



HOKKAIDO UNIVERSITY

Title	Signaling Events of the Rim101 Pathway Occur at the Plasma Membrane in a Ubiquitination-Dependent Manner
Author(s)	Obara, Keisuke; Kihara, Akio
Citation	Molecular and Cellular Biology, 34(18), 3525-3534 https://doi.org/10.1128/MCB.00408-14
Issue Date	2014-09-15
Doc URL	https://hdl.handle.net/2115/57670
Type	journal article
File Information	Obara_MCB_2014.pdf



**Signaling events of the Rim101 pathway occur at the plasma
membrane in a ubiquitination-dependent manner**

Keisuke Obara and Akio Kihara[#]

Laboratory of Biochemistry, Faculty of Pharmaceutical Sciences, Hokkaido
University, Sapporo, Japan

Running Head: Rim101 signaling at the plasma membrane

[#]Address correspondence to Akio Kihara, kihara@pharm.hokudai.ac.jp.

Word count for Materials and Method: 657 words

Word count for the rest of the text: 4,288 words

16 ABSTRACT

17 In yeast, external alkalization and alteration in plasma membrane lipid asymmetry
18 are sensed by the Rim101 pathway. It is currently under debate whether the signal
19 elicited by external alkalization is transduced to downstream molecules at the plasma
20 membrane or *via* endocytosis of the sensor protein Rim21 at the late endosome. We
21 found that the downstream molecules, including the arrestin-related protein Rim8,
22 calpain-like protein Rim13, and scaffold protein Rim20, accumulated at the plasma
23 membrane upon external alkalization, and the accumulation was dependent on
24 Rim21. Snf7, an ESCRT III subunit also essential for the Rim101 pathway, localized
25 to the plasma membrane, in addition to the late endosome, under alkaline conditions.
26 Snf7 at the plasma membrane, but not at the late endosome, was shown to be
27 involved in Rim101 signaling. In addition, the Rim101 pathway was normally
28 activated, even when endocytosis was severely impaired. Considering this
29 information on whole, we propose that Rim101 signaling proceeds at the plasma
30 membrane. We also found that activity of the ubiquitin ligase Rsp5 was required for
31 recruiting the downstream molecules to the plasma membrane, suggesting that
32 ubiquitination mediates Rim101 signaling at the plasma membrane.

33

33 INTRODUCTION

34 Adaptation to external pH is vital for natural growth and for the pathogenicity of
35 certain microorganisms, so understanding the mechanisms for such adaptation is
36 important in biology, medicine, and agriculture. In the filamentous fungi *Aspergillus*
37 *nidulans* and the budding yeast *Saccharomyces cerevisiae*, alkalization of the
38 external environment is sensed by the Rim101 pathway (1-3). This pathway regulates
39 the expression of alkaline-responsive genes that encode secreted enzymes, permeases,
40 and proteins involved in intracellular pH homeostasis, which allows adaptation to the
41 alkaline conditions (4-7). In the Rim101 pathway, a sensor complex composed of the
42 integral membrane proteins Rim21, Dfg16, and Rim9 monitors ambient pH (8-10).
43 Rim21 acts as a sensor molecule, while Dfg16 and Rim9 are involved in the
44 stabilization and delivery of Rim21 to the plasma membrane (11). Upon exposure to
45 external alkalization, the sensor complex is endocytosed toward the vacuole and
46 degraded (11).

47 Two possible models have been proposed for the site of Rim101 signaling. One
48 is that the alkaline pH signal is transduced to the late endosome from the plasma
49 membrane *via* endocytosis of the sensor complex (12-14). Theoretically, at the late
50 endosome, downstream molecules are recruited from the cytosol to assemble and
51 form a protein complex that can proteolytically activate the transcription factor
52 Rim101 (13, 14). These downstream molecules include the endosomal sorting
53 complex required for transport (ESCRT) III subunit Snf7, the Bro1 family protein
54 Rim20, and the calpain-like cysteine protease Rim13. Several ESCRT I, II, and III

subunits that usually function at the endosomal membrane are required but ESCRT 0 subunits such as Vps27 are dispensable for Rim101 signaling (15-17). The hypothesis that Rim101 signaling proceeds at the endosome is mainly based on observations of yeast *vps4Δ* cells. Vps4 is an AAA-ATPase involved in the formation of intraluminal vesicles at the late endosome. Vps4 catalyzes the disassembly and membrane release of ESCRT III including its subunit Snf7, so in *vps4Δ* cells ESCRT III subunits accumulate at the late endosome (18). Rim20 also accumulates at the late endosome in *vps4Δ* cells, and the Rim101 pathway is constitutively activated in these cells even without external alkalization (12). Such findings led to the suggestion that Rim20 accumulated at the late endosome functions in Rim101 signaling. However, it is still unknown whether the Rim20 accumulation is a normal process that also occurs in WT cells in response to external alkalization or is just an aberrant event observed in *vps4Δ* cells.

The other model is that Rim101 signaling proceeds at the plasma membrane by recruiting downstream molecules to the plasma membrane. A recent study in *A. nidulans* showed that Vps23, an ESCRT I subunit involved in Rim101 signaling, and PalC, a Rim20 homolog, are recruited to the plasma membrane upon external alkalization (19). It is also reported in *S. cerevisiae* that overexpressed Rim8, an arrestin-related protein essential for Rim101 signaling, accumulates at the plasma membrane (20). In this situation, Vps23 is recruited to the plasma membrane in a Rim8-dependent manner. These studies cast doubt on the model that Rim101 signaling proceeds at the late endosome. However, localization of Rim8 and Vps23

in *S. cerevisiae* was analyzed in cells overexpressing both from high-copy plasmids, under conditions in which the Rim101 pathway is not activated (20). Thus, the essential need still remains to analyze their localization in native conditions, *i.e.*, in WT cells at endogenously expressed levels and under conditions in which the Rim101 pathway is activated. Moreover, it is still unclear whether endocytosis of the sensor complex is required for mediating the signal to downstream molecules or if its sole purpose is turnover of the sensor complex after transducing the signal.

Rim8 is known to be ubiquitinated, probably by Rsp5, a Nedd4-type ubiquitin ligase (20). However, ubiquitination of Rim8 is not essential for the Rim101 pathway, and the levels of ubiquitinated Rim8 do not change before and after external alkalization (20). Therefore, whether ubiquitination is essential for Rim101 signaling is still a matter of debate.

In the plasma membrane lipid bilayer, lipid asymmetry is maintained by energy-consuming inward and outward movement of lipid molecules, termed flip and flop, respectively. Yeast cells lacking all enzymes responsible for catalyzing phospholipid flip are inviable, indicating that maintenance and proper regulation of lipid asymmetry are essential for cell viability. We previously reported that the Rim101 pathway senses alterations in lipid asymmetry, as well as alkalization of the external environment, through the sensor protein Rim21 (11, 21). How a single pathway can sense both perturbations is currently unknown.

In the work presented here, we carefully monitored the localization of proteins involved in Rim101 signaling, expressed in WT cells from their own promoters at

the original loci, during a response to alkaline conditions. Considering those findings together with other biochemical data presented here, we concluded that Rim101 signaling in *S. cerevisiae* proceeds at the plasma membrane by an ordered recruitment of downstream molecules to the plasma membrane, and not at the late endosome *via* endocytosis of the sensor complex. We also found that ubiquitination of protein(s) other than Rim8 by Rsp5 mediates Rim101 signaling at the plasma membrane by recruiting downstream molecules to the plasma membrane.

106 MATERIALS AND METHODS

107 **Yeast culture and media.** *Saccharomyces cerevisiae* strains used in this study are
108 listed in Table 1. Yeast cells were grown at 30 °C to log phase in synthetic complete
109 (SC) medium (2% D-glucose, 0.67% yeast nitrogen base without amino acids, 0.5%
110 casamino acids, 20 mg/L tryptophan, 20 mg/L uracil, and 20 mg/L adenine-sulfate).
111 Alkaline treatment was performed by adding 1 M Tris-HCl (pH 8.0) to culture
112 medium at a final concentration of 100 mM. A 500 mM stock solution of
113 3-indoleacetic acid (IAA, Nacalai Tesque, Kyoto, Japan) was prepared in ethanol and
114 added to the medium at a final concentration of 500 µM. For the alkaline treatment
115 of the temperature-sensitive *rsp5-1* mutant at a restrictive temperature, *rsp5-1* cells
116 and corresponding WT cells were cultured at 38 °C for 90 min before alkaline
117 treatment.

118
119 **Genetic manipulation.** Gene disruption was performed by replacing the entire
120 coding region of the gene with a marker gene. Chromosome fusion of HA, GFP,
121 2xGFP, mCherry, mRFP, or HA-AID to the relevant gene sequence to label the
122 C-terminus of each protein of interest was performed using PCR-based gene
123 disruption and modification (22). The sequence encoding a respective tag, the *ADHI*
124 termination sequence, and a marker sequence was amplified by PCR from a pFA6a
125 vector series (22) with a primer set containing the homologous region of the target
126 gene. For the chromosomal fusion of AID to the N-terminus, the sequence encoding
127 a marker sequence, *ADHI* promoter, and AID tag was amplified by PCR from a

pCT1 vector (a kind gift from Dr. H. Nakatogawa, Tokyo Institute of Technology, Japan) with a primer set containing the homologous region of the target gene. Amplified cassettes were inserted directly into the chromosome by homologous recombination. Correct integrations of the fusion constructs were confirmed by PCR and immunoblot analysis.

Plasmid construction. To construct the plasmid for the expression of Rim8-HA in yeast cells, a DNA fragment containing the *RIM8* promoter sequence, the *RIM8* coding sequence, the *HA* sequence, and the *ADHI* terminator sequence was amplified by PCR from genomic DNA of YOK2279 (*RIM8-HA*) to have *EcoRI* sites at both 5' and 3' ends. The amplified fragment was cloned into the *EcoRI* site of pRS316 (23) to generate pOK476. The *K521R* mutation was introduced into pOK476 using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) to produce pOK573. To express Rim8-HA or Rim8-HA (K521R) from plasmids containing the *TRP1* marker gene, *EcoRI* fragments of pOK476 and pOK573 were transferred to pRS314 (23), producing pOK574 and pOK576, respectively. The plasmid for the expression of HA-Rim101 (pFI1) was a kind gift from Dr. T. Maeda (University of Tokyo, Japan).

Immunoblot analysis. Proteins were separated by SDS-PAGE and transferred to an ImmobilonTM polyvinylidene difluoride membrane (Millipore, Billerica, MA) as described previously (24). The membrane was incubated with an anti-HA (16B12,

Covance, Princeton, NJ; or TANA2, Medical & Biological Laboratories, Nagoya, Japan), anti-GFP (JL-8, Takara Bio, Shiga, Japan), anti-CPY (Molecular Probes, Eugene, OR), anti-AID (BioROIS, Tokyo, Japan), or anti-Pgk1 (Molecular Probes) antibody. Immunodetection was performed using an ECL plus (GE Healthcare Biosciences, Piscataway, NJ) or Western Lightning ECL Pro system (PerkinElmer Life Sciences, Waltham, MA) with a bioimaging analyzer (LAS4000, Fuji Photo Film, Tokyo, Japan) or X-ray films.

Microscopy. The intracellular localization of GFP-, mRFP-, and mCherry-tagged proteins was monitored using a fluorescence microscope (DM5000B, Leica Microsystems, Wetzlar, Germany). To monitor the progression of endocytosis, cells in log phase were loaded with 1 μ M FM4-64 (Molecular Probes), a lipophilic fluorescent dye, for 15 min, then were washed and resuspended with medium and incubated for an additional 15 min. Sodium azide was added to a final concentration of 20 mM, and cells were kept on ice until subjected to microscopy. For better visualization of Snf7-mRFP signals at the plasma membrane and FM4-64 signals in endocytosis-defective cells (*i.e.*, *end3 Δ* cells and IAA-treated *LAS17-HA-AID* cells), images were digitally enhanced using the “Levels adjustment” command in Photoshop software (Adobe, San Jose, CA) without altering the midtones.

RESULTS

Rim20 accumulates at the plasma membrane in response to external alkalization and alterations in lipid asymmetry. We first analyzed the localization of Rim20 before and after external alkalization by observing Rim20-GFP chromosomally expressed in yeast cells of the SEY6210 background. Fusion of GFP to the C-terminus of Rim20 did not affect the function of Rim20, since normal processing of Rim101 was observed in cells expressing Rim20-GFP that had been exposed to alkali (Fig. S1). Contrary to the proposed model that Rim20 functions at the late endosome, multiple Rim20 foci were formed at the plasma membrane but not at the late endosome, after external alkalization (Fig. 1A). Rim20 foci at the plasma membrane increased in number with prolonged culture in alkaline medium. After the cells were transferred back to SC medium (pH 4.5), the majority of the Rim20 foci disappeared, indicating that they were not dead end structures but were reversible assemblies of Rim20. Importantly, similar Rim20 foci were not observed in *rim21Δ* cells, in which the alkaline sensor Rim21 is absent. These results indicate that accumulation of Rim20 at the plasma membrane is an ordinary event occurring downstream of the alkaline sensor Rim21, and not a non-specific response unrelated to alkaline sensing. We confirmed that Rim20 accumulated at the plasma membrane but not at internal organelles, by analyzing its co-localization with the plasma membrane protein Pma1 (Fig. 1B) and also by immunoelectron microscopy (Fig. S2A). Similar accumulation occurred in yeast cells of other strain backgrounds, namely BY4741 and YPH499 (Figs. S2B and 7), indicating that this is a general

process in yeast and not a strain-dependent unusual process. The Rim20 accumulation was dependent on an arrestin-related protein Rim8 (Fig. 1C), which is considered to bind to Rim21 (16, 20, 25).

The Rim101 pathway senses alterations in lipid asymmetry of the plasma membrane as well as external alkalization. Thus, we monitored Rim20 foci in *lem3Δ* cells, in which lipid asymmetry is disturbed and the Rim101 pathway is activated in normal acidic medium (21). In normal acidic medium, Rim20 foci were formed more readily in *lem3Δ* cells than in WT cells (Fig. 1D), indicating that Rim20 accumulates at the plasma membrane in response to altered lipid asymmetry. The accumulation in *lem3Δ* cells was less prominent than in alkaline-treated WT cells, probably because activation of the Rim101 pathway in *lem3Δ* cells in normal acidic medium is much weaker than in alkali-treated WT cells (11, 21).

Arrestin-related protein Rim8 and calpain-like protein Rim13 accumulate at the plasma membrane upon external alkalization. We next monitored the localization of Rim8 in yeast cells by tagging its C-terminus with 2xGFP by chromosomal fusion and examining the cells microscopically. Before alkaline treatment, Rim8 was mostly dispersed in the cytosol (Fig. 2A). After 20 min of external alkalization, Rim8 significantly accumulated at the plasma membrane (Fig. 2B). Accumulation was transient, and Rim8 foci became less prominent when observed after 60 and 90 min of external alkalization. Similar to the accumulation observed for Rim20, accumulation of Rim8 at the plasma membrane was

213 Rim21-dependent.

214 Localization of Rim13, a calpain-like protein considered to be involved in the
215 cleavage of Rim101, was also monitored. Rim13 was tagged with 2xGFP by
216 chromosomal fusion, expressed in yeast cells, and examined using fluorescence
217 microscopy. Rim13 also accumulated at the plasma membrane upon external
218 alkalization, and the accumulation was dependent on Rim21 (Fig. 2C). As observed
219 with Rim20 but not Rim8, accumulation of Rim13 was not transient but was
220 enhanced by prolonged incubation in the alkaline medium.

221

222 **Snf7 at the plasma membrane plays an essential role in Rim101 signaling.** Snf7
223 is a subunit of the ESCRT-III complex, which is involved in the sorting of
224 transmembrane proteins into the late endosome (18). Snf7 is recruited from the
225 cytoplasm to the endosomal membranes by the sequential actions of ESCRT I and II
226 complexes (18). Interestingly, Snf7 is also required for the Rim101 pathway (17), a
227 fact that supports the possibility that Rim101 signaling proceeds at the late endosome.
228 We analyzed the localization of Snf7 in yeast cells grown under alkaline conditions
229 by labeling the Snf7 on its C-terminus with mRFP using chromosomal fusion and
230 examining the cells microscopically. The processing of Rim101 was found to be
231 normal in *SNF7-mRFP* cells (Fig. S1), while it is totally abolished in *snf7* Δ cells (21),
232 which indicated that the Snf7-mRFP fusion protein retained its normal Rim101
233 signaling function. Consistent with previous reports, a Snf7 signal was detected at
234 the late endosome before external alkalization (Fig. 3A). However, upon external

alkalization, a portion of Snf7 was recruited to the plasma membrane, although the Snf7 signal at the late endosome was still dominant.

With multiple localizations observed for Snf7, it was difficult to clarify at exactly which location Snf7 actually participates in Rim101 signaling. To overcome this difficulty, we deleted *RIM21* and analyzed the localization of Snf7 and activation of the Rim101 pathway. The plasma membrane localization of Snf7 was totally abolished in the *rim21Δ* cells, while its localization at the late endosome was unaffected (Fig. 3B). Under these conditions, the Rim101 pathway was not activated at all, as indicated by absence of processed Rim101 (Fig. 3C). This suggests that recruitment of Snf7 to the plasma membrane is required for Rim101 signaling.

We also analyzed the localization of Snf7 and activation of the Rim101 pathway in cells lacking *VPS27*. Vps27 acts as an initial factor that directly recruits ESCRT I, and therefore indirectly ESCRT II and III, to the late endosome (26, 27). In untreated *vps27Δ* cells, the Snf7 signal at the late endosome was considerably reduced (Fig. 3B). In *vps27Δ* cells exposed to alkaline medium, the Snf7 signal was mostly detected at the plasma membrane and not at the late endosome. However, under these conditions the Rim101 pathway was normally activated (Fig. 3C), which strongly suggests that Snf7 at the late endosome is dispensable for the Rim101 pathway.

We next examined the vacuolar protein sorting pathway in *vps27Δ* and *rim21Δ* cells. In this pathway, soluble vacuolar proteases such as carboxypeptidase Y (CPY) are synthesized in the ER and delivered to the vacuole *via* the Golgi apparatus and

the late endosome. In *vps27Δ* cells, some of the p2 (Golgi) form of CPY was mis-sorted to the extracellular space (Fig. 3D), as others have reported (28). In contrast, in *rim21Δ* cells virtually all of the CPY was detected intracellularly as the mature vacuolar form, similar to results in WT cells. These results suggest that Snf7 at the plasma membrane is dispensable for the vacuolar protein sorting pathway. Taken together, these findings indicate that, under alkaline conditions, Snf7 localizes to the plasma membrane in addition to the late endosome, and that Snf7 at the plasma membrane is involved in the Rim101 pathway.

We subsequently determined co-localization of Rim20 with Snf7, Rim13, or Rim8 at the plasma membrane after external alkalization (Fig. S2C). After 60 min of alkali-treatment, Snf7 was widely spread over the plasma membrane and a part of it co-localized with Rim20. Rim13 also co-localized with Rim20 at the plasma membrane, but the co-localization of Rim8 was not prominent due to its transient localization to the plasma membrane (Fig. 2). Since the physical interaction of these proteins has been previously demonstrated (29), it is conceivable that they indeed function together at the plasma membrane.

Endocytosis is dispensable for the Rim101 pathway. Rim21 is endocytosed and degraded in the vacuole following external alkalization (11). We next investigated whether endocytosis is essential for mediating the signal of external alkalization or just occurs to promote turnover of Rim21 after it has transduced the signal to downstream molecules. To clarify this point, activation of the Rim101 pathway was

examined in cells lacking the *END3* gene, which encodes an EH domain-containing protein involved in endocytosis (30). In *end3Δ* cells, the Rim101 pathway was normally activated upon external alkalization (Fig. 4A), although endocytosis was severely impaired (Fig. S3A). We found endocytosis to be impaired by other conditions as well, *i.e.*, following the transient degradation of Las17, an actin assembly factor involved in endocytosis (31), using the auxin-inducible degron (AID) system. In this system, proteins tagged with the AID tag are specifically polyubiquitinated and degraded after treatment with a phytohormone auxin (32). We found that Las17-HA-AID was degraded (Fig. 4B) by treatment with 3-indoleacetic acid (IAA), a typical auxin, and endocytosis was severely impaired (Fig. S3B). Even under these conditions, the Rim101 pathway was normally activated upon external alkalization (Fig. 4B). These results indicate that endocytosis is not required for the Rim101 signaling.

Activity of a ubiquitin ligase Rsp5 is essential for the Rim101 pathway. The arrestin-like protein Rim8 is known to be ubiquitinated, most likely by the ubiquitin ligase Rsp5 (20). However, introduction of a mutation into the putative ubiquitination site of Rim8 (K521R mutation) does not affect the Rim101 pathway, although ubiquitinated Rim8 is no longer detected (20). This indicates that Rim8 ubiquitination is dispensable for the Rim101 pathway. We confirmed that this variant does not receive ubiquitination and that the Rim101 pathway was not impaired in *rim8Δ* cells expressing this variant (data not shown). However, whether

ubiquitination itself is essential for the Rim101 signaling is still unclear. To examine this point, we employed the temperature-sensitive *rsp5-1* mutant (33). In the *rsp5-1* mutant cultured at the permissive temperature of 30 °C, the Rim101 pathway was normally activated upon external alkalization (Fig. 5A). In contrast, when the *rsp5-1* mutant was initially cultured for 90 min at the restrictive temperature of 38 °C, activation of the Rim101 pathway by alkaline treatment was abolished, indicating that Rsp5 activity is essential for the Rim101 pathway. We also examined the involvement of Rsp5 in the Rim101 pathway using the AID system. Cells expressing AID-Rsp5 were pre-treated with IAA or vehicle and then exposed to alkaline medium. In IAA-treated *AID-RSP5* cells, the Rim101 pathway was severely impaired (Fig. 5B). These results indicate that Rsp5 activity is essential for the Rim101 pathway, which in turn strongly suggests that ubiquitination is involved in Rim101 signaling.

We next investigated if ubiquitination is facilitated when the Rim101 pathway is active. Although ubiquitination of Rim8 is not essential for the Rim101 pathway, changes in the ubiquitination of Rim8 may represent ubiquitination activity of Rsp5 under alkaline conditions. As shown in Fig. 5C, the levels of ubiquitinated Rim8 did not differ between 0 and 20 min of external alkalization. However, in cells lacking the *DOA4* gene encoding a deubiquitination enzyme, ubiquitinated Rim8 accumulated with alkaline treatment, indicating that the ubiquitination-deubiquitination cycle of Rim8 is facilitated in alkaline conditions.

We next investigated the function of Rim8 and its ubiquitination in the Rim101

pathway using the Rim8 K521R variant, which does not undergo ubiquitination (20). First, accumulation of Rim20 at the plasma membrane was examined. In *rim8Δ* cells carrying an empty vector, Rim20 accumulation at the plasma membrane was totally abolished even after external alkalization (Fig. 6A). In contrast, in *rim8Δ* cells expressing the K521R variant, Rim20 foci were induced normally at the plasma membrane upon external alkalization. This observation indicates that the Rim8 protein, but not its ubiquitination, is essential for Rim20 accumulation at the plasma membrane.

Arrestins are often involved in endocytosis of plasma membrane proteins through being ubiquitinated and/or facilitating ubiquitination of the target plasma membrane proteins (34). In the Rim101 pathway, the sensor protein Rim21 is internalized from the plasma membrane and degraded in the vacuole following external alkalization (11). This process can be monitored by detecting decreases in Rim21 levels, *e.g.*, by immunoblotting. As shown in Fig. 6B, Rim21-2xGFP levels were reduced in *rim8Δ* cells expressing either Rim8 or the Rim8-K521R variant after 60 min of exposure to alkaline medium. This indicates that Rim8 ubiquitination is not required for the endocytosis of Rim21. On the other hand, in *rim8Δ* cells carrying an empty vector, Rim21 levels were not reduced, indicating that the Rim8 protein itself is required for Rim21 turnover. At present, it is not clear whether Rim8 is directly involved in the endocytosis of Rim21 or just facilitates Rim21 endocytosis indirectly through progressing Rim101 signaling.

345 **Ubiquitination by Rsp5 mediates Rim101 signaling at the plasma membrane.**

346 We next searched for the process in which Rsp5-mediated ubiquitination is actually
347 involved. We first examined Rim20 accumulation in *rsp5-1* cells. In *rsp5-1* cells
348 cultured at 38 °C, Rim20 did not accumulate at the plasma membrane even after
349 external alkalization (Fig. 7A). Transient degradation of AID-Rsp5 by IAA treatment
350 also severely impaired Rim20 recruitment to the plasma membrane in response to
351 external alkalization (Fig. 7B). These results indicate that Rsp5-mediated
352 ubiquitination is required for the accumulation of Rim20 at the plasma membrane
353 under alkaline conditions. Interestingly, we noticed that, during examination under a
354 fluorescence microscope at room temperature, the formation of Rim20 foci at the
355 plasma membrane was restored in *rsp5-1* cells originally cultured at 38 °C (Fig. S4).
356 The recovery of the Rim20 plasma membrane accumulation was rapid: Rim20
357 accumulation at the plasma membrane was nearly absent at the beginning of the
358 examination yet became prominent (similar to that observed in WT cells) within 150
359 sec of serial observation of the same view. This recovery is presumably due to the
360 restoration of Rsp5 activity during incubation at room temperature. This observation
361 suggests that recruitment of Rim20 to the plasma membrane proceeds rapidly after
362 Rsp5-mediated ubiquitination.

363 We next examined the accumulation of Rim8 at the plasma membrane in
364 response to external alkalization in *AID-RSP5* cells. After 20 min of alkaline
365 treatment, accumulation of Rim8 was observed at the plasma membrane in
366 *AID-RSP5* cells, even after AID-Rsp5 was degraded by IAA treatment (Fig. 7C and

367 D). The accumulation in the IAA-treated *AID-RSP5* cells appeared to be more
368 prominent than in WT cells or mock-treated *AID-RSP5* cells. This is likely because
369 Rim8 expression is negatively regulated by the Rim101 pathway, and impairment of
370 the Rim101 pathway in IAA-treated *AID-RSP5* cells induced Rim8 expression. This
371 observation indicates that Rsp5-mediated ubiquitination is involved in processes
372 other than recruitment of Rim8 to the plasma membrane.
373

373 **DISCUSSION**

374 **Rim101 signaling proceeds at the plasma membrane.** It has been an open question
375 whether Rim101 signaling proceeds at the plasma membrane or at the endosome *via*
376 endocytosis of the sensor protein Rim21. In the work presented here, we carefully
377 analyzed the localization of the downstream molecules, expressed from their own
378 promoters at the original loci, and concluded that Rim101 signaling takes place at the
379 plasma membrane. Evidence that supports our conclusion follows. 1) Proteins that
380 function downstream of the sensor protein Rim21, such as Rim8, Rim20, and Rim13,
381 accumulated at the plasma membrane but not at the late endosome upon external
382 alkalization (Figs. 1 and 2). 2) Alteration in the lipid asymmetry of the plasma
383 membrane, which also stimulates the Rim101 pathway, also led to an accumulation
384 of Rim20 at the plasma membrane (Fig. 1D). 3) Accumulation of the downstream
385 proteins at the plasma membrane was completely dependent on Rim21, indicating
386 that the accumulation is a *bona fide* process occurring downstream of the sensor
387 protein rather than a non-specific process unrelated to Rim101 signaling (Figs. 1 and
388 2). 4) Snf7 also accumulated at the plasma membrane upon external alkalization (Fig.
389 3). Snf7 is a subunit of the ESCRT III complex, which usually functions at the late
390 endosome. Upon external alkalization, a portion of Snf7 was recruited to the plasma
391 membrane (Fig. 3). Importantly, Snf7 at the plasma membrane, but not that at the
392 late endosome, was shown to be involved in Rim101 signaling. 5) The Rim101
393 pathway was normally activated, even when endocytosis was inhibited (Fig. 4). All
394 of these evidences strongly support the contention that Rim101 signaling is mediated
395 at the plasma membrane through recruitment of the downstream molecules to the

plasma membrane, rather than proceeding at the late endosome *via* endocytosis of the sensor protein.

We previously reported that the sensor protein Rim21 is endocytosed and degraded in the vacuole upon external alkalization (11). Given this fact and the results presented here, we consider that Rim21 is internalized for its turnover after transducing the signal to the downstream molecules at the plasma membrane, and not to transduce the signal to the late endosome.

The model stating that Rim101 signaling proceeds at the late endosome is largely based on observations in a *vps4Δ* mutant. In the *vps4Δ* mutant, Rim20 constitutively accumulates at the late endosome, and the Rim101 pathway is activated even without external alkalization (12, 13). These results suggested that downstream molecules accumulated at the late endosome are involved in the Rim101 pathway. Our results indicate that this is not the case for WT cells. Furthermore, we found that in *vps4Δ* cells not exposed to alkaline conditions, a portion of Rim20 and Snf7 accumulated at the plasma membrane in addition to accumulating at the late endosome (Fig. S5), which supports our conclusion that Rim101 signaling occurs at the plasma membrane.

Rim8 transiently localized to the plasma membrane upon external alkalization (Fig. 2). A similar finding was also reported in *C. albicans* (16). Given the fact that Rim8 physically interacts with Vps23, an ESCRT I subunit (20), Rim8 may be directly involved in the recruitment of ESCRT I subunits to the plasma membrane; hence, once the ESCRT I complex has recruited the ESCRT II complex to the

plasma membrane, Rim8 may be dissociate from the plasma membrane together with the ESCRT I complex. Since Rim8 is presumed to bind to Rim21 (15, 16, 20), it is also possible that Rim8 dissociates with Rim21. In contrast, accumulation of Snf7, Rim20, and Rim13 persisted even after 90 min of alkaline exposure, when Rim21 was mostly degraded (11) and the majority of Rim8 had dissociated from the plasma membrane (Fig. 2A). It is known that Snf7 can self-assemble without assistance from other proteins (35). Thus, Snf7 might accumulate continuously and autonomously once an “on” signal emitted from Rim21 was transduced to it, and this in turn might facilitate the persistent recruitment of Rim20 and Rim13. It is worth noting that accumulation of Rim20 was resolved after re-acidification of the medium (Fig. 1A), even when most of the Rim21 had been degraded and Rim8 had dissociated from the plasma membrane. This suggests that some unknown “off” signal is emitted by external acidification and reaches Snf7, probably in a manner independent of Rim21 and Rim8.

Ubiquitination by Rsp5 mediates Rim101 signaling at the plasma membrane.

Rim8 is known to be ubiquitinated. However, ubiquitination of Rim8 is not essential for the Rim101 pathway and the level of ubiquitinated Rim8 does not alter upon external alkalization (20). Thus, whether ubiquitination itself is essential for the Rim101 signaling or not has been in question. Here, we determined, using the level of ubiquitinated-Rim8 as an indicator, that the ubiquitination-deubiquitination cycle is facilitated under alkaline conditions (Fig. 5C). Moreover, we demonstrated that

activity of Rsp5, a NEDD4-type ubiquitin ligase, is essential for the Rim101 pathway (Fig. 5A and B). Thus, it is highly likely that ubiquitination by Rsp5 of some protein(s) other than Rim8 plays an essential role in Rim101 signaling. Ubiquitination of plasma membrane proteins and/or arrestins is often implicated in the endocytosis of the plasma membrane proteins for their degradation (34). We demonstrated that ubiquitination and the arrestin-related protein Rim8 are involved in recruiting downstream molecules to the plasma membrane to mediate Rim101 signaling emitted by the sensor protein Rim21 (Figs. 6 and 7). Thus, our work provides a novel paradigm for the function of ubiquitination and arrestin, *i.e.*, signal transduction at the plasma membrane. An important future step will be to identify the target of Rsp5-mediated ubiquitination.

Rim20 foci localizing at the plasma membrane serves as a novel indicator of the Rim101 pathway. Processed Rim101, which is easily detectable by immunoblotting, has been utilized as a reliable indicator for the activation of the Rim101 pathway (8). However, the proteolytic activation of Rim101 occurs at the final step of Rim101 signaling. To dissect Rim101 signaling into its elementary steps, the use of other indicators may be necessary. For this purpose, the accumulation of Rim8, Rim13, Snf7, and Rim20 at the plasma membrane will be a novel, useful indicator. In particular, the presence of Rim20 foci may be the most convenient indicator, since Rim20-GFP foci are much brighter at the plasma membrane than foci of Rim8-2xGFP, Rim13-2xGFP, or Snf7-mRFP. Using Rim20 foci as an indicator, we

could clarify that Rsp5-mediated ubiquitination of some protein other than Rim8 occurs before, and is a prerequisite for, recruitment of Rim20 to the plasma membrane. For further upstream processes, recruitment of Rim8 to the plasma membrane will be an ideal indicator. With this indicator, we revealed that the Rsp5-mediated ubiquitination of some protein other than Rim8 occurs independently of Rim8 recruitment or after Rim8 is recruited to the plasma membrane (Fig. 7C). Imaging of these proteins in combination with immunoblotting of the processed Rim101 will be convenient assays for monitoring the progression of each elementary step in Rim101 signaling.

ACKNOWLEDGMENTS

We thank Dr. T. Maeda (Institute of Molecular and Cellular Biosciences, the University of Tokyo) for providing the HA-Rim101 plasmid (pFI1), Dr. H. Nakatogawa (Frontier Research Center, Tokyo Institute of Technology) for the template vector for N-terminal tagging of AID tag (pCT1), and Dr. F. Abe (College of Science and Engineering, Aoyama Gakuin University) for the *rsp5-1* strain. The template plasmid for the AID tag was provided by the National Bio-Resource Project (NBRP) of the MEXT, Japan. We are grateful to Dr. E. A. Sweeney and Dr. T. Toyokuni for scientific editing of the manuscript. This work was supported by a Grant-in-Aid for Scientific Research (C) (25440038) and Grant-in-Aid for Young Scientists (B) (23770135) to KO and a Grant-in-Aid for Challenging Exploratory Research (25650059) to AK from the Japan Society for the Promotion of Science (JSPS). This work was also supported by The Naito Foundation Subsidy for Promotion of Specific Research Projects to KO from The Naito Foundation.

REFERENCES

1. **Davis DA.** 2009. How human pathogenic fungi sense and adapt to pH: the link to virulence. *Curr. Opin. Microbiol.* **12**:365-370.
2. **Peñalva MA, Arst HN, Jr.** 2002. Regulation of gene expression by ambient pH in filamentous fungi and yeasts. *Microbiol. Mol. Biol. Rev.* **66**:426-446.
3. **Peñalva MA, Arst HN, Jr.** 2004. Recent advances in the characterization of ambient pH regulation of gene expression in filamentous fungi and yeasts. *Annu. Rev. Microbiol.* **58**:425-451.
4. **Causton HC, Ren B, Koh SS, Harbison CT, Kanin E, Jennings EG, Lee TI, True HL, Lander ES, Young RA.** 2001. Remodeling of yeast genome expression in response to environmental changes. *Mol. Biol. Cell* **12**:323-337.
5. **Lamb TM, Mitchell AP.** 2003. The transcription factor Rim101p governs ion tolerance and cell differentiation by direct repression of the regulatory genes *NRG1* and *SMP1* in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **23**:677-686.
6. **Lamb TM, Xu W, Diamond A, Mitchell AP.** 2001. Alkaline response genes of *Saccharomyces cerevisiae* and their relationship to the RIM101 pathway. *J. Biol. Chem.* **276**:1850-1856.
7. **Serrano R, Ruiz A, Bernal D, Chambers JR, Ariño J.** 2002. The transcriptional response to alkaline pH in *Saccharomyces cerevisiae*: evidence for calcium-mediated signalling. *Mol. Microbiol.* **46**:1319-1333.
8. **Barwell KJ, Boysen JH, Xu W, Mitchell AP.** 2005. Relationship of *DFG16* to the Rim101p pH response pathway in *Saccharomyces cerevisiae* and *Candida albicans*. *Eukaryot. Cell* **4**:890-899.
9. **Li W, Mitchell AP.** 1997. Proteolytic activation of Rim1p, a positive regulator of yeast sporulation and invasive growth. *Genetics* **145**:63-73.
10. **Tréton B, Blanchin-Roland S, Lambert M, Lepingle A, Gaillardin C.** 2000. Ambient pH signalling in ascomycetous yeasts involves homologues of the *Aspergillus nidulans* genes *palF* and *palH*. *Mol. Gen. Genet.* **263**:505-513.
11. **Obara K, Yamamoto H, Kihara A.** 2012. Membrane protein Rim21 plays a central role in sensing ambient pH in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **287**:38473-38481.

- 519 12. **Boysen JH, Mitchell AP.** 2006. Control of Bro1-domain protein Rim20
520 localization by external pH, ESCRT machinery, and the *Saccharomyces*
521 *cerevisiae* Rim101 pathway. *Mol. Biol. Cell* **17**:1344-1353.
- 522 13. **Hayashi M, Fukuzawa T, Sorimachi H, Maeda T.** 2005. Constitutive
523 activation of the pH-responsive Rim101 pathway in yeast mutants defective
524 in late steps of the MVB/ESCRT pathway. *Mol. Cell. Biol.* **25**:9478-9490.
- 525 14. **Xu W, Mitchell AP.** 2001. Yeast PalA/AIP1/Alix homolog Rim20p
526 associates with a PEST-like region and is required for its proteolytic cleavage.
527 *J. Bacteriol.* **183**:6917-6923.
- 528 15. **Calcagno-Pizarelli AM, Hervas-Aguilar A, Galindo A, Abenza JF,**
529 **Peñalva MA, Arst HN, Jr.** 2011. Rescue of *Aspergillus nidulans* severely
530 debilitating null mutations in ESCRT-0, I, II and III genes by inactivation of
531 a salt-tolerance pathway allows examination of ESCRT gene roles in pH
532 signalling. *J. Cell Sci.* **124**:4064-4076.
- 533 16. **Gomez-Raja J, Davis DA.** 2012. The β -arrestin-like protein Rim8 is
534 hyperphosphorylated and complexes with Rim21 and Rim101 to promote
535 adaptation to neutral-alkaline pH. *Eukaryot. Cell* **11**:683-693.
- 536 17. **Xu W, Smith FJ, Jr., Subaran R, Mitchell AP.** 2004. Multivesicular
537 body-ESCRT components function in pH response regulation in
538 *Saccharomyces cerevisiae* and *Candida albicans*. *Mol. Biol. Cell*
539 **15**:5528-5537.
- 540 18. **Babst M, Katzmman DJ, Estepa-Sabal EJ, Meerloo T, Emr SD.** 2002.
541 Escrt-III: an endosome-associated heterooligomeric protein complex required
542 for mvb sorting. *Dev. Cell* **3**:271-282.
- 543 19. **Galindo A, Calcagno-Pizarelli AM, Arst HN, Jr., Peñalva MA.** 2012. An
544 ordered pathway for the assembly of fungal ESCRT-containing ambient pH
545 signalling complexes at the plasma membrane. *J. Cell Sci.* **125**:1784-1795.
- 546 20. **Herrador A, Herranz S, Lara D, Vincent O.** 2010. Recruitment of the
547 ESCRT machinery to a putative seven-transmembrane-domain receptor is
548 mediated by an arrestin-related protein. *Mol. Cell. Biol.* **30**:897-907.
- 549 21. **Ikeda M, Kihara A, Denpoh A, Igarashi Y.** 2008. The Rim101 pathway is
550 involved in Rsb1 expression induced by altered lipid asymmetry. *Mol. Biol.*
551 *Cell* **19**:1922-1931.
- 552 22. **Longtine MS, McKenzie A, 3rd, Demarini DJ, Shah NG, Wach A,**

- 553 **Brachat A, Philippsen P, Pringle JR.** 1998. Additional modules for
554 versatile and economical PCR-based gene deletion and modification in
555 *Saccharomyces cerevisiae*. *Yeast* **14**:953-961.
- 556 23. **Sikorski RS, Hieter P.** 1989. A system of shuttle vectors and yeast host
557 strains designed for efficient manipulation of DNA in *Saccharomyces*
558 *cerevisiae*. *Genetics* **122**:19-27.
- 559 24. **Yamagata M, Obara K, Kihara A.** 2011. Sphingolipid synthesis is involved
560 in autophagy in *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.*
561 **410**:786-791.
- 562 25. **Herranz S, Rodríguez JM, Bussink HJ, Sánchez-Ferrero JC, Arst HN,**
563 **Jr., Peñalva MA, Vincent O.** 2005. Arrestin-related proteins mediate pH
564 signaling in fungi. *Proc. Natl. Acad. Sci. U. S. A.* **102**:12141-12146.
- 565 26. **Bilodeau PS, Urbanowski JL, Winistorfer SC, Piper RC.** 2002. The
566 Vps27p Hse1p complex binds ubiquitin and mediates endosomal protein
567 sorting. *Nat. Cell Biol.* **4**:534-539.
- 568 27. **Piper RC, Cooper AA, Yang H, Stevens TH.** 1995. VPS27 controls
569 vacuolar and endocytic traffic through a prevacuolar compartment in
570 *Saccharomyces cerevisiae*. *J. Cell Biol.* **131**:603-617..
- 571 28. **Robinson JS, Klionsky DJ, Banta LM, Emr SD.** 1988. Protein sorting in
572 *Saccharomyces cerevisiae*: isolation of mutants defective in the delivery and
573 processing of multiple vacuolar hydrolases. *Mol. Cell. Biol.* **8**:4936-4948.
- 574 29. **Ito T, Chiba T, Ozawa R, Yoshida M, Hattori M, Sakaki Y.** 2001. A
575 comprehensive two-hybrid analysis to explore the yeast protein interactome.
576 *Proc. Natl. Acad. Sci. U. S. A.* **98**:4569-4574.
- 577 30. **Chvatchko Y, Howald I, Riezman H.** 1986. Two yeast mutants defective in
578 endocytosis are defective in pheromone response. *Cell* **46**:355-364.
- 579 31. **Lechler T, Li R.** 1997. In vitro reconstitution of cortical actin assembly sites
580 in budding yeast. *J. Cell Biol.* **138**:95-103.
- 581 32. **Nishimura K, Fukagawa T, Takisawa H, Kakimoto T, Kanemaki M.**
582 2009. An auxin-based degron system for the rapid depletion of proteins in
583 nonplant cells. *Nat. Methods* **6**:917-922.
- 584 33. **Abe F, Iida H.** 2003. Pressure-induced differential regulation of the two
585 tryptophan permeases Tat1 and Tat2 by ubiquitin ligase Rsp5 and its binding
586 proteins, Bul1 and Bul2. *Mol. Cell. Biol.* **23**:7566-7584.

587 34. **Lin CH, MacGurn JA, Chu T, Stefan CJ, Emr SD.** 2008. Arrestin-related
588 ubiquitin-ligase adaptors regulate endocytosis and protein turnover at the cell
589 surface. *Cell* **135**:714-725.

590 35. **Hanson PI, Roth R, Lin Y, Heuser JE.** 2008. Plasma membrane
591 deformation by circular arrays of ESCRT-III protein filaments. *J. Cell Biol.*
592 **180**:389-402.

593 36. **Brachmann CB, Davies A, Cost GJ, Caputo E, Li J, Hieter P, Boeke JD.**
594 1998. Designer deletion strains derived from *Saccharomyces cerevisiae*
595 S288C: a useful set of strains and plasmids for PCR-mediated gene disruption
596 and other applications. *Yeast* **14**:115-132.

597 37. **Winzeler EA, Shoemaker DD, Astromoff A, Liang H, Anderson K,**
598 **Andre B, Bangham R, Benito R, Boeke JD, Bussey H, Chu AM, Connelly**
599 **C, Davis K, Dietrich F, Dow SW, El Bakkoury M, Foury F, Friend SH,**
600 **Gentalen E, Giaever G, Hegemann JH, Jones T, Laub M, Liao H,**
601 **Liebundguth N, Lockhart DJ, Lucau-Danila A, Lussier M, M'Rabet N,**
602 **Menard P, Mittmann M, Pai C, Rebischung C, Revuelta JL, Riles L,**
603 **Roberts CJ, Ross-MacDonald P, Scherens B, Snyder M,**
604 **Sookhai-Mahadeo S, Storms RK, Veronneau S, Voet M, Volckaert G,**
605 **Ward TR, Wysocki R, Yen GS, Yu K, Zimmermann K, Philippsen P,**
606 **Johnston M, Davis RW.** 1999. Functional characterization of the *S.*
607 *cerevisiae* genome by gene deletion and parallel analysis. *Science*
608 **285**:901-906.
609

FIGURE LEGENDS

FIG 1 Rim20 accumulates at the plasma membrane of yeast cells in response to external alkalization and alteration in lipid asymmetry. (A) YOK3248 (*RIM20-EGFP*) and YOK3249 (*RIM20-EGFP rim21Δ*) cells were subjected to fluorescence microscopy after external alkalization and re-acidification. Bar, 5 μm. (B) YOK3334 (*RIM20-EGFP PMA1-mCherry*) cells were examined after 60 min of external alkalization. Arrowheads, Rim20-GFP foci co-localized with Pma1-mCherry. Bar, 5 μm. (C) YOK3248 and YOK3306 (*RIM20-EGFP rim8Δ*) cells were examined before and after 60 min of external alkalization. Bar, 5 μm. (D) YOK3248 and YOK3265 (*RIM20-EGFP lem3Δ*) cells in log phase were subjected to fluorescence microscopy. Bar, 5 μm. The number of Rim20 foci at the plasma membrane were counted and are presented in the right panel. Values are the means ± SD from three independent experiments (**, $P < 0.01$; Student's t test).

FIG 2 Rim8 and Rim13 accumulate at the plasma membrane in yeast cells exposed to alkaline medium. (A) YOK3410 (*RIM8-2xEGFP*) and YOK3411 (*RIM8-2xEGFP rim21Δ*) cells were subjected to fluorescence microscopy after exposure to alkaline medium for the indicated time. Bar, 5 μm. (B) The percentage of the cells with clear Rim8 foci at the plasma membrane is presented. Values are the means ± SD from three independent experiments (**, $P < 0.01$; Student's t test). (C) YOK3271 (*RIM13-2xEGFP*) and YOK3272 (*RIM13-2xEGFP rim21Δ*) cells were subjected to fluorescence microscopy after exposure to alkaline medium for the indicated time. Bar, 5 μm.

632

633 **FIG 3** Snf7 localized to the plasma membrane is involved in the Rim101 pathway.

634 (A) YOK3250 (*SNF7-mRFP*) cells were subjected to fluorescence microscopy after

635 exposure to alkaline medium for the indicated time. Images digitally enhanced to

636 highlight the Snf7-mRFP signal at the plasma membrane are also shown. Snf7 foci at

637 the plasma membrane are indicated by arrowheads. Bar, 5 μ m. (B) YOK3251

638 (*SNF7-mRFP rim21 Δ*) and YOK3418 (*SNF7-mRFP vps27 Δ*) cells were examined as

639 in (A) before and 60 min after external alkalization. Images of YOK3251 cells

640 digitally enhanced to highlight the Snf7-mRFP signal are also shown. Bars, 5 μ m.

641 (C) BY4741 (WT), 1150 (*rim21 Δ*), and 5381 (*vps27 Δ*) cells harboring pFI1

642 (*HA-RIM101*) were grown to log phase and exposed to alkaline medium for 20 min,

643 then total lysates were prepared. Immunoblotting on total lysates was performed with

644 an anti-HA or, to demonstrate uniform protein loading, anti-Pgk1 antibody. FL and

645 Δ C, full-length and processed Rim101, respectively. (D) Total lysates were prepared

646 from BY4741, 1150, and 5381 cells in log phase and were subjected to

647 immunoblotting as in (C). Proteins in the extracellular fraction were also analyzed. I

648 and E, intracellular and extracellular fractions, respectively. p2 and m, p2 (Golgi)

649 and mature (vacuolar) form of CPY, respectively.

650

651 **FIG 4** Endocytosis is not essential for the Rim101 pathway. (A) Total lysates were

652 prepared from BY4741 (WT), 1150 (*rim21 Δ*), and MYY312 (*end3 Δ*) cells harboring

653 pFI1 (*HA-RIM101*) before and 20 min after external alkalization. Immunoblotting

was performed with an anti-HA antibody. Two independent *end3Δ* clones (#1 and #2) were analyzed. FL and ΔC, full-length and processed Rim101, respectively. (B) YOK3073 (WT), YOK3094 (*rim21Δ*), YOK3159 (*LAS17-HA-AID*) cells in log phase were pre-treated with 500 μM IAA or ethanol (mock) for 1 h, and exposed to alkaline medium for 20 min. Total cell lysates were subjected to immunoblotting with an anti-HA antibody. FL and ΔC, full-length and processed Rim101, respectively.

FIG 5 Rsp5-mediated ubiquitination is essential for the Rim101 pathway. (A) YPH499 (WT) and FAY51R (*rsp5-I*) cells harboring pFI1 (*HA-RIM101*) were grown to log phase at 30 °C then further cultured for 90 min at 30 °C or 38 °C. Cells were then exposed to alkaline medium at 30 °C or 38°C for 20 min. Total cell lysates were subjected to immunoblotting with an anti-HA or, to demonstrate uniform protein loading, anti-Pgk1 antibody. FL and ΔC, full-length and processed Rim101, respectively. (B) YOK3073 (WT), YOK3094 (*rim21Δ*), and YOK3393 (*AID-RSP5*) cells harboring pFI1 grown to log phase were pre-treated with 500 μM IAA or ethanol (mock) for 2 h, then exposed to alkaline medium for 20 min. Total cell lysates were subjected to immunoblotting with an anti-HA, anti-AID, or, to demonstrate uniform protein loading, anti-Pgk1 antibody. FL and ΔC, full-length and processed Rim101, respectively. (C) YOK2649 (*RIM8-HA*) and YOK2658 (*RIM8-HA doa4Δ*) cells in log phase were exposed to alkaline medium for 20 min. Total cell lysates were subjected to immunoblotting with an anti-HA or, to

676 demonstrate uniform protein loading, anti-Pgk1 antibody. Ub, ubiquitin.

677

678 **FIG 6** Ubiquitination of Rim8 is not essential for the Rim101 pathway. (A)
679 YOK3306 (*RIM20-EGFP rim8Δ*) cells harboring an empty vector (vec), pOK476
680 (*RIM8-HA*), or pOK573 (*RIM8-HA (K521R)*) were subjected to fluorescence
681 microscopy after 60 min of exposed to alkaline medium. Bar, 5 μm. (B) YOK3384
682 (*RIM21-2xEGFP rim8Δ*) cells harboring an empty vector, pOK574 (*RIM8-HA*), or
683 pOK576 (*RIM8-HA (K521R)*) in log phase were exposed to alkaline medium for 60
684 min. Total cell lysates were subjected to immunoblotting with an anti-GFP or, to
685 demonstrate uniform protein loading, anti-Pgk1 antibody.

686

687 **FIG 7** Ubiquitination is essential for formation of Rim20 foci. (A) YOK3342
688 (*RIM20-EGFP*) and YOK3343 (*rsp5-1 RIM20-EGFP*) were grown to log phase at 30
689 °C, further cultured for 90 min at 30 °C or 38 °C, and exposed to alkaline medium at
690 30 °C or 38 °C. After 60 min of alkaline treatment, cells were subjected to
691 fluorescence microscopy. (B) YOK3403 (*AID-RSP5 RIM20-EGFP*) cells in log
692 phase were pre-treated with 500 μM IAA or ethanol (mock) for 2 h, exposed to
693 alkaline medium for 60 min, and subjected to fluorescence microscopy. (C)
694 YOK3412 (*RIM8-2xEGFP*) and YOK3413 (*AID-RSP5 RIM8-2xEGFP*) cells in log
695 phase were pre-treated with 500 μM IAA or ethanol (mock) for 2 h, exposed to
696 alkaline medium for 20 min, and subjected to fluorescence microscopy. (D)
697 YOK3413 cells were cultured as in (C), and total cell lysates were prepared at each

698 time point. Immunoblotting was performed with an anti-AID or, to demonstrate
699 uniform protein loading, anti-Pgk1 antibody.

700 **TABLE 1.** Yeast strains used in this study

701	Strain	Genotype	Source
702	SEY6210	<i>MATa his3 leu2 ura3 trp1 lys2 suc2</i>	(28)
703	YOK2027	SEY6210, <i>rim21Δ::KanMX4</i>	(11)
704	YOK3248	SEY6210, <i>RIM20-EGFP::TRP1</i>	This study
705	YOK3249	SEY6210, <i>RIM20-EGFP::TRP1 rim21Δ::KanMX4</i>	This study
706	YOK3265	SEY6210, <i>RIM20-EGFP::TRP1 lem3Δ::KanMX4</i>	This study
707	YOK3334	SEY6210, <i>RIM20-EGFP::TRP1 PMA1-mCherry::KanMX6</i>	This study
708	YOK3306	SEY6210, <i>RIM20-EGFP::TRP1 rim8Δ::KanMX4</i>	This study
709	YOK3266	SEY6210, <i>RIM20-EGFP::TRP1 vps4Δ::URA3</i>	This study
710	YOK3410	SEY6210, <i>RIM8-2xEGFP::KanMX6</i>	This study
711	YOK3411	SEY6210, <i>RIM8-2xEGFP::KanMX6 rim21Δ::NatNT2</i>	This study
712	YOK3271	SEY6210, <i>RIM13-2xEGFP::KanMX6</i>	This study
713	YOK3272	SEY6210, <i>RIM13-2xEGFP::KanMX6 rim21Δ::NatNT2</i>	This study
714	YOK3250	SEY6210, <i>SNF7-mRFP::NatNT2</i>	This study
715	YOK3251	SEY6210, <i>SNF7-mRFP::NatNT2 rim21Δ::KanMX4</i>	This study
716	YOK3598	SEY6210, <i>SNF7-mRFP::NatNT2 vps4Δ::HIS3</i>	This study
717	YOK3408	SEY6210, <i>SNF7-mRFP::NatNT2 RIM20-EGFP::TRP1</i>	This study
718	YOK3527	SEY6210, <i>RIM20-mCherry::NatNT2 RIM8-2xEGFP::KanMX6</i>	This study
719	YOK3528	SEY6210, <i>RIM20-mCherry::NatNT2 RIM13-2xEGFP::KanMX6</i>	This study
720	YOK3418	SEY6210, <i>SNF7-mRFP::NatNT2 RIM20-EGFP::TRP1</i>	This study
721		<i>vps27Δ::KanMX4</i>	

722	YOK2279	SEY6210, <i>RIM8-HA::KanMX6</i>	This study
723	YOK2649	SEY6210, <i>RIM8-HA::NatNT2</i>	This study
724	YOK2658	SEY6210, <i>RIM8-HA::NatNT2 doa4Δ::KanMX4</i>	This study
725	YOK2562	SEY6210, <i>rim8Δ::KanMX4</i>	This study
726	YOK3073	SEY6210, <i>P_{ADH}-OsTIR1-myc::URA3</i>	This study
727	YOK3094	SEY6210, <i>P_{ADH}-OsTIR1-myc::URA3 rim21Δ::KanMX</i>	This study
728	YOK3159	SEY6210, <i>P_{ADH}-OsTIR1-myc::URA3 LAS17-HA-AID::KanMX6</i>	This study
729	YOK3393	SEY6210, <i>P_{ADH}-OsTIR1-myc::URA3 P_{ADH}-AID-RSP5::NatNT2</i>	This study
730	YOK3403	SEY6210, <i>P_{ADH}-OsTIR1-myc::URA3 P_{ADH}-AID-RSP5::NatNT2</i>	This study
731		<i>RIM20-EGFP::TRP1</i>	
732	YOK3412	SEY6210, <i>P_{ADH}-OsTIR1-myc::URA3 RIM8-2xEGFP::KanMX6</i>	This study
733	YOK3413	SEY6210, <i>P_{ADH}-OsTIR1-myc::URA3 P_{ADH}-AID-RSP5::NatNT2</i>	This study
734		<i>RIM8-2xEGFP::KanMX6</i>	
735	YOK3384	SEY6210, <i>RIM21-2xEGFP::KanMX6 rim8Δ::NatNT2</i>	This study
736	BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	(36)
737	YOK3427	BY4741, <i>RIM20-EGFP::KanMX6</i>	This study
738	YOK3428	BY4741, <i>RIM8-2xEGFP::KanMX6</i>	This study
739	YOK3429	BY4741, <i>RIM13-2xEGFP::KanMX6</i>	This study
740	1150	BY4741, <i>rim21Δ::KanMX4</i>	(37)
741	5381	BY4741, <i>vps27Δ::KanMX4</i>	(37)
742	MYY312	BY4741, <i>end3Δ::KanMX4</i>	This study
743	YPH499	<i>MATa his3Δ200 lys2-801 ade2-101 leu2Δ1ura3-52 trp1Δ63</i>	(23)

744	FAY51R	YPH499, <i>rsp5-1</i>	(33)
745	YOK3342	YPH499, <i>RIM20-EGFP::TRP1</i>	This study
746	YOK3343	YPH499, <i>rsp5-1 RIM20-EGFP::TRP1</i>	This study

747

Figure 1

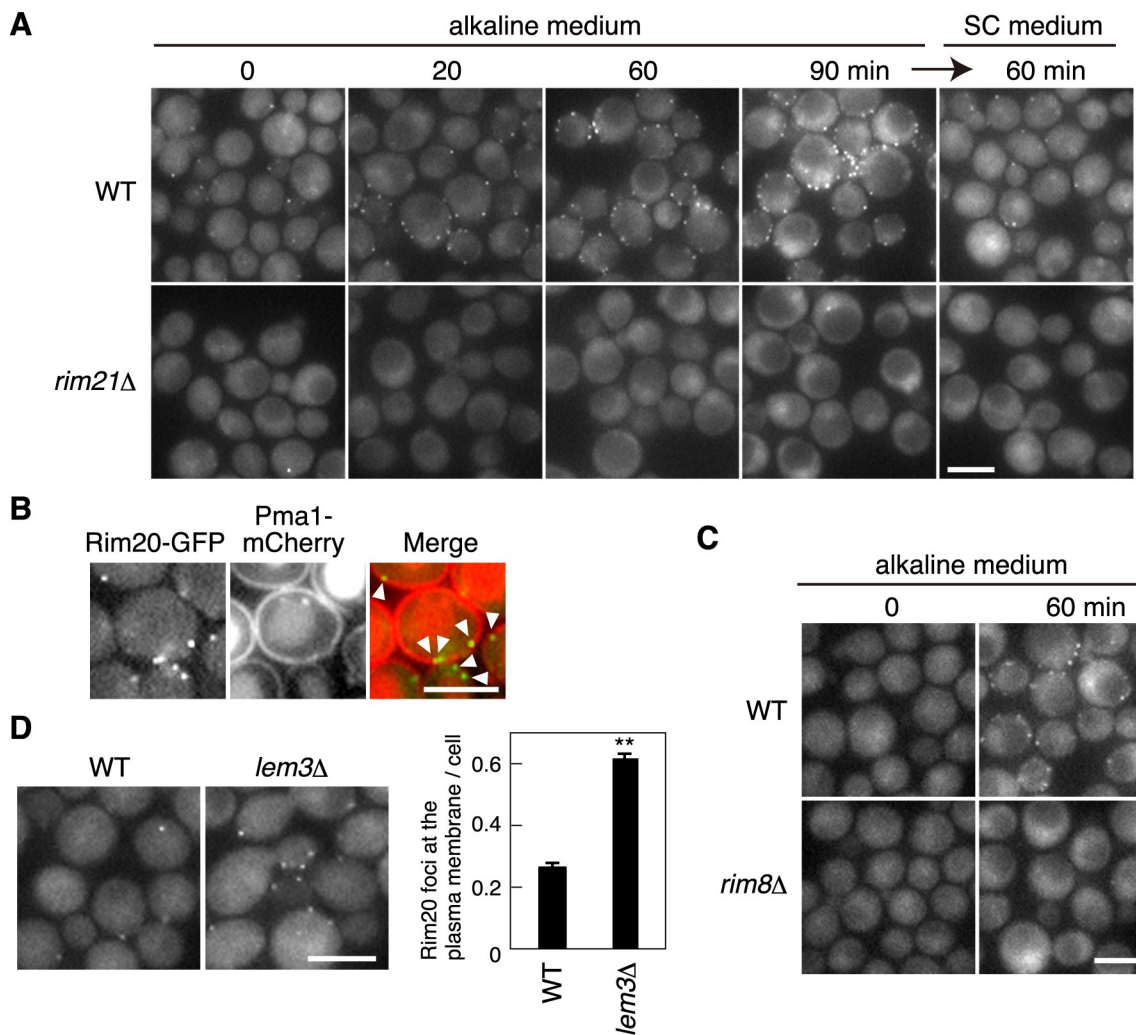
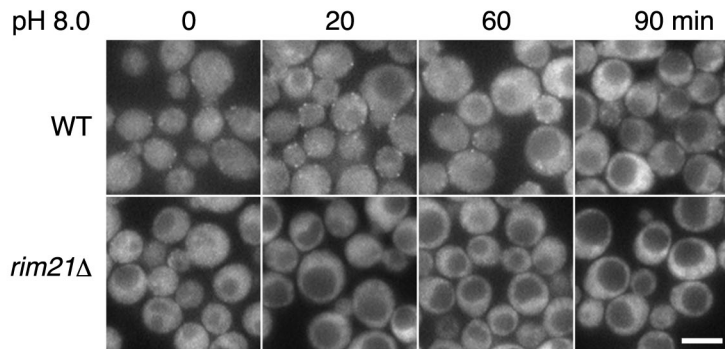
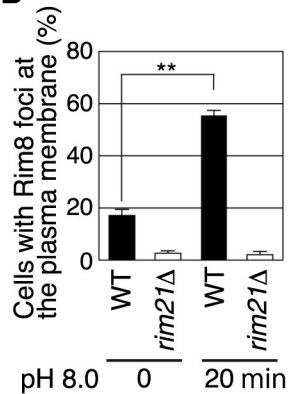


Figure 2

A



B



C

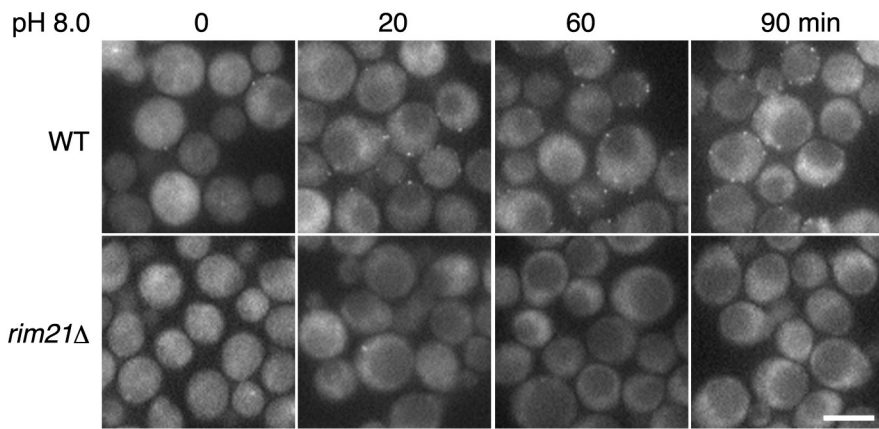


Figure 3

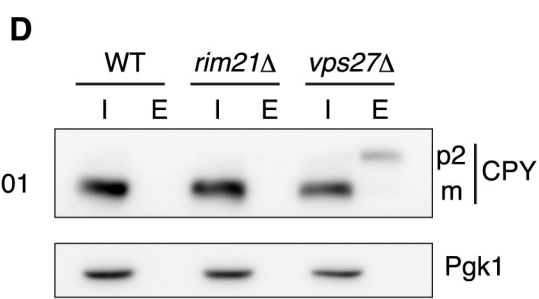
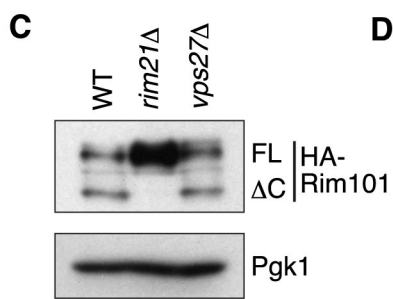
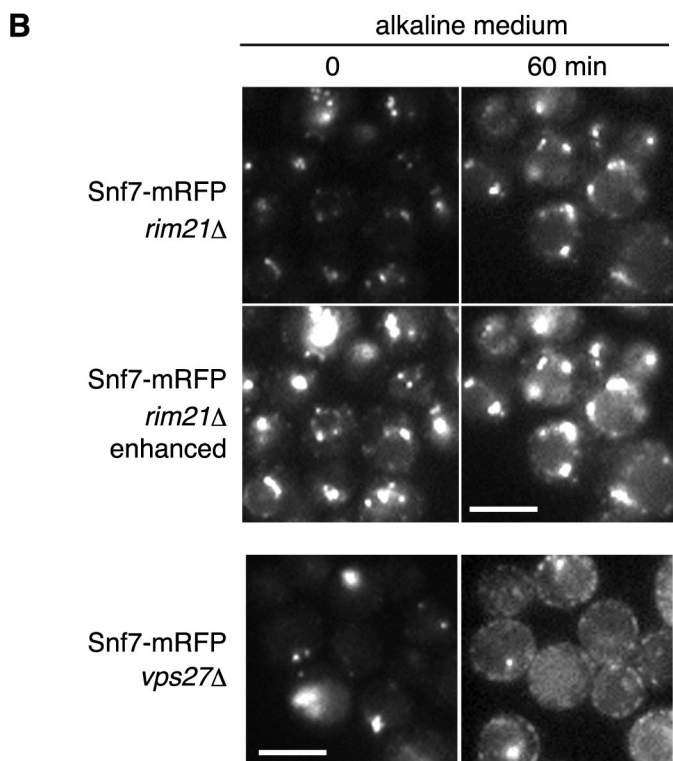
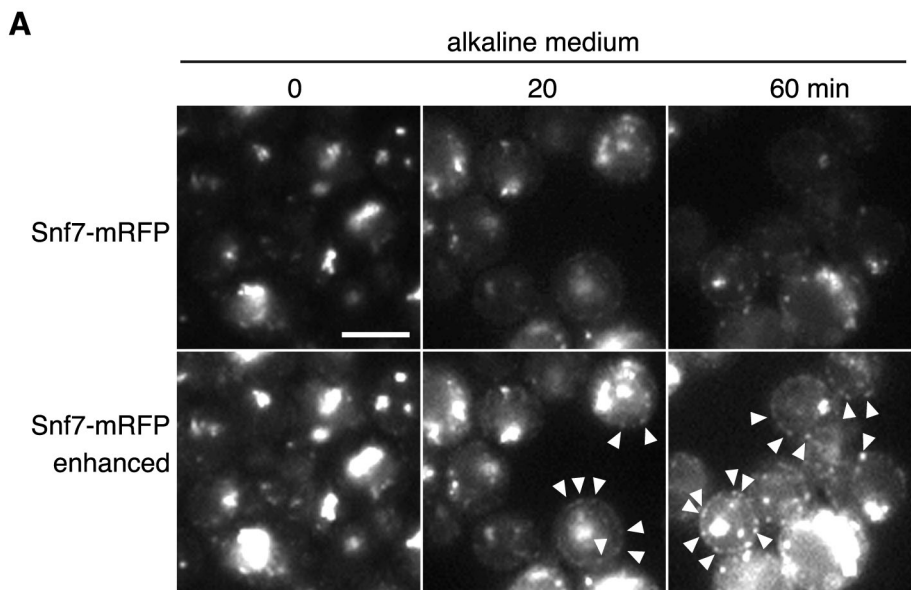
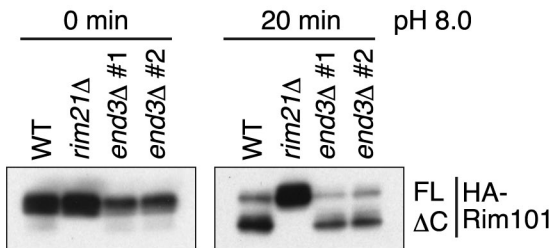


Figure 4

A



B

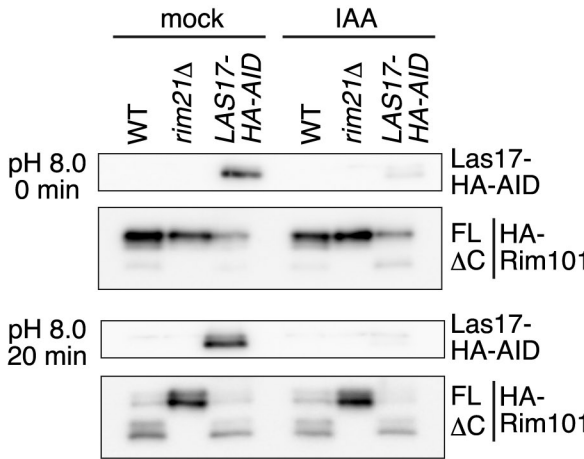
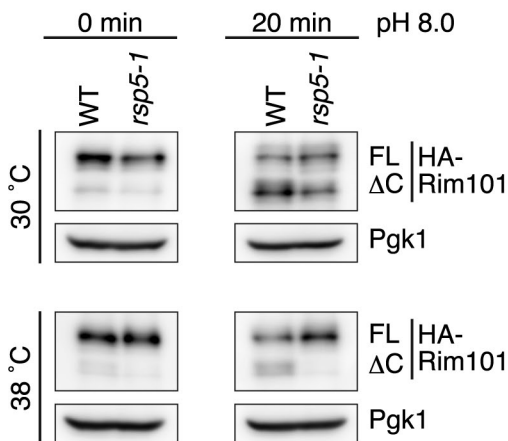
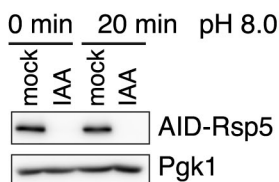
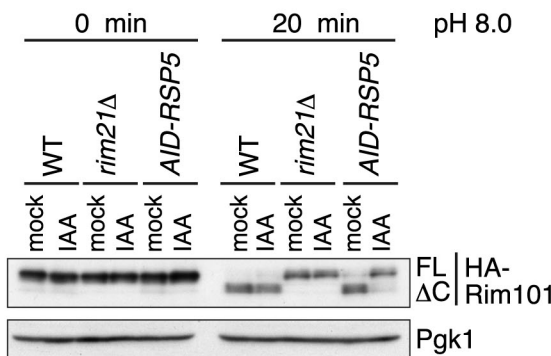


Figure 5

A



B



C

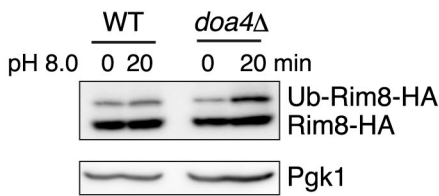
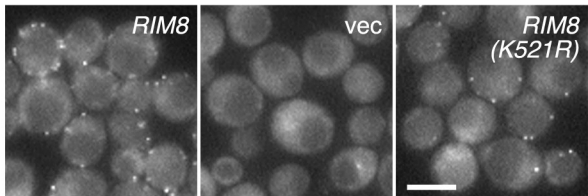


Figure 6

A *RIM20-GFP*



B

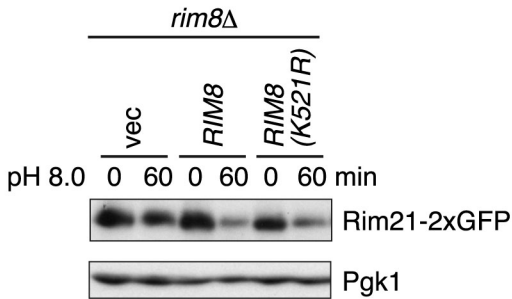
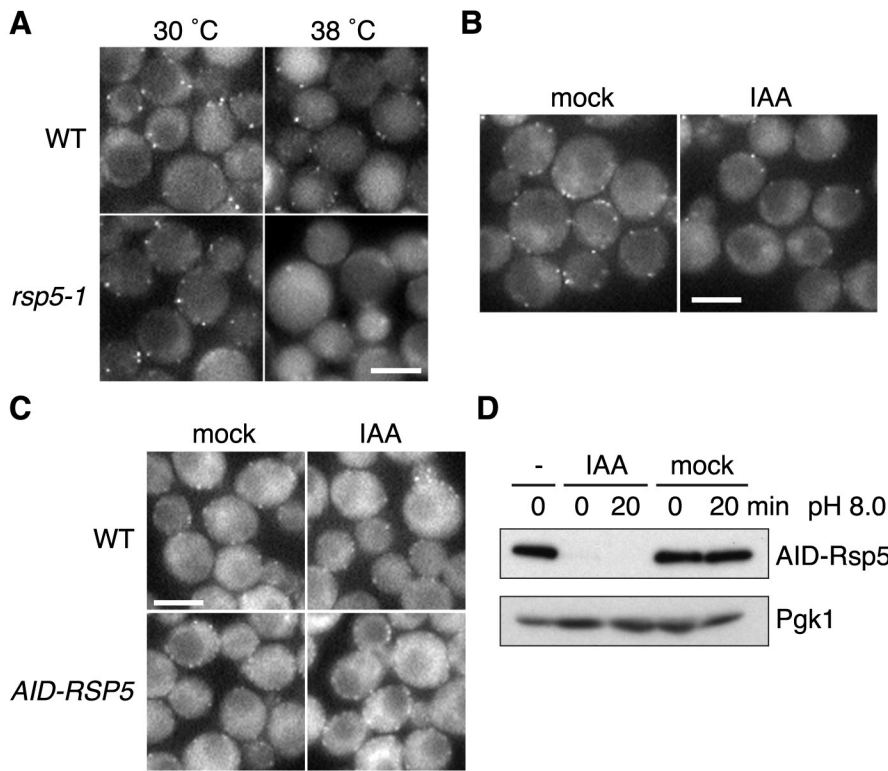


Figure 7



SUPPLEMENTAL METHOD

Immunoelectron Microscopy. YOK3248 (*RIM20-GFP*) cells at log phase were exposed to alkaline medium for 90 min, sandwiched between gold disks, and frozen in liquid propane using Leica EM CPC (Leica Microsystems, Wetzlar, Germany). The frozen cells were freeze-substituted with acetone containing 2% distilled water. Samples were dehydrated with anhydrous ethanol and infiltrated with LR White resin (London Resin, Hampshire, UK). The resin was polymerized under ultraviolet light. Ultrathin sections were prepared with an ultramicrotome (Ultracut UCT, Leica Microsystems). Rim20-GFP was detected using anti-GFP antibody (Abcam, Cambridge, UK) and goat anti-Rabbit IgG antibody conjugated with 10 nm gold particle (British BioCell International, Cardiff, UK) under a transmission electron microscope (JEM-1400Plus, JOEL, Tokyo, Japan).

Figure S1

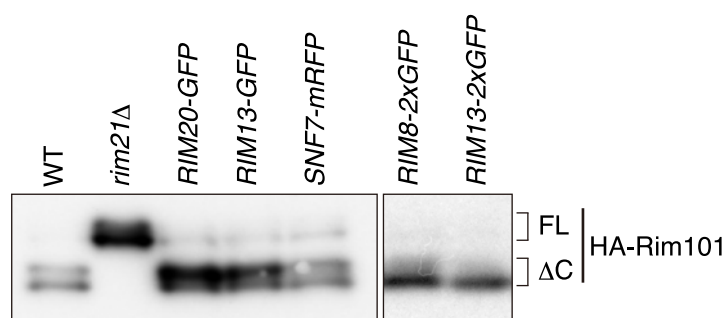


FIG S1 Labeling Rim20, Rim8, Rim13, and Snf7 with GFP or mRFP does not interfere with their function. SEY6210 (WT), YOK2027 (*rim21Δ*), YOK3248 (*RIM20-EGFP*), YOK3263 (*RIM13-EGFP*), YOK3250 (*SNF7-mRFP*), YOK3410 (*RIM8-2xEGFP*), and YOK3271 (*RIM13-2xEGFP*) cells harboring pFI1 (a vector for expressing *HA-RIM101* in yeast cells) were exposed to alkaline medium for 20 min, then total cell lysates were prepared. Proteins from cell lysates were separated by SDS-PAGE and transferred to Immobilon™ polyvinylidene difluoride membrane. Immunoblotting for the HA-Rim101 protein was performed using an anti-HA antibody. Normal processing of HA-Rim101 from its full-length inactive form (FL) into its active form (ΔC) indicates that the Rim101 pathway is functioning normally in cells exposed to alkaline medium.

Figure S2

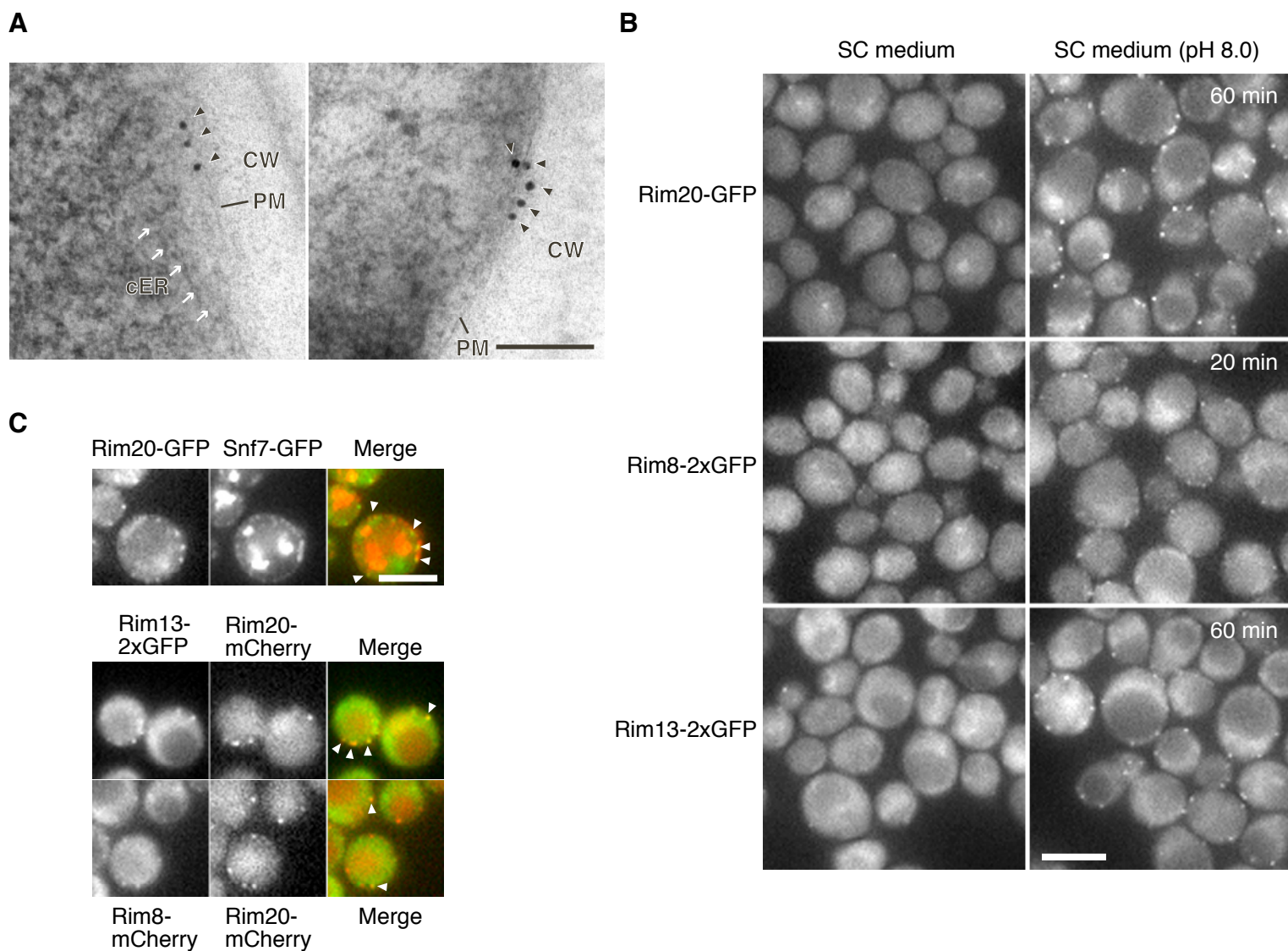


FIG S2 Rim20, Rim8, and Rim13 accumulate at the plasma membrane. (A) YOK3248 (*RIM20-GFP*) cells at log phase were exposed to alkaline medium for 90 min and subjected to immunoelectron microscopy using an anti-GFP antibody (see SUPPLEMENTAL METHOD). CW, cell wall; PM, plasma membrane; cER, cortical ER; Arrow, cortical ER; Arrowhead, gold particle; Bar, 100 nm. (B) YOK3427 (*RIM20-EGFP*), YOK3428 (*RIM8-2xEGFP*), and YOK3429 (*RIM13-2xEGFP*) cells, all sharing the BY4741 background, were grown to log phase then were subjected to fluorescence microscopy before and after alkaline treatment. Bar, 5 μ m. (C) YOK3408 (*RIM20-EGFP SNF7-mRFP*), YOK3528 (*RIM13-2xEGFP RIM20-mCherry*), and YOK3527 (*RIM8-2xEGFP RIM20-mCherry*) cells at log phase were exposed to alkaline medium for 60 min and subjected to fluorescence microscopy. Bar, 5 μ m.

Figure S3

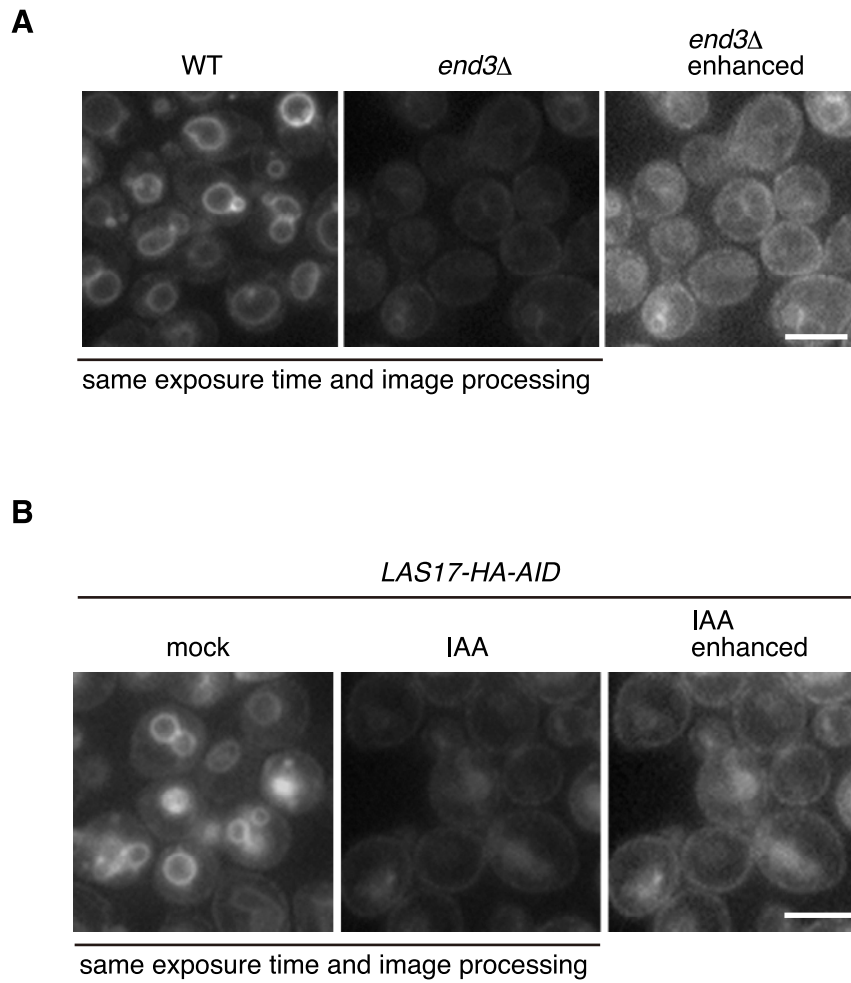


FIG S3 Endocytosis is impaired in *end3Δ* and IAA-treated *LAS-HA-AID* cells. (A) BY4741 (WT) and MYY312 (*end3Δ*) cells in log phase were loaded with 1 μ M FM4-64 for 15 min, then were washed and resuspended with SC medium, and further incubated for an additional 15 min. Sodium azide was added to a final concentration of 20 mM and cells were kept on ice until subjected to microscopy. An image of MYY312 cells digitally enhanced to more clearly visualize the FM4-64 signal is also shown. Bar, 5 μ m. (B) YOK3159 (*LAS17-HA-AID*) cells in log phase were treated with 500 μ M IAA or ethanol for 1 h. Cells were processed as in (A) except that after the wash cells were resuspended with SC medium containing 500 μ M IAA or ethanol. Bar, 5 μ m.

Figure S4

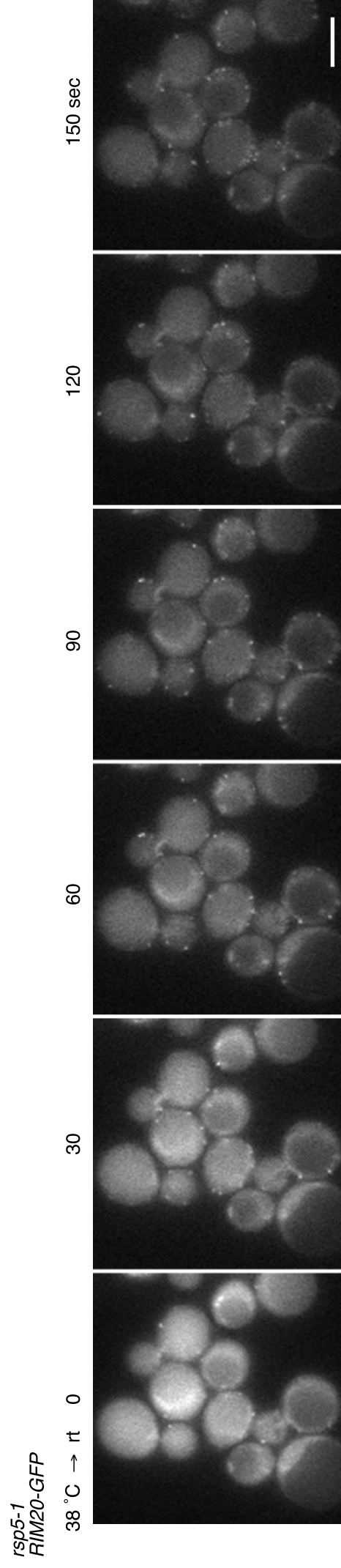


FIG S4 Rim20 foci at the plasma membrane are restored during examination of *rsp5-1* cells that were cultured at a restrictive temperature. YOK3343 (*rsp5-1 RIM20-EGFP*) cells were cultured at 30 °C to log phase, then transferred to 38 °C for 90 min, and exposed to alkaline medium at 38 °C. After 60 min of alkaline treatment, cells were immediately examined under a fluorescence microscope at room temperature (rt). Serial images taken at intervals of 30 sec are shown. Bar, 5 μ m.

Figure S5

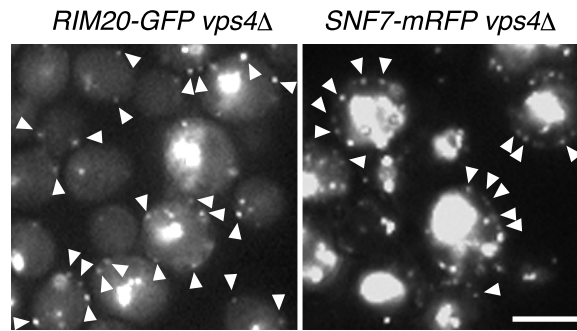


FIG S5 Rim20 and Snf7 accumulate at the plasma membrane in addition to the late endosome in *vps4Δ* cells even without external alkalization. YOK3266 (*RIM20-EGFP vps4Δ*) and YOK3598 (*SNF7-mRFP vps4Δ*) cells in log phase were subjected to fluorescence microscopy. Arrowheads, Rim20 and Snf7 foci at the plasma membrane. Bar, 5 μ m.