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Genetics

Suppressors of *nmt1-181*, a conditional lethal allele of the *Saccharomyces cerevisiae* myristoyl-CoA:protein N-myristoyltransferase gene, reveal proteins involved in regulating protein N-myristoylation

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ABSTRACT **Several essential *Saccharomyces cerevisiae* proteins require myristate to be covalently bound to their amino-terminal glycine for biological activity. Protein N-myristoylation is catalyzed by myristoyl-CoA:protein N-myristoyltransferase, Nmt1p. *nmt1-181* encodes a mutant enzyme with a $Gly^{451} \rightarrow$ Asp substitution. nmt181p has a reduced affinity for myristoyl-CoA and produces global defects in protein N-myristoylation at ≥30°C. *nmt1-181* results in growth arrest at various stages of the cell cycle within 1 hr after cells are shifted to ≥30°C and lethality within 8 hr. The growth-arrest phenotype and loss of viability do not require components of the mating pathway and are associated with lysis sensitivity that may be related to undermyristoylation of two protein phosphatases, Ppz1p and Ppz2p. Growth can be rescued at 30°C by adding myristate or sorbitol to the medium or by removing inosine. Cells can be rescued at 37°C by overexpressing nmt1-181p or Nmt1p or by adding myristate to the medium. Selection of high-copy suppressors of the myristate auxotrophy and lethality observed at 37°C yielded only *NMT1*, whereas six unlinked *s*uppressors of the *m*yristoylation *d*efect (*SMD1–6*) were obtained when the screen was conducted at 30°C. The protein products of three *SMD* loci were identified: (*i*) cdc39-Δ1.7p, which transactivates *NMT1*; (*ii*) Fas1p, the β subunit of the fatty acid synthetase complex, activates *FAS2*'s promoter and increases myristoylation of Gpa1p; and (*iii*) Pho5p, the major secreted acid phosphatase produced by this yeast. *PHO5* is normally induced when yeast are grown in phosphate-depleted medium. Removal of inorganic phosphate from the medium also rescues *nmt1-181* cells at 30°C. *PHO5*'s mechanism of suppression of *nmt1-181* appears to involve, at least in part, activation of *FAS2* transcription and a resulting effect on *FAS1* expression. There is an inverse relationship between cellular N-myristoyltransferase and secreted acid phosphatase activities. These observations provide a potential mechanism for coupling phosphate metabolism with the regulation of myristoyl-CoA synthesis and protein N-myristoylation.**

Saccharomyces cerevisiae myristoyl-CoA:protein N-myristoyltransferase (Nmt1p) transfers the myristoyl group from myristoyl-CoA to the amino-terminal glycine residue of ≈12 cellular proteins (1). These proteins include Gpa1p, the α subunit of a heterotrimeric G protein involved in the mating-factor signal transduction pathway (2, 3), plus two functionally interchangeable ADP ribosylation factors, Arf1p and Arf2p (4). Gpa1p and the Arf proteins are essential and require myristate for expression of their biological functions. Thus, it is not surprising that disruption of *NMT1* produces recessive lethality (5).

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Nmt1p has a sequential ordered reaction mechanism (1). The apoenzyme binds myristoyl-CoA with high affinity (K_d = 15 nM) (6). Formation of a myristoyl-CoA/Nmt1p binary complex is required for generation of a functional peptide binding site. After catalysis, CoA and then myristoylpeptide are released.

nmt1-181 encodes a mutant acyltransferase. The $Gly^{451} \rightarrow$ Asp substitution in nmt181p produces a temperature-dependent reduction in its affinity for myristoyl-CoA and growth arrest at temperatures ≥30°C (7). Arrest appears to be associated with defects in the acylation of multiple cellular proteins. *nmt1-181* cells can be rescued at 37°C by overexpressing Nmt1p or nmt181p or by supplementing the medium with myristate (C14:0). C12:0, C13:0, or saturated fatty acids of chain length >14 are ineffective (7–9). nmt181p's increased requirement for myristoyl-CoA at nonpermissive temperatures has been exploited to study factors that regulate the size and/or accessibility of cellular myristoyl-CoA pools. *S. cerevisiae* possesses two principal pathways for generating myristoyl-CoA. Myristoyl-CoA represents ≈5% of the acyl-CoA produced by the fatty acid synthetase (Fas) complex. In addition, C14:0 is an active substrate for three of the yeast's four known acyl-CoA synthetases—Faa1p, Faa2p, and Faa4p (10, 30). The growth of isogenic strains containing *NMT1* or *nmt1-181* and various combinations of *faa1*, *faa2*, *faa3*, and *faa4* null alleles has been characterized on rich medium with or without myristate supplementation, in the presence or absence of a specific inhibitor of Fas (cerulenin), at 24–37°C (10). These studies indicate that nmt181p can use myristoyl-CoA generated from both Fas and Faa1p, Faa2p, and Faa4p. Faa1p and Faa4p appear to be the acyl-CoA synthetases responsible for activating imported myristate and can supply sufficient myristoyl-CoA to sustain growth of *nmt1-181* cells even when the *de novo* (Fas) pathway is blocked. Faa2p appears to be able to activate endogenous pools of myristate (10, 30). It is the only yeast acyl-CoA synthetase that, when overexpressed, can rescue growth (at 30°C) in medium without myristate. This suggests that pools of myristate and/or the acyl-CoA synthetases themselves may be functionally compartmentalized in *S. cerevisiae*. The results obtained with overexpression of Faa2p stimulated us to search for other high-copy suppressors of the growth arrest and myristate auxotrophy of *nmt1-181* cells observed at 30–37°C.

MATERIALS AND METHODS

Strains. YB332 (*MAT***a**, *NMT1*, *ura3-52*, *his*Δ*200*, *ade2-101*, *lys2-801*, *leu2-3,112*), YB336 (*MAT***a**, *nmt1-181*, *ura3-52*, *his*Δ*200*, *ade2-101*, *lys2-801*, *leu2-3,112*), YB338 (*MAT***a**,

Abbreviations: CAT, chloramphenicol acetyltransferase; ORF, open reading frame.

NMT1, ste4Δ::LEU2, ura3-52, hisΔ200, ade2-101, lys2-801, leu2-3,112), and YB342 (*MATa, nmt1-181, ste4Δ::LEU2, ura3-52, hisΔ200, ade2-101, lys2-801, leu2-3,112*) are described in ref. 8. YB427 (*MATa, nmt1-181, ura3, his3, ade2, ade3, leu2, trp1*) was constructed by conventional methods. Strains were grown at 24–37°C on YPD (1% yeast extract/2% peptone/2% dextrose) agar plates with or without fatty acids, cerulenin (25 μM), sorbitol (0.5 M), and KH_2PO_4 (0.5%, wt/vol). Phosphate-depleted medium was prepared according to ref. 11.

Selection. YB336 was transformed with a high-copy YEp-based genomic library (12) and replica plated at 24°C on selective medium to calculate transformation efficiencies and at 30°C and 37°C on YPD to identify suppressors of *nmt1-181*. Thirty thousand transformants were obtained, corresponding to 10- to 15-fold coverage of the yeast genome. Plasmid-dependent rescue at 30°C and 37°C was established by reversion to the *nmt1-181* phenotype on 5-fluoroorotic acid-containing plates. The 12 plasmids which functioned as suppressors at 37°C contained *NMT1*. The 18 plasmids that functioned as suppressors at 30°C were digested with various restriction endonucleases and found to contain six distinct genomic DNA inserts.

Secondary Assays of Suppressors of *nmt1-181*. Mating-pathway induction was measured (13) in the isogenic strains YB332 (*NMT1*) and YB336 (*nmt1-181*) transformed with a YEp-*FUS1/lacZ* construct. This recombinant DNA was made by ligating the *Pst* I–*Sal* I *FUS1/lacZ* fragment from pSL307 (14) to pRS425. All assays were performed in duplicate (two independent experiments) and the results were averaged. Nucleotides −1925 (relative to the initiator ATG codon) to +603 of *NMT1* were attached to the chloramphenicol acetyltransferase (CAT) reporter gene, *cat*, in pBM2072 at its *Sph* I site by using a linker (5′-GACGTCCATG-3′). [Note that pBM2072 is a derivative of pBM1974 (15) which was generated by introducing a copy of the *TRP1* gene.] *NMT1/cat* was integrated at the *GAL4* locus of YM1368 (*NMT1*) and YB427 (*nmt1-181*). Reporter activity was assayed in cell lysates (16). Assays were performed in duplicate in two separate experiments and the results were averaged.

Acid Phosphatase Assays. Strains YB332 and YB336, with or without episomes, were grown in to midlogarithmic phase in the appropriate selective medium, and acid phosphatase activity was measured in 50-μl aliquots of the culture (17). All assays were done in quadruplicate in two to five independent experiments and the results were averaged.

RESULTS AND DISCUSSION

Further Phenotypic Characterization of *nmt1-181* Strains. The *nmt1-181* allele can produce lethality. If exponentially growing cultures are shifted from 24°C to 37°C, no viable colonies are observed 24 hr later. Growth arrest occurs within 1 hr after shifting to 30–37°C and is associated with increased cell size. Cells remain viable at 37°C for 8 hr but are dying by 12 hr (data not shown).

Integration of an *NMT1/cat* fusion gene into the *GAL4* locus of isogenic *nmt1-181* and *NMT1* strains revealed similar levels of *NMT* transcription during growth on YPD medium at 24°C and up to 4 hr after shifting to 30–37°C (data not shown). Moreover, the steady-state levels of Nmt1p and nmt181p (7) are comparable in isogenic strains grown at 24°C and 1–4 hr after shifting to 30–37°C. These observations suggest that changes in the levels of protein N-myristoylation do not serve as a feedback mechanism to control *NMT1* transcription or the concentration of its protein product.

nmt1-181 produces undermyristoylation of Gpa1p (8). This leads to constitutive activation of the pheromone response pathway, arrest in the G_1 stage of the cell cycle, and increased transcriptional activity of mating-specific genes (e.g., *FUS1*). To investigate the contribution of components of this signaling pathway to the growth arrest at 30–37°C, we introduced a *ste4* null allele into *nmt1-181* and *NMT1* cells. (Ste4p and Ste18p are the β and γ subunits of the G protein involved in the mating cascade; the Ste4p/Ste18p dimer transduces the signal produced by binding of pheromone to its receptor.) *ste4Δ* does not affect the time course of growth arrest, the length of time *nmt1-181* cells remain viable at 30–37°C, or the time course or the extent of lethality (data not shown). These data suggest that components of the mating pathway are not required to produce the growth arrest or the loss of viability of *nmt1-181* cells.

The increase in cell size observed shortly after shifting to 30–37°C raised the possibility of associated defects in the structural stability of the cell wall. Two, functionally interchangeable, type 1-related protein phosphatases, Ppz1p and Ppz2p, have recently been identified on the basis of their ability to suppress the cell lysis produced by a null allele of the yeast protein kinase C gene, *PKC* (18). *ppz1Δ/ppz2Δ* cells have lysis defects which are similar to the defects observed in *pkcΔ* cells. These defects can be suppressed by growth in 0.5 M sorbitol (18). Octapeptides representing the amino-terminal sequences of Ppz1p and Ppz2p are excellent substrates for Nmt1p *in vitro* (Table 1). Moreover, addition of 0.5 M sorbitol to YPD medium rescues the growth defect of *nmt1-181* cells at 30°C (Fig. 1*A*) but not at 37°C (data not shown). To directly assess the lysis sensitivity of isogenic *NMT1* and *nmt1-181* strains, cells were grown at 24°C in YPD/0.5 M sorbitol then resuspended in either water or 0.5 M sorbitol, incubated at 24°C or 35°C for 4 hr, and then plated on YPD at 24°C to assess loss of viability. Cells with *NMT1* showed no loss of viability during the 4-hr incubation in water (compared with sorbitol). *nmt1-181* cells had a 2-fold reduction in viability at 24°C in water and an 8-fold reduction at 35°C. Together, these findings suggest that Ppz1p and Ppz2p are Nmt1p substrates, that they are undermyristoylated in

Table 1. *In vitro* assay of the substrate and inhibitor activities of octapeptides derived from known or predicted Nmt1p substrates

Peptide (protein)	Single-point assay: V_{max},* pmol/(min·mg)	Substrate		Inhibitor	
		V_{max},† pmol/(min·mg)	K_m, nM	IC_{50},‡ nM	K_i,‡ nM
GAAPSKIV (Cnb1p)	22,000 ± 458	17,000 ± 1,300	20 ± 10	20	7 ± 2
GCTVSTQT (Gpa1p)	91,000 ± 14,000	24,500 ± 2,300	2100 ± 100	ND	ND
GNSSSKSS (Ppz1p)	40,000 ± 1,500	6,100 ± 2,800	32 ± 1	≤200	ND
GNSGSKQH (Ppz2p)	67,000 ± 3,300	18,000 ± 7,800	480 ± 100	≤200	ND
GRTTSEGI (Pho4p)	≤50	ND	ND	90,000	95,000 ± 18,000

*This assay is described in refs. 1 and 6. Reaction mixtures contained 0.23 μM [^{3}H]myristoyl-CoA, 182 μm peptide, and 1–100 ng of purified *Escherichia coli*-derived Nmt1p. Assays were performed in triplicate on at least two occasions. ND, not determined.

†Values from Lineweaver–Burk double reciprocal plots.

‡Determined with a well-characterized Nmt1p substrate, GNAAAARR-amide.

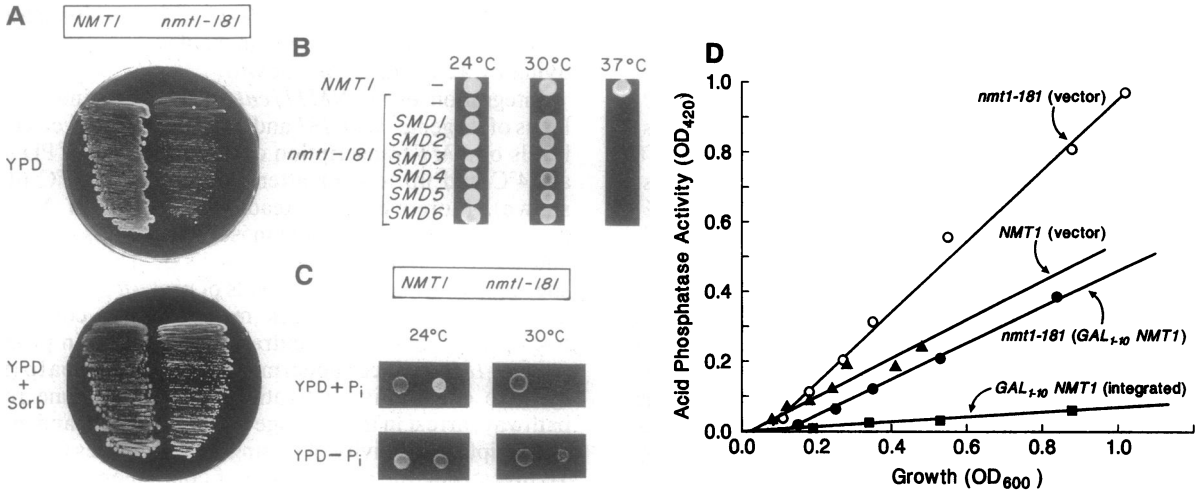

FIG. 1. Phenotype of *nmt1-181* cells. (*A*) Addition of 0.5 M sorbitol to YPD medium rescues the growth of *nmt1-181* cells at 30°C. (*B*) *SMD1–6* produce different degrees of rescue of *nmt1-181* cells on YPD at 30°C. (*C*) Addition of inorganic phosphate (0.5% KH_2PO_4) exacerbates the temperature-sensitive growth defect of an *nmt1-181* strain on YPD. (*D*) Inverse relationship between cellular N-myristoyltransferase activity and medium acid phosphatase activity.

nmt1-181 cells, and that loss of their acyl chain may contribute to the growth-arrest phenotype produced by *nmt1-181*.

Based on these results, we adopted the following strategy for selecting and initially characterizing suppressors of *nmt1-181*. High-copy suppressors were selected on YPD at 30°C and 37°C. If suppressors functioned by increasing cellular myristoyl-CoA pools or increasing the activity of nmt181p, they should be able to increase myristoylation of Gpa1p (8) and thereby decrease the activity of the *FUS1* promoter (measured by the level of expression of a YEp-based *FUS1/lacZ* fusion gene). If suppressors increase nmt181p activity by increasing the activity of the *NMT1* promoter, they should be able to increase transcription of a *NMT1/cat* fusion gene.

Selection of Suppressors of *nmt1-181* at 30°C and 37°C. All the recombinant YEp plasmids which were identified as suppressors of the myristoylation defect (*SMD*) produced by *nmt1-181* at 37°C contained *NMT1*. A repeat screen conducted at 30°C on YPD plates yielded six distinct recombinant plasmids (*SMD1–6*; Table 1). They produced different degrees of rescue (Fig. 1*B*). *nmt1-181* cells containing *SMD2* showed the greatest viability under the selection conditions and approached the level of growth of *NMT1* strains. Moreover, it was the only *SMD* which was able to rescue growth at temperatures up to 34°C (data not shown).

Cerulenin is a specific inhibitor of Fas and can be used to block *de novo* myristoyl-CoA production. Both *NMT1* and *nmt1-181* cells are not viable on YPD plus cerulenin at 24–37°C (10). Addition of 125 μM myristate to YPD/cerulenin medium fully rescues growth of *nmt1-181* strains at 30°C. We lowered the dose of myristate below this threshold value (to 60 μM) and observed the ability of the *SMD*-containing episomes to influence growth of *nmt1-181* cells on YPD/cerulenin at 30°C. Only *SMD1* and *SMD6* produced a growth advantage compared with a reference strain containing the YEp plasmid without insert (data not shown).

As noted above, suppressors that function by increasing the size or accessibility of cellular myristoyl-CoA pools and/or that affect the abundance or activity of nmt181p should increase the amount of myristoyl-Gpa1p and thereby lower *FUS1* transcription. This set of assumptions is based on the observation that adding as little as 10 μM myristate to YPD reduced transcription of a *FUS1/lacZ* fusion gene in *nmt1-181* cells to levels approaching those found in isogenic *NMT1* cells (when grown at 30°C in the absence of α factor; see Table 2). Table 2 shows the β-galactosidase activity found in lysates prepared from *nmt1-181* cells that had been incubated at 24°C to midlogarithmic phase in YPD (without α factor) and then shifted for 2 hr to 30°C. All of the *SMD*

Table 2. Characterization of multicopy suppressors of *nmt1-181*

	Chr	λ clone*	ORF	β-Gal from *FUS1/lacZ*,† (Miller units)		CAT from *NMT1/cat*, % control‡
				− α	+ α	
NMT1 (strain YM1368)				3	464	100
nmt1-181						
No myristate				93	293	100
10 μM myristate				4		
SMD1	VII	4155/4379	ND	62	ND	ND
SMD2	XI	3845	*FAS1*	8	ND	100
SMD3	II	6156	*PHO5*	83	ND	100
SMD4	XII	5864/7002	ND	42	ND	ND
SMD5	VII	1381/7971	ND	61	ND	ND
SMD6	III	3712	*cdc39-Δ1.7*	64	ND	210

Chr, chromosome; ORF, open reading frame; ND, not determined.
*λ clones are numbered according to M. Olsen and L. Riles (personal communication). These phage DNAs can be obtained from the American Type Culture Collection.
†β-Galactosidase activity in the presence or absence of 5 μM α factor. Miller units are defined according to ref. 13.
‡Control was strain YM1368 (ref. 15).

plasmids reduced *FUS1/lacZ* expression to values below that observed in the reference *nmt1-181* strain transfected with the YEp plasmid without insert. The greatest suppression was conferred by *SMD2*.

Expression of a *NMT1/cat* fusion gene was the same in *NMT1* and *nmt1-181* strains when they were grown to midlogarithmic phase in YPD at 24°C or 4 hr after they had been shifted to 30°C (data not shown). Therefore, we assayed the effects of *SMD1–6* on CAT production in *NMT1* cells during midlogarithmic phase of growth on selective medium at 30°C. *SMD6* was the only suppressor which produced an unequivocal, albeit modest, 2-fold increase in CAT activity (Table 2).

SMD6's ability to increase production of CAT and reduce production of β-galactosidase suggests that it functions by increasing the activity of the *NMT1* promoter. In contrast, *SMD2*'s inability to influence *NMT1/cat* activity indicates that its mechanism does not involve changes in transcription of *nmt1-181*. Rather, its ability to decrease expression of *FUS1/lacZ* (presumably through increased myristoylation of Gpa1p) suggests that it functions by influencing myristoyl-CoA pool size/accessibility and/or the activity of nmt181p itself. The notion that *SMD2* affects nmt181p activity directly can be discounted, but not dismissed, by the fact that it is unable to rescue growth at 30°C when *de novo* fatty acid biosynthesis is blocked with cerulenin and the YPD medium is supplemented with myristate.

All six *SMD* loci were physically mapped by probing filters containing (*i*) electrophoretically separated yeast chromosomes and (*ii*) a mapped library of λ phage which incorporate >90% of the *S. cerevisiae* genome. Three *SMD* suppressors were characterized further (Table 2).

***SMD6* Is a Truncated Version of *CDC39*.** *SMD6* maps to chromosome III and λ clone 3712. From the published sequence of chromosome III we concluded that *SMD6* contained all or parts of ORFs YCR93W, YCR94W, and YCR95C (19). Subclones containing each of these ORFs were then tested for their ability to rescue *nmt1-181*. The results allowed *SMD6* to be assigned to ORF YCR93W (*CDC39*). *CDC39* is thought to function as a negative regulator of the basal transcriptional activity of a wide variety of genes (20), yet *SMD6* appears to function, at least in part, by increasing the transcriptional activity of *NMT1*'s promoter.

SMD6 lacks codons specifying amino acids 1–573 of the 2108-residue Cdc39p protein (*cdc39-Δ1.7*). The first in-frame methionine codon would correspond to residue 601 of Cdc39p, although we have not established that this codon is the one selected for initiation of translation. YEp and CEN plasmids containing the wild-type *CDC39* allele are unable to suppress the phenotype of *nmt1-181* at 30°C (data not shown), indicating that the activity of *SMD6* is dependent upon removal of a portion of the amino terminus of Cdc39p. However, this truncated protein appears to be able to complement functions encoded by wild-type Cdc39p. For example, *cdc39-1* is a mutant allele that causes constitutive activation of the mating pathway (21, 22). *SMD6* can complement the temperature-sensitive growth defect exhibited by *NMT1 cdc39-1* cells (data not shown). Finally, although *SMD6* has the ability to suppress the basal activity of the mating pathway (Table 2), its rescue of *nmt1-181* must involve N-myristoylproteins other than just Gpa1p; i.e., *SMD6* can produce equivalent rescue of isogenic *nmt1-181*, *STE4*, and *nmt1-181 ste4Δ* strains (data not shown).

***SMD2* Is *FAS1*.** *SMD2* maps to λ clone 3845 on chromosome XI. Analysis of restriction endonuclease digests of this DNA and partial sequencing allowed us to conclude that *SMD2* encodes Fas1p, which specifies the pentafunctional β subunit of the $\alpha_6\beta_6$ Fas complex (23).

The genes in the *de novo* pathway for acyl-CoA synthesis, *FAS1*, *FAS2*, and *ACC1* (*FAS3*) are coordinately regulated at the level of transcription (24). This regulation is produced, in part, by soluble lipid precursors. For example, inositol and choline repress *FAS* transcription through interactions that involve cis-acting $UAS_{INO/FAS/ICE}$ elements (23). Additional cis-acting elements mediate the repression of *FAS* transcription observed when wild-type strains are grown in medium supplemented with fatty acid. This repression appears to depend upon activation of imported fatty acids to their acyl-CoA derivatives (25). Yet other elements appear to be responsible for producing increases in *FAS1* promoter activity when *FAS2* is overexpressed (24). A final level of coordinate regulation involves extravacuolar proteolytic degradation of Fas β subunits which have not been incorporated into functional Fas complexes (26).

To determine whether *SMD2*'s rescue of *nmt1-181* was due, at least in part, to activation of the *FAS2* promoter, we surveyed the levels of *FAS2/lacZ* expression in isogenic *NMT1* and *nmt1-181* strains, using a dual plasmid system. Each strain was transformed with *SMD2* (which includes ≈1 kb of the *FAS1* promoter and its entire 6-kb ORF) or the parental YEp vector without insert. A low-copy YCp-*FAS2/lacZ* construct (pSCFAS2 in ref. 24) was then introduced into these cells. There were no detectable differences in β-galactosidase activity in cell lysates prepared from *NMT1*(YCp-*FAS2/lacZ*) and *nmt1-181*(YCp-*FAS2/lacZ*) strains harboring the parental YEp vector (two experiments performed during midlogarithmic phase of growth at 24°C in selective medium without myristate). YEp-*SMD2* (*FAS1*) produced a 100 ± 10% increase in cellular β-galactosidase activity in the *nmt1-181* strain and a smaller, yet reproducible, increase in the isogenic *NMT1* strain (range, 10–50%; two and four experiments, respectively, each done in duplicate).

Chirala (24) has shown that introduction of a YEp plasmid containing *FAS2* under the control of its own promoter into a wild-type strain of yeast results in a 2-fold increase in expression of the endogenous *FAS1* gene. Thus, our results are consistent with the notion that introduction of multiple copies of *SMD2* (*FAS1*) into *nmt1-181* cells causes increases in the production of Fas1p and Fas2p, the formation of increased amounts of functional $\alpha_6\beta_6$ Fas complexes, increased *de novo* myristoyl-CoA production, and improvement in the ability of nmt181p to acylate essential myristoylproteins (including Gpa1p). The *FAS1*, *FAS2*, and/or *FAS3* genes remain responsive to known modulators of their transcriptional activity despite the defects in protein N-myristoylation associated with *nmt1-181*. For example, omission of inositol from synthetic medium, enhances the growth of *nmt1-181* strains at 24–30°C (data not shown).

***SMD3* Is *PHO5*.** *SMD3* maps to λ clone 6156 on chromosome II. We determined the ORFs contained in the 8-kb *SMD6* plasmid from results generated during systematic sequencing of chromosome II (27). The suppressor function of *SMD3* could be assigned to *PHO5* on the basis of the ability of its ORF to suppress the *nmt1-181* phenotype when placed in a low-copy YCp plasmid under the control of the *GAL1-10* promoter (data not shown) and by *SMD3*'s ability to increase acid phosphatase activity in the medium 3-fold. Pho5p is the major, repressible secreted acid phosphatase produced in *S. cerevisiae*. It provides inorganic phosphate from a variety of substrates to cellular metabolic pathways when phosphate concentrations in the medium are low (28).

Addition of 0.5% KH_2PO_4 to synthetic medium suppresses *PHO5* (acid phosphatase activity is reduced by a factor of 10–15 in both *NMT1* and *nmt1-181* strains; four experiments). Addition of inorganic phosphate also exacerbates the temperature-sensitive growth defect of an *nmt1-181* strain on YPD. Conversely, depletion of phosphate from YPD produces a rescue of *nmt1-181* at 30°C which is equivalent to that obtained with *SMD3* (*PHO5*) (Fig. 1*C*). Two observations indicate that these growth characteristics are directly attributable to changes in *PHO5* expression. First, *nmt1-181* cells

with *SMD3* (but not with the YEp vector alone) are rescued at 30°C even when grown in synthetic medium supplemented with 0.5% KH_2PO_4. Second, Pho80p is a cyclin homolog that functions together with Pho85p (a kinase related to $p34^{cdc2/CDC28}$) to phosphorylate and inactivate Pho4p, a transcription factor needed for transactivation of *PHO5* (29). Deletion of *PHO80* produces constitutive activation of the *PHO5* promoter. Introduction of a *pho80* null allele in an *nmt1-181* strain rescues growth at 30°C even on medium containing phosphate (data not shown).

These observations raise two mechanistic questions. How does *PHO5* expression influence protein N-myristoylation in *nmt1-181* cells? Do changes in cellular N-myristoyltransferase activity affect *PHO5* expression? The answer to the first question may involve components of the *de novo* fatty acid biosynthetic pathway. Conditions that result in induction of *PHO5* (growth at 24°C on synthetic medium without phosphate) produce a very modest (10%) increase in *FAS2/lacZ* expression in an *nmt1-181* strain (control, growth in 0.5% KH_2PO_4). However, this increase is amplified 10-fold when a second YEp plasmid, containing *FAS1* under the control of its own promoter, is added to cells already containing the *FAS2/lacZ* episome.

The more modest nature of the change in *FAS2/lacZ* expression produced by *PHO5* (*SMD3*) compared with *FAS1* (*SMD2*) can be directly correlated with the extent of the rescue produced by these *SMD* genes and their ability to increase myristoylation of Gpa1p (Table 2), lending further support to the idea that their mechanism of suppression of the *nmt1-181* phenotype involves alterations in *de novo* myristoyl-CoA production.

The answer to the second question is yes—changes in cellular N-myristoyltransferase activity do appear to affect *PHO5* expression. *nmt1-181* cells grown at 24°C on synthetic medium produced 2-fold higher acid phosphatase activity in the medium than isogenic *NMT1* cells. Introduction of a centromeric plasmid containing *NMT1* under the control of the *GAL1-10* promoter into *nmt1-181* cells produced a 2-fold decrease in secreted acid phosphatase activity when the cells were grown on synthetic medium containing 2% galactose, to levels which were equivalent to those observed in an isogenic *NMT1* strain grown under the same conditions (Fig. 1*D*). The inverse relationship between cellular N-myristoyltransferase activity and *PHO5* expression was further established by results obtained with a strain containing an *nmt1* null allele that was complemented by integrating *GAL1–10/NMT1* into the *URA3* locus. Growing this strain in galactose-containing medium produced a 70-fold increase in the steady-state level of Nmt1p and a 5-fold decrease in acid phosphatase activity compared to a reference isogenic wild-type strain (four experiments; e.g., see Fig. 1*D*).

The levels of expression of *PHO5* may be determined directly by an N-myristoylprotein. Both Pho4p, a basic–helix–loop–helix protein, and Pho2p, a homeodomain protein, are required to transactivate *PHO5* (28, 29). The amino-terminal sequence of Pho4p, GRTTSEGI, contains several features that make it a potential substrate for Nmt1p—i.e., a Gly^1 and a Ser^5. However, its acidic glutamic residue at position 6 is generally not favored by the enzyme (1). GRTTSEGI is not a good substrate for Nmt1p *in vitro* (Table 1), although examples are known where the determinants for recognition are contained in sequences beyond the amino-terminal eight residues (1). If Pho4p (or another regulator of *PHO5*) is an Nmt1p substrate, then its levels of phosphorylation and myristoylation may determine the level of *PHO5* expression. As noted above, hyperphosphorylation of Pho4p is associated with repression of *PHO5* (29). Our data are compatible with the notion that nonmyristoylated Pho4p may be better able to transactivate *PHO5* than myristoylated Pho4p. Undermyristoylation of Pho4p could serve at least two functions: to induce *PHO5* and to provide a sensor for decreased acylation of other cellular N-myristoylproteins. Pho5p's effects on myristoyl-CoA metabolism (via its effects on Fas production) would allow appropriate adjustments to be made that increase N-myristoyltransferase activity.

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