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Direct Sorting of the Yeast Uracil Permease to the Endosomal System Is Controlled by Uracil Binding and Rsp5p-dependent Ubiquitylation

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The yeast uracil permease, Fur4p, is downregulated by uracil, which is toxic to cells with high permease activity. Uracil promotes cell surface Rsp5p-dependent ubiquitylation of the permease, signaling its endocytosis and further vacuolar degradation. We show here that uracil also triggers the direct routing of its cognate permease from the Golgi apparatus to the endosomal system for degradation, without passage via the plasma membrane. This early sorting was not observed for a variant permease with a much lower affinity for uracil, suggesting that uracil binding is the signal for the diverted pathway. The *FUI1*-encoded uridine permease is similarly sorted for early vacuolar degradation in cells exposed to a toxic level of uridine uptake. Membrane proteins destined for vacuolar degradation require sorting at the endosome level to the intraluminal vesicles of the multivesicular bodies. In cells with low levels of Rsp5p, Fur4p can be still diverted from the Golgi apparatus but does not reach the vacuolar lumen, being instead missorted to the vacuolar membrane. Correct luminal delivery is restored by the biosynthetic addition of a single ubiquitin, suggesting that the ubiquitylation of Fur4p serves as a specific signal for sorting to the luminal vesicles of the multivesicular bodies. A fused ubiquitin is also able to sort some Fur4p from the Golgi to the degradative pathway in the absence of added uracil but the low efficiency of this sorting indicates that ubiquitin does not itself act as a dominant signal for Golgi-to-endosome trafficking. Our results are consistent with a model in which the binding of intracellular uracil to the permease signals its sorting from the Golgi apparatus and subsequent ubiquitylation ensures its delivery to the vacuolar lumen.

INTRODUCTION

Studies in the budding yeast, *Saccharomyces cerevisiae*, have made a major contribution to demonstrating the involvement of ubiquitylation in the trafficking of integral membrane proteins (Hicke, 2001). The endocytosis of numerous plasma membrane proteins has been shown to be mediated by ubiquitylation. The ubiquitin ligase Rsp5p seems to be the only ligase involved in the ubiquitylation of yeast plasma membrane proteins. This enzyme is the only member of the Nedd4 protein family of ubiquitin ligases known in yeast (Rotin *et al.*, 2000). After removal from the plasma membrane, receptor and transporter proteins tagged with ubiquitin transit via endosomes and are ultimately delivered to the lumen of the vacuole, where they are degraded by vacuolar proteases. Delivery to the interior of the vacuole requires sorting in the late endosome compartment to internal vesicles resulting from invagination of the endosomal membrane, giving rise to multivesicular bodies (MVB; Piper and Luzio, 2001). Ubiquitin was recently shown to play a crucial role in this sorting step, in which proteins entering vesicles are separated from those that remain on the endosomal membrane. Those remaining on the membrane are found on the vacuolar membrane after fusion of the MVB with the vacuole (Katzmann *et al.*, 2001; Reggiori and Pelham, 2001; Urbanowski and Piper, 2001). These studies essentially concerned biosynthetic proteins such as the carboxypeptidase S Cps1p and the polyphosphatase Phm5p, which are transported from the Golgi apparatus to the vacuolar lumen. The Golgi-located ubiquitin ligase Tul1p is required for the ubiquitylation of these cargoes, to ensure their correct delivery to the interior of the vacuole (Reggiori and Pelham, 2002).

Plasma membrane transporters are delivered to the cell surface by the secretory pathway. It was recently shown that the efficiency of delivery of some transporters may be regulated by nutritional conditions. Newly synthesized permeases may be routed, entirely or in part, to the degradative vacuolar pathway without passing via the cell surface (Arvan *et al.*, 2002). One well-known example is that of the general amino acid permease, Gap1p, which can be diverted from the late secretory pathway to the vacuolar pathway, depending on the nitrogen source (Roberg *et al.*, 1997; Soetens *et al.*, 2001). The ubiquitylation of Gap1p is required for its delivery to the vacuole from the late secretory pathway (Helliwell *et al.*, 2001; Soetens *et al.*, 2001). Ubiquitylation is also required for the endocytosis of this transporter under similar nutrient conditions (Springael and Andre, 1998), and the ubiquitin ligase Rsp5p plays an essential role in both processes. The tryptophan permease Tat2p is also sorted from the Golgi apparatus to the vacuole rather than the cell surface under conditions of nutrient deprivation

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Table 1. Strains used in this study

Name	Genotype	Origin
27061b	*MAT***a** *ura3 trp1*	Galan *et al.* (1996)
27064b	*MAT***a** *ura3 trp1npi1*	Galan *et al.* (1996)
27081a	*MATα ura3 trp1npi2*	Springael and Andre (1998)
33125a	*MAT***a** *ura3 trp1pep12::KanMX4*	De Craene *et al.* (2001)
OS27-1	*MAT***a** *ura3 bul1::Kan MX4 bul2::KanM4*	Soetens *et al.* (2001)
MOB51	*MAT***a** *ura3 trp1 pep4::KanMX4*	This study
MOB52	*MAT***a** *ura3 trp1 end3::KanMX4*	This study
MOB53	*MAT***a** *ura3 trp1 doa4::KanMX4*	This study
JM01	*MAT***a** *ura3 trp1 npi1 doa4::KanMX4*	This study
BY4742	*MATα his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0	Euroscarf
BY4742tul1Δ	*MATα his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *tul1::KanMX4*	Euroscarf

All strains, with the exception of BY4742 and its derivative *tul1*Δ, are isogenic to the wild-type strain Σ1278b except for the mutations stated.

(Beck *et al.*, 1999) or in the presence of high tryptophan levels (Umebayashi and Nakano, 2003). A member of the Nramp family of metal transporters, Smf1p, is targeted to the vacuole in the presence of metal ions but accumulates at the cell surface under conditions of metal starvation (Liu and Culotta, 1999b). Similarly, trafficking of the ferrichrome transporter, Arn1p, depends on the exposure of the cells to ferrichrome (Kim *et al.*, 2002).

Levels of the *FUR4*-encoded uracil permease are also subject to posttranslational control, with Fur4p levels decreasing in response to uracil (Séron *et al.*, 1999). Downregulation has also been observed under adverse conditions, such as the inhibition of protein synthesis (Volland *et al.*, 1994), suggesting that uracil of exogenous or catabolic origin downregulates the cognate permease to prevent the accumulation of excess intracellular uracil-derived nucleotides. We have shown that uracil accelerates degradation by increasing the efficiency of ubiquitylation of the permease, signaling its internalization (Galan *et al.*, 1996; Séron *et al.*, 1999). Permease ubiquitylation, which requires prior phosphorylation, is mediated by the Rsp5p ubiquitin ligase and occurs on two target lysines (Hein *et al.*, 1995; Marchal *et al.*, 1998; Marchal *et al.*, 2000). We show here that uracil negatively controls the exocytic trafficking of its cognate permease by diverting it from the Golgi apparatus to the vacuole. Our results suggest that the Rsp5p-dependent ubiquitylation of Fur4p is required for the sorting of this protein to the internal vesicles of MVB, allowing its delivery to the vacuolar lumen.

MATERIALS AND METHODS

Media and Growth Conditions

The S. *cerevisiae* strains used in this study are listed in Table 1. Yeast strains were transformed as described by Gietz *et al.* (1992). Cells were grown at 30°C in YPD medium (1% yeast extract, 2% peptone, 2% glucose) or in YNB minimal medium containing 0.5% ammonium sulfate, 0.17% yeast nitrogen base (Difco, Detroit, MI) without ammonium and amino acids and supplemented with 0.1% casamino-acids (Difco). The carbon source was 2% glucose, 2% galactose plus 0.02% glucose, or 2% lactate plus 0.05% glucose. Exogenous uracil and uridine were added at concentrations of 40 μg/ml and 80 μg/ml, respectively, corresponding to essentially identical millimolar concentrations.

Strain and Plasmid Construction

Yeast strains derived from the 27061b strain with deletions of the *END3, PEP4,* or *DOA4* gene were obtained by replacement of the entire coding region with the geneticin resistance gene *KanMX4* (Wach, 1996). An ORF replacement cassette with long flanking homology regions was amplified by PCR and used to transform yeast cells. The integration of the *KanMX4* marker into the correct locus in geneticin-resistant cells was confirmed by PCR analysis of chromosomal DNA.

The plasmid Yep46FUR4 (2 μ, *TRP1, FUR4*), carrying the *FUR4* gene under the control of its promoter, was constructed from a 2.6-kb *Bam*H1-*Kpn*I fragment containing the *FUR4* gene amplified by PCR, using genomic DNA as template, and inserted into the Yep46 vector. The centromeric plasmid pFL38GalFUR4-GFP, carrying the *URA3* gene and a *FUR4* fusion gene, encoding Fur4p with a C-terminal GFP tag, under the control of the *GAL10* promoter, was constructed in a previous study and designated pFL38gF-GFP (Marchal *et al.*, 2002). To obtain Fur4-GFP variants, we used a PCR-based in vivo gap repair method (Papa *et al.*, 1999). A DNA fragment including the mutations of interest in the *FUR4* coding sequence and encompassing the *GAL10* promoter sequence and the *FUR4* sequence downstream from the single *Bsu*36I restriction site was amplified by PCR, using p195gF derivative plasmids (Marchal *et al.*, 1998) as templates. Yeast cells were then cotransformed with pFL38GalFUR4-GFP, cleaved at the *Bsu*36I restriction site, and the fragments amplified by PCR. We obtained pFL38GalFUR4^{KR}-GFP and pFL38GalFUR4^{SA}-GFP, encoding Fur4-GFP variants in which K38, K41, and K60 were replaced by R and S 42, 43, 45, 55, and 56 were replaced by A. PFL38GalFUR4^{272}-GFP was obtained in a similar manner, using the appropriate plasmid (Urban-Grimal *et al.*, 1995) as a PCR template. It encodes a Fur4-GFP variant in which K272 is replaced by E. The K272E mutation was also introduced in the SA variant, giving rise to pFL38GalFUR4^{SA,272}-GFP. In all cases, the changes were confirmed by sequencing. To tag the *FUI1*-encoded uridine permease with GFP, we replaced the 3′ flanking sequence of *FUI1* at the chromosomal locus, including the stop codon, with a PCR-amplified fragment containing the sequence encoding the GFP(S65T) variant and the KanMX module as a selection marker (Longtine *et al.*, 1998). The PCR fragment was generated by amplification with appropriate oligonucleotides, using pFA6a-GFP(S65T)-kanMX6 as the template. The fragment was introduced into wild-type cells and clones resistant to geneticin were checked for correct integration by PCR analysis of chromosomal DNA. The *FUI1-GFP* fusion was then cloned, by gap repair, in pFL38, to put it under the control of the *GAL10* promoter, thereby generating pFL38GalFUI1-GFP.

An in-frame ubiquitin was introduced at the N-terminus of Fur4-GFP in pFL38GalFUR4-GFP by the gap repair technique. The ubiquitin used for the fusion already contained mutations K 6, 11, 27, 29, 48, and 63 R (Arnason and Ellison, 1994) and the mutation G76V, to prevent its removal, was added in this construct. A PCR-amplified DNA fragment encompassing the *GAL10* promoter sequence and the *FUR4* coding sequence downstream from the single *Bsu*36I restriction site, including the coding sequence for mutated ubiquitin, was obtained by sequential PCR-fusion using the appropriate oligonucleotides and templates. Cells were cotransformed with pFL38GalFUR4-GFP cleaved at the *Bsu*36I restriction site and the PCR-amplified fragment, to obtain pFL38GalUb-FUR4-GFP. The same mutated ubiquitin was also introduced into the appropriate plasmid to generate pFL38GalUb-FUR4^{KR}-GFP, pFL38GalUb-FUR4^{272}-GFP and pFL38GalUb-FUI1-GFP. In pFL38GalUb-FUI1-GFP, the single *Afl*II restriction site within pFL38GalFUI1-GFP was used. All chimeric constructs were checked by sequencing.

Uracil Uptake

Uracil uptake was measured as previously described (Volland *et al.*, 1994), by incubating a 1-ml culture of exponentially growing cells with 5×10^{-6} M radiolabeled uracil for 20 s at 30°C. Cells were quickly filtered through Whatman GF/C filters, which were then washed twice with ice-cold water and counted for radioactivity. Fur4p activity was measured various times after the addition of cycloheximide (100 μg/ml) as previously described (Volland *et al.*, 1994), to determine the rate of internalization of plasma membrane-located Fur4p.

Fluorescence Microscopy and Vacuole Staining

We added 2% glucose and 10 μM cell tracker blue CMAC (10 mM stock solution in DMSO, Molecular Probes, Eugene, OR) to cells cultured in YNB medium with galactose. Cells were incubated for an additional 15 min, then centrifuged for 20 s to increase cell density by a factor of 10, and washed twice with YNB medium in the same conditions. Cells were viewed immediately, without fixing, under a fluorescence microscope and images were acquired with a digital camera.

Protein Extracts and Western Blotting

Total protein extracts were prepared by the NaOH-TCA lysis technique (Volland *et al.,* 1994). Plasma membrane-enriched fractions were prepared as previously described (Dupre and Haguenauer-Tsapis, 2001) except that cells were broken in a "One Shot" Cell Disrupter (Constant Systems LDT, Daventry, Northants, United Kingdom) at maximum pressure. Proteins in sample buffer were heated at 37°C, resolved by SDS-PAGE in 10% acrylamide gels using tricine buffer, and transferred to nitrocellulose membranes. The membranes were probed with monoclonal anti-GFP antiserum (Roche, Applied Sciences, Indianapolis, IN), polyclonal antibodies against the plasma membrane H^+ ATPase Pma1p (a gift from C. Slayman) and monoclonal antibodies against Vat2p, a subunit of V-ATPase (Molecular Probes). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G was used as the secondary antibody (Sigma, St. Louis, MO) and was detected by enhanced chemiluminescence (ECL).

Equilibrium Density Gradient

Membrane proteins were separated by equilibrium density centrifugation on a continuous 20–60% sucrose gradient, essentially as previously described (Kölling and Hollenberg, 1994). Exponentially growing cultures were arrested by adding 10 mM sodium azide. Cells (50 A600 U) were harvested by centrifugation, washed once in the presence of 10 mM azide, and disrupted in a "One Shot" Cell Disrupter with 0.5 ml of 10 mM Tris-HCl buffer, pH 7.6, containing 10% wt/wt sucrose, 10 mM EDTA, 25 mM freshly prepared *N*-ethylmaleimide and protease inhibitors (Complete Cocktail; Roche). Cell lysates, made up to a volume of 0.7 ml with the same buffer were centrifuged at 3000 rpm for 3 min and 0.3-ml aliquots of the cleared extracts were layered onto 11.1 ml 20–60% linear sucrose gradients made up in 10 mM Tris-HCl (pH 7.6) containing 10 mM EDTA. Samples were centrifuged for 18 h at 100,000 × *g* in an SW41 rotor (Beckman, Berkeley, CA). We collected 0.8-ml fractions from the top of the gradient and made the volume up to 1.6 ml with distilled water, and proteins were precipitated by adding 160 μl of 100% TCA and incubating on ice for 60 min. Proteins were then pelleted by centrifugation and resuspended in 10 μl of 1 M Tris base + 40 μl of 2× sample buffer and heated for 10 min at 37°C. The proteins in each fraction were analyzed by Western blotting, as described above.

RESULTS

Newly Synthesized Uracil Permease Can Be Diverted from the Secretory Pathway to the Vacuole

The steady state level of plasma membrane transporters is highly regulated. It was recently shown that these proteins not only display regulated turnover at the plasma membrane, but also regulated delivery to the cell surface under specific physiological conditions. We investigated whether exogenous uracil, which downregulates Fur4p at the plasma membrane, also affected the fate of newly synthesized permease. Because GFP fusions have proved very useful for the analysis of membrane protein distribution in living cells, we used a GFP-tagged version of the uracil permease placed under the control of a promoter that could be regulated, the *GAL10* promoter, to follow the intracellular trafficking of this protein.

We first investigated whether *end3*Δ cells, impaired in the internalization step of endocytosis (Benedetti *et al.,* 1994) and producing Fur4-GFP after galactose induction, were indeed protected against the endocytosis promoted by uracil. We determined the distribution of Fur4-GFP in wild-type and *end3*Δ cells cultured on galactose and then in the presence of glucose and uracil for a additional 2 h (Figure 1A). Exponentially growing *end3*Δ cells displayed fluorescence only at the cell surface, whereas wild-type cells displayed fluorescence both at the plasma membrane and within the vacuole. The presence of fluorescence in the vacuole probably resulted from the basal endocytosis of Fur4p, which has been shown to occur (Séron *et al.,* 1999). After glucose repression and further growth in the presence of uracil, the plasma membrane of wild-type cells no longer displayed fluorescence, indicating that Fur4-GFP had been internalized and delivered to the vacuolar lumen, although not yet entirely degraded. In contrast, permease at the cell surface of *end3*Δ cells was not internalized further in the presence of uracil, showing that Fur4p was largely resistant to endocytosis in these conditions. We therefore used *end3*Δ cells to investigate the effect of uracil on intracellular trafficking of the permease. *End3*Δ cells were cultured with lactate as the carbon source. Galactose was then added to the medium to induce permease synthesis in the presence or absence of uracil. Uracil was taken up by these cells at the very start of Fur4-GFP synthesis, albeit at a modest rate, thanks to the presence of the chromosomally encoded Fur4p in these cells. After 2 h of synthesis, Fur4-GFP was typically found almost exclusively on the plasma membrane in the absence of uracil, whereas substantial fluorescence within cells was also observed after exposure to uracil (Figure 1B). In cells exposed to uracil, intracellular permease was distributed similarly to CMAC stain, a useful marker of the vacuolar lumen. Because the endocytic pathway is efficiently blocked in *end3*Δ cells, we concluded that uracil triggered the sorting of Fur4p from an internal compartment to the vacuole, without passage via the plasma membrane. Thus, uracil targets a portion of its specific transporter to the vacuole, ensuring regulated degradation without trafficking via the cell surface. It should be noted that very similar patterns of vacuolar staining were obtained in *PEP4* and *pep4*Δ cells, which are defective in vacuolar protease activities (our unpublished results). The vacuolar fluorescence observed is readily accounted for by the relative resistance of GFP to vacuolar proteases, as shown by Western blot analysis (Figure 1C). We therefore used *PEP4* cells for all subsequent experiments.

We investigated whether Fur4p was delivered to the vacuole via the vacuolar protein sorting (VPS) pathway. Deletion of the endosomal SNARE protein Pep12p is known to block the VPS pathway by preventing the fusion of Golgi-derived vesicles with the late endosome (Gerrard *et al.,* 2000). *Pep12*Δ cells were transformed with pFL38GalFUR4-GFP. All permease newly synthesized in *pep12*Δ cells in the absence of uracil was delivered to the plasma membrane, as in wild-type cells (Figure 1B). In *pep12*Δ cells exposed to uracil, no intracellular fluorescence was observed in the vacuole and instead, a punctate pattern of fluorescence was observed, probably corresponding to Golgi-derived vesicles. We checked that this fluorescent material indeed corresponded to permease trafficking along the biosynthetic pathway and not from the cell surface, by repeating the experiment with a variant of Fur4p resistant to endocytosis. $Fur4^{KR}p$, which lacks the lysines essential for ubiquitylation at the cell surface, is resistant to endocytosis in wild-type cells (Marchal *et al.,* 2000), as is the corresponding GFP fusion protein (see the control shown Figure 3A). Very similar fluorescence patterns were obtained with *pep12*Δ cells producing wild-type permease and endocytosis-resistant permease (Figure 1B). In cells exposed to uracil, Fur4p is therefore diverted from the Golgi apparatus to the vacuole via the VPS pathway.

Cell fractionation experiments were carried out to analyze in more detail the distribution of permease between intracellular compartments and the cell surface. Extracts from *end3*Δ cells allowed to produce Fur4-GFP for 2 h in the presence or absence of exogenous uracil were subjected to

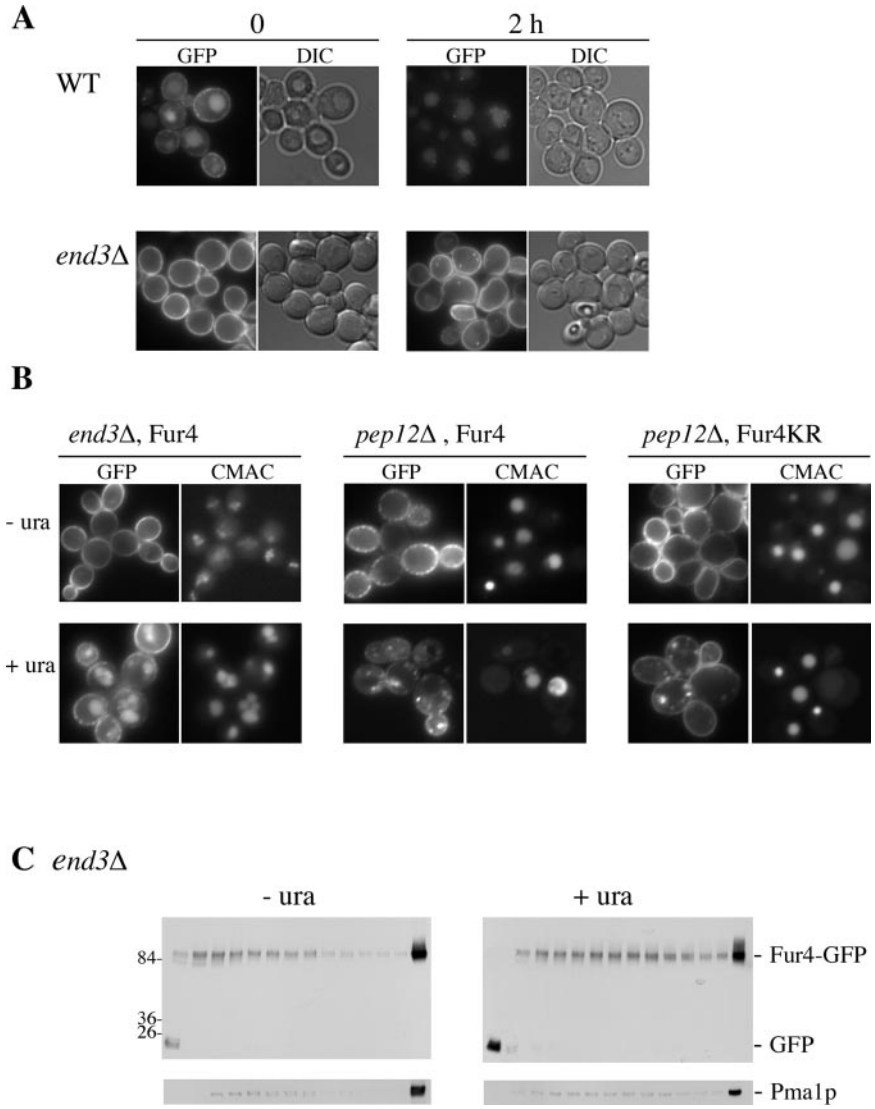

Figure 1. Uracil triggered delivery of its specific permease to the vacuole via the VPS pathway. (A) Wild-type and *end3*Δ cells transformed with pFL38GalFUR4-GFP were cultured on galactose to midexponential growth phase. Glucose and uracil were then added and cells were cultured for an additional 2 h. Cells withdrawn from cultures before (0) and after (2 h) the addition of glucose and uracil were examined for fluorescence and with Nomarski optics. (B) *End3*Δ cells transformed with pFL38 GalFUR4-GFP and *pep12*Δ cells transformed with either pFL38 GalFUR4-GFP or pFL38 GalFUR4^{KR}-GFP were cultured with lactate and then with added galactose, in the absence or presence of uracil. The cellular distribution of newly synthesized permease was assessed, over 2 h for *end3*Δ cells and 3 h for *pep12*Δ cells to take into account their very slow growth, by fluorescence microscopy, after staining the vacuoles with CMAC as described in MATERIALS AND METHODS. (C) Extracts from *end3*Δ cells that had produced Fur4-GFP for 2 h in the absence or presence of uracil were fractionated on equilibrium sucrose density gradients. Sucrose gradient concentration increases from left to right in the figure. Aliquots of the various fractions were analyzed by western immunoblotting for Fur4-GFP/GFP and Pma1p. Molecular weight markers are indicated in kDa.

centrifugation on sucrose density gradients and fractions were analyzed by immunoblotting with anti-GFP antibodies (Figure 1C). We detected a strong signal, corresponding to Fur4-GFP, in the highest density fraction, in the presence and absence of uracil. The H^+ ATPase Pma1p, used as a plasma membrane protein marker, was also mainly detected in this fraction. The presence of Fur4-GFP in intermediate fractions can be accounted for in part by minor contamination with fragments of the plasma membrane, because Pma1p also gave a faint signal in these fractions. However, the presence of Fur4-GFP in these fractions is also consistent with the possible presence of this protein in endosomes or other intermediate organelles along the secretory or Golgi-to-vacuole pathways. The low-molecular-weight species present in the low-density fractions corresponds to GFP resistant to vacuolar proteases, as previously reported (Dupre and Haguenauer-Tsapis, 2003). This protein was present in much larger amounts in the presence of uracil than in its absence, because of the diversion of Fur4-GFP to the vacuole. We roughly estimated the proportions of material present in the vacuole and plasma membrane by western blotting, using several dilutions of the lowest and highest density fractions from this and another sucrose gradient experiment. We found that, in the presence of uracil, the intensity of the vacuolar GFP signal was similar to that of the plasma membrane Fur4-GFP signal, whereas it was only 10–20% as strong in the absence of uracil. Thus, ~30–40% of the permease appears to have been diverted by uracil to an early degradative pathway. This estimate was confirmed by an independent approach. We measured uracil uptake in *end3*Δ cells after 2 h of Fur4-GFP synthesis in the presence and absence of uracil. Although activity measurements are not entirely reliable for cells cultured in the presence of exogenous uracil, even if these cells are carefully washed, the addition of uracil appeared to decrease activity by 40–50%. Uracil may therefore decrease the delivery of its specific permease to the plasma membrane by a factor of up to two.

The FUI1*-encoded Uridine Permease Is Similarly Sorted for Early Vacuolar Degradation in Cells Exposed to Uridine*

Uridine is another precursor of pyrimidine nucleotides that yeast cells are able to take up from the external environment (Wagner *et al.*, 1998). We found that adding uridine to cells overproducing the specific *FUI1*-encoded uridine permease resulted in a decrease in growth rate (our unpublished re-

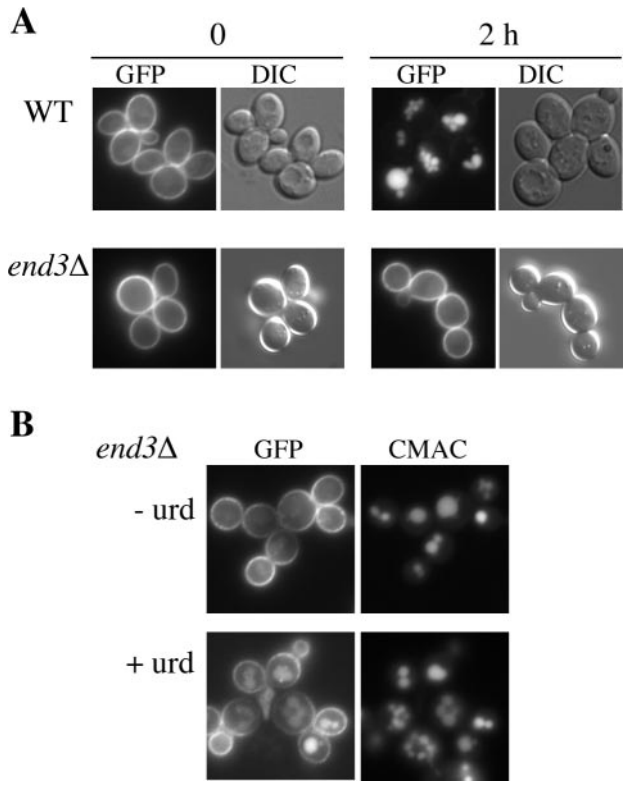

Figure 2. Uridine promoted the direct routing of newly synthesized uridine permease to the vacuole. (A) WT and *end3*Δ cells producing Fui1-GFP under control of the Gal10 promoter, were cultured on galactose to midexponential growth phase. Glucose (2%) and uridine were then added and cells cultured for an additional 2 h. Cells withdrawn from cultures before (0) and after (2 h) the addition of glucose and uridine were examined for fluorescence and with Nomarski optics. (B) Fui1-GFP was produced for 2 h in *end3*Δ cells in the absence or presence of uridine (urd). Permease distribution (GFP) and vacuolar staining (CMAC) were assessed.

sults). We therefore thought that uridine might control Fui1p trafficking, preventing harmful uridine uptake. Galactose-induced Fui1-GFP accumulated at the cell surface in wild-type cells, in which only faint vacuolar fluorescence was observed, suggesting that the constitutive turnover of Fui1-GFP is probably lower than that of Fur4-GFP (Figure 2A and Figure 1A, respectively). After the addition of glucose to block permease synthesis and further growth for 2 h in the presence of uridine, plasma membrane fluorescence had disappeared and the vacuoles had become highly fluorescent, indicating that Fui1-GFP had been internalized and delivered to the vacuolar lumen (Figure 2A). *End3*Δ cells producing Fui1-GFP were subjected to the same glucose shut-off procedure. As expected, uridine permease, which was located entirely at the cell surface in these cells cultured on galactose, was not internalized further in the presence of uridine (Figure 2A). Thus, the plasma membrane-located Fui1p was turned over similarly to other permeases, by means of endocytosis and vacuolar degradation. We then investigated the possible effects of uridine on newly synthesized Fui1p by inducing the production of Fui1-GFP in the presence or absence of uridine in *end3*Δ cells. Fui1-GFP synthesized in the absence of uridine was present almost exclusively at the cell surface, whereas the presence of uridine resulted in additional fluorescence in the vacuole (Figure 2B). Uridine therefore routed a portion of Fui1p directly to the degradative pathway, without the necessity of passing via the plasma membrane. This effect is similar to that of uracil on Fur4p and appeared to be substrate specific as no direct targeting of Fui1p to the vacuole was observed in cells taking up large amounts of uracil (our unpublished results).

A Lack of Ubiquitylation Prevents Delivery of the Diverted Fur4p to the Vacuolar Lumen

Ubiquitylation was recently shown to be an important signal in post-Golgi sorting for both biosynthetic vacuolar proteins, such as Cps1p (Katzmann *et al.*, 2001; Reggiori and Pelham, 2001), and plasma membrane transporters, such as Gap1p (Helliwell *et al.*, 2001; Soetens *et al.*, 2001), and Tat2p (Beck *et al.*, 1999; Umebayashi and Nakano, 2003). The same *cis*- and *trans*-elements are involved in the ubiquitylation of Gap1p at the cell surface and in intracellular compartments (Soetens *et al.*, 2001; Springael and Andre, 1998). We have previously shown that the cell surface ubiquitylation of Fur4p requires the ubiquitin ligase Rsp5p and that lysines 38 and 41 are the main ubiquitin acceptors (Galan *et al.*, 1996; Marchal *et al.*, 2000). Permease phosphorylation, occurring within an N-terminal PEST region of Fur4p, is also required for cell surface ubiquitylation. The SA variant of uracil permease, in which all five serine residues included in the PEST region are replaced by alanines, is very poorly phosphorylated, and is thus strongly stabilized at the plasma membrane (Marchal *et al.*, 1998). We therefore investigated the possible involvement of Rsp5p and of the relevant serine and lysines of Fur4p in direct routing of the permease to the vacuole. We first checked that the GFP-tagged versions of the SA and KR variants were resistant to endocytosis in wild-type cells. Galactose-induced Fur4^{SA}-GFP and Fur4^{KR}-GFP accumulated only at the cell surface (Figure 3A). After the addition of glucose to block permease synthesis and further growth for 2 h in the presence of uracil, the plasma membrane remained fluorescent and no intracellular fluorescence was detected, showing that these variant permeases were indeed resistant to endocytosis. We then checked whether the SA variant was normally sorted to the endosomal pathway when synthesized in the presence of uracil. The SA variant was partly delivered to the vacuolar lumen (Figure 3B), and behaved very similarly to the wild-type uracil permease in *end3*Δ cells (Figure 1B). Because the SA variant is itself resistant to endocytosis, the fraction present within the vacuole of wild-type cells must have arrived at this location without passing via the plasma membrane. Thus, the phosphorylation of Fur4p, at least on the serine residues within the PEST sequence, is not important for the diverted pathway. This result is consistent with our previous observations that phosphorylation of the uracil permease occurs at a late stage in the secretory pathway (Volland *et al.*, 1992), and depends on the casein kinase I isoforms, Yck1p and Yck2p (Marchal *et al.*, 2000), two peripheral plasma membrane proteins (Vancura *et al.*, 1994). We also found that uracil permease was generally less phosphorylated in cells cultured in the presence of uracil than in those cultured in its absence (Séron *et al.*, 1999). This may be due to some of the permease not undergoing phosphorylation because of premature targeting to the vacuole.

We also produced Fur4^{SA}-GFP in *npi1/rsp5* mutant cells, which have very low levels of Rsp5p (Springael and Andre, 1998) and thus display impaired cell surface ubiquitylation. Under conditions resulting in the direct routing of uracil permease in the endosomal pathway, we observed fluorescence not only at the plasma membrane and in the vacuolar lumen, but also at the vacuolar membrane (Figure 3B). Fur4-GFP produced in the *npi1* mutant strain gave very similar

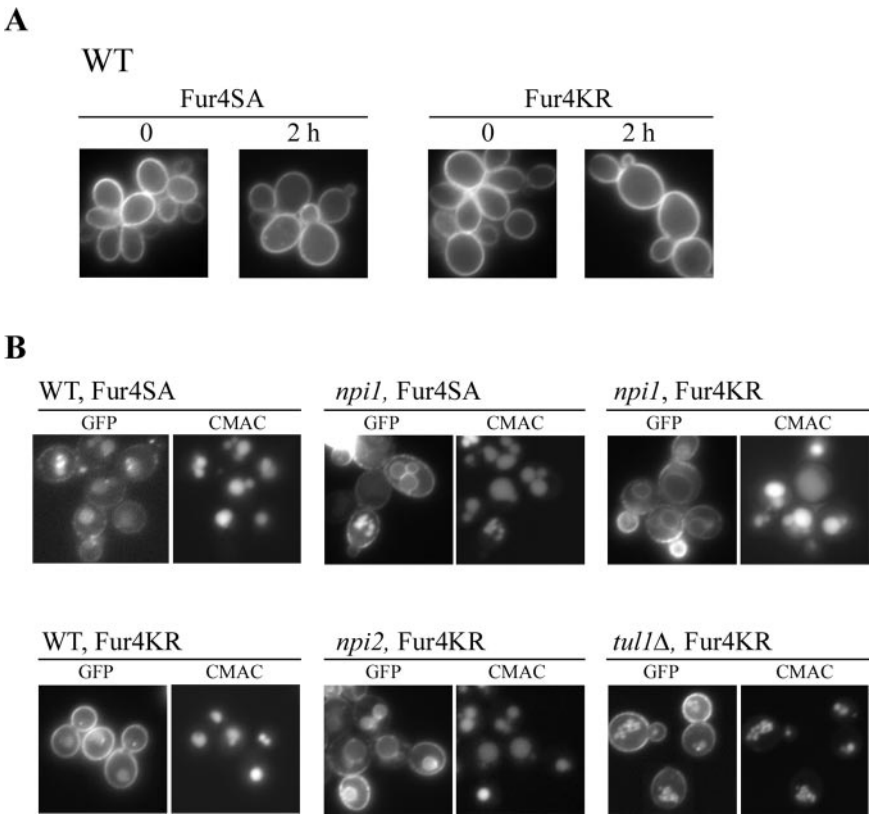

Figure 3. The delivery of uracil permease to the vacuolar lumen was compromised in cells with impaired ubiquitylation. (A) Wild-type cells producing either the SA or the KR variant of Fur4-GFP under control of the Gal10 promoter were used. Cells were cultured with galactose to midexponential growth phase. Glucose and uracil were then added and the cells cultured for an additional 2 h. Cells withdrawn from cultures before (0) and after (2 h) the addition of glucose and uracil were examined for fluorescence. (B) GFP-tagged versions of SA or KR variant of Fur4p were produced for 2 h in the presence of uracil, in cells first grown on lactate as a carbon source, with galactose then added to start permease synthesis. Wild-type, *npi1/rsp5*, *npi2/doa4,* and *tul1*Δ mutants were used. Permease distribution (GFP) and vacuolar staining (CMAC) were assessed.

pictures (unpublished data). This suggests that Rsp5p is required for the entry of Fur4p into the internal vesicles of the MVB and its subsequent delivery to the lumen of the vacuole but that residual ligase activity in *npi1* cells may be sufficient for some MVB sorting. We investigated whether lysines 38 and 41, which are essential for the Rsp5p-dependent ubiquitylation of uracil permease at the cell surface (Marchal *et al.,* 2000), were also involved in the diverted pathway. We looked at the subcellular distribution of fluorescence after production of the KR variant of Fur4-GFP in the presence of uracil in *npi1* cells. The diverted variant permease was detected only at the vacuolar membrane and did not enter the vacuolar lumen (Figure 3B). This finding is consistent with the idea that Rsp5p exerts an effect on Fur4p directly by altering the ubiquitylation status of this protein. We also directly assessed the ubiquitylation state of the permease (see below). The behavior of the KR variant also indicated that, in addition to their involvement in internalization at the plasma membrane, lysines 38 and 41 are probably sites of ubiquitylation for MVB sorting. However, we observed that the KR variant was delivered to the vacuolar lumen in wild-type cells (Figure 3B). Hence, in addition to lysines 38 and 41, one or more other lysines probably act as sites of uracil permease ubiquitylation, ensuring the efficient entry of this protein into MVB, at least in cells with no major defect in the ubiquitylation system. This interpretation was confirmed using *npi2/doa4* mutant cells (Springael *et al.,* 1999), which have low ubiquitin levels and hence display impairment of various ubiquitin-dependent processes (Swaminathan *et al.,* 1999). Fur4-GFP, when produced in *npi2* cells, reached the vacuolar lumen (unpublished data) as it did in wild-type cells, whereas the KR variant was clearly present in the vacuolar membrane in addition to the lumen (Figure 3B). The use of *doa4*Δ cells rather than *npi2* cells, which have a point mutation affecting a conserved region of the ubiquitin isopeptidase Doa4p (Springael *et al.,* 1999), did not lead to more pronounced missorting of uracil permease (unpublished data). The decrease in the ubiquitin pool is therefore less crucial for the diverted pathway than for permease endocytosis, as wild-type uracil permease was strongly stabilized at the plasma membrane in *npi2/doa4* mutant cells (Galan and Haguenauer-Tsapis, 1997). Overall, these results suggest that poorly ubiquitylated Fur4p, regardless of the reason for this defect, can be diverted from the Golgi apparatus toward the vacuole, but cannot efficiently enter the MVB and is therefore detected within the vacuolar membrane. Such a location has already been reported for the ABC transporter Ste6p and the tryptophan permease Tat2p in *doa4* cells (Beck *et al.,* 1999; Losko *et al.,* 2001).

The Golgi-located ubiquitin ligase Tul1p has been shown to be required for the ubiquitylation and sorting of Cps1p or Phm5p to internal vesicles within MVB. In *tul1*Δ cells, Cps1p and Phm5p were found to be ultimately located on the vacuolar membrane rather than in the lumen (Reggiori and Pelham, 2002). We investigated whether this ligase was also involved in the intracellular trafficking of permeases. Production of the GFP-tagged KR variant in the *tul1*Δ strain in the presence of uracil revealed no accumulation in the limiting membrane of the vacuole (Figure 3B). Instead, this variant was found to be delivered to the vacuolar lumen, as for wild-type Fur4p in *tul1*Δ and the KR variant in parental cells (unpublished data). This indicates that the Tul1p ligase is not important for the sorting of permeases within the MVB. This is consistent with the role previously attributed to Tul1p in the recognition of polar transmembrane domains within abnormal proteins and biosynthetic precursors (Reggiori and Pelham, 2002). Proteins with many membrane-

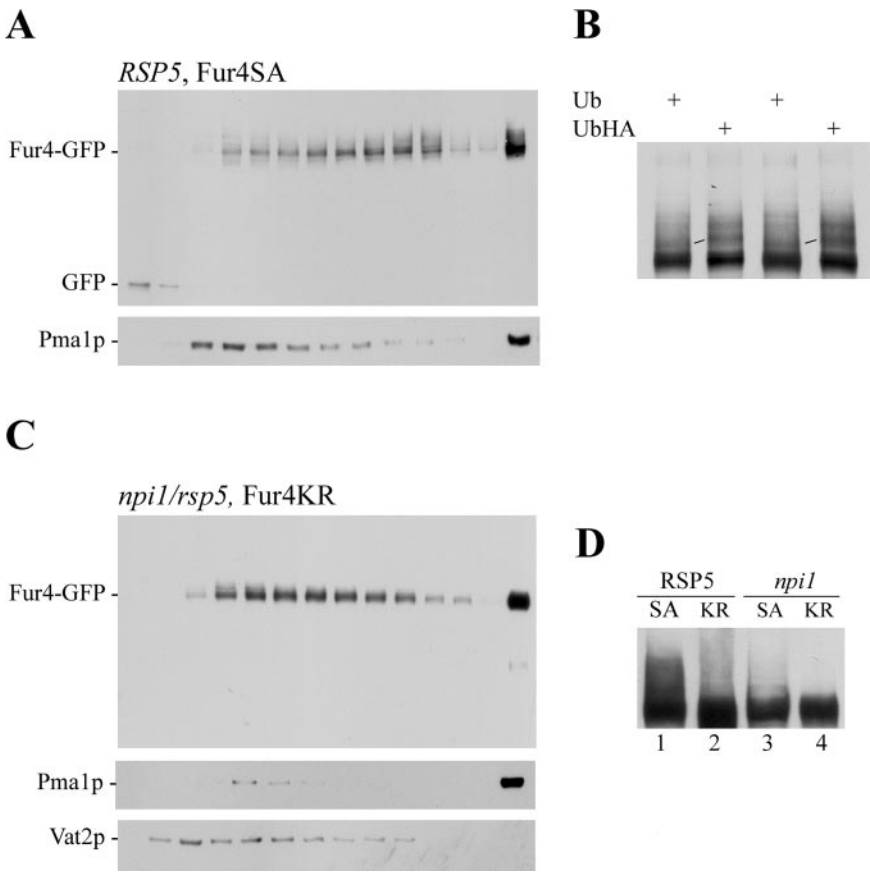

Figure 4. Uracil permease is ubiquitylated during trafficking from the Golgi apparatus to the vacuole, in a Rsp5p-dependent manner. The *DOA4* gene had been deleted in the cells used here, which harbored either the wild-type or a mutant version (*npi1*) of the *RSP5* gene. The cells were cotransformed with plasmid Yep96, encoding normal ubiquitin under the control of the Cup1 promoter (Ellison and Hochstrasser, 1991), and either pFL38GalFUR4^{SA}-GFP or pFL38GalFUR4^{KR}-GFP. Cells were first cultured with lactate as the carbon source, and CuSO4 (100 μM) and galactose were then added and the cells cultured for 2 h in the presence of uracil. Protein extracts were prepared and fractionated on equilibrium sucrose density gradients. (A) Aliquots of the various fractions from *RSP5* cells producing the SA variant of Fur4-GFP were analyzed by Western immunoblotting for Fur4-GFP, GFP, and Pma1p. Sucrose gradient concentration increases from left to right in the figure. (B) The eight central fractions of the gradient shown in A were pooled and analyzed in parallel to equivalent fractions from the same cells that have produced HA-tagged ubiquitin (from the Yep112 plasmid; Ellison and Hochstrasser, 1991) instead of normal ubiquitin. A small line indicates the increase in the molecular weight of a ubiquitin conjugate. (C) As for A except that fractions of gradients from extracts of *npi1* mutant cells producing Fur4^{KR}-GFP were analyzed by Western immunoblotting for Fur4-GFP, GFP, Pma1p, and Vat2p, a marker of the vacuolar membrane. (D) Pools of the eight central fractions of gradients from RSP5 and *npi1* cells producing the SA or KR variant of Fur4-GFP were analyzed by immunoblotting with anti-GFP antibodies.

spanning domains, such as Fur4p, are thought to acquire a folding pattern in which the few charged residues present in the hydrophobic transmembrane domains are not exposed to the lipid environment.

We carried out experiments to determine directly the ubiquitylation status of Fur4p sorted to the endosomal pathway in cells harboring either wild-type or mutant *RSP5* genes. To facilitate the detection of Ub-permease conjugates, we used cells lacking the Doa4p ubiquitin isopeptidase, which is involved in the deubiquitylation of endocytic (Dupre and Haguenauer-Tsapis, 2001) and biosynthetic substrates (Katzmann *et al.*, 2001; Reggiori and Pelham, 2001). A normal pool of ubiquitin was restored by producing ubiquitin in a copper-regulated manner. We used the SA variant rather than the wild-type permease, to minimize ubiquitylation of the cell surface-located permease and its subsequent endocytosis. Extracts were prepared from *doa4*Δ cells allowed to synthesize ubiquitin and Fur4^{SA}-GFP for 2 h in the presence of uracil and fractionated by sedimentation on a density gradient. In *doa4*Δ cells used in these conditions, permease trafficking to the vacuole was frequently retarded, possibly because of a toxic effect of copper, but this situation made it easier to detect potential ubiquitin conjugates. On immunoblots (Figure 4A), a smear was detected just above the main Fur4-GFP signal in intracellular fractions. To check whether this smear indeed correspond to ubiquitin conjugates, we carried out the same experiment in cells producing HA-tagged ubiquitin, and pools of the internal fractions from cells producing normal or HA-tagged ubiquitin were analyzed by immunoblotting (Figure 4B). We observed a difference in electrophoretic migration for bands above the main signal and the presence of HA-tagged ubiquitin resulted in an increase in the molecular weight of at least the first species that very likely corresponded to a mono-ubiquitylated permease. Therefore, permease was ubiquitylated during trafficking from the Golgi apparatus to the vacuole. The ubiquitylation status of the diverted permease was then analyzed in *npi1doa4*Δ cells producing Fur4^{KR}-GFP and normal ubiquitin (Figure 4C). Vacuolar fractions did not display any proteolytic GFP in agreement with the lack of fluorescence inside the vacuole (Figure 3B). A substantial amount of permease was again found in intermediate fractions; this distribution is consistent with the KR variant being able to reach the limiting membrane of the vacuole but not the lumen of *npi1* cells (Figure 3B). Importantly, the extent of ubiquitylation of Fur4^{KR}-GFP that has been diverted appeared to be very low, (Figure 4C), compared with that seen for the SA variant in *RSP5* cells (Figure 4A), suggesting that the sorting to the endosomal pathway efficiently occurred despite a severe impairment in the ubiquitylation level of the permease. The overall levels of ubiquitylation of the diverted permease, whether that permease was the SA variant, which behaved like the wild-type protein, or the KR variant, produced in *RSP5* or *npi/rsp5* cells were better compared by Western blot analysis after pooling the internal fractions (Figure 4D). The KR variant produced in cells with no defect in the ubiquitylation machinery displayed a much lower level of ubiquitylation that the SA permease (compare lane 2 with lane 1). This suggests that K38 and K41 are the main acceptor sites in Fur4p for ubiquitylation during the diverted pathway. Normal delivery to the vacuolar lumen may however be ensured by a rather modest ubiquitylation

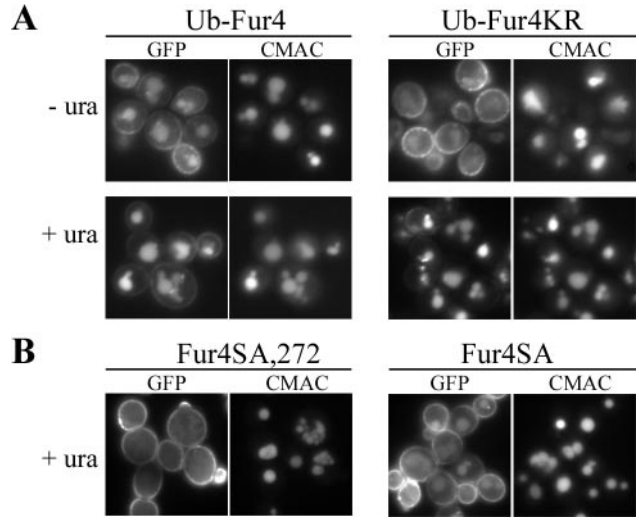

Figure 5. The binding of uracil is the main signal for the efficient sorting of Fur4p to the endosomal system. (A) *End3*Δ cells transformed with pFL38GalUb-FUR4-GFP or pFL38GalUb-FUR4^{KR}-GFP were cultured with lactate, and the medium was then supplemented with galactose to induce the synthesis of GFP-tagged permease. After 2 h in the absence or presence of uracil, permease fluorescence (GFP) and vacuole staining (CMAC) were assessed. (B) WT cells cotransformed with Yep46FUR4 and either pFL38GalFUR4^{SA,272}-GFP or pFL38GalFUR4^{SA}-GFP were subjected to galactose induction in the presence of uracil and examined as in A.

of alternative lysines (Figure 3B). In *npi1/rsp5* cells, residual levels of ubiquitin conjugates were still present for the diverted SA permease (lane 3), whereas such conjugates appeared under the limit of detection for the diverted KR variant (lane 4). This latter result is consistent with fluorescence analysis (Figure 3B), showing that, in *npi1* cells, the KR variant may reach the limiting membrane of the vacuole but not the lumen, whereas some wild-type Fur4p reached the vacuolar lumen. A low level of Rsp5p ubiquitin ligase, limiting the ubiquitylation of the permease, may therefore account for its impaired sorting at the MVB level. However, we cannot exclude the possibility that Rsp5p has additional effects on elements involved in MVB formation.

Exposure to Substrate Is the Main Signal for Sorting to the Vacuole

We further analyzed the role of ubiquitin in marking permeases for direct sorting from the Golgi apparatus to the vacuole and investigated whether ubiquitylation was sufficient for such sorting by adding ubiquitin to the N-terminus of the GFP-tagged Fur4p. We fused to GFP-permeases a variant ubiquitin with all its relevant lysine residues mutated to arginine (Arnason and Ellison, 1994), to ensure that no additional ubiquitin was added to the ubiquitin moiety. We also modified the added ubiquitin further, by replacing glycine 96 with a valine, to prevent this molecule from being removed from the chimera. Ub-Fur4-GFP synthesized in the absence of uracil in *end3*Δ cells was partitioned between the cell surface and the vacuole (Figure 5A). We estimated the proportion of Ub-permease delivered to the plasma membrane in these conditions by measuring uracil uptake in *end3*Δ mutant cells producing either Ub-Fur4-GFP or Fur4-GFP and cultured in the absence of uracil. We obtained an activity ratio of 75–80% for cells producing the Ub-chimera with respect to cells producing the normal permease, indicating that there was 20–25% vacuolar diversion. Diversion to the vacuole, which was rather modest in the absence of uracil, became the predominant pathway in cells exposed to uracil (Figure 5A). Thus, a single ubiquitin can mediate some vacuolar sorting, but it is not sufficient for efficient targeting to the vacuole in the absence of the physiological signal. Nonubiquitylable ubiquitin was also fused to the KR variant of Fur4-GFP and the chimeric protein produced in *end3*Δ cells. Newly synthesized Ub-Fur4^{KR}-GFP behaved identically to Ub-Fur4-GFP: it was targeted to the vacuole in only moderate amounts in the absence of uracil but was very efficiently targeted to the vacuole after exposure to uracil (Figure 5A). The biosynthetic addition of ubiquitin to the N-terminus of Fui1-GFP resulted in similar behavior, with the Ub-chimeric permease efficiently directed to the VPS pathway only in uridine-treated cells. Thus, uracil itself, or uridine, seems to be the dominant signal in the secretory pathway for decreasing the amount of permease delivered to the cell surface.

We tested this hypothesis using a Fur4p mutant (Fur4K272E) in which lysine 272, lying in a transmembrane segment, was replaced by a glutamic acid. This mutant has been shown to have a very low affinity for uracil and only residual transport activity (Urban-Grimal *et al.*, 1995). A GFP-tagged version of this mutant protein was constructed and, as expected, Fur4^{272}-GFP was found to be located at the cell surface and did not transport uracil (our unpublished results). Because the chromosomally encoded uracil permease alone cannot increase uracil levels to such a point that the permease is sorted to the vacuole, another plasmid, encoding an active form of uracil permease, was introduced into cells. The presence of the two plasmids appeared to be very toxic to *end3*Δ cells. This difficulty was overcome by introducing the SA mutation into Fur4^{272}-GFP, to obtain a permease resistant to endocytosis, making it possible to use wild-type cells. Cells producing active Fur4p under the control of its own promoter from a multicopy plasmid were grown with lactate as the carbon source, with subsequent addition of uracil and galactose to induce synthesis of either Fur4^{SA,272}-GFP or Fur4^{SA}-GFP, used as a control. The mutation of residue 272 almost entirely prevented diversion of the permease to the vacuolar pathway as the vacuoles showed only very faint labeling with this mutant permease (Figure 5B). This strongly suggests that the direct binding of intracellular uracil to its specific transporter is indeed the signal for sorting of the permease to the endosomal pathway for early degradation. However, an Ub-Fur4 chimera mutated for residue 272, Ub-Fur4^{272}-GFP, was diverted to the MVB pathway in the absence of added uracil, to the same extent as Ub-Fur4-GFP. This indicates that early sorting of the permease may also occur in the absence of uracil binding, at least for a protein with a fused ubiquitin moiety.

Mono-ubiquitylation Is Not Sufficient for the Rapid Internalization of Uracil Permease at the Plasma Membrane or for the Efficient Sorting of This Protein to MVB

We investigated whether the biosynthetic addition of ubiquitin restored normal turnover to a variant uracil permease lacking its ubiquitin acceptor lysines, resulting in stabilization at the plasma membrane. In wild-type cells growing exponentially on galactose, Fur4^{KR}-GFP was located exclusively at the plasma membrane whereas Ub-Fur4^{KR}-GFP was partitioned between the plasma membrane and vacuole (Figure 6A). The fluorescence observed within the vacuoles of cells producing the Ub-chimera can be accounted for, at least in part, by some diversion of this protein from the exocytic pathway, even in the absence of added uracil (see

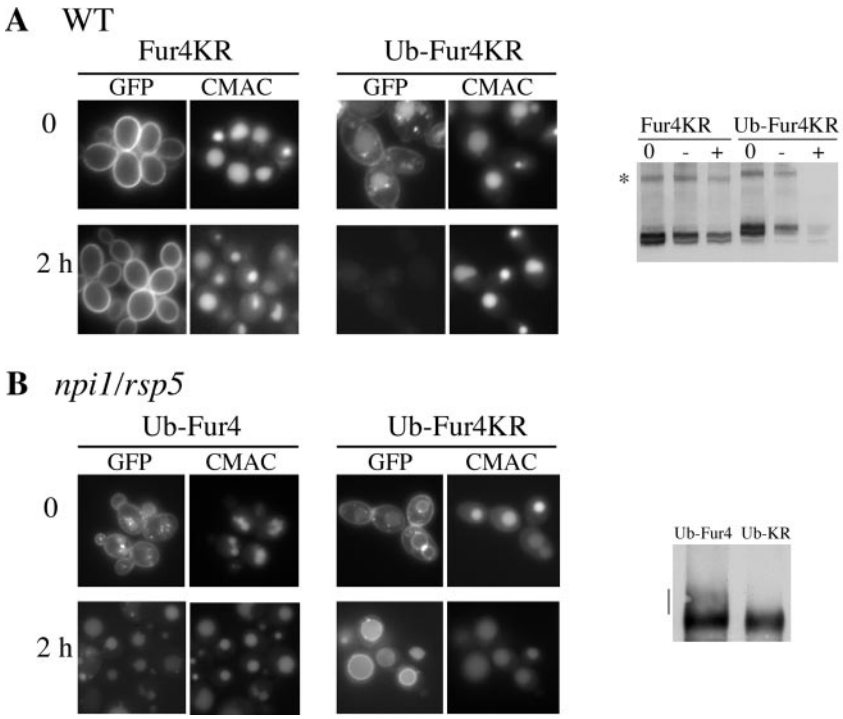

Figure 6. Polyubiquitylation is required for efficient trafficking along the endocytic pathway and subsequent vacuolar degradation. (A) WT cells producing either Fur4^{KR}-GFP or Ub-Fur4^{KR}-GFP under control of the Gal10 promoter were supplemented with glucose and the cells cultured for an additional 2 h in the absence or presence of uracil. Cells withdrawn from cultures before (0) and after the addition of glucose + uracil (2 h) were labeled with CMAC and examined to determine permease distribution (GFP) and the pattern of vacuolar staining (CMAC). Total protein extracts from cells withdrawn before (0) and after the addition of glucose for 2 h in the absence (−) or presence (+) of uracil were analyzed by Western blotting with anti-GFP antibodies. Potential SDS-resistant dimers of permease are indicated by an asterisk. (B) *npi1/rsp5* cells producing either Ub-Fur4-GFP or Ub-Fur4^{KR}-GFP were cultured with galactose to midexponential growth phase, then allowed to grow for 2 h in the presence of glucose and uracil, and examined as in A for GFP and CMAC fluorescence. Membrane-enriched fractions from these cells were prepared before the addition of glucose and analyzed by immunoblotting with anti-GFP antibodies. A small vertical line indicates potential ubiquitin conjugates.

Figure 5). After adding glucose to block synthesis of the permease and allowing cells to grow for an additional period in the presence of uracil, fluorescence disappeared from both the plasma membrane and vacuole, indicating that the fused ubiquitin restored uracil-triggered endocytosis and subsequent vacuolar degradation of the KR variant (Figure 6A). Western blot analysis of total protein extracts confirmed that the fusion of ubiquitin to the N-terminus of the molecule led to proteolytic breakdown of the KR variant (Figure 6A). Because the KR variant without fused ubiquitin was present principally at the cell surface, it seems that alternative lysines of Fur4p do not act as targets for ubiquitylation at the plasma membrane. We therefore suggest that monoubiquitylation, at least that involving in a nonremovable fused ubiquitin, allows both endocytosis and correct delivery to the interior of the vacuole. We investigated whether the rates of these processes were affected by the level of ubiquitylation, by comparing the rate of internalization of the wild-type and KR variant forms of Ub-permease chimeras. We did this by determining the rate at which uracil uptake activity was lost after the addition of cycloheximide (Table 2). Under these high turnover conditions, Ub-Fur4-GFP was internalized several times more rapidly than Ub-Fur4^{KR}-GFP. This indicates that polyubiquitylation of the permease resulted in much more efficient endocytosis than the mere addition of an N-terminal nonubiquitylable ubiquitin. The presence of an N-terminal-fused ubiquitin resulted in upregulation of the endocytosis of the wild-type uracil permease (Table 2), showing that the number of ubiquitin moieties, rather than their precise sites of attachment, is the key element for acceleration of the rate of internalization in endocytosis. Our results are consistent with conclusions drawn from studies with the yeast **a**-factor receptor, indicating that multiubiquitylation increases the rate of internalization (Roth and Davis, 2000).

Table 2. Half-time (min) of the internalization step of endocytosis of GFP-tagged uracil permease derivatives

Strains	Fur4p	Fur4^{KR}p	Ub-Fur4p	Ub-Fur4^{KR}p
WT	40	>180	20	75
npi1	>180	>180	75	70

Uracil uptake was measured various times after the addition of cycloheximide and the time corresponding to a 50% loss of activity is reported.

We further analyzed the intracellular trafficking of ubiquitin permease chimeras by checking whether normal sorting to MVB was restored by biosynthetically added ubiquitin in conditions in which Rsp5p levels were very low. Figure 6B shows the distribution of Ub-Fur4-GFP and Ub-Fur4^{KR}-GFP produced in *npi1/rsp5* mutant cells. During exponential growth in the absence of uracil, these two proteins were partitioned between the cell surface and intracellular compartments. The vacuolar lumen contained both proteins but the KR variant was also detected at the vacuolar membrane, indicating impairment of its sorting at the MVB level. Blocking the synthesis of permease and allowing the cells to grow for a additional 2 h in the presence of uracil resulted in the complete removal from the cell surface of the permease to which a ubiquitin had been fused, whether or not the target lysines were present (Figure 6B). The absence of target lysines in the Ub-chimera did not further delay the internalization step in *npi1* cells (Table 2). However, the KR variant was clearly present at the vacuolar membrane, indicating that its delivery to the vacuolar lumen was still largely impaired. One possible reason for this is that the ubiquitylation of some of the lysines in the permease is required for efficient sorting to the MVB. If Rsp5p levels were normal, it might be possible to overcome the absence of the normal target lysines, possibly by adding ubiquitin to alternative lysines, but this is not possible in mutants with low Rsp5p

levels. Western blotting of membrane-enriched fractions from *npi1* cells (Figure 6B) revealed that additional ubiquitin was probably added to Ub-Fur4-GFP but not to Ub-Fur4^{KR}-GFP. Thus, in the absence of polyubiquitylation, the permease was not efficiently sorted to vesicles and became permanently resident in the vacuolar membrane after fusion of the MVB with the vacuole. Because some permease nonetheless reached the vacuolar lumen, the deficiency may lie in the sorting step being limiting, resulting in a fraction of the permease being trapped at the endosomal membrane and subsequently at the vacuolar membrane.

DISCUSSION

The plasma membrane transporters of *S. cerevisiae* play a critical role in controlling growth rate and the tight regulation of their removal from the cell surface is a key feature in the capacity of this organism to adapt rapidly to changing nutrient availability. We previously demonstrated the negative control exerted by uracil over its own transport. In particular, uracil decreases the stability of its specific permease, Fur4p, by promoting the Rsp5p-dependent ubiquitylation of this protein at the cell surface (Séron *et al.*, 1999). This provides a signal for the internalization and delivery of Fur4p to the vacuole for degradation. We suggest that intracellular uracil from catabolic or external sources directly controls the fate of Fur4p, preventing the uptake of excess pyrimidine nucleotide precursors, which would be harmful to cells. The data reported here show that uracil also affects the exocytic trafficking of Fur4p by inducing the direct routing of this protein from the Golgi apparatus to the endosomal system for degradation without the necessity of passing via the plasma membrane. Similarly, exogenous uridine promotes the direct routing of its specific permease, Fui1p. A very efficient mechanism for responding to rapid changes in the nutritional state of nucleotide precursors is thus provided through the control of early protein sorting and cell surface protein stability. The metal transporter Smf1p provides another example of a transporter negatively regulated by its own substrate. Smf1p is not directed to the cell surface if manganese is plentiful but accumulates at the plasma membrane under conditions of manganese starvation (Liu and Culotta, 1999b). There are three closely related Smf metal transporters, operating at different locations within the cell, on a variety of metals, some of which are toxic. This system requires sophisticated regulation (Portnoy *et al.*, 2000).

Our results show that ubiquitin plays an essential role in the entry into MVB of Fur4p diverted from the late secretory pathway to the vacuole. Direct analysis of the level of ubiquitylation of Fur4p prematurely targeted for degradation showed that the permease is ubiquitylated during direct routing to the vacuole. *Npi1* mutation, which results in very low levels of the ubiquitin ligase Rsp5p, impaired the delivery of Fur4p, which accumulated at the vacuolar membrane rather than in the lumen. This impairment was even more marked if the *npi1* mutation was combined with a *cis*-acting Fur4 KR mutation, and may be accounted for by a lack of ubiquitylation of the permease. Thus, the same *cis*- and *trans*-elements, Rsp5p and lysine residues at positions 38 and 41 are used for both the downregulation of uracil permease levels at the cell surface (Galan *et al.*, 1996; Marchal *et al.*, 2000) and the sorting of this protein from the biosynthetic pathway to the MVB. In contrast, phosphorylation of the serine residues within a PEST sequence of Fur4p that is important for ubiquitylation at the cell surface (Marchal *et al.*, 1998) does not seem to be required for normal entry into MVB. Our data also show that alternative lysines can serve as ubiquitin acceptors for Fur4p in the diverted pathway, whereas this is not the case at the plasma membrane. Rsp5p may recognize relevant lysines for the ubiquitylation of intracellular permease in a less specific way, different from the process of recognition at the plasma membrane. The delivery of the general amino acid permease Gap1p to the cell surface appears to be controlled by the quality of the external nitrogen source. Rich nitrogen sources, such as $NH4^+$ and glutamate, trigger the sorting of this permease exclusively to the vacuole, without passing via the plasma membrane (Roberg *et al.*, 1997; Soetens *et al.*, 2001). The features of Gap1p ubiquitylation important for the intracellular targeting of this molecule to the vacuole have been characterized. It is striking that the elements implicated in the intracellular ubiquitylation of Gap1p are identical to those involved in the ubiquitylation that signals its endocytosis. Both events require Rsp5p, the same Gap1 target lysines and the Bul proteins (Helliwell *et al.*, 2001; Soetens *et al.*, 2001). Bul1p and Bul2p were first identified as proteins that bind Rsp5p (Yashiroda *et al.*, 1996). The overproduction of Bul proteins results in the sorting of Gap1p to the vacuole regardless of the nitrogen source and it has been suggested that the Bul proteins specify the intracellular polyubiquitylation of Gap1p, resulting in its efficient sorting to the vacuole (Helliwell *et al.*, 2001). The double mutant *bul1*Δ bul2Δ has the inverse phenotype, causing Gap1p to be rerouted to the plasma membrane (Helliwell *et al.*, 2001; Soetens *et al.*, 2001). Similarly ubiquitylation of the tryptophan permease Tat2p is markedly decreased in *bul1*Δ cells and Tat2p appears to be targeted to the plasma membrane of these mutant cells even at high tryptophan concentrations, which promote the transport of Tat2p to the vacuole of wild-type cells (Umebayashi and Nakano, 2003). GFP-tagged Fur4p or its KR variant, newly synthesized in *bul1*Δ bul2Δ cells in the absence or presence of uracil, behaved similarly in these cells and in parental cells, and no difference was observed in the extent to which permeases were diverted (unpublished data). Although some of our data indicate that polyubiquitylation is more efficient than monoubiquitylation for internalization of uracil permease into MVB, we obtained no evidence that Bul proteins were involved in this process. Control of the cell surface delivery of transporters may involve different partners according to whether amino acids or pyrimidine nucleotide precursors are being transported, because of the different physiological significance of these molecules. The early degradation of Fur4p prevents the uptake of an excess of nucleotide precursors, which would be detrimental to the cells, and would be expected to be rapid and irreversible. Amino acid uptake involves many permeases classified according to their response to the source of nitrogen and regulation of the trafficking of these permeases makes it possible to optimize the use of all available nutrients. A pool of intracellular Gap1p exists under various growth conditions and it has been suggested that this pool is a type of physiological storage system (Helliwell *et al.*, 2001). Conversely, Fur4p is located exclusively at the cell surface in the absence of endocytosis (see Figure 1A). Sorting decisions concerning the early degradation of Gap1p may therefore require additional factors that are not required for Fur4p disposal.

The precise location, between the Golgi apparatus and the endosomes, at which the intracellular sorting of uracil permease destined for the interior of the vacuole occurs is unclear. The identification of this site is hampered by the finding that a subset of exocytic cargoes do transit through an endosomal compartment before reaching the cell surface,

but can be rerouted to the classical exocytic pathway if this route is blocked, as in *vps* mutants (Gurunathan *et al.,* 2002; Harsay and Schekman, 2002). It has been suggested that the plasma membrane H^+ATPase, Pma1p, reaches the cell surface directly from the Golgi apparatus (Gurunathan *et al.,* 2002; Harsay and Schekman, 2002), but it is not yet determined whether plasma membrane transporters follow a route that passes through endosomes. Our results, obtained in *pep12*Δ cells, which display impaired vesicle fusion with late endosomes, must be considered within this context. Some permease newly synthesized in the presence of uracil was found in small vesicles in *pep12* cells. This site would be expected for material blocked on its route from the Golgi apparatus to the endosomes. The sorting of Fur4p triggered by uracil occurred at the Golgi level in *pep12* cells but it is impossible to determine from this observation whether such sorting occurs in the Golgi apparatus or in the endosomes in wild-type cells.

Another unanswered question concerns the site of Fur4p ubiquitylation in the direct route to the vacuole. Vacuolar proteins, such as the membrane precursor of Cps1p, receive ubiquitin before the Pep12 compartment (Katzmann *et al.,* 2001). Tul1p, the ubiquitin ligase involved in this process, is a Golgi-resident transmembrane protein (Reggiori and Pelham, 2002). We showed that Tul1p was not involved in the sorting of Fur4p, and therefore probably not in its ubiquitylation, whereas Rsp5p-dependent ubiquitylation was clearly required for Fur4p sorting to the MVB. Staining for Rsp5p gives a punctate pattern at the plasma membrane and at perivacuolar sites, probably corresponding to late endosomal structures (Wang *et al.,* 2001). Although this location is consistent with the ubiquitylation event occurring at the endosome rather than at the Golgi level, it is not definitive proof of this as a small amount of Rsp5p in the vicinity of the Golgi apparatus may have escaped detection. Our findings extend the list of known functions of Rsp5p in the intracellular trafficking of membrane-bound proteins. The role of ubiquitin ligases of the Rsp5/Nedd4 family may have been conserved throughout evolution. Recent data highlighted a role of *Drosophila* Nedd4 in the downregulation of a receptor of the nervous system (Murphey and Godenschwege, 2002). Ubiquitin-dependent events may result in the sorting of the receptor to the endosomal pathway without passing via the plasma membrane (Keleman *et al.,* 2002)

We showed that Fur4p newly synthesized in the presence of uracil can reach the vacuole even if ubiquitylation is severely impaired. In such cases, it is present at the vacuolar membrane rather than in the vacuolar lumen. A vacuolar membrane location has also been demonstrated for the tryptophan permease Tat2p, in *npi1* cells, after rapamycin treatment, which is known to induce the sorting of Tat2p for early degradation (Beck *et al.,* 1999). These findings suggest that regardless of where ubiquitylation occurs, it is required for delivery into the vacuolar lumen. This is consistent with the crucial role assigned to ubiquitin in directing the sorting of proteins to MVB luminal vesicles through recognition by several Vps proteins carrying Ub-binding domains (Katzmann *et al.,* 2002; Donaldson *et al.,* 2003). In contrast, it is not consistent with ubiquitin being sufficient signal in itself to govern the routing of permeases to the plasma membrane or the degradative pathway. The presence of a nonubiquitylable ubiquitin moiety fused to Fur4p indeed results in some diversion for early degradation, but most of this Ub-permease chimera is still delivered to the cell surface. Similarly, the fusion of a ubiquitin moiety to the C-terminus of both mutant α-factor receptor Ste2p (Terrell *et al.,* 1998) and **a**-factor receptor Ste3p (Roth and Davis, 2000) was not pointed out to prevent the delivery of these proteins to the plasma membrane. In contrast, for certain other proteins, such as the plasma membrane SNARE protein Snc1p, a single fused ubiquitin appears to be sufficient to route the protein to the endosomes (Reggiori and Pelham, 2002). In all these chimeras, the attached ubiquitin lacked one or several lysines preventing its extension into a polyubiquitin chain and whether the same results would be obtained with chimeric multispanning proteins containing wild-type ubiquitin remains unanswered. Anyhow, intrinsic features independent of the ability to accept ubiquitin seem to be required for the efficient targeting to the vacuole of proteins normally destined for the plasma membrane.

We previously suggested that intracellular uracil binds to a site on the cytoplasmic domain of the permease, inducing a conformational change (Séron *et al.,* 1999). Such binding probably occurs in the biosynthetic pathway in cells exposed to uracil because a mutant permease strongly deficient in binding was not sorted to the endosomal pathway. Uracil binding may favor a particular folding pattern. Various mechanisms may take into account differences in the folding patterns of membrane proteins. Interaction with various lipids is undoubtedly one of the most important of these mechanisms, and sorting may be achieved by favoring the segregation or exclusion of proteins in particular lipid microdomains. The yeast plasma membrane contains lipid raft microdomains enriched in sphingolipids and ergosterol (Bagnat *et al.,* 2000), and it has recently been shown that Fur4p at the cell surface is associated with rafts (Dupre and Haguenauer-Tsapis, 2003). Furthermore, Fur4p has been found to be associated with detergent-resistant membranes in *sec18* mutant cells, suggesting that it enters lipid rafts early in the secretory pathway (Dupre and Haguenauer-Tsapis, 2003). It is possible that the binding of uracil to the permease disturbs the association of the permease with lipid rafts, preventing its packaging into post-Golgi vesicles destined for the cell surface. It was very recently reported that the association of Tat2p with the detergent-insoluble membrane rafts is required for plasma membrane delivery and if lipid rafts are altered, as in *erg6*Δ cells, Tat2p is missorted to the vacuole (Umebayashi and Nakano, 2003). This mechanism is similar to that proposed for Pma1–7, an H^+ATPase mutant that is misfolded at nonpermissive temperature and targeted directly to the vacuole rather than the cell surface. Wild-type ATPase has indeed been shown to be incorporated into lipid rafts after leaving the endoplasmic reticulum whereas the mutant displays impaired raft association that may prevent its sorting to the plasma membrane (Bagnat *et al.,* 2001).

We present evidence that a mutant form of Fur4p with a very low affinity for uracil is not targeted to the vacuole in the presence of uracil. In contrast, in cases of defective ubiquitylation, Fur4p reaches the vacuole but is mostly missorted to the vacuolar membrane. Thus, uracil, acting as a ligand, seems to be the principal element signaling the sorting of its specific permease from the secretory pathway to the vacuole and ubiquitylation appears to occur subsequently. Mutant forms of the Smf1p metal transporter with probable deficiencies in ligand-binding were also found not to be sorted from the Golgi apparatus to the degradative pathway (Liu and Culotta, 1999a). However, the potential involvement of ubiquitin-dependent events in the trafficking of this transporter was not reported. Our conclusions concerning the role of ubiquitylation in regulating the exocytosis of Fur4p somehow differ from the mechanism proposed for Gap1p and Tat2p trafficking in which impaired ubiquitylation results in rerouting to the cell surface, preventing

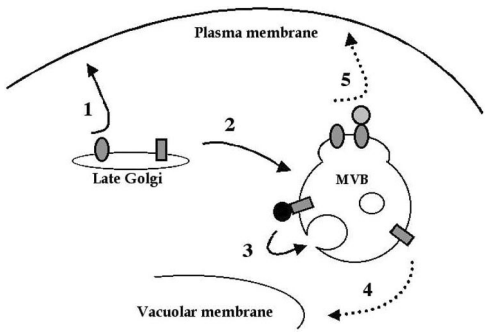

Figure 7. Presumed sorting steps for newly synthesized uracil permease. At the Golgi level, free permease (oval) is targeted to the cell surface (1), whereas uracil-liganded permease (rectangle) is sorted to the VPS pathway (2). An induced change in the conformation of Fur4p may modify its association with lipid rafts, accounting for its sorting to either plasma membrane or endosomes. On delivery to the endosome, uracil-liganded Fur4p may be efficiently ubiquitylated (●) and sorted to inward budding vesicles of MVB (3), resulting in its degradation in the vacuolar lumen or it may be maintained at the surface of MVB due to defective ubiquitylation, resulting in its becoming a permanent resident of the vacuolar membrane (4). It may be retrieved by the recycling pathway to the plasma membrane (5) if uracil is removed or in cases of underubiquitylation (○). If the sorting decision governed by uracil takes place only at the endosome level, the various possible routes from that location remain valid.

sorting to the vacuole (Helliwell *et al.*, 2001; Soetens *et al.*, 2001). In contrast, a very residual ubiquitylation seems sufficient for uracil-induced sorting of Fur4p to the endosomal pathway, although we cannot completely exclude that a process of rerouting to the plasma membrane occurs, in a small extent, for poorly ubiquitylated Fur4p. Figure 7 depicts a model fitting our results including the fact that a fused ubiquitin, acting in part as a signal, did increase the extent of permease degradation. Fur4p that has bound its ligand is sorted at the Golgi level, possibly by being excluded from rafts domains. Once it reaches the endosomes, Fur4p may still have different fates. It is efficiently segregated in internal membranes of the MVB for lumen release if ubiquitylation proceeds rapidly and extensively. Alternatively, in cases of poor ubiquitylation, the permease is not recognized by the protein complexes that bind ubiquitylated cargoes and remains on the external membrane of endosomes. From there, depending on uracil concentration, the permease may be delivered to the limiting membrane of the vacuole after the fusion of mature MVB with vacuoles or packaged into budding vesicles, which are thought to ensure transport in an endosome-to-plasma membrane pathway. Vacuolar delivery and export to the cell surface may compete kinetically. It should be noted that the ubiquitylation of the permease, which is crucial in determination of its fate, is a reversible process and that Rsp5p and Doa4p, which are involved in the ubiquitylation/deubiquitylation of Fur4p (Galan *et al.*, 1996; Dupre and Haguenauer-Tsapis, 2001), are both found in the vicinity of endosomes (Amerik *et al.*, 2000; Wang *et al.*, 2001). In addition, ligand binding, the extent of which may change rapidly, may in turn modify the distribution of Fur4p in particular lipid microdomains and/or the ability of this protein to accept ubiquitin. In this model, a low level of ligand, including low intracellular levels of uracil of catabolic origin, might lead to efficient sorting of the permease to the endosomal system, but the final extent of permease degradation would be controlled by the level of ubiquitylation. Some permease reaching the endosomes might escape targeting to the vacuole and reach the plasma membrane from the endosomal compartment. Our preliminary data indicate that permease transported away from the cell surface via the endocytic pathway might also escape vacuolar degradation by being recycled to the plasma membrane. A second decision step at the level of the endosomes, common to permeases from both the exocytic and endocytic pathways, would add flexibility to control of the amount of permease reaching the cell surface, giving a higher quality control of plasma membrane content.

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