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

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ABSTRACT

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

INTRODUCTION

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

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

55 subunits that usually function at the endosomal membrane are required but ESCRT 0
56 subunits such as Vps27 are dispensable for Rim101 signaling (15-17). The
57 hypothesis that Rim101 signaling proceeds at the endosome is mainly based on
58 observations of yeast *vps4*Δ cells. Vps4 is an AAA-ATPase involved in the
59 formation of intraluminal vesicles at the late endosome. Vps4 catalyzes the
60 disassembly and membrane release of ESCRT III including its subunit Snf7, so in
61 *vps4*Δ cells ESCRT III subunits accumulate at the late endosome (18). Rim20 also
62 accumulates at the late endosome in *vps4*Δ cells, and the Rim101 pathway is
63 constitutively activated in these cells even without external alkalization (12). Such
64 findings led to the suggestion that Rim20 accumulated at the late endosome functions
65 in Rim101 signaling. However, it is still unknown whether the Rim20 accumulation
66 is a normal process that also occurs in WT cells in response to external alkalization
67 or is just an aberrant event observed in *vps4*Δ cells.

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

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

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

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

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

99 the original loci, during a response to alkaline conditions. Considering those findings
100 together with other biochemical data presented here, we concluded that Rim101
101 signaling in *S. cerevisiae* proceeds at the plasma membrane by an ordered
102 recruitment of downstream molecules to the plasma membrane, and not at the late
103 endosome *via* endocytosis of the sensor complex. We also found that ubiquitination
104 of protein(s) other than Rim8 by Rsp5 mediates Rim101 signaling at the plasma
105 membrane by recruiting downstream molecules to the plasma membrane.
106

MATERIALS AND METHODS

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

Genetic manipulation. Gene disruption was performed by replacing the entire coding region of the gene with a marker gene. Chromosome fusion of HA, GFP, 2xGFP, mCherry, mRFP, or HA-AID to the relevant gene sequence to label the C-terminus of each protein of interest was performed using PCR-based gene disruption and modification (22). The sequence encoding a respective tag, the *ADH1* termination sequence, and a marker sequence was amplified by PCR from a pFA6a vector series (22) with a primer set containing the homologous region of the target gene. For the chromosomal fusion of AID to the N-terminus, the sequence encoding a marker sequence, *ADH1* 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 *ADH1* terminator sequence was amplified by PCR from genomic DNA of YOK2279 (*RIM8-HA*) to have *Eco*RI sites at both 5' and 3' ends. The amplified fragment was cloned into the *Eco*RI 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, *Eco*RI 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 Immobilon^{TM} 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 μM 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 *rim21*Δ 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 *lem3*Δ 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 *lem3*Δ cells than in WT cells (Fig. 1D), indicating that Rim20 accumulates at the plasma membrane in response to altered lipid asymmetry. The accumulation in *lem3*Δ cells was less prominent than in alkaline-treated WT cells, probably because activation of the Rim101 pathway in *lem3*Δ 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

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

237 With multiple localizations observed for Snf7, it was difficult to clarify at exactly
238 which location Snf7 actually participates in Rim101 signaling. To overcome this
239 difficulty, we deleted *RIM21* and analyzed the localization of Snf7 and activation of
240 the Rim101 pathway. The plasma membrane localization of Snf7 was totally
241 abolished in the *rim21*Δ cells, while its localization at the late endosome was
242 unaffected (Fig. 3B). Under these conditions, the Rim101 pathway was not activated
243 at all, as indicated by absence of processed Rim101 (Fig. 3C). This suggests that
244 recruitment of Snf7 to the plasma membrane is required for Rim101 signaling.

245 We also analyzed the localization of Snf7 and activation of the Rim101 pathway
246 in cells lacking *VPS27*. Vps27 acts as an initial factor that directly recruits ESCRT I,
247 and therefore indirectly ESCRT II and III, to the late endosome (26, 27). In untreated
248 *vps27*Δ cells, the Snf7 signal at the late endosome was considerably reduced (Fig.
249 3B). In *vps27*Δ cells exposed to alkaline medium, the Snf7 signal was mostly
250 detected at the plasma membrane and not at the late endosome. However, under
251 these conditions the Rim101 pathway was normally activated (Fig. 3C), which
252 strongly suggests that Snf7 at the late endosome is dispensable for the Rim101
253 pathway.

254 We next examined the vacuolar protein sorting pathway in *vps27*Δ and *rim21*Δ
255 cells. In this pathway, soluble vacuolar proteases such as carboxypeptidase Y (CPY)
256 are synthesized in the ER and delivered to the vacuole *via* the Golgi apparatus and

257 the late endosome. In *vps27*Δ cells, some of the p2 (Golgi) form of CPY was
258 mis-sorted to the extracellular space (Fig. 3D), as others have reported (28). In
259 contrast, in *rim21*Δ cells virtually all of the CPY was detected intracellularly as the
260 mature vacuolar form, similar to results in WT cells. These results suggest that Snf7
261 at the plasma membrane is dispensable for the vacuolar protein sorting pathway.
262 Taken together, these findings indicate that, under alkaline conditions, Snf7 localizes
263 to the plasma membrane in addition to the late endosome, and that Snf7 at the plasma
264 membrane is involved in the Rim101 pathway.

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

273

274 **Endocytosis is dispensable for the Rim101 pathway.** Rim21 is endocytosed and
275 degraded in the vacuole following external alkalization (11). We next investigated
276 whether endocytosis is essential for mediating the signal of external alkalization or
277 just occurs to promote turnover of Rim21 after it has transduced the signal to
278 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 *end3*Δ 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 *rim8*Δ cells expressing this variant (data not shown). However, whether

301 ubiquitination itself is essential for the Rim101 signaling is still unclear. To examine
302 this point, we employed the temperature-sensitive *rsp5-1* mutant (33). In the *rsp5-1*
303 mutant cultured at the permissive temperature of 30 ˚C, the Rim101 pathway was
304 normally activated upon external alkalization (Fig. 5A). In contrast, when the *rsp5-1*
305 mutant was initially cultured for 90 min at the restrictive temperature of 38 ˚C,
306 activation of the Rim101 pathway by alkaline treatment was abolished, indicating
307 that Rsp5 activity is essential for the Rim101 pathway. We also examined the
308 involvement of Rsp5 in the Rim101 pathway using the AID system. Cells expressing
309 AID-Rsp5 were pre-treated with IAA or vehicle and then exposed to alkaline
310 medium. In IAA-treated *AID-RSP5* cells, the Rim101 pathway was severely
311 impaired (Fig. 5B). These results indicate that Rsp5 activity is essential for the
312 Rim101 pathway, which in turn strongly suggests that ubiquitination is involved in
313 Rim101 signaling.
314 We next investigated if ubiquitination is facilitated when the Rim101 pathway is
315 active. Although ubiquitination of Rim8 is not essential for the Rim101 pathway,
316 changes in the ubiquitination of Rim8 may represent ubiquitination activity of Rsp5
317 under alkaline conditions. As shown in Fig. 5C, the levels of ubiquitinated Rim8 did
318 not differ between 0 and 20 min of external alkalization. However, in cells lacking
319 the *DOA4* gene encoding a deubiquitination enzyme, ubiquitinated Rim8
320 accumulated with alkaline treatment, indicating that the
321 ubiquitination-deubiquitination cycle of Rim8 is facilitated in alkaline conditions.
322 We next investigated the function of Rim8 and its ubiquitination in the Rim101

323 pathway using the Rim8 K521R variant, which does not undergo ubiquitination (20).
324 First, accumulation of Rim20 at the plasma membrane was examined. In *rim8*Δ cells
325 carrying an empty vector, Rim20 accumulation at the plasma membrane was totally
326 abolished even after external alkalization (Fig. 6A). In contrast, in *rim8*Δ cells
327 expressing the K521R variant, Rim20 foci were induced normally at the plasma
328 membrane upon external alkalization. This observation indicates that the Rim8
329 protein, but not its ubiquitination, is essential for Rim20 accumulation at the plasma
330 membrane.

331 Arrestins are often involved in endocytosis of plasma membrane proteins through
332 being ubiquitinated and/or facilitating ubiquitination of the target plasma membrane
333 proteins (34). In the Rim101 pathway, the sensor protein Rim21 is internalized from
334 the plasma membrane and degraded in the vacuole following external alkalization
335 (11). This process can be monitored by detecting decreases in Rim21 levels, *e.g.*, by
336 immunoblotting. As shown in Fig. 6B, Rim21-2xGFP levels were reduced in *rim8*Δ
337 cells expressing either Rim8 or the Rim8-K521R variant after 60 min of exposure to
338 alkaline medium. This indicates that Rim8 ubiquitination is not required for the
339 endocytosis of Rim21. On the other hand, in *rim8*Δ cells carrying an empty vector,
340 Rim21 levels were not reduced, indicating that the Rim8 protein itself is required for
341 Rim21 turnover. At present, it is not clear whether Rim8 is directly involved in the
342 endocytosis of Rim21 or just facilitates Rim21 endocytosis indirectly through
343 progressing Rim101 signaling.
344

Ubiquitination by Rsp5 mediates Rim101 signaling at the plasma membrane. We next searched for the process in which Rsp5-mediated ubiquitination is actually involved. We first examined Rim20 accumulation in *rsp5-1* cells. In *rsp5-1* cells cultured at 38 ˚C, Rim20 did not accumulate at the plasma membrane even after external alkalization (Fig. 7A). Transient degradation of AID-Rsp5 by IAA treatment also severely impaired Rim20 recruitment to the plasma membrane in response to external alkalization (Fig. 7B). These results indicate that Rsp5-mediated ubiquitination is required for the accumulation of Rim20 at the plasma membrane under alkaline conditions. Interestingly, we noticed that, during examination under a fluorescence microscope at room temperature, the formation of Rim20 foci at the plasma membrane was restored in *rsp5-1* cells originally cultured at 38 ˚C (Fig. S4). The recovery of the Rim20 plasma membrane accumulation was rapid: Rim20 accumulation at the plasma membrane was nearly absent at the beginning of the examination yet became prominent (similar to that observed in WT cells) within 150 sec of serial observation of the same view. This recovery is presumably due to the restoration of Rsp5 activity during incubation at room temperature. This observation suggests that recruitment of Rim20 to the plasma membrane proceeds rapidly after Rsp5-mediated ubiquitination.

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

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

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 *vps4*Δ mutant. In the *vps4*Δ 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 *vps4*Δ 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. alnicans* (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

418 plasma membrane, Rim8 may be dissociate from the plasma membrane together with
419 the ESCRT I complex. Since Rim8 is presumed to bind to Rim21 (15, 16, 20), it is
420 also possible that Rim8 dissociates with Rim21. In contrast, accumulation of Snf7,
421 Rim20, and Rim13 persisted even after 90 min of alkaline exposure, when Rim21
422 was mostly degraded (11) and the majority of Rim8 had dissociated from the plasma
423 membrane (Fig. 2A). It is known that Snf7 can self-assemble without assistance from
424 other proteins (35). Thus, Snf7 might accumulate continuously and autonomously
425 once an "on" signal emitted from Rim21 was transduced to it, and this in turn might
426 facilitate the persistent recruitment of Rim20 and Rim13. It is worth noting that
427 accumulation of Rim20 was resolved after re-acidification of the medium (Fig. 1A),
428 even when most of the Rim21 had been degraded and Rim8 had dissociated from the
429 plasma membrane. This suggests that some unknown "off" signal is emitted by
430 external acidification and reaches Snf7, probably in a manner independent of Rim21
431 and Rim8.
432
433 **Ubiquitination by Rsp5 mediates Rim101 signaling at the plasma membrane.**
434 Rim8 is known to be ubiquitinated. However, ubiquitination of Rim8 is not essential
435 for the Rim101 pathway and the level of ubiquitinated Rim8 does not alter upon
436 external alkalization (20). Thus, whether ubiquitination itself is essential for the
437 Rim101 signaling or not has been in question. Here, we determined, using the level
438 of ubiquitinated-Rim8 as an indicator, that the ubiquitination-deubiquitination cycle
439 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

462 could clarify that Rsp5-mediated ubiquitination of some protein other than Rim8
463 occurs before, and is a prerequisite for, recruitment of Rim20 to the plasma
464 membrane. For further upstream processes, recruitment of Rim8 to the plasma
465 membrane will be an ideal indicator. With this indicator, we revealed that the
466 Rsp5-mediated ubiquitination of some protein other than Rim8 occurs independently
467 of Rim8 recruitment or after Rim8 is recruited to the plasma membrane (Fig. 7C).
468 Imaging of these proteins in combination with immunoblotting of the processed
469 Rim101 will be convenient assays for monitoring the progression of each elementary
470 step in Rim101 signaling.
471

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609 **FIGURE LEGENDS**

610 **FIG 1** Rim20 accumulates at the plasma membrane of yeast cells in response to
611 external alkalization and alteration in lipid asymmetry. (A) YOK3248
612 (*RIM20-EGFP*) and YOK3249 (*RIM20-EGFP rim21*Δ) cells were subjected to
613 fluorescence microscopy after external alkalization and re-acidification. Bar, 5 μm.
614 (B) YOK3334 (*RIM20-EGFP PMA1-mCherry*) cells were examined after 60 min of
615 external alkalization. Arrowheads, Rim20-GFP foci co-localized with
616 Pma1-mCherry. Bar, 5 μm. (C) YOK3248 and YOK3306 (*RIM20-EGFP rim8*Δ)
617 cells were examined before and after 60 min of external alkalization. Bar, 5 μm. (D)
618 YOK3248 and YOK3265 (*RIM20-EGFP lem3*Δ) cells in log phase were subjected to
619 fluorescence microscopy. Bar, 5 μm. The number of Rim20 foci at the plasma
620 membrane were counted and are presented in the right panel. Values are the means ±
621 SD from three independent experiments (**, $P < 0.01$; Student's *t* test).

622

623 **FIG 2** Rim8 and Rim13 accumulate at the plasma membrane in yeast cells exposed
624 to alkaline medium. (A) YOK3410 (*RIM8-2xEGFP*) and YOK3411 (*RIM8-2xEGFP*
625 *rim21*Δ) cells were subjected to fluorescence microscopy after exposure to alkaline
626 medium for the indicated time. Bar, 5 μm. (B) The percentage of the cells with clear
627 Rim8 foci at the plasma membrane is presented. Values are the means ± SD from
628 three independent experiments (**, $P < 0.01$; Student's *t* test). (C) YOK3271
629 (*RIM13-2xEGFP*) and YOK3272 (*RIM13-2xEGFP rim21*Δ) cells were subjected to
630 fluorescence microscopy after exposure to alkaline medium for the indicated time.
631 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 μm. (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 μm.
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 ΔC, 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 *end3*Δ clones (#1 and #2) were analyzed. FL and ΔC, full-length and processed Rim101, respectively. (B) YOK3073 (WT), YOK3094 (*rim21*Δ), 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 (*rsp5-1*) 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 (*rim21*Δ), 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 doa4*Δ) 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 rim8*Δ) 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 rim8*Δ) 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

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

TABLE 1. Yeast strains used in this study

Strain	Genotype	Source
SEY6210	*MATα his3 leu2 ura3 trp1 lys2 suc2*	(28)
YOK2027	SEY6210, *rim21*Δ*::KanMX4*	(11)
YOK3248	SEY6210, *RIM20-EGFP::TRP1*	This study
YOK3249	SEY6210, *RIM20-EGFP::TRP1 rim21*Δ*::KanMX4*	This study
YOK3265	SEY6210, *RIM20-EGFP::TRP1 lem3*Δ*::KanMX4*	This study
YOK3334	SEY6210, *RIM20-EGFP::TRP1 PMA1-mCherry::KanMX6*	This study
YOK3306	SEY6210, *RIM20-EGFP::TRP1 rim8*Δ*::KanMX4*	This study
YOK3266	SEY6210, *RIM20-EGFP::TRP1 vps4*Δ*::URA3*	This study
YOK3410	SEY6210, *RIM8-2xEGFP::KanMX6*	This study
YOK3411	SEY6210, *RIM8-2xEGFP::KanMX6 rim21*Δ*::NatNT2*	This study
YOK3271	SEY6210, *RIM13-2xEGFP::KanMX6*	This study
YOK3272	SEY6210, *RIM13-2xEGFP::KanMX6 rim21*Δ*::NatNT2*	This study
YOK3250	SEY6210, S*NF7-mRFP::NatNT2*	This study
YOK3251	SEY6210, *SNF7-mRFP::NatNT2 rim21*Δ*::KanMX4*	This study
YOK3598	SEY6210, *SNF7-mRFP::NatNT2 vps4*Δ*::HIS3*	This study
YOK3408	SEY6210, *SNF7-mRFP::NatNT2 RIM20-EGFP::TRP1*	This study
YOK3527	SEY6210, *RIM20-mCherry::NatNT2 RIM8-2xEGFP::KanMX6*	This study
YOK3528	SEY6210, *RIM20-mCherry::NatNT2 RIM13-2xEGFP::KanMX6*	This study
YOK3418	SEY6210, *SNF7-mRFP::NatNT2 RIM20-EGFP::TRP1*	This study
	*vps27*Δ*::KanMX4*	

YOK2279	SEY6210, *RIM8-HA::KanMX6*	This study
YOK2649	SEY6210, *RIM8-HA::NatNT2*	This study
YOK2658	SEY6210, *RIM8-HA::NatNT2 doa4*Δ*::KanMX4*	This study
YOK2562	SEY6210, *rim8*Δ*::KanMX4*	This study
YOK3073	SEY6210, P_{ADH}*-OsTIR1-myc::URA3*	This study
YOK3094	SEY6210, P_{ADH}*-OsTIR1-myc::URA3 rim21*Δ*::KanMX*	This study
YOK3159	SEY6210, P_{ADH}*-OsTIR1-myc::URA3 LAS17-HA-AID::KanMX6*	This study
YOK3393	SEY6210, P_{ADH}*-OsTIR1-myc::URA3* P_{ADH}*-AID-RSP5::NatNT2*	This study
YOK3403	SEY6210, P_{ADH}*-OsTIR1-myc::URA3* P_{ADH}*-AID-RSP5::NatNT2*	This study
	RIM20-EGFP::TRP1	
YOK3412	SEY6210, P_{ADH}*-OsTIR1-myc::URA3 RIM8-2xEGFP::KanMX6*	This study
YOK3413	SEY6210, P_{ADH}*-OsTIR1-myc::URA3* P_{ADH}*-AID-RSP5::NatNT2*	This study
	RIM8-2xEGFP::KanMX6	
YOK3384	SEY6210, *RIM21-2xEGFP::KanMX6 rim8*Δ*::NatNT2*	This study
BY4741	*MAT**a** his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*	(36)
YOK3427	BY4741, *RIM20-EGFP::KanMX6*	This study
YOK3428	BY4741, *RIM8-2xEGFP::KanMX6*	This study
YOK3429	BY4741, *RIM13-2xEGFP::KanMX6*	This study
1150	BY4741, *rim21*Δ*::KanMX4*	(37)
5381	BY4741, *vps27*Δ*::KanMX4*	(37)
MYY312	BY4741, *end3*Δ*::KanMX4*	This study
YPH499	*MAT**a** his3*Δ*200 lys2-801 ade2-101 leu2*Δ*1ura3-52 trp1*Δ*63*	(23)

FAY51R	YPH499, *rsp5-1*	(33)
YOK3342	YPH499, *RIM20-EGFP::TRP1*	This study
YOK3343	YPH499, *rsp5-1 RIM20-EGFP::TRP1*	This study

Figure 1

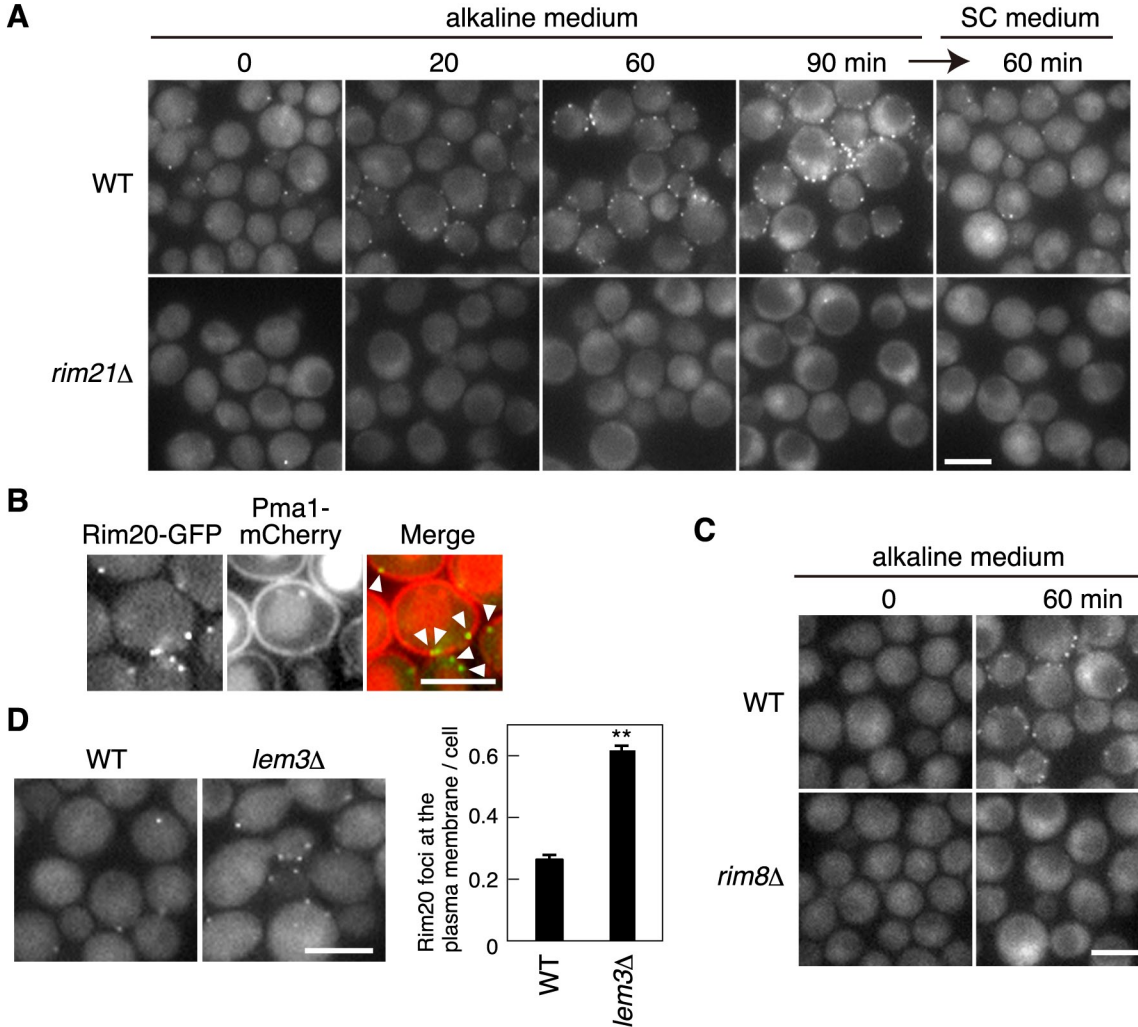

A
alkaline medium
SC medium
0
20
60
90 min
60 min
WT
rim21Δ
B
Rim20-GFP
Pma1-mCherry
Merge
C
alkaline medium
0
60 min
WT
rim8Δ
D
WT
lem3Δ
Rim20 foci at the plasma membrane / cell
0
0.2
0.4
0.6
**
WT
lem3Δ

Figure 2

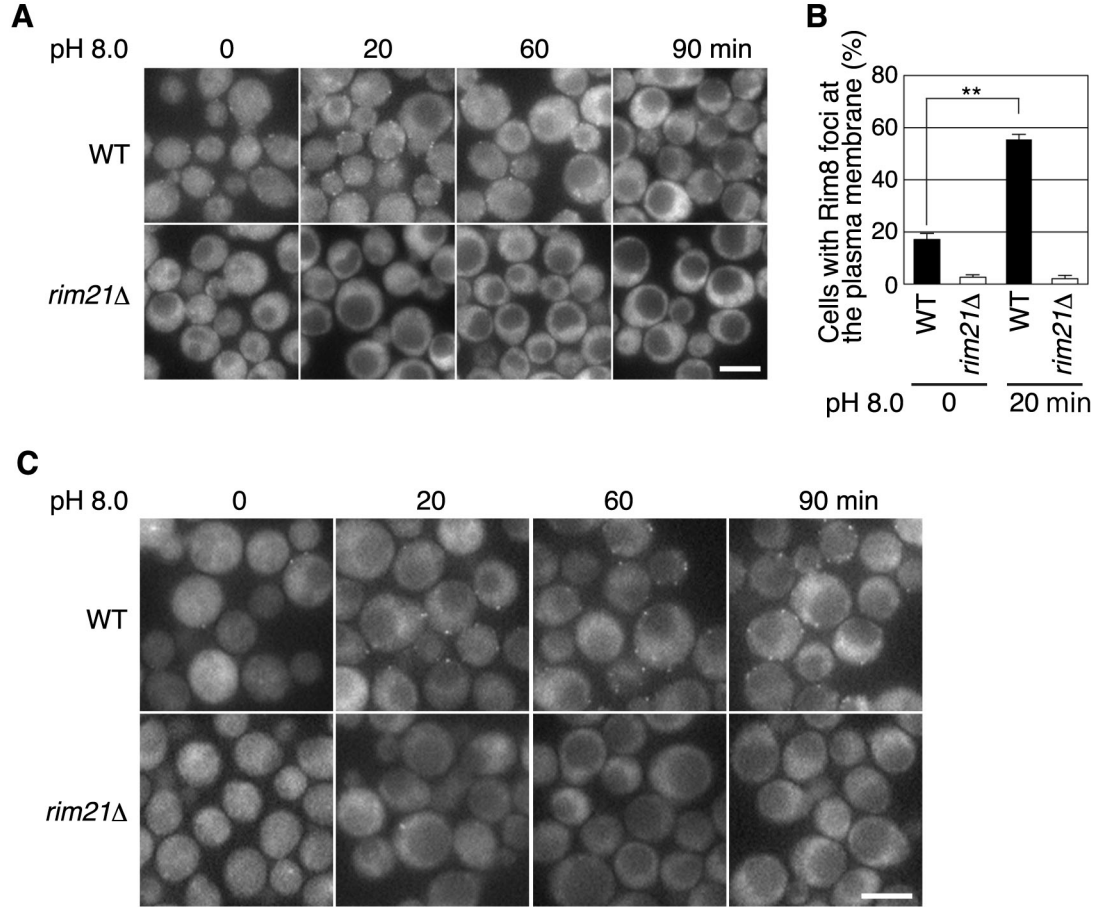

A
pH 8.0
0
20
60
90 min
WT
rim21Δ
B
Cells with Rim8 foci at the plasma membrane (%)
80
60
40
20
0
**
WT
rim21Δ
WT
rim21Δ
pH 8.0
0
20 min
C
pH 8.0
0
20
60
90 min
WT
rim21Δ

Figure 3

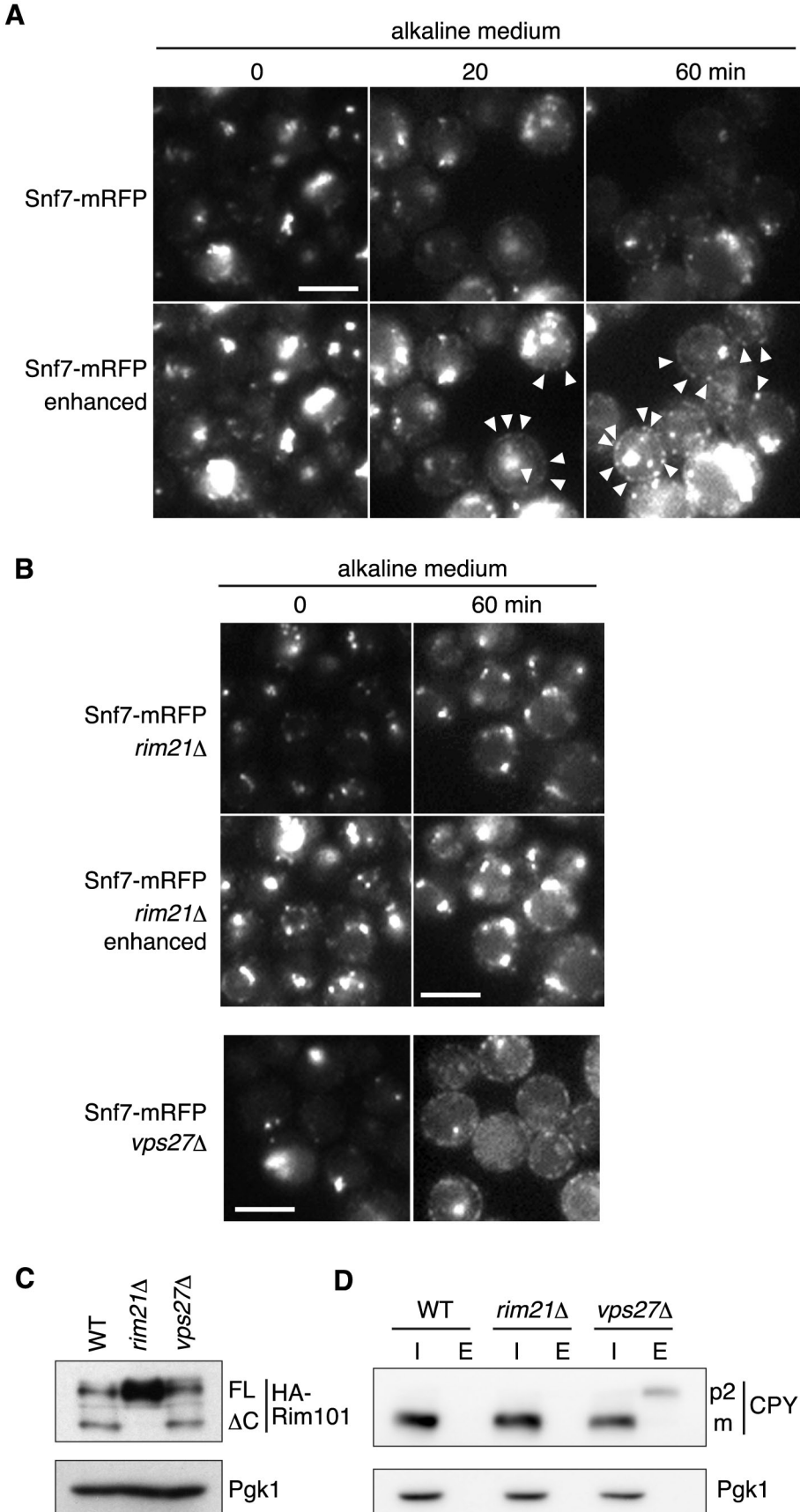

A
alkaline medium
0
20
60 min
Snf7-mRFP
Snf7-mRFP
enhanced
B
alkaline medium
0
60 min
Snf7-mRFP
rim21Δ
Snf7-mRFP
rim21Δ
enhanced
Snf7-mRFP
vps27Δ
C
WT
rim21Δ
vps27Δ
FL
ΔC
HA-
Rim101
Pgk1
D
WT
rim21Δ
vps27Δ
I
E
I
E
I
E
p2
m
CPY
Pgk1

Figure 4

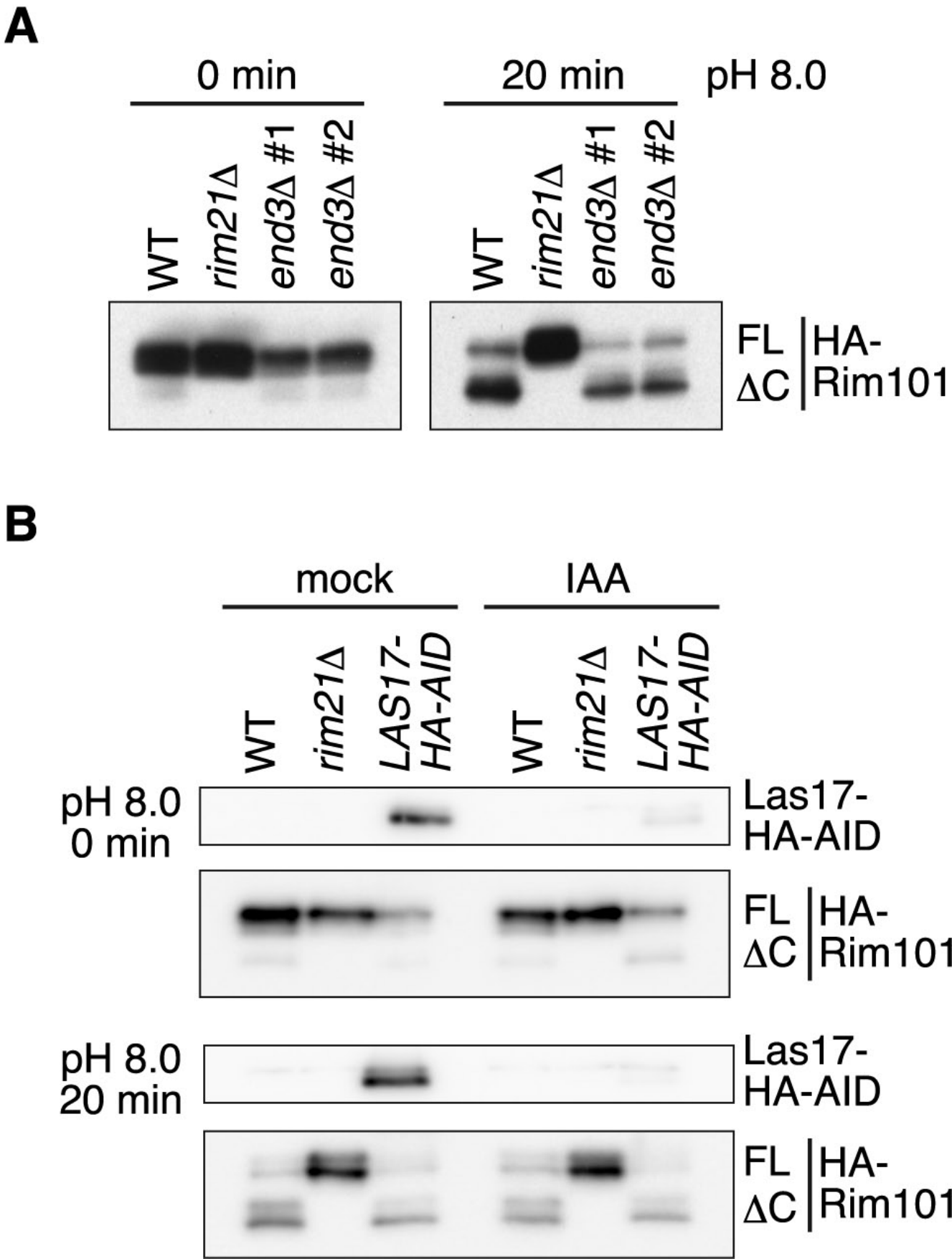

A
0 min
20 min
pH 8.0
WT
rim21Δ
end3Δ #1
end3Δ #2
WT
rim21Δ
end3Δ #1
end3Δ #2
FL
ΔC
HA-Rim101
B
mock
IAA
WT
rim21Δ
LAS17-HA-AID
WT
rim21Δ
LAS17-HA-AID
pH 8.0
0 min
Las17-HA-AID
FL
ΔC
HA-Rim101
pH 8.0
20 min
Las17-HA-AID
FL
ΔC
HA-Rim101

Figure 5

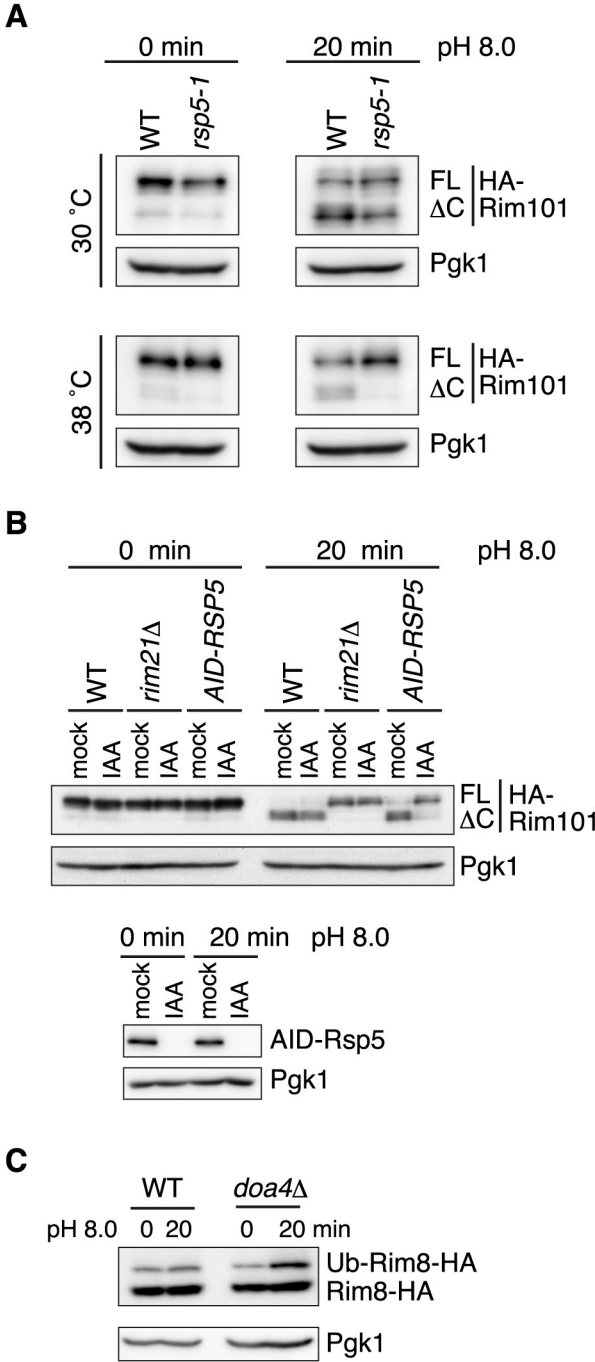

A
0 min
20 min
pH 8.0
WT
rsp5-1
WT
rsp5-1
30 °C
FL
ΔC
HA-Rim101
Pgk1
38 °C
FL
ΔC
HA-Rim101
Pgk1
B
0 min
20 min
pH 8.0
WT
rim21Δ
AID-RSP5
WT
rim21Δ
AID-RSP5
mock
IAA
FL
ΔC
HA-Rim101
Pgk1
0 min
20 min
pH 8.0
mock
IAA
AID-Rsp5
Pgk1
C
WT
doa4Δ
pH 8.0
0 20
0 20 min
Ub-Rim8-HA
Rim8-HA
Pgk1

Figure 6

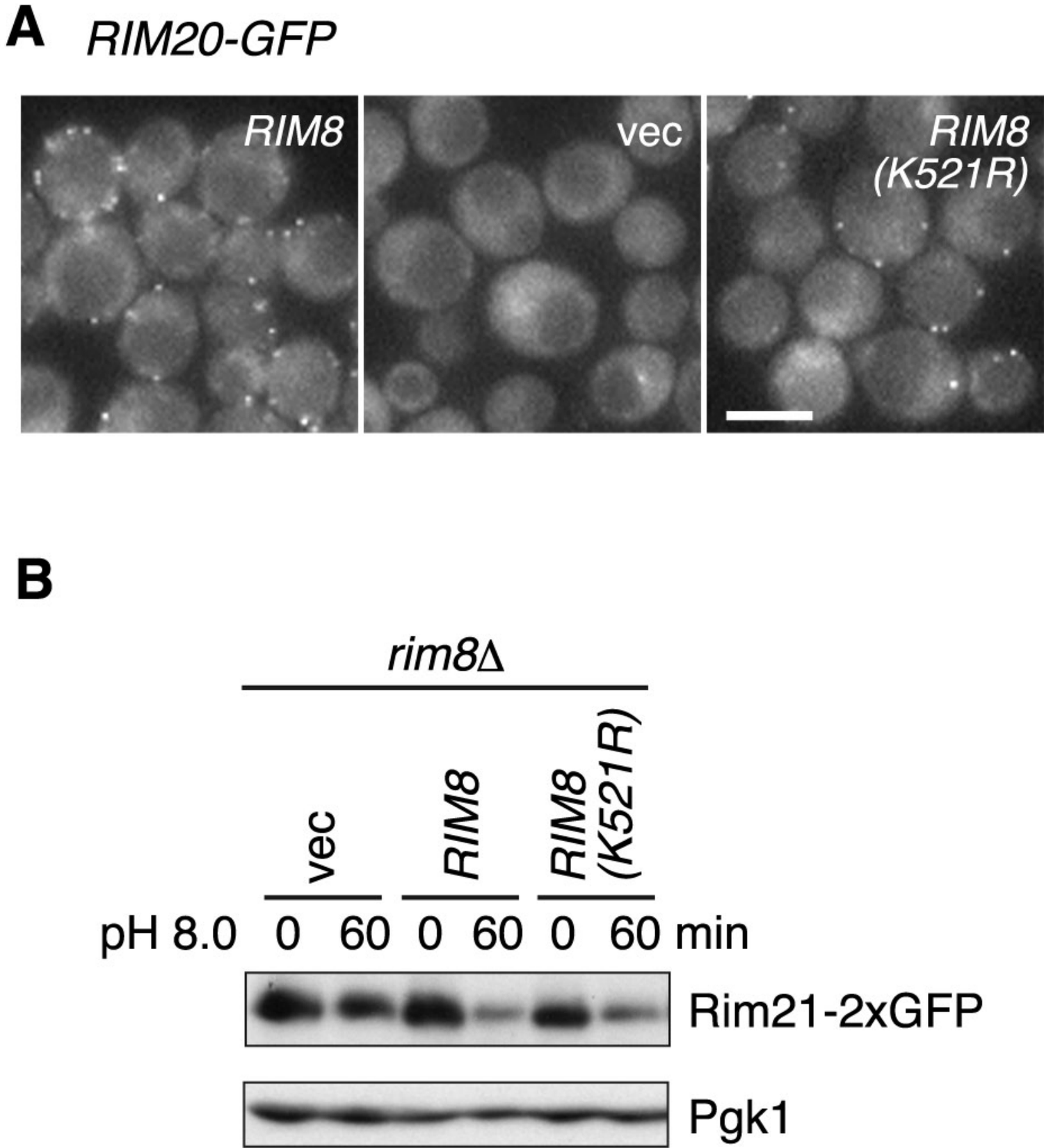
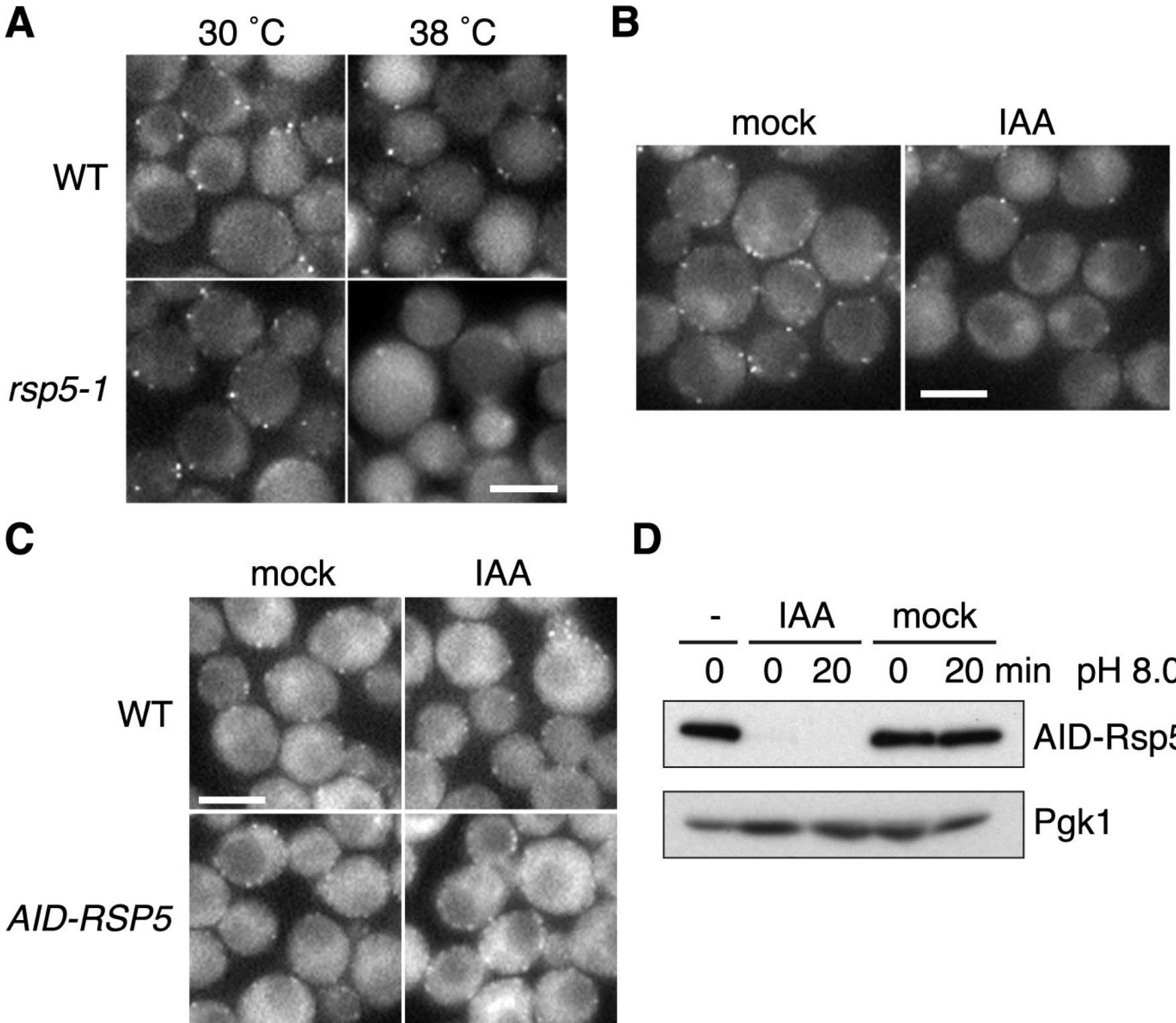

A
RIM20-GFP
RIM8
vec
RIM8
(K521R)
B
rim8Δ
vec
RIM8
RIM8
(K521R)
pH 8.0
0
60
0
60
0
60
min
Rim21-2xGFP
Pgk1

Figure 7


A
30 ˚C
38 ˚C
WT
rsp5-1
B
mock
IAA
C
mock
IAA
WT
AID-RSP5
D
-
IAA
mock
0
0
20
0
20 min
pH 8.0
AID-Rsp5
Pgk1

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

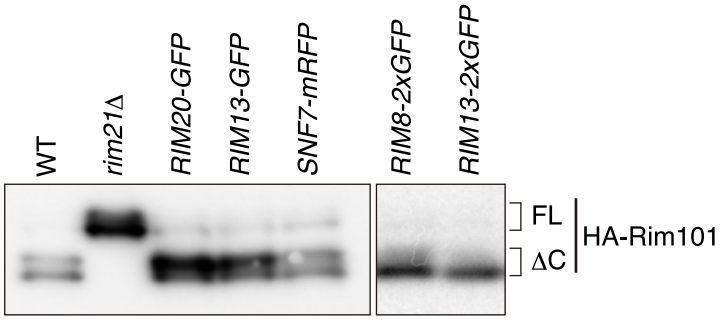


FIG S1 Labeling Rim20, Rim8, Rim13, and Snf7 with GFP or mRFP does not interfere with their function. SEY6210 (WT), YOK2027 (*rim21*Δ), YOK3248 (*RIM20-EGFP*), YOK3263 (*RIM13-EGFP*), YOK3250 (*SNF7-mRFP*), YOK3410 (*RIM8-2xEGFP*), and YOK3271 (*RIM13-2xEGFP*) cells harboring pFI1 (a vector for expressing *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

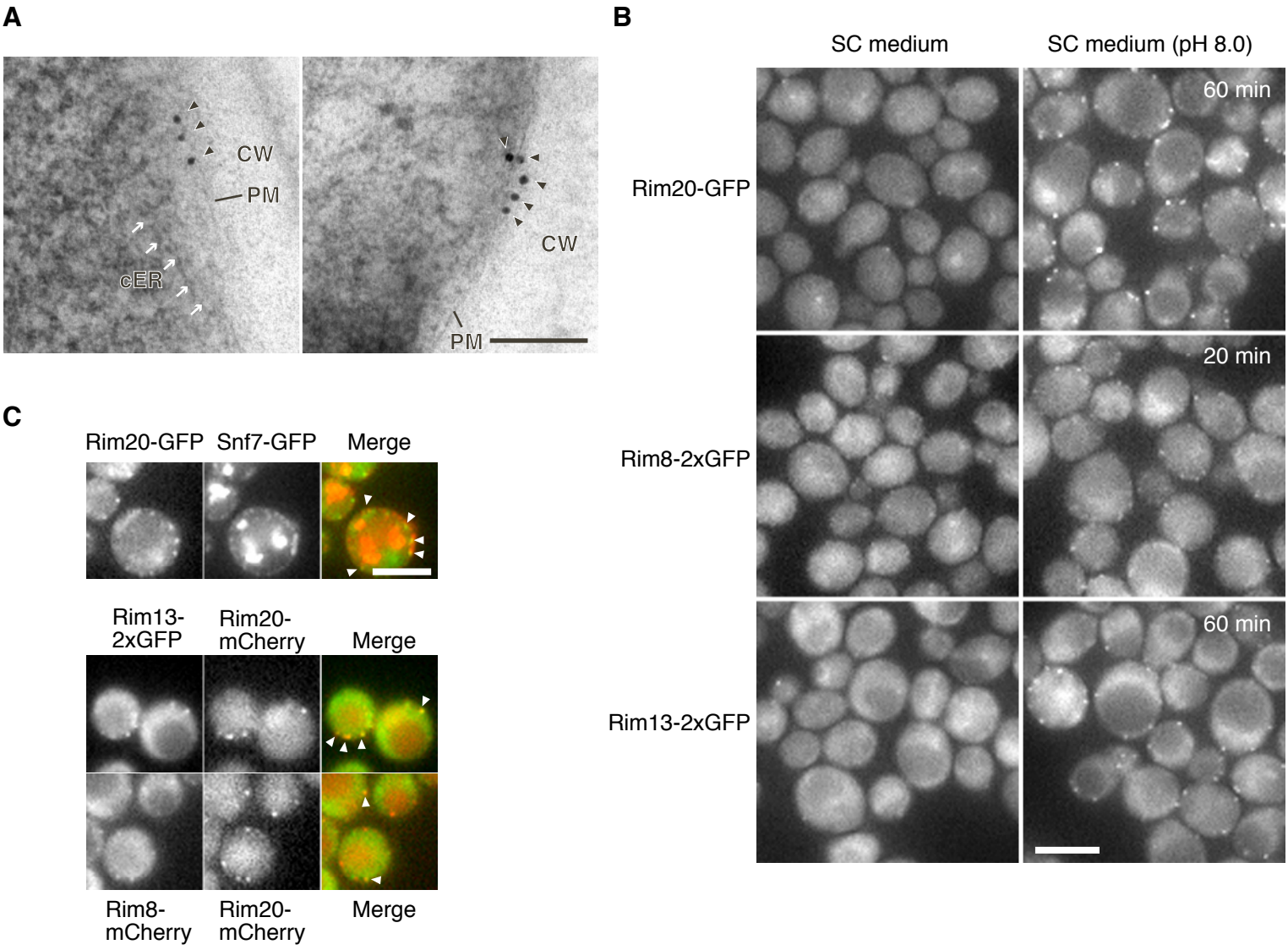


FIG S2 Rim20, Rim8, and Rim13 accumulate at the plasma membrane. (A) YOK3248 (*RIM20-GFP*) cells at log phase were exposed to alkaline medium for 90 min and subjected to immunoelectron microscopy using an anti-GFP antibody (see SUPPLEMENTAL METHOD). CW, cell wall; PM, plasma membrane; cER, cortical ER; 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 μm. (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 μm.

Figure S3

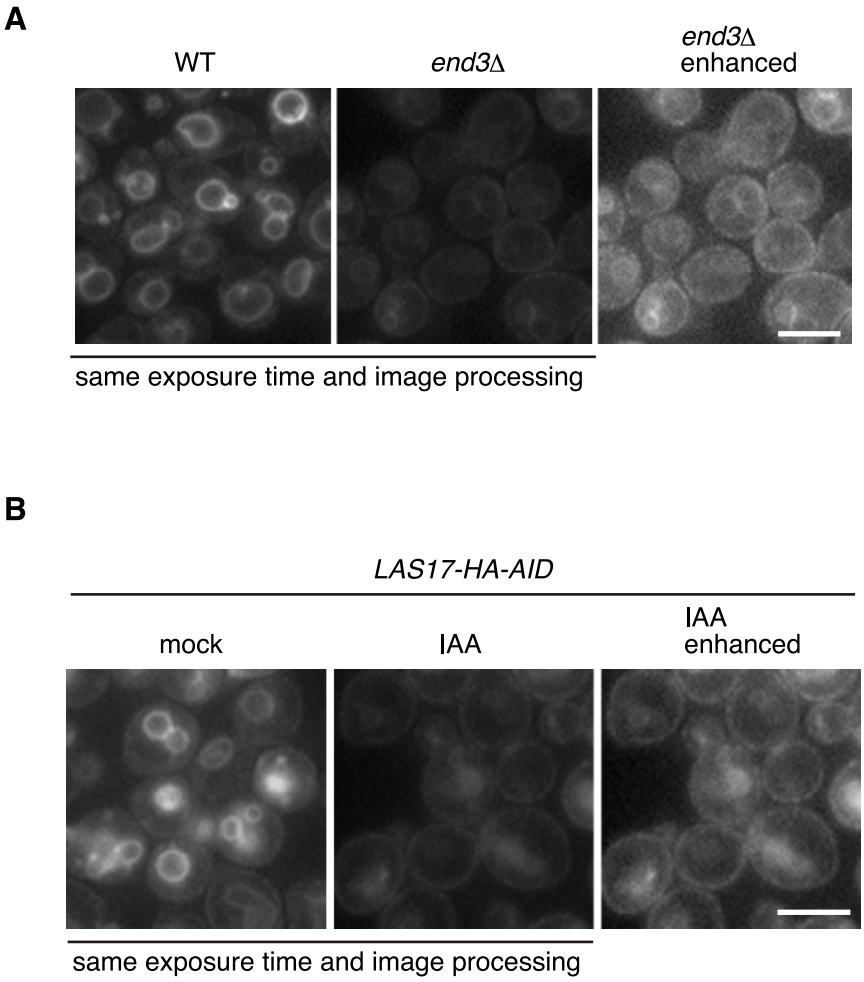


FIG S3 Endocytosis is impaired in *end3*Δ and IAA-treated *LAS-HA-AID* cells. (A) BY4741 (WT) and MYY312 (*end3*Δ) cells in log phase were loaded with 1 μM FM4-64 for 15 min, then were washed and resuspended with SC medium, and further incubated for an additional 15 min. Sodium azide was added to a final 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 μm. (B) YOK3159 (*LAS17-HA-AID*) cells in log phase were treated with 500 μM 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 μM IAA or ethanol. Bar, 5 μm.

Figure S4

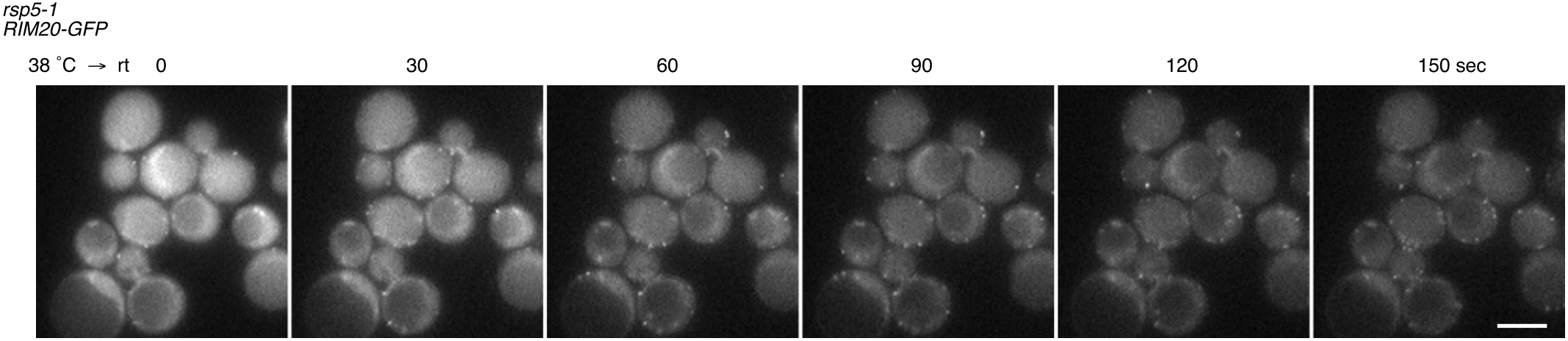


FIG S4 Rim20 foci at the plasma membrane are restored during examination of *rsp5-1* cells that were cultured at a restrictive temperature. YOK3343 (*rsp5-1 RIM20-EGFP*) cells were cultured at 30 ˚C to log phase, then transferred to 38 ˚C for 90 min, and exposed to alkaline medium at 38 ˚C. After 60 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 μm.

Figure S5

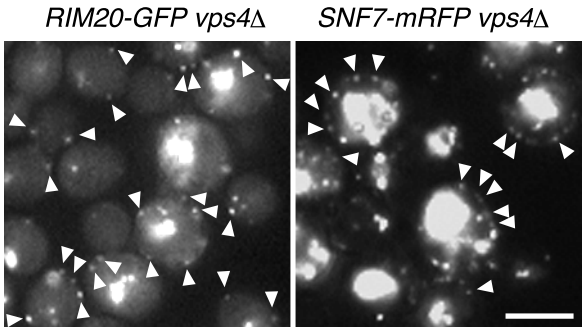


FIG S5 Rim20 and Snf7 accumulate at the plasma membrane in addition to the late endosome in *vps4*Δ cells even without external alkalization. YOK3266 (*RIM20-EGFP vps4*Δ) and YOK3598 (*SNF7-mRFP vps4*Δ) cells in log phase were subjected to fluorescence microscopy. Arrowheads, Rim20 and Snf7 foci at the plasma membrane. Bar, 5 μm.