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Review

The role of ubiquitin in down-regulation and intracellular sorting of membrane proteins: insights from yeast

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Abstract

Ubiquitination is a versatile tool used by all eukaryotic organisms for controlling the stability, function, and intracellular localization of a wide variety of proteins. Two of the best characterized functions of protein ubiquitination are to mark proteins for degradation by cytosolic proteasome and to promote the internalization of certain plasma membrane proteins via the endocytotic pathway, followed by their degradation in the vacuole. Recent studies of membrane proteins both in yeast and mammalian cells suggest that the role of ubiquitin may extend beyond its function as an internalization signal in that it also may be required for modification of some component(s) of the endocytotic machinery, and for cargo protein sorting at the late endosome and the Golgi apparatus level. In this review, I will attempt to bring together what is currently known about the role of ubiquitination in controlling protein trafficking between the yeast plasma membrane, the *trans*-Golgi network, and the vacuole/lysosome.

Keywords: Ubiquitin; Proteolysis; Vacuole; Membrane protein; Sorting; Yeast

1. Introduction

Ubiquitination (or ubiquitylation) is the posttranslational conjugation of ubiquitin to specific lysine residues in a multitude of eukaryotic proteins. Ubiquitin is a 76-amino-acid polypeptide that is highly conserved and expressed in all eukaryotic cells. When conjugated to proteins, it often serves to target them for degradation. The conjugation of ubiquitin to proteins is a process that involves the sequential transfer of the ubiquitin moiety to substrate proteins through the E1–E2–E3, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-ligase (E3) thiol ester cascade culminating in the formation of an amide (isopeptide) bond between the C-terminal glycine Gly-76 of ubiquitin and the å-amino group of specific lysine residues within the substrate protein (for reviews see Refs. [1–3]). Specificity in substrate recognition resides largely at the level of E3s and an additional degree of combinatorial specificity may arise from specific E2–E3 interactions. The E3 ubiquitin ligases facilitate the recognition of the target protein by E2 or directly transfer the ubiquitin to the substrate. Monoubiquitinated proteins are then often (but not always) further modified by the attachment of additional ubiquitin molecules to lysine residues on ubiquitin itself, giving rise to polyubiquitin (multiubiquitin) chains. Since ubiquitin is a long-lived protein in wild-type yeast cells [4], it can be recycled from ubiquitin–protein conjugates by the action of specific thiol proteases named deubiquitination enzymes (Dub's), or isopeptidases, and thereby reused in multiple rounds of the ubiquitin cycle (for reviews see Refs. [1,5]).

Attachment of ubiquitin to substrate proteins has distinct mechanistic roles in two different intracellular proteolytic pathways. One well-established role for the covalent linkage of ubiquitin is to mark cytosolic and nuclear proteins, and those proteins that are subjected to endoplasmic reticulum-associated hydrolytic degradation, by the 26S proteasome. The 26S proteasome is a large barrel-shaped multicatalytic protease complex (for reviews see Refs. [6–8]). Polyubiquitin chains composed of at least four ubiquitin monomer units linked by isopeptide bonds between Lys-48 of ubiquitin molecules and the C-terminal carboxyl group of the following ubiquitin are found attached to most 26S proteasome substrates. These polyubiquitin chains facilitate the

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binding of substrate proteins to the proteasome [9]. Ubiquitination of many cell-surface nutrient and ion transporters and signal-transducing receptors, however, appears to play no role in proteasomal breakdown; rather ubiquitination of these proteins serves as a signal for their internalization via the endocytotic pathway and subsequent proteolysis in the lysosome/vacuole (reviewed in Refs. [7,10–12]). In all known cases of yeast plasma membrane proteins, a single ubiquitin moiety or di- to tri-ubiquitin chains, in which ubiquitin molecules may be linked through Lys-63, appear to suffice to trigger their internalization into the interior of the cell. In addition, several recent studies indicate that ubiquitin also regulates events at three distinct intracellular destinations, including sorting of proteins into inward-budding vesicles in the late endosome/prevacuolar compartment (LE/PVC), which give rise to multivesicular bodies (MVB; reviewed in Refs. [13–17]), endosome fusion events between steps of internalization and sorting of proteins at the MVB [18], and sorting of proteins at the *trans*-Golgi network (TGN) (reviewed in Refs. [13,15,16]). This review is confined largely to the current understanding of the role of ubiquitination in the down-regulation of plasma membrane proteins. To keep the review down to a reasonable size, the discussion is focused, with a few exceptions, on the yeast *Saccharomyces cerevisiae.*

2. Ubiquitin-dependent internalization of yeast plasma membrane proteins

Ubiquitination is implicated as an internalization signal for most (if not all) yeast endogenous plasma membrane proteins that are endocytosed and ultimately degraded in the vacuole (Table 1). The view of a close link between ubiquitination, endocytosis, and vacuolar degradation emerged mainly from three types of observation (reviewed in Refs. [7,12]). (i) Both internalization by endocytosis and proteolysis in the vacuole are strongly impaired in cells defective in the proper subsets of enzymes of ubiquitination (see the E2s and E3 Rsp5p in the Table 1) and deubiquitination machinery (Section 3.2), respectively, and/or in wild-type cells expressing protein variants in which ubiquitination sites are mutated. (ii) Increased levels of ubiquitin–protein conjugates are often found at the cell surface of endocytosis-deficient cells, suggesting that ubiquitination precedes endocytosis and the process of ubiquitination occurs in close proximity to the plasma membrane. (iii) Degradation of many plasma membrane proteins is strongly reduced in mutants lacking key vacuolar protease activities but not in mutants carrying defective catalytic or regulatory 26S proteasome subunits.

The endocytotic pathway is defined as a time-, temperature-, and energy-dependent system of membrane traffic

Table 1
Current list of endocytosed yeast proteins

Protein	Function	E2	E3	Ubiquitination pattern	Half-time (min)	Modes of endocytosis/degradation	References
Ste2	α-Factor receptor	Ubc1/4/5	Rsp5	Mono (several lysines)	6–7.5 60	Substrate-induced Constitutive	[19–21]
Ste3	**a**-Factor receptor	Ubc4/5		Mono + di + tri (three lysines)	15–20	Substrate-induced and constitutive	[22,23]
Ste6	**a**-Factor transporter	Ubc4/5		Poly	15–40	Constitutive	[24]
Pdr5	Multidrug transporter				60–90	Constitutive	[25]
Mal11	Maltose transporter	Ubc4/5	Rsp5	Mono	45–100	Glucose-induced under nitrogen starvation	[26,27]
Mal61	Maltose transporter	Ubc1/4/5	Rsp5	Mono		Glucose-induced under nitrogen starvation	[28]
Gal2	Galactose transporter	Ubc1/4/5	Rsp5	Mono (several lysines)	60	Glucose-induced under nitrogen starvation	[29,30]
Hxt6/7	Glucose transporters		Rsp5		45	Glucose-induced under nitrogen starvation	[31]
Fur4	Uracil transporter		Rsp5	Mono + di + tri (two lysines; K-63-linked)	40–150	Constitutive and induced (under a set of adverse conditions)	[32,34]
Gap1	General amino acid transporter		Rsp5	Mono + di + tri (two lysines; K-63 linked)	45–50	Ammonium- or glutamate-induced	[34–36]
Tat2	Tryptophan transporter		Rsp5	Poly	30	Induced by nitrogen starvation or rapamycin	[37]
Can1[a]	Basic amino acid transporter		Rsp5			Constitutive (?)	[38]
Bap2	Branched amino acid transporter		Rsp5			Constitutive and induced by poor nitrogen source or rapamycin	[39]
Zrt1	Zinc transporter	Ubc4/5	Rsp5	Mono + di (one lysine)	40	Substrate-induced	[40]
Alr1	Magnesium transporter (?)		Rsp5			Substrate-induced	[41]
Smf1	Manganese (heavy metal) transporter				10–20	Metal-induced in a Bsd2p- dependent way	[42]
Pho84	Phosphate transporter					Induced by phosphate starvation	[43]

[a] Transporter from *Candida albicans* expressed in *Saccharomyces cerevisiae*.

from the plasma membrane to the vacuole/lysosome. During this process all eukaryotic cells internalize extracellular fluids and portions of the plasma membrane (including associated proteins and lipids) by invagination of the plasma membrane at specialized sites. This leads to formation of vesicles, by which some internalized compounds are delivered to the vacuole/lysosome through at least two endocytotic compartments, early endosome (EE) and LE/PVC, or recycled to the plasma membrane (for reviews see Refs. [44–46] and Sections 3.1 and 3.2). Because the LE/PVC serves as the transient destination for some proteins that travel from the TGN to the vacuole, endosomes have a crucial role in coordinating transport between the plasma membrane, the TGN, and the vacuole (Fig. 1).

For most of the yeast plasma membrane proteins that are constitutively internalized in the endocytotic system, the rate of internalization is modulated by a change in a variety of parameters, such as binding of substrates or other ligands, nutrient availability, temperature, or stress conditions [47–49]. Accelerated internalization of the general amino acid transporter Gap1p by addition of ammonium to proline-grown cells [35], or internalization of sugar-specific transporters Gal2p, Mal11p, and Mal61p, induced by glucose added to galactose- and maltose-grown cells, [29,50,51], respectively, are examples of endocytosis markedly accelerated or triggered by nutrient changes. The transporters Zrt1p [52], or Fur4p [47,49], are examples of proteins, whose rates of internalization are dramatically increased by their own substrates. The only exception to this rule seems to be the case of **a**-factor receptor Ste3p, whose constitutive internalization is rapid and not significantly affected by the presence of its ligand, **a**-factor [53].

2.1. Signals for internalization

Internalization of the plasma membrane proteins by endocytosis is a selective process and usually relies on signal(s) within their cytoplasmatically disposed amino acid sequences. Two categories of internalization signals, (probably) ubiquitin-independent and ubiquitin-dependent, have been described for eukaryotic proteins. In higher eukaryotes many plasma membrane proteins display short, discrete peptidyl sequences that direct their internalization by endocytosis. Among the most frequently occurring targeting signals are those that contain a dileucine pair or tyrosine-based sequences with the consensus sequence NPxY or YxxZ, where x is any amino acid, and Z is a hydrophobic amino acid such as leucine (for review see Ref. [54]). Both the above types of internalization signals are most probably ubiquitin-independent and appear to interact with the components of the coats of endocytotic invaginations (pits), including clathrin chains and dynamin, via the AP-2 adaptor complex, which are thought to recruit proteins into the sites of endocytosis (for review see Ref. [55]).

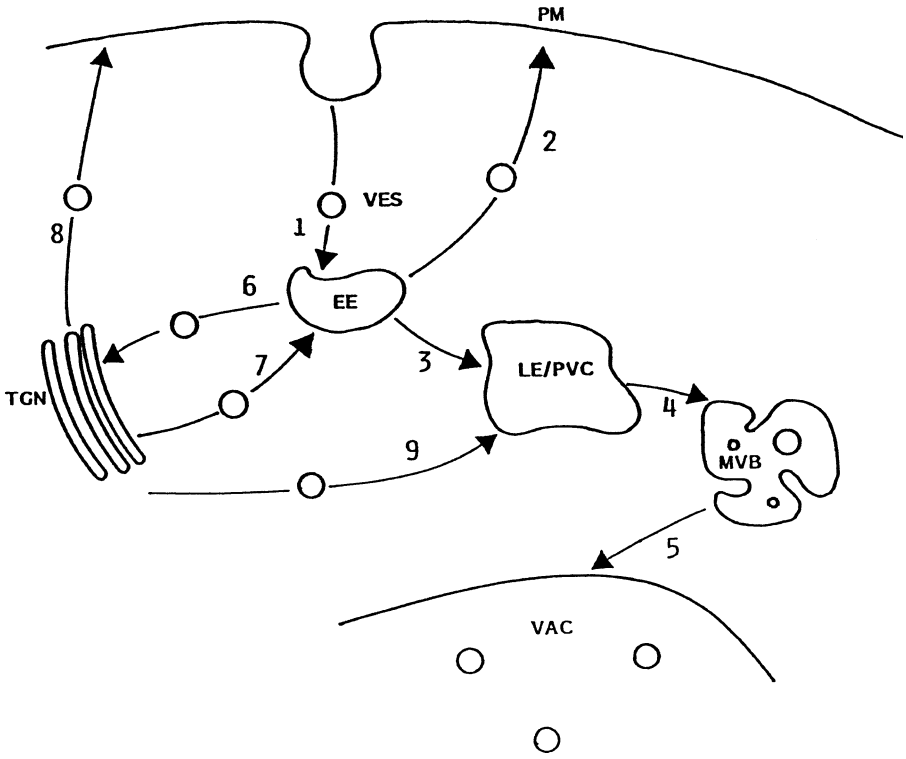

Fig. 1. Overview of organelles participating in the traffic pathways in the yeast endosomal system. All relevant compartments are shown: the plasma membrane (PM), the vesicle (VES), an early endosome (EE), the late endosome/prevacuolar compartment (LE/PVC), the multivesicular bodies (MVB), the vacuole (VAC), and the *trans*-Golgi network (TGN). Protein trafficking may involve transport vesicles (steps 1, 2, 6, 7, 8, 9), maturation of one organelle into another (step 4), or direct fusion of the MVB compartment with the vacuole (step 5). The nature of trafficking between the EE and LE/PVC (step 3) is unclear.

Current evidence from yeasts indicates that they express homologues of the key players for clathrin-mediated endocytosis, including the light and heavy clathrin chains, proper adaptor complex, and dynamin, and that yeast clathrin chains are involved in some nonessential way in the internalization step of endocytosis. Despite the presence of these homologues, no classic adaptor complexes or dynamin-like proteins have been shown to participate in endocytosis (for reviews see Refs. [46,56]). Moreover, the NPFxD motif of Ste3p [57], its poorly conserved version GPFAD near the C terminus of Ste2p [20], and the dileucine motif in Gap1p and Mal61p [58,59] were identified as the only candidates for ubiquitin-independent, clathrin-facilitated endocytotic targeting signals in *S. cerevisiae.* Both motifs in the Ste2p and Ste3p receptors were shown to mediate efficient internalization of receptor versions that cannot be ubiquitinated [20,57]. Interestingly, the actin-associated protein Sla1p was recently found to serve as an adaptor for NPFxD-based endocytotic targeting signals, suggesting its possible function in linking NPFxD-containing cargo to the clathrin-based machinery in *S. cerevisiae* instead of classic adaptors [60]. Finally, the C-terminal dileucine motif is required (together with the motif EEKAI and the last 11 amino acids) for ammonium-induced internalization and turnover of Gap1p [58]. The dileucine motif is also required for glucose-induced inactivation and degradation of Mal61p [57]. However, it is not clear whether these motifs function together with or independently of the ubiquitination signals (see below).

The sequence motif ^{331}SINNDAKSS^{339} within the regulatory cytoplasmatically located region of Ste2p has been shown to be necessary and sufficient for ligand-induced internalization of the receptor version that has lost approximately two thirds of its C-terminal tail due to protein truncation at residue 345 [61]. An important clue to the function of this motif came from the observations that its only lysine (Lys-337) becomes ubiquitinated in an α-factor-dependent fashion and that this modification is necessary for internalization of truncated Ste2p [19]. However, when Lys-337 is substituted for Arg in the full-length protein, only a minor effect on its turnover was observed, suggesting that the receptor might contain multiple sequence motifs that independently facilitate internalization. Indeed, in addition to Lys-337, the cytoplasmic tail of full-length Ste2p contains seven other lysine residues, six of which also probably serve as ubiquitin acceptors. However, Lys-374 appears to be the preferred ubiquitin-acceptor site. The simultaneous substitution of all seven lysines in the Ste2p cytoplasmic tail for arginine residues results in a loss of its ubiquitination and a strong defect in internalization, even in the context of full-length protein [20]. The DAKTI motif, partly resembling the SINNDAKSS endocytotic signal, was identified in the Ste6p [62]. This motif lies within the D-box (*d*estruction box), a sequence of about 100 amino acids in the Ste6p linker region, which connects the two homologous halves of the protein. An inverse relationship between the stability of different deletion variants of Ste6p and the degree of their ubiquitination was found. Together with the fact that a Lys-to-Arg mutation within the DAKTI motif has only a minor influence on Ste6p degradation, it suggests that other sequence motifs within the D-box are required for efficient Ste6p internalization and degradation. One of the two Lys residues required for Fur4p ubiquitination is located within the EYKSS sequence, another DAKSS-like motif [63]. Likewise, two Lys residues within the ERKS and EYKS sequences, together with three other Lys residues, all present in the N-terminal 31 amino acids, appear to be essential for ubiquitination and proteolysis of Tat2p in rapamycin-treated cells [37]. Together with the finding of DAKSS-like motifs in other yeast plasma membrane transporters [58,63], it suggests that the lysine residues within the [(D/E)xK(S/T)] motifs serve as common targets for the ubiquitination of these proteins.

Other ubiquitin acceptor sites were mapped either within or very close to the PEST-like sequences of Fur4p [64], Ste3p [65], Ste6p [62], and Mal61p [59]. The PEST regions have been shown to serve as destruction signals in a variety of proteins which undergo ubiquitin-dependent proteasosomal degradation [66]. Although PEST sequences share no primary sequence identity, they are typically rich in proline, aspartate, glutamate, serine, and threonine residues, and are bordered by positively charged amino acids that are, however, disallowed within them. Interestingly, the PEST-like sequences in Fur4p, Ste3p, and Ste6p do not score strongly as PEST sequences due to the paucity of proline residues within them. The PEST sequences of Fur4p and Mal61p are located in the cytoplasmically oriented N-terminal regions, aa 42–59 [64] and aa 48–79 [59], respectively. The PEST-like sequence of Ste3p, minimally defined as a sequence of 36 amino acids, was mapped within the Ste3p C-terminal cytoplasmic part (aa 414–449; [65]) and an acidic sequence of 61 amino acids rich in Ser and Thr residues constitutes a part of the Ste6p D-box (see above). Two lysine residues (Lys-38 and Lys-41) adjacent to the PEST-like sequence of Fur4p were shown to represent the target sites for ubiquitination [63]. The three Lys residues in the PEST-like sequence of Ste3p exhibit a redundancy; both the ubiquitination and the constitutive endocytosis of the receptor are severely diminished only when all three Lys residues were substituted for Arg residues [23]. Although deletion of the classical PEST sequence of Mal61p (as the only one containing Pro residues) strongly diminish its ubiquitination and glucose-induced turnover, the identity of ubiquitin-acceptor Lys residues is unknown [59].

Taken together, the yeast internalization signals appear mostly to be ubiquitin-dependent, whereas the role of ubiquitin in the internalization of mammalian proteins is much less clear.

2.2. Components of the ubiquitination machinery

Further data arguing that ubiquitin acts as a signal for internalization of proteins emerged from examinations of

the fate of a number of plasma membrane proteins in yeast mutant strains deficient in proteins of the ubiquitination machinery. It thus appeared that of the 11-member family of E2s [1–3], two or three members of the Ubc1p/Ubc4p/Ubc5p triad of ubiquitin-conjugating enzymes with partially overlapping functions (but not other E2s) are required for ubiquitination of proteins such as Ste2p [19], Ste3p [22], Ste6p [24], Mal61p [28], Gal2p [30], or Zrt1p [40] (see also the Table 1). In the case of Fur4p, neither ubiquitination nor endocytosis of Fur4p is affected in *ubc1Δ* and *ubc4Δubc5Δ* cells. Because the loss of function in *ubc1Δubc4Δubc5Δ* cells is lethal [67]), the data suggest that if the triad of Ubc1p/Ubc4p/Ubc5p is involved in ubiquitination of a uracil transporter, the presence of only one of them is sufficient [33].

In contrast to our limited knowledge of E2 enzymes involved in ubiquitination of certain yeast plasma membrane proteins, the ubiquitination of all studied proteins (Table 1 and Refs. [21,26,28,30–32,34,35,37–41]) requires the HECT ubiquitin ligase Rsp5p/Npi1p. The role of Rsp5p (Npi1p) in ubiquitination and endocytosis of yeast plasma membrane proteins was first demonstrated for Gap1p and Fur4p transporters in *npi1* mutant cells carrying a promoter mutation that express less than 10% of the wild-type enzyme [35]. Subsequently, Rsp5p has been shown to affect a broad range of other ubiquitin-dependent cellular events, suggesting that it can have a multitude of diverse membrane-bound as well as nuclear substrates (reviewed in Ref. [12]). Examples of cellular functions ascribed to Rsp5p are depicted in Fig. 2.

Rsp5p is encoded by an essential *RSP5* gene [68] and with the Nedd4p, its mammalian orthologue, is the best characterized member of a large subfamily of E3 ubiquitin ligases that form a thiol ester bond with ubiquitin during the ubiquitination reaction [12,68]. The vital function of Rsp5p is the ubiquitination of the Spt23p and Mga2p transcription factors, which activate transcription of the *OLE1* gene [69], which encodes Δ9 fatty acid desaturase, an ER-bound enzyme required for the synthesis of palmitooleic and oleic acids. Thus, the essential function of Rsp5p is associated with providing cells with unsaturated fatty acids.

All proteins of this family share a common modular structure. They contain a variable N terminus, a C2 domain and two to four WW domains (three in Rsp5p) between the C2 and the HECT domain in the C-terminal part (Fig. 3). C2 domains span about 120 amino acid residues and are thought to regulate the function of proteins by mediating their interactions with membrane phospholipids and/or proteins, mostly in a Ca^{2+}-dependent fashion (reviewed in Ref. [70]). WW domains are evolutionarily conserved, 30- to 40-amino-acid-long globular modules that facilitate protein–protein interactions. Polyproline sequences, such as PPxY, PGM/PPR, or PPLP, bind WW domains (for review see Ref. [71]) as well as phosphoserine (or phosphothreonine) residues in certain peptides and proteins [72]. Their name refers to two conserved tryptophan (W) residues that are spaced 20–22 amino acids apart. The catalytic HECT domain (*h*omologous to the *E*6-AP *C t*erminus) of about 350 amino acids forms a thiol ester bond with ubiquitin via Cys-777 [68].

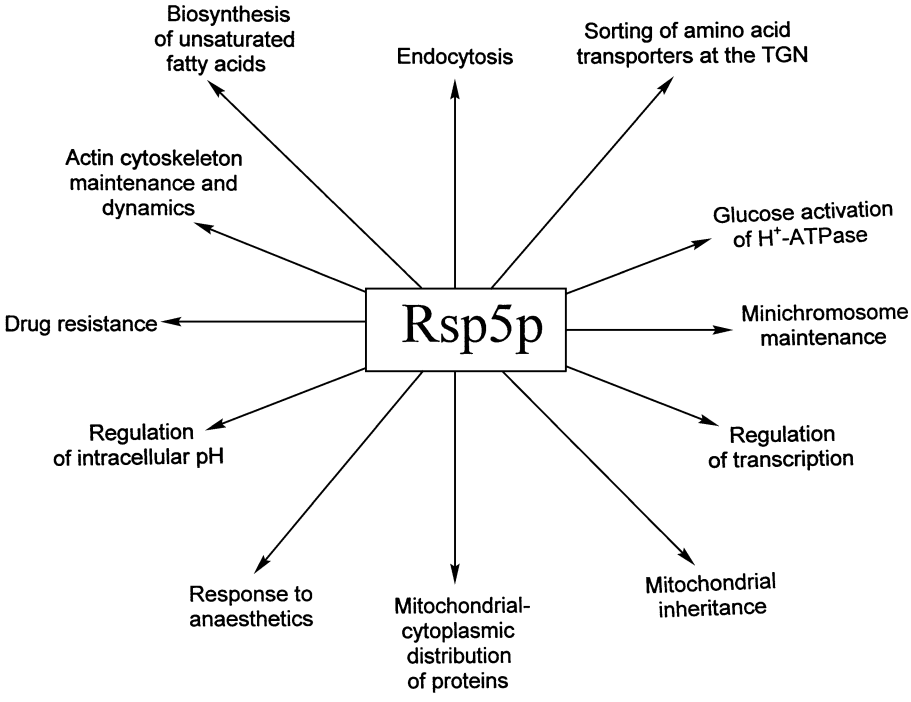

Fig. 2. Examples of the known functions of Rsp5p.

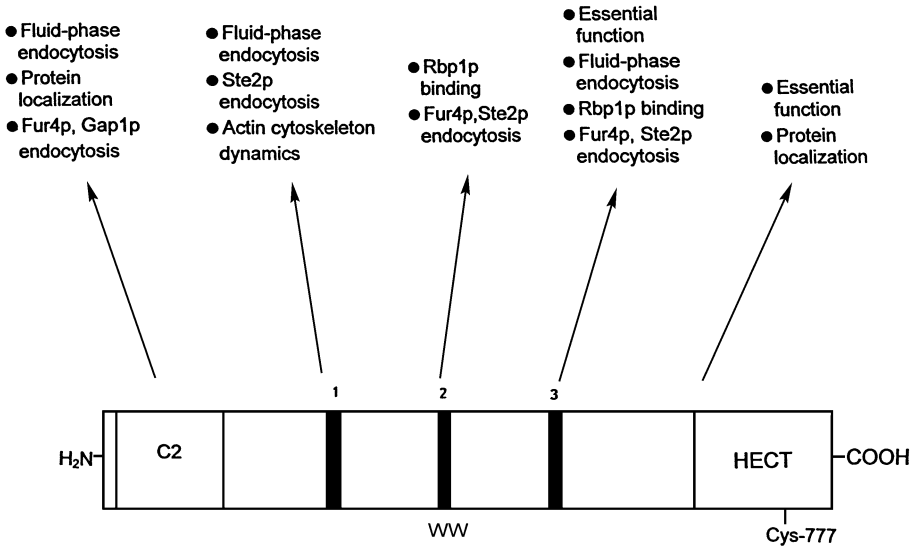

Fig. 3. Schematic representation of Rsp5p domain structure, including C2, WW1 to WW3, and HECT domains. Each Rsp5p domain is shown with its associated functions. Cys-777 is an active-site cysteine that forms a critical thiol ester bond with ubiquitin.

Cell fractionation studies [73–75] have shown that Rsp5p is almost completely associated with larger structures, representing cell organelles and/or protein complexes (perhaps including homomeric Rsp5p complexes [73]) that fractionate with organelles, and that both the protein–protein and C2 domain–membrane interactions are responsible for its associations with these structures. Using immunogold electron microscopy, Rsp5p has been localized in uniformly distributed punctate complexes, some of which reside at the plasma membrane invaginations and in a perivacuolar compartment(s) [75], e.g., at sites involved primarily in ubiquitin-dependent endocytosis. Moreover, the observation of perivacuolar localization of Rsp5p dependency on its catalytic activity as well as functional Sla2p/End4p protein, the component of the actin cytoskeleton implicated in endocytosis, led to the suggestion that active Rsp5p might be routed to the endosome via the endocytotic pathway [75]. Intriguingly, Rsp5p was not found in the nucleus [74,75] even though it is well documented that at least two nuclear proteins, the largest subunit of RNA polymerase II (Rbp1p) and a subunit of replication protein A (Rfa1p), are its substrates (for review see Ref. [12]. Therefore, there is the possibility that nuclear proteins move outside the nucleus prior to ubiquitination.

Recently, a number of structure–function studies of individual domains of Rsp5p have been performed (Fig. 3). There is now consensus that the C2 domain of Rsp5p is not required for the vital function of this enzyme [76]. In cells with the entire C2 domain deleted, a severe defect in the transport of fluid-phase markers to the vacuole was observed [73]. However, the internalization and breakdown (but not ubiquitination) of Fur4p [75] and Gap1p [77] was only partially impaired, and the rate of α-factor-induced internalization of Ste2p was not affected [73]. GFP-Rsp5p fusion studies and electron microscopy identified the C2 domain of Rsp5p as an important determinant of its localization to membrane invaginations and perivacuolar structures [75]. To determine which (if any) WW domains of Rsp5p are required for its essential function in vivo, and endocytotic function, a variety of point mutations in conserved residues of the ligand-binding pocket in each domain and some truncated versions were analyzed. It thus appeared that WW3 and HECT domains of Rp5p are sufficient to provide its essential function under normal growth conditions [69,74]. In the same set of mutant cells it was also found that the WW2 and WW3 domains are required for Fur4p endocytosis [74], all three WW domains for ubiquitination and internalization of the Ste2p [73], and WW1 and WW3 for fluid-phase endocytosis [71,73]. These latter observations indicate that individual WW domains or their different subsets may play distinct regulatory roles in selecting proteins as endocytotic cargo.

2.3. Phosphorylation-dependent ubiquitination

Several yeast plasma membrane proteins, including some of those that are known to undergo ubiquitin-dependent internalization and subsequent degradation in the vacuole, such as Ste2p, Ste3p, Fur4p, and Mal61p, are phosphorylated at multiple serine and/or threonine residues [22,51,78–80]. Phosphorylation is thought to occur upon arrival of the proteins at the plasma membrane. The role of phosphorylation in the control of stability of membrane proteins is only partially understood. The Ste2p cytoplasmic tail is constitutively phosphorylated and α-factor binding induces hyperphosphorylation of the receptor [78]. Studies of the C-terminally truncated version of Ste2p revealed that substitution of all serine residues within its SINNDAKSS internalization signal for alanine residues leads to a Ste2p species which is not phosphorylated, either constitutively or

ligand-dependent, and consequently, it is not ubiquitinated or internalized [19,81]. Likewise, replacement of all five serine residues with alanines within the N-terminal hydrophilic PEST-like sequence of Fur4p or its entire deletion results in protein stabilization at the plasma membrane, probably due to the nearly complete loss of phosphorylation at the cell surface and poor ubiquitination [64]. The findings of efficient degradation of Fur4p and Ste2p variants, in which all serines within the PEST-like sequence of Fur4p and SINNDAKSS of Ste2p were substituted for glutamic acid, are consistent with the view that their phosphorylation, rather than mere presence of serines, is required for efficient protein proteolysis [64,81]. On the other hand, phosphorylation of Ste2p is independent of its ubiquitination [19]. All in all, the data suggest that phosphorylation of Ste2p and Fur4p serine residues precedes and is required for efficient ubiquitination and degradation of both.

Further data in support of the role of phosphorylation in regulation of the stability of the plasma membrane proteins came from the examinations of the fate of Ste2p, Ste3p, Fur4p, and Pdr5p in mutant cells lacking the structurally and functionally redundant pair of type I casein kinases (CK1), Yck1p and Yck2p [64,81–83]. *YCK1*- and *YCK2*-encoded proteins are essential Ser/Thr-specific protein kinases [84] that are probably anchored to the plasma membrane by prenylation of their C-terminal dicysteinyl motifs [85].

Using a *yck*^{ts} mutant strain, which bears a temperature-sensitive allele of *YCK2* (*yck2-2*^{ts}) and a null allele of *YCK1* (*yck1*Δ) [86], Hicke et al. [81] showed that both the constitutive and α-factor-induced ubiquitination, internalization, and breakdown of Ste2p are abrogated in this mutant strain due to defective phosphorylation of the receptor. Likewise, the lack of phosphorylation of Fur4p [64] and Ste3p [82] at PEST-like sequences in the *yck*^{ts} strain correlated with diminished levels of ubiquitination and rates of degradation. Interestingly, an opposite role of phosphorylation was found for the multidrug ABC-transporter Pdr5p [83]. In this case the phosphorylation protects Pdr5p in some way against vacuolar proteolysis.

How phosphorylation by the Yck1p/Yck2p pair regulates ubiquitination of the plasma membrane proteins is unclear. The ability of WW domains of Rsp5p to serve as a phosphoserine-binding module [72] implies that direct interaction between Rsp5p and phosphorylated transporter/receptor might be one of the mechanisms of protein selection, such as Ste2p and Fur4p, for ubiquitin. Alternatively, phosphorylation could induce a conformational change, leading to unmasking a region within the protein that is then recognizable by the ubiquitination machinery. There is also the possibility that the plasma membrane proteins interact indirectly with the ubiquitination machinery, e.g., through some adaptor proteins.

Increasing evidence suggests that Yck1p and Yck2p activities might also have additional roles at multiple stages of protein trafficking. For example, the instability of Fur4p and loss of stability of its non-phosphorylatable PEST variants observed in *yck*^{ts} cells suggest involvement of Yck activity in the negative control of some *trans*-acting components that participate in internalization events [63]. Since additional possible functions of Yck's concern the post-internalization events, they will be discussed in Section 3.2.

The Npr1p is another Ser/Thr protein kinase involved in the posttranscriptional control of at least two amino acid transporters, Gap1p and Tat2p. However, Npr1p is a central player in nitrogen-regulated intracellular trafficking of transporters, and thereby its role will be discussed in more detail in Section 3.3.

2.4. Types of cargo ubiquitination

One of the most probable ways to explain how the cell surface proteins escape the recognition and degradation by the 26S proteasome consists in their type of ubiquitination. Indeed, proteins with at least four ubiquitin moieties conjugated through Lys-48 are usually targeted to the proteasome [9], whereas the number of ubiquitin molecules conjugated to the plasma membrane proteins targeted to the vacuole for degradation is always below the limit for recognition by the proteasome [10–12]. The patterns of yeast plasma membrane protein ubiquitination vary (Table 1). The Ste6p, Pdr5p, Gal2p, and Tat2p proteins were reported to exhibit a complex set of ubiquitin conjugates [24,25,29,37]. Except for Gal2p, which becomes monoubiquitinated on several lysine residues in response to glucose addition [30], it remains to be elucidated whether the observed ubiquitination patterns result from protein modifications by ubiquitin chains of different length, or from the addition of single ubiquitin molecules on multiple lysine residues. As concerns the remaining plasma membrane proteins examined, e.g., Fur4p [33], Gap1p [35,36], Mal11p [27], Ste2p [19], Ste3p [23], and Zrt1p [40] (see also the Table 1), a small number of lysines (one to three) appear to be modified by one to three ubiquitin molecules.

2.5. Ubiquitin can serve as an internalization signal

Several mechanisms by which ubiquitin can induce internalization of a subset of cell surface proteins, followed by their degradation in the vacuole, have been suggested. For instance, ubiquitination might trigger internalization either by inducing movement of an endocytotic cargo into subdomains of the plasma membrane destined for active endocytosis [87] or via multimerization of modified proteins. Other ways in which ubiquitination could facilitate internalization would be that ubiquitination induces a conformational change in the target protein that unmasks an internalization signal [87] or that ubiquitin itself is the internalization signal [7,19,87]. Although no experimental data which support the first three alternative mechanisms have been described, several pieces of evidence support the latter possibility. The fusion of a single copy of ubiquitin in

frame to a Ste2p receptor tail that lacks all cytoplasmic lysines promotes receptor internalization and degradation [20]. Likewise, the Ste3p-ubiquitin fusion, in which ubiquitin is fused to the receptor in place of the PEST-like endocytotic signal, appears to undergo internalization and proteolysis in the vacuole [65]. These results suggest that ubiquitin alone, i.e., with no required contribution from additional protein sequences or signals, is sufficient for triggering uptake. Consistent with these findings, Pma1p is destabilized when fused in frame to the D-box of Ste6p [62], or to the PEST-like sequence of Ste3p [65], or ubiquitin itself [88]. All these chimeras, containing the highly stable plasma membrane H^+-ATPase Pma1p [89], appear to be ubiquitinated and subsequently rapidly degraded in the vacuole.

Importantly, alanine-scanning mutagenesis has revealed two hydrophobic surface patches, surrounding Phe-4 and Ile-44, in the three-dimensional structure of the folded ubiquitin polypeptide that appear to play specific roles in proteolysis. Phe-4 and surrounding amino acids appear to be required for endocytosis, whereas Ile-44 and surrounding hydrophobic amino acids are required for both endocytosis and proteasome recognition [88,90].

2.6. Sorting machinery

In addition to ubiquitinating yeast endocytotic cargo, Rsp5p also appears to be involved in the regulation of the machinery that executes and/or regulates endocytosis. This view has emerged principally from the observations showing that internalization of Ste2p-ubiquitin fusion protein requires functional Rsp5p although it carries ubiquitin [73] and that the fluid-phase endocytosis, for which there is no protein cargo to be a substrate for ubiquitination, depends on Rsp5p [73,74]. This led to the proposal that Rsp5p-dependent ubiquitination of some *trans*-acting component(s) of the endocytotic machinery might be required for the internalization step of endocytosis. Consistent with this model are also findings showing that Fur4p and Gap1p are ubiquitinated, but not properly internalized, in the *rsp5* mutant, which contained the mutant Rsp5p with deleted C2 domain [75,77].

The breakthrough in the search for proteins that could link internalization and post-internalization endocytotic machineries to ubiquitin was achieved recently by identification and functional characterization of several yeast proteins and their mammalian homologues bearing one of four ubiquitin-binding domains. They include UIM (*u*biquitin-*i*nteracting *m*otif), UBA (*ub*iquitin-*a*ssociated) domain, UBC (*ub*iquitin-*c*onjugating) domain, and CUE (*c*oupling of *u*biquitin conjugation to *E*R degradation) domain that enable the proteins to bind noncovalently to ubiquitin [91–93]. Most proteins of this class have elaborate modular domain architecture that assists them in binding other factors, such as lipids and other proteins. The UIM motif is a sequence of about 20 amino acids that consists of a block of acidic residues followed by a highly conserved ZxxAxxxSxxD/E core, in which Z denotes a large hydrophobic residue. This core probably forms an α-helix embedded in different protein folds. UIM, often present in doublet or triplet arrays in proteins, was initially identified in the 5Sa/Rpn10p subunit of the 19S regulatory subcomplex of the proteasome that directly interacts with polyubiquitin chains. The UBA and CUE domains of about 45 conserved residues are predicted to adopt similar three-helix bundle structures in solution and their function to directly bind to ubiquitin was established for several UBA and CUE proteins that became modified in vivo with ubiquitin ranging from a single copy to longer chains [18,92,94,95]. Cue1p, a yeast protein known to recruit the soluble E2 Ubc7p to an ER-associated complex [96], was the founding member of the CUE domain family with eight members in *S. cerevisiae* [17,93], in which (M)FP (P is invariant) sequence and conserved dileucine-like motif are part of the CUE ubiquitin-binding surface. Remarkably, the CUE domains, like UBA and UIM domains, require ubiquitin Ile-44 for interaction, but not ubiquitin Phe-4 ([18,94]; see Section 2.5); UIM and CUE domains might also be required to monoubiquitinate the endocytotic proteins within which they are found [94,95]. Finally, the UBC-like domain has homology to E2s but lacks a critical active-site cysteine, necessary for its function as an E2 [97].

A recent database search put yeast epsins Ent1p and Ent2p, Vps27p, Hse1p, Ede1p, Vps9p, and Vps23p on the list of UIM, UBA, CUE, and UBC-like protein candidates for ubiquitin-binding receptors [18,91–94] (Fig. 4). These proteins are known or supposed to be involved in controlling the proper execution of endocytosis (epsins, Ede1p [98,99]), endosomal protein sorting (Vps27p, Hse1p, Vps23p [94,100,101]), and endosomal vesicle fusion (Vps9p [18]).

Shih et al. [102] demonstrated that UIM motifs found in yeast Ent1p, Ent2p, and Vps27p, and the UBA motif in Ede1p are required for the binding of a single ubiquitin molecule and protein internalization. Both UIMs in Ent1p are necessary and sufficient for direct binding of ubiquitin through its Ile-44, required for both the endocytosis and proteasome recognition [88,90]). In addition, UIMs of Ent1p and Ent2p appear to be important for the internalization of the Ste2p receptor into endocytotic vesicles at the plasma membrane, an event that is also partially dependent on Ede1p. The finding that deletions of both UIMs of Ent1p, as well as of Ede1p, either alone or in combination, do not affect ubiquitination of the Ste2p suggests that these proteins are required downstream of cargo modification by ubiquitin. The putative roles of Vps9p, Vps27p, Hse1p, and Vps23p will be discussed in Sections 3 and 3.2.

Several ideas concerning the putative *cis* and in *trans* functions of UIMs and/or CUE domains have been, or can be, proposed [18,94,95,103]. These domains might be recruited to and interact with ubiquitinated endocytotic cargo and/or ubiquitinated *trans*-acting components of the

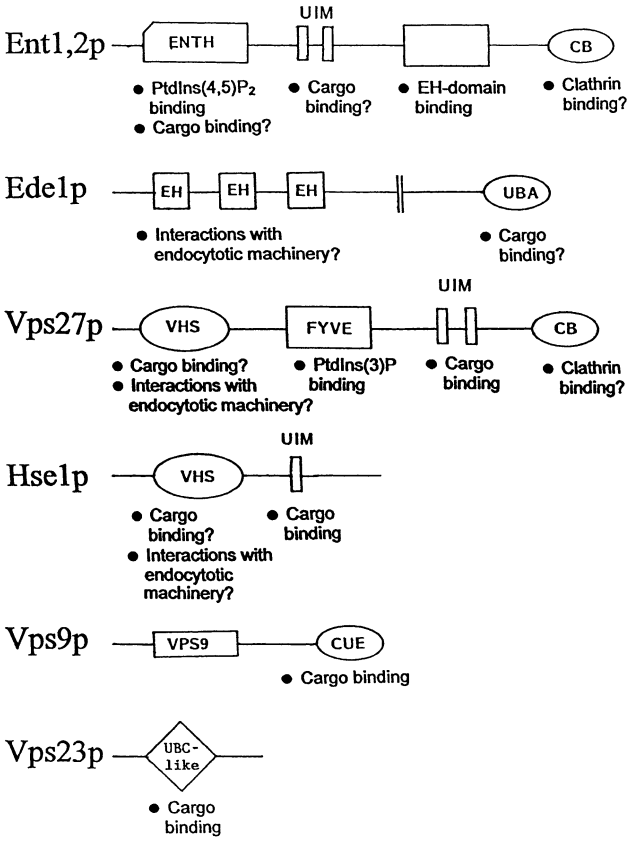

Fig. 4. Modular organization of the yeast UIM, UBA, UBC-like and CUE domain-containing proteins. Each protein domain is shown with its known or putative binding partner. Abbreviations: ENTH, *e*psin *N*-*t*erminal *h*omology domain; EH, *E*ps15 *h*omology domain; VHS, *V*ps27-*H*rs-*S*tam domain; FYVE, *F*ab1-*Y*OTB-*V*ac1-*E*EA1 domain; CB, *c*argo-*b*inding domain; VPS9, *Vps9p* catalytic domain; UIM, *u*biquitin-*i*nteraction *m*otif; UBA, *ub*iquitin-*a*ssociated domain; UBC-like, *ub*iquitin-*c*onjugating enzyme-like domain; CUE, *c*oupling of *u*biquitin conjugation to *E*R degradation.

endocytotic machinery to coordinate cargo sorting and vesicle formation. Alternatively, these motifs might regulate in *cis* availability of domains surrounding them, such as specific phosphoinositide-binding domains ENTH and FYVE of epsins and Vps27p, for example, for interaction with other proteins or lipids. Another possibility is that UIM and CUE domains recognize the ubiquitin moiety on E2 or E3 enzymes and facilitate its transfer to another protein or to the region of the same protein that is, however, distinct from the UIM (CUE) itself. However, it is also possible that the above ubiquitin-binding domains interact with E2 or E3 enzymes independently of binding to ubiquitin. Finally, UIMs also might protect in *cis* monoubiquitinated proteins from becoming polyubiquitinated.

3. Ubiquitination in intracellular events

Once yeast plasma membrane proteins have been selectively packed into endocytotic vesicles and internalized, other mechanisms must operate to determine the ultimate fate of endocytosed cargo. Among them two post-internalization processes, involving fusion of vesicles originating from the plasma membrane and protein-sorting at the MVB (Section 3.2), appear to depend on ubiquitin and ubiquitin-interacting proteins.

Recent findings indicate that fusion of plasma membrane-derived vesicles with some compartment upstream of MVB is promoted by direct interaction of monoubiquitin with the CUE domain of Vps9p, a yeast guanine nucleotide exchanging factor [18,94]. Importantly, this interaction also requires E3 ubiquitin ligase Rsp5p [94] and results in CUE domain-dependent monoubiquitination of Vps9p. Using a Ste2p-ubiquitin fusion protein, Donaldson et al. [18] revealed that impairing the interaction between monoubiquitin and Vps9p, either deleting the *VPS9* gene or mutating ubiquitin, results in cytoplasmic rather than vacuolar accumulation of the fusion protein. In addition, it appears that the traffic defect caused by mutating ubiquitin can be rescued by deletion of the Vps9p CUE domain. Thus, it seems probable that the CUE domain negatively regulates Vps9p activity until ubiquitin positively regulates it via interaction with the CUE domain [18,94]. In what precise manner ubiquitin binding or ubiquitination might modulate Vps9p remains to be determined.

3.1. Recycling from the early endosome to the plasma membrane

The first sorting decision takes place at the next endocytotic organelle, EE, where the subset of proteins that recycle back to the plasma membrane is separated from proteins en route to the vacuole for degradation. In the yeast *S. cerevisiae* direct evidence for such a recycling mechanism was provided only recently by developing a new recycling assay [104]. This assay discovered a rapid recycling (half-time of about 10 min) of internalized fluid-phase endocytosis marker dye FM4-64 to the cell surface. To date, the endocytotic recycling is thought to participate in trafficking of Chs3p, a subunit of the cell-wall-biosynthetic chitin synthase [105], the exocytotic v-SNARE Snc1p [106], Ste6p [107], and Ste3p [108,109]. The Chs3p translocates between two pools, the chitin deposition on the cell surface and an intracellular structure called chitosome. Snc1p functions in the recycling pathway between the plasma membrane and the early endosomes. No evidence for involvement of ubiquitin in endocytosis or recycling of Chs3p and/or Snc1p has been provided. In the case of Ste6p, only a smaller fraction of constitutively internalized Ste6p recycles back to the plasma membrane, while the rest of it is targeted to the vacuole for degradation [107]. Interestingly, two distinct endocytotic modes, constitutive and **a**-factor-dependent, which use different internalization signals, partially overlapping *trans*-acting factors, differing in the requirement for ubiquitin, and leading to different fates of the protein, were established for Ste3p. Constitutive

endocytosis of Ste3p requires a PEST-like sequence as internalization signal, ubiquitin, and ultimately leads to the degradation of the receptor in the vacuole [22,53,65]. In contrast, the **a**-factor-induced Ste3p endocytosis requires the NPFxD motif as an internalization signal, is ubiquitin-independent, and is primarily associated with recycling of the receptor to the plasma membrane [65,108,109]. Thus, Ste3p (and possibly also Ste6p) represents proteins that may undergo ubiquitin-dependent redistribution from the recycling pathway to the MVB-sorting pathway (Section 3.2) and degradation.

Four putative protein components of the yeast recycling machinery, viz. Rcy1p, Skp1p, and two v-SNAREs (Tlg1p and Tlg2p), were identified to date. The Rcy1p (*recy*cling 1) protein contains two motifs that may provide clues to its function, e.g., a C-terminal prenylation motif CAAx and an N-terminal F-box motif [101], through which proteins usually bind to the Skp1p, one of the core components of SCF ubiquitin ligase complex, an E3 which ubiquitinates multiple proteins destined for degradation (see also in Section 4; reviewed in Ref. [111]). Indeed, the CAAx motif appears to be required for proper localization of Rcy1p to the plasma membrane. However, although Rcy1p binds Skp1p in a way that is essential for recycling to occur, no other known components of SCF complexes are known to associate [110]. Thus, the Rcy1p–Skp1p complex probably does not function as E3 ubiquitin ligase.

3.2. The MVB sorting pathway

In the next step of endocytosis, internalized proteins that are not recycled back are targeted from the EE to the LE/PVC, where they encounter vacuolar resident proteins and transporters diverted from their normal route both arriving from the TGN. At the level of LE/PVC, the second sorting decision is made, by which proteins routed to the vacuolar lumen are diverted from proteins of the limiting (outer) vacuolar membrane (Fig. 5). This sorting event requires

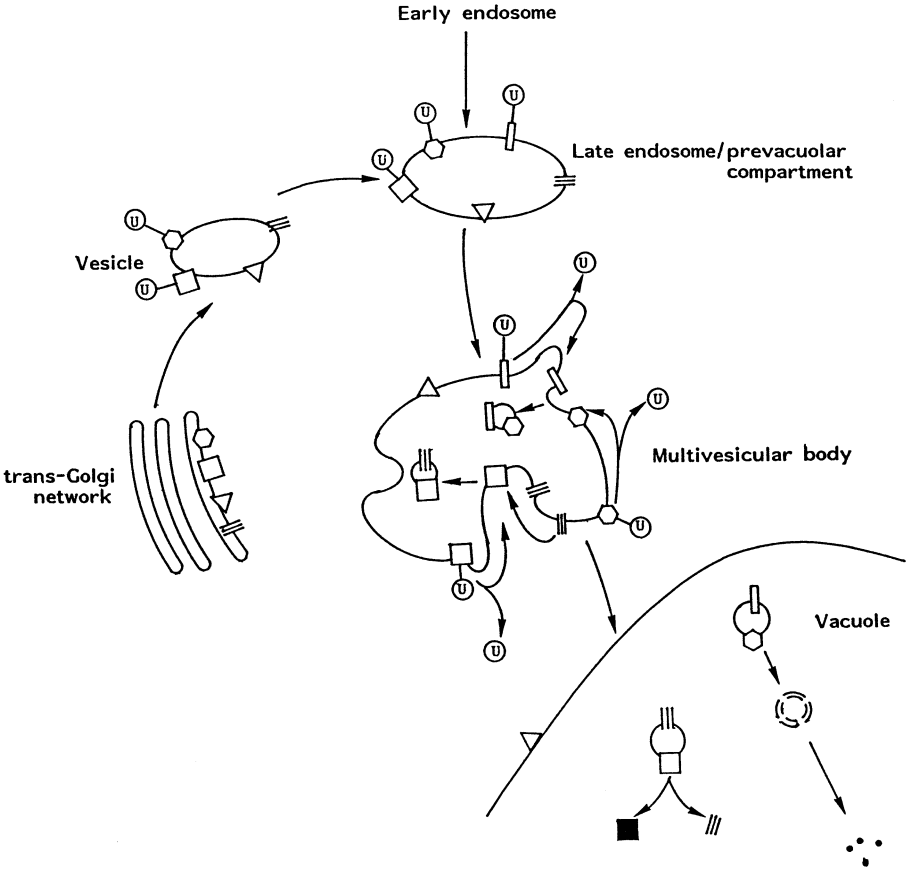

Fig. 5. Overview of the role of ubiquitination in protein delivery from the TGN and LE/PVC to the vacuole. Precursor of carboxypeptidase S (squares) and Gap1p, whose traffic to the plasma membrane is blocked in cells growing on a rich nitrogen source (hexagons), are ubiquitinated after their exit from TGN, while proteins destined for the vacuolar membrane (Vph1p; triangles) and the vacuolar lumen (Sna3p; three parallel lines) are not ubiquitinated. All these proteins are routed to the LE/PVC, where they encounter ubiquitinated membrane transport proteins and receptors delivered from the plasma membrane (hexagons, rectangles, Gap1p, Ste3p). In the next step, the proteins are sorted in the MVB vesicles in a process facilitated by class E Vps protein complexes (not shown), except for Vph1p, which remains in the noninvaginated MVB membrane. Before the proteins enter the MVB, ubiquitin is split off by Doa4p. Subsequently, direct fusion of MVB with the vacuole results in the delivery of Vph1p to the vacuolar membrane, and the MVB vesicle content to vacuolar lumen. In the vacuolar lumen, proteolysis of receptors and transport proteins as well as maturation of carboxypeptidase S proceeds in a vacuolar-dependent fashion, whereas Sna3p is not degraded. Mature carboxypeptidase S is shown as black squares; U stands for ubiquitin.

generation of MVB; invaginations in the limiting membrane of the LE/PVC bud into its lumen, forming internal vesicles, which give rise to the MVB [112] (reviewed in Refs. [13–17]). Membrane proteins destined for degradation in the vacuolar lumen, such as Ste2p, or undergoing specialized processing (precursor of carboxypeptidase S; pCps1p), are sorted into these vesicles, while protein constituents of the limiting vacuolar membrane, such as the Vph1p subunit of the vacuolar H+-ATPase, or iron transporter Fth1p, are excluded from them. The protein sorting into vesicles is thought to be an irreversible event, and the mature MVBs loaded with them fuse directly with the vacuole, delivering their intraluminal vesicles into the vacuolar lumen for degradation by lipase(s). The protein cargo is released and degraded (in the case of certain membrane proteins), or processed (in the case of pCps1p) via *PEP4*-dependent proteolysis machinery [112]. In contrast, proteins retained in the limiting membrane of the MVBs are ultimately incorporated into the limiting vacuolar membrane.

Studies in yeast have identified a family of proteins that are required for endosomal sorting. Mutants defective in any of at least 17-member-family of class E vacuolar protein-sorting (*VPS*) genes fail to transport biosynthetic and endocytotic cargoes to the vacuole. Instead, they accumulate protein cargoes in a large perivacuolar and multilamellar late endosome-like compartment, referred to as the class E compartment (for review see Ref. [113]). Current data suggest that the class E compartment is primarily defective in cargo sorting to internal vesicles, apparently as a consequence of its inability to mature into the MVB [112].

The first studies implicating (mono)ubiquitination as a specific signal for protein cargo sorting into the internal membranes of MVBs have come from studies of two vacuolar enzymes, Cps1p [114,115] and polyphosphatase Phm5p [115], that follow the same pathway. The pCps1p is delivered directly from the TGN to the endosomal system, where it is selectively sorted to the invaginating vesicles of the MVBs [114]. Monoubiquitination of pCps1p on Lys-8 in the 19-amino-acid cytoplasmic region is necessary for its binding to ESCRT-I complex (see below) that targets it subsequently into the luminal vesicles of MVBs [114]. When monoubiquitination of pCps1p is prevented by mutation of Lys-8 to Arg, the precursor remains on the outer surface of the MVBs and ends up on the limiting vacuolar membrane. After delivery to the lumen of the vacuole, pCps1p is proteolytically processed, an event that liberates the mature soluble and active form of the enzyme from its transmembrane anchor [116]. In this context, Reggiori and Pelham [115] have shown that Phm5p (and also Cps1p) is ubiquitinated and when Lys-6 of its cytoplasmic region is mutated, the enzyme is missorted to the vacuolar membrane. In contrast, the Sna3p, another membrane protein reported in Ref. [115], is unique in that it enters intraluminal vesicles of MVBs in ubiquitin-independent fashion, implying a function of an additional sorting mechanism. Additional studies demonstrating the importance of ubiquitin mediated sorting into MVBs have focused on the vacuolar membrane-resident proteins Vph1p and Fth1p, the Vps10p sorting receptor for carboxypeptidase Y that normally recycles between the LE and the Golgi network, and a mutant form of Pep12p. When ubiquitin was covalently linked to the Vph1p, Fth1p, and Vps10p, the proteins were redirected to intralumenal vesicles and, in addition, ubiquitin fusion in frame to the cytoplasmic region of Vps10p was shown to be sufficient to target this chimera to the MVBs [117]. In addition, introduction of a polar residue into the cytoplasmic portion of the transmembrane domain (TMD) of LE/PVC-resident Pep12p induces its tagging with ubiquitin, which serves subsequently as a signal for sorting of this mutant Pep12p into MVBs [118]. Remarkably, ubiquitination of the mutant Pep12p, as well as Cps1p and Phm5p, two biosynthetic membrane proteins containing moderately polar TMDs, appears to be mediated by a novel E3 ubiquitin ligase, Tul1p, that acts in concert with the E2 Ubc4p [119]. Tul1p is a transmembrane protein that has a RING-finger domain, resides in the Golgi, and seems to recognize proteins by their TMDs. Taken together with the inducibility of *TUL1* gene by conditions that trigger "unfolded protein response", one role of Tul1p seems to be in the recognition of misfolded or unassembled membrane proteins within the lipid bilayer and tagging them for transport to and proteolysis in the vacuole. Such proteins would be prone to aggregate [119]. However, removal of Tul1p causes only partial defect in the ubiquitination of the above proteins and, thereby, at least one other E3 must also be capable to act on these proteins. Whether the Rsp5p, also involved in TGN to endosome transport of some proteins (Section 3.3), or other E3 ubiquitin ligases have a role in this process remains to be shown.

Although ubiquitination is required for sorting of most protein cargoes into the MVB, it is less clear whether also the reverse reaction, i.e., deubiquitination, is necessary for proper sorting. Doa4p (Ubp4p) is the most extensively characterized member of a large family of deubiquitination enzymes (17 members in *S. cerevisiae*) [1,5], responsible for removing of ubiquitin from ubiquitin–protein conjugates targeted to the 26S proteasome [120,121] and vacuole [4,121] for proteolysis. Swaminathan et al. [121] have shown that cells deleted for *DOA4* have decreased levels of free ubiquitin, presumably due to the accumulation of ubiquitin–protein conjugates. Subsequently, six distinct *DID* (Doa4p-independent degradation) genes, all encoding class E Vps proteins, were identified and shown to be responsible for maturation of a LE/PVC into MVBs [4]. Importantly, *DID1*, *DID3*, *DID4*, and *DID6* are allelic to *SNF7*, *VPS24*, *VPS2* encoding proteins of the ESCRT-III complex, and *VPS4*, respectively (see below). Moreover, Doa4p, which exhibits a diffuse cytoplasmic/nuclear distribution in wild-type cells, partly relocated in some class E *vps* mutants to the LE/PVC-like class E compartment adjacent to the vacuole [4]. Taken together with the fact that inactivation of genes important for endocytosis and

vacuolar proteolysis significantly reduced ubiquitin depletion in *doa4*Δ cells [121], Doa4p appears to play a crucial role at the LE/PVC (before invagination) in the recovery of ubiquitin from plasma membrane proteins and biosynthetic cargo proteins en route to the vacuole (Fig. 5), thus contributing significantly to homeostasis of ubiquitin. It is, however, still unclear whether the Doa4p function is necessary for proper endosomal function because defects observed in *doa4*Δ cells for distinct proteins differ significantly [107,114,122].

Remarkably, the accumulation of the endocytosed Fur4p in the LE/PVC of *yck*^{ts} cells, induced by inhibition of protein synthesis, led to the suggestion that Yck1p/Yck2p-mediated phosphorylation of Fur4p or some downstream component of the endocytotic machinery might increase the endosome-to-vacuole traffic of the Fur4p [123]. Because the status of Fur4p phosphorylation is similar, independent of whether the transporter is present at the plasma membrane, the LE/PVC, or the vacuole, the Yck-dependent phosphorylation must regulate the activity of some other endocytotic component(s). These observations have resulted in a model in which Yck kinases are endocytosed and subsequently phosphorylate component(s) of the endocytotic pathway, and recycle back to the plasma membrane [123].

Recently, four class E Vps protein complexes, named ESCRT-I to ESCRT-III (*e*ndosomal *s*orting *c*omplex *r*equired for *t*ransport) and Vps27p/Hse1p, all acting sequentially in ubiquitin-dependent protein cargo sorting into the internal membranes of MVBs, were reported [114,124,125]. ESCRT-I is a cytosolic complex comprised of three proteins (Vps23p/28p/37p) [114] that is recruited transiently to the endosomal membrane. The Vps23p subunit of ESCRT-I has a UBC-like domain (see Section 2.6) and in complex with Vps28p and Vps37p, it binds (directly or indirectly) ubiquitinated cargo, and initiates its sorting into the MVB vesicles [114]. ESCRT-II is also a soluble heterotrimeric complex consisting of Vps22p/25p/36p [124] recruited transiently to the endosome membrane. In contrast, the four structurally related components of the ESCRT-III (Vps2p/20p/24p and Snf7p) are monomeric in the cytosol and form a large oligomer only when recruited to the endosomal membrane. ESCRT-III contains two functionally distinct subcomplexes, Vps20p/Snf7p and Vps2p/24p [125]. ESCRT-II interacts with the ESCRT-III Vps20p/Snf7p subcomplex to promote assembly of multiple copies of ESCRT-III on the membrane and thereby serves to recruit at least two additional proteins, Doa4p and Vps4p, an AAA-type ATPase. The Doa4p removes ubiquitin from the cargo prior to entry into these MVB vesicles and, after completion of MVB sorting, the Vps4p dissociates all ESCRT complexes from the membrane. Genetic data showing that the recruitment of ESCRT-III to membranes needs ESCRT-II and overexpression of ESCRT-I can partially substitute for loss of ESCRT-II function (but not vice versa) suggests that ESCRT-I activates ESCRT-II to initiate the formation of ESCRT-III.

The components of ESCRT-I, II, and III complexes, together with the Vps4p, account for only 11 of 17 known class E Vps proteins, suggesting the existence of even more class E Vps protein-containing complexes. One attractive candidate is a complex of two UIM-containing proteins, Vps27p and Hse1p [101] (Fig. 4). The complex localizes to the endosomal compartment (s), where it plays roles in the formation of vacuolar luminal membranes and sorting of ubiquitinated proteins into them. The ubiquitin binding by the complex is strictly dependent on both UIM domains of Vps27p. Whether the ESCRT-I and Vps27p/Hse1p complexes recognize ubiquitinated protein sequentially in the budding process, or whether they recognize ubiquitin in a different context to fulfill a different function, remains to be determined.

3.3. Sorting of amino acid transporters at the Golgi apparatus

Strong evidence suggesting that ubiquitination also regulates sorting of at least two plasma membrane transporters Tat2p and Gap1p at the level of TGN or endosome in response to the quality of the nitrogen source in the growth medium is now available. In cells growing on poor nitrogen sources (proline, urea) the Gap1p is expressed at the plasma membrane in an active and stable form [126]. Upon addition of ammonium (or glutamate), the Gap1p is ubiquitinated, internalized by endocytosis, and degraded in the vacuole. However, in cells growing in a medium containing glutamate [127,128] or ammonium [126], highly preferred nitrogen sources, Gap1p is directly targeted from the TGN to the vacuole for degradation without ever being delivered to the plasma membrane (Fig. 5). In contrast, Tat2p is expressed at the plasma membrane in cells growing on rich nitrogen sources, whereas it is targeted from the TGN directly to the vacuole for degradation during nitrogen (carbon) starvation or rapamycin treatment [37]. When present at the plasma membrane, Tat2p is internalized by endocytosis and degraded in the vacuole in response to nitrogen- or rapamycin-triggered starvation conditions [37]. Addition of any amino acid to the growth medium results in an increase of Gap1p sorting to the vacuole, regardless of the ability of these compounds to be used as a nitrogen source [129]. Thus, the Gap1p sorting machinery seems to respond to amino-acid-like compounds, rather than to the overall nitrogen nutritional status in the cell. The exact mechanism through which exogenous amino acids act is not known. However, if the suggested possibility that they act as allosteric effectors of the Gap1p sorting machinery decreasing transporter capacity is correct [129], it could provide an explanation for the old phenomenon, known as trans-inhibition. This phenomenon was based on the observation that cells grown or preincubated in the presence of an amino acid exhibit a decrease in the activity of the corresponding transporter [130].

Detailed studies of the events leading to loss of plasma membrane and internal Gap1p by degradation in the vacuole

revealed three lines of evidence that ubiquitination of both pools shares the same *cis*- and *trans*-acting elements. (i) The Gap1p must be ubiquitinated on at least one of the two acceptor lysine residues in its cytosolic N-terminal part [126]. (ii) Ubiquitination requires essential Rsp5p [34,35,126], at least one of two redundant, closely related Bul proteins, Bul1p and Bul2p [125,127], which interact with Rsp5p [131,132], and functional C-terminal tail of Gap1p, containing a complex internalization signal (Section 2.1; [35,126]). (iii) The requirement for Doa4p [36,126] and a novel protein, Bro1p/Npi3p/Asi6p/Vps31p [133–135], in the regulation of Gap1p trafficking, has also been shown. Bro1p appears to be required for efficient ubiquitination and turnover of Gap1p, Fur4p, and Hxt6p/Hxt7p [135]. Genetic data [134,135], the ability of Rsp5p to bind Bul1p and Bul2p [132,134], and the possible association of Bro1p with two members of class E Vps proteins (Snf7p/Vps32p, Vps4p) revealed by mass spectrometry [136] led to the suggestion that Bro1p, Rsp5p, Bul1p, and Bul2p might constitute a complex involved in ubiquitin-dependent control of at least certain transporters in distinct cell compartments [135].

Helliwell et al. [128] have reported that sorting of Gap1p in the TGN can be regulated by polyubiquitination. Thus, in cells overexpressing either Bul1p or Bul2p, the Gap1p is polyubiquitinated and routed from the TGN to the vacuole regardless of nitrogen source quality. However, in the *bul1Δbul2Δ* mutant cells the reduced formation of polyubiquitinated Gap1p is accompanied by increased amounts of monoubiquitinated form of Gap1p, and the transporter is delivered to the plasma membrane more efficiently than in wild-type cells. Bul1p and Bul2p must affect Gap1p sorting through their interactions with Rsp5p because a point mutation in *BUL1* gene, which specifically abolishes the ability of the mutant Bul1p to bind Rsp5p [131], affects this event [126]. These data led to suggestion that polyubiquitination is the key determinant for Gap1p trafficking from the TGN to the vacuole. In contrast, neither increased accumulation of monoubiquitinated Gap1p in *bul1Δbul2Δ* cells nor polyubiquitination of a transporter independent of nitrogen source quality was observed by others [128]. Instead, the Gap1p has been shown to be polyubiquitinated only in response to ammonium added to urea (proline)-grown cells [36]. In addition, a nonessential role for Gap1p polyubiquitination in routing of the transporter to the vacuole was also found [128]. These and other published data [128] imply that deciphering of the exact role for mono- vs. polyubiquitination in the regulation of Gap1p trafficking will require further studies.

Npr1p, a Ser/Thr-specific protein kinase, is another key player in nitrogen source-regulated membrane trafficking of Gap1p and Tat2p [37,137]. The absence of functional Npr1p affects the fate of both the plasma membrane and the internal pools of Gap1p in the same way as ammonium does in wild-type cells. In addition, Npr1p itself is subject to Tor signaling pathway-dependent phosphorylation in cells growing on a rich or ammonium-containing medium, while it is dephosphorylated under the conditions of nitrogen- or rapamycin-induced starvation or growth on proline [37]. Thus, nonphosphorylated Npr1p is required for the stabilization of Gap1p at the cell surface and targets internal Gap1p to the cell surface [126]. Remarkably, the Tat2p transporter is regulated by Npr1p in the opposite fashion, i.e., it is stable under conditions when Npr1p is phosphorylated [37]. Gap1p is highly phosphorylated in wild-type cells growing on proline [138] and its phosphorylation is reduced in *npr1Δ* cells [137]. Nevertheless, the fact that it is still highly phosphorylated in *npr1Δnpi1* double mutant cells indicates that the target of the Npr1p may not be Gap1p itself.

4. Signaling pathways involved in proteolysis of sugar transporters

Sugar transporters inducible by their own substrates, such as Mal11p and Mal61p [26,28,50], Gal2p [29], and Hxt6p and Hxt7p [31], belong to the family of plasma membrane proteins, whose degradation occurs upon arrest of cytosolic protein synthesis in combination with a readily fermentable carbon sources such as D-glucose. Their degradation takes place by a mechanism called glucose or catabolite inactivation due to apparent analogy with the catabolite inactivation of gluconeogenic enzymes [139].

Each extracellular signal must be registered by the cell and, thereafter, the signal must be transduced and ultimately transformed into a biochemical response. For glucose signaling in yeast, at least three principal signal transduction pathways exist. These pathways include the Hxk2p–Snf1p pathway involved in repression (reviewed in Refs. [140–143]), the cAMP–protein kinase A pathway (reviewed in Refs. [142,143]), and the Snf3p–Rgt2p pathway controlling the induction of *HXT* genes (reviewed in Refs. [140–144]). There is now evidence that some protein components of these pathways are also involved in ubiquitin-dependent proteolysis of the sugar transporters Mal61p and Gal2p, in response to glucose addition. Degradation of Mal61p in the vacuole requires two distinct glucose signaling pathways [145–147]. The pathway predominantly responsible for proteolysis of Mal61p is a glucose-transport-independent pathway, in which glucose activates a low-affinity glucose sensor Rgt2p at the plasma membrane. Rgt2p generates a signal that is transmitted through the downstream effector Grr1p [145]. The second pathway requires the rapid transport via glucose transporters Hxt and subsequent Hxk2p-dependent phosphorylation of glucose. Some yet unknown glucose metabolite generates a signal that is then transmitted possibly through the Snf1p protein kinase system [144–147].

Several lines of evidence indicate that the glucose-transport-dependent signaling pathway also generates the major signal for initiation of Gal2p proteolysis [148]. To trigger Gal2p degradation, at least the transport and phosphoryla-

tion of glucose and/or glucose-related sugars appear to be prerequisite for generation of the proper signal. The strict requirement for sugar phosphorylation suggests that the sensing mechanism probably does not operate at the level of sugar transport. A very similar pattern of responses to the sugars was also found for Mal61p [146,149]. Consistent with the above view, Hxk2p is required for induction of Gal2p turnover. Hxk2p is a glucokinase that, in addition to its catalytic function, i.e., phosphorylation of intracellular glucose and related sugars, plays a pivotal role in the control of the expression of a variety of genes, including itself (reviewed in Ref. [150]).The signal generated in this way is subsequently transduced through Reg1p and Grr1p, two downstream components of the glucose signaling pathway. How these proteins contribute to Gal2p degradation remains a puzzle. Reg1p is a regulatory subunit of protein phosphatase type I (PP1p) that targets its catalytic subunit Glc7p to proteins involved in Hxk2p–Snf1p and Snf3p–Rgt2p pathways (reviewed in Refs. [141,144]. Reg1p seems to directly inactivate Snf1p kinase of the Hxk2p–Snf1p pathway by its dephosphorylation in the presence of high glucose concentrations [141]. However, whether this PP1p is also a component of the Gal2p and Mal61p degradation pathways in which PP1p acts via Snf1p, or whether it affects the fate of Gal2p and Mal61p in an indirect manner, remains to be elucidated. Some data suggest that PP1p might activate a protein kinase required for phosphorylation of transporters and thereby targets them for degradation [147]. Grr1p contains an F-box motif and, as a component of SCF^{Grr1} complex (*S*kp1p-*C*ullin/Cdc53p-*F*-box), which belongs to a class of E3 ubiquitin ligases, it plays key roles in a variety of cell events, including transduction of the glucose signal generated by the Snf3p–Rgt2p pathway. It is thus possible that Gal2p proteolysis is prevented in *grr1* null cells due to block in SCF^{Grr1}-dependent degradation of some factor. The factor might be a negative component of the signaling pathway or a positive component that needs to undergo ubiquitin-requiring processing to be activated by glucose. Interestingly, two E3 ubiquitin-ligases, Rsp5p and SCF^{Grr1}, are needed for Gal2p and Mal61p turnover. However, a nearly complete block of Gal2p and Mal61p in either *rsp5* or *grr1* single mutants implies that they function at distinct steps of protein turnover.

Remarkably, proteolysis of fructose-1,6-bisphosphatase, a key gluconeogenic enzyme that is degraded by the 26S proteasome after polyubiquitination [151–153], requires most (if not all) protein components of the above glucose-transport-dependent pathway [148]. Thus, one signaling pathway initiates two different proteolytic mechanisms of catabolite inactivation, proteasomal proteolysis and endocytosis, followed by proteolysis inside the vacuole.

The chemical nature of the signal(s) initiating glucose-induced turnover of Mal61p, Gal2p, and FBPase is unknown. The ATP/AMP ratio or its change [154], glucose-6-phosphate, UDP-glucose, or derivatives of trehalose were suggested as putative candidates for this function [80,149].

Since arrest of protein synthesis by nitrogen starvation or cycloheximide addition stimulates general protein turnover [155], it has been proposed that glucose-induced proteolysis of the maltose transporter might be mainly due to the nonspecific mechanism in which glucose metabolism provides an energy source rather than to a specific mechanism controlled by glucose [156]. However, proteolysis of sugar transporters depends on their specific amino acid sequences [59,157], the kinetics and extent of proteolysis of individual transporters differ, the Mal61p degradation requires a functional Rgt2p glucose sensor [145], the capability of some readily fermentable sugars to induce Gal2p and Mal61p proteolysis differ [146,148], and, for example, Gal2p is stable in cells starved for nitrogen even when galactose metabolism provides sufficient energy [148]. Taken together, these data suggest that degradation of sugar transporters is a specific process rather than a simple sequestration of bulky membrane proteins.

5. Concluding remarks

The past few years clarified some of the far-reaching functions of ubiquitin other than proteasome-dependent proteolysis. One of such functions is a ubiquitin-dependent control of endocytotic and biosynthetic protein cargo trafficking en route to the vacuole. An important landmark in the recent progress has been made especially by identifying the components of the above machineries, including a network of ubiquitinated and ubiquitin-binding proteins. Despite that, however, many goals remain to be solved in the future, for instance: (i) Since nearly 2% of the genome of *S. cerevisiae* is associated with genes encoding proteins of ubiquitin metabolism, it is clear that many other ubiquitin-dependent factors and motifs will be identified. (ii) Another major challenge is to understand how the different proteins or their families communicate with each other and coordinate the events for successfully transporting cargo to the vacuole. Little is known about, for instance, how ubiquitin acts in the selection of protein for endocytosis, in its sorting into any endosome-derived structure, or in determination of its correct destination. (iii) Little is also known regarding the signaling pathways that stimulate ubiquitin-dependent degradation of a certain proteins to enable cells to adapt to changing environmental conditions. Since cellular mechanisms are often conserved from yeast to multicellular organisms, knowledge gained from yeast can lead to a better understanding of similar mechanisms also in higher eukaryotes (reviewed in Refs. [10,12,13,17,87,158]).

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