Relationship of DFG16 to the Rim101p pH Response Pathway in Saccharomyces cerevisiae and Candida albicans†

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Many fungal pH responses depend upon conserved Rim101p/PacC transcription factors, which are activated by C-terminal proteolytic processing. The means by which environmental pH is sensed by this pathway are not known. Here, we report a screen of the Saccharomyces cerevisiae viable deletion mutant library that has yielded a new gene required for processed Rim101p accumulation, DFG16. An S. cerevisiae dfg16Δ mutant expresses Rim101p-repressed genes at elevated levels. In addition, Candida albicans dfg16Δ/dfg16Δ mutants are defective in alkaline pH-induced filamentation, and their defect is suppressed by expression of truncated Rim101-405p. Thus, Dfg16p is a functionally conserved Rim101p pathway member. Many proteins required for processed Rim101p accumulation are members of the ESCRT complex, which functions in the formation of multivesicular bodies (MVBs). Staining with the dye FM4-64 indicates that the S. cerevisiae dfg16Δ mutant does not have an MVB defect. We find that two transcripts, PRY1 and ASN1, respond to mutations that affect both the Rim101p and MVB pathways but not to mutations that affect only one pathway. The S. cerevisiae dfg16Δ mutation does not affect PRY1 and ASN1 expression, thus confirming that Dfg16p function is restricted to the Rim101p pathway. Dfg16p is homologous to Aspergillus nidulans PalH, a component of the well-characterized PacC processing pathway. We verify that the previously recognized PalH homolog, Rim21p, also functions in the S. cerevisiae Rim101p pathway. Dfg16p is predicted to have seven membrane-spanning segments and a long hydrophilic C-terminal region, as expected if Dfg16p were a G-protein-coupled receptor.

The recognition of environmental cues and presentation of an appropriate response are central to the survival of microorganisms. The range of possible responses is broad and may affect metabolic activities, organelle biogenesis, cell division, or differentiation. For pathogens, environmental response pathways are typically critical for virulence. Our interests are in how diverse responses are coordinated and how coordination mechanisms may have evolved.

For the yeast Saccharomyces cerevisiae, the environmental pH affects growth as well as differentiation to permit invasive growth or meiotic sporulation. Among gene products that are required for adaptation to alkaline pH, haploid invasive growth, and sporulation is the zinc finger transcription factor Rim101p (19, 22, 31, 32). Microarray analysis and chromatin immunoprecipitation studies (18) have shown that S. cerevisiae Rim101p functions as a repressor through the target site TG...
is required to promote invagination of the limiting vesicular membrane to create an MVB. Eight ESCRT subunits (Snf7p/Vps32p, Vps20p, Snf8p/Vps22p, Vps25p, Vps36p, Vps23p, Vps26p, and Vps37p), which form what has been called the core ESCRT complex (3), function in both MVB formation and Rim101p processing (17, 37). Other proteins required for MVB formation and trafficking, including Vps27p, Vps2p, Vps24p, Vps4p, Bro1p, Doa4p, and Vps60p, are not required for Rim101p processing (17, 37). Two-hybrid studies (13) and functional analysis (35, 37) have led to the model that the core ESCRT subunits may bridge the interaction between the protease Rim13p and the substrate complex Rim20p-Rim101p (37).

Here, we report the characterization of a new gene that is required for Rim101p processing in S. cerevisiae. Its role is conserved, as evidenced by analysis of its C. albicans homolog. Our findings provide new insight into the Rim101p/PacC pathway and its relationship to ESCRT subunit function.

MATERIALS AND METHODS

Strains and media. The haploid S. cerevisiae deletion strain libraries were derived from the parental strain, BY4741 (MATa his3Δ1 lys2Δ0 met15Δ0 ura3Δ0) and BY4742 (MATa his3Δ1 lys2Δ0 met15Δ0 ura3Δ0), were purchased from Invitrogen (Carlsbad, CA). Strain YKB167 was derived from the RIM101-HA2 epo- tagged strain WXY169 (36). The dfg16Δ::kanMX4 yeast deletion clone (Invitrogen) was introduced by PCR product-directed gene disruption using genomic DNA from the dfg16Δ:: kanMX4 yeast deletion clone (Invitrogen) as a template along with the primers TCT TTT TGT TTT TTC GGG GTG (forward) and TGC CAG AAT GAC GGA TTA TTA TAT ATC GGG TTT TGT TAG GAC GAA (reverse). All C. albicans strains were derived from strain BWP17 (uras3::XmnI43 ura3Δ::XmnI43 his1::HisG his1::HisG arg1::HisG ura3::HisG) through standard transformation methods (35). The dfg16Δ::URA3/dfg16Δ::ARG4 strain, KBC033, was generated by PCR product-directed gene disruption using the primers AGA ACG AGA TCG AAA AAG TAA TAA CAA CTA CTT TCC TAC TCG ACA CGT T (forward) and AAG CTA CAC AAA TAA TTG CTC TTG CAC GAG CAC TAA AAG AAA TAA TAC CTA CAA CTA CTT TCC TAC TCG ACA CGT AGG (reverse) and was verified by PCR using the primer pair ATT TCT GGT TAC AAT ATG CAG GGA TGT GAA TTC CCA (reverse); for

Transformation methods (35). The dfg16Δ::URA3/dfg16Δ::ARG4 strain, KBC033, was generated by PCR product-directed gene disruption using the primers AGA ACG AGA TCG AAA AAG TAA TAA CAA CTA CTT TCC TAC TCG ACA CGT T (forward) and AAG CTA CAC AAA TAA TTG CTC TTG CAC GAG CAC TAA AAG AAA TAA TAC CTA CAA CTA CTT TCC TAC TCG ACA CGT AGG (reverse) and was verified by PCR using the primer pair ATT TCT GGT TAC AAT ATG CAG GGA TGT GAA TTC CCA (reverse); for the construction and characterization of this fusion gene will be reported elsewhere (W. Xu and A. P. Mitchell, unpublished results).

Suppression studies were carried out using plasmids pDDBD1 (RIM101) and pDDBD1 (RIM101-405) as described previously (4).

β-Galactosidase assays. A 0.45-μm 85-nm nitrocellulose membrane (Millipore Corporation, Bedford, MA) was placed on a selective plate and a YPD plate for replica plating of each of the plates of transformations before incubation overnight at 30°C. Membranes were removed from the plates and placed at –80°C for 1 h to permeabilize the cells. Disks of 2.5 mm filter paper (Whatman) were soaked in 3 ml Z buffer (60 mM NaHPO4 [anhydrous], 60 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4) containing 35 μl 5-bromo-4-chloro-3-indoly β-D-galactoside (50 μg μl–1 stock solution in dimethylformamide), and the membranes were placed on top. Membranes were incubated for 1 h at 30°C. The reaction was stopped by removing the membranes from the filter paper, and results were scored immediately.

Immunodots. Cells were grown overnight in selective medium at 30°C and used to inoculate YPD at an optical density at 600 nm (OD600) of 0.25. After two doublings, cells were pelleted, resuspended at an OD600 of 50 in 3× Laemmli buffer, vortexed with glass beads, and boiled for 5 min. After centrifugation, 20 to 60 μl of the supernatant was fractionated on a 9% sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose. For V5 epo detection, the filter was probed with anti-V5-horseradish peroxidase antibody (Invitrogen) (1:5,000 dilution in phosphate-buffered saline-Tween). For hemagglutinin (HA) epitope detection, the filter was probed with anti-β-human papillomavirus peroxidase antibody (3F10; Roche Diagnostics, Indianapolis, IN) (1:10,000 dilution in phosphate-buffered saline-Tween). Peroxidase activity was visualized using ECL detection reagents (Amersham, Piscataway, NJ).

Microarrays and Northern blotting were performed as previously described (18). Analysis was performed using the Affymetrix Microarray Suite, version 5, analysis program. Data were manipulated with Microsoft Excel worksheet functions.

Northern probes were generated by PCR using BY4741 genomic DNA as a template with the following oligonucleotide pairs: for PRY1, TGC AAG GCG TAC TGT ATG TCC (forward) and CGG GTG CTT AAC TAC TGA TGA (reverse); for ASNI, GAC ACT ATC ACT GCA TCC ACA (forward) and ATT TCA TCG TGA CTT TCA CC (reverse); for ENO1, CCA AGC ACT TCG TTA ACA ACA (forward) and GAA CTG CTA AAA ATG ATG (reverse); for the construction and characterization of this fusion gene will be reported elsewhere (W. Xu and A. P. Mitchell, unpublished results).

Membrane staining. Staining with N-(3-triethylammoniumpropyl)-4-(α-dihex- yaminophenyl-hexatrienyl)-pyridinium dibromide (FM4-64; Molecular Probes) followed the procedure previously described by Amerik et al. (1), with slight modifications. Cells were grown for two doublings to mid-log phase in 10 ml YPD medium at 30°C and then harvested and resuspended in 166 μl YPD to which 0.4 μl 16 mM FM4-64 in dimethyl sulfoxide was added. The tubes were wrapped in foil and incubated at 30°C for 20 min on a shaker. The cells were harvested, washed once with 200 μl YPD medium, resuspended in 200 μl YPD medium, and incubated at 30°C for 60 min on a shaker. Membrane staining was visualized immediately after the second incubation by fluorescence microscopy with a Nikon Eclipse E800 microscope equipped with a Plan Apo 100×/1.4 objective. Images were processed with Improvision software.
RESULTS

Screen for Rim101p repression and processing defects. To identify new genes that may function in the Rim101p pathway, we screened the *S. cerevisiae* haploid deletion strain library with a Rim101p-repressible reporter plasmid. The plasmid contains four PaC sites inserted between the upstream activation sequence and TATA regions of a *CYC1-lacZ* fusion (18). This *CYC1_pac-lacZ* reporter gene is expressed at much lower levels expected (18). The remaining 25 strains had deletions of genes included the deletion mutant lacking the corepressor Tup1p, as previously known Rim101p pathway genes and all eight deletion mutants lacking core ESCRT subunits. The group also included the deletion mutant lacking the corepressor Tup1p, as expected (18). The remaining 25 strains had deletions of genes not associated previously with the Rim101p pathway.

We used a URA3-V5-RIM101 fusion gene to determine whether any repression-defective deletion strains may be defective in Rim101p processing. This fusion gene consists of an epitope-tagged URA3-V5 gene fused in frame to *RIM101* codons 501 to 628, which specify the Rim101p C-terminal segment, and is expressed from the *RIM101* 5' region. Immunoblot showed that the wild-type strain contained both processed and unprocessed forms of Ura3-V5-Rim101p (Fig. 1A, lane 2), whereas a control rim20Δ strain contained only the unprocessed form of the protein (lane 3), thus indicating that Rim101p repression activity is not required for Rim101p processing.

Among the deletion strains, accumulation of Ura3-V5-

### TABLE 1. Results of *CYC1_pac-lacZ* reporter screen

| Name     | Alias       | ORF* | Description |
|----------|-------------|------|-------------|
| Rim101   | RIM1        | YHL027W | Transcriptional repressor, response to pH, sporulation, meiosis |
| Rim8     | PAL3        | YGL045W | Regulator of IME2, Rim101 pathway member |
| Rim9     | YMR063W     | YMR154C | Regulator of IME2, Rim101 pathway member, probably a transmembrane protein |
| Rim13    | CPE1        | YMR154C | Cysteine-type endopeptidase involved in Rim101p processing |
| Rim20    | YOR275C     | YOR275C | Regulator of IME2, Rim101 pathway member, scaffold protein that interacts with Rim20p and Snf7p |
| Rim21    | PAL2        | YNL294C | Regulator of IME2, Rim101 pathway member, probably a transmembrane protein |
| Spy22    | VPS22       | YCL008C | Vacuolar protein sorting, ESCRT-I |
| VPS28    | VPS28       | YPL065W | Vacuolar protein sorting, ESCRT-I |
| Spr2     | SRN10, VPS37| YLR119W | Vacuolar protein sorting, ESCRT-I |
| VPS36    | VAC2, VPL11, GRD12 | YLR417W | Vacuolar protein sorting, ESCRT-II |
| VPS25    | VPS25       | YJR102C | Vacuolar protein sorting, ESCRT-II |
| VPS20    | VPS20       | YLR002C | Vacuolar protein sorting, ESCRT-II |
| Snf7     | DID1, VPS32 | YLR025W | Vacuolar protein sorting, ESCRT-III |
| TUP1     | AAA1, AER2, AMM1, CRT4, CYC9, FLK1, RXO4, SFL2, UMR7 | YCR084C | General repressor of transcription (with Snf6p); mediates glucose repression |
| ATG21    | MAI1        | YPL100W | Autophagy-related vacuolar protein involved in processing/maturation |
| BRR1     | YPR057W     | RNA binding, spliceosome assembly |
| CIT1     | CS1, LYS6   | YNR010C | Citrate synthase |
| Cka2     | YOR063W     | Casein kinase II alpha subunit |
| Ckb1     | YGL019W     | Beta (38-kDa) subunit of protein kinase CK2 |
| DFG16    | ECM41, ZRG11| YOR030W | Defective in flocculent growth |
| Dla2     | FUN38, SWA3 | YOR080W | Digs into agar |
| DRS2     | YAL029C     | Integral membrane Ca\(^{2+}\)-ATPase |
| FUN12    | YAL035W     | GTPase activity, translation initiation factor activity |
| GPH1     | YPR160W     | Glycogen phosphorylase |
| GRR1     | CAT80, COT2, SSU2 | YJR090C | F-box protein component of the SCF ubiquitin-ligase complex |
| Ies6     | YEL044W     | Protein associates with INO80 chromatin remodeling complex under low-salt conditions |
| Sit4     | LGN4        | YDL047W | Similar to catalytic subunit of bovine type 2A protein phosphatase |
| SPE1     | ORD1, SPE10 | YKL184W | Ornithine decarboxylase |
| SPE2     | YOR063W     | S-Adenosylmethionine decarboxylase |
| SPE3     | YPL065W     | Putrescine aminopropyltransferase |
| Spt3     | YDR392W     | Subunit of the SAGA and SAGA-like transcriptional regulatory complexes |
| Srb8     | GIG1, NUT6, SSN5 | YCR081W | Negative regulation of transcription from PolII promoter |
| Ssn6     | CRT8, CYC8  | YBL112C | General repressor of transcription (with Cyc8p); also acts as part of a transcriptional coactivator complex that recruits the SWI/SNF and SAGA complexes to promoters |
| Taf14    | SWP29, TAF30, TFG3, ANCl | YPL129W | Subunit (30 kDa) of TFIIID, TFIF, and SWI/SNF complexes |
| THI6     | YPL214C     | Thiamine biosynthetic bifunctional enzyme |
| Ubr1     | PTR1        | YGR184C | Ubiquitin-protein ligase |
| VAC8     | YEB3        | YEL013W | Vacuolar membrane protein that interacts with Atg13p, required for cytoplasm-to-vacuole targeting (CVT) pathway |
| Ygr122w  | YGR122W     | YGR122W | Unknown |
| Ypr116w  | YPR116W     | YPR116W | Uncharacterized |

* ORF, open reading frame.
Rim101p forms fell into three categories (Fig. 1). In the first category, both unprocessed and processed forms were apparent. This group included drs2Δ, cka2Δ, dia2Δ, thi6Δ, atg21Δ, vac8Δ, ubr1Δ, ypr116wΔ, cit1Δ, fun12Δ, ckb1Δ, tup1Δ, grr1Δ, ssn6Δ, spe1Δ, spe2Δ, and spe3Δ deletion strains. These genes may be required for processed Rim101p repressor activity or DNA binding ability. In the second category, overall levels of Ura3-V5-Rim101p were low. This group included tag1Δ, spt3Δ, ygr122wΔ, gph1Δ, brr1Δ, srb8Δ, sir4Δ, and ies6Δ deletion strains. The low protein level may represent decreased transcription, translation, protein stability, or, perhaps, plasmid stability. The final category comprised strains that accumulated only unprocessed Ura3-V5-Rim101p. The dfg16Δ strain clearly had this property (Fig. 1A, lane 6). The ygr122wΔ and gph1Δ strains might fit into this category as well (Fig. 1A, lanes 15 and 17), but their low levels of Ura3-V5-Rim101p made it difficult to distinguish processed Ura3-V5-Rim101p from a faint background band. These results indicate that Dfg16p may be required for Rim101p processing.

We used two approaches to confirm that the dfg16Δ deletion and not a secondary mutation causes a defect in processed Rim101p accumulation. First, the Ura3-V5-Rim101p plasmid was transformed into an independently constructed dfg16Δ strain from the MATα deletion library. The transformant also accumulated only unprocessed Ura3-V5-Rim101p (data not shown). This result argues that the dfg16Δ mutation is the cause of the defect. Second, we introduced a dfg16Δ mutation into a strain expressing functional epitope-tagged Rim101-HA2p and analyzed processing on an immunoblot (Fig. 1C). The DFG16 parent strain expressed primarily processed Rim101-HA2p of ~90 kDa, whereas the dfg16Δ mutant expressed only unprocessed Rim101-HA2p of ~98 kDa. Therefore, the dfg16Δ mutation does not simply affect the Ura3-V5-Rim101p fusion protein, it affects native Rim101p as well. We conclude that Dfg16p is required for accumulation of processed Rim101p.

**Requirement for Dfg16p in Rim101p pathway function.** If Dfg16p is required for processed Rim101p accumulation, then dfg16Δ and rim101Δ mutants should have similar phenotypes. One promising indication is that Dfg16p, like Rim101p, is known to be required for haploid invasive growth (24). In order to investigate Dfg16p function in control of Rim101p-responsive genes, we performed microarray analysis on the dfg16Δ mutant in parallel with the isogenic wild-type and rim101Δ strains. In addition, we included rim21Δ and snf7Δ deletion strains. Rim21p has several similarities to Dfg16p (see Discussion) and has been implicated in the Rim101p/PacC pathways in *A. nidulans* and *Y. lipolytica* (11, 25). However, it has not been characterized in *S. cerevisiae*. Snf7p is of interest as an ESCRT subunit that functions in both the Rim101p processing pathway and the MVB pathway (17, 37), a point that is elaborated upon below. Because the Rim101p pathway is responsible for adaptation to alkaline conditions in yeast, this analysis was carried out on RNA that had been isolated from yeast grown in standard YPD medium (pH 6.6) and then shifted to alkaline YPD medium (pH 8) for approximately 4 h. (The entire data set is available in the supplemental material.)

We found that 14 of 16 genes that had been up-regulated in rim101Δ mutants in the SK-1 and YC11 strain backgrounds (18) were up-regulated in the rim101Δ strain analyzed here. These 14 genes include all genes known to be repressed directly by Rim101p: YJR061W, YOR389W, YPL277C, RIM8, PRB1, NRG1, and SMP1 (18). However, we did not detect increased expression in the rim101Δ strain of CTS1, which we had previously detected only in SK-1 strains, or YPL088W. We also found that 11 of 17 genes that had been down-regulated in the previously studied rim101Δ mutants were down-regulated in the rim101Δ strain analyzed here. The weaker correspondence among down-regulated genes may reflect the fact that they are regulated by Rim101p indirectly.

If Dfg16p is required for Rim101p processing, then we expect that the genes whose expression is altered by a rim101Δ mutation will be similarly altered by a dfg16Δ mutation. We focused on the 25 genes discussed above that respond to a rim101Δ mutation in all *S. cerevisiae* strain backgrounds examined thus far. A plot of their expression ratios (Fig. 2A) shows that the majority of transcripts responded similarly to the dfg16Δ and rim101Δ mutations (Pearson coefficient, 0.987). The results with rim21Δ and snf7Δ strains showed a similar correlation with the rim101Δ strain (Pearson coefficients of
0.979 and 0.970, respectively [Fig. 2B and C]). NRG1 and SMP1 are the two repression targets whose function in Rim101p-dependent responses has been demonstrated (18), and we verified their increased expression in each of the mutants through Northern analysis (Fig. 3, lanes 1 to 5 and 11 to 15). These results indicate that Dfg16p, like Rim21p and Snf7p, is required for Rim101p-dependent effects on expression of native S. cerevisiae genes.

Relationship of Dfg16p and Rim21p to the MVB pathway. The fact that many gene products are required for both the MVB and Rim101p pathways raises the question of whether Dfg16p and Rim21p may be required for MVB pathway function. We addressed this question through comparison of live-cell staining with the lipophilic dye FM4-64. This dye stains the vacuole of wild-type cells vividly but accumulates in prevacuolar class E compartments in MVB pathway mutants (16, 34). Control wild-type and rim101Δ strains displayed vacuolar staining, as indicated by comparison of Nomarski images (Fig. 4A and C), in which the vacuole appears as a large indentation in the middle of the cell, and FM4-64 fluorescence images (Fig. 4B and D), in which the periphery of the indentation is fluorescent. The known MVB-defective snf7Δ mutant showed pronounced accumulation of FM4-64 in compartments surrounding the vacuole and little vacuolar staining (Fig. 4E and F). The dfg16Δ and rim21Δ mutants showed clear vacuolar staining patterns (Fig. 4G to J) very similar to those of the wild-type and rim101Δ strains. These results argue that Dfg16p and Rim21p are not required for MVB pathway function.

We sought to develop an independent criterion that might be diagnostic of MVB pathway defects. Hughes et al. have shown that large-scale mutant gene expression profiles are useful indicators of functional relationships among genes, even if the genes in question are not transcription factors themselves (12). Therefore, we turned to our microarray results to identify transcripts that respond to the snf7Δ mutation and not the rim101Δ mutation, with the rationale that these transcripts might be solely responsive to MVB pathway defects. We found 103 up-regulated transcripts and 222 down-regulated transcripts with these properties. We focused on two genes, PRY1 and ASN1, whose signal intensities indicated that they would be detectable by Northern analysis. PRY1 was expressed at fourfold-higher levels in the snf7Δ mutant than in the wild type (Fig. 5A, lanes 1 and 5, and B). Also, ASN1 was expressed at 3.5-fold-lower levels in the snf7Δ mutant than in the wild type (Fig. 5A, lanes 11 and 15, and B). A rim101Δ mutation had little effect on expression of these genes (Fig. 5A, lanes 2 and 12, and B). These results argue that PRY1 and ASN1 respond
to Snf7p through a mechanism that is not solely dependent upon Rim101p function.

To determine the relationship of the Snf7p-responsive genes to the MVB pathway, we examined the effects of four MVB pathway-defective mutations (Fig. 5). The \( \text{vps20}^- \) and \( \text{vps25}^- \) mutants expressed \( \text{PRY1} \) and \( \text{ASN1} \) at levels similar to that of the \( \text{snf7}^- \) mutant. Vps20p and Vps25p are ESCRT subunits that function in both the MVB and Rim101p pathways (37). In contrast, \( \text{vta1}^- \) and \( \text{vps4}^- \) mutants expressed \( \text{PRY1} \) and \( \text{ASN1} \) similarly to the wild type. Vps4p functions only in the MVB pathway and not in the Rim101p pathway (17, 37). Vta1p functions in the MVB pathway (28, 39) and has not been tested for a role in the Rim101p pathway. However, we found that the \( \text{vta1}^- \) mutant failed to derepress \( \text{NRG1} \) and \( \text{SMP1} \) (Fig. 3, lanes 6, 7, 16, and 17) and failed to express \( \text{CYC1}_{\text{puri}}-\text{lacZ} \) in our initial screen, thus indicating that Vta1p is not required for Rim101p function. These results indicate that \( \text{PRY1} \) and \( \text{ASN1} \) respond to mutations that cause combined defects in the MVB and Rim101p pathways.

We used Northern analysis to determine whether Dfg16p and Rim21p govern \( \text{PRY1} \) and \( \text{ASN1} \) expression (Fig. 5). Transcript levels of \( \text{PRY1} \) and \( \text{ASN1} \) were unaffected in \( \text{dfg16}^- \) and \( \text{rim21}^- \) strains. These observations indicate that Dfg16p and Rim21p are functionally distinguishable from the ESCRT subunits that function in both the MVB and Rim101p pathways. These findings support the conclusion that Dfg16p and Rim21p are not required for both Rim101p and MVB pathway function.

**Conservation of Dfg16p function in C. albicans.** The \( C. \) albicans ORF19.881 (IPF9013) gene product is that organism’s closest homolog of \( S. \) cerevisiae Dfg16p. (We refer to the \( C. \) albicans gene here as \( \text{DFG16} \) based on the results below.) To determine whether this protein is required for \( C. \) albicans Rim101p pathway function, we examined the phenotype of \( C. \) albicans \( \text{dfg16}^- \) deletion strains. In alkaline media, \( C. \) albicans produces hyphae, and this response depends upon Rim101p (4, 27). As expected, the wild-type reference strain displayed filamentous growth around the periphery of colonies on pH 8 plates, and a \( \text{rim101}^-/\text{rim101}^- \) mutant did not (Fig. 6A and D). We observed that a \( \text{dfg16}^-/\text{dfg16}^- \) mutant failed to

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**FIG. 4.** Staining of vacuolar and prevacuolar compartments with FM4-64. Wild-type (WT) and mutant \( S. \) cerevisiae strains (A to J) and \( C. \) albicans strains (K to P), as indicated to the left of the micrographs, were stained with FM4-64. Cells were visualized with visible Nomarski optics (A, C, E, G, I, K, M, and O). FM4-64 fluorescence was visualized for the same fields (B, D, F, H, J, L, N, and P). All images are shown at the same magnification.
We focused here on DFG16 because the mutant’s defects in haploid invasive growth (24) and processed spermidine auxotrophy (unpublished results). Thus, we expect that the results will be sufficiently reliable to make them useful.

DFg16p function. We focused here on DFG16 because the mutant’s defects in haploid invasive growth (24) and processed Rim101p accumulation resemble other Rim101p pathway mutant defects. These observations, combined with microarray and Northern analysis for S. cerevisiae, and with mutant and suppressor analysis in C. albicans, indicate clearly that DFG16p functions in the Rim101p pathway.

**DISCUSSION**

We describe here a new S. cerevisiae Rim101p pathway gene, *DFG16*, and show that its function is conserved in *C. albicans*. It is one of three predicted membrane proteins that function in the Rim101p pathway and, as such, is a candidate for an environmental sensor that promotes Rim101p processing. Recent findings indicate that the Rim101p and MVB pathways intersect, and FM4-64 staining indicates that DFG16p does not function in the MVB pathway. We have borrowed the “compendium” strategy of Hughes et al. (12) on a small scale to develop a new criterion for genes at the Rim101p-MVB pathway intersection. These findings are of interest in providing new insight into MVB pathway function. They also invite speculation about the evolutionary pressures that co-opted the complex ESCRT machinery to participate in what might otherwise have been a simple protease-substrate reaction.

**Rim101p pathway gene identification.** Our screen employed a *CYC1-prc-lacZ* reporter that is a direct assay for Rim101p function (18). The screen might have been simplified by using functional profiling results (9) to select the subset of strains that are sensitive to both NaCl and alkaline pH (7, 19). Unfortunately, *rim101Δ* mutations have a mild effect on these phenotypes in the S288c genetic background that is the platform for the deletion collection. Our screen of 84% of the deletion library led to the clear identification of one new gene that is required for processed Rim101p accumulation, *DFG16*. It also implicated two genes, *YGR122W* and *GPH1*, that may have a more complex relationship to Rim101p, perhaps affecting both processing and expression. Finally, it has provided numerous candidate genes that may govern *RIM101* gene expression and Rim101p repression activity. These areas that have received little attention. The overall results of the screen are preliminary, but promising signs of veracity are the cases in which known functionally related genes yielded similar mutant phenotypes. Examples include *CKA2-CKB1, TUP1-SSN6, and SPE1-SPE2-SPE3*. In addition, the *spe1Δ, spe2Δ*, and *spe3Δ* repression defects were reversed by supplementation of their spermidine auxotrophy (unpublished results). Thus, we expect that the results will be sufficiently reliable to make them useful.

**yield filamentous growth, and this ability was restored by an ectopic copy of the wild-type DFG16 gene (Fig. 6B and C). Similar results were obtained with two additional *dfg16Δ*, *dfg16Δ* deletion strains that had been constructed independently (data not shown). Therefore, *C. albicans* DFG16 is required for filamentation in this alkaline medium.**

If the requirement for DFG16 in filamentation reflects a Rim101p pathway defect, then filamentation should be restored by introduction of the *RIM101-405* allele. This allele specifies a C-terminally truncated product that suppresses filamentation defects of all tested Rim101p pathway mutants (4, 17, 37). We found that a copy of *RIM101-405* restored filamentation to the *dfg16Δ*/*dfg16Δ* mutant, much as it did to a control *rim101Δ/rim101Δ* mutant (Fig. 6H and E). The suppression was not simply a result of increased overall *RIM101* gene dosage, because a copy of wild-type *RIM101* had no effect on *dfg16Δ*/dfg16Δ* filamentation (Fig. 6I). These two results were verified with the two independent *dfg16Δ*/dfg16Δ* deletion strains (data not shown). Function of the wild-type *RIM101* copy was verified by its ability to complement the *rim101Δ* mutant (Fig. 6F). These results support the conclusion that Dfg16p functions in the *C. albicans* Rim101p pathway.

To assess whether *C. albicans* Dfg16p may function in the MVB pathway, we again compared live-cell FM4-64 staining. The control wild-type *C. albicans* strain showed vacuolar staining (Fig. 4K and L). A control snf7/snf7 strain showed little vacuolar staining (Fig. 4M and N). These findings are in keeping with the extensive analysis by Kullas et al. (17). The *dfg16Δ/dfg16Δ* strain showed clear vacuolar staining (Fig. 4O and P). These results argue that Dfg16p is not required for MVB pathway function in *C. albicans*.
Dfg16p has some noteworthy features that frame a simple hypothesis for its mechanistic function. The Dfg16p sequences of *S. cerevisiae* and *C. albicans* are predicted by EMBOSS and SPLIT programs to have seven membrane-spanning segments and a long hydrophilic C-terminal region (see http://db.yeastgenome.org/cgi-bin/protein/protein?sgdid/H11005 and http://split.pmfst.hr/split/). The EMBOSS prediction for *S. cerevisiae* Dfg16p includes a signal sequence as well. Either predicted architecture is shared with G-protein-coupled receptors (GPCRs), leading to the hypothesis that Dfg16p may function as such a receptor. Dfg16p does not fall into a recognized GPCR subclass (15), so this model is quite speculative at present. However, it makes two simple, testable predictions. First, Rim8p (25, 31), which has homology to GPCR-interacting proteins of the β-arrestin family (20), may interact with Dfg16p to govern its localization or activity. Second, there may be a G protein that relays a Dfg16p-dependent signal. Therefore, while our findings do not establish a mechanistic role for Dfg16p, they provide a new framework to guide further investigation.

Rim21p may have seven transmembrane segments as well (25), though EMBOSS and SPLIT analysis programs predict that it has only six such segments. Nonetheless, the fact that Dfg16p and Rim21p are predicted membrane proteins suggests that they may function together, perhaps alongside the third predicted membrane protein, Rim9p. The closest *A. nidulans* homolog of Rim21p is PalH (*E* value, 8.0e-96; 68.3% aligned), as has long been appreciated (11). Interestingly, the closest *A. nidulans* homolog of Dfg16p is also PalH (*E* value, 2.0e-88; 59.1% aligned). Our results here confirm that Rim21p is an *S. cerevisiae* Rim101p pathway component, so the homology of both Rim21p and Dfg16p to PalH seems to be meaningful. Whether either *S. cerevisiae* protein, or perhaps both together, carries out a function equivalent to that of *A. nidulans* PalH is an interesting question. Given that there are homodimeric GPCRs (23), it seems possible that heterodimeric GPCRs may exist as well. One thought is that Dfg16p and Rim21p function as a heterodimeric receptor.

**Functional interaction of MVB and Rim101p pathways.** It has seemed likely that the core ESCRT subunits that govern both MVB and Rim101p pathways may have additional unique functions (3, 28). For example, Bowers et al. (3) showed that almost all of the core ESCRT mutants are hypersensitive to LiCl and CaCl₂. Sensitivity to LiCl is shared with Rim101p pathway mutants (18), but neither Rim101p pathway mutants nor other MVB pathway mutants are hypersensitive to CaCl₂. In addition, Shiflett et al. (28) showed that almost all core
ESCRT mutants are resistant to the cell wall inhibitor calcofluor white, unlike other MVB pathway mutants. Our findings that PRY1 is up-regulated and that ASN1 is down-regulated in three core ESCRT mutants, but not in rim101Δ or vps4Δ mutants, strengthens the case for a unique role of core ESCRT subunits. Four conditions, nitrogen depletion, amino acid starvation, stationary phase, and postdiauxic growth, cause an increase in PRY1 expression and a decrease in ASN1 expression in wild-type strains (6, 8). A simple inference is that the core ESCRT mutants respond to nitrogen or carbon limitation after a shift to pH 8, the conditions under which we examined gene expression. Indeed, the core ESCRT subunits Snf7p and Snf8p were first characterized genetically for their role in SUC2 derepression in response to glucose limitation (33, 38), an independent indication that they may affect a carbon-sensing pathway. We suggest two simple models to explain this unique role. One model is that the core ESCRT subunits function in a third pathway in addition to the MVB and Rim101p pathways: glucose- or nitrogen-sensing pathways are good candidates. This model is intriguing because it implies that the core ESCRT complex coordinates diverse cellular responses. A second model is that the MVB and Rim101p pathways have a redundant function, perhaps in nutrient limitation responses. Thus, defects in either pathway alone do not affect PRY1 and ASN1 expression because the other pathway provides a compensating function. However, a defect in both pathways, as is caused by core ESCRT subunit mutations, eliminates the compensating function. However, a defect in both pathways, as is caused by core ESCRT subunit mutations, eliminates the compensating function. Therefore, a defect in both pathways, as is caused by core ESCRT subunit mutations, eliminates the compensating function. Therefore, a defect in both pathways, as is caused by core ESCRT subunit mutations, eliminates the compensating function. Therefore, a defect in both pathways, as is caused by core ESCRT subunit mutations, eliminates the compensating function. Therefore, a defect in both pathways, as is caused by core ESCRT subunit mutations, eliminates the compensating function.

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