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V-ATPase-dependent luminal acidification is required for endocytic recycling of a yeast cell wall stress sensor, Wsc1p

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ABSTRACT

Wsc1p is a major cell wall sensor protein localized at the polarized cell surface. The localization of Wsc1p is maintained by endocytosis and recycling from endosomes back to the cell surface, but changes to the vacuole when cells are subjected to heat stress. Exploiting this unique property of Wsc1p, we screened for yeast single-gene deletion mutants exhibiting defects in Wsc1p trafficking. By expressing 3GFP-tagged Wsc1p in mutants with deleted genes whose function is related to intracellular trafficking, we identified 5 gene groups affecting Wsc1p trafficking, impaired respectively in endocytic internalization, multivesicular body sorting, the GARP complex, endosomal maturation/vacuolar fusion, and V-ATPase. Interestingly, deletion of the *VPH1* gene, encoding the V_o subunit of vacuolar-type H^+-ATPase (V-ATPase), led to mis-localization of Wsc1p from the plasma membrane to the vacuole. In addition, disruption of other V-ATPase subunits (*vma* mutants) also caused defects of Wsc1p trafficking and vacuolar acidification similar to those seen in the *vph1Δ* mutant. Moreover, we found that deletion of the *VPS26* gene, encoding a subunit of the retromer complex, also caused a defect in Wsc1p recycling and mis-localization of Wsc1p to the vacuole. These findings clarified the previously unidentified Wsc1p recycling pathway and requirement of V-ATPase-dependent luminal acidification for Wsc1p recycling.

1. Introduction

Endocytosis and recycling of membrane proteins are important processes regulating various intracellular signaling pathways, such as those responsible for receptor-mediated proliferation, differentiation, and cell wall integrity. Upon internalization from the plasma membrane, cargo proteins are usually delivered to the early endosome, known as the sorting endosome, and sorted to recycling endosomes that bring the cargo back to the plasma membrane, or to late endosomes/multivesicular bodies (MVBs) en route to the lysosome/vacuole for degradation [1,2]. While the role of the recycling endosome in protein recycling has been extensively clarified, another route that involves retrograde transport from the endosome to the trans-Golgi network (TGN), resulting in access to the secretory pathways, has also been characterized in recent years [3].

Cell wall integrity (CWI) signaling is known to be regulated by constitutive endocytosis and recycling of cell wall sensor proteins that detect and transmit information on cell wall status to the Rho1-mediated CWI signaling pathway [4]. In yeast, the cell wall sensor proteins include Wsc1p with the homologous proteins Wsc2-4p and Mid2p, and its homologue Mtl1p [4]. Wsc1p is normally localized to the polarized cell surface at 24 °C, but after cell wall stress imposed by heat shock (shift to 37 °C), the principal site of Wsc1p localization changes from the plasma membrane to vacuoles to down-regulate the CWI signaling pathway [4,5]. Deletion of *SLA1*, or expression of a mutant form of Sla1p lacking the SHD1 domain, which acts as an adaptor for the $NPFX_{(1,2)}D$ endocytic targeting signal, blocks Wsc1p internalization and results in defective polarized deposition of the cell wall with increased sensitivity to perturbation of cell wall synthesis [5]. These findings show that $NPFX_{(1,2)}D$-mediated endocytosis is responsible for directing Wsc1p into an endocytosis and recycling pathway necessary for maintaining cell wall polarity in yeast. However, the mechanism of temperature-regulated Wsc1p sorting is little understood.

Abbreviations: VPS, vacuolar protein sorting; PVC, prevacuolar compartment; MVB, multivesicular body; ESCRT, endosomal sorting complex required for transport; GARP, Golgi-associated retrograde protein.

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Vacuolar-type H^+-ATPase (V-ATPase) is a highly conserved proton pump responsible for acidification of intracellular organelles, such as the lysosome/vacuole, Golgi apparatus, and endosomes, and works together with other ion channels and transporters to maintain pH homeostasis in all eukaryotic cells [6,7]. Eukaryotic V-ATPase is a multi-subunit complex composed of an integral membrane protein, V_o, and a peripheral membrane protein, V_1. Deletion of genes encoding any V-ATPase subunit (*vma* mutants) results in a well defined set of growth defects in yeast, including sensitivity to elevated pH and calcium concentration, inability to grow on non-fermentable carbon sources, and sensitivity to a variety of heavy metals [8]. Previous studies have also suggested a requirement for V-ATPase activity in the intracellular protein trafficking pathway, including endocytosis, vacuolar protein sorting and protein recycling [9,10], but the role of V-ATPase in protein trafficking is still largely unclear.

In this study we screened yeast single-gene deletion mutants exhibiting defects in Wsc1p trafficking and identified the *VPH1* gene, encoding the V_o subunit of vacuolar-type H^+-ATPase (V-ATPase), deletion of which leads to mis-localization of Wsc1p from the plasma membrane to the vacuole. Other V-ATPase gene mutants (*vma* mutants) also exhibited a phenotype similar to that of the *vph1Δ* mutant. We further demonstrate the functional interaction between the retromer complex and V-ATPase-dependent luminal acidification in the Wsc1p recycling pathway.

2. Materials and methods

2.1. Yeast strains and growth conditions

The yeast strains used in this study are listed in Supplementary Table 2. All strains were grown in standard rich medium (YPD) or synthetic medium (SM) supplemented with 2% glucose and appropriate amino acids.

2.2. Plasmids and strain construction

The triple GFP was integrated at the C terminus of the *WSC1* gene as follows: The 3GFP fragment was subcloned into BamHI- and NotI-digested pBlueScript II SK (pBS-3GFP), and the HIS3MX6 was amplified by PCR using pFA6a-GFP (S65T)-HIS3MX6 as a template, and inserted into the NotI and SacII sites of pBS-3GFP to give pBS-3GFP-HIS3 (pBS-3GFP-HIS3). To create an integration plasmid, a fragment of the *WSC1* ORF (nt 721-1134) was generated by PCR and cloned into the BamHI site of pBS-3GFP-HIS3. To integrate 3GFP at the C-terminus of the *WSC1* gene, the integration plasmid was linearized by ApaI and transformed into yeast. GFP, mCherry, and pHluorin were introduced by standard PCR-based method.

2.3. Fluorescence labeling of α-factor and endocytosis assays

Fluorescence labeling of α-factor was performed as described previously [11]. For endocytosis assays, cells were grown to an OD600 of ~0.5 in 0.5 ml YPD, briefly centrifuged, resuspended in 100 μl SM containing 2% glucose and amino acids, and then added 5 μM Alexa Fluor 594-α-factor. After incubation for 20 min at 25 °C, the cells were washed three time with SM, resuspended in 20 μl SM containing 2% glucose and amino acids and observed by microscopy.

2.4. Fluorescence microscopy

Fluorescence microscopy was performed using an Olympus IX81 microscope equipped with a ×100/NA 1.40 (Olympus) objective and Orca-AG cooled CCD camera (Hamamatsu), using Metamorph software (Universal Imaging).

3. Results

3.1. Screening for yeast mutants defective in Wsc1p trafficking

Wsc1p is a cell wall stress sensor and a putative upstream regulator of the CWI signaling pathway [4]. At 24 °C, Wsc1p is localized primarily at the plasma membrane, but changes its localization to the vacuole when the cell wall is subjected to heat shock stress (a shift to 37 °C) [4,5]. To perform in-depth characterization of Wsc1p localization, we tagged endogenous Wsc1p with three tandem copies of GFP (3GFP) at the C terminus, and examined their localization in living cells. Consistent with prior studies using Wsc1-GFP [5], Wsc1-3GFP displayed a mostly polarized cell surface distribution along with some intracellular localization at 25 °C, and changed its distribution to the vacuole upon a temperature shift to 37 °C (Fig. 1A) [5]. To identify proteins required for Wsc1p trafficking, we extracted 251 genes that were possibly related to intracellular protein trafficking from the *Saccharomyces* Genome Database, and expressed Wsc1-3GFP in cells lacking each of the individual genes (Supplemental Table 1). Each of the mutant cells expressing Wsc1-3GFP was grown to early logarithmic phase in YPD medium at 25 °C, exposed or unexposed to heat shock at 37 °C for 2 h, and then studied for Wsc1p localization using epifluorescence and differential interference contrast (DIC) microscopy. This screening identified 24 mutants that exhibited differences in Wsc1-3GFP localization from wild-type cells at 25 and/or 37 °C. These genes were categorized into at least five groups (Table 1). Six of these genes (Group A in Table 1) – *END3*, *RVS161*, *SAC6*, *SLA1*, *SLA2*, and *VRP1* – encoded proteins that have been implicated in endocytic internalization [12]. In cells depleted of these genes, Wsc1-3GFP was localized to the plasma membrane at both 25 and 37 °C (Fig. 1B and Table 1), suggesting defective internalization of Wsc1p in these strains. Another six genes (Group B in Table 1) – *VPS20*, *VPS25*, *VPS27*, *VPS32*, *VPS36*, and *SNF8* – belonged to the class E *vps* (vacuolar protein sorting) family, one of the *vps* mutant subgroups, which exhibits a modest degree of secretion of newly synthesized carboxypeptidase Y (CPY) [13]. The class E *vps* mutants accumulate an exaggerated endosomal/prevacuolar compartment (class E compartment/PVC) that contains endocytosed markers, such as FM4-64 and Alexa Fluor 594-α-factor (A594-α-factor), as well as vacuolar proteins and Golgi membrane proteins that are unable to recycle back to the Golgi complex [13,14]. Wsc1-3GFP also accumulated in the class E compartments, labeled by A594-α-factor, adjacent to the vacuole in these mutants at both 25 and 37 °C (Figs. 1C, 2A, and Table 1). Three genes (Group C in Table 1) – *VPS51*, *VPS52*, and *VPS53* – encoded subunits of the GARP (Golgi-associated retrograde protein) complex, which is a protein complex involved in the recycling of proteins from endosomes to the late Golgi [15]. In the *vps51Δ*, *vps52Δ*, and *vps53Δ* mutants, Wsc1-3GFP showed punctate localization in the cytosol (Fig. 1D and Table 1). Partial co-localization with mCherry-tagged Pep4p, a vacuolar aspartyl protease, and previous observations indicating that the *vps51Δ*, *vps52Δ*, and *vps53Δ* mutants have fragmented vacuoles suggest that some of the Wsc1p is localized to the fragmented vacuoles (Fig. 2B) [16,17]. In mutants with deletion of eight genes (Group D in Table 1), including *VPS11*, *VPS33*, *VPS41*, *VAM3*, *CCZ1*, and *MON1*, which encode proteins required for endosomal maturation and vacuolar fusion processes [18], Wsc1-3GFP displayed a punctate distribution in the cytosol (Figs. 1E and 2C). Taken together, these results demonstrate that Wsc1p is recycled by the GARP complex-mediated endocytic recycling pathway in which endocytosed proteins

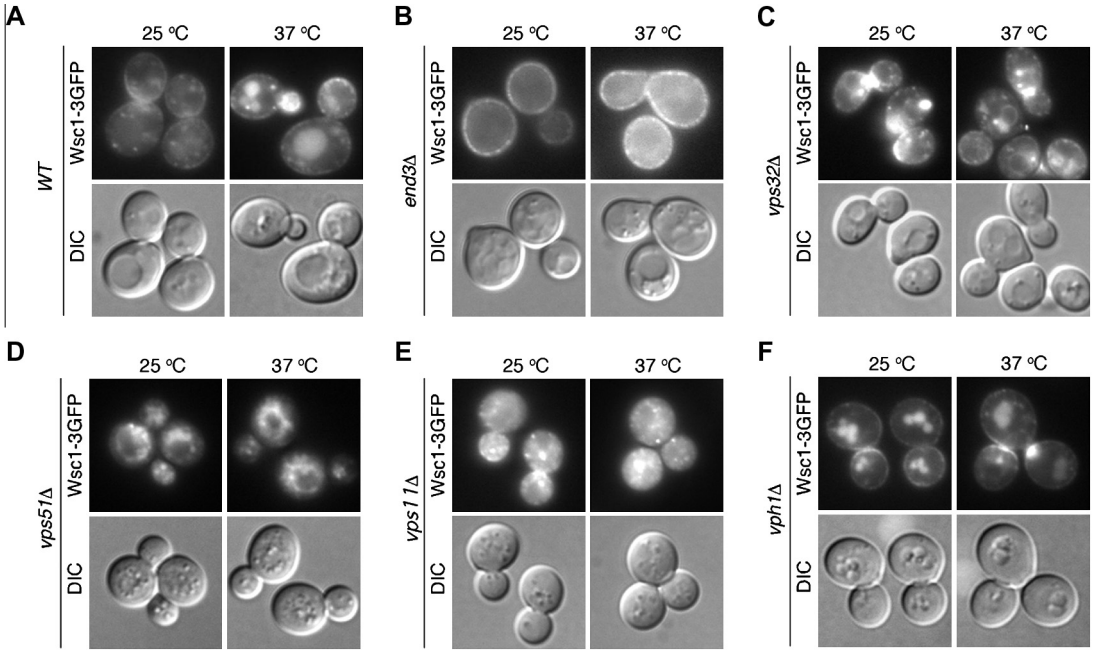

Fig. 1. Different localization of Wsc1-3GFP observed in different mutant cells. (A) Temperature dependent localization of Wsc1-3GFP in wild-type cells. Wild-type cells expressing Wsc1-3GFP were grown to early logarithmic phase in YPD medium at 25 °C, treated with or without heat shock at 37 °C for 2 h, and observed by epifluorescence and differential interference contrast (DIC) microscopy. (B–F) Localization of Wsc1-3GFP in indicated mutant strains. Each mutant cell expressing Wsc1-3GFP were grown and observed as described in (A).

Table 1
Summary of Wsc1-GFP screening.

Group	Gene	Alias	Function (Homology)	Wsc1-3GFP localization
A	*END3*	*YNL084C*	EH-domain Protein	Plasma membrane
	RVS161	*YCR009C*	Amphyphysin	Plasma membrane
	SAC6	*YDR129C*	Fimbrin	Plasma membrane
	SLA1	*YBL007C*	Actin related protein	Plasma membrane
	SLA2	*YNL243W*	Actin related protein	Plasma membrane
	VRP1	*YLR337W*	WASP interacting protein	Plasma membrane
B	*VPS20*	*YMR077C*	ESCRT-III subunit	Class E compartment
	VPS25	*YJR102C*	ESCRT-II subunit	Class E compartment
	VPS27	*YNR006W*	ESCRT-0 subunit	Class E compartment
	VPS32	*YLR025W*	ESCRT-III subunit	Class E compartment
	VPS36	*YLR417W*	ESCRT-II subunit	Class E compartment
	SNF8	*YPL002C*	ESCRT-II subunit	Class E compartment
C	*VPS51*	*YKR020W*	GARP complex subunit	Fragmented vacuoles and punctate structures
	VPS52	*YDR484W*	GARP complex subunit	Fragmented vacuoles and punctate structures
	VPS53	*YJL029C*	GARP complex subunit	Fragmented vacuoles and punctate structures
D	*VPS5*	*YOR069W*	Retromer subunit	Punctate structures
	VPS11	*YMR231W*	CORVET/HOPS subunit	Punctate structures
	VPS33	*YLR396C*	CORVET/HOPS subunit	Punctate structures
	VPS34	*YLR240W*	Phosphatidylinositol 3-kinase	Punctate structures
	VPS41	*YDR080W*	HOPS subunit	Punctate structures
	CCZ1	*YBR131W*	Ypt7p GEF	Punctate structures
	MON1	*YGL124C*	Ypt7p GEF	Punctate structures
	VAM3	*YOR106W*	Vacuolar t-SNARE	Punctate structures
E	*VPH1*	*YOR270C*	V-ATPase V_o subunit	Vacuole

are transported to late endosomal/prevacuolar compartments and recycled backed to the cell surface through the *trans*-Golgi compartment.

Another gene, *VPH1*, encodes a subunit of yeast V-ATPase that is a multi-subunit complex composed of two domains: a peripheral V_1 domain containing the ATPase activity, and a membrane-bound V_o domain responsible for translocation of protons across the membrane [6]. In the *vph1Δ* mutant, Wsc1-3GFP exhibited vacuolar localization at both 25 and 37 °C (Fig. 1F and Table 1). To clarify whether decreased localization of Wsc1p at the plasma membrane in *vph1Δ* cells is caused by a defect of recycling or simply by defective transport from the TGN to the plasma membrane, we inhibited endocytic internalization by deleting the *END3* gene [12], and then examined the localization of Wsc1p. As shown in Fig. 2D, most of the Wsc1p was localized to the plasma membrane, and localization to the vacuole was significantly decreased. This result supports the idea that Wsc1p can reach the plasma membrane, but is not efficiently recycled in *vph1Δ* cells.

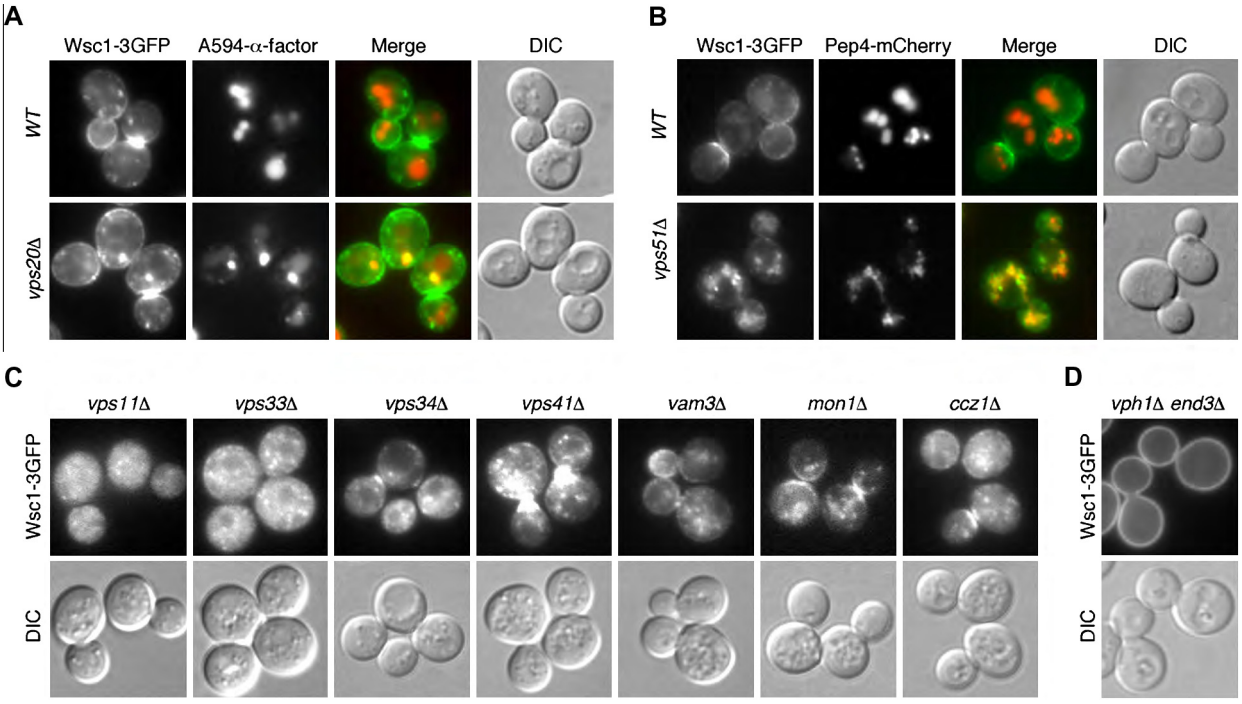

Fig. 2. Localization of Wsc1-3GFP in mutant cells. (A) Localization of Wsc1-3GFP in Group B mutants. Cells expressing Wsc1-3GFP were labeled with A594-α-factor as described in Section 2. Images were acquired at 20 min after washing out unbound A594-α-factor and warming the cells to 25 °C. (B) Localization of Wsc1-3GFP and Pep4-mCherry in Group C mutants. Cells expressing Wsc1-3GFP and Pep4-mCherry were grown to early to mid-logarithmic phase in YPD medium at 25 °C and observed by fluorescence microscopy. (C) Localization of Wsc1-3GFP in Group D mutants. (D) Localization of Wsc1-3GFP in *vph1Δ end3Δ* cells.

3.2. Effects of disruption of V-ATPase subunits on the protein recycling

Vph1p is an approximately 100-kDa membrane (V_o) subunit (subunit a) of yeast vacuolar ATPase (V-ATPase) [6]. Yeast V-ATPase is composed of 14 subunits, including a catalytic V_1 domain of peripherally associated proteins (Vma1, 2, 4, 5, 7, 8, 10, and 13) and a proton-translocating V_o domain of integral membrane proteins (Vma3, 6, 9, 11, 16, and Vph1p) [6]. Genes encoding vacuolar V-ATPase subunits are called *VMA* (vacuolar membrane ATPase) genes. It is reported that disruption of any of these subunit genes results in a very similar pH-dependent, calcium-sensitive growth phenotype [6]. To examine whether Wsc1p recycling is also impaired by deletion of genes encoding other subunits of V-ATPase, we expressed Wsc1-3GFP in cells depleted of several V-ATPase subunits (Vma2p, subunit B; Vma7p, subunit F; Vma3p, subunit c; Vma6p, subunit d) and observed its localization at 25 °C. Similarly to the *vph1Δ* mutant, in these *vma* mutants, the Wsc1-3GFP signal was observed predominantly in the vacuole (Fig. 3A and D). We next determined whether deletion of *VMA* genes would result in loss of vacuolar acidification by observing the fluorescence of a pH-sensitive GFP variant, pHluorin, whose fluorescence is quenched at pH values below 6.0 [19]. We fused pHluorin at the C terminus of Ste2p, a yeast pheromone receptor, which is constitutively internalized, incorporated into MVBs, and transported to the vacuole lumen [20], and observed the fluorescence in the *vph1* and *vma* mutants. While the fluorescence of Ste2-pHluorin was almost invisible in the vacuole of wild-type cells, the *vph1* and *vma* mutants exhibited strong fluorescence in their vacuoles (Fig. 3B), indicating defective vacuolar acidification of these mutants. To further address the functional requirement of vacuolar V-ATPase in the protein recycling pathway, we examined the effect of *vma* mutants on trafficking of another recycling marker, GFP-Snc1, an exocytic v-SNARE that is endocytosed, transiently localized to early endosomes, and recycled back to the plasma membrane via the *trans*-Golgi compartment [21]. It has been shown that mutations affecting endosome-mediated trafficking often cause mis-localization of Snc1p from the plasma membrane to endosomal or vacuolar compartments [21]. Intriguingly, in the *vph1* and *vma* mutants, localization of GFP-Snc1 was partly shifted to the vacuole, in contrast to its predominant localization at the plasma membrane in wild-type cells (Fig. 3C and E).

3.3. Retromer deletion leads to mis-sorting of Wsc1p to different organelles

In the screening performed in this study, we also identified the *VPS5* gene, which encodes a subunit of the retromer complex, as a Group D gene (Fig. 4A and Table 1). The retromer is a heteropentameric complex composed of a membrane-associated sorting nexin dimer (Vps5p, Vps17p) that binds to phosphatidylinositol 3-phosphate-enriched endosomal membranes and a trimeric Vps29-Vps35-Vps26 sub-complex that participates in cargo recognition [3]. Since the retromer complex is known to be important for the retrograde transport of recycling cargos from endosomes to the TGN, we next examined whether Wsc1p recycling is also impaired by deletion of each retromer subunit. In the *vps5Δ* and *vps17Δ* mutants (data not shown), Wsc1p showed similar localization to Group D mutants, displaying a punctate distribution in the cytosol (Fig. 4A, *left panels*). In contrast, the *vps29Δ* and *vps35Δ* mutants (data not shown) exhibited localization of Wsc1p in the prevacuolar endosomal compartments (Fig. 4A, *middle panels*). Interestingly, deletion of the *VPS26* gene led to mis-localization of Wsc1p from the plasma membrane to the vacuole at 25 °C, similarly to *vma* mutants (Fig. 4A, *right panels*). The phenotypic similarity observed in *vma* and *vps26* mutants motivated us to further examine the functional interaction between the Vps26p and V-ATPase. We first examined the localization of Vps26p in V-ATPase-defective mutants, but the localization was not affected in *vma2Δ* and *vph1Δ* cells (Fig. 4B). We

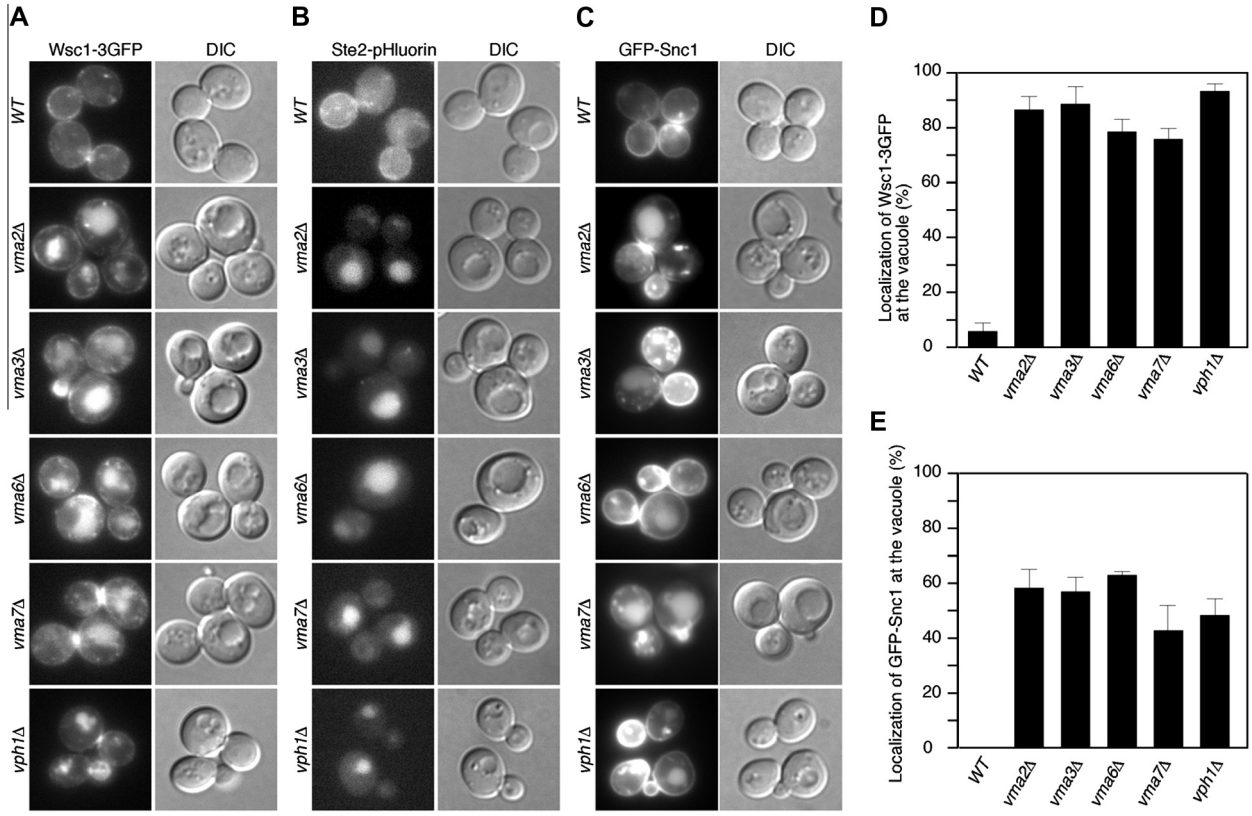

Fig. 3. Effects of *vma* mutants on the protein recycling. (A) Localization of Wsc1-3GFP at 25 °C in wild-type and *vma* mutant cells. Wild-type or each mutant cells expressing Wsc1-3GFP were grown to early logarithmic phase in YPD medium at 25 °C and observed by epifluorescence and differential interference contrast (DIC) microscopy. (B) Wild-type and *vma* mutant cells expressing Ste2-pHluorin were grown to early logarithmic phase in YPD medium at 25 °C and observed as described in (A). The pHluorin is sensitive in pH and loses its fluorescence when transported to the acidic vacuole. All images were captured under identical exposure conditions. (C) Cells expressing GFP-Snc1 were grown to early logarithmic phase in YPD medium at 25 °C, and observed by epifluorescence and differential interference contrast (DIC) microscopy. (D, E) Quantification of Wsc1-3GFP (D) or GFP-Snc1 (E) localizing at the vacuole in wild-type and mutant cells. Error bars indicated the standard deviation (SD) from at least three experiments.

next overexpressed the *VPS26, VPS29,* or *VPS35* gene, encoding the retromer complex subunit, in *vma1Δ, vma3Δ,* or *vma5Δ* cells, but no recovery of Wsc1p localization in these cells was observed (Supplementary Fig. 1). Previous comprehensive studies of genetic interactions revealed that *VPS26* and other retromer genes exhibit multiple interactions with genes encoding V-ATPase proteins (Fig. 4C) [22]. Additionally, we found that the *vma3Δ* or *vma10Δ* mutant exhibits a slightly additive growth defect when combined with the *vps26Δ* mutant, relative to each single mutant alone (Fig. 4D). To compare effect of these mutations on Wsc1p localization more detail, we categorized the Wsc1p localization into three classes; vacuole only, vacuole and the plasma membrane, or the plasma membrane only. Quantitative analysis revealed that a combination of the *vps26Δ* and *vma3Δ* mutations facilitates vacuolar localization of Wsc1p (Fig. 4E and F), suggesting that V-ATPase-dependent luminal acidification might be related to the function of Vps26p, which recognizes and recycles cargos from the endosome to the TGN. We also examined whether a known suppressor of the *VPS26* mutant is able to reverse Wsc1p mis-localization in the *vps26Δ* or *vma3Δ* mutant. A previous study reported that the *VPS26* gene has positive interaction with the *GLO3* gene, encoding an Arf GTPase-activating protein, during protein folding in the endoplasmic reticulum [23]. Interestingly, deletion of the *GLO3* gene markedly suppressed the mis-localization of Wsc1p in the *vps26Δ* mutant (Fig. 4E and F), although no apparent recovery of Wsc1p localization was observed in the *vma3Δ glo3Δ* double mutant (Fig. 4E and F). This result appears to suggest a potential role of Glo3p in the retromer-mediated protein recycling pathway.

4. Discussion

In this study we used a 3GFP-tagged Wsc1p to probe the pathway of recycling from the plasma membrane and the sorting events that are involved. By expressing Wsc1-3GFP in mutants null for various genes related to intracellular trafficking, we found 24 mutants that were categorized into at least five groups. Group A included mutants that are known to block endocytic internalization (Fig. 4G) [12]. In agreement with previous observation [5], Wsc1-3GFP was primarily localized in a depolarized manner at the plasma membrane in each endocytic mutant, suggesting the importance of endocytosis in Wsc1p trafficking. Group B included the class E *vps* mutants that are characterized by formation of the class E compartment [13]. Piao et al. also reported that Wsc1-3GFP accumulates in the class E compartment present in *vps27Δ* cells [5]. Accumulation of Wsc1p in the class E compartment suggests that these mutants have defects in Wsc1p trafficking out of the PVC (Fig. 4G). Group C included genes encoding subunits of the GARP complex, Vps51p, Vps52p, and Vps53p, which might be required for recycling of Wsc1p from endosomes to the late Golgi (Fig. 4G). In *vps51Δ*, *vps52Δ,* and *vps53Δ* mutants, Wsc1-3GFP seems to be localized to fragmented vacuoles and cytoplasmic punctate dots. Since the GARP complex mediates tethering and fusion of endosome-derived vesicles to the trans-Golgi network, these cytoplasmic dots may represent vesicles that cannot fuse with the *trans*-Golgi network; in these mutants, retrieval of Wsc1-3GFP to the Golgi complex might be blocked, resulting in its delivery to the vacuole via vesicular intermediates (Fig. 4G). Wsc1p recycling was also interrupted in cells depleted of Vps11p

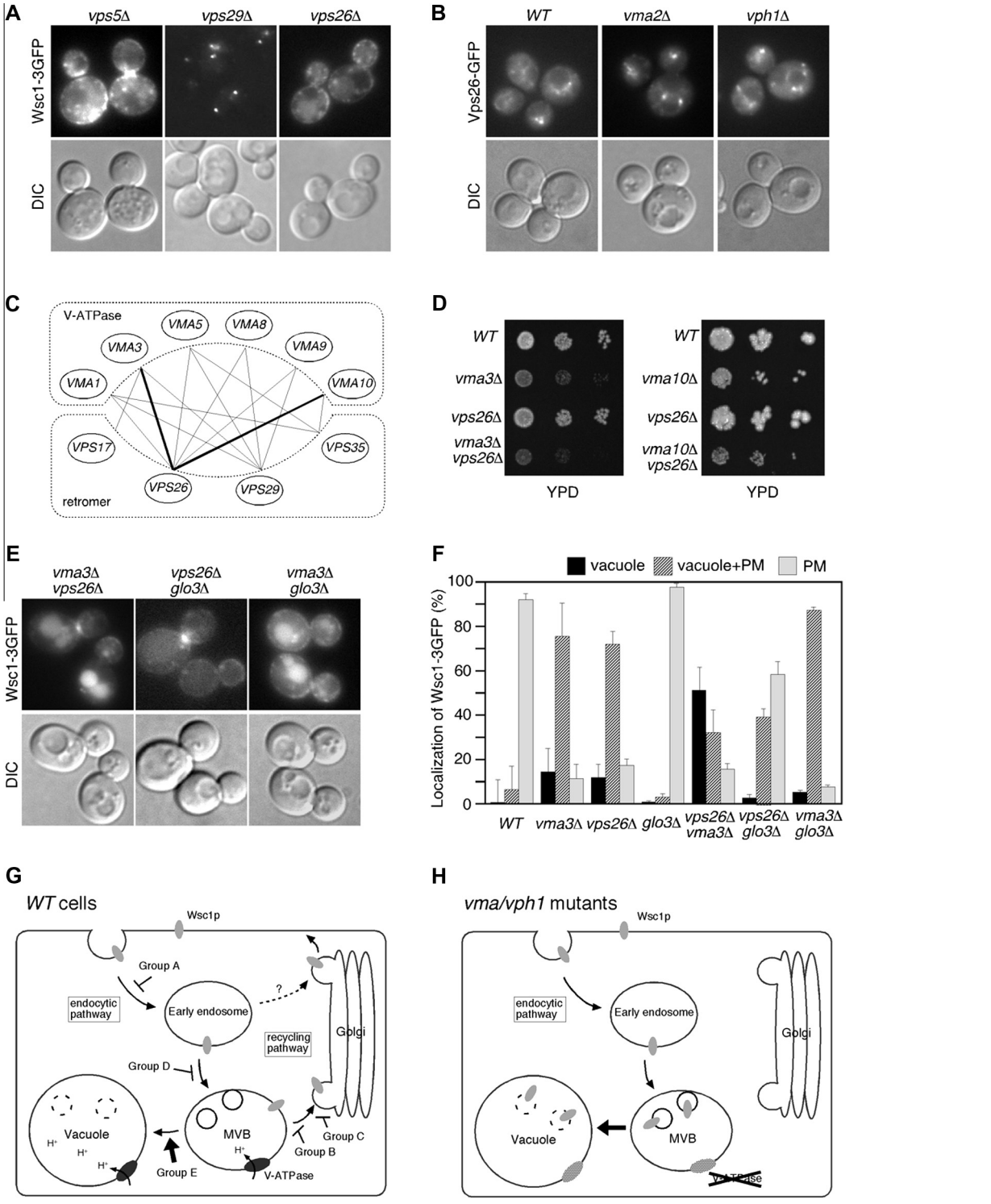

Fig. 4. Localization of Wsc1-3GFP in the retromer mutants. (A, B) Localization of Wsc1-3GFP (A) or Vps26-GFP (B) in indicated mutant strains. Each mutant cell expressing Wsc1-3GFP were grown to early logarithmic phase in YPD medium at 25 °C, and observed by fluorescence microscopy. (C) Genetic interaction map between the V-ATPase subunit genes and the retromer subunit genes. Thick lines show genetic interaction identified in this study. (D) *vma3Δ* or *vma10Δ* mutant exhibits an additive growth defect when combined with *vps26Δ* mutant. A dilution series of cells were plated on YPD medium and incubated at 25 °C. (E) Effect of double mutant on Wsc1p localization. Cells expressing Wsc1-3GFP were grown to early logarithmic phase in YPD medium at 25 °C and observed by epifluorescence and DIC microscopy. (F) Localization of Wsc1-3GFP was categorized into three classes; vacuole only, vacuole and the plasma membrane, or the plasma membrane only. Data are means ± SD from at least three experiments, with >50 cells counted for each strain per experiment. (G, H) Model for role of yeast V-ATPase in Wsc1p recycling. In wild-type cells, Wsc1-3GFP is endocytosed to early endosome, transported MVB, and recycled to the plasma membrane through the Golgi (G). In *vma*/*vhp1* mutants, Wsc1p is probably missorted into vesicles in the MVB, resulting in vacuolar localization (H).

or Vps33p, subunits of the CORVET/HOPS complex, or Vps41p, a subunit of the HOPS complex. Recent studies have demonstrated the importance of the CORVET and HOPS complexes in endosomal maturation and vacuolar fusion [18]. Thus, it seems reasonable to consider that Wsc1p is transported to the TGN after maturation of the early endosome to the late endosome (Fig. 4G). These results,

taken together with previous observations, have clarified the previously unidentified Wsc1p recycling pathway, in which endocytosed Wsc1p is transported to the late endosome/MVB, sorted at the MVB, transported to the TGN mediated by the GARP complex, and finally recycled back to the cell surface from the TGN (Fig. 4G).

We found that disruption of V-ATPase subunits caused a defect in Wsc1p recycling and mis-localization of Wsc1p to the vacuole (Fig. 4H). Thus, V-ATPase-dependent luminal acidification seems to be critical for cargo sorting at early-to-late endosomes. We also demonstrated that Wsc1-3GFP accumulates in the prevacuolar endosomal compartments or vacuole in the retromer mutants, indicating that the retromer complex is required for Wsc1p recycling. This finding is coincident with previous observations that membrane proteins, transported from the prevacuolar endosomal compartment to the TGN by the retromer complex, become mislocalized to the late endosome or lysosome when lacking their retromer recognition motifs [24,25]. Interestingly, the localization of Wsc1-3GFP was affected differently in each of the retromer mutant cell types. Among the 5 retromer components, only *vps26Δ* mutant cells clearly demonstrated vacuolar localization of Wsc1-3GFP. The *vps5Δ* and *vps17Δ* mutants have been classified into class B *vps* mutants that display fragmented vacuoles, and thus, Wsc1p might reside in the PVCs of fragmented vacuoles in these mutants [13]. In *vps29Δ* and *vps35Δ* cells, Wsc1-3GFP was localized to the late endosomal compartments, distinctly from the Class E compartment. These observations suggest that Wsc1p recycling is disrupted in the endosomal compartment of the retromer mutants. Further study will be required in order to clarify these differences observed among retromer mutant cells. In addition, the next important issue for study is whether loss of V-ATPase-dependent luminal acidification affects recognition of Wsc1p by the retromer complex in the endosomal compartment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.008.

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