The yeast multidrug transporter Pdr5 of the plasma membrane is ubiquitinated prior to endocytosis and degradation in the vacuole

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Abstract **We have recently demonstrated that the Pdr5 ATP binding cassette multidrug transporter is a short-lived protein, whose biogenesis involves cell surface targeting followed by endocytosis and delivery to the vacuole for proteolytic turnover [Egner, R., Mahé, Y., Pandjaitan, R., and Kuchler, K. (1995) Mol. Cell. Biol. 15, 5879–5887]. Using c-*myc* epitope-tagged ubiquitin, we now have shown that Pdr5 is a ubiquitinated plasma membrane protein in vivo. Ubiquitination of Pdr5 was detected in both wild type and conditional *end4* mutants defective in endocytic vesicle formation. Likewise, the Ste6 a-factor pheromone transporter, which represents another short-lived ABC transporter whose turnover requires vacuolar proteolysis, was also found to be ubiquitinated, and ubiquitin-modified Ste6 massively accumulated in *end4* mutants at the restrictive temperature. By contrast, the plasma membrane ATPase Pma1, a long-lived and metabolically very stable protein, was found not to be ubiquitinated. Our results imply a novel function for ubiquitin in protein trafficking and suggest that ubiquitination of certain short-lived plasma membrane proteins may trigger their endocytic delivery to the vacuole for proteolytic turnover.**

Key words: Yeast; ABC transporter; Proteolysis; Ubiquitin; Endocytosis; Vacuole

1. Introduction

The yeast Pdr5 ATP binding cassette (ABC) multidrug transporter is a integral plasma membrane protein [1,2] consisting of twelve predicted membrane spanning helices and two ATP binding domains putatively facing the cytoplasm [3,4]. Overexpression of *PDR5* leads to the development of multidrug resistance (MDR) to a variety of structurally and functionally unrelated cytotoxic compounds [3–6]. Thus, Pdr5 is a functional yeast counterpart [7] of mammalian Mdr1 or *P*-glycoprotein that is associated with MDR development in tumor cells [8].

The steady state levels of Pdr5 in the plasma membrane are maintained by endocytosis and delivery of Pdr5 to the vacuole, where it is degraded in a proteinase A-dependent manner [2]. Endocytosis was demonstrated to be a prerequisite for the delivery to the vacuole of several plasma membrane proteins, including the Ste2 and Ste3 mating pheromone receptors [9,10], the Fur4 uracil permease [11], the Itr1 inositol permease [12] and the Ste6 a-factor pheromone transporter [13,14].

Interestingly, it was recently demonstrated that the short-lived Ste6 ABC transporter accumulates in a ubiquitinated form in the plasma membrane of conditional endocytosis mutants [13]. Ubiquitin, a polypeptide of 76 amino acids that is covalently attached to protein substrates via an isopeptide bond between its α-carboxyl group amino acid and lysine ε-amino groups of appropriate acceptor proteins, is required for the degradation of certain short-lived soluble proteins (reviewed in [15]). The proteolytic breakdown of multi-ubiquitinated proteins is mediated by the 26S proteasome, a ATP-dependent multicatalytic enzyme complex containing the 20S proteasome as the catalytic core particle, found both in the cytoplasm and the nucleus of eukaryotic cells (reviewed in [16,17]). In yeast, several examples of multi-ubiquitinated, soluble proteins or proteins whose turnover requires a functional proteasome have been reported. For instance, turnover of the transcription factor *MAT*α2 [18], the yeast cyclins Cln2 [19], Cln3 [20] and Clb2 [21], the Gcn4 transcriptional regulator [22], and the fructose-1,6-bisphosphatase [23] is mediated by the ubiquitin/proteasome pathway.

The existence of ubiquitinated membrane proteins such as the mammalian T cell antigen receptor [24] and the cystic fibrosis transmembrane conductance regulator (CFTR) [25] was recently demonstrated. Interestingly, the proteolytic breakdown of the immature, endoplasmic reticulum-localized CFTR, as well as a mutated yeast endoplasmic reticulum membrane protein, Sec61, depends on a functional ubiquitin/proteasome pathway [25–27]. However, ubiquitin–protein conjugates of membranous origin have also been detected in vacuoles of yeast cells lacking vacuolar proteinases [28]. Likewise, immunoelectron microscopy [29,30] and subcellular fractionation studies [31] showed that both ubiquitin and ubiquitinated proteins are found in lysosomes of mammalian cells. Strikingly, upon ligand binding, the mammalian platelet-derived growth factor receptor-β was shown to be subject to multi-ubiquitination, followed by endocytosis and subsequent delivery to the lysosomes for degradation [32].

As we have previously shown that the turnover of the short-lived yeast plasma membrane transporter Pdr5 requires a functional vacuole [2], we have set out to identify potential signals responsible for vacuolar delivery of Pdr5. In this report, we show that the Pdr5 plasma membrane ABC transporter is ubiquitinated both in wild type and in *end4* endocytosis mutants. Interestingly, the long-lived plasma membrane ATPase Pma1 is not ubiquitinated under the same conditions. These results suggest a novel role for ubiquitin in protein trafficking within the yeast endocytic pathway, in addition to its established function in the degradation of multi-ubiquitinated proteins mediated by the proteasome.

2. Materials and methods

2.1. Media, culture conditions and strain construction

Rich medium (YPD) and synthetic media supplemented with auxotrophic components to maintain plasmids were prepared essen-

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tially as described elsewhere [33]. Cultures were grown at 24°C except where otherwise indicated. Overproduction of ubiquitin under the control of the *CUP1* promoter was induced by adding $CuSO_4$ to 100 μM final concentration. The *S. cerevisiae* strains YAL21 (*MATa ade2-1^{oc} his leu2 trp1-1 ura3*) and YAL22 (*MATa ade2-1^{oc} his3-11,-15 leu2 trp1-1 ura3 end4*) used in this study were isogenic segregants obtained from a cross between CB005 (*MATα can1-100 ade2-1^{oc} his3-11,-15 leu2-3,-112 trp1-1 ura3-;1 Δpep4::LEU2*) (obtained from R. Fuller) and RH268-1C (*MATa his4 leu2 ura3 bar1-1 end4*) [34].

2.2. Plasmids

The 2 μ-based ubiquitin expression vector YEp96 containing a synthetic yeast ubiquitin gene under the control of the *CUP1* promoter, and the corresponding plasmid YEp105 with an additional c-*myc* epitope at the N-terminus of the ubiquitin gene, were previously described [35]. Plasmid pYKS18 carrying a *Haemophilus influenza* hemeagglutinin (HA) epitope-tagged *PDR5* gene was described elsewhere [4]. Transformation of yeast cells used standard methods [33].

2.3. Antisera

The generation of polyclonal rabbit antibodies against a glutathione-*S*-transferase–Pdr5 fusion protein that contained 183 amino acids of the N-terminal part of Pdr5 (amino acids 4–187) was described elsewhere [2]. The polyclonal antiserum against fatty acid synthase (Fas) complex was raised in rabbits using SDS-denatured fatty acid synthase purified from yeast [36]. Anti-Ste6 antiserum #9384 used for immunoprecipitation and anti-Ste6 antibodies for immunoblots were described elsewhere [13,37]. Monoclonal antibodies recognizing the c-*myc* epitope and the HA epitope were previously described, respectively [38,39]. Polyclonal anti-Pma1 antiserum #838 was kindly provided by R. Serrano.

2.4. Yeast cell extracts, immunoprecipitation and immunoblotting

4 OD_{600} equivalents of cells were harvested and washed once with cold 10 mM NaN_3. After repelleting, the cells were resuspended in 50 μl cold breaking buffer (50 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1 mM phenylmethansulfonylfluoride, 5 mM *N*-ethyl maleimide) and an equal volume of cold glass beads was added. Cell lysis was achieved by vortexing the mixture 4 times for 1 min with 1 min on ice in between. After adding 50 μl breaking buffer containing 2% SDS, cell lysis was continued for another 2 min and the cell extracts were cleared by centrifugation at 13,000 × *g* for 5 min.

For immunoblotting, 10 μl lysate were diluted 1-fold with sample buffer (40 mM Tris-HCl, pH 6.8, 8 M urea, 5% SDS, 0.1 mM EDTA, 2% β-mercaptoethanol, 0.01% bromphenol blue) and heated at 37°C for 10 min. The proteins were resolved by SDS-PAGE on a 7.5% gel and transferred to nitrocellulose membranes as previously described [37]. Membranes were incubated for 1 h in blocking buffer (25 mM Tris-HCl, pH 7.4, 140 mM NaCl, 5% fat-free dry milk, 0.1% Triton X-100, 10 mM NaN_3) and proteins on immunoblots were visualized using the ECL chemoluminescence detection [40]. Antisera dilutions in 10 ml blocking buffer were 1/20,000 for anti-Pdr5, 1/10,000 for anti-Fas, 1/5,000 for anti-Ste6, 1/30,000 for anti-Pma1 and 1/10,000 for monoclonal anti-c-*myc* 9E10 antibodies. Goat anti-rabbit and goat anti-mouse horseradish peroxidase conjugates (Oncogene Science) were used as secondary antibodies at a 1/10,000 dilution.

For immunoprecipitation, extracts were diluted with 20 vols. of IP buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 4 mM EDTA, 1% Triton X-100, 2 mM NaN_3) and insoluble material was removed by centrifugation (13,000 × *g*, 10 min). Polyclonal anti-Ste6 (#9384, 1/200), anti-Fas (1/200), anti-Pma1 (1/400) or monoclonal anti-HA 12CA5 antibodies (1/200) were added to the supernatant and immunoprecipitation was performed as previously described [37]. Immunoprecipitates corresponding to 1 OD_{600} of cells were loaded onto a 7.5% SDS polyacrylamide gel and subjected to immunoblotting as described above.

3. Results

3.1. The plasma membrane protein Pdr5 is ubiquitinated

To elucidate a potential role for ubiquitin in the endocytosis of the Pdr5 multidrug transporter, we have employed a functional c-*myc* epitope-tagged version of ubiquitin [35]. Cells of the wild type YAL21 and the *end4* mutant strain YAL22, both of which carrying plasmid pYKS18 containing a HA-tagged variant of Pdr5 that is functionally indistinguishable from au-

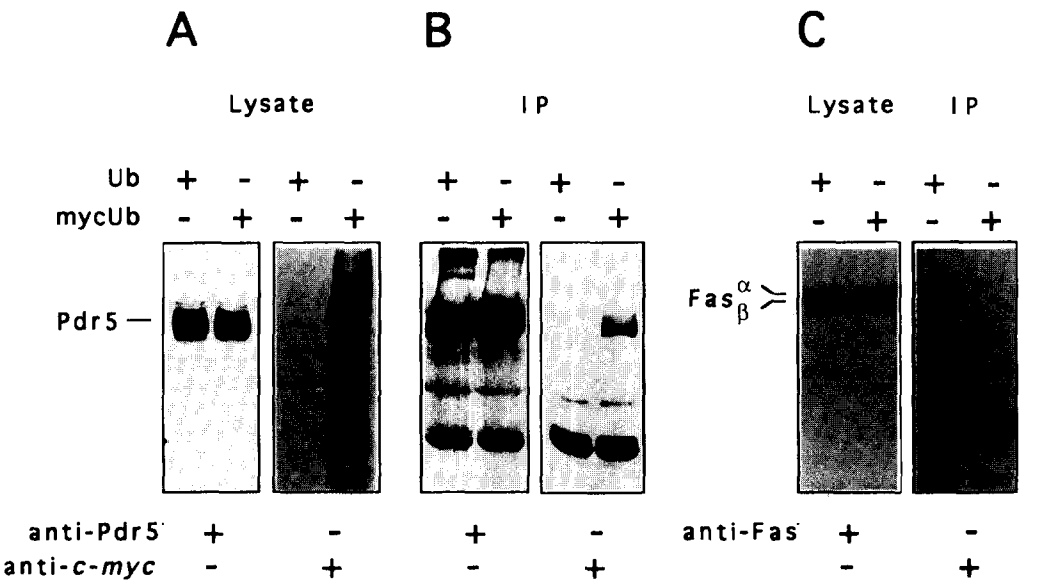

Fig. 1. The integral plasma membrane protein Pdr5 is ubiquitinated in vivo. Strain YAL21 [pYKS18] harboring a HA-tagged Pdr5 gene was transformed either with plasmid YEp96 carrying a ubiquitin gene (Ub) or plasmid YEp105 containing a c-*myc* tagged version of ubiquitin (mycUb). (A) Total protein extracts of strains YAL21 [pYKS18 YEp96] and YAL21 [pYKS18 YEp105] corresponding to 0.4 OD_{600} cells were separated by SDS-PAGE on a 7.5% gel and analyzed by immunoblotting with polyclonal anti-Pdr5 antiserum and monoclonal anti-c-*myc* 9E10 antibodies. (B) Cell lysates of strains YAL21 [pYKS18 YEp96] and YAL21 [pYKS18 YEp105] corresponding to 1 OD_{600} of cells were subjected to immunoprecipitation as described in section 2. Immunoprecipitates (IP) of Pdr5 with monoclonal anti-HA 12CA5 antibodies were analyzed on immunoblots with polyclonal anti-Pdr5 antiserum and for ubiquitination with anti-c-*myc* 9E10 antibodies, respectively. (C) Cell lysates were subjected to immunoblotting with anti-Fas antiserum against fatty acid synthase complex (Fas). Immunoprecipitates (IP) of Fas were analyzed on immunoblots with anti-c-*myc* 9E10 antibodies.

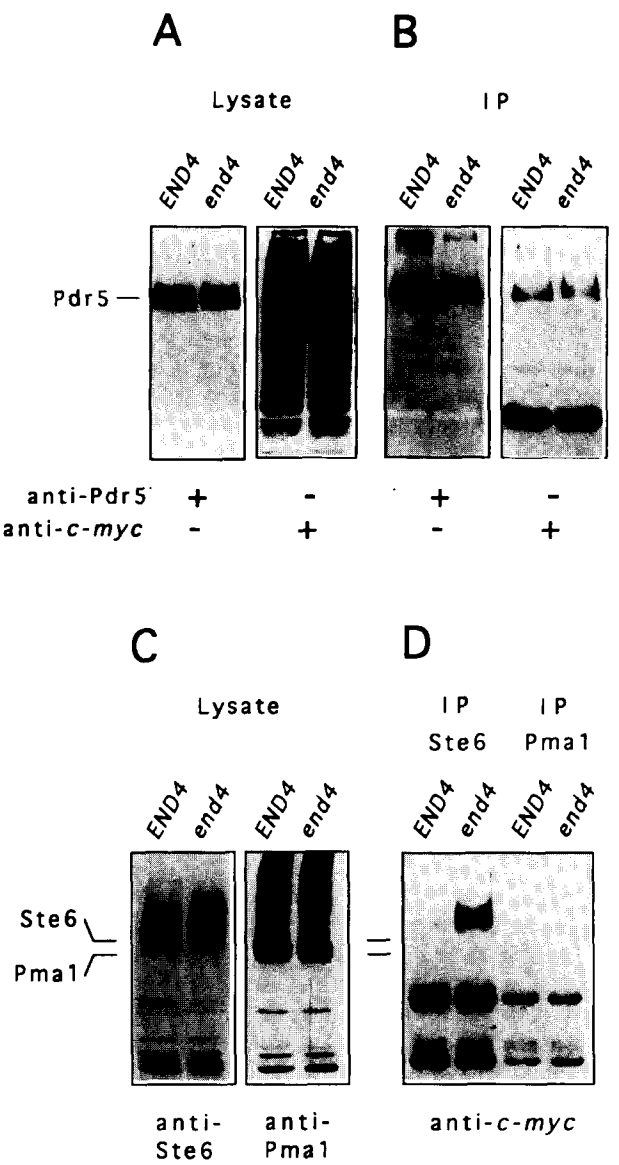

Fig. 2. Pdr5 and Ste6, but not Pma1, are ubiquitinated in wild type and in *end4* mutants. Cells of wild type strain YAL21 [pYKS18 YEp105] and *end4* mutant strain YAL22 [pYKS18 YEp105] were grown at 24°C to an OD_{600} of 1.5. Ubiquitin expression was induced with $CuSO_4$ 30 min before shifting cells to 37°C for 1.5 h. Cell lysis and immunoprecipitation were performed as described above. (A) Analysis of cell lysates from *END4* wild type and *end4* mutant cells on immunoblots for Pdr5 (anti-Pdr5) and ubiquitinated proteins (anti-c-*myc* 9E10). (B) Immunoprecipitation of HA-tagged Pdr5 of *END4* wild type and *end4* mutant cells with anti-HA 12CA5 antibodies. Detection of Pdr5 and ubiquitinated Pdr5 in the immunoprecipitates (IP) was performed by immunoblotting with anti-Pdr5 and anti-c-*myc* 9E10 antibodies, respectively. (C) Immunodetection of Ste6 and Pma1 in cell lysates of *END4* wild type and *end4* mutant cells (anti-Ste6 or anti-Pma1). (D) Visualization of ubiquitinated Ste6 after immunoprecipitation of the protein (antiserum #9384) and blotting of Pma1 immunoprecipitates (antiserum #838) with anti-c-*myc* 9E10 antibodies, respectively.

thentic Pdr5 [2,4], were transformed with either ubiquitin expressing plasmid YEp96 or with YEp105 expressing the c-*myc* tagged version of ubiquitin under the control of the *CUP1* promoter. Cells were grown in synthetic medium to an OD_{600} of 1.5 and the expression of plasmid-encoded ubiquitin was induced by adding Cu^{2+} ions for 60 min before harvesting cells. Cell lysates were prepared and the levels of Pdr5 and ubiquitin expression were determined by immunoblotting with anti-Pdr5 antiserum and anti-c-*myc* 9E10 antibodies, respectively (Fig. 1A).

Equal amounts of Pdr5 protein were found in the control cells carrying the ubiquitin plasmid (YEp96; Ub) and in the transformant containing the c-*myc* tagged ubiquitin (YEp105; mycUb). Further, the c-*myc* 9E10 antibodies recognized no proteins in the total lysate of the control cells (Ub), except one faint single band of low molecular mass. In the c-*myc* ubiquitin expressing cells (mycUb), a ladder of bands over the entire molecular mass spectrum was detectable, reflecting cellular proteins covalently conjugated to c-*myc* tagged ubiquitin.

Next, HA-tagged Pdr5 from cell lysates of the control transformant (Ub) and the cells containing c-*myc* ubiquitin (mycUb) was immunoprecipitated with monoclonal anti-HA 12CA5 antibodies and analyzed on immunoblots with polyclonal anti-Pdr5 antiserum and anti-c-*myc* 9E10 antibodies (Fig. 1B). Total HA-tagged Pdr5 was detectable in same amounts in the control transformant (Ub) and in the cells with c-*myc* tagged ubiquitin (mycUb). In contrast, when analyzed for ubiquitinated proteins with c-*myc* 9E10 antibodies, only a signal for the c-*myc* ubiquitin containing cells corresponding to the molecular mass of Pdr5 including a smear to higher molecular mass was detected. Since the anti-c-*myc* 9E10 antibody recognizes only proteins that are covalently conjugated to c-*myc* ubiquitin, Pdr5 must therefore be linked to ubiquitin, demonstrating that the Pdr5 belongs to a subclass of plasma membrane proteins that is a target for ubiquitination in vivo.

In a further control experiment, we immunoprecipitated the α and β subunits of the fatty acid synthase (Fas), a soluble and long-lived $\alpha_6\beta_6$ enzyme complex of the cytoplasm [36]. As shown in Fig. 1C, no ubiquitination of the Fas complex was detectable on the immunoblot when probed with anti-c-*myc* 9E10 antibodies. The ability of the anti-Fas antibodies to immunoprecipitate the Fas complex was verified in a separate experiment with ^{35}S-radiolabelled Fas (data not shown).

3.2. *Pdr5 and Ste6, but not Pma1, are ubiquitinated prior to endocytosis*

To test if Pdr5 ubiquitination requires a functional endocytic pathway, we compared the formation of Pdr5-ubiquitin conjugates in the wild type strain YAL21 (*END4* [pYKS18 YEp105]) and the endocytosis mutant YAL22 (*end4* [pYKS18 YEp105]), both of which carried the plasmids with HA-tagged Pdr5 and the c-*myc* tagged ubiquitin. After growing the cells to an OD_{600} of 1.5, the expression of ubiquitin was induced by adding $CuSO_4$ to 100 mM for 30 min before shifting the cells for 90 min to the restrictive temperature of 37°C. The expression levels of Pdr5 and the total amounts of ubiquitinated proteins were measured in the cell lysates of wild type and *end4* mutants (Fig. 2A). Pdr5 was again immunoprecipitated with monoclonal anti-HA 12CA5 antibodies, followed by immunoblotting of the precipitated protein using polyclonal anti-Pdr5 antibodies. As shown in Fig. 2B (right panel), the amount of ubiquitinated Pdr5 appeared to be nearly the same in the wild type *END4* cells and the *end4* mutant cells. However, the ratio of ubiquitinated Pdr5 (Fig. 2B, right panel) to total Pdr5 (Fig. 2B, left panel) was higher in the *end4* mutant at the restrictive temperature than

the same ratio for the *END4* wild type. Hence, this showed accumulation of ubiquitinated Pdr5 in the *end4* mutant after blocking endocytosis, suggesting a function for ubiquitin in mediating endocytosis of Pdr5.

The Ste6 **a**-factor transporter was the first yeast membrane protein shown to be ubiquitinated [13]. Ubiquitinated Ste6 accumulates at the plasma membrane of *end4* mutants at the restrictive temperature [13], where it is stabilized against proteolysis [14]. Thus, as a positive control, we have also immunoprecipitated Ste6 from the same wild type and *end4* mutant lysates using polyclonal anti-Ste6 antibodies (serum #9384). Ste6-ubiquitin conjugates were then detected on immunoblots using anti-c-*myc* 9E10 antibodies (Fig. 2D). In the wild type strain, ubiquitinated Ste6 was nearly undetectable. However, in the *end4* mutant, Ste6-ubiquitin conjugates of higher molecular mass were drastically enriched after blocking the endocytic pathway for 1.5 h. The apparent immunoreactive smear to higher molecular mass could indicate multi-ubiquitination of Ste6. To control for Ste6 expression levels, Ste6 was also immunodetected in total extracts from both the wild type and the *end4* mutant as shown in Fig. 2C.

Ste6 and Pdr5, both of which are rather short-lived proteins and degraded in the vacuole, are ubiquitinated membrane proteins in yeast. To test if a long-lived cell surface protein such as the plasma membrane ATPase Pma1 is also subject to covalent ubiquitination modification, we have also analyzed whether or not Pma1 is ubiquitinated (Fig. 2C and D). However, Pma1, which has a half-life of more than 10 h [41], was not ubiquitinated under the experimental conditions used, since c-*myc* 9E10 antibodies did not allow for detection of any immunoprecipitated Pma1 (Fig. 2D). The capability of anti-Pma1 antiserum #838 for immunoprecipitation was tested with ^{35}S-labeled Pma1 in a separate experiment (data not shown). These results provide evidence that long-lived plasma membrane proteins such as Pma1 are not subject to a post-translational ubiquitin modification, and suggest a novel function for ubiquitin in promoting or facilitating the endocytic delivery of a subset of cell surface proteins to the vacuole for terminal degradation.

4. Discussion

The Pdr5 multidrug transporter and the Ste6 **a**-factor pheromone transporter are both membrane proteins of the ABC transporter superfamily [7]. As shown in this report, the proteolytic turnover of these transporters appears to involve a ubiquitin modification, because both Pdr5 and Ste6 are ubiquitinated prior to endocytosis and vacuolar degradation. It was previously shown that the turnover of both Ste6 and Pdr5 does not depend on the ubiquitin/proteasome pathway, since the half-lifes of neither Ste6 (R. Egner and K. Kuchler, unpublished results) nor Pdr5 [2] are affected in proteasomal *pre1-1* and *pre1-1 pre2-1* mutants. A drastic metabolic stabilization of Ste6 [13,14] and Pdr5 [2] can only be brought about by impaired proteolytic activities of the vacuole or by blocking endocytosis. Furthermore, as in the case of Ste6, mutations in genes encoding ubiquitin conjugating enzymes (*ubc4 ubc5*) also lead to an increased metabolic stability of Ste6 [13].

Pdr5– and Ste6–ubiquitin conjugates accumulate at different rates in the plasma membrane of *end4* mutants at the restrictive temperature (Fig. 2B and D), which most likely reflects the different cellular localization of Ste6 and Pdr5 in the steady state. While Pdr5 is a resident plasma membrane protein [2], Ste6 is mainly associated with an intracellular, Golgi-like compartment [13,14]. If ubiquitination of Ste6 takes place at the cell surface, where Ste6 is found only transiently en route to the vacuole [13], an endocytic block would result in a significant accumulation of ubiquitinated Ste6 at the cell surface. Indeed, while ubiquitin-modified Ste6 was detectable only at very low levels in the wild type strain, presumably because of low steady state levels of Ste6 in the plasma membrane, Ste6-ubiquitin conjugates massively accumulated in the *end4* mutant at the restrictive temperature (Fig. 2D). In contrast, Pdr5 seems to be permanently available for ubiquitination, since Pdr5 was already found in the ubiquitinated state in the wild type strain, and because Pdr5 is localized in the plasma membrane in the steady state (Fig. 2B). The Pdr5 half-life of 60–90 min [2] is about six-fold higher than the one of Ste6, which was determined to be about 10–15 min [13], providing a plausible explanation for our observation that blocking the endocytic pathway for 90 min did not result in a drastic accumulation of Pdr5–ubiquitin conjugates (Fig. 2B).

Ubiquitin–protein conjugates of membranous origin were previously found in the vacuole of yeast cells deficient in vacuolar proteolysis [28]. Besides the ubiquitinated membrane proteins Pdr5 and Ste6, the Ste2 and Ste3 mating pheromone receptors [9,10] and the Fur4 uracil permease [11] are also endocytosed for degradation in the vacuole. Although endocytosis signals are still ill-defined, a potential signal for regulated endocytosis was identified in the Ste2 α-factor pheromone receptor in the C-terminal sequence DAKSS [10]. Deletion of the C-terminal tail of Ste2 up to amino acid 345 and a simultaneous amino acid exchange to DA*R*SS leads to a complete lack of regulated Ste2 endocytosis [10]. In the case of the Fur4 uracil permease, a 'destruction box' like nine amino acid sequence, which was originally found in the sea urchin B cyclin as a signal for protein ubiquitination [42], is believed to be required for ubiquitination and endocytosis. A point mutation in this sequence stabilizes Fur4 against degradation under nutritional stress conditions [43]. Interestingly, it was recently demonstrated that N-starvation-induced metabolic instability of Fur4, depends on a functional *NPI1* gene that encodes a E3-type ubiquitin–protein ligase [44] closely related to a family of mammalian E6-AP ubiquitin–protein ligases [45]. Indeed, consistent with our idea that ubiquitination can trigger endocytosis, Fur4 was recently also shown to be ubiquitinated and delivered to the vacuole in response to N-starvation (R. Hagenuauer-Tsapis, personal communication).

Taken together, these results and our data provide evidence that a covalent ubiquitin modification can not only function as a signal for protein degradation by the 26S proteasome [15], but could also trigger the vacuolar delivery and subsequent turnover of short-lived plasma membrane proteins in yeast. Thus, it is tempting to speculate that ubiquitination of yeast membrane proteins may be a signal for membrane protein trafficking and/or for the remodeling of the cell surface in general. A possible functional role for ubiquitin in the yeast endocytic pathway was indeed suggested by the earlier observation that overexpression of ubiquitin suppresses the lethality of certain clathrin-deficient yeast strains [46]. It will be important to identify the signals required for the ubiquitination of ABC transporters such as Pdr5 and Ste6 and other plasma membrane

proteins, and to determine if differences in ubiquitination patterns and/or multi-ubiquitination [47] determine the life-span of proteins and target proteins for either vacuolar or proteasomal turnover.

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Note added in proof

During the review process of this manuscript, it was demonstrated that ubiquitination of the Ste2 α-factor pheromone receptor stimulates its endocytosis, showing that ubiquitination of the pheromone receptor represents an endocytosis signal (L. Hicke and H. Riezman (1995) Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis, Cell, in press).