cis- and trans-Acting Localization Determinants of pH Response Regulator Rim13 in Saccharomyces cerevisiae

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The Rim101/PacC pathway governs adaptation to alkaline pH in many fungi. Output of the pathway is mediated by transcription factors of the Rim101/PacC family, which are activated by proteolytic cleavage. The proteolytic complex includes scaffold protein Rim20 and endosome-associated subunits of the endosomal sorting complex required for transport (ESCRT). We provide here evidence that Saccharomyces cerevisiae Rim13, the protease that is implicated in Rim101 cleavage, is associated with the Rim20-ESCRT complex, and we investigate its regulation. Rim13-GFP is dispersed in cells grown in acidic medium but forms punctate foci when cells encounter alkaline conditions. A vps4Δ mutant, which accumulates elevated levels of endosomal ESCRT, also accumulates elevated levels of Rim13-GFP foci, independently of external pH. In the vps4Δ background, mutation of ESCRT subunit Snf7 or of Rim20 blocks the formation of Rim13 foci, and we found that Rim13 and Rim20 are colocalized. The Rim13 ortholog PalB of Aspergillus nidulans has been shown to undergo ESCRT and membrane association through an N-terminal MIT domain, but Rim13 orthologs in the Saccharomyces clade lack homology to this N-terminal region. Instead, there is a clade-limited C-terminal region, and we show that point mutations in this region prevent punctate localization and impair Rim13 function. We suggest that RIM13 arose from its ancestral gene through two genome rearrangements. The ancestor lost the coding region for its MIT domain through a 5′ rearrangement and acquired the coding region for the Saccharomyces-specific functional equivalent through a 3′ rearrangement.

The ability to adapt to diverse environmental conditions is critical for the survival of free-living microorganisms. The response to an environmental change is typically orchestrated by signal transduction pathways. Functional outputs may include both transcriptional and posttranscriptional changes that alter microbe physiology, establishing a state that improves growth under the altered environmental conditions (1, 9, 29).

Our focus is the adaptation of fungi to changes in environmental pH. A signal transduction pathway, the Rim101/PacC pathway, is broadly conserved among fungi (6, 23). It mediates diverse cellular behaviors that include salt tolerance and sporulation in Saccharomyces cerevisiae, secondary metabolite production in A. nidulans, and pathogenicity in Candida albicans, Aspergillus fumigatus, and Cryptococcus neoformans (6, 21–23). Many environmental response pathways comprise mitogen-activated protein kinase modules, but the Rim101/PacC pathway uses a different signaling mechanism. The key pathway output is the activation of a transcription factor, called Rim101 or PacC, by C-terminal proteolytic cleavage. The cleavage reaction is tied to endocytic vesicle metabolism. Recent observations indicate that other signaling pathways are also intimately connected to vesicle metabolism (19, 20). The Rim101/PacC pathway may serve as a model for this emerging regulatory paradigm.

The Rim101/PacC signaling pathway may be viewed as two modules (18). The upstream module is the “sensing complex,” which includes transmembrane proteins Dfg16, Rim21, and Rim9, along with the β-arrestin homolog Rim8. Signal recognition results in activation of Rim8 through phosphorylation and ubiquitination (11, 12). Activation of Rim8 stimulates function of the downstream module, which is the “proteolytic complex.” This complex includes scaffold protein Rim20, protease homolog Rim13, endosomal ESCRT, and nascent transcription factor Rim101. Rim20 binds to the Rim101 C-terminal region and promotes its cleavage by Rim13. The N-terminal region of Rim101 includes three zinc fingers. Rim101 functions as a transcriptional repressor in S. cerevisiae (16), while many Rim101/PacC proteins in other fungi are transcriptional activators (23, 24).

The functional connection of the proteolytic complex to endocytic vesicles is indicated by four lines of evidence. First, Rim101 cleavage depends upon several endocytic vesicle proteins, including Snf7, that are subunits of the ESCRT complex (10, 15, 26, 32). Second, Rim101 cleavage becomes independent of the upstream sensing complex in strains that hyperaccumulate ESCRT complexes, such as vps4Δ mutants (10, 15, 26, 32). Third, two-hybrid studies indicate that Rim20 and Rim13 can each interact with Snf7 (14). Fourth, Rim20-GFP is localized to punctate structures when the Rim101 pathway is activated by alkaline conditions (3), and these structures have properties of endocytic vesicles: they are stained by the lipophilic dye FM4-64, they are labeled by Snf7-RFP, and they hyperaccumulate in a vps4Δ mutant strain. In addition, punctate localization of Rim20-GFP depends upon Snf7 and several other ESCRT subunits. Therefore, activation of the Rim101 proteolytic complex is thought to occur through association of Rim20 with the ESCRT complex on endocytic vesicle surfaces.

Rim13 was first thought to associate with the Rim20-ESCRT complex because it interacts with Snf7 in two-hybrid assays (14). Strong evidence in support of this association comes from a recent study of the A. fumigatus Rim13 ortholog, PalB (25). PalB has an N-terminal MIT domain, a feature found in ESCRT-interacting
proteins (13). The study showed that PalB is membrane associated and that it interacts directly with ESCRT subunit Vps24. Both of these interactions depend upon the PalB MIT domain. In addition, PalB function is partially impaired by an MIT domain deletion, and full function of PalB lacking its MIT domain was recovered when it was expressed as a protein fusion to Vps24. This fusion restores membrane association as well, likely by restoring association with the ESCRT complex. This study clearly established a key role for the PalB MIT domain in Rim101/PacC pathway signaling and in PalB-ESCRT association.

Rim13 is distinct from PalB in lacking an MIT domain. In addition, prior two-hybrid and coimmunoprecipitation studies indicate that Rim13 interacts with Snf7 rather than Vps24 (14, 30). Here, we have used a combination of fluorescence microscopy and mutational analysis to define the localization of Rim13 and its functional determinants. Our results support the model that Rim13 is associated with the ESCRT complex and provide insight into the genetic and environmental regulation of Rim13-ESCRT association.

### MATERIALS AND METHODS

#### Strains, plasmids, and growth conditions

The *S. cerevisiae* strains and plasmids used in the present study are listed in Tables 1 and 2, respectively.

#### TABLE 1 Yeast strains used in this study

| Strain | Genotype | Source or reference |
|--------|-----------|---------------------|
| JBY46  | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rim13Δ0-GFP-HIS3MX6 | This study |
| JBY115 | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rim20-GFP-HIS3MX6 vps4Δ::URA3 | This study |
| JBY209 | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rim20-GFP-HIS3MX6 rim13Δ::KANMX4 | This study |
| SSY1   | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RIM13-GFP-HIS3MX6 | ATCC (BY4741) |
| SSY14  | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RIM13-GFP-HIS3MX6 vps4Δ::KANMX4 | This study |
| SSY22  | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RIM13-GFP-HIS3MX6 vps4Δ::KANMX4 | This study |
| SSY24  | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RIM13-GFP-HIS3MX6 vps4Δ::KANMX4 | This study |
| SSY25  | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RIM13-GFP-HIS3MX6 vps4Δ::KANMX4 | This study |
| SSY27  | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RIM13-GFP-HIS3MX6 vps4Δ::KANMX4 | This study |
| SSY28  | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RIM13-GFP-HIS3MX6 vps4Δ::KANMX4 | This study |
| SSY30  | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RIM13-GFP-HIS3MX6 vps4Δ::KANMX4 | This study |
| SSY32  | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RIM13-GFP-HIS3MX6 vps4Δ::KANMX4 | This study |
| SSY52  | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RIM13-GFP-HIS3MX6 vps4Δ::KANMX4 snf7Δ::LEU2 | This study |
| SSY61  | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RIM13-GFP-HIS3MX6 vps4Δ::KANMX4 rim101Δ::KANMX4 | This study |
| SSY69  | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RIM13-GFP-HIS3MX6 vps4Δ::KANMX4 rim101Δ::KANMX4 | This study |
| SSY81  | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RIM13-GFP-HIS3MX6 vps4Δ::KANMX4 | This study |
| SSY83  | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RIM13-GFP-HIS3MX6 vps4Δ::KANMX4 | This study |
| SSY109 | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RIM13-GFP-HIS3MX6 vps4Δ::KANMX4 | ATCC; Yeast Ko Bank |
| SSY110 | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RIM13-GFP-HIS3MX6 vps4Δ::KANMX4 | ATCC; Yeast Ko Bank |

#### TABLE 2 Plasmids used in this study

| Plasmid | Construct details | Source or reference |
|---------|-------------------|---------------------|
| pPS46   | pRS316            | 3                   |
| pPS22   | pJB11 (URA3-V5-RIM101) | 2                   |
| pPS36   | RIM13 promoter-RIM13-GFP-ADH1 terminator,500 in pRS316 at BamHI site | This study |
| pPS40   | RIM13 promoter-RIM13-GFP-ADH1 terminator,500 in pRS316 at BamHI site | This study |
| pPS73   | RIM13 promoter-RIM13-GFP-ADH1 terminator,500 in pRS316 at BamHI site | This study |
| pPS74   | RIM13 promoter-RIM13-GFP-ADH1 terminator,500 in pRS316 at BamHI site | This study |
| pPS75   | RIM13 promoter-RIM13-GFP-ADH1 terminator,500 in pRS316 at BamHI site | This study |
| pPS76   | RIM13 promoter-RIM13-GFP-ADH1 terminator,500 in pRS316 at BamHI site | This study |
| pPS77   | RIM13 promoter-RIM13-GFP-ADH1 terminator,500 in pRS316 at BamHI site | This study |
| pPS78   | RIM13 promoter-RIM13-GFP-ADH1 terminator,500 in pRS316 at BamHI site | This study |
| pPS79   | RIM13 promoter-RIM13-GFP-ADH1 terminator,500 in pRS316 at BamHI site | This study |
| pPS80   | RIM13 promoter-RIM13-GFP-ADH1 terminator,500 in pRS316 at BamHI site | This study |
| pPS81   | RIM13 promoter-RIM13-GFP-ADH1 terminator,500 in pRS316 at BamHI site | This study |
| pPS82   | RIM13 promoter-RIM13-GFP-ADH1 terminator,500 in pRS316 at BamHI site | This study |
| pPS84   | RIM13 promoter-RIM13-GFP-ADH1 terminator,500 in pRS316 at BamHI site | This study |
| pPS85   | RIM13 promoter-RIM13-GFP-ADH1 terminator,500 in pRS316 at BamHI site | This study |
| pPS86   | RIM13 promoter-RIM13-GFP-ADH1 terminator,500 in pRS316 at BamHI site | This study |
| pPS87   | RIM13 promoter-RIM13-GFP-ADH1 terminator,500 in pRS316 at BamHI site | This study |
| pPS88   | RIM13 promoter-RIM13-GFP-ADH1 terminator,500 in pRS316 at BamHI site | This study |
| pPS89   | RIM13 promoter-RIM13-GFP-ADH1 terminator,500 in pRS316 at BamHI site | This study |
| pPS90   | RIM13 promoter-RIM13-GFP-ADH1 terminator,500 in pRS316 at BamHI site | This study |
| pPS91   | RIM13 promoter-RIM13-GFP-ADH1 terminator,500 in pRS316 at BamHI site | This study |
All strains were created in the BY4741 background, and liquid cultures and plates were incubated at 30°C unless noted otherwise.

Strains SSY1 and SSY18, bearing Rim13-GFP-His3MX6 in wild-type (WT) and \( \text{vps4} \Delta \) backgrounds, respectively, were created using a previously described method (17) that tags green fluorescent protein (GFP) to the C termini of open reading frames in the genome. Full-length and truncated versions of Rim13-GFP-3×FLAG, strains SSY22 to SSY32, were generated using primer-design and selection adapted from a published protocol (7).

The Rim13-GFP fusion constructs on plasmids were generated by PCR amplifying genomic Rim13-GFP from SSY1 including 500 bp of upstream promoter region followed by gap repair into the BamHI site of plasmids pRS515 or pRS316. A total of 500 bp of ADH1 terminator was subcloned downstream of the GFP stop codon in both plasmids (pSS36 and pSS40, respectively). Plasmids containing constructs with amino acids 650 to 727 and amino acids 700 to 727 of Rim13 fused to GFP were subcloned by gap repair into pSS40, replacing the full-length reading frame with specific 3′ sequences as indicated above. The Rim13-tdTomato-bearing plasmid, pSS88, was generated by replacing GFP in plasmid pSS36 with PCR-amplified tdTomato (Clontech, Mountain View, CA) via gap repair (27, 28).

Serial alanine-scanning point mutations of two adjacent amino acids in the C-terminal sequence of Rim13 were generated by PCR mutagenesis. Specifically, two sets of PCRs were performed to amplify the regions upstream and downstream of the mutation independently, with slight overlap at the ends. Overlap primers, which included the mutant sequence that changes the specified amino acid pair into an alanine pair, were designed such that the 3′ end of the reverse primer of the upstream PCR was complementary to the 5′ end of the forward primer of the downstream PCR. The upstream and downstream PCR products were purified and mixed in equal volume. These products recombined in \textit{vivo} and were used as a template for a secondary PCR, yielding the full-length gene product. The PCR product and plasmid were recombined by gap repair. Expected sequences of all of the point mutation constructions were confirmed by DNA sequencing. Plasmid pSS89, bearing the truncated Rim101-511 and including 500 bp 5′ and 3′ of the start codon, was created by overlap PCR, as discussed above, with the overlap primers, including the truncation region with genomic DNA as a template.

\textbf{pH shift microscopy assays.} Strains were grown overnight at 30°C in YPD or selective medium (MPBio, Solon, OH [8]) as appropriate, diluted, and then grown for two or three doublings to obtain an early-log-phase culture with an optical density at 600 nm (OD\(_{600}\)) between 0.4 and 0.6. Then, 200 \( \mu \)l of these cells was placed on concanavalin A (1-mg/ml stock solution; Sigma, St. Louis, MO)-coated 35-mm glass-bottom culture dishes (MatTek, Ashland, MA). After allowing for 5 to 10 min of adherence to the glass bottom at 30°C, the cells were washed with prewarmed complete synthetic medium (CSM), followed by the addition of filter-sterilized CSM-Leu medium (8), which is selective for the Rim13 fusion plasmid, and then diluted and grown for two to three doublings to obtain an early-log-phase culture (OD\(_{600}\) of ca. 0.4 to 0.6). Next, 200 \( \mu \)l of these cells was placed on a concanavalin A-coated 35-mm glass-bottom culture dish and allowed to adhere for 5 to 10 min at 30°C. After adherence, the cells were washed with 5 ml of prewarmed CSM-Leu, followed by the addition of filter-sterilized CSM-Leu for live imaging. Imaging was performed under a Zeiss LSM 510 Meta/DuoScan inverted spectral confocal microscope using a Plan-Apochromat 100×/1.4NA oil immersion objective lens. Two laser lines of 488 and 561 nm were used for excitation. z-stack images at 0.5-\( \mu \)m intervals were acquired using Zen 2009 software with simultaneous acquisition of both channels (green and red fluorescence) with appropriate filter sets. z-stacks were then processed using ImageJ with uniform background subtraction and summing stacks to a single composite image. A single pixel-wide line was drawn across selected fluorescence foci for both channels, and colocalization of GFP and tdTomato fluorescence was quantified using the Plot Profile plugin feature on ImageJ. The fluorescence intensity units (gray value) were plotted on a standard graph to identify overlapping fluorescence intensities for Rim20-GFP and Rim13-tdTomato.

\textbf{Immunoblots.} Cells transformed with plasmid pKJB11 (2), specifying the URA3-V5-RIM101 gene under the control of the RIM101 native 5′ region, were grown overnight in CSM diluted to obtain to early-log-phase culture with an OD\(_{600}\) between 0.4 and 0.6. The cells were collected, resuspended in 100 \(\mu \)l of 0.2 M NaOH and 100 \(\mu \)l of H\(_2\)O, and incubated at room temperature for 30 min. An equal volume of reducing sample buffer was added, vortexed with glass beads, and boiled for 5 min. After centrifugation, an equal volume of the supernatant was fractionated on a SDS–10% PAGE gel and transferred to nitrocellulose membrane. The filter was probed with mouse anti-V5 antibody (Invitrogen, Carlsbad, CA; 1:3,000 dilution in TBST), followed by anti-mouse horseradish peroxidase-conjugated antibody (Invitrogen; 1:3,000 dilution in 3% nonfat dry milk in TBST), and was developed with chemiluminescence detection reagents.
A similar protocol was used for all immunoblots. Anti-GFP, N Terminal (Sigma; 1:1,000 dilution in nonfat dry milk in TBST) and anti-FLAG (Sigma; 1:3,000 dilution in nonfat dry milk in TBST) immunoblots were performed according to the protocol described above.

Quantitative reverse transcription-PCR. Three independent transformants of JBY209 (rim13Δ) cells transformed with pSS46, pSS40, pSS76, or pSS77 were grown overnight in CSM-Ura, diluted into fresh YPD to an OD₆₀₀ of 0.2, and grown at 30°C with shaking to an OD₆₀₀ of 0.75. Equal amounts of cells were harvested by filtration, flash-frozen, and stored at −80°C. RNA isolation was performed by using the Qiagen RNeasy minikit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. A total of 1 µg of DNase-treated RNA was used to synthesize cDNA using Bio-Rad iScript reverse transcription Supermix (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. The final reaction volume of 20 µl was diluted to 50 µl, and 6 µl was used for quantitative real-time PCR (equivalent of 0.12 µg of starting RNA) using the Bio-Rad iQ SYBR green Supermix (Bio-Rad). Primers located at the 3′ end of genes NRG1 (forward, CGGAAGCAAAAGACAGATCC; reverse, TTGCAAGCAATTATCATGTGC), RIM101 (forward, TGAGTAAAGCTG GCGATTGAA; reverse, TGGTCAAGATGCGAACTGAG), SMP1 (forward, ACAGGTGTACAGCCACTCC; reverse, GGTGTTCCGGAATT TGGC), and YPL277c (forward, GAAAAGCAATTGGACATGG; reverse, TGTGAGTGCTGTTGCATTGA) were designed using Primer3 (http://frodo.wi.mit.edu/primer3/) and used in the reaction described above. Transcript levels were normalized against TDH3 expression (forward, GTCGATGTCTCCGTTGTTGA; reverse, ACCAGGAGACCAACT TGACG), and gene expression changes were calculated by the ΔΔCₚ method. The data analysis was conducted using Bio-Rad iQ5 software 2.0.

RESULTS

Rim13 localization. The protease homolog Rim13 is required for C-terminal cleavage and activation of Rim101, a critical regulator of adaptation to alkaline pH growth conditions. In order to understand how Rim13 may be regulated, we visualized its accumulation and localization with a genomic RIM13-GFP fusion allele. The allele was functional, because the strain did not display sensi-
tivity to NaCl and LiCl seen with rim13 mutants (data not shown). Cells grown in acidic medium (pH 4) displayed a faint uniform GFP signal (Fig. 1A). Cells shifted to alkaline medium (pH 8.3) displayed occasional GFP foci (Fig. 1B). These observations indicate that Rim13 undergoes localization to punctate structures after cells encounter alkaline conditions.

Stress responses can be transient because they promote adaptation that alleviates stress. We reasoned that Rim13 localization responses may be transient and thus quantified cells with Rim13 foci after a shift from acidic to alkaline conditions (Fig. 2A). The fraction of cells with Rim13-GFP foci increased within 2 min after exposure to alkaline conditions and continued to rise until 8 min. The fraction of cells with foci then dropped to a steady-state level (~10% of cells) that was slightly greater than in acidic conditions. The Rim13 foci seemed similar to Rim20 foci that appear in cells in alkaline media (3). In fact, a time course analysis of Rim20-GFP localization revealed that the initial kinetics of accumulation of Rim20-GFP and Rim13-GFP foci are similar (Fig. 2A and B).

**FIG 3** Rim13-GFP localization dependence on Rim20 and Snf7. (A) Log-phase vps4Δ Rim13-GFP, snf7Δ vps4Δ Rim13-GFP, and rim20Δ vps4Δ Rim13-GFP cells grown in YPD were imaged in CSM. Scale bar, 5 μm. (B) The numbers of cells displaying fluorescent foci were quantified.

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**FIG 4** Control of Rim13 localization by Rim101 function. (A) RIM13-GFP cells were compared to rim101Δ RIM13-GFP cells at 10 min after a shift from YPD to CSM plus 0.1 M HEPES (pH 8.3) through fluorescence microscopy. (B) The number of cells displaying fluorescent foci after the shift in panel A was quantified. (C) Complementation analysis was performed by comparing rim101Δ RIM13-GFP cells carrying an empty vector or a RIM101-511 construct at 10 min after a shift from YPD to CSM plus 0.1 M HEPES (pH 8.3) by fluorescence microscopy. (D) The number of cells displaying fluorescent foci after the shift in panel C was quantified. Scale bar, 5 μm.
However, Rim20-GFP foci appear in a larger fraction of cells, and their steady-state levels persist for longer than Rim13-GFP foci. Therefore, both Rim13 and Rim20 foci are induced by alkaline conditions, but Rim13 foci are more transient than Rim20 foci.

We used two approaches to further examine the relationship between Rim13 and Rim20 foci: we compared their genetic requirements, and we sought to determine whether the foci colocalize. Previous studies have shown that Rim20 foci accumulate at elevated levels, independently of external pH, in a vps4Δ mutant background (3). Vps4 is required for disassembly of the endosome-associated ESCRT complex with which Rim20 associates, so the accumulation of Rim20 foci in the vps4Δ mutant presumably represents the increased abundance of ESCRT complexes. We observed that Rim13-GFP foci also accumulate at elevated levels in a vps4Δ mutant when grown at acidic or alkaline pH (Fig. 1C and D). The ESCRT-III subunit Snf7 is required for Rim20 foci to form, and we observed that levels of Rim13-GFP foci were greatly reduced by an snf7Δ mutation in the vps4Δ background (Fig. 3). In addition, the levels of Rim13-GFP foci were greatly reduced by a rim20Δ mutation in the vps4Δ background (Fig. 3). Therefore, Rim13 and Rim20 foci are regulated in parallel by Vps4 and Snf7.

Previous studies indicate that Rim101 is formally a negative regulator of Rim20 foci (3). To determine whether this relationship extends to Rim13, we introduced a rim101Δ mutation into a Rim13-GFP strain. The rim101Δ mutation caused Rim13-GFP foci to accumulate at elevated levels after a shift to alkaline pH (Fig. 6A and B). This phenotype was reversed by introduction of an activated Rim101-511 allele (Fig. 4C and D), a functional Rim101 derivative lacking the Rim20-binding region (31). This result indicates that Rim101 functions to reduce accumulation of Rim13-GFP foci. The negative regulation of Rim13 and Rim20 foci by Rim101 is another parallel feature of their genetic control.

To determine whether Rim13 and Rim20 may reside in the same complex, we visualized a Rim13-tdTomato fusion in a Rim20-GFP vps4Δ strain. In most cells, the Rim20-GFP signal (Fig. 2C) overlapped substantially with the Rim13-tdTomato signal (Fig. 2D and E). Quantitative scans of several regions confirmed that the signals overlapped considerably (Fig. 2F). These results indicate that many Rim13 foci correspond to the same structures as Rim20 foci in a vps4Δ strain.

**Structural determinants of Rim13 localization.** The Rim13 sequence includes an N-terminal cysteine protease domain and a large C-terminal region that lacks functionally informative homology. To determine whether the latter region may be required for biological function, we characterized a series of C-terminal truncation derivatives (Fig. 5A). Each derivative had an epitope tag that permitted verification of their accumulation (Fig. 5C). No Rim13 truncation derivative was functional, as assayed by processing a Rim101 reporter (Fig. 5B) or by the ability to grow on high-salt medium (Fig. 5D). This result indicates that Rim101 functions to reduce accumulation of Rim13-GFP foci. The negative regulation of Rim13 and Rim20 foci by Rim101 is another parallel feature of their genetic control.

To define key residues required for Rim13 localization, we created alanine substitution derivatives for pairs of residues within the segment from residues 700 to 727 in the context of a Rim13-GFP strain. Each derivative had an epitope tag that permitted verification of their accumulation (Fig. 5C). No Rim13 truncation derivative was functional, as assayed by processing a Rim101 reporter (Fig. 5B) or by the ability to grow on high-salt medium (Fig. 5D). This result indicates that Rim101 functions to reduce accumulation of Rim13-GFP foci. The negative regulation of Rim13 and Rim20 foci by Rim101 is another parallel feature of their genetic control.

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**FIG 5** Functional role of the Rim13 C-terminal region. A rim13Δ strain carrying Rim13 truncation derivatives, each with a C-terminal 3×FLAG tag, was assayed for Rim13 expression and function. (A) A diagram depicts the C-terminal truncation derivatives. (B) Cells carrying each Rim13 truncation were transformed with a URA3-V5-RIM101 processing reporter plasmid (2), were grown overnight in YPD, diluted and grown to log phase, harvested, lysed, and assayed by immunoblotting with anti-V5 antibody. (C) Anti-FLAG antibody immunoblots were performed on the same extracts as in panel B to detect Rim13 derivatives. The asterisks indicate background proteins that are independent of any introduced FLAG tag. (D) Growth of serial dilutions of each strain on YPD plates with 1 M NaCl was used to determine Rim13 function. In panels B, C, and D, the samples are displayed as follows: lane 1, WT; lane 2, rim13Δ; lane 3, Rim131-550-3Δ; lane 4, Rim131-500-3Δ; lane 5, Rim131-450-3Δ; lane 6, Rim131-400-3Δ; lane 7, Rim131-350-3Δ; lane 8, Rim131-300-3Δ; lane 9, full-length Rim131-3×FLAG.
wild type, as indicated by an anti-GFP Western blot (Fig. 7C). We then examined localization of each fusion and quantified the fraction of cells displaying punctate localization. Mutations in two adjacent segments, L707-F708, and V709-G710, caused a significant decrease in the numbers of Rim13-GFP foci (Fig. 7B). We then examined localization of each fusion and quantified the fraction of cells displaying punctate localization. Mutations in two adjacent segments, L707-F708, and V709-G710, caused a significant decrease in the numbers of Rim13-GFP foci (Fig. 7B). We then examined localization of each fusion and quantified the fraction of cells displaying punctate localization. Mutations in two adjacent segments, L707-F708, and V709-G710, caused a significant decrease in the numbers of Rim13-GFP foci (Fig. 7B). We then examined localization of each fusion and quantified the fraction of cells displaying punctate localization. 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Rim13 is dependent upon Rim20. This is the specific functional observation that suggests that, after cells encounter alkaline conditions, Rim20 is recruited to the ESCRT complex first, and Rim13 subsequently follows. Association of Rim13 with Rim20-ESCRT may be governed by a regulated event or process in addition to Rim20-ESCRT binding, because Rim13 foci appear transiently after an alkaline shift, whereas the presence of Rim20 foci is sustained. This finding can explain our observation that Rim20-ESCRT association, promoted by absence of Bro1, is not sufficient to permit Rim101 processing in the absence of sensing complex activity (4). Specifically, our data show that Rim20 foci accumulate at much higher levels than Rim13 foci, thus arguing that Rim20-ESCRT association is not sufficient for proteolytic complex formation.

Rim101 itself is likely to be a component of the proteolytic complex, because it binds to Rim20 and is presumably the substrate for Rim13 (6, 18, 23). However, Rim101 is not required for the formation of Rim13 foci. Rather, Rim13 foci hyperaccumulate in a rim101Δ mutant. Restoration of normal levels of Rim13 foci by Rim101-511, a functional Rim101 derivative lacking the Rim20-binding region (31), suggests that it is Rim101 function, rather than Rim101-proteolytic complex interaction, that governs levels of proteolytic complex accumulation. For example, Rim101 may promote an adaptation to alkaline pH that leads to reduced proteolytic complex accumulation.

The sequences that mediate the localization of Rim13 are distinct from those of its A. nidulans ortholog PalB. In fact, Rim13 lacks an N-terminal segment homologous to the PalB MIT domain, and PalB lacks a C-terminal segment homologous to the Rim13 localization sequence. Inspection of syntenic relationships among ascomycetes, using the Yeast Gene Order Browser (5), provides a rationale for this peculiar Rim13-PalB relationship. The rationale stems from the impact of genome rearrangements underlying Rim13 phylogeny. In the proposed common ancestor of the ascomycetes, Rim13 is flanked by YDL089W and YDL090C. In S. cerevisiae, Rim13 is flanked by YMR153W and YMR155W, which are not homologous to YDL089W and YDL090C. This comparison suggests that the Rim13 locus underwent both 5' and 3' rearrangements during evolution of S. cerevisiae. We can imagine that the 5' rearrangement occurred first, resulting in loss of the MIT domain. Penalva and coworkers have shown that PalβΔMIT retains considerable functional activity (25), so this intermediate Rim13-like gene may have retained partial function. The 3' rearrangement may then have provided a sequence that strengthened interaction of Rim13 with Rim20-ESCRT, thus bypassing the need for an MIT domain.

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