80, 82, 129, 143, 160, 177]. In these vacuolar mutants, a subset of these proteins, which undergo constitutive internalization, were also detected in the vacuole/lysosome using immunostaining. For example, the Fur4p transporter accumulates in the vacuoles of *pep4* cells as an entire protein [36]. This indicates that uracil permease does not undergo proteolytic processing on its way from the plasma membrane to the vacuole, suggesting that the proteasome is not involved even in partial proteolysis of the protein.

Thus, it clearly appears that many yeast plasma membrane proteins undergo regulated cell-surface ubiquitination which triggers their entry into the endocytic pathway, followed by their targeting for vacuolar degradation (reviewed in [13, 59, 60]). The key player in this post-translational modification appears to be the ubiquitin-protein ligase Rsp5p, which has been shown to be involved in constitutive and regulated ubiquitination of a growing list of proteins (*see* Table 1). It is noteworthy that the arginine permease of *C. albicans* is also internalized in an *RSP5*-dependent manner when expressed in *S. cerevisiae* [99]. This general role of the proteins of the Nedd4/Rsp5p family in ubiquitin-dependent endocytosis extends to yeast species other than *S. cerevisiae.* It was recently demonstrated that Pub1, one of the two Rsp5p homologues identified in fission yeast [53], is essential for ammonium-induced downregulation of amino acid permeases [78]. The pleiotropic effect of a pub1-1 mutation suggested that Pub1 might control the turnover rate of multiple transporters [141].

Despite the fact that Rsp5p is clearly required for the in vivo ubiquitination of a growing list of yeast plasma membrane proteins, a direct interaction between any of

[1] Contradictory results have been reported for Ste6p, with the description that in addition to vacuolar degradation, Ste6p could [91], or not [81], also undergo proteasomal degradation.

**Table 2.** Ubiquitination motifs of plasma membrane proteins

| Protein | Sequence | References |
|---|---|---|
| Yeast | | |
| Ste2p (truncated) | SINNDAKSS (C-t) Includes a critical lysine | [62] |
| Ste3p | Large PEST-like (58 AA) (C-t) (including target lysines) | [136;138) |
| Ste6p | Large acidic A-box (52AA) including a DAKTI signal (internal linker region) | [81] |
| Fur4p | PEST-like (20 AA) (N-t) Preceded by two target lysines | [97–98] |
| Tat2p | 31AA (N-t) including critical lysines | [7] |
| Zrt1p | A critical lysine (AA 195) in an internal loop | [41] |
| Mammalian | | |
| αβγENaC | xPPxY (PY motif) (Nedd4-WW domain binding sites) | [164] |
| GHR | SWVEFIELD (UbE motif) | [45] |
| PDGFR | C terminal Tyr- | [77; 103, 104] |
| EGFR | phosphorylation sites | [90;180] |
| CSF-1R | c-Cbl-SH2 domain binding sites?) | [88] |

these cell surface proteins and Rsp5p has not yet been demonstrated, and data on in vitro ubiquitination have not been reported for any of these substrates.

In agreement with an involvement of Rsp5p in the ubiquitination of plasma membrane proteins, ubiquitination and/or endocytosis of a subset of these Rsp5p targets have been shown to be partly or strongly impaired in strains deleted for one or two of the Ubcp belonging to the *UBC1/4/5* family (Table 1), which display in vitro interactions with Rsp5p [147]. These three genes are differentially regulated during growth. Two of the corresponding genes are dispensable for viability, as long as the third one is present, but cells disrupted for both *UBC4* and *UBC5* exhibit extremely slow growth properties and stress sensitivity [150, 151] At least in one case, that of Fur4p, neither ubiquitination nor endocytosis of the protein was found to be impaired in *ubc4*Δ*ubc5*Δ or *ubc1*Δ cells [35], suggesting that if *UBC1/4/5* family is involved in ubiquitination, expression of only one member is sufficient.

## Role of Nedd4 in Regulating Ubiquitination, Endocytosis and Degradation of Mammalian Cell Surface Proteins

Perhaps the best characterized example of a plasma membrane protein regulated by Nedd4 is the epithelial $Na^+$ channel (ENaC). ENaC is responsible for salt and fluid reabsorption in the distal nephron, distal colon and lung epithelia, and abnormal elevation of its activity is associated with hypertension. The channel is composed of 3 partially homologous subunits (αβγ) [16, 17], each containing a proline-rich region at its C terminus [139], which includes a PY motif (xPPxY) [146, 164]. Deletion or mutation of the PY motifs of β or γ ENaC cause Liddle's syndrome, a hereditary form of arterial hypertension [50, 51, 74, 154, 169] characterized by an abnormal increase in ENaC activity which is caused by increased retention and opening of the channel at the cell surface [32, 145, 146, 155]. These PY motifs serve as binding sites for the WW domains of Nedd4, and mutations that cause Liddle's syndrome also abrogate binding to this E3 enzyme [164]. In accord with its ability to associate with Nedd4, ENaC was found to be regulated by ubiquitination, which takes place primarily on a cluster of Lys residues at the N terminus of the γ (but also on the α) subunit; replacing these lysines with arginines leads to impaired channel ubiquitination and increase in channel numbers (channel retention) at the cell surface [165]. The final proof that Nedd4 is indeed a suppressor of ENaC came recently, when it was demonstrated that overexpression of this E3 leads to an inhibition of channel activity by reducing the number of channels at the cell surface, an effect not seen upon overexpression of a catalytically inactive Nedd4 (bearing C-S mutation in the Hect domain) or just the Nedd4-WW domains alone. Importantly, the inhibition by Nedd4 is impaired in channels lacking one or all of their PY motifs [2, 44, 53], demonstrating that Nedd4 is an inhibitor of ENaC which exerts its (Hect domain-dependent) effect by binding of its WW domains to the PY motifs of the channel, and that its ability to suppress the channel is impaired in Liddle's syndrome.

In addition to the WW domains responsible for substrate specificity of Nedd4, the intracellular localization of this protein likely plays a role in the choice of its substrates as well. Recent studies have demonstrated that the C2 domain of Nedd4, which binds membrane and phospholipids in a $Ca^{2+}$-dependent fashion, is responsible for localizing Nedd4 to the plasma membrane in response to elevation of intracellular $Ca^{2+}$ levels [126]. In polarized MDCK cells, this localization is primarily apical [126], which is the location of ENaC. Indeed ENaC activity is inhibited upon increasing intracellular $Ca^{2+}$ concentrations [75, 118]. Interestingly, the capacity of Nedd4 to "choose" the apical membrane in these cells is conferred by the ability of its C2 domain to associate in a $Ca^{2+}$-dependent manner with annexin XIIIb, a protein enriched in apical rafts [125].

The regulation of ENaC by Nedd4 may represent a more general mechanism for the control of cell surface proteins which possess PY motifs. In support of this notion, the cardiac voltage-gated $Na^+$ channel (rH1),

which contains a PY-motif at its cytoplasmic C-terminus, is negatively regulated by Nedd4 when expressed in *Xenopus* oocytes [1]. Other potential candidates include various PY motifs-containing plasma membrane proteins, or those that interact with an adapter protein that in turn binds Nedd4. An example of the former may be Integral membrane protein Deleted in Digeorges syndrome (IDD) [178], which was found to interact with one of Nedd4-WW domains in an expression library screen (N. Pham & D. Rotin, *unpublished results*). An example of the latter is the Insulin-like Growth Factor Receptor (IGF-1R), which binds Grb10 [106], which in turn binds Nedd4 [105]. Indeed, IGF-IR (but not Grb10) was shown to be ubiquitinated [105, 148], raising the possibility that the association with Grb10 may allow Nedd4 to access the IGF1 receptor.

## Signals for Ubiquitination/Endocytosis of Plasma Membrane Proteins Downregulated by the Nedd4/Rsp5p E3s

While the mode of interaction between Rsp5p and its plasma membrane substrates is still unclear, as indicated above, the presence of PY motifs in ENaC allows regulation of channel stability at the cell surface by direct binding of Nedd4-WW domains, presumably leading to ubiquitination of the channel by this E3 enzyme. Thus, the PY motifs can be viewed as internalization signals. Indeed, these motifs have been proposed to serve as internalization motifs which target the channel for clathrin-mediated endocytosis [153, 155], with subsequent channel degradation primarily in the lysosome [165]. This provides further support for the notion that ubiquitination and endocytosis of ENaC are linked, as seen for the yeast permeases and receptors.

The classical endocytic signals identified in mammalian plasma membrane proteins (e.g., YxxΦ [Φ = bulky hydrophobic amino acid such as Leu], NPxY, di-Leu) are known to bind specific subunits of the AP2 adapter complex (e.g., YxxΦ) [134]. Rare cases of similar signals have been identified in yeast. One is a linear sequence with aromatic residues, NPFTD, that is required for ligand-dependent, clathrin-dependent, endocytosis of the a-factor receptor [170]. A second case is a di-Leu motif required for endocytosis of the Gap1p permease [56]. In neither case are the potential interacting molecules identified. All other cases reported so far involve ubiquitination signals, as detailed hereafter. Early extensive work was devoted to determining the endocytic signals in the α-factor receptor, by tracing α-factor internalization of truncated or mutated receptors. Surprisingly, a nonclassical signal, $SINNDAK_{337}SS$, was found to be necessary and sufficient for endocytosis of a C-terminally truncated Ste2p [135]. Within this sequence, the K337 residue was found to be necessary. Many additional data on the fate of this truncated receptor now lead to a better understanding of these early observations. Ste2p undergoes basal constitutive phosphorylation. α-factor binding induces a conformational change, leading to rapid (within 5 min) hyperphosphorylation on Ser and Thr residues [128]. Serines of the SINNDAKSS sequence are required for constitutive receptor phosphorylation, which is a prerequisite for ligand-stimulated hyper-phosphorylation, at or near the SINNDAKSS sequence. This phosphorylation event requires the activity of the casein kinases I homologues, Yck1p and Yck2p, suggesting that these kinases play a direct or indirect role in phosphorylating the receptor. Ligand-induced hyperphosphorylation of Ste2p precedes and regulates ubiquitination which occurs on K337 in the context of the truncated receptor [61, 62, 172], and is one of the major ubiquitination sites in the full length receptor [172].

A similar motif to the SINNDAKSS sequence, DAKTI, was identified in Ste6p. This motif is within a 117-amino acid long linker region connecting the two homologous halves of this ABC transporter, that was found to be important for the fast constitutive turnover of this protein, hence its name, "D (destabilizing) box." The D-box contains an acidic region, 52 amino acids long (including the DAKTI motif), which is required for ubiquitination of the receptor. Even though the DAKTI motif seems to be an important part of this signal, a Lys to Arg mutation within it had minor effect on Ste6p turnover, suggesting the involvement of additional amino acids. Accordingly, when used as a transferable signal, the entire D-box is required for triggering ubiquitination and destabilization of the plasma membrane $H^+$-ATPase [81].

The importance of acidic regions, and more precisely PEST-like sequences, as ubiquitination signals, was further illustrated by studies on Ste3p [138]. PEST sequences, i.e., sequences enriched in Pro, Glu, Ser and Thr, and bordered by positively charged residues, have been identified by statistical means as signals for protein instability, and some of them have indeed been demonstrated to be involved in the ubiquitination of regulatory short-lived proteins [58]. The C-terminal tail of Ste3p contains two regions that are responsible for constitutive and pheromone-induced endocytosis, respectively. The NPFTD signal described above, required for a-factor-dependent internalization was proposed to act as an endocytic signal independently of ubiquitination [170], although it was also established that a-factor induced Ste3p ubiquitination [137]. A 58-residue region is important for constitutive endocytosis, including a 36-residue long PEST-like sequence which is necessary and sufficient for ubiquitination and fast turnover of the receptor [138]. Extensive mutagenesis within this sequence has shown that the signal for ubiquitination is large and complex,

potentially composed of redundant functional elements. A truncated form of the receptor, lacking a region including the a-factor dependent endocytic signal, undergoes phosphorylation in the PEST-like region, that depends on Yck1p/Yck2p. This modification is required for subsequent ubiquitination of the receptor [31] which occurs on three lysines within the PEST sequence that function redundantly, with one of them playing a predominant role [136].

Another example of the critical role of a PEST-like sequence for ubiquitination of a plasma membrane protein has been observed in the case of Fur4p. This protein undergoes constitutive turnover in growing cells, and accelerated turnover in the presence of excess substrate [149], or under stress conditions [36, 177]. Fur4p carries in its N-terminus a 20-residue long PEST-like sequence (KSSGSNITTEVYEASSFEEK) that plays a critical role in its constitutive and accelerated turnover[2]. Replacing the 5 serines in this sequence by alanines progressively impairs phosphorylation and ubiquitination, and stabilizes Fur4p at the plasma membrane, whereas the progressive replacement of the 5 alanines by glutamic acid residues, which mimic phosphorylation, promotes the reverse effect. These data suggest that Ser residues within this PEST sequence are phosphoacceptors, and that phosphorylation within this sequence is a prerequisite for Fur4p ubiquitination [98]. At least two serines in this PEST region are potential phosphorylation sites for casein kinase I, and phosphorylation and ubiquitination of Fur4p is indeed partly dependent on Yck1p/Yck2p [97]. It was demonstrated that Fur4p is ubiquitinated on only two target lysines [35], both closely preceding the PEST sequence [97]. This situation is reminiscent of that described for the soluble IκBα, where ubiquitination takes place on two adjacent lysines located only 10–14 residues away from the two Ser residues which are part of a short phosphorylation-dependent recognition element for the IκB-ubiquitin ligase [185].

Hence, most of the prevailing “endocytic” signals identified in yeast plasma membrane proteins correspond to signals required for ubiquitination of these proteins. So far, the only exceptions are possibly the NPFTD signal in Ste3p [170], the C-terminal di-Leu motif and nearby sequences in the Gap1p permease. Point mutations of the di-Leu signal, or small deletions in this region impair endocytosis but not ubiquitination of the Gap1p permease, suggesting an involvement of this region of the protein in the endocytic process at a step subsequent to ubiquitination [56, 160]. The identified ubiquitination signals are acidic sequences, rather long, most of them carrying redundant information. A few examples are already available, either with target Lys residues included or closely linked to the possible recognition signals. Obviously, these sequences do not include any PY motifs which could accommodate direct binding of Rsp5p-WW domains. A common theme of the above ubiquitination signals, at least for Ste2p, Fur4p and Ste3p, is that their acidity is potentially modulated by phosphorylation events [62, 98], and [31]. A serine critical for glucose-induced Mal61p downregulation has also been identified, although its role in ubiquitination is not known [15], and most if not all yeast receptors and transporters are indeed phosphorylated [27, 83, 100, 117, 123, 128, 137, 163, 176]. A phosphorylation-based recognition signal is an appealing hypothesis, since ubiquitination and/or endocytosis of so many receptors/transporters is most often regulated by ligands, substrates, and/or changes in external nutrients [7, 42, 52, 62, 82, 129, 137, 149, 160][3]. Moreover, in the specific case of Ste2p, it was clearly demonstrated that the ligand induces hyperphosphorylation of the receptor, which in turn triggers its ubiquitination. How to reconcile these observations with the probable general involvement of Rsp5p in the ubiquitination process? Might all these proteins interact via their phosphorylated serines with one or more of the WW domains of Rsp5p, in accord with a recent report suggesting that Nedd4-WW domains can bind a phosphoserine-containing peptide [93]? Although appealing, a caution must be exercised, because recent work has demonstrated that high affinity interactions between specific phosphorylated serines in RNA polymerase II and the Pin1-WW domain involves an Arg in Pin1-WW that is absent from all the Rsp5p-WW domains (J. Noel, *personal communication*). However, lower affinity interactions with Rsp5p-WW3 domain, which contains an Asn instead of this Arg, may be possible (J. Noel, *personal communication*). Alternatively, there may be other motifs, yet unknown, interacting with WW domains. It is also possible that these plasma membrane proteins indirectly interact with Rsp5p, through adapter proteins. Regardless of the mechanism, it is striking that mutations in conserved residues within the Rsp5p-WW2 or -WW3 domains, proposed to be involved in binding to PY motifs [20, 96], result in a slight or very strong inhibition of Fur4p internalization, respectively (B. Gajewska and T. Zoladek, *personal communication*). Irrespective of the mode of interaction (direct via the Rsp5p-WW domains,

[2] A point mutation in a sequence similar to the destruction box of mitotique cyclines, located in a central loop of the protein, was first found to partly impair stress-induced permease turnover [37], but the effect of this mutation was found to depend on genetic background. Moreover, further elucidation of the role of this region appears difficult because more extensive modifications within it leads to misfolding of the protein, and its failure to reach the plasma membrane background (J.M. Galan, *personal communication*).

[3] It is striking that ammonium regulates in an opposite way the turnover of the two amino acid permeases Gap1p and Tat2p [7, 160].

or indirect), it remains to be shown whether the Hect domain that has to interact with the ubiquitination sites is also involved in substrate recognition. The C2 domain is likely not involved in substrate recognition, but rather in trafficking and endocytosis of Rsp5p/Nedd4 (*see below*).

### Type of Ubiquitin Modification in Plasma Membrane Proteins

Understanding why most ubiquitinated plasma membrane proteins escape recognition and subsequent degradation by the proteasome was one of the first questions arising from the observation of ubiquitin-dependent endocytosis. The occurrence of another type of ubiquitin modification was an appealing hypothesis. Recognition of ubiquitinated proteins by the proteasome subunit 5a requires formation of a polyubiquitin chain at least four subunits long, with linkages involving ubiquitin Lys48 [122]. But alternative type of ubiquitin chains were reported, both in vitro, and in vivo in yeast, involving, in the latter case, ubiquitination on Lys29 and 63 [5, 158].

Mammalian cell surface proteins display different types of ubiquitin modification. The ζ subunit of the T-cell receptor, or the FcεRI, are modified with very few ubiquitin moieties [18, 119], whereas some receptors like Growth Hormone Receptor (GHR), Epidermal Growth Factor Receptor (EGFR), or Platelet Derived Growth Factor Receptor (PDGFR), appear to be modified with multiple ubiquitins [103, 167]. Likewise, ENaC is polyubiquitinated [165], although its status of ubiquitination at the cell surface is not yet known. Similar to mammalian cell surface proteins, the state and nature of ubiquitination of yeast plasma membrane proteins vary (Table 1). The Ste6p, Gal2p and Tat2p transporters all display high molecular weight ubiquitin-conjugates [7, 66, 80, 91]. Whether these modifications correspond to the formation of ubiquitin chains, or the addition of single ubiquitins on multiple lysines, remains unknown. On the other hand, for Ste2p, pulse-chase experiments following the addition of α-factor to endocytosis-defective mutants had revealed the transient formation of mono to tri-ubiquitinated forms of the receptor [61]. A truncated receptor carrying a unique target Lys residue was observed to undergo mono-ubiquitination, sufficient to signal receptor internalization. Data obtained on numerous variant receptors suggested that full length receptor could undergo mono-ubiquitination on several lysines, two of them serving as strong acceptor sites [172]. Physical mapping of the ubiquitin attachment sites in Ste3p was investigated by site-directed mutagenesis and CNBr cleavage. This work demonstrated that Ste3p undergoes ubiquitination on three target lysines modified both with single ubiquitin, as well as with short di- or tri-ubiquitin chains [136]. Analysis of the mode of ubiquitination of several transporters (Fur4p, Gap1p, Zrt1p) [35, 41, 162] revealed a similar scenario, whereby a small number of target lysines (one to three) accept one to three ubiquitin moieties (Table 1), i.e., the number of ubiquitins conjugated is too small to permit proteasomal recognition.

Characterization of the type of ubiquitin linkage has been carried out more precisely with the Fur4p and Gap1p transporters, using cells deficient for the *DOA4/NPI2* encoded ubiquitin isopeptidase. These mutant cells have greatly reduced amounts of intracellular free ubiquitin [162, 168], and are therefore impaired in ubiquitination and subsequent endocytosis of numerous cell-surface proteins. Both ubiquitination and internalization can be rescued by overproduction of ubiquitin [35, 162, 172]. The ubiquitination pattern of the Fur4p and Gap1p was analyzed in *doa4/npi2* cells overexpressing either wild-type or variant ubiquitins incompetent for the formation of K29, K48 or K63-linked ubiquitin chains. It was observed that both transporters carry two target Lys residues, that can accept up to two (or three for Gap1p) ubiquitin residues, linked through ubiquitin Lys63 [35, 162]. For both transporters, the addition of one ubiquitin on the two target lysines (mono-ubiquitination) appears sufficient for some basal endocytosis, but the formation of Lys63-linked short ubiquitin chains is required for efficient internalization[4]. In Gap1p, the specific shift from mono- to "poly"-ubiquitination is rapid and is observed within 5 min after the addition of ammonium to the medium [162], which suggests an ammonium-induced modification of monoubiquitinated Gap1p, eliciting its polyubiquitination.

As it appears that the formation of short K63-linked ubiquitin chains cannot target for proteasome recognition, several questions remain outstanding: What are the molecular determinants of poly-ubiquitination? Is this modification Rsp5p-dependent, as is the fixation of the first ubiquitin? Alternatively, is there a specific E4, as recently reported for the formation of K29-linked ubiquitin chains [79]? It is tempting to speculate that this specific ubiquitin-modification is a property of Rsp5p. In favor of this hypothesis is the observation that mitochondrial inheritance defect in *rsp5* mutants could be rescued by overexpression of wild-type ubiquitin, but not K63R mutant of ubiquitin [33], implicating K63 ubiquitin chains in this other Rsp5p-dependent process. On the other hand, it is puzzling that Rsp5p is also required for UV-induced Rbp1p ubiquitination, leading to proteasomal degradation of this enzyme [179], a process expected to involve the addition of Lys48-linked ubiquitin

[4] There is indirect evidence that it might be the same for cadmium transporter, and arginine permease, since *doa4*Δ cells exhibit hypersensitivity to Cd and canavanine (transported by the latter), that can be relieved by overproduction of wild-type ubiquitin, but not of the variant K63R [168].

chains. Similarly, Smurf1-dependent ubiquitination of Smad1 leads to its degradation by the proteasome [191], as does Pub1 in the case of cdc25 [112]. It is thus possible that enzymatic properties of proteins of Rsp5p/Nedd4 family would be modulated by various adapters, or be dependent on the target proteins.

## Ubiquitin as an Internalization Signal

It had been suggested that ubiquitin could facilitate internalization by promoting dimerization or aggregation of receptors [67], or by inducing movement of a protein into membrane subdomains of active endocytosis [60]. No data have yet been provided in support of either hypothesis. Alternative models proposed for understanding the role of ubiquitin as an internalization signal were either that ubiquitination might induce structural changes in the modified proteins, unmasking internalization signals [60], or that ubiquitin itself may constitute an internalization signal binding to some component of the internalization machinery [13, 60, 61]. The latter hypothesis appears by far the most likely, at least in yeast, given the sparse examples of identified functional internalization motifs in yeast plasma membrane proteins in addition to the ubiquitination motifs. Recent data obtained on Ste2p and Ste3p clearly support this hypothesis. The fusion of ubiquitin in frame with truncated Ste2p that lack C-terminal ubiquitination sites restores the ability of these receptors to promote α-factor mediated internalization [152]. Similarly, a variant Ste3p lacking the PEST-like region fused in frame with ubiquitin undergoes rapid endocytosis and vacuolar turnover [136]. An in-frame expressed ubiquitin lacking its own lysines was as efficient as wild-type ubiquitin in mediating endocytosis of Ste2p, as judged by the rate of α-factor internalization, indicating that further ubiquitination of the hybrid protein was not required. Ste2p-ubiquitin chimera can promote α-factor internalization in the absence of almost the entire receptor tail. This suggests that ubiquitin does not unmask cryptic endocytic signals in the receptor tail, but instead constitutes itself a signal for endocytosis of the protein. This was further supported by the observation that fusion of ubiquitin in-frame to the stable $[H^+]$-ATPase Pma1p stimulates its endocytosis [152]. The turnover time of this chimera decreased from 11 to 3 hours. It should be noted, however, that Pma1p fused to the D box of Ste6p, or the PEST-sequence of Ste3p displayed more rapid turnover ($t_{1/2}$ ~30 min) [81, 138]. This might indicate that efficient endocytosis of Pma1p requires more extensive ubiquitination, as is the case for Fur4p and Gap1p. In agreement with these observations, various Ste3-ubiquitin variants, including a fusion protein that retains the potential for ubiquitination of the PEST-like sequence, internalize faster than wild type Ste3p, suggesting that multiple ubiquitin moieties either as a chain, or as mono-ubiquitin added onto separate lysines, may serve to augment the rate of uptake [136].

Extensive Ala scanning has demonstrated that the three-dimensional structure of ubiquitin is important to promote endocytosis of Ste2p, with a specific role played by two hydrophobic surface patches [152]. These overall data suggest that a specific receptor that recognizes ubiquitin might play a role in the internalization process. The affinity of such a putative receptor for ubiquitinated proteins could be correlated to the number of ubiquitin moieties, K63-linked ubiquitin residues giving possibly an optimal signal for internalization of larger proteins. Such a putative receptor should be sequestered away from free ubiquitin, and from ubiquitin chains of K63 type, that were recently recognized to be involved in processes distinct from endocytosis [33, 64, 157].

The existence of a ubiquitin receptor in internalization of mammalian plasma membrane proteins has not yet been demonstrated or proposed. In the case of GHR, it has been shown that ubiquitination of the receptor itself is not required for ubiquitin-dependent internalization (*see below*), suggesting that in this specific case, ubiquitination might not simply serve as an internalization signal. Hence, it is possible that ubiquitination of yeast plasma membrane proteins, and ubiquitination of at least some mammalian plasma membrane proteins might play distinct roles. Further illustration of these distinct features of both classes of proteins is evident by the massive accumulation of ubiquitin-conjugates of some yeast plasma membrane proteins in mutants impaired for the internalization step of endocytosis, whereas ubiquitination of GHR or EGFR is abolished under conditions leading to inhibition of their internalization [38, 47].

## Ubiquitination/Endocytosis in Mammalian Receptors Downregulated by Non-Hect E3s

In addition to the regulation of ubiquitin-mediated endocytosis of transporters, receptors and channels by Nedd4/Rsp5p described above, the internalization and degradation of several cell surface proteins, mostly described in multicellular organisms, are also regulated by ubiquitination, likely not involving Nedd4. Prominent examples include the GHR, the T cell receptor (TCR) and several growth factor receptor tyrosine kinases (RTKs), such as the EGFR, PDGFR and Colony Stimulating Factor –1 Receptor (CSF1-R) (*see* Tables 1 and 2 for partial lists). The E3s involved are not always known, but recent exciting work has suggested that RING finger domains serve as E3s and are likely involved in the ubiquitination of several RTKs. The best example of such a RING finger-containing regulator of RTK ubiquitination is c-Cbl.

c-Cbl is a 120 kDa protein which contains a variant SH2 domain, a RING finger, a Pro rich region and a Leu

zipper. It was originally identified as a cellular homologue of the oncogene v-Cbl, which lacks the C terminus region, i.e., the RING finger, Pro-rich tracts and Leu zipper [12, 87]. c-Cbl is a negative regulator of several RTKs, including the EGF, PDGF and CSF-1 receptors [77, 88, 90, 180] and its *C. elegans* homologue, Sli-1 is a suppressor of Let23, the worm EGFR homologue [189]. c-Cbl exerts its inhibitory effect by binding via its variant SH2 domain to tyrosine-phosphorylated RTKs, inducing receptor ubiquitination and endocytosis [39, 88, 90, 95], thus leading to the removal of the receptor from the cell surface and termination of ligand-induced signaling. This effect is dependent on the presence of intact RING finger domain of c-Cbl, as it is prevented in v-Cbl, in c-Cbl bearing a small deletion (70Z/3 cbl) within the RING finger, or a point mutation (C381A) in one of the conserved cysteins of the domain [90, 180, 189]; the 70Z deletion appears to abolish interactions with UbcH7, suggesting that this deleted region is needed for interaction with the E2 [188]. Moreover, the RING finger on its own is sufficient to self-ubiquitinate in vitro (in the presence of E1 and E2-Ubc4) and to mediate ubiquitination of the PDGFR when attached to the SH2 domain [77], suggesting that the RING finger of c-Cbl possesses E3 activity (likely by promoting transfer of ubiquitin from an E2 to the substrate), as recently demonstrated for RING fingers of other proteins [92, 108]. The SH2 domain is necessary for binding to the tyrosine-phosphorylated (ligand-activated) receptor, in accord with an earlier report demonstrating impaired ubiquitination and endocytosis of PDGFR bearing mutations in some of its tyrosine-phosphorylation sites [104]. Interestingly, a recent report [89] has demonstrated that tyrosine phosphorylation of c-Cbl itself is necessary for its ability to ubiquitinate the EGFR. Ligand binding to the EGFR resulting in autophosphorylation on Tyr 1045 leads to recruitment of c-Cbl via its SH2 domain, phosphorylation of Tyr 371 on c-Cbl by the EGFR leading to activation of the adjacent RING finger, and EGFR ubiquitination and degradation. Thus, c-Cbl contains both the substrate recognition domain (SH2) and the E3 domain (RING finger) within the same protein, conceptually resembling Nedd4 which also possesses adjacent substrate recognition (WW) and E3 (Hect) domains. It should be emphasised that the role of c-Cbl in endocytosis is controversial, as the same report has suggested that it may act in sorting EGFR for recycling or transport to the lysosome [89], while other investigators proposed that c-Cbl-dependent CSF-1R multiubiquitination occurs prior to internalization [88].

In the above examples, tyrosine phosphorylation sites on growth factor receptors may be viewed as signals not only for the initiation of signal transduction cascades, but also for ubiquitination and endocytosis, which usually leads to termination of signaling. Another example in which tyrosine phosphorylation (although indirect) is involved in receptor endocytosis is the T cell receptor (TCR). The TCR is ubiquitinated on cytosolic Lys residues of its multiple subunits [18]. This ubiquitination is dependent on receptor-mediated activation of tyrosine kinases (e.g., lck) [19], and the ubiquitinated receptor is internalized and degraded by the lysosomes and not by the proteasome [13].

A well-studied plasma membrane protein in which ubiquitination and endocytosis are linked is the growth hormone receptor (GHR), a cytokine receptor lacking intrinsic kinase activity which recruits the JAK2 tyrosine kinase to mediate its signaling. Ligand binding induces receptor ubiquitination and endocytosis, which are blocked in E1-deficient cells [167], indicating the two events are tied. The receptor C terminal tail contains two motifs involved in endocytosis: a ubiquitin-dependent endocytic motif (UbE) (DSWVEFIELD) functional in the intact receptor [45], and a ubiquitination-independent di-Leu motif (DTDRLL) which directs endocytosis of a partially truncated receptor, but has no role in internalization of the full length receptor [46]. Interestingly, removal of all Lys residues from the C terminal tail of the receptor, which abolishes direct receptor ubiquitination, does not impede receptor internalization, while mutating the UbE motif inhibits both ubiquitination and endocytosis, suggesting that the UbE motif recruits the ubiquitination system rather than directs ubiquitination of the receptor [45]. It is possible, therefore, that an activated E2/E3 (not yet identified) binds to the UbE motif, or that an adapter protein or complex which interacts with the UbE motif may itself become the target of ubiquitination and hence mediate internalization of the GHR. The cytosolic C terminus of GHR may be partially clipped and degraded by the proteasome before receptor internalization, while the rest of the receptor becomes susceptible to lysosomal degradation following endocytosis [175]. Interestingly, the UbE motif has been detected in several other proteins, including receptors known to be ubiquitinated (e.g., EGFR, PDGFR, prolactin receptor, neu).

## Machinery Involved in Internalization of Ubiquitinated Proteins: A Role for Rsp5p?

Much remains to be learned about the mechanisms that govern internalization of ubiquitinated proteins. Whether the same machinery is involved in endocytosis of ubiquitinated and nonubiquitinated proteins in mammals is still mostly unknown. In addition to identification of a hypothetical ubiquitin adapter/receptor, the molecular details of internalization in yeast remain generally surprisingly obscure, in spite of the wealth of genetic data that have revealed fundamental aspects previously unnoticed in mammals; the requirement for actin and some

actin-associated proteins, and the involvement of certain lipids [40, 109, 181]. Unlike mammalian cells in which extensive knowledge has been accumulated on clathrin-dependent endocytosis, as well as on nonclathrin pathways [102], the role of clathrin appears more obscure in yeast. Deletion of the gene encoding the clathrin heavy chain (*CHC1*), or thermosensitive mutation in this gene (*chc1-ts*) cause only 50% inhibition in the internalization of Ste2p and Ste3p [40], or the maltose transporter [120]. Moreover, endocytosis of the Fur4p transporter is unaffected in the thermosensitive *chc1-ts* cells (A. Gratias and R. Haguenauer-Tsapis, *unpublished results*). Deletion of the genes encoding proteins similar to the mammalian adapter AP2 subunits does not cause endocytic defects, and there is no evidence indicating complex formation, nor plasma membrane localization, of these proteins [68]. This correlates well with the lack of Tyr-based endocytic signals in yeast.

Although some similar players involved in endocytosis have been identified in both yeast and mammals, they do not necessarily perform the same functions. The mammalian Eps15 is involved in endocytosis through its association with the α subunit of AP2 [11] and is localized to the neck of clathrin-coated buds and vesicles. Eps15 undergoes multiple post-translational modifications, including EGF-induced monoubiquitination [174]. The yeast Pan1p displays some homology with Eps15 (EH domains, coiled-coil domain, and C-terminal proline-rich region) but lacks an AP2 binding site, and colocalizes with cortical actin patches [171]. It is not known whether Pan1p also undergoes ubiquitination. *pan1-ts* mutants are strongly defective in endocytosis. They display impaired organization of the actin cytoskeleton, and deeper plasma membrane invaginations than the control wild type cells [182]. Pan1p interacts with several proteins that are necessary for endocytosis: End3p in yeast [10], and yAP180 (the yeast homologue of the clathrin assembly polypeptide AP180), the epsin homologues Ent1p and Ent2p and the synaptojanin homologue, Slj1p in both yeast and mammals [142, 181, 183] and therefore has been proposed to function as a multivalent adapter required for endocytosis [181]. The observation of a genetic interaction between *PAN1* and *RSP5* [192] was the first indication of a possible role of Rsp5p in endocytosis beyond its role in ubiquitination of endocytosed proteins. Pan1p indeed carries several putative PY motifs which may allow biochemical interactions with the Rsp5p WW domains. A possible involvement of Rsp5p in endocytosis *per se* is supported by several other observations. *RSP5* also displays genetic interaction with *END5/MDP2/VRP1* (verprolin) [192], another gene required for proper actin cytoskeleton organization and endocytosis [110], and with Ede1p, the yeast protein that displays the closest similarity to Eps15, which also lacks AP2 binding sites, and is required for efficient endocytosis [34]. *mdp1/rsp5* ts mutants are impaired in fluid phase endocytosis [192], a phenotype also observed in *rsp5* mutants carrying point mutations in conserved residues within the WW3 domain which are known to interact with PY motifs (B. Gajewska and T. Zoladek, *personal communication*). It was observed that Rsp5p is required for endocytosis of the Ste2p-ubiquitin chimera lacking ubiquitination signals, suggesting that Rsp5p-dependent ubiquitination of unknown targets could be required for proper endocytosis (R. Dunn and L. Hicke, *personal communication*). Furthermore, cells expressing Rsp5p lacking its C2 domain have normally ubiquitinated Gap1p, but subsequent internalization of this permease is impaired, suggesting that the C2 domain is directly involved in the internalization process, or mediates interaction with components required for this event [161]. Indeed, a GFP-tagged Rsp5p was recently detected both near the plasma membrane and in intracellular structures, but deletion of the C2 domain promoted delocalization (G. Wang and J. Huibregtse, *personal communication*). Moreover, electron microscopy analysis of Nedd4 endogenously expressed in MDCK cells revealed it is found not only in apical rafts and at the apical membrane, but also in endosomes [125]. Whether Nedd4 presence in endosomes reflect its co-internalization with its substrate(s), or whether it actually stimulates substrate internalization not only by ubiquitination but also by other means, for example involving its C2 domain, remains to be shown. Interestingly, a recent report has demonstrated that binding of the second C2 domain of synaptotagmin to AP-2 in clathrin-coated pits [190] is enhanced in the presence of a YxxΦ, a classic endocytic signal [55]. The PY motif of each ENaC chain is adjacent to a highly conserved YxxL motif (xP-PxYxxL). Although it is not known whether the Nedd4-C2 domain can bind AP-2, it is tempting to speculate that if it does, the interaction may be enhanced by the YxxL motifs of ENaC.

Further insights into this hypothetical function of Rsp5p/Nedd4 in the internalization step of endocytosis will require identification of its partners, knowledge of the domains involved in the various interactions, and precise localization data.

## Conclusion

The accumulating evidence suggests that Rsp5p plays a critical role in regulating ubiquitin-mediated endocytosis of numerous yeast plasma membrane proteins, and recent evidence implicate Nedd4 in a similar function for some mammalian cell surface proteins. It remains to be clarified whether different subcellular localization and/or different partners may explain why and how Nedd4-related E3s are involved in the latter function, or in ubiquitination of cytoplasmic proteins leading to their degradation

by the proteasome. Concerning the endocytic function, it is clear that key questions need to be answered in order to understand the mechanism(s) by which ubiquitin signals endocytosis and by which Rsp5p/Nedd4 is involved in this process. First, if ubiquitin itself contains sufficient determinants to dictate endocytosis (e.g., hydrophobic patches), what do they interact with (e.g., components of the endocytic machinery or adapter proteins) to facilitate endocytosis in yeast, and does a similar scenario also exists in mammalian cells? Second, how is free ubiquitin, abundant in cells, not interfering with this ubiquitin-mediated endocytosis? Is the type of ubiquitin linkage (e.g., K63) critical for this selectivity, and if so, is it also prevalent in mammalian cells? Third, does Rsp5p directly bind and directly ubiquitinate yeast plasma membrane proteins which lack PY motifs? How are the interactions triggered in the case of regulated ubiquitination? Do the relevant target proteins all undergo ligand-induced phosphorylation, or conformational changes potentiating their interaction with Nedd4/ Rsp5p? Fourth, in mammalian cells, other ubiquitination systems in addition to the Hect-containing Nedd4 (e.g., RING finger E3s) have been proposed to control ubiquitin-mediated endocytosis. How are these different ubiquitination systems operating to achieve a similar goal? Answering at least some of these questions will facilitate our understanding of ubiquitin-mediated endocytosis.

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## *Note Added in Proof*

Since submission of this manuscript, it was published that monoubiquitination is sufficient to signal internalization of the maltose transporter (Lucero et al., 2000. *J. Bacteriol.* **182:**241–253).