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The Conserved ATPase Get3/Arr4 Modulates the Activity of Membrane-Associated Proteins in *Saccharomyces cerevisiae*

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ABSTRACT

The regulation of cellular membrane dynamics is crucial for maintaining proper cell growth and division. The Cdc48-Npl4-Ufd1 complex is required for several regulated membrane-associated processes as part of the ubiquitin–proteasome system, including ER-associated degradation and the control of lipid composition in yeast. In this study we report the results of a genetic screen in *Saccharomyces cerevisiae* for extragenic suppressors of a temperature-sensitive *npl4* allele and the subsequent analysis of one suppressor, *GET3/ARR4*. The *GET3* gene encodes an ATPase with homology to the regulatory component of the bacterial arsenic pump. Mutants of *GET3* rescue several phenotypes of the *npl4* mutant and transcription of *GET3* is coregulated with the proteasome, illustrating a functional relationship between *GET3* and *NPL4* in the ubiquitin–proteasome system. We have further found that Get3 biochemically interacts with the *trans*-membrane domain proteins Get1/Mdm39 and Get2/Rmd7 and that Δ*get3* is able to suppress phenotypes of *get1* and *get2* mutants, including sporulation defects. In combination, our characterization of *GET3* genetic and biochemical interactions with *NPL4*, *GET1*, and *GET2* implicates Get3 in multiple membrane-dependent pathways.

INTRACELLULAR membranes such as the nuclear envelope and the compartments of the secretory pathway are the key feature that distinguishes the eukaryotic cell from bacteria. Along with the evolution of membrane-bound organelles came the need for intricate and highly regulated mechanisms to control the composition and movements of these membranes in response to environmental changes and during complex cellular events such as the cell cycle or meiosis (Howe and McMaster 2001; reviewed in Albertson *et al.* 2005).

The highly conserved Cdc48-Npl4-Ufd1 complex is an important regulator of several membrane-associated cellular processes in eukaryotic cells. In one critical role, the Cdc48-Npl4-Ufd1 complex participates in the clearing of aberrantly folded proteins from the endoplasmic reticulum (ER) through ER-associated degradation (ERAD) (Bays *et al.* 2001). During ERAD, misfolded ER proteins are retrotranslocated to the cytosol where they are ubiquitinated and degraded by the proteasome (reviewed in Romisch 2005). The increased level of certain ER proteins that occurs if this process is prevented can cause drastic alterations in the organization of the ER compartment (Wright *et al.* 2003). The Cdc48-Npl4-Ufd1 complex also functions to regulate intracellular membranes in yeast through a pathway that controls production of unsaturated fatty acids. Specifically, the Cdc48-Npl4-Ufd1 complex directs the ubiquitin–proteasome-dependent cleavage and activation of two ER-membrane anchored transcription factor precursors, Spt23 and Mga2 (Hoppe *et al.* 2000; Hitchcock *et al.* 2001). Once released from the membrane, Spt23 and Mga2 activate transcription of the *OLE1* gene (Zhang *et al.* 1999; Chellappa *et al.* 2001), which encodes a fatty acid desaturase enzyme (Stukey *et al.* 1990). Thus, not only is the Cdc48-Npl4-Ufd1 complex important for the proteasome-dependent degradation and processing of protein substrates at the ER membrane, but it is also required for the regulation of cellular unsaturated fatty acid (UFA) content, and in turn membrane fluidity, through transcriptional control of *OLE1*. A further requirement has been shown for Cdc48-Npl4-Ufd1 in postmitotic nuclear membrane assembly in higher eukaryotes (Hetzer *et al.* 2001).

Forward genetic screens in the yeast *Saccharomyces cerevisiae* have been critical in elucidating *NPL4* function in ERAD (Bays *et al.* 2001) and in *OLE1* regulation (Hoppe *et al.* 2000; Hitchcock *et al.* 2001). As such, we sought to learn more about *NPL4* function and membrane dynamics by performing a screen for extragenic suppressors of a temperature-sensitive *npl4* mutant. In this study, we present the full panel of genes that we identified as extragenic suppressors of an *npl4* mutation. In

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TABLE 1

***Saccharomyces cerevisiae* strains used in this study**

Strain	Genotype	Source
PSY3322	*MAT*α *ura3-52 leu2Δ1 trp1Δ63*	This study
PSY3323	*MAT*α *ura3-52 leu2Δ1 trp1Δ63 npl4-1*	This study
PSY3383	*MAT***a** *ura3-52 leu2Δ1 his3Δ200 trp1Δ63 npl4-1 cue1:Tn [LEU2]*	This study
PSY3384	*MAT***a** *ura3-52 leu2Δ1 his3Δ200 trp1Δ63 npl4-1 vps27:Tn [LEU2]*	This study
PSY3385	*MAT***a** *ura3-52 leu2Δ1 his3Δ200 trp1Δ63 npl4-1 srn2:Tn [LEU2]*	This study
PSY3386	*MAT***a** *ura3-52 leu2Δ1 his3Δ200 trp1Δ63 npl4-1 get3:Tn [LEU2]*	This study
PSY3091	*MAT***a** *ura3-52 leu2Δ1 trp1Δ63 npl4-1 spt23:Tn [LEU2]*	Hitchcock *et al.* (2001)
PSY3387	*MAT***a** *ura3-52 leu2Δ1 his3Δ200 trp1Δ63 npl4-1 ifh1:Tn [LEU2]*	This study
PSY3388	*MAT***a** *ura3-52 leu2Δ1 his3Δ200 trp1Δ63 npl4-1 prp6:Tn [LEU2]*	This study
PSY3389	*MAT***a** *ura3-52 leu2Δ1 his3Δ200 trp1Δ63 npl4-1 cbp80:Tn [LEU2]*	This study
PSY3390	*MAT***a** *ura3-52 leu2Δ1 his3Δ200 trp1Δ63 Δget3::HIS3*	This study
PSY3391	*MAT*α *ura3-52 leu2Δ1 trp1Δ63 Get3tn-3HA [KanMX]*	This study
PSY3392	*MAT***a** *ura3-52 leu2Δ1 his3Δ200 trp1Δ63 npl4-1 Δget3::HIS3*	This study
PSY3393	*MAT*α *ura3-52 leu2Δ1 trp1Δ63 Δget1::KanMX*	This study
PSY3394	*MAT*α *ura3-52 leu2Δ1 trp1Δ63 Δget2::KanMX*	This study
PSY3395	*MAT*α *ura3-52 leu2Δ1 trp1Δ63 Δget1::KanMX npl4-1*	This study
PSY3396	*MAT*α *ura3-52 leu2Δ1 his3Δ200 npl4Δ::NPL4-sGFP [URA3] Get3-tevProA [KanMX]*	This study
PSY3066	*MAT*α *ura3-52 leu2Δ1 his3Δ200 trp1Δ63 npl4Δ::NPL4-sGFP::URA3*	This study
PSY3397	*MAT*α *ura3-52 leu2Δ1 trp1Δ63 Get3-EGFP [KanMX]*	This study
PSY3398	*MAT*α *ura3-52 leu2Δ1 his3Δ200 trp1Δ63 Get3-EGFP [KanMX] Δget1::LEU2*	This study
PSY3399	*MAT*α *ura3-52 leu2Δ1 his3Δ200 trp1Δ63 Get3-EGFP [KanMX] Δget2::HIS3*	This study
PSY3400	*MAT*α *ura3-52 leu2Δ1 his3Δ200 Get3-EGFP [KanMX] Δget1::LEU2 Δget2::HIS3*	This study
PSY3164	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 Δget3::KanMX*	This study
PSY1930	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Winzeler *et al.* (1999)
PSY3402	*MAT*α *ura3-52 leu2Δ1 trp1Δ63 CPY**	This study
PSY3403	*MAT*α *ura3-52 leu2Δ1 trp1Δ63 npl4-1 CPY**	This study
PSY3404	*MAT***a** *ura3-52 leu2Δ1 his3Δ200 trp1Δ63 Δget3::HIS3 CPY**	This study
PSY3405	*MAT*α *ura3Δ leu2Δ Δget3::LEU2 Δget2::KanMX*	This study
PSY3406	*MAT*α *ura3-52 leu2Δ1 his3Δ200 Δget3::HIS3 Δget1::KanMX*	This study
PSY3407	*MAT***a**/*MAT*α *ho::hisG/ho::hisG lys2/lys2 ura3/ura3 leu2/leu2 his3/his3 trp1ΔFA/trp1ΔFA*	Benjamin *et al.* (2003)
PSY3408	*MAT***a**/ *MAT*α *ho::hisG/ho::hisG lys2/lys2 ura3/ura3 leu2/leu2 his3/his3 trp1ΔFA/trp1ΔFA Δget3::KanMX/Δget3::KanMX*	This study
PSY3409	*MAT***a**/*MAT*α *ho::hisG/ho::hisG lys2/lys2 ura3/ura3 leu2/leu2 his3/his3 trp1ΔFA/trp1ΔFA Δget2::TRP1/Δget2::TRP1*	This study
PSY3410	*MAT***a**/*MAT*α *ho::hisG/ho::hisG lys2/lys2 ura3/ura3 leu2/leu2 his3/his3 trp1ΔFA/trp1ΔFA Δget3::KanMX/Δget3::KanMX Δget2::TRP1/Δget2::TRP1*	This study

addition, we present extensive characterization of one gene identified as an *npl4* suppressor, the *GET3/ARR4* gene. *GET3* encodes a highly conserved ATPase with homology to ArsA, the regulatory component of the bacterial arsenic export pump (Boskovic *et al.* 1996), and to a human protein of unknown function, hASNA-I (Kurdi-Haidar *et al.* 1996). Get3 has been suggested to have roles in cellular resistance to stress (Shen *et al.* 2003), in metal ion homeostasis (Metz *et al.* 2006), and, in complex with Get1 and Get2, in protein sorting via the secretory pathway (Schuldiner *et al.* 2005).

Here we present evidence that *GET3* displays functional interactions with *NPL4* in the context of the ubiquitin–proteasome pathway. We further illustrate interactions of *GET3* with *GET1* and *GET2* during sporulation, a process in which dynamic new growth of cellular membranes is required for proper spore and spore wall formation. In particular, we have demonstrated that Get3 can play an antagonistic role in both settings, leading to the proposal that Get3 is a functionally conserved regulator of membrane-associated proteins.

MATERIALS AND METHODS

Yeast strains and manipulations: Standard yeast methods and media were used (Guthrie and Fink 1991). The genotypes of all strains used in this study are provided in Table 1. The *npl4-1* mutant strains are FY23-backcrossed strains derived from PSY825 and PSY826, which were previously described (DeHoratius and Silver 1996). Null alleles and C-terminal tags were integrated using PCR-based techniques (Baudin *et al.* 1993; Knop *et al.* 1999). *CPY** was integrated into wild-type (WT), *npl4-1*, and Δ*get3 npl4-1* strains using two-step gene replacement (Adams *et al.* 1997) of plasmid pRS306-*prc1-1* as described (Knop *et al.* 1996).

Screens for extragenic suppressors of *npl4-1*: *npl4-1* cells were mutagenized with either ethyl methanesulfonate (EMS) or an mTn-*lacZ/LEU2* transposon library (Burns *et al.* 1994). Methods for the EMS screen and cloning of *UBC7* and *CUE1* have been described (Hitchcock *et al.* 2003). Suppressing

mutations in *DOA4* and *UBP3* were identified by transformation with a CEN-based *URA3*-marked plasmid library containing yeast genomic DNA fragments (Rose *et al.* 1987) and subsequent analysis of the rescuing plasmids pPS2915, pPS2918, and pPS2931. Transposon mutagenesis was performed essentially as described (Seifert *et al.* 1986). Two micrograms of *Not*I-digested DNA were transformed into *npl4-1* cultures, and colonies able to grow at the nonpremissive temperature of 30° were observed at the rate of 5×10^{-5}. The location of insertion was identified for nine of these colonies by vectorette PCR, as described (Kumar and Snyder 2000).

ERAD assay: Cells were grown to $OD_{600} = 0.5$–1.0, pelleted, and resuspended in 3 ml YPD containing 100 ug/ml cycloheximide. Samples were removed at each time point and prepared as described (Johnson *et al.* 1995) for immunoblotting with anti-CPY (Molecular Probes, Eugene, OR). Equal protein loading was verified by subsequent staining with amido black.

Northern blot analysis: Cultures were grown in YPD at 25° to log phase and then split such that half continued growth at 25° while half were shifted to 37° for 2 hr. All samples were frozen and total RNA was prepared in parallel. Northern blotting and analysis were performed as described (Hitchcock *et al.* 2001).

Cell fractionation: Get3-EGFP cells were grown at 30° to logarithmic phase in YPD. Subcellular fractionation and solubilization of the P13 fraction were performed essentially as described (Munoz-Centeno *et al.* 1999). To determine the fractionation profile of Get3, equal cell volumes were analyzed by Western blot with antibodies to GFP (Seedorf *et al.* 1999) and Sec62.

Affinity purification and mass spectrometry: Get3-TEV-protein A and control cells expressing protein A from plasmid pPS1973 were grown in media lacking leucine to logarithmic phase. Microsomes were prepared essentially as described (Baker *et al.* 1990). After homogenization of spheroplasts, the membrane fraction was isolated by centrifugation at 13,000 rpm for 15 min. The pellet was resuspended and washed in B88 buffer (20 mm HEPES, pH 6.8, 150 mm KOAc, 5 mm $Mg(OAc)_2$, 250 mm sorbitol). Equal protein amounts were resuspended in IPPT-150 buffer (10 mm Tris pH 8.0, 150 mm NaCl, 0.5% Triton X-100) supplemented with 0.5 mm DTT, 0.5 mm PMSF, and protease inhibitors, and proteins were extracted by rocking at 4° for 1–5 hr.

Affinity purification was performed overnight at 4° using 30 μl of IgG Sepharose bead slurry (Pharmacia/Amersham, Piscataway, NJ), 50 μl of 80% glycerol, and 500 μl of extracted proteins. After washing with IPPT-150 buffer (as above except 0.1% Triton), cleavage with TEV protease (GIBCO, Grand Island, NY) was performed in IPPT-150 supplemented with 0.5 mm EDTA and 1 mm DTT at 14°. After SDS–PAGE, silver-stained bands were excised from the gel and analyzed by MALDI-TOF mass spectrometry at the Southern Alberta Mass Spectrometry Centre (Calgary, AB, Canada).

Transcriptional profiling and genomic analysis: Expression profiling was carried out in triplicate, with swapping of fluor orientation, from Δ*get3* and WT cells grown at 30° in YPD to early logarithmic phase. Total RNA preparation, cDNA preparation, and hybridization were performed as described (Casolari *et al.* 2004). Up- and downregulated genes were defined as genes with fold change ≥1.5 and *P*-value ≤0.05 as determined by Rosetta Resolver. Analysis for enrichment of functional classes was performed using FuncAssociate (Berriz *et al.* 2003). Data mining for *GET3* transcription was performed using Cluster and TreeView software from Eisen *et al.* (1998) and tools available on the Saccharomyces Genome Database (Dolinski *et al.* 2006).

Sporulation experiments: Synchronous sporulation of homozygous SK1 diploid cells was performed using previously described methods (Padmore *et al.* 1991; Huang *et al.* 2005). Samples were monitored throughout sporulation to ensure good synchrony and efficiency. Each sample was fixed and stained with DAPI before observation by light and fluorescence microscopy (Grether and Herskowitz 1999). For quantification, images were recorded of 36-hr samples using a Nikon microscope equipped with a DAPI filter (Chroma Technology, Brattleboro, VT) and a 100× DIC (Nomarski) objective. At least 200 cells were scored for each strain. Spore viability was determined by tetrad dissection after sporulation for 2–4 days on 1% potassium-acetate plates.

RESULTS

Identification of *npl4-1* extragenic suppressors: Several phenotypes affecting cellular membranes have been observed in the *npl4-1* mutant allele, including defects in ERAD, and in nuclear envelope structure and nucleocytoplasmic transport likely due to the misregulation of *OLE1* (DeHoratius and Silver 1996; Bays *et al.* 2001). To learn more about these effects, we conducted a genetic screen for extragenic suppressors of the temperature-sensitive lethality of this *npl4* mutant at 30°. *npl4-1* cells were mutagenized with either EMS or transposon-mutagenized yeast genomic DNA, and mutants capable of growth at 30° were isolated, characterized, and cloned. This analysis resulted in the identification of 11 genes whose mutation was capable of rescuing growth at the nonpermissive temperature (Table 2). Suppressing mutations isolated by EMS mutagenesis fell into five distinct complementation groups, four of which were identified by various techniques (materials and methods). An additional 7 genes with suppressing mutations were isolated as transposon insertions and were cloned by vectorette PCR. One gene, *CUE1*, was isolated by both techniques. None of the isolated suppressors restored wild-type growth to the *npl4-1* mutant or were able to rescue at higher temperatures (Figure 1B and data not shown), suggesting that in each case the deficits of this mutation were only partially corrected.

Mutations in genes affecting several pathways, including ERAD, secretion, and gene expression, were able to ameliorate the temperature-sensitive lethality of *npl4-1* (Table 2). Two of the suppressors are active in ERAD (*CUE1* and *UBC7*) (Biederer *et al.* 1997) and have already been reported by our lab as suppressors of *npl4-1* (Hitchcock *et al.* 2003). All four suppressors in the secretory pathway class have also been implicated in ubiquitin-dependent processes: *DOA4* and *UBP3* have direct roles in deubiquitination at secretory vesicles (Amerik *et al.* 2000; Cohen *et al.* 2003a), whereas *VPS27* and *SRN2/VPS37* are involved in the sorting and degradation of ubiquitinated proteins via the multivesicular body (MVB) pathway (Katzmann *et al.* 2001; Bilodeau *et al.* 2002). Genes in the third class of *npl4-1* extragenic suppressors affect transcription (*SPT23*, *IFH1*) (Zhang *et al.* 1999; Dula and Holmes 2000; Schawalder *et al.*

TABLE 2

Suppressors of *npl4-1*

Gene		Cellular activity	Allele(s)
		ERAD	
YMR264W	*CUE1*[a,c]	Docking of Ubc7 in ERAD	Transposon after AA72, EMS (5)
YMR022W	*QRI8/UBC7*[a,c]	Ubiquitin-conjugation to ERAD substrates	EMS (16)
		Secretion	
YNR006W	*VPS27*	Sorting of ubiquitinated proteins in MVB pathway	Transposon after AA260
YLR119W	*SRN2/VPS37*	Sorting of ubiquitinated proteins in MVB pathway	Transposon after AA44
YDR069C	*DOA4*[c]	Deubiquitination in endosome to vacuole transport	EMS (8)
YER151C	*UBP3*[c]	Deubiquitination in vesicle transport	EMS (2)
		Transcription/RNA modulation	
YKL020C	*SPT23*[b]	Activation of *OLE1* transcription	Transposon after AA710
YLR223C	*IFH1*	Chromatin assembly and silencing	Transposon after AA155
YBR055C	*PRP6*	Pre-mRNA splicing (U4/U6-U5 snRNP)	Transposon after AA793
YMR125W	*STO1/CBP80*	Nuclear mRNA cap-binding protein	Transposons (2) in noncoding regions
YDL100C	*GET3*[c]	Homolog of bacterial arsenite transporter	Transposon after AA215

Suppressors generated by EMS or transposon mutagenesis are listed by functional class. Several were isolated multiple times, as indicated. For mutants isolated from the transposon screen, the integration site is listed.

[a] Previously published in Hitchcock *et al.* (2003).

[b] Previously published in Hitchcock *et al.* (2001).

[c] Null allele was tested and also found to rescue growth of *npl4-1*.

2004; Wade *et al.* 2004) or have roles in RNA processing (*PRP6*, *STO1*) (Abovich *et al.* 1990; Das *et al.* 2000). The rescue of *OLE1* transcription in *npl4-1* cells by truncations of *SPT23* and *MGA2* has been reported previously (Hitchcock *et al.* 2001); however, the links between the remaining members of class 3 and Npl4 and/or ubiquitin–proteasome function are unclear.

In addition to these genes with known functions, we identified a suppressing mutation in the *YDL100C* open reading frame (ORF), which had no reported functions at the time of the screen. *YDL100C* has since been named *ARR4* for its homology to the bacterial ArsA protein (Shen *et al.* 2003) and, more recently, *GET3* (Schuldiner *et al.* 2005). We chose to pursue the characterization of this gene as a means to gain insight into both Get3 and Npl4 function.

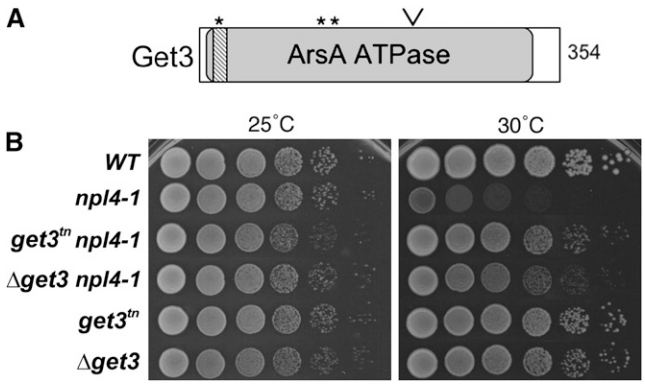

Figure 1.—*get3* mutants suppress the temperature-sensitive lethality of *npl4-1*. (A) Diagram of the Get3 protein. The site of truncation in the *get3*^{tn} allele is shown (∨) along with the p-loop ATP-binding site (striped box). Predicted myristoylation sites are marked with asterisks. (B) *get3* mutants rescue *npl4-1* growth at 30°. Wild-type (WT), *npl4-1*, *get3*, and double-mutant strains were grown to log phase and then serially diluted and plated to rich media at 25° and 30° for 2 days.

***get3* mutants suppress *npl4-1*-associated phenotypes:** The *get3* mutant isolated as a suppressor of *npl4-1* is a truncation produced by transposon insertion and is designated *get3*^{tn}. The transposon insertion adds two amino acids and a premature stop codon after K215 of Get3, generating a truncated protein product consisting of the first 60% of Get3 (Figure 1A). This region contains the P-loop ATP-binding site and several predicted myristoylation sites. We observed that both *get3*^{tn} and the null allele Δ*get3* were able to suppress *npl4-1* temperature-sensitive lethality at 30° (Figure 1B). However, phenotypic differences between the two alleles suggested that the isolated transposon insertion does not generate a null allele; specifically, Δ*get3* is a weaker suppressor of *npl4-1* and has a more marked growth defect in cells that are wild type for *NPL4* than does the *get3*^{tn} allele (Figure 1B).

We next tested whether mutants of *get3* rescued *npl4-1* phenotypes in addition to temperature-sensitive lethality, including a diminished capacity for ERAD and misregulation of *OLE1* transcription (Figure 2). To test for function of the ERAD pathway in *get3 npl4-1* cells, a mutant allele of carboxypeptidase Y (CPY*) that is subject to rapid degradation via the ERAD system was integrated into the *npl4-1* and *get3* mutant strains. Cells

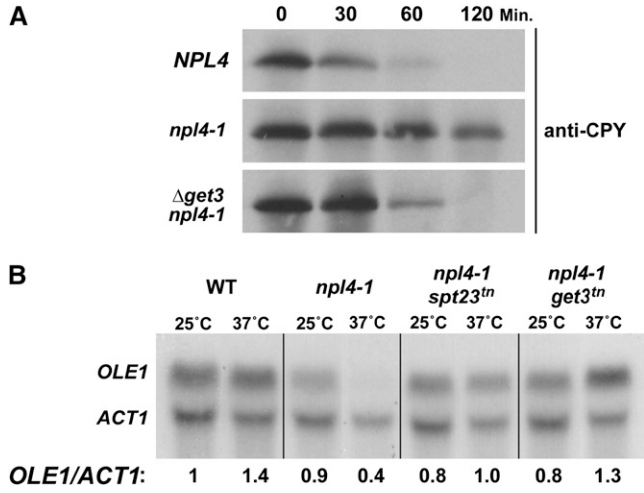

Figure 2.—*get3* mutants suppress *npl4-1* phenotypes. (A) Δ*get3* rescues ERAD-mediated CPY* degradation in *npl4-1* cells. Wild-type (*NPL4*), *npl4-1*, and *npl4-1* Δ*get3* mutant cells were grown to log phase and protein synthesis was halted by treatment with cyclohexamide. Samples were collected 30, 60, and 120 min after cyclohexamide treatment, separated by SDS–PAGE, and subjected to Western blot analysis with anti-CPY antibodies. (B) *get3*^{tn} restores *OLE1* transcription in *npl4-1* cells. Northern analysis was performed on total RNA isolated from wild type (WT), *npl4-1*, or *npl4-1* with the suppressing mutations *spt23*^{tn} or *get3*^{tn}, using *OLE1*- and *ACT1*-specific DNA probes. Cells were either continuously grown at 25° or shifted to 37° for 2 hr (labeled as 25° and 37°, respectively) prior to RNA purification. The ratio of *OLE1*/*ACT1* signal for each sample is given (at the bottom) relative to that for the WT 25° sample.

expressing CPY* were treated with cyclohexamide to halt protein synthesis, and levels of this protein remaining at various time points after treatment were determined by Western blot analysis with anti-CPY antibodies. As expected, CPY* was degraded rapidly in wild-type cells with a half-life of ~30 min (Figure 2A). In contrast, the half-life of CPY* was significantly extended in *npl4-1* cells, as has been previously reported (Bays *et al.* 2001). Interestingly, the *get3* null and transposon alleles both displayed partial rescue of the *npl4-1* ERAD defect (Figure 2A and data not shown), decreasing the half-life of the CPY* protein in *npl4-1* cells to <1 hr. We found that the Δ*get3* allele does not have a strong defect or enhancement of ERAD in cells that are wild type for *NPL4* (supplemental material at http://www.genetics.org/supplemental/).

To determine whether *get3* mutation was able to rescue *OLE1* transcription in *npl4-1* cells, we performed Northern blot analysis using probes for *OLE1*, and for *ACT1* (actin) as a loading control. Total RNA was analyzed from *npl4-1* and *get3* single- and double-mutant cells, shifted to the nonpermissive temperature of 37° for 2 hr. After shift to 37°, *OLE1* transcript levels were observed to increase in wild-type cells by ~40% and decrease in the *npl4-1* mutant by ~50% compared to the unshifted samples (Figure 2B; Hitchcock *et al.* 2001). We found that the transposon allele of *get3* was able to rescue *OLE1* transcription to wild-type levels (Figure 2B). It appears that this effect is specific to the *get3*^{tn} allele, since Δ*get3* does not restore *OLE1* transcription in *npl4-1* cells; however, Δ*get3* does block the elevation of *OLE1* transcript levels seen at 37° in *NPL4* cells (supplemental material at http://www.genetics.org/supplemental/). We then compared the ability of *get3*^{tn} to rescue *OLE1* transcription with that of a dominant activating truncation of Spt23 (*spt23*^{tn}), which encodes a transcription factor that activates *OLE1* gene expression (Zhang *et al.* 1999). Surprisingly, we found that the rescue of *OLE1* transcript level in *npl4-1* is more robust in the *get3* mutant than in the *spt23* mutant.

***GET3* is coregulated with the proteasome and the Cdc48-Npl4-Ufd1 complex:** Given that genes with similar function often exhibit transcriptional coregulation, we used genomic analyses of available transcriptional profiling data to examine *GET3* expression and coregulation with other yeast genes under various growth conditions. First, the transcriptional profiles of all yeast genes under conditions including specific cell cycle stage, diauxic shift, and sporulation were clustered as described (materials and methods; Eisen *et al.* 1998). The genes showing the most similar expression with *GET3* under these conditions, shown in Figure 3, include a significant number of genes encoding proteasomal components ($P < 0.001$ by FuncAssociate) and those encoding the Cdc48-Npl4-Ufd1 complex. Supporting this result, we found that *GET3* also clustered with proteasome-encoding genes using a different computational method (Jelinsky *et al.* 2000). These genomic data fit well with the genetic interaction we found between *GET3* and *NPL4* and provide evidence indicating functional connections between *GET3* and the ubiquitin–proteasome pathway.

The strongest coregulation observed between *GET3*, *NPL4*, and the proteasome components occurs under conditions relating to sporulation (Figure 3). Expression of nearly all the genes in this cluster is elevated early in sporulation and under altered expression of *NDT80* (Figure 3), which encodes a master regulator for transcriptional activation of middle sporulation-specific genes (Xu *et al.* 1995; Chu and Herskowitz 1998; Hepworth *et al.* 1998). Looking more specifically at the regulation of *GET3* during sporulation in existing data sets for genomewide expression during sporulation in SK1 cells (Chu *et al.* 1998; Primig *et al.* 2000), we found that *GET3* mRNA expression is slightly elevated shortly after induction of sporulation. The set of 20 genes with the most similar expression pattern to *GET3* during sporulation, according to the Saccharomyces Genome Database (Dolinski *et al.* 2006), includes genes encoding several proteasome subunits (data not shown) and is significantly enriched for genes involved in ubiquitin-dependent protein catabolism ($P < 0.001$ by FuncAssociate).

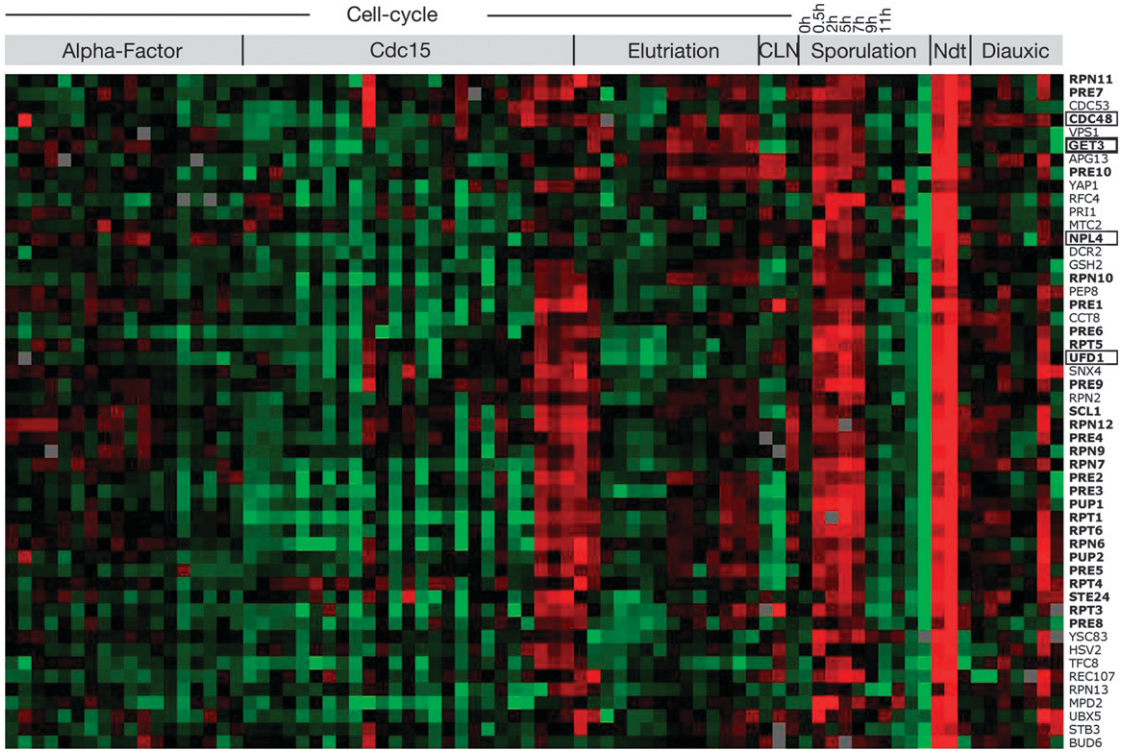

FIGURE 3.—*GET3* is coregulated with genes encoding components of the proteasome and the Cdc48-Npl4-Ufd1 complex. GET3 (thick box) is similarly expressed with many protein-degradation genes (boldface type) and genes encoding the Cdc48/Npl4/Ufd1 complex (thin boxes). The data set of expression profiling under various conditions from EISEN *et al.* (1998) was analyzed by hierarchical clustering using Average Link correlation (uncentered), and the GET3-containing cluster from analysis with TreeView software is shown. Treatments analyzed include cell cycle time courses, the diauxic shift, a time course during sporulation, and the altered expression of NDT80.

Get3 membrane localization depends on Get1 and Get2: We next examined the subcellular localization and biochemical characteristics of the Get3 protein. For these analyses, DNA sequence encoding an EGFP tag was integrated in frame at the 3′ end (translated C terminus) of the genomic locus of *GET3*; the resulting *GET3-EGFP* strain exhibits no growth defect (data not shown), indicating that the EGFP tag does not disrupt Get3 function. Upon examination by live-cell fluorescence microscopy, Get3-EGFP was observed at the ER/nuclear membrane and in the cytoplasm (Figure 4A). This finding was consistent with published reports for Get3 localization in rich media (HUH *et al.* 2003; SCHULDINER *et al.* 2005).

To biochemically confirm that a population of Get3 associates with cellular membranes, cells expressing Get3-EGFP were lysed under gentle, nondetergent conditions and fractionated by two successive rounds of centrifugation (MATERIALS AND METHODS). Proteins copurifying with the isolated membrane and soluble fractions were separated by SDS–PAGE and probed with anti-GFP antibodies to visualize Get3-EGFP and with antibodies to Sec62, an integral ER-membrane protein. This analysis revealed that the majority of Get3 protein sedimented with the nuclei and ER membranes in the P13 membrane fraction (Figure 4B), consistent with our observation of a membrane-associated population of Get3-EGFP by fluorescence microscopy. We also observed a population of Get3 that remained soluble, even upon ultracentrifugation (S100 fraction), confirming our visualization of a cytoplasmic pool of Get3 by microscopy (Figure 4B).

On the basis of sequence predictions and its homology to ArsA (BOSKOVIC *et al.* 1996), the Get3 protein is not expected to contain a *trans*-membrane domain, raising the question of how Get3 associates with cellular membranes. To assess the nature and extent of Get3 membrane association, cellular membranes purified from Get3-EGFP-expressing cells (P13 fraction) were resuspended in various buffers, incubated for 10 min on ice, and repelleted. Proteins released into the soluble phase (S) as well as those remaining in the membrane pellet (P) were analyzed by Western blot as above. We observed that Get3-EGFP remained tightly membrane associated in the presence of high salt and high pH; only treatment with a detergent was able to solubilize Get3 to the supernatant (Figure 4C).

On the basis of our microscopic and biochemical analysis of Get3 subcellular localization, we hypothesized that Get3 was likely to interact with one or more proteins embedded in the ER/nuclear membrane. To identify these potential proteins, we affinity purified

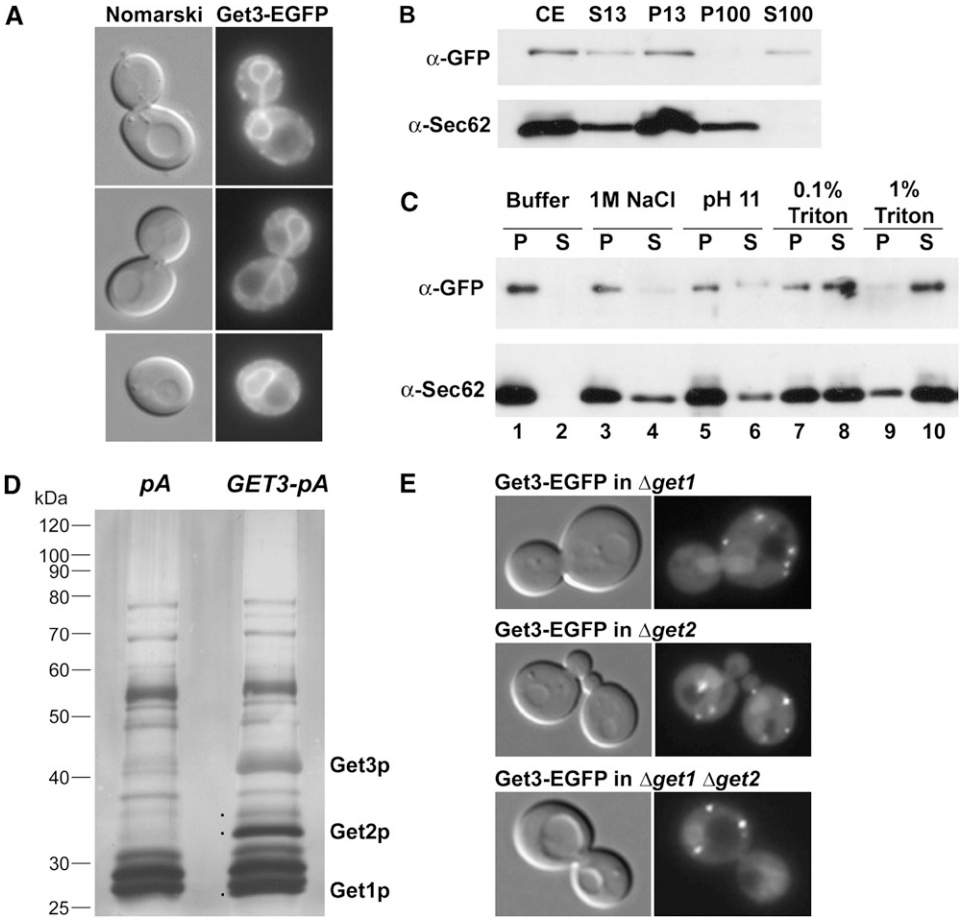

FIGURE 4.—ER-membrane localization of Get3 requires Get2 and Get1. (A) Get3-EGFP localizes to the nuclear/ER membrane in rich media, by live cell fluorescence microscopy. Corresponding Nomarski image of cells is shown to the left. (B) Get3-EGFP cofractionates with both soluble and membrane-bound fractions. Cellular extract (CE) was separated into pellet and supernatant fractions following centrifugation at 13,000 rpm (P13 and S13, respectively). The S13 fraction was then subjected to ultracentrifugation at 100,000 rpm and separated into pellet and supernatant fractions (P100 and S100). Corresponding volumes from each isolated fraction were separated by SDS–PAGE and Western blotted with anti-GFP or anti-Sec62 antibodies as indicated. (C) The P13-associated fraction of Get3-EGFP is tightly membrane associated. The P13 fraction as in B was washed with either buffer alone (lanes 1 and 2) or buffer with 1 M NaCl (lanes 3 and 4), 0.2 M Na_2CO_3 pH 11 (lanes 5 and 6), 0.1% Triton X-100 (lanes 7 and 8), or 1% Triton X-100 (lanes 9 and 10). The samples were then recentrifuged and separated into pellet (P, odd lanes) or supernatant fractions (S, even lanes), which were analyzed by Western blotting as above. (D) Get3 biochemically copurifies with Get2 and Get1. Solubilized membranes from cells expressing Get3-TEV-proteinA (*GET3-pA*) or protein A alone (*pA*) were incubated with IgG Sepharose beads. After extensive washing, bound proteins were treated with TEV protease. Proteins released by this treatment were separated by SDS–PAGE and visualized by silver staining. Specific bands marked with a dot were excised for analysis by MALDI-TOF mass spectrometry and two of these bands were identified as Get2 and Get1 as labeled. The protein band corresponding to Get3 is also indicated. (E) Get3-EGFP mislocalizes in the absence of *GET2* and/or *GET1*. Get3-EGFP was visualized by live-cell fluorescence microscopy in Δ*get1*, Δ*get2*, or Δ*get1* Δ*get2* cells grown in rich media to logarithmic phase. Images are representative of analysis of more than one clone for each genotype.

protein A-tagged Get3 (Get3-ProA) from solubilized cell membranes by binding to IgG Sepharose. Proteins copurifying with Get3-ProA were eluted by TEV protease cleavage, separated by SDS–PAGE, and detected by silver staining. Two protein bands that were specifically observed in the Get3-ProA purification, and not in a negative control purification from cells expressing protein A alone, were present in similar quantities as Get3-ProA itself (Figure 4D). These were identified by MALDI-TOF mass spectrometry to be Get1 and Get2. A fainter band running at 35 kDa was also observed, but unfortunately this interactor was of insufficient quantity to identify. Further increasing our confidence that Get1 and Get2 represent true Get3-binding partners, these two proteins were also found among the proteins purified with Get3 in a large-scale study (Ho *et al.* 2002). Despite their colocalization at the ER/nuclear membrane (HITCHCOCK *et al.* 2001; HUH *et al.* 2003), no physical interactions were observed between Get3 and members of the Npl4 complex by Western blot (data not shown).

To test the possibility that Get3 membrane localization is mediated by interaction with the *trans*-membrane domain-containing proteins Get1 and Get2, we monitored Get3-EGFP localization in Δ*get1* and Δ*get2* single- and double-mutant cells. Strikingly, Get3-EGFP was completely absent from the ER membrane in deletions of either *GET1* or *GET2* (Figure 4E). Instead it was found in punctate sites in the cytoplasm, which are likely Golgi compartments as suggested by SCHULDINER *et al.* (2005). Conversely, we found that the localization of Get1-EGFP and Get2-EGFP was unaffected by the absence of Get3 (supplemental material at http://www.genetics.org/supplemental/). Thus, under these conditions the ER-membrane localization of Get3 is dependent upon the presence of both Get2 and Get1; however, even in the absence of Get1 and Get2, Get3 appears to retain some capacity to interact with other cellular membranes.

Sporulation genes are misregulated in a *get3* mutant: As a complementary approach to identify pathways

TABLE 3

Misregulation of sporulation genes in Δ*get3*

Class	No. genes up in Δ*get3*	No. genes in class	*P*-value
Metabolic	2	51	0.39
Early I	7	61	9.87*E*-04
Early II	5	56	0.013
Early–mid	4	86	0.19
Middle	18	156	9.83*E*-08
Mid–late	3	58	0.19
Late	2	9	0.022
Sporulation	41	477	3.40*E*-12

The numbers of genes upregulated during each stage of sporulation (as defined by Chu *et al.* 1998) and in Δ*get3* are listed. *P*-values for significance of overlap were determined using Fisher's exact test.

affected by *GET3*, transcriptional profiling of Δ*get3* cells was performed. Total RNA was purified from WT and Δ*get3* cells, differentially labeled, and competitively hybridized to microarrays spotted with cDNAs representing ~6200 predicted yeast ORFs. Statistical analysis of WT *vs.* Δ*get3* signal for each ORF led to the identification of 265 genes whose transcription is upregulated and 345 genes whose transcription is downregulated in Δ*get3* relative to WT cells, as defined in materials and methods (complete data set available in supplemental material at http://www.genetics.org/supplemental/). The most striking finding from this analysis was that the set of genes upregulated in Δ*get3* is significantly enriched for genes whose expression increases during sporulation ($P = 3.4 \times 10^{-12}$, by Fisher's exact test). Table 3 shows the breakdown of these sporulation- and Δ*get3*-induced genes into stages of transcriptional activation during sporulation, on the basis of the classifications made by Chu *et al.* (1998). Although a large fraction of genes involved in all stages of sporulation was induced in Δ*get3* cells, enrichment of genes involved in the middle and early I stages was the most significant ($P = 9.83 \times 10^{-8}$ and $P = 9.87 \times 10^{-4}$, respectively; Table 3).

Given the genetic interactions between *GET3* and *NPL4*, we sought to compare the set of genes with altered transcription in Δ*get3* mutant cells with those whose transcription is altered in *npl4-1* mutant cells (Auld *et al.* 2006). Surprisingly, the genes with altered transcription in Δ*get3* and *npl4-1* strains showed no significant similarity, except in the activation of genes encoding heat-shock and other stress response proteins (data not shown). Interestingly, however, the transcription of *GET3* was significantly induced in *npl4-1* ($P = 4.33 \times 10^{-3}$). In all, this transcriptional profiling analysis supports the existence of a functional connection between *GET3* and *NPL4*, but also suggests that *GET3* may function independently of *NPL4* in affecting the expression of sporulation-specific genes.

Sporulation phenotypes of GET complex and *npl4-1* mutants: We found the misregulation of sporulation genes in Δ*get3* to be of particular interest given *GET3*'s strong transcriptional coregulation with *NPL4* and other ubiquitin–proteasome system genes during this process (see above and Figure 3). Furthermore, both the Get3 interactors Get1 and Get2 (Figure 4 and Schuldiner *et al.* 2005) have been implicated in sporulation by a large-scale study (Enyenihi and Saunders 2003). Taken together, these data suggested that the GET complex may have a role, perhaps in conjunction with Npl4 and the ubiquitin–proteasome system, in sporulation. To investigate this model, diploid yeast cells homozygous for *npl4-1*, Δ*get3*, Δ*get2*, or Δ*get1* (in the synchronously sporulating SK1 strain background) were induced to sporulate, and meiotic divisions were assayed by microscopic analysis of DAPI-stained samples throughout the time course. The terminal phenotype was then recorded by fluorescence and Nomarski microscopy 36 hr after induction. We found that the timing and occurrence of meiotic divisions in the GET complex deletion strains were not significantly altered compared to those in a wild-type strain (supplemental material at http://www.genetics.org/supplemental/). In fact, no strong sporulation defect was apparent in the Δ*get3* and *npl4-1* SK1 strains, even after characterization of the terminal sporulation phenotype (Figure 5B and data not shown).

In contrast to Δ*get3* and *npl4-1* cells, we observed a striking defect in the terminal sporulation phenotype of Δ*get2* and Δ*get1* mutant cells (Figure 5, A and B). Whereas the majority of wild-type cells generated tetrads with four spores "impenetrable" to DAPI when stained using the conditions described (materials and methods), only 20% of Δ*get2* cells exhibited this phenotype. Instead, the majority of Δ*get2* diploid cells produced "partial penetrable" tetrads, which contain one or more spores that are refractile, but immature looking and penetrable to DAPI (Figure 5, A and B). The Δ*get2* strain also exhibited a mild increase in "multinucleate" cells, those with multiple or fragmented nuclei but with no refractile spores. A similar, though more severe, phenotype was observed in the Δ*get1* strain (Figure 5A): only ~10% of Δ*get1* cells produced complete tetrads after 36 hr in sporulation medium, whereas ~40% displayed the multinucleate phenotype. A significant number (~30%) of Δ*get1* diploids also formed abnormal refractile spores similar to those seen in Δ*get2*, suggesting that these genes function within the same process. All strains tested had a similar percentage of "single-nucleus" cells that have not undergone sporulation, indicating that the fraction of cells initiating sporulation was not significantly different in the various strains (Figure 5B and data not shown). Tetrad dissection was performed to analyze the viability of spores produced in Δ*get1* and Δ*get2* strains. Overall, 79.7% of Δ*get2* spores (287/360 spores) and 86.3% of Δ*get1* spores (335/388)

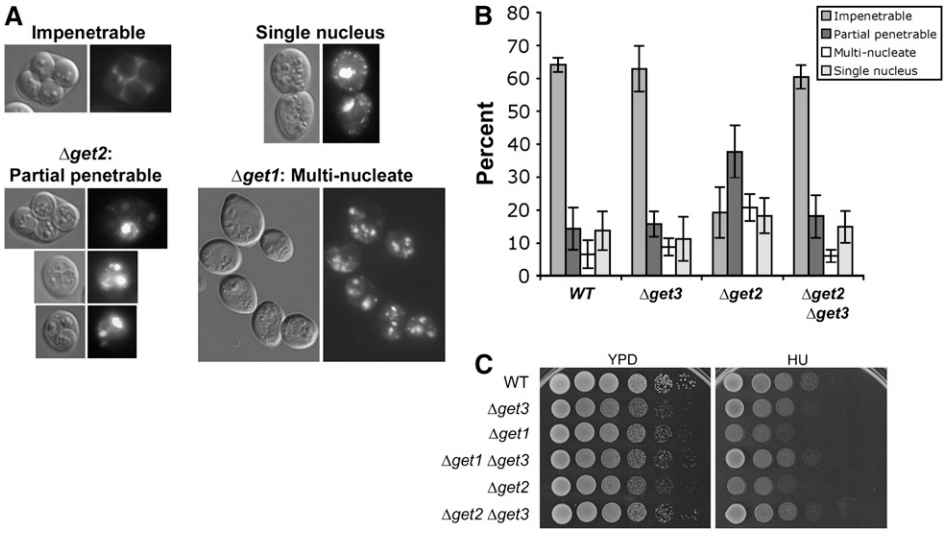

FIGURE 5.—Δ*get3* rescues phenotypes of Δ*get2* and Δ*get1* cells. (A) Representative sporulated cells displaying wild-type or defective terminal sporulation phenotypes. Synchronously sporulated SK1 cells were fixed 36 hr after shift to sporulation medium (SPM) and nuclei were stained with DAPI. Nomarski (left) and DAPI (right) images representative of the indicated terminal (36 hr) sporulation phenotypes are shown. (B) Δ*get3* rescues terminal sporulation defects displayed by Δ*get2* cells. Synchronously sporulated wild-type (WT), Δ*get3*, Δ*get2*, and Δ*get3* Δ*get2* homozygous diploid yeast (SK1 background) were stained with DAPI, and cells falling into each of the four categories shown in A were counted. The average percentage of each phenotype is graphed with error bars depicting the standard deviation over three separate experiments. At least 200 cells were counted for each strain per experiment. (C) Δ*get3* suppresses the HU sensitivity of Δ*get2* and Δ*get1* cells. Wild-type (WT), Δ*get3*, Δ*get1*, Δ*get2*, and double-mutant strains were grown to log phase and then serially diluted and plated to YPD or media containing 150 mM hydroxyurea at 25° for 2 days.

from tetrads with normal appearance were viable, compared with 99.5% viability of WT spores (199/200).

These observed phenotypes of Δ*get1* and Δ*get2* spores (Figure 5A) are similar to those previously described for *spo11-1* mutant diploids, which undergo the meiotic divisions but fail to package all of their spores correctly (Klapholz *et al.* 1985). Since Spo11 is important in catalyzing double-strand breaks necessary for recombination during meiosis (Keeney *et al.* 1997), this similarity suggested that meiotic recombination may be affected in GET complex mutants. To test this hypothesis, the frequency of heteroallele recombination in Δ*get1* and Δ*get2* was analyzed by a return-to-growth time-course experiment during the first 8 hr after induction of sporulation. Interestingly, we observed no significant defect in either mutant (data not shown). An alternative explanation for these phenotypes, in keeping with previous proposals of spore wall defects in Δ*get1* (Enyenihi and Saunders 2003), is that the GET complex is important for spore packaging. For further characterization of the mutant phenotypes, we also examined the localization of Don1-GFP, a marker for the leading edge of the prospore membrane (Knop and Strasser 2000), in our mutant strains. Prospore membrane growth appeared normal as well, suggesting that *GET1* and *GET2* function at a later step in spore development.

Δ*get3* suppresses phenotypes of Δ*get2* and Δ*get1*: Given that *get3* mutants are capable of suppressing phenotypes of *npl4-1* cells, we asked whether Δ*get3* might also be able to suppress the sporulation defect displayed by Δ*get2* cells. To this end, we generated a homozygous diploid Δ*get2* Δ*get3* double mutant in the SK1 strain background and analyzed its terminal sporulation phenotype as described above. Strikingly, we found that the deletion of *get3* completely rescued the significant sporulation defect of Δ*get2* (Figure 5B). The sporulation defect of Δ*get1* was also rescued by Δ*get3* (data not shown).

To investigate the extent of these epistatic interactions, we sought to determine whether Δ*get3* could rescue other defects displayed by Δ*get2* and/or Δ*get1*. Previous studies have demonstrated that Δ*get2* is sensitive to the DNA-damaging agents hydroxyurea (HU) and methyl methanesulfonate (MMS) (Zewail *et al.* 2003). To test whether Δ*get3* is able to rescue the HU sensitivity of Δ*get2* cells, we compared the growth of single- and double-deletion strains of the GET complex on rich media plates containing HU. As expected, Δ*get2* cells were sensitive to HU, showing a growth defect of at least two orders of magnitude as compared to wild-type cells (Figure 5C). We found that Δ*get1* cells were similarly sensitive to HU, whereas Δ*get3* cells were only slightly more sensitive than wild-type cells (less than one order of magnitude, Figure 5C). Interestingly, the HU sensitivity of both Δ*get2* and Δ*get1* cells was rescued by Δ*get3*, such that the double mutants displayed the Δ*get3* phenotype (mild HU sensitivity, Figure 5C). In all, this genetic analysis has revealed that Δ*get3* suppresses both the terminal sporulation phenotype of Δ*get2* cells and the HU sensitivity of Δ*get2* and Δ*get1* cells.

DISCUSSION

In this study we have characterized the yeast *GET3/ARR4*, a highly conserved gene encoding an ATPase whose bacterial homolog regulates arsenic transport (Rosen *et al.* 1995). We isolated a truncation of *get3* in a genetic screen for suppressors of an *npl4* mutant, along with mutations in several other genes involved in

ubiquitin-dependent events in protein trafficking through the secretory system. We have demonstrated that mutants of *get3* rescue multiple defects in this *npl4* mutant and that *GET3* exhibits transcriptional coregulation with genes encoding proteasome components. These results combine to suggest that *GET3* can antagonize the pathways of Cdc48-Npl4-Ufd1 complex activity in the ubiquitin–proteasome system. Further characterization of *GET3*, independent of its interaction with *NPL4*, included biochemical and localization studies demonstrating that the ER-membrane localization of Get3 depends on the presence of both Get1 and Get2. Transcriptional profiling and phenotypic analyses then illustrated a role for the GET complex in sporulation. Intriguingly, Get3 appears to have a negative role in this process, as evidenced by our discovery that Δ*get3* can reverse the sporulation phenotypes of *get1* and *get2* mutants. In combination, we have demonstrated that *GET3* can modulate pathways requiring either the Npl4 complex or the GET complex.

Several lines of evidence from this and other studies suggest that Get3 may act in a regulatory capacity in these pathways. First, the Get3 protein itself is not required for the processes of sporulation or ERAD (Figure 5B and supplemental Figure S2 at http://www.genetics.org/supplemental/), yet it modulates these pathways in mutants of the GET complex genes and *npl4*, respectively (Figures 5B and 2A). This type of interaction implies a regulatory relationship that could be mediated by the ATPase domain of Get3 (Schuldiner *et al.* 2005; Shen *et al.* 2003). The physical interaction between Get3, Get2, and Get1 (Figure 4D) supports the possibility of a direct action of Get3 on these proteins. Finally, the homology of Get3 to a bacterial ATPase that regulates the channel for transport of arsenic across the plasma membrane (Boskovic *et al.* 1996) supports the idea that the yeast *GET3* may have a conserved function with its bacterial homolog in the regulation of membrane-associated proteins.

A screen for suppressors of *npl4*: The set of genes isolated as suppressors of *npl4-1* reveals several insights into Npl4 function and membrane dynamics (Table 2). First, *CUE1* and *UBC7* are important for the ubiquitination step of ERAD (Biederer *et al.* 1997) and deletions in these genes reverse the accumulation of ubiquitinated proteins at the ER membrane in *npl4-1* cells (Hitchcock 2003); thus our isolation of these particular ERAD components suggests this accumulation as one reason for temperature-sensitive lethality in *npl4-1* cells. Additional suppression likely occurs in these mutants through increased half-life of the Ole1 protein (Hitchcock 2003), which is a documented ERAD substrate (Braun *et al.* 2002). Second, the isolation of a class of secretory pathway genes suggests intimate connections between the phenotypes of the *npl4* mutant and secretory function. The mechanism of rescue by these suppressors is not clear but could be caused by a reduction in ER protein load or by the diversion of more proteins to alternative degradation systems such as the vacuole (Spear and Ng 2003). Finally, the involvement of many of these secretory pathway suppressors in ubiquitin-dependent events illustrates the importance of degradation pathways in maintaining proper function of the membranes of the secretory system.

It is more difficult to interpret potential mechanisms of *npl4* phenotype suppression by the RNA-processing genes *PRP6* and *CBP80*. The *npl4* mutants were initially isolated on the basis of their ability to block nuclear transport (DeHoratius and Silver 1996). Thus these RNA-processing mutants may alleviate an RNA transport defect, now presumed to be caused by the membrane defects in *npl4-1* cells; alternatively, they may increase the half-life of *OLE1* or *npl4-1* transcripts.

***GET3* and the ubiquitin–proteasome system:** Several plausible explanations can be envisioned for *get3*-mediated suppression of *npl4-1* phenotypes. One possibility is that this suppression is due to activity of the GET complex in retrograde transport from the Golgi to the ER (Schuldiner *et al.* 2005) as part of the secretory pathway. The identification in our screen of other genes affecting secretion, including one (*UBP3*) that also participates in this type of retrograde transport (Cohen *et al.* 2003b), supports this model. However, our data suggest that Get3 has a more specific function relating to the ubiquitin–proteasome system and that it is this function that explains the ability of *get3* mutants to partially rescue *npl4-1* cells. First, given our understanding of the transcriptional regulation of *OLE1*, it is difficult to explain how an ER–Golgi transport defect would rescue the *OLE1* transcription defect of *npl4-1* cells more potently than a dominant activating truncation of the *OLE1* transcription factor Spt23 (Figure 2B). Second, the coregulation of *GET3* with genes encoding the Npl4 complex and components of the proteasome (Figure 3) suggests a functional interaction between *GET3* and this pathway. Finally—and most convincingly—*GET1* and *GET2*, which are required along with *GET3* for retrograde transport, are not coregulated with the proteasome (Figure 3 and data not shown) and are unable, when deleted, to suppress *npl4-1* temperature sensitivity (data not shown). If the ability of Δ*get3* to suppress *npl4-1* were due to its activity in the secretory pathway, it would be expected that *GET1* and *GET2* would display identical genetic interactions with *NPL4*. Thus, our finding that *GET3* has a unique ability to antagonize *NPL4* activities indicates a functional connection between Get3 and the ubiquitin–proteasome system, independent of its interactions and functions with the GET complex.

The GET complex in sporulation: In our studies of Get3 as part of a complex with Get1 and Get2 we have focused on its role in sporulation, a line of study suggested by our transcriptional profiling of Δ*get3* and by documented sporulation defects of Δ*get1/mdm39* and

Δ*get2/rmd7* (Enyenihi and Saunders 2003). This function of Get3 is likely distinct from its interactions with the ubiquitin–proteasome system, as discussed above.

Several hypotheses can be proposed for the mechanism of GET complex function in sporulation. The first possibility is that the sporulation defects we observed in Δ*get1* and Δ*get2* mutants are a result of defects in retrograde protein transport in the secretory pathway. Given the requirement of the GET complex for proper localization of proteins within the ER and Golgi (Schuldiner *et al.* 2005), and the importance of these compartments to prospore membrane and spore wall formation (Neiman 2005), these structures could be affected in the *get1* and *get2* mutant strains. The increased HU sensitivity and DAPI penetrability of Δ*get2* spores may reflect membrane or spore wall defects, supporting this model.

The sporulation phenotypes we observed are somewhat different from the previously described defects in spore wall formation (Δ*get1*) and meiotic division (Δ*get2*) (Enyenihi and Saunders 2003). While preliminary data suggest that there may be spore wall defects, we observed no defect in meiotic divisions (supplemental material at http://www.genetics.org/supplemental/). It is possible that this discrepancy is due to strain differences, particularly in the timing and efficiency of sporulation. Our detailed analysis of Δ*get1* and Δ*get2* mutants in synchronously sporulated SK1 cells, especially the observed increases in partial penetrable tetrads, fragmented nuclei, and nonviable spores, suggests that the GET complex may act at a postmeiosis step to allow proper spore packaging (Figure 5 and results).

Our genetic analysis of the sporulation phenotypes of GET complex mutants provides evidence that the sporulation defects of Δ*get2* may not be due to the secretory activity of the complex. In the secretory pathway, the presence of *GET3* is required for the retrograde transport of proteins meant to reside in the ER (Schuldiner *et al.* 2005). In contrast, *GET3* itself is not required for sporulation, but this process cannot occur correctly when the complex is disrupted by the absence of *GET2* (Figure 5B). The rescue of sporulation and HU-sensitive phenotypes of Δ*get1* and Δ*get2* in Δ*get3* illustrates that in addition to cooperating with Get2 and Get1 in the secretory pathway, in other cases Get3 is capable of antagonizing the function of Get1 and Get2.

The evolution of *GET3* function: The bacterial homolog of *GET3*, ArsA, is the cytoplasmic regulatory subunit of the pump required to export arsenic through the plasma membrane (Rosen *et al.* 1995). One potential hypothesis suggested by our work is that this activity as a regulatory ATPase for membrane-associated proteins is conserved in the yeast protein Get3. Through our analysis of the *GET3* gene as a suppressor of *npl4-1* we have shown that it can antagonize the pathways of Npl4 complex activity, while experiments independent of this interaction have shown a similar capacity for Get3 with Get1 and Get2. Thus, in combination, our studies of *GET3* have illustrated its role in two different cellular pathways: proteasome-dependent events at the ER membrane through Cdc48-Npl4-Ufd1 and sporulation through Get1 and Get2. Interestingly, a recent study has shown a similar interaction between Get3 and the intracellular CLC chloride-transport protein, Gef1 (Metz *et al.* 2006), suggesting metal ion homeostasis as yet a third pathway subject to potential regulation by Get3.

In summary, Get3 has been implicated, through this and other studies, in such seemingly disparate cellular pathways as the ubiquitin–proteasome system, secretion, and sporulation. We propose the regulation of intracellular membrane composition and organization to be the fundamental connection among these activities, and future studies of Get3 will undoubtedly further our understanding of its role in the membrane dynamics of the eukaryotic cell.

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