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Quality Control of Plasma Membrane Proteins by *Saccharomyces cerevisiae* Nedd4-Like Ubiquitin Ligase Rsp5p under Environmental Stress Conditions

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In *Saccharomyces cerevisiae*, when a rich nitrogen source such as ammonium is added to the culture medium, the general amino acid permease Gap1p is ubiquitinated by the yeast Nedd4-like ubiquitin ligase Rsp5p, followed by its endocytosis to the vacuole. The arrestin-like Bul1/2p adaptors for Rsp5p specifically mediate this process. In this study, to investigate the downregulation of Gap1p in response to environmental stresses, we determined the intracellular trafficking of Gap1p under various stress conditions. An increase in the extracellular ethanol concentration induced ubiquitination and trafficking of Gap1p from the plasma membrane to the vacuole in wild-type cells, whereas Gap1p remained stable on the plasma membrane under the same conditions in *rsp5*^{A401E} and Δ*end3* cells. A ^{14}C-labeled citrulline uptake assay using a nonubiquitinated form of Gap1p (Gap1p$^{K9R/K16R}$) revealed that ethanol stress caused a dramatic decrease of Gap1p activity. These results suggest that Gap1p is inactivated and ubiquitinated by Rsp5p for endocytosis when *S. cerevisiae* cells are exposed to a high concentration of ethanol. It is noteworthy that this endocytosis occurs in a Bul1/2p-independent manner, whereas ammonium-triggered downregulation of Gap1p was almost completely inhibited in Δ*bul1/2* cells. We also found that other environmental stresses, such as high temperature, H_2O_2, and LiCl, also promoted endocytosis of Gap1p. Similar intracellular trafficking caused by ethanol occurred in other plasma membrane proteins (Agp1p, Tat2p, and Gnp1p). Our findings suggest that stress-induced quality control is a common process requiring Rsp5p for plasma membrane proteins in yeast.

In eukaryotic cells, there are known to be protein quality-control mechanisms that remove aberrant polypeptides generated as a consequence of errors in transcription, translation, folding, and environmental stresses (1, 2). Misfolded membrane proteins are normally eliminated by endoplasmic reticulum (ER) quality control, in which the misfolded proteins are retrotranslocated into the cytoplasm and ubiquitinated, followed by degradation via the ubiquitin proteasome system (3, 4). In contrast, it is believed that plasma membrane proteins such as permeases and receptors with limited conformational defects can escape ER, localize at the plasma membrane, and be eliminated by lysosomal degradation, which serves as post-ER quality control (5–7). However, it is poorly understood how the post-ER quality control described above is involved in degradation of aberrant plasma membrane proteins generated by environmental stresses.

The budding yeast *Saccharomyces cerevisiae* is a unicellular eukaryote that is exposed to various stresses, including a high concentration of ethanol, freezing, desiccation, and high osmotic pressure during fermentation processes (8). It is important to clarify the quality-control mechanism of yeast plasma membrane proteins because these proteins would be directly exposed to such extracellular stress conditions. In particular, ethanol damages the cell membrane and functional proteins, gradually reducing cell viability and leading to cell death during fermentation (8–10). Therefore, the use of ethanol-resistant yeast strains with enhanced quality control of plasma membrane proteins would enable more efficient production of bioethanol and alcoholic beverages.

In mammalian cells, a variety of ubiquitin ligases recognize different plasma membrane proteins prior to endocytosis (11–13), but yeast endocytosis proceeds mostly through ubiquitination by the HECT-type ubiquitin ligase Rsp5p (14, 15). The essential protein Rsp5p is the only *S. cerevisiae* member of the Nedd4 family; it contains a membrane-binding C2 domain; three substrate-recognizing WW domains, which bind short peptide sequences called PY motifs (XPXY); and the catalytic HECT domain, which ligates ubiquitin to the target protein (16). The general amino acid permease Gap1p has been used as a model of ubiquitin-dependent trafficking of plasma membrane proteins (17). When yeast cells are cultivated on a poor nitrogen source, such as urea, allantoin, or proline, arrestin-like proteins Aly1p and Aly2p promote endosomal recycling of Gap1p, which is localized at the plasma membrane in a stable form (18). In contrast, after addition of a good nitrogen source, such as ammonium, Gap1p is rapidly ubiquitinated by Rsp5p, endocytosed, sorted into the multivesicular endosomes (MVEs), and finally degraded in the vacuoles (19, 20). It should be noted, however, that Rsp5p does not directly bind and ubiquitinate Gap1p and other several permeases that lack PY motifs. Instead, the adaptor proteins containing PY motifs mediate ubiquitination of these proteins by Rsp5p (21, 22). The arrestin-like redundant adaptor proteins Bul1p and Bul2p (Bul1/2p) are required for endocytosis of Gap1p in response to nitrogen availability (21). Recently, Merhi and André showed that ubiquitination of Gap1p localized at the plasma membrane is regulated via phosphorylation of Bul1/2p mediated by protein kinase

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TABLE 1 Yeast strains used in this study

Strain	Genotype	Source or reference
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf
TSY259	BY4741 *rsp5*^{A401E}	24
TSY262	BY4741 *bul1*::*hphNT1*	24
TSY263	BY4741 *bul2*::*natNT2*	24
TSY264	BY4741 *bul1*::*hphNT1 bul2*::*natNT2*	24
TSY260	BY4741 *end3*::*natNT2*	24
ESY001	BY4741 *gap1*::*kanMX4*	This study
YSY001	BY4741 *bul3*::*kanMX4*	This study
YSY002	BY4741 *bul1*::*hphNT1 bul3*::*kanMX4*	This study
YSY003	BY4741 *bul2*::*natNT2 bul3*::*kanMX4*	This study

Npr1p and association of the 14-3-3 protein to the phosphorylated site (23). We also proposed a novel mechanism for ubiquitination of Gap1p, in which Rsp5p is phosphorylated and inactivated when a poor nitrogen source is provided to the culture medium (24).

We previously found that Gap1p in *rsp5*^{A401E} (i.e., the A-to-E change at position 401 encoded by *rsp5*) cells, which is a loss-of-function mutant, remained stable and active on the plasma membrane and was not ubiquitinated even when the mutant was grown on ammonium (25). Interestingly, *rsp5*^{A401E} cells showed much more sensitivity to a variety of stresses that may induce protein denaturation, such as high growth temperature in a rich medium, heat shock treatment, and a high concentration of ethanol, than wild-type cells (25). This suggests that aberrant plasma membrane proteins generated by such environmental stresses are also ubiquitinated by Rsp5p and eliminated from the plasma membrane via endocytosis. However, the mechanism by which plasma membrane proteins, including Gap1p, are endocytosed and degraded under these stress conditions remains unknown.

We show here that, under severe ethanol stress conditions, Gap1p underwent ubiquitination and endocytosis. Exposure to a high concentration of ethanol also induced endocytosis of other substrate proteins localized on the plasma membrane. Thus, this study reports the quality control mechanism of plasma membrane proteins in *S. cerevisiae* cells under environmental stress conditions.

MATERIALS AND METHODS

Strains, culture media, and plasmids. All of the yeast strains used in the present study were derivatives of *S. cerevisiae* BY4741. The strains are listed in Table 1. Several gene knockout strains were obtained from the Euroscarf gene knockout collection or constructed by homologous recombination of antibiotic-resistant genes (*kanMX4*, *hphNT1*, and *natNT2*). *Escherichia coli* strain DH5α or OneShot *ccdB* survival T1 phage-resistant cells (Invitrogen) were used to construct vectors or subclone the yeast gene. The media used for growth of *S. cerevisiae* were a nutrient medium YPD (2% glucose, 1% yeast extract, 2% peptone) (26) and a synthetic minimal medium SD (2% glucose, 0.17% yeast nitrogen base without amino acids and ammonium sulfate [Difco Laboratories]) (26) containing 0.5% $(NH_4)_2SO_4$ (SD+Am) or 0.1% allantoin (SD+Alt) as the sole nitrogen source. An alternative synthetic minimal medium SG contains 2% galactose as the carbon source instead of glucose in SD medium. *E. coli* cells were grown in Luria-Bertani medium containing ampicillin (50 μg/ml). If necessary, 2% agar was added to solidify the medium.

The plasmids used to examine localization of plasma membrane proteins were constructed with Gateway recombination cloning technology (Life Technologies). Plasmid pRS416-P$_{GAL1}$-*ccdB*-yEGFP was prepared as the Gateway destination vector in the following way. A P$_{GAL1}$ fragment was obtained from pYM-N24 (27) by SacI/XbaI digestion. Plasmid pRS416-P$_{ADH1}$-*ccdB*-yEGFP (24) was digested with the same restriction enzymes set and ligated with the P$_{GAL1}$ fragment. The *GAP1*, *AGP1*, *GNP1*, and *TAT2* genes were amplified by PCR from yeast genomic DNA, and Gateway entry vectors were generated by a BP reaction with the amplified fragments and pDONR221. LR reactions were performed with the destination and entry vectors to construct the plasmids pRS416-P$_{GAL1}$-*GAP1*-yEGFP, pRS416-P$_{GAL1}$-*AGP1*-yEGFP, pRS416-P$_{GAL1}$-*TAT2*-yEGFP, and pRS416-P$_{GAL1}$-*GNP1*-yEGFP. To express Gap1p variants, plasmids pRS416-P$_{GAL1}$-*GAP1*^{K9R}-yEGFP, pRS416-P$_{GAL1}$-*GAP1*^{K16R}-yEGFP, and pRS416-P$_{GAL1}$-*GAP1*$^{K9R/K16R}$-yEGFP were constructed using a QuikChange II XL site-directed mutagenesis kit (Stratagene).

To detect ubiquitination of Gap1p, two plasmids (pMK088 carrying P$_{CUP1}$-Myc-Ubi and pMK089 carrying P$_{CUP1}$-Ubi) (28) were kindly provided from M. Kitabatake (Kyoto University).

Stress treatments. Yeast cells cultivated in SD+Am at 25°C for 35 h were washed with distilled water, suspended in SG+Alt at an optical density at 600 nm (OD_{600}) of 0.3, and cultivated at 25°C for 24 h to induce expression of Gap1p. After centrifugation, the cells were suspended in SD+Alt at an OD_{600} of 0.3 and cultivated at 25°C for 3 h to shut off the expression of Gap1p. Ethanol (final concentrations of 10% or 15%), H_2O_2 (0.25 mM), or LiCl (100 mM) was added to the cell suspension, followed by incubation at 25°C for indicated periods. For high-temperature conditions, the incubation was performed at 40°C. Fifty millimolar $(NH_4)_2SO_4$ and distilled water were used as the positive and negative controls for the endocytosis of plasma membrane proteins, respectively.

Fluorescence microscopy. Yeast cells harboring the plasmids containing the *GAP1-yEGFP* fusion gene were treated under various stress conditions as described above and subjected to 40 μM FM4-64 (Biotium) for 15 min to visualize the vacuole. The cells were then harvested, washed with SD+Alt medium three times, incubated 30 min at 25°C, and concentrated by a factor of 10 by centrifugation. Cells were viewed immediately without fixation under a fluorescence microscope (Axiovert 200M; Carl Zeiss), and images were captured with an HBO 100 microscope illuminating system (Carl Zeiss) digital camera and processed using Adobe Photoshop Elements 2.0 (Adobe Systems). The results presented are based on our observations of >100 cells.

Immunoprecipitation and Western blotting. The cells were suspended in 200 μl of an sodium dodecyl sulfate (SDS) buffer (1% SDS, 45 mM Na-HEPES [pH 7.5], 80 mM *N*-ethylenemaleimide [NEM], 0.4 mM phenylmethylsulfonyl fluoride, and 4% protease inhibitor cocktail [Sigma-Aldrich]) and disrupted by glass beads. The lysates were diluted with 700 μl of a Triton buffer (1.5% Triton X-100, 150 mM NaCl, 50 mM Na-HEPES [pH 7.5], 5 mM Na-EDTA, 10 mM NEM, and 4% protease inhibitor cocktail) and centrifuged at 800 × *g* for 15 min. Immunoprecipitation was carried out with 10 μl of equilibrated anti-green fluorescent protein (anti-GFP) magnetic beads (Medical & Biological Laboratories) for 1.5 h at 4°C by a rotator. The beads were washed five times with 1% Triton X-100 in phosphate-buffered saline. The immunoprecipitates were solubilized with incubation in a 5-fold concentration of the sample buffer (Tris-HCl [pH 8.0], 2% SDS, 0.0125% bromophenol blue, and 2.25% glycerol) for 15 min at 65°C. The samples were loaded on a 10.5% or an 8% SDS-polyacrylamide gel. The Gap1p protein was detected by an anti-GFP mouse antibody (Roche Diagnostics) at 1:2,000 dilutions. The ubiquitin-conjugated proteins were detected by an anti-c-Myc mouse antibody (Santa Cruz Biotechnology) at 1:1,000 dilutions. The GAPDH protein was detected by an anti-GAPDH rabbit antibody (Nordic Immunology) at 1:10,000 dilutions.

Measurement of Gap1p activity. The Gap1p activity was measured as the initial uptake rate of radioactive L-citrulline by intact yeast cells (29). Yeast cells were collected by centrifugation, washed with distilled water, and suspended in SD medium at OD_{600} of 0.8. ^{14}C-radiolabeled L-citrulline was added to the cell suspension at final concentrations of 4 μM. One hundred microliters of the sample was withdrawn at an interval of 20 s and filtrated with a glass fiver filter (GC-50, 25 mm diameter; Advantec). After

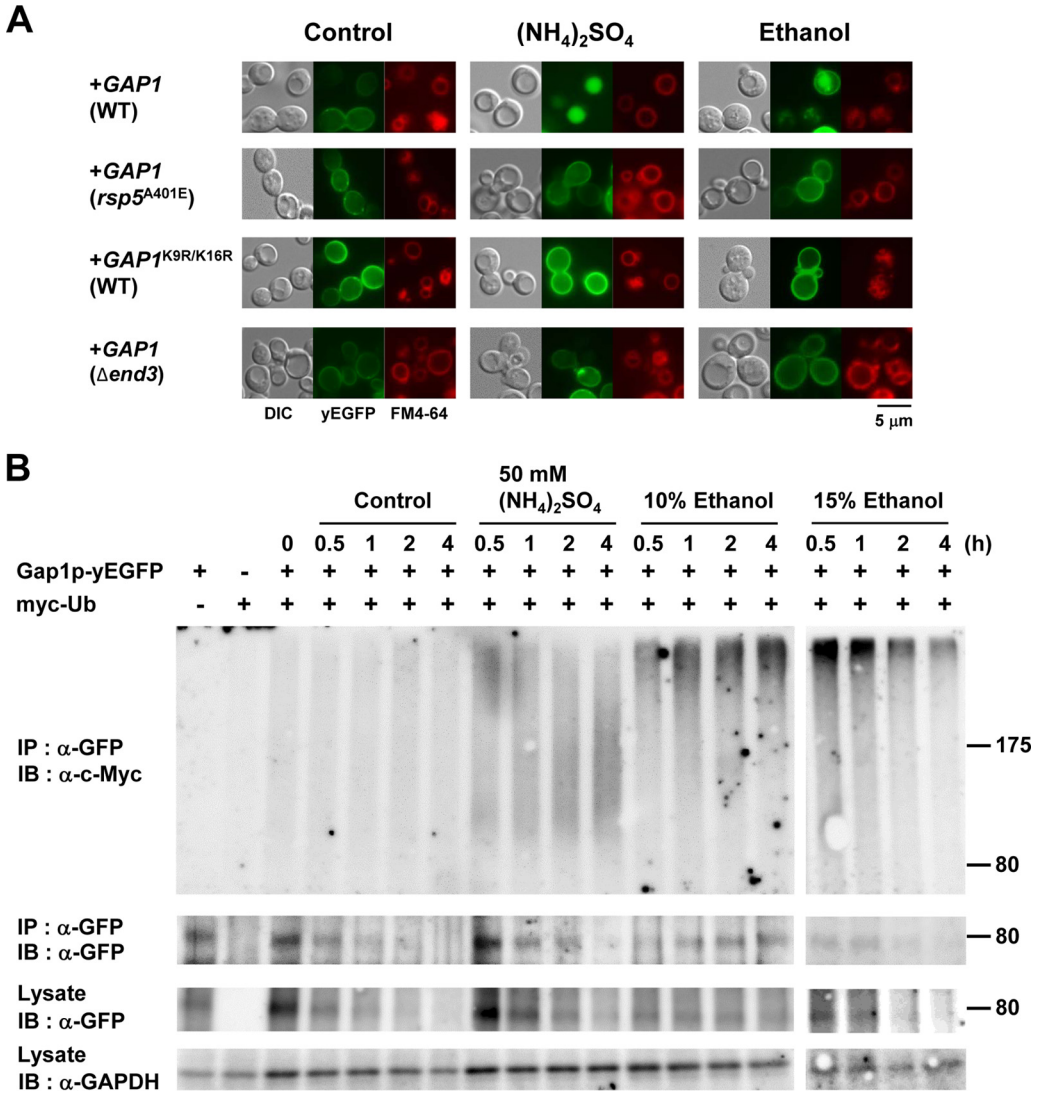

FIG 1 Endocytosis and ubiquitination of Gap1p under ethanol stress conditions. (A) Subcellular localization of the yEGFP-fused Gap1 proteins. *S. cerevisiae* wild-type (BY4741), *rsp5*^{A401E}, and Δ*end3* cells harboring pRS416-P$_{GAL1}$-*GAP1*, or pRS416-P$_{GAL1}$-*GAP1*$^{K9R/K16R}$ were grown in SG+Alt medium to express Gap1p. After the expression was shut off in SD+Alt medium, the cells were treated with 50 mM $(NH_4)_2SO_4$, 15% ethanol, or an equal amount of distilled water as a control for 2 h and observed with the fluorescence microscope for the localization of yEGFP-fused proteins. Cell morphology was observed through differential interference contrast (DIC), and vacuolar membranes were stained with FM4-64. Bar, 5 μm. (B) Immunoblot analysis of the yEGFP-fused Gap1 proteins. *S. cerevisiae* wild-type cells harboring pRS416-P$_{GAL1}$-*GAP1*-yEGFP were grown in SG+Alt medium to express Gap1p. After the expression was shut off in SD+Alt medium and c-Myc-tagged ubiquitin was ectopically induced from the *CUP1* promoter by adding 0.1 mM $CuSO_4$ for 2 h, the cells were treated with 10 or 15% ethanol, and the same conditions as described above for 0.5, 1, 2, or 4 h and were subjected to immunoprecipitation and Western blot analysis with anti-yEGFP, anti-c-Myc, and anti-GAPDH antibodies. Whole-cell lysates used as input for the immunoprecipitation are shown (two bottom panels). The cells harboring the empty vector pRS416 (Gap1p-yEGFP:−) or expressing nontagged ubiquitin (myc-Ub:−) were used as negative controls (two left lanes). Molecular mass standards in kilodaltons are shown at the right.

a washing step with 30 ml of cold distilled water and drying, the ^{14}C radioactivity was measured by a liquid scintillation counter (LS6500; Beckman Coulter). Permease activities were calculated in pmol per min per OD_{600}.

RESULTS

A high concentration of ethanol induces ubiquitination and endocytosis of Gap1p. When a good nitrogen source is provided to the culture medium, Gap1p is ubiquitinated by Rsp5p and finally sorted into the vacuole (20, 30). To examine the localization of Gap1p under ethanol stress condition, we constructed plasmid harboring *yEGFP*-fused *GAP1* under the control of the *GAL1* promoter. Yeast cells that carry this plasmid were cultivated in SG+Alt medium to express Gap1p-yEGFP and to prevent its degradation and then in SD+Alt medium to retain Gap1p-yEGFP on the plasma membrane. The localization of Gap1p-yEGFP was then observed after ethanol treatment by a fluorescence microscope. As shown in Fig. 1A, Gap1p-yEGFP was clearly delivered into the vacuole in wild-type cells after incubation with $(NH_4)_2SO_4$ for 2 h, an observation consistent with a previous study (31). After addition of 15% ethanol to the medium, a significant amount of enhanced GFP (EGFP) signal accumulated in a

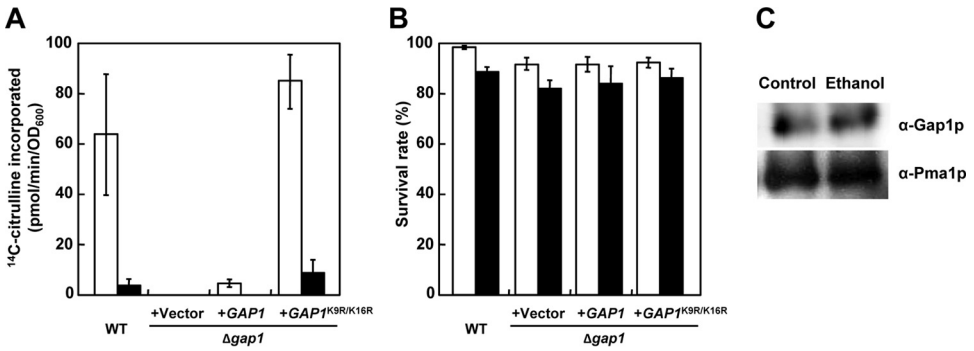

FIG 2 Dysfunction of Gap1p under ethanol stress conditions. (A) Permease activity of Gap1p. *S. cerevisiae* wild-type and Δ*gap1* cells harboring pRS416-P$_{GAL1}$(vector), pRS416-P$_{GAL1}$-*GAP1*, or pRS416-P$_{GAL1}$-*GAP1*$^{K9R/K16R}$ were cultivated in SG+Alt medium and treated with or without 15% ethanol. Gap1p activity was measured by incorporation of ^{14}C-L-citrulline in SD medium at intervals of 20 s. Permease activities were calculated as the initial rate of ^{14}C-L-citrulline uptake per OD$_{600}$ of the cells. Black and white bars represent the results obtained with and without ethanol treatment, respectively. The values are the means and standard deviations of three independent experiments. (B) Cell viability assay. The cells used in the above-described experiment were stained by trypan blue, and the numbers were counted by using a hemocytometer. Survival rates were expressed as percentages of the number of living cells after 15% ethanol treatment for 30 min at 25°C relative to that before ethanol treatment. Black and white bars represent the results with and without ethanol treatment, respectively. The values are the means and standard deviations of three independent experiments. (C) Immunoblot analysis of the yEGFP-fused Gap1 proteins in yeast cells. *S. cerevisiae* Δ*gap1* cells harboring pRS416-P$_{GAL1}$-*GAP1*$^{K9R/K16R}$ were treated with or without 15% ethanol for 30 min at 25°C and subjected to Western blot analysis to determine the Gap1p levels using anti-Gap1p antibody. Pma1p was used as a loading control.

punctate structure stained by FM4-64, suggesting that Gap1p was localized in the vacuoles or the early or late endosomes. We previously isolated the *rsp5*^{A401E} strain having a loss-of-function mutation in the *RSP5* gene, in which ubiquitination and endocytosis of Gap1p are severely impaired even in the presence of ammonium (25). In the present study, Gap1p-yEGFP was localized on the plasma membrane of *rsp5*^{A401E} cells under ethanol stress conditions (Fig. 1A), suggesting that endocytosis of Gap1p is also dependent on ubiquitination mediated by Rsp5p in the presence of a high concentration of ethanol. It is known that ubiquitination occurs at two lysine residues at positions 9 and 16 of Gap1p, and the nonubiquitinated Gap1p variant (Gap1p$^{K9R/K16R}$) is constitutively localized on the plasma membrane, even after shifting to a rich nitrogen source (21). We found that Gap1p$^{K9R/K16R}$ was stable on the plasma membrane even after ethanol treatment, supporting the idea that ubiquitination of Gap1p is required for its endocytosis under ethanol stress conditions. The Δ*end3* cells having a severe defect in endocytosis (32) also showed the plasma membrane localization of Gap1p-yEGFP under ethanol stress conditions.

Furthermore, Western blot analysis detected the ubiquitinated forms of Gap1p-yEGFP in wild-type cells treated with 10% or 15% of ethanol (Fig. 1B), whereas Gap1p$^{K9R/K16R}$-yEGFP was not ubiquitinated (see Fig. S1 in the supplemental material). Notably, ubiquitinated Gap1p under ethanol stress conditions was detected as a higher-molecular weight band than that observed after $(NH_4)_2SO_4$ treatment, suggesting that Gap1p is highly polyubiquitinated and/or aggregated after exposure to ethanol. In the presence of 15% ethanol, the low-mobility form of Gap1p appeared faster than in the presence of 10% ethanol. This suggests that severe ethanol stress induces high polyubiquitination or aggregation of Gap1p. Taken together, these data raise the possibility that Gap1p denatured by ethanol is recognized and ubiquitinated by Rsp5p, in a somehow different manner from Gap1p when a rich nitrogen source is added, and removed from the plasma membrane via endocytosis requiring End3p. Since the Gap1 expression was shut off by glucose before and during exposure to ethanol stress, trafficking of newly synthesized Gap1p proteins to the plasma membrane hardly occurred.

Ethanol causes dysfunction of Gap1p on the plasma membrane. To demonstrate the quality control process of Gap1p under ethanol stress conditions described above, denaturation or dysfunction of Gap1p on the plasma membrane needs to be confirmed. As for cytoplasmic proteins, proteotoxic stresses was reported to lead to quick formation of ER-associated puncta (33). There has been, however, no direct evidence of denaturation of plasma membrane proteins by ethanol *in vivo*. Therefore, we measured the permease activity to obtain insight into the dysfunction of Gap1p. L-Citrulline, a nonstandard amino acid, is reported to be incorporated only through Gap1p in *S. cerevisiae* (34). We examined the effect of ethanol stress on Gap1p activity *in vivo* measured by the incorporation of ^{14}C-labeled L-citrulline. In wild-type cells, the Gap1p activity after the addition of 15% ethanol was dramatically decreased compared to that without stress (Fig. 2A). However, since Gap1p is internalized via endocytosis after ethanol treatment in wild-type cells, the decrease in Gap1p activity might be due to there being less Gap1p on the plasma membrane. Therefore, we next used Δ*gap1* cells expressing a nonubiquitinated form of Gap1p (Gap1p$^{K9R/K16R}$) under the control of the *GAL1* promoter, because this Gap1p variant remained stable on the plasma membrane even after exposure to ethanol as described above (Fig. 1A). As we expected, before cells were exposed to ethanol stress, L-citrulline uptake was not detected in Δ*gap1* cells carrying the vector only. Gap1p activity in Δ*gap1* cells overexpressing the wild-type *GAP1* gene was significantly lower than in wild-type cells, suggesting that overexpression of Gap1p caused its rapid endocytosis. In contrast, yeast cells overexpressing *GAP1*$^{K9R/K16R}$ displayed a Gap1p activity similar to that in wild-type cells in the absence of ethanol, but this activity was markedly decreased after exposure to 15% ethanol for 30 min, as observed in wild-type cells. Even in this case, we could not exclude the possibility that the quantity of the Gap1p variant and/or total cellular activity might affect its permease activity. As shown in Fig. 2B, there were no significant differences in survival rates among yeast cells tested

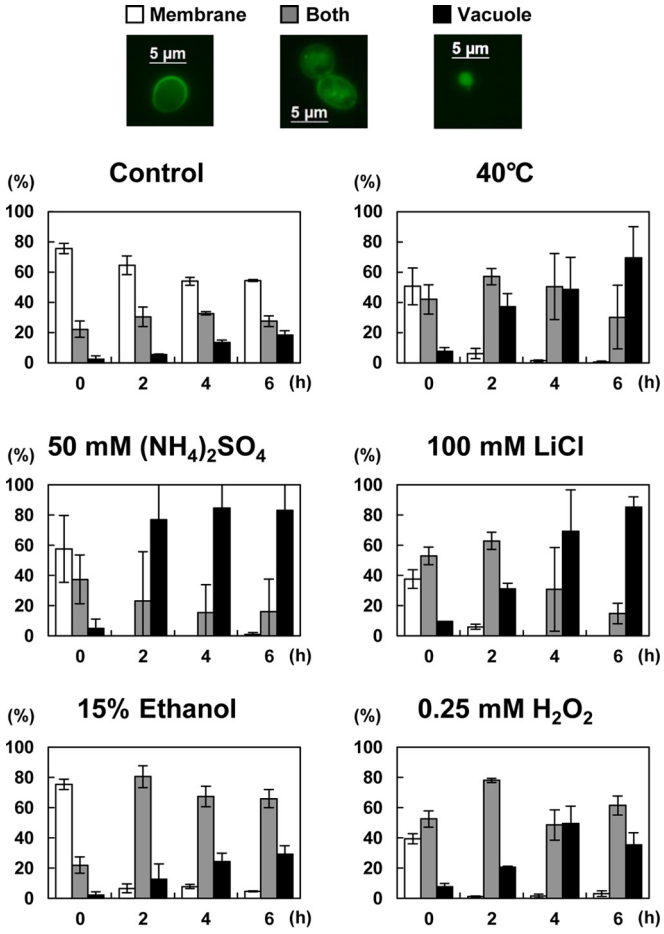

FIG 3 Trafficking of Gap1p under various stress conditions. *S. cerevisiae* wild-type cells harboring pRS416-P_{GAL1}-*GAP1*-yEGFP were grown in SG+Alt medium to express Gap1p. After the expression was shut off in SD+Alt medium, the cells were treated for indicated time with various stresses: water (control), 50 mM $(NH_4)_2SO_4$, 15% ethanol, high temperature (40°C), 100 mM LiCl, and 0.25 mM H_2O_2. The subcellular localization of Gap1p-yEGFP was observed with the fluorescence microscope, and the percentages of the cells showing each localization pattern were calculated from 100 to 200 cells. White, gray, and black bars represent the percentages of the cells in which Gap1p is localized on the plasma membrane, both of on the plasma membrane and in the vacuoles, and in the vacuoles, respectively. The values are the means and standard deviations of two independent experiments. Photographs show the representative subcellular localization patterns corresponding to the categories described above. Bar, 5 μm.

with or without ethanol treatment. Furthermore, Western blot analysis showed that the expression level of $Gap1p^{K9R/K16R}$ in the presence of 15% ethanol was almost the same as that under nonstress conditions (Fig. 2C). These results provide evidence demonstrating that ethanol stress directly causes dysfunction of Gap1p on the plasma membrane.

Other environmental stresses also induce endocytosis of Gap1p. Given that ethanol stress inactivated Gap1p and caused endocytosis via ubiquitination in the Rsp5p-dependent manner, we examined the localization of Gap1p-yEGFP under various stress conditions. Figure 3 shows the intracellular localization pattern of Gap1p after exposure to 15% ethanol, high temperature (40°C), 100 mM LiCl, and 0.25 mM H_2O_2. When $(NH_4)_2SO_4$ was added to the culture medium, Gap1p-yEGFP rapidly accumulated in the vacuoles in >50% of the cells. In contrast, under ethanol stress conditions, a large quantity of cells showed localization of Gap1p both on the plasma membrane and in the vacuole. Although the percentage of cells in which Gap1p was completely sorted into the vacuoles gradually increased by addition of ethanol, Gap1p-yEGFP certainly accumulated in the intracellular punctate structures. Furthermore, we found that high temperature, H_2O_2, and LiCl stresses also induced endocytosis of Gap1p. These results indicate that Gap1p is endocytosed in response to various environmental stresses, as well as ethanol stress.

The Bul proteins do not affect endocytosis of Gap1p under ethanol stress conditions. There are two types of Rsp5p-triggered trafficking of Gap1p: one dependent on redundant adaptor proteins Bul1/2p and one independent of them (31). In the present study, we examined the involvement of Bul1/2p and a newly identified Bul family protein Bul3p (35) on endocytosis of Gap1p under both conditions of a rich nitrogen source and ethanol stress. When $(NH_4)_2SO_4$ was added to the medium, both Δ*bul1* and Δ*bul2* cells showed the localization patterns of Gap1p similar to that of wild-type cells, while endocytosis of Gap1p was severely inhibited in Δ*bul1/2* cells (Fig. 4A). These results suggest the functional redundancy of Bul1/2p, which is consistent with the findings of previous reports (21, 31). In a previous report, it was concluded that both Bul1/2p-dependent and -independent pathways contribute to endocytosis of Gap1p in a rich nitrogen medium that contains Casamino Acids (31). As previously reported (23), our results also indicate that $(NH_4)_2SO_4$ added to the culture medium induces predominantly Bul1/2p-dependent endocytosis of Gap1p. In addition, in wild-type, Δ*bul1*, and Δ*bul2* cells, the Δ*bul3* mutation seemed to derepress endocytosis of Gap1p; disruption of *BUL3* enhanced the trafficking of Gap1p to the vacuole with a poor nitrogen source, but did not exhibit any significant effects with $(NH_4)_2SO_4$ (Fig. 4A). In contrast, disruption of *BUL3* promoted endocytosis of Gap1p in Δ*bul1/2* cells with or without $(NH_4)_2SO_4$. These results suggest that the Bul3 protein negatively affects Bul1/2p-independent endocytosis of Gap1p, which might occur at a low level under nonstress conditions in wild-type cells.

Under ethanol stress conditions, however, there was no significant difference in the Gap1p localization between wild-type and all *bul* mutant cells, suggesting that endocytosis of Gap1p proceeds through a Bul1/2/3p-independent manner in response to ethanol stress. It is known that ubiquitination of the lysine residue at either position 9 or position 16 is sufficient for Bul1/2p-dependent endocytosis of Gap1p, whereas only the lysine residue at position 16 contributes to the Bul1/2p-independent pathway (21). To examine the effect of the Bul1/2p-dependent pathway on Gap1p endocytosis under ethanol stress conditions, three types of Gap1p variants—K9R, K16R, and K9R/K16R—were expressed in Δ*bul1/2* cells (Fig. 4B). The sorting of $Gap1p^{K9R}$ occurred at almost the same level of that of wild-type Gap1p, whereas significant decreases were observed in the sorting of $Gap1p^{K16R}$ and $Gap1p^{K9R/K16R}$ to the vacuoles. These results also support the hypothesis that ethanol triggers Bul1/2p-independent endocytosis of Gap1p. In fact, it was recently shown that the arrestin-related adaptor protein Art1p is involved in Rsp5p-mediated endocytosis of the lysine permease Lyp1p under high-temperature stress conditions (36).

Ethanol also induces endocytosis of other plasma membrane proteins. We wondered whether a high concentration of ethanol induces endocytosis of other plasma membrane proteins, as well

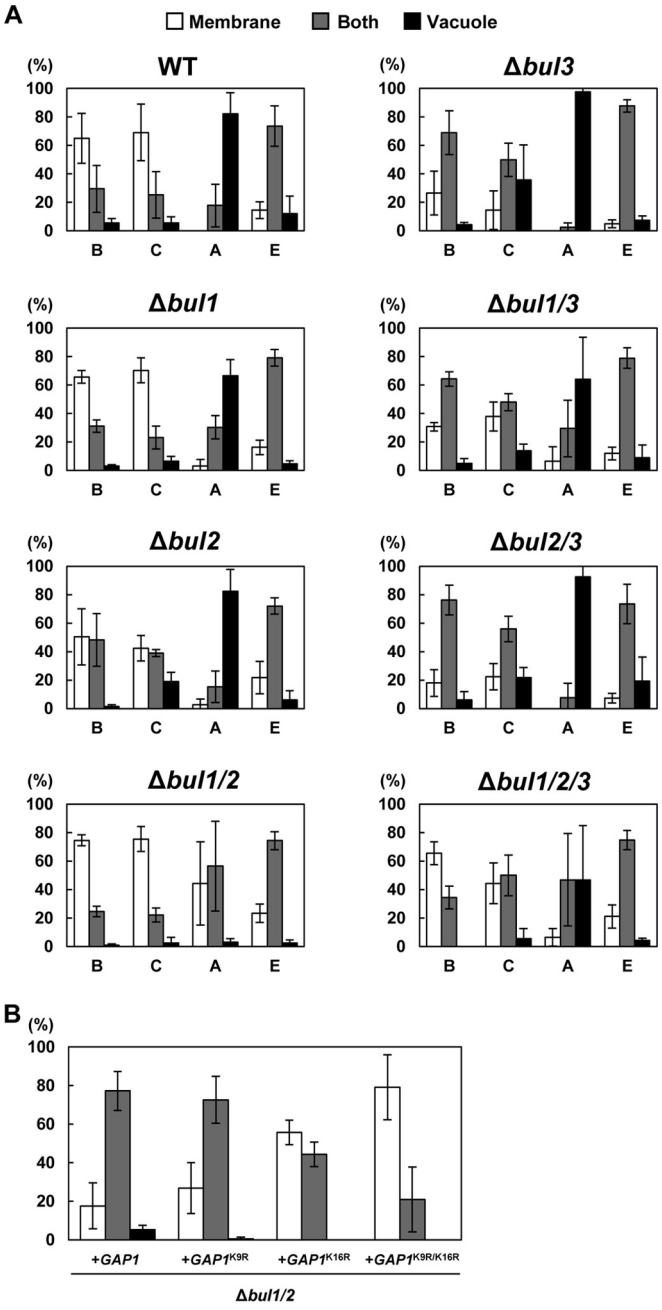

FIG 4 Effect of Bul proteins on trafficking of Gap1p under ethanol stress conditions. (A) *S. cerevisiae* wild-type, Δ*bul1*, Δ*bul2*, Δ*bul1/2*, Δ*bul3*, Δ*bul1/3*, Δ*bul2/3*, or Δ*bul1/2/3* cells harboring pRS416-P$_{GAL1}$-*GAP1*-yEGFP were grown in SG+Alt medium to express Gap1p. After the expression was shut off in SD+Alt medium, the cells were treated for 3 h with or without 15% ethanol. Distilled water and 50 mM $(NH_4)_2SO_4$ were used as the negative and positive controls, respectively. Columns: B, before stress treatment; C, negative control; A, $(NH_4)_2SO_4$; E, ethanol. The values are the means and standard deviations of three independent experiments. (B) *S. cerevisiae* Δ*bul1/2* cells harboring pRS416-P$_{GAL1}$-*GAP1*-yEGFP, pRS416-P$_{GAL1}$-*GAP1*^{K9R}-yEGFP, pRS416-P$_{GAL1}$-*GAP1*^{K16R}-yEGFP, or pRS416-P$_{GAL1}$-*GAP1*$^{K9R/K16R}$-yEGFP were grown in SG+Alt medium. After the expression was shut off in SD+Alt medium, the cells were treated for 4 h with 15% ethanol. For the other conditions and information, please refer to the legend for Fig. 3. The values are the means and standard deviations of three independent experiments.

as Gap1p. Accordingly, we next analyzed endocytosis of other plasma membrane proteins, Agp1p, Tat2p, and Gnp1p, under ethanol stress conditions (Fig. 5). Agp1p is a low-affinity, broad-specificity amino acid permease and is induced by uncharged amino acids, such as tryptophan and phenylalanine (37). Tat2p is a high-affinity tryptophan permease and is transported from the Golgi apparatus to the vacuole or endocytosed from the plasma membrane at a high concentration of tryptophan (38, 39). Gnp1p is a high-affinity glutamine permease and is constitutively transported to the vacuole in yeast cells expressing the hyperactive variant of Rsp5p (Rsp5p^{T357A}) (24, 40). Remarkably, all of the plasma membrane proteins described above were sorted into the vacuoles in response to ethanol. Agp1p was strictly delivered to the vacuole compared to Gap1p, whereas Tat2p and Gnp1p were partially internalized similar to Gap1p (Fig. 4A). These results suggest that yeast cells possess common quality control mechanisms for plasma membrane proteins involving ubiquitination, followed by removal from the plasma membrane via endocytosis under various stress conditions.

DISCUSSION

Many works have attempted to demonstrate the quality control functions of plasma membrane proteins using temperature-sensitive mutants of transporters and receptors, such as Pma1p (41), α-factor receptors (42), the arginine permease Can1p (5), and cystic fibrosis transmembrane conductance regulator CFTR (6). Recently, Apaja et al. (7) constructed CD4 receptor chimeras containing a temperature-sensitive bacteriophage λ domain in their cytoplasmic region, which is a sophisticated system applicable to various plasma membrane proteins. However, these studies assumed that the quality control mechanism for plasma membrane proteins escaped from the ER quality control involving ER-associated degradation (ERAD). In contrast, we hypothesize here that generation of aberrant proteins under various stress conditions such as a high concentration of ethanol and high temperature directly induces their ubiquitination on the plasma membrane by Rsp5p. Our hypothesis includes the mechanisms that occur after ubiquitination: ubiquitinated plasma membrane proteins are endocytosed and subsequently degraded in the vacuoles, whereas ubiquitinated cytosolic proteins are degraded by the ubiquitin/proteasome system (3, 4). To prove this, we first elucidated that Gap1p on the plasma membrane was ubiquitinated and sorted into the vacuoles in the presence of 10 or 15% ethanol (Fig. 1 and 3). It was confirmed that a high concentration of ethanol induced dysfunction of Gap1p on the plasma membrane, using a nonubiquitinated variant of Gap1p (Fig. 2). We particularly focused on ethanol stress in the present study because it is one of the most serious environmental stresses for yeast cells during fermentation processes involved in alcoholic beverages and bioethanol (8–10). During sake brewing or very high gravity fermentation for bioethanol production, yeast cells are exposed to >15% ethanol, in which other microorganisms cannot survive (8–10). It is noteworthy that yeast cells can still respond to 15% ethanol and change the ubiquitination status of Gap1p (Fig. 1).

Our results also showed that similar phenomena occurred in other plasma membrane proteins, Agp1p, Tat2p, and Gnp1p (Fig. 5), suggesting that the ethanol stress-induced quality control is a general mechanism requiring Rsp5p for plasma membrane proteins in *S. cerevisiae*. This is the first report to examine the quality control mechanism of plasma membrane proteins after exposure

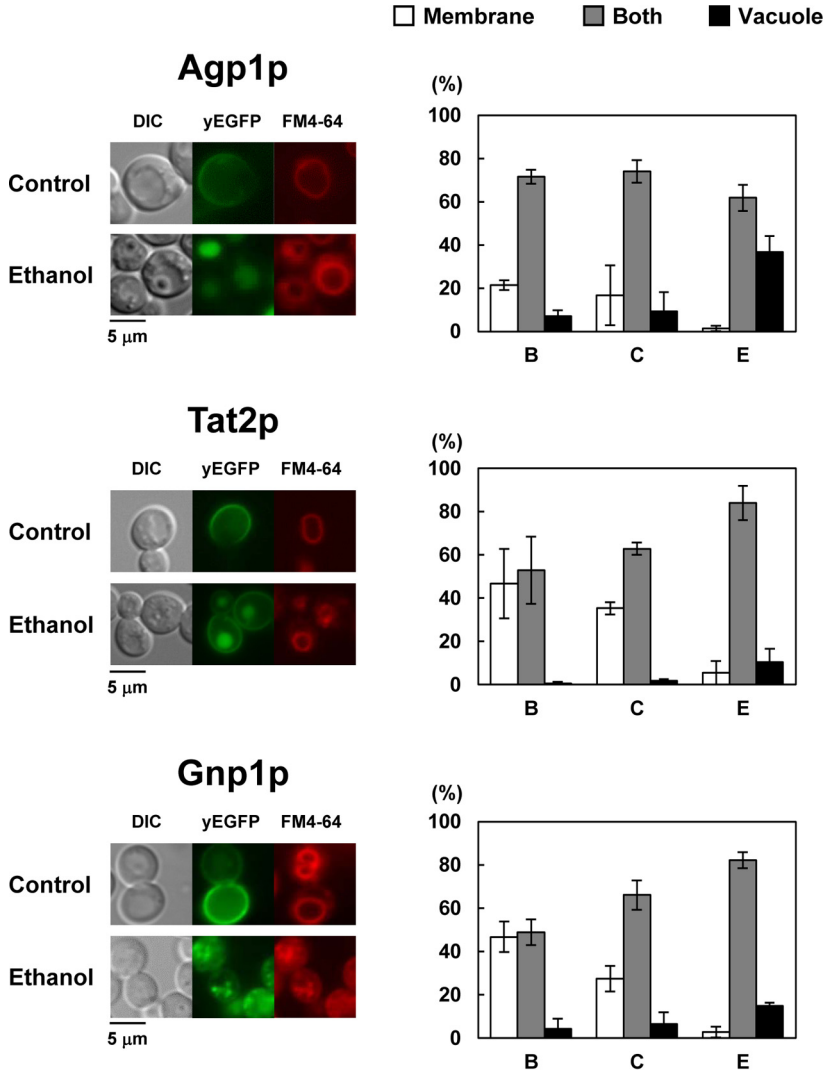

FIG 5 Trafficking of other plasma membrane proteins under ethanol stress conditions. Expression vectors for other plasma membrane proteins—pRS416-P_{GAL1}-*AGP1*-yEGFP, pRS416-P_{GAL1}-*TAT2*-yEGFP, and pRS416-P_{GAL1}-*GNP1*-yEGFP—were constructed by the same procedure as that for Gap1p, and their subcellular localizations were determined. For the other conditions and information, please refer to the legend of Fig. 4. Photographs show the representative of the subcellular localization of each transporter with or without ethanol stress for 3 h. Cell morphology was observed through DIC, and vacuolar membranes were stained with FM4-64. Bar, 5 μm. Columns: B, before stress treatment; C, negative control, E, ethanol. The values are the means and standard deviations of three independent experiments.

to severe ethanol stress, which might in part contribute to the improvement of fermentation properties of *S. cerevisiae*. Recently, Keener and Babst (43) reported quality control of the yeast high-affinity uracil importer Fur4p, which is one of the substrates for Rsp5p. They elucidated that Fur4p was localized into the vacuoles after treatment with not only uracil but also high temperature and H_2O_2. It was suggested that a common mechanism for substrate-induced trafficking and quality control of Fur4p is involved in the loop interaction domain-degron system (43). However, no particular adaptor proteins have been identified for the downregulation of Fur4p.

In addition to ethanol stress, we found that high-temperature, H_2O_2, and LiCl stresses also induced endocytosis of Gap1p (Fig. 3), suggesting that quality control of Gap1p mediated by Rsp5p is a common process regardless of the type of stress. Further study is necessary for elucidation of the molecular mechanisms, including the adaptor proteins required for endocytosis of Gap1p under these stress conditions. Among the stresses tested in the present study, LiCl treatment caused the largest sorting of Gap1p to the vacuoles. It is probable that high temperature and H_2O_2 induce protein denaturation due to their physicochemical properties, but the LiCl-triggered endocytosis of Gap1p might be due to the stress responses to cations such as the *HOG1* and calcineurin pathways (44). Otherwise, based on a previous report that monovalent cations, including Li^+, induce conformational change of glucose oxidase (45), LiCl may directly lead to Gap1p denaturation on the plasma membrane.

Nedd4-family E3 ligases, including Rsp5p, require the adaptor proteins to bind their targets that have no PY motif. Until now, various adaptor proteins, such as arrestin-like Bul proteins and yeast arrestins, have been identified in *S. cerevisiae* (11, 46). It is intriguing to reveal the specificity of these adaptor proteins to-

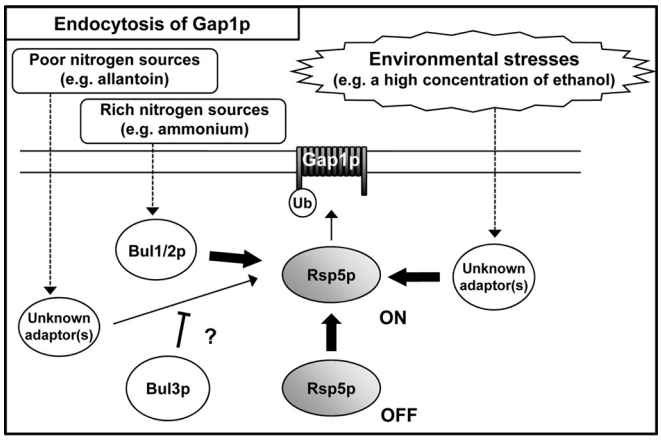

FIG 6 Schematic proposed model for the Gap1p endocytosis in response to environmental signals. In the presence of a poor nitrogen source, such as allantoin, endocytosis of Gap1p occurs at a low level by unknown adaptor protein(s) or is negatively regulated by Bul3p. When a rich nitrogen source, such as $(NH_4)_2SO_4$, is added to the culture medium, Bul1/2p binds to Rsp5p to predominantly accelerate the ubiquitination and the endocytosis of Gap1p. Under such conditions, the effects of unknown adaptor(s) and Bul3p may be too weak to be observed. Only when both Bul1/2p are absent, the endocytosis status of Gap1p may be dependent on the non-Bul adaptor protein(s) and Bul3p. In contrast, when Gap1p suffers from denaturation or dysfunction under environmental stresses, such as a high concentration of ethanol, alternative unknown adaptor protein(s) may be involved in endocytosis of Gap1p in a Bul1/2/3p-independent manner.

ward different environments in the quality control mechanism of plasma membrane proteins. There are known to be two types of Rsp5p-triggered endocytosis of Gap1p, one dependent on and another independent of Bul1/2 (31). The Bul1/2p-dependent process is involved in the trafficking from the Golgi to the vacuole while the MVEs are delivered to the vacuole through Bul1/2p-independent ubiquitination (31). As previously reported, endocytosis of Gap1p from the plasma membrane occurs via both Bul1/2p-dependent and independent pathways when Casamino Acids are supplied to the culture medium (31). Here, we determined the localization patterns of Gap1p using fluorescence microscopy and found evidence suggesting that endocytosis of Gap1p proceeds via the Bul1/2p-dependent pathway when $(NH_4)_2SO_4$ was added to the medium but via the Bul1/2p-independent pathway under ethanol stress conditions, based on the results for the Δ*bul1/2* mutant and the K16 variants of Gap1p (Fig. 4).

Another finding in the present study is that disruption of *BUL3* led to derepression of endocytosis under negative-control conditions and enhancement of Bul1/2p-independent endocytosis observed in Δ*bul1/2* cells (Fig. 4A), suggesting a novel physiological role for Bul3p. Bul3p has been identified as a third member of the Bul family proteins based on structural similarities (35). However, the similarity is rather low, and disruption of the *BUL3* gene did not confer a significant difference in endocytosis of the known Bul1/2p-targeted permeases, such as Smf1p, Gap1p, and Can1p, although the direct interaction of Bul3p with Rsp5p has been observed (35). Given the hypothesis that Bul3p acts as a suppressor of Bul1/2p-independent ubiquitination, we propose here a new model for Gap1p trafficking involving interaction with non-Bul adaptor proteins (Fig. 6). When a poor nitrogen source (e.g., allantoin) is provided or under the conditions lacking both Bul1/2p, endocytosis of Gap1p seems to occur at a very low level. Although its physiological role is still unknown, Bul3p is supposed to inhibit such a Bul1/2p-independent process (Fig. 6). Previously reported interaction between Bul3p and Rsp5p (35) might competitively prevent from interaction with the unknown adaptors. If this model is correct, Bul3p might be identified as a novel negative regulator of Rsp5p-dependent ubiquitination. It is also noted that this inhibitory mechanism does not work under ethanol stress conditions, because disruption of *BUL3* did not significantly affect endocytosis of Gap1p in any of the tested strains (Fig. 4A).

Taken together, we here discovered Bul1/2p-dependent and -independent endocytosis of plasma membrane proteins in response to various stresses, namely, the plasma membrane quality-control mechanism. To further elucidate this, (i) phenotypic analysis of the adaptor protein mutants under multiple stress conditions and (ii) biochemical analysis to identify substrate-specific adaptor protein(s) should be intensively performed. Such studies would illuminate the multifunctionality of yeast Nedd4-like ubiquitin ligase Rsp5p and contribute to understanding how specifically and cooperatively the stress response systems are regulated in eukaryotic cells.

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