

Versatile role of the yeast ubiquitin ligase Rsp5p in intracellular trafficking

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Abstract

The ubiquitin ligase (E3) Rsp5p is the only member of the Nedd4 family of E3s present in yeast. Rsp5p has several proteasome-independent functions in membrane protein trafficking, including a role in the ubiquitylation of most plasma membrane proteins, leading to their endocytosis. Rsp5p is also required for the ubiquitylation of endosomal proteins, leading to their sorting to the internal vesicles of multivesicular bodies (MVBs). Rsp5p catalyzes the attachment of non conventional ubiquitin chains, linked through ubiquitin Lys63, to some endocytic and MVB cargoes. This modification appears to be required for efficient sorting, possibly because these chains have a greater affinity for the ubiquitin-binding domains present within endocytic or MVB sorting complexes. The mechanisms involved in the recognition of plasma membrane and MVB substrates by Rsp5p remain unclear. A subset of Rsp5/Nedd4 substrates have a "PY motif" and are recognized directly by the WW domains of Rsp5p. Most Rsp5p substrates do not carry PY motifs, but some may depend on PY-containing proteins for their ubiquitylation by Rsp5p, consistent with the latter acting as specificity factors or adaptors. As in other ubiquitin-conjugating systems, these adaptors are also Rsp5p substrates and undergo ubiquitin-dependent trafficking. In this review, we discuss recent examples illustrating the role of Rsp5p in membrane protein trafficking and providing new insight into the regulation of this E3 by adaptor proteins.

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Introduction

Ubiquitylation was initially described as promoting proteasomal degradation, and has since been shown to regulate other processes, including DNA repair, signaling and trafficking. In this review, we will focus on the role of the yeast ubiquitin protein ligase Rsp5p in several steps of intracellular trafficking.

Protein ubiquitylation is a reversible posttranslational modification in which the 76-amino acid peptide ubiquitin is covalently linked, via its COOH-terminal glycine, to the ϵ -amino group of the lysine residues of target proteins. This process involves sequential — E1 (ubiquitin-activating), E2 (ubiquitin-conjugating or UBC) and E3 (ubiquitin-protein ligase) — enzyme activities, with the E3 responsible for target recognition [1]. E3 enzymes form several families, the two main families being RING-finger and HECT E3 proteins. RING-finger E3s act as scaffolds, bringing E2 and the substrate into contact. HECT-domain E3s are directly involved in catalysis: the activated ubiquitin is transferred from the E2 to an internal Cys residue on the E3 and is then conjugated to a lysine residue within the target. As ubiquitin itself carries several lysines (K6, 11, 27, 29, 33, 48 and K63), multiubiquitin chains are frequently formed. K48-linked multiubiquitin chains, at least four ubiquitin units long, are potent signals leading to recognition and degradation by the 26S proteasome. K63-linked multiubiquitin chains are involved in other functions, including DNA repair, the activation of specific kinases and endocytosis. Finally, ubiquitylation is reversible, and ubiquitin can be removed by deubiquitylation enzymes (DUBs) [1].

HECT-containing E3s include the family of Nedd4/Nedd4-like ubiquitin ligases, of which there are nine in humans and one in *Saccharomyces cerevisiae* — the essential protein Rsp5p. These proteins have a modular structure: an N-terminal C2 domain (mainly known as a lipid-binding domain), two to four WW domains (protein-protein interaction modules that bind short recognition motif called PY motif: [L/P]PxY), and a catalytic COOH-terminal HECT domain. Rsp5p has multiple functions, including roles in mRNA export [2], or the processing of transcription factors [3]. Its function in trafficking has already been documented (Reviewed in [4-7]). Here, we summarize recent data and discuss some of the questions that have been raised concerning the mode of action of Rsp5p in trafficking.

Role of Rsp5p in the internalization step of endocytosis

The role of Rsp5p in trafficking was initially discovered in a genetic screen investigating the ammonium-induced downregulation ("inactivation") of yeast amino-acid transporters. Several *npi* (nitrogen permease inactivator) mutants had been isolated in which the general amino-acid permease Gap1p was not inactivated by ammonium. Cloning and sequencing of the *NPI1* gene showed that it encoded the ubiquitin ligase Rsp5p. Both Gap1p and another transporter, the Fur4p uracil permease, were then shown to undergo Rsp5p-dependent ubiquitylation at the plasma membrane, a modification required for their internalization and subsequent proteasome-independent vacuolar degradation. Mutation of the target lysines in these transporters strongly impaired internalization of these proteins (reviewed in [4, 8]). Rsp5p-mediated ubiquitylation and internalization appear to be a general feature of most plasma membrane proteins in yeast [4, 9] (Fig. 1). Ubiquitin-dependent endocytosis also

occurs in mammalian cells, mediated either by Nedd4/Nedd4-like E3s or by RING-finger E3s, but this process is less general in mammals than in yeast [4, 6, 7, 9].

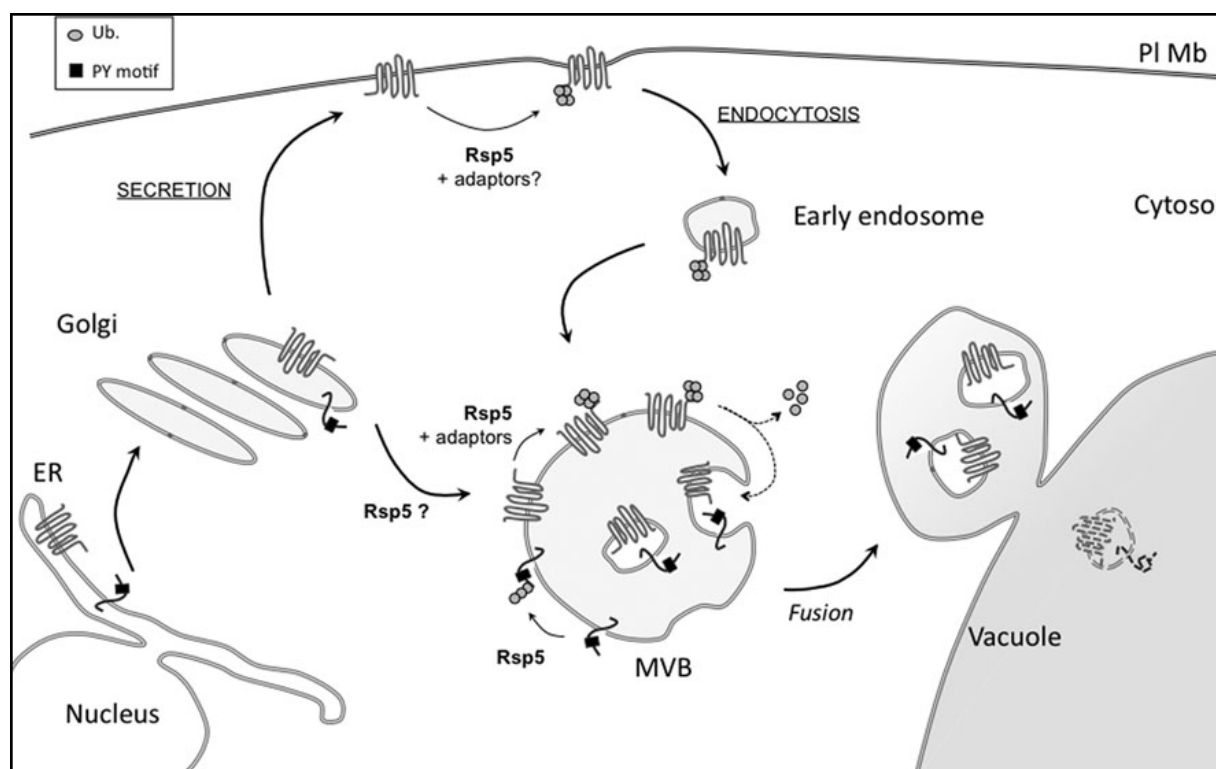


Fig 1. Multiple roles of Rsp5p in protein trafficking. A multispanning transporter targeted to the plasma membrane undergoes phosphorylation (not shown) followed by Rsp5p-dependent ubiquitylation (UbK63-linked oligoubiquitylation), triggering its internalization. The same transporter can be directly routed to the endosomal pathway, therefore following the VPS (vacuolar protein sorting) pathway. This pathway can be used by other proteins, such as a membrane PY-motif (black rectangle)-containing protein. Both proteins undergo Rsp5p-dependent ubiquitylation in their sorting form the Golgi apparatus to endosomes. Whereas the transporter displays adapter-dependent ubiquitylation at MVB, the PY-motif containing protein interacts directly with Rsp5p, leading to its ubiquitylation (UbK63-linked chains). Ubiquitylated proteins originating from the Golgi- or the plasma membrane undergo deubiquitylation prior to their sorting to MVB internal vesicles. After fusion of MVBs with the vacuole, these proteins are degraded by vacuolar proteases.

The observation of Rsp5p-mediated endocytosis in yeast constituted a major step towards deciphering the mechanisms involved in the internalization step of endocytosis in yeast, but it also raised new questions, concerning substrate recognition in particular. The sodium channel ENaC, one of the first plasma membrane substrates of Nedd4 to be described in mammals, carries several PY motifs that are recognized by Nedd4 through a PY-WW interaction [10]. This mode of recognition has often been considered to be general, and the WW domains of Rsp5/Nedd4 E3s are often presented as substrate recognition modules. Indeed, some Rsp5p substrates carry PY motifs, and display PY-WW based interaction with Rsp5p [11], but this is not the case for endocytic substrates from the plasma

membrane [4]. It has been suggested that PY-containing proteins may act as Rsp5p-substrate adapters. The only PY-containing proteins characterized so far that interact with Rsp5p and regulate the endocytosis of a subset of plasma membrane proteins are the homologous, cytosolic proteins Bul1/2. These proteins are required for the Rsp5p-mediated ubiquitylation and internalization of several endocytic substrates [12-14]. However, the role of these proteins, which have also been presented as E4 enzymes (involved in the extension of ubiquitin chains) [15] remains unclear. The mechanism underlying Bul1/2 function remains to be defined, and other potential adapters at the plasma membrane remain to be identified. There is also an additional level of complexity concerning the recognition of Rsp5p plasma membrane substrates. In addition to basal endocytosis, most yeast plasma membrane proteins display regulated ubiquitylation and internalization. For example, the ubiquitylation and internalization of Gap1p, Tat2p, and Ctr1p are triggered by ammonium, high tryptophan, and high copper concentrations, respectively [8]. It is tempting to hypothesize that phosphorylation events may play a role in substrate recognition: indeed, the phosphorylation of Ste2p (triggered by its ligand, α -factor) and Fur4p is required for the ubiquitylation of these proteins [4, 9]. This posttranslational modification may result in conformational changes, rendering certain lysines accessible for ubiquitylation. It remains unclear whether such conformational changes may trigger the direct interaction of Rsp5p with substrates, or with potential adapters.

The first observations of ubiquitin-dependent internalization rapidly led to questions concerning the mechanism by which these substrates escaped recognition by the proteasome. The use of ubiquitin point mutants (mutation of Lys into Arg) showed that Fur4p and Gap1p were modified by short ubiquitin chains (2 to 3 ubiquitin moieties long, oligoubiquitylation) linked *via* their Lys63 residues [16, 17], and that this modification was required for efficient endocytosis. By contrast, the α -factor receptor Ste2p has been reported to undergo primarily monoubiquitylation [18]. Two other yeast transporters were recently shown to be modified by Rsp5p, with the attachment of UbK63-linked ubiquitin chains ([19], and Erpapazoglou et al, submitted). These observations are consistent with recent studies showing that Rsp5p preferentially assembles this type of ubiquitin chain *in vitro* and *in vivo* [20]. A number of plasma membrane transporters or cell surface proteins also undergo modification by Lys63-linked ubiquitin chains in mammalian cells [6, 21-23]. This type of ubiquitylation is carried out by Nedd4 in the case of the dopamine DAT transporter [6].

The role of Rsp5p in sorting to multivesicular bodies

In addition to its role as an internalization signal at the plasma membrane, ubiquitylation is also required for the sorting of membrane proteins originating from the Golgi apparatus or the plasma membrane to the internal vesicles of multivesicular bodies (MVBs) [24]. Yeast MVB cargoes have been shown to undergo ubiquitylation. Inhibition of this ubiquitylation abolishes MVB sorting, resulting in the recovery of cargoes at the vacuolar membrane rather than in the vacuolar lumen, after the fusion of MVBs with the vacuole (reviewed in [24]). Genetic analyses in yeast have led to the discovery of four protein complexes — the “endosomal sorting complexes required for transport” (ESCRTs 0 to III) — conserved from yeast to humans and involved in the recognition of ubiquitylated cargoes and their sorting to the internal vesicles of MVBs. ESCRTs 0, I and II each carry at least one

subunit bearing a ubiquitin-binding domain [24]. Extensive characterization of the ESCRT complexes preceded identification of the E3 involved in the ubiquitylation of MVB cargoes, which turned out to be Rsp5p [25, 26, 27]. The MVB substrates of Rsp5p identified to date include two vacuolar enzymes, the carboxypeptidase S1 Cps1p and the polyphosphate phosphatase Phm5p, a membrane protein of unknown function, Sna3p, and several transporters directly sorted from the Golgi apparatus to the vacuole, bypassing plasma membrane delivery, under certain nutrient/substrate conditions [8, 11, 24, 28, 29]. Nedd4 and the Nedd4-like E3 AIP4/Itch have also been shown to be involved in the ubiquitylation of a few MVB cargoes and lysosomal proteins [30, 31].

The observation of Rsp5p-dependent ubiquitylation at MVBs raises questions similar to those raised following observation of the role of this E3 in endocytic internalization. How are substrates recognized? With the exception of Sna3p, most of the known substrates do not carry PY motifs, and a requirement for PY-containing adapters for Rsp5p recruitment has been predicted. Indeed, the PY-containing membrane protein Bsd2p, identified in a genetic screen for its role in trafficking of the manganese transporter Smf1p [32], is involved in the sorting of a few MVB cargoes, including Smf1p [33]. The correct sorting of Smf1p to MVBs involves the association of Bsd2p with two additional PY-containing membrane proteins, Tre1/2 [34].

Potential Rsp5p adapters have recently been identified, based on the wealth of information deduced from genomic and proteomic approaches, and the use of protein microarray ubiquitylation assays *in vitro* [35, 36]. Ear1p (endosomal adapter of Rsp5p), another endosomal PY-containing membrane protein, has been identified as both a binding partner and a substrate of Rsp5p. Deletion of *EAR1*, when combined to that of its redundant homolog, *SSH4*, led to a trafficking defect. In the double mutant the Rsp5p-dependent ubiquitylation and MVB sorting of several cargoes is impaired, including Phm5p or transporters such as Gap1p and the siderophore transporter Sit1p, when directly routed to the endosomal pathway. No defect in Smf1p MVB sorting, which depends on the Bsd2/Tre1/2 adaptors [33, 34], was observed. Other MVB cargoes, either directly fused to ubiquitin, or containing PY motifs enabling their direct interaction with and ubiquitylation by Rsp5p were also sorted to MVBs independently of Ear1p/Ssh4p. Thus, Ear1p/Ssh4p appears to be essential for the MVB sorting of a specific set of cargoes by acting on their ubiquitylation status [37]. It remains unclear how Ear1p and Ssh4p promote close connections between Rsp5p and its MVB substrates. Both proteins carry a B30.2/SPRY domain, known to be involved in protein-protein interactions [38] and that could be involved in this interaction. An alternate mechanism has been proposed for Bsd2p/Tre1/Tre2-dependent Smf1p ubiquitylation. The Tre1/2 proteins recognize Smf1p, probably through interaction with polar residues in transmembrane regions, while simultaneously binding Bsd2p. Rsp5p could be brought into close contact with Smf1p through interaction of its WW domain with the PY motifs of Bsd2/Tre1/Tre2 [33]. Adapters of Nedd4/Nedd4-like proteins probably play a similar role in mammals, as a number of PY-containing proteins, partners of Nedd4/Nedd4-like proteins have been reported to be involved in the recruitment of these E3s to the Golgi apparatus/endosomes [39]. Interaction of the C2 domain of Rsp5p/Nedd4-like proteins with phospholipids, including phosphoinositides in particular, probably reinforces adapter-mediated recruitment to a specific membrane [10, 25]

What type of ubiquitylation is associated with and required for MVB sorting? Cps1p was initially reported to undergo monoubiquitylation. Ubiquitin (with its main target Lys mutated) fused in frame to lysine-less Cps1p or Phm5p has been shown to restore MVB sorting [24, 40]. These findings led to the hypothesis that monoubiquitylation is the signal required for MVB sorting. However, ubiquitin-binding domains present in proteins of the ESCRT machinery have low affinity for monoubiquitin [41]. Plasma membrane proteins ubiquitylated at the cell surface by Rsp5p are still oligoubiquitylated when they arrive at the MVBs [42]. The MVB cargo Sna3p was recently reported to undergo Rsp5p-mediated polyubiquitylation at a single target Lys, with up to seven or eight ubiquitin moieties linked via Lys63 (Fig. 1). This pattern of ubiquitylation was altered in cells unable to form Lys63-linked ubiquitin chains [11]. For another cargo, Sit1p, MVB sorting was profoundly affected in cells unable to form Lys63-linked ubiquitin chains (Erapapazoglou et al, submitted). However, this may be due to an indirect effect of these ubiquitin chains on the MVB machinery.

The observation that Rsp5p modifies some MVB and endocytic substrates with Lys63-linked ubiquitin chains is consistent with the recent discovery that Rsp5p and the DUB Ubp2p are present within the same complex, and the demonstration that these enzymes preferentially assemble and disassemble, respectively, Lys63-linked ubiquitin chains [20, 43]. A requirement for Lys63-linked ubiquitin chains at the MVB is also consistent with the demonstration that the mammalian DUB enzyme AMSH, which specifically disassembles UbK63 chains [44], is partly endosomal, associates with ESCRT proteins, and is activated by an ESCRT-0 component (STAM) [45].

Little is known about the mechanisms underlying the possible function of Lys63-linked ubiquitin chains as internalization signals at the plasma membrane and MVBs, but several proteins carrying ubiquitin binding domains, such as the UIM (ubiquitin-interacting motif) or UBA (ubiquitin-associated domain), play a key role in trafficking. Lys63-linked ubiquitin chains have an open, linear conformation [46] in which the hydrophobic patch centered on ubiquitin Ile44 is readily accessible for ligand binding. A clue to the possible role of these chains in trafficking is provided by the observation that the UBA domain of Ede1p, a protein involved in the internalization step of endocytosis, and the UIM domain of Hrs/Vps27, an ESCRT-0 component, have a specific or strong affinity for LysK63-linked ubiquitin chains [41, 47].

Rsp5p and Golgi-to-endosome sorting

In addition to its role in ubiquitin-mediated internalization at the cell surface and sorting to MVBs, a role for Rsp5p in the exit of transporters from the Golgi apparatus and their transfer to the endosomal pathway for vacuolar degradation has also been reported. Regulation of the internalization rate is not the only mechanism by which eukaryotic cells monitor the steady-state abundance of transporters at the plasma membrane. The fate of newly synthesized transporters within the secretory pathway can be regulated by diverting the protein to the lysosome/vacuole without passing by the plasma membrane, in response to environmental conditions. Typical physiological situations inducing this type of control mechanism include hormonal regulation, changes in substrate concentration or the availability of alternative nutrients. Examples in mammals include insulin-mediated trafficking of the glucose transporter GLUT4. The first example identified in yeast was Gap1p, which is sorted to the

vacuolar pathway in an Rsp5p-dependent manner if synthesized in the presence of rich source of ammonium. Tat2p, another amino-acid transporter, displays a similar sorting pattern in the presence of high tryptophan concentrations. In an *rsp5* mutant, Gap1p and Tat2p are recovered at the plasma membrane under conditions known to lead to their vacuolar sorting in wild-type cells. It was thus suggested that Rsp5p is required for the Golgi exit of these transporters, in addition to being required for internalization and sorting to MVBs (reviewed in [8]).

The role of ubiquitin and Rsp5p in the regulated sorting of plasma membrane transporters was recently reconsidered in the case of the siderophore transporter Sit1p. Newly synthesized Sit1p is sorted to the plasma membrane or to the endosomal/vacuolar pathway, depending on the presence or absence of its substrate, ferrioxamine B (FOB) [48]. Following its production in the absence of FOB in *rsp5Δ* cells, Sit1p was recovered at the vacuolar membrane. Thus, it was able to exit the Golgi apparatus, but was not sorted to MVBs. Kinetic experiments on hypomorphic *rsp5* cells demonstrated that Sit1p exited the Golgi apparatus and was sorted to endosomes, but the impairment of MVB sorting resulted in a redistribution from endosomes to the plasma membrane (Erpapazoglou et al, submitted). Consistent with the hypothesis that ubiquitylation is not required for Golgi exit into the endosomal pathway, non ubiquitylable mutant forms of Cps1p, Phm5p and Sna3p are trapped at the vacuolar membrane. However, these proteins are not recovered at the plasma membrane. The plasma membrane redistribution of endosomal proteins appears therefore possible only for transporters [19, 28], perhaps because they carry appropriate plasma membrane sorting signals.

Therefore, ubiquitylation does not appear essential *per se* for the exit from the Golgi apparatus of some MVB cargoes. However, Rsp5p has been shown to function at the Golgi/endosome interface when analyzing the intracellular fate of some MVB cargoes, including PY-containing proteins. This function of Rsp5p was clearly different from its enzymatic function at MVBs. Mutations impairing the interaction of Sna3p with Rsp5p led to deficiencies in the sorting of Sna3p, which was recovered in small structures (Golgi vesicles/endosomes?) upstream from MVBs, whereas a non ubiquitylable form of Sna3p was targeted to the vacuolar membrane [11]. The physical interaction between Sna3p and Rsp5p appears to be crucial for Sna3p sorting to the endosomal pathway. The function of Rsp5p in Sna3p sorting as a mere consequence of a strong interaction would be similar to that fulfilled by its homolog in humans, Nedd4, in the Golgi-to-lysosome trafficking of the lysosome-associated protein transmembrane 5 (LAPTM5), which is sorted from the Golgi apparatus to lysosomes following the binding of its PY motifs to Nedd4 WW domains [31].

Conclusion and perspectives

Rsp5p plays a pleiotropic role in trafficking, and its functions are representative of the trafficking functions of Nedd4/Nedd4-like proteins in mammals [6, 7]. The role of Rsp5p in sorting at the plasma membrane and MVBs is based principally on its function in ubiquitylation. In addition, interaction with Rsp5p, apparently acting as a scaffold, seems to play a crucial role in the Golgi-to-endosome sorting of some PY-containing proteins. An increasing number of MVB cargoes of Rsp5p are thought to be recognized by PY-containing adapters, and PY-containing proteins have also been shown to be required for the ubiquitylation and internalization of a few plasma membrane proteins.

The mechanisms of action of the PY-containing Rsp5p adapters identified to date remain unclear. Depending on the cargo, an adapter may function alone with a single substrate or in combination with other adapters for another substrate. Additional adapters will undoubtedly be discovered in the future, possibly from the long list of PY-containing proteins shown to interact with Rsp5p in genomic and proteomic studies [35, 36]. The four Rsp5p partners shown to function as adapters for the ubiquitylation of MVB cargoes are themselves ubiquitylated and sorted to MVBs [34, 37]. It remains unclear whether this modification is important for the function of these adapters.

Consistent with studies showing a preference for Rsp5p in the assembly of K63-linked ubiquitin chains [20, 43], the list of endocytic and MVB cargoes modified by K63-linked ubiquitin chains is continuing to increase ([8, 11, 19], and Erpapazoglou et al, submitted). Similarly, the first example of a Nedd4 endocytic substrate displaying this type of ubiquitylation has also been reported [6]. The structural features of Rsp5p/Nedd4 accounting for this preference toward the synthesis of ubiquitin-Lys63 linked chains remain to be determined, as does the precise role of these chains in sorting processes. Rsp5p has also been described to promote monoubiquitylation, and even the production of K48-linked ubiquitin chains, ultimately leading to the proteasomal degradation of the target. How is the same E3 able to promote different types of ubiquitylation? The monoubiquitylation of some Rsp5p substrates — particularly endocytic substrates — may result from the trimming of pre-existing UbK63-linked ubiquitin chains by Ubp2p, which associates with Rsp5p and specifically disassembles this type of chain [43]. For Rsp5p- and Nedd4- or Nedd4-like-dependent polyubiquitylation by Lys63-, Lys29- [49] or Lys48-linked chains, it remains unclear whether the chain specification depends on the substrates themselves, requires the involvement of particular adapters and/or E4, or the binding of preformed ubiquitin chains.

Finally, new functions of Rsp5p/Nedd4 E3s in trafficking are likely to emerge. Genetic and cell biology evidence has already been obtained for a link between Rsp5p and the actin cytoskeleton [50] and these findings are supported by proteomic evidence indicating that Rsp5p interacts with numerous proteins involved in actin cytoskeleton organization. Future investigations should improve our knowledge of the corresponding underlying functions.

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References

- 1 Glickman, M. H. and Ciechanover, A. (2002) The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev* **82**, 373-428
- 2 Rodriguez, M. S., Gwizdek, C., Haguenaue-Tsapis, R. and Dargemont, C. (2003) The HECT ubiquitin ligase Rsp5p is required for proper nuclear export of mRNA in *Saccharomyces cerevisiae*. *Traffic* **4**, 566-575
- 3 Hoppe, T., Matuschewski, K., Rape, M., Schlenker, S., Ulrich, H. D. and Jentsch, S. (2000) Activation of a membrane-bound transcription factor by regulated ubiquitin/proteasome-dependent processing. *Cell* **102**, 577-586
- 4 Dupre, S., Urban-Grimal, D. and Haguenaue-Tsapis, R. (2004) Ubiquitin and endocytic internalization in yeast and animal cells. *Biochim Biophys Acta* **1695**, 89-111
- 5 Horak, J. (2003) The role of ubiquitin in down-regulation and intracellular sorting of membrane proteins: insights from yeast. *Biochim Biophys Acta* **1614**, 139-155
- 6 Miranda, M. and Sorkin, A. (2007) Regulation of receptors and transporters by ubiquitination: new insights into surprisingly similar mechanisms. *Mol Interv* **7**, 157-167
- 7 Staub, O. and Rotin, D. (2006) Role of ubiquitylation in cellular membrane transport. *Physiol Rev* **86**, 669-707
- 8 André, B. and Haguenaue-Tsapis, R. (2004) Membrane trafficking of yeast transporters: mechanisms and physiological control of downregulation. *Topics in Curr. Genet.* **9**, 273-323
- 9 Hicke, L. and Dunn, R. (2003) Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Annu Rev Cell Dev Biol* **19**, 141-172
- 10 Rotin, D., Staub, O. and Haguenaue-Tsapis, R. (2000) Ubiquitination and endocytosis of plasma membrane proteins: role of Nedd4/Rsp5p family of ubiquitin-protein ligases. *J Membr Biol* **176**, 1-17
- 11 Stawiecka-Mirota, M., Pokrzywa, W., Morvan, J., Zoladek, T., Haguenaue-Tsapis, R., Urban-Grimal, D. and Morsomme, P. (2007) Targeting of Sna3p to the endosomal pathway depends on its interaction with Rsp5p and multivesicular body sorting on its ubiquitylation. *Traffic* **8**, 1280-1296
- 12 Liu, J., Sitaram, A. and Burd, C. G. (2007) Regulation of copper-dependent endocytosis and vacuolar degradation of the yeast copper transporter, Ctr1p, by the Rsp5 ubiquitin ligase. *Traffic* **8**, 1375-1384
- 13 Soetens, O., De Craene, J. O. and Andre, B. (2001) Ubiquitin is required for sorting to the vacuole of the yeast general amino acid permease, Gap1. *J Biol Chem* **276**, 43949-43957
- 14 Umabayashi, K. and Nakano, A. (2003) Ergosterol is required for targeting of tryptophan permease to the yeast plasma membrane. *J Cell Biol* **161**, 1117-1131
- 15 Helliwell, S. B., Losko, S. and Kaiser, C. A. (2001) Components of a ubiquitin ligase complex specify polyubiquitination and intracellular trafficking of the general amino acid permease. *J Cell Biol* **153**, 649-662
- 16 Galan, J. M. and Haguenaue-Tsapis, R. (1997) Ubiquitin lys63 is involved in ubiquitination of a yeast plasma membrane protein. *Embo J* **16**, 5847-5854
- 17 Springael, J. Y., Galan, J. M., Haguenaue-Tsapis, R. and Andre, B. (1999) NH₄⁺-induced down-regulation of the *Saccharomyces cerevisiae* Gap1p permease involves its ubiquitination with lysine-63-linked chains. *J Cell Sci* **112** (Pt 9), 1375-1383
- 18 Shih, S. C., Sloper-Mould, K. E. and Hicke, L. (2000) Monoubiquitin carries a novel internalization signal that is appended to activated receptors. *Embo J* **19**, 187-198
- 19 Kim, Y., Deng, Y. and Philpott, C. C. (2007) GGA2- and ubiquitin-dependent trafficking of Arn1, the ferrichrome transporter of *Saccharomyces cerevisiae*. *Mol Biol Cell* **18**, 1790-1802
- 20 Kee, Y., Lyon, N. and Huibregtse, J. M. (2005) The Rsp5 ubiquitin ligase is coupled to and antagonized by the Ubp2 deubiquitinating enzyme. *Embo J* **24**, 2414-2424
- 21 Duncan, L. M., Piper, S., Dodd, R. B., Saville, M. K., Sanderson, C. M., Luzio, J. P. and Lehner, P. J. (2006) Lysine-63-linked ubiquitination is required for endolysosomal degradation of class I molecules. *Embo J* **25**, 1635-1645
- 22 Geetha, T., Jiang, J. and Wooten, M. W. (2005) Lysine 63 polyubiquitination of the nerve growth factor receptor TrkA directs internalization and signaling. *Mol Cell* **20**, 301-312
- 23 Li, J. G., Haines, D. S. and Liu-Chen, L. Y. (2008) Agonist-promoted Lys 63-linked polyubiquitination of the human kappa opioid receptor is involved in receptor down-regulation. *Mol Pharmacol*
- 24 Katzmann, D. J., Odorizzi, G. and Emr, S. D. (2002) Receptor downregulation and multivesicular-body sorting. *Nat Rev Mol Cell Biol* **3**, 893-905
- 25 Dunn, R., Klos, D. A., Adler, A. S. and Hicke, L. (2004) The C2 domain of the Rsp5 ubiquitin ligase binds membrane phosphoinositides and directs ubiquitination of endosomal cargo. *J Cell Biol* **165**, 135-144

- 26 Katzmann, D. J., Sarkar, S., Chu, T., Audhya, A. and Emr, S. D. (2004) Multivesicular body sorting: ubiquitin ligase Rsp5 is required for the modification and sorting of carboxypeptidase S. *Mol Biol Cell* **15**, 468-480
- 27 Morvan, J., Froissard, M., Haguenaue-Tsapis, R. and Urban-Grimal, D. (2004) The ubiquitin ligase Rsp5p is required for modification and sorting of membrane proteins into multivesicular bodies. *Traffic* **5**, 383-392
- 28 Blondel, M. O., Morvan, J., Dupre, S., Urban-Grimal, D., Haguenaue-Tsapis, R. and Volland, C. (2004) Direct sorting of the yeast uracil permease to the endosomal system is controlled by uracil binding and Rsp5p-dependent ubiquitylation. *Mol Biol Cell* **15**, 883-895
- 29 Reggiori, F. and Pelham, H. R. (2002) A transmembrane ubiquitin ligase required to sort membrane proteins into multivesicular bodies. *Nat Cell Biol* **4**, 117-123
- 30 Levy, F., Muehlethaler, K., Salvi, S., Peitrequin, A. L., Lindholm, C. K., Cerottini, J. C. and Rimoldi, D. (2005) Ubiquitylation of a melanosomal protein by HECT-E3 ligases serves as sorting signal for lysosomal degradation. *Mol Biol Cell* **16**, 1777-1787
- 31 Pak, Y., Glowacka, W. K., Bruce, M. C., Pham, N. and Rotin, D. (2006) Transport of LAPT5 to lysosomes requires association with the ubiquitin ligase Nedd4, but not LAPT5 ubiquitination. *J Cell Biol* **175**, 631-645
- 32 Liu, X. F. and Culotta, V. C. (1999) Post-translation control of Nramp metal transport in yeast. Role of metal ions and the BSD2 gene. *J Biol Chem* **274**, 4863-4868
- 33 Hettema, E. H., Valdez-Taubas, J. and Pelham, H. R. (2004) Bsd2 binds the ubiquitin ligase Rsp5 and mediates the ubiquitination of transmembrane proteins. *Embo J* **23**, 1279-1288
- 34 Stimpson, H. E., Lewis, M. J. and Pelham, H. R. (2006) Transferrin receptor-like proteins control the degradation of a yeast metal transporter. *Embo J* **25**, 662-672
- 35 Gupta, R., Kus, B., Fladd, C., Wasmuth, J., Tonikian, R., Sidhu, S., Krogan, N. J., Parkinson, J. and Rotin, D. (2007) Ubiquitination screen using protein microarrays for comprehensive identification of Rsp5 substrates in yeast. *Mol Syst Biol* **3**, 116
- 36 Hesselberth, J. R., Miller, J. P., Golob, A., Stajich, J. E., Michaud, G. A. and Fields, S. (2006) Comparative analysis of *Saccharomyces cerevisiae* WW domains and their interacting proteins. *Genome Biol* **7**, R30
- 37 Leon, S., Erpapazoglou, Z. and Haguenaue-Tsapis, R. (2008) Ear1p/Ssh4p confer substrate specificity to the ubiquitin ligase Rsp5p for cargo sorting at multivesicular bodies *Mol Biol Cell* **in press**
- 38 Woo, J. S., Suh, H. Y., Park, S. Y. and Oh, B. H. (2006) Structural basis for protein recognition by B30.2/SPRY domains. *Mol Cell* **24**, 967-976
- 39 Shearwin-Whyatt, L., Dalton, H. E., Foot, N. and Kumar, S. (2006) Regulation of functional diversity within the Nedd4 family by accessory and adaptor proteins. *Bioessays* **28**, 617-628
- 40 Reggiori, F. and Pelham, H. R. (2001) Sorting of proteins into multivesicular bodies: ubiquitin-dependent and -independent targeting. *Embo J* **20**, 5176-5186
- 41 Barriere, H., Nemes, C., Du, K. and Lukacs, G. L. (2007) Plasticity of polyubiquitin recognition as lysosomal targeting signals by the endosomal sorting machinery. *Mol Biol Cell* **18**, 3952-3965
- 42 Dupre, S. and Haguenaue-Tsapis, R. (2001) Deubiquitination step in the endocytic pathway of yeast plasma membrane proteins: crucial role of Doa4p ubiquitin isopeptidase. *Mol Cell Biol* **21**, 4482-4494
- 43 Kee, Y., Munoz, W., Lyon, N. and Huibregtse, J. M. (2006) The deubiquitinating enzyme Ubp2 modulates Rsp5-dependent Lys63-linked polyubiquitin conjugates in *Saccharomyces cerevisiae*. *J Biol Chem* **281**, 36724-36731
- 44 McCullough, J., Clague, M. J. and Urbe, S. (2004) AMSH is an endosome-associated ubiquitin isopeptidase. *J Cell Biol* **166**, 487-492
- 45 Clague, M. J. and Urbe, S. (2006) Endocytosis: the DUB version. *Trends Cell Biol* **16**, 551-559
- 46 Varadan, R., Assfalg, M., Haririnia, A., Raasi, S., Pickart, C. and Fushman, D. (2004) Solution conformation of Lys63-linked di-ubiquitin chain provides clues to functional diversity of polyubiquitin signaling. *J Biol Chem* **279**, 7055-7063
- 47 Raasi, S., Varadan, R., Fushman, D. and Pickart, C. M. (2005) Diverse polyubiquitin interaction properties of ubiquitin-associated domains. *Nat Struct Mol Biol* **12**, 708-714
- 48 Froissard, M., Belgareh-Touze, N., Dias, M., Buisson, N., Camadro, J. M., Haguenaue-Tsapis, R. and Lesuisse, E. (2007) Trafficking of siderophore transporters in *Saccharomyces cerevisiae* and intracellular fate of ferrioxamine B conjugates. *Traffic* **8**, 1601-1616
- 49 Chastagner, P., Israel, A. and Brou, C. (2006) Itch/AIP4 mediates Deltex degradation through the formation of K29-linked polyubiquitin chains. *EMBO Rep* **7**, 1147-1153
- 50 Kaminska, J., Gajewska, B., Hopper, A. K. and Zoladek, T. (2002) Rsp5p, a new link between the actin cytoskeleton and endocytosis in the yeast *Saccharomyces cerevisiae*. *Mol Cell Biol* **22**, 6946-6948