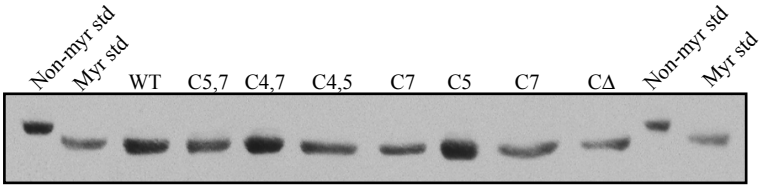
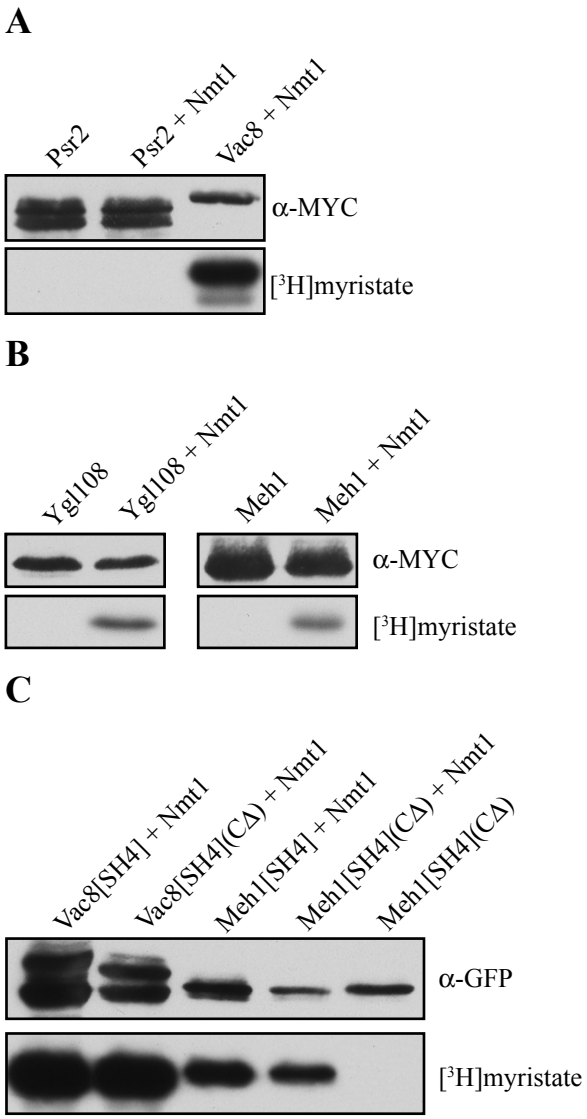
Supplemental Figure S1



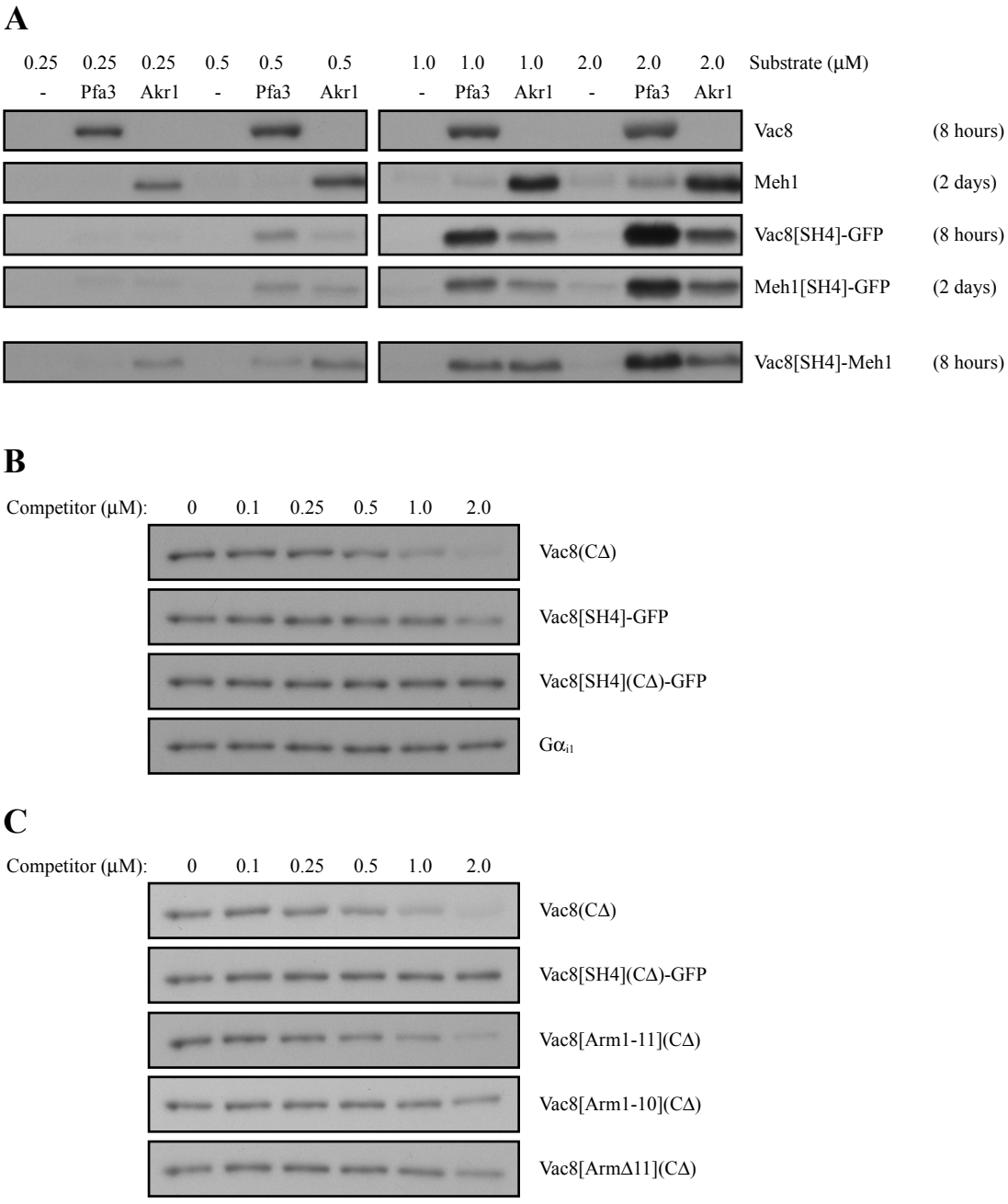
Supplemental Figure S1. ***N*-myristoylation status of Vac8 mutants assessed by mobility shift.** Vac8-myc WT and cysteine mutants were partially purified from *E. coli* coexpressing Nmt1 as described in Experimental Procedures. The proteins were resolved on a 7.5% acrylamide gel with 4 M urea and analyzed by anti-myc immunoblot. Non-myristoylated and *N*-myristoylated standards were prepared from soluble lystates of *E. coli* expressing Vac8-myc in the absence or presence of Nmt1. The partially purified proteins have a similar mobility to that of the myristoylated standard suggesting that the Vac8-myc WT and cysteine mutant preparations are fully myristoylated.

Supplemental Figure S2



Supplemental Figure S2. ***N*-myristoylation of substrates in *E. coli*.** (A) *E. coli* BL21(DE3) were transformed with pML1164 (*PSR2-MYC-6xHIS*), pML1164 and pBB131 (*NMT1*), or pML658 (*VAC8-MYC*) and pBB131. Cultures (50 ml) were grown to OD_{600} = 0.4 and induced with 0.3 mM IPTG in the presence of 1 µg/ml chloramphenicol for 60 min at 30˚C. Each culture (1 ml) was incubated with 1.2 nmol (50 µCi) [^{3}H]myristic acid for 4 h at 30˚C. Cells were collected, suspended in 100 µl lysis buffer (50 mM Tris, pH 8.0, 1 mM EDTA, 1 mM DTT, 2 mg/ml lysozyme, 1x PTT), incubated on ice for 30 min and fractionated at 200,000g for 20 min. The soluble fraction was collected and equal volumes from each condition were separated by SDS-PAGE and prepared for fluorography. Vac8-myc, but not Psr2-myc-6xHis, was radiolabeled with [^{3}H]myristate. Psr2-myc-6xHis is present in lanes 1 and 2, and Vac8-myc is present in lane 3 as shown by anti-myc immunoblot. (B) Ygl108 and Meh1 were analyzed for N-myristoylation as described in A. Both incorporated [^{3}H]myristate when coexpressed with Nmt1 in *E. coli*. (C) Vac8[SH4], Vac8[SH4](CΔ), Meh1[SH4], and Meh1[SH4](CΔ) were analyzed for N-myristoylation as described in A. All incorporated [^{3}H]myristate when coexpressed with Nmt1 in *E. coli*.

Supplemental Figure S3



Supplemental Figure S3. (A) **Representative fluorograph from data in Figure 4 and 5b**. Increasing amounts of myr-Vac8, myr-Meh1, myr-Vac8[SH4]-GFP , myr-Meh1[SH4]-GFP, myr-Vac8[SH4]-Meh1, or myr-Meh1[SH4]-Vac8 were incubated with [^{3}H]Palm-CoA and either no enzyme, 10 nM Pfa3-Flag, or 10 nM Akr1-Flag. Reactions were processed for fluorography and exposed to film for the time indicated to the right of each panel. (B) **Representative fluorograph from data in Figure 6a**. Increasing amounts of myr-Vac8(CΔ), myr-Vac8[SH4]-GFP, myr-Vac8[SH4](CΔ)-GFP, or myr-Gα_{i1} were incubated with 0.1 μM myr-Vac8, [^{3}H]Palm-CoA, and partially purified Pfa3-6xHis-Flag. Reactions were processed for fluorography and exposed to film for 4 h. (C) **Representative fluorograph from data in Figure 6b**. Increasing amounts of myr-Vac8(CΔ), myr-Vac8[SH4](CΔ)-GFP, myr-Vac8[Arm1-11](CΔ), myr-Vac8[Arm1-10](CΔ),or myr-Vac8[ArmΔ11](CΔ were incubated with 0.1 μM myr-Vac8, [^{3}H]Palm-CoA, and partially purified Pfa3-6xHis-Flag. Reactions were processed for fluorography and exposed to film for 4 h.

STable I. 100% values for densitometry in Figure 4 & 5B

Figure 4 - Substrate	**Expt**	**Arbitrary Units**
A. Myr-Vac8	1	3008
	2	3430
	3	6311
B. Myr-Vac8[SH4]-GFP	1	32072
	2	25512
	3	38416
C. Myr-Meh1	1	14036
	2	10444
	3	9660
D. Myr-Meh1[SH4]-GFP	1	4708
	2	8916
	3	4876
Figure 5 - Substrate	**Expt**	**Arbitrary Units**
B. Myr-Meh1[SH4]-Vac8	1	4932
	2	3991
	3	2667

STable II. 100% values for densitometry in Figure 6A.

Competitor	**Expt**	**Arbitrary Units**
Myr-Vac8(CΔ) (●)	1	1791
	2	4198
	3	6374
Myr-Vac8[SH4]-GFP (▲)	1	1291
	2	3894
	3	6093
Myr-Vac8[SH4](CΔ)-GFP (■)	1	2183
	2	4352
	3	6480
Myr -Gα_{i1} (◆)	1	2797
	2	3911
	3	5863

STable III. 100% values for densitometry in Figure 6B.

Competitor	**Expt**	**Arbitrary Units**
Myr-Vac8(CΔ) (●)	1	3674
	2	1913
	3	4849
Myr-Vac8[SH4](CΔ)-GFP (■)	1	3880
	2	2107
	3	4949
Myr-Vac8[Arm1-11](CΔ) (△)	1	3815
	2	2068
	3	4497
Myr-Vac8[Arm1-10](CΔ) (◇)	1	4002
	2	1956
	3	4775
MyrVac8[ArmΔ11](CΔ) (□)	1	3870
	2	1966
	3	4830