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Regulatory Subunit Myristoylation Antagonizes Calcineurin Phosphatase Activation in Yeast*[S]

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Background: Stimulation of calcineurin phosphatase activity is required for diverse cellular responses to calcium signals.
Results: Disruption of calcineurin regulatory subunit myristoylation causes constitutive activation of calcineurin-dependent gene transcription in yeast.
Conclusion: Myristoylation of calcineurin B limits phosphatase activation by intracellular calcium signals.
Significance: Our findings reveal that *N*-myristoyltransferase activity regulates the calcium sensitivity of calcineurin activation.

The Ca^{2+}/calmodulin-stimulated protein phosphatase calcineurin is a critical component of Ca^{2+} signaling cascades in eukaryotic cells. Myristoylation of the regulatory subunit of calcineurin (CNB) is conserved from yeast to humans. Here, we show that CNB myristoylation antagonizes phosphatase activation in yeast. Disruption of CNB myristoylation by mutation of the myristoylated glycine triggered constitutive expression of a calcineurin-dependent reporter gene and enhanced calcineurin-dependent phenotypes. Basal phosphatase activity was also increased in *nmt1–181* yeast with reduced *N*-myristoyltransferase activity. Our findings are the first demonstration of a functional role for CNB myristoylation and reveal the importance of Nmt1 in modulating cellular calcineurin activation.

Ca^{2+} is a ubiquitous second messenger utilized by cells to orchestrate responses to diverse environmental stimuli and developmental cues. Cellular responses to Ca^{2+} signals are determined by the amplitude, duration, frequency, and localization of Ca^{2+} transients via activation of downstream Ca^{2+}-dependent signaling molecules (1–3). An important determinant of Ca^{2+} signaling output in eukaryotes is the Ca^{2+}- and calmodulin-stimulated protein phosphatase calcineurin. Calcineurin regulates numerous cellular processes, including cell proliferation, differentiation, survival, and death, via its ability to regulate the activity of transcription factors, ion channels, and cell signaling molecules (4).

A key target of calcineurin in mammals is the nuclear factor of activated T cells (NFAT)[2] family of transcription factors. Activation of NFAT by calcineurin-mediated dephosphorylation is necessary for lineage specification in embryonic stem cells (5). Calcineurin-NFAT signaling is also essential for the formation and function of the cardiovascular, nervous, immune, and musculoskeletal systems (6, 7). Calcineurin-NFAT signaling must be appropriately regulated *in vivo*. Mice expressing an NFAT allele with increased affinity for calcineurin exhibit developmental abnormalities (8). Alterations in calcineurin activity have been linked to multiple human diseases including cardiac hypertrophy (9, 10), cancer (11, 12), and neurodegeneration (13). Inhibition of calcineurin phosphatase activity underlies the therapeutic action of the immunosuppressants tacrolimus and cyclosporine A, which have been extensively administered to patients to prevent organ transplant rejection (14). Binding of immunosuppressant/immunophilin complexes to calcineurin inhibits phosphatase activity, thereby preventing T-cell activation.

Calcineurin is a heterodimer composed of a catalytic subunit CNA and a smaller regulatory subunit CNB that confers Ca^{2+} responsiveness to phosphatase activation (15). Full phosphatase activation further requires the recruitment of Ca^{2+}/calmodulin to displace the autoinhibitory domain of CNA from the catalytic core. Thus, calcineurin activation by intracellular Ca^{2+} transients requires the coordinated action of two Ca^{2+}-binding proteins. Given the diversity of Ca^{2+} signals occurring in specific cell types during development, additional mechanisms must exist to control the spatial and temporal dynamics of calcineurin signaling *in vivo*. Endogenous factors such as regulator of calcineurin (RCAN), A-kinase-anchoring protein 79 (AKAP79), and superoxide dismutase 1 (SOD1) interact with calcineurin to modulate phosphatase activity, localization, and stability (16, 17). Substrate competition also determines the output of calcineurin signaling *in vivo* (18). The regulation of calcineurin function via calcineurin-substrate, calcineurin-scaffolding protein, and calcineurin-inhibitory factor interactions has recently been reviewed (19). In contrast, the contribution of calcineurin protein modifications in shaping phosphatase activity *in vivo* remains largely unknown.

Ca^{2+} signaling cascades are highly conserved from yeast to humans. As observed for mammalian calcineurin, stimulation of phosphatase activity in yeast requires Ca^{2+}/calmodulin and

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[S] This article contains supplemental Figs. 1 and 2.
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[2] The abbreviations used are: NFAT, nuclear factor of activated T cells; CDRE, calcineurin-dependent reporter gene; CNB, calcineurin B subunit; CNA, calcineurin A subunit; Nmt, *N*-myristoyltransferase; RCAN, regulator of calcineurin; Crz1, calcineurin-responsive zinc-finger; YPD, yeast extract peptone dextrose medium; SD, synthetic complete medium; cmd, calmodulin; GCAP1, guanylyl cyclase-activating protein; RetGC, retinal guanylyl cyclase.

TABLE 1
Yeast strains used in this study

Yeast strain	Relevant genotype	Source
K603	*cnb1*Δ	Cunningham and Fink (51)
JGY41	*cmd1–3*	Geiser *et al.* (38)
YB336	*nmt1–181*	Johnson *et al.* (20)
YB332	*NMT1*	Johnson *et al.* (20)
TK101	*cnb1*Δ, *nmt1–181*	This study

is sensitive to inhibition by immunosuppressant/immunophilin complexes or regulator of calcineurin overexpression (4, 21). The regulatory subunit of yeast calcineurin is encoded by a single gene, *CNB1* (22). The catalytic subunit is encoded by two genes, *CNA1* and *CNA2* (23). Yeast lacking functional calcineurin due to genetic deletion of either *CNB1* (*cnb1*Δ) or both *CNA1* and *CNA2* (*cna1*Δ *cna2*Δ) are viable, but exhibit reduced survival following exposure to environmental stress (24). Calcineurin-mediated responses to cell stress requires activation of the transcription factor Crz1 (calcineurin-responsive zinc finger) (25, 26), which is regulated in a manner similar to mammalian NFAT. In the absence of Ca^{2+} signaling, Crz1 is phosphorylated and cytoplasmic. Ca^{2+} signaling stimulates calcineurin-dependent Crz1 dephosphorylation, leading to Crz1 accumulation in the nucleus where it activates transcription of genes required for calcium homeostasis and stress response (27). We are taking advantage of the yeast model system to identify cellular mechanisms regulating phosphatase activity. As a consequence of our mutational analysis of the evolutionarily conserved serine residue located within the Cnb1 myristoylation consensus site sequence, we have discovered a role for Cnb1 myristoylation in preventing constitutive calcineurin activity in yeast.

EXPERIMENTAL PROCEDURES

Yeast Culture—All yeast culture and transformations were conducted using standard techniques. Yeast rich medium (YPD) or synthetic medium (SD) were purchased from Clontech. Agar, amino acids, and salts were purchased from Difco and Sigma. YPD medium was buffered to pH 5.5 by the addition of 0.5 M succinic acid as indicated. Yeast strains used in this study are noted in Table 1.

Cloning and Mutagenesis—Yeast CNB1 genomic DNA plasmid pYDZ3 was a kind gift of J. Heitman (28). *CNB1* genomic region was cloned into pRS313 BamHI-XhoI to generate pTJK100. QuikChange site-directed mutagenesis (Stratagene) of pTJK100 was conducted according to the manufacturer's instructions to generate mutant *CNB1* alleles: *CNB1-S6A*, GACCATCCACAATTTTGGCAGGAGCAGCACCCAT; *CNB1-S6D*, GACCATCCACAATTTTGTCAGGAGCAGCACCCAT; *CNB1G2A*, CATTTTTATTTCTTAAAATGGCTGCTGCTCCTTCCAAAATTG; *CNB1-G2E*, CATTTTTATTTCTTAAAATGGAAGCTGCTCCTTCCAAAATTG. Constructs were verified by DNA sequencing.

Ion Tolerance Assays—Yeast strains were inoculated into YPD supplemented with a range of LiCl or $MnCl_2$ concentrations as indicated. Yeast were grown as 0.18-ml cultures in 96-well flat bottom dishes in a 30 °C incubator without shaking for 2 days. The A_{600} was measured using a BioTek μQuant plate reader following resuspension of yeast.

β-Galactosidase Assays—Yeast cultures were grown overnight in SD lacking histidine and uracil, pelleted, and resuspended in YPD (pH 5.5) medium supplemented with $CaCl_2$ as indicated and plated in 24-well dishes. Following 4 h incubation at 30 °C, β-galactosidase activity was measured as described previously (21).

Protein Analysis—Yeast were grown overnight in SD-His medium, pelleted, resuspended in YPD, and cultured an additional 4 h. Yeast were quantitated by A_{600}, and an equal number of yeast were harvested for protein extraction as described previously (21). Proteins were separated by SDS-PAGE on 4–20% Precise protein gels (Thermo Scientific) and subjected to Western blotting using anti-Cnb1 (28). For membrane association studies, *cnb1*Δ yeast expressing wild type Cnb1, Cnb1G2A, or Cnb1G2E were grown in YPD (pH 5.5) for 4 h before harvesting by bead beating in ice-cold Hepes buffer (20 mM, pH 7.2) with 2 mM EDTA, 1 mM DTT, 1 mM PMSF, and 1× Halt protease and phosphatase inhibitor mixture. Lysates were clarified at 5000 rpm microcentrifugation for 5 min and then subjected to high-speed ultracentrifugation (100,000 × *g*) for 30 min. Samples were separated by SDS-PAGE and probed with anti-Cnb1p. Total extract sample was collected after initial clarification, prior to 2-fold dilution of extracts for ultracentrifugation, and is thus loaded on gel at 2×.

RESULTS

To identify cellular mechanisms regulating calcineurin activity, we initiated a mutational analysis of the regulatory subunit of calcineurin in yeast. Initially, we focused on mutating the 8 serine residues conserved between yeast Cnb1 and human CNB because serine residues are potential sites for protein phosphorylation. During our analysis, we observed that mutation of the serine residue located within the myristoylation consensus sequence (Ser-6) increased basal calcineurin activity. Site-directed mutagenesis of *CNB1* was conducted to mutate Ser-6 of Cnb1 to either alanine (Cnb1-S6A) or aspartic acid (Cnb1-S6D) to generate potential phosphoryl-defective and phosphoryl-mimic alleles. The functional consequence of the Ser-6 mutations was subsequently determined by assaying the ability of the mutant Cnb1 to restore calcineurin activity to yeast in which the chromosomal *CNB1* gene has been deleted (*cnb1*Δ).

Calcineurin activity in yeast is readily quantitated by measuring expression of the calcineurin-dependent reporter gene *CDRE-lacZ* (calcineurin-dependent response element), composed of four copies of the minimal binding domain of the calcineurin-dependent transcription factor Crz1 (26). Ca^{2+} influx triggered by the addition of extracellular $CaCl_2$ leads to robust stimulation of *CDRE-lacZ* activity. *Cnb1*Δ yeast harboring the *CDRE-lacZ* reporter gene were transformed with low-copy centromere-based plasmids expressing wild type Cnb1, Cnb1-S6A, or Cnb1-S6D under control of the endogenous *CNB1* promoter. Calcineurin activity was assessed by stimulating yeast with a range of added extracellular $CaCl_2$ (0–100 mM) to initiate Ca^{2+} signaling. Four hours after stimulation, yeast were harvested for quantitative β-galactosidase assays to measure *CDRE-lacZ* activity. As expected, Ca^{2+} influx triggered by the addition of extracellular $CaCl_2$-stimulated *CDRE-lacZ* activity in a dose-dependent manner in yeast expressing wild type Cnb1

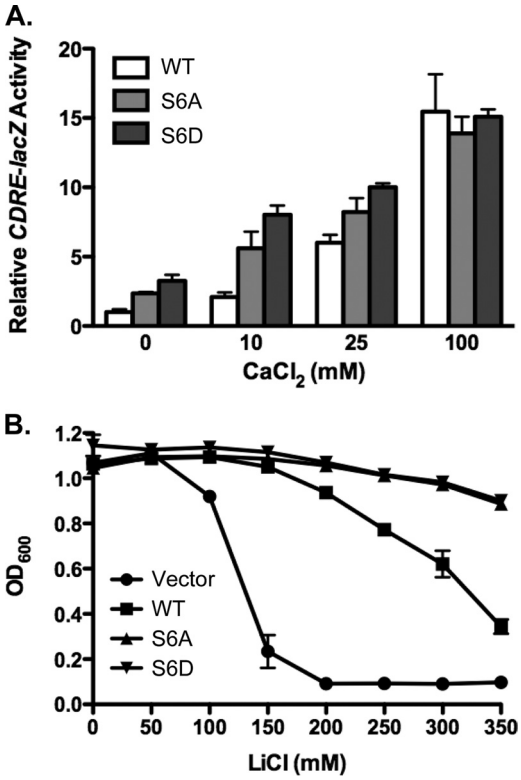

FIGURE 1. **Conserved serine S6 modulates calcineurin activity.** The ability of Cnb1-S6A and Cnb1-S6D mutants to complement *cnb1*Δ yeast was assayed using calcineurin-dependent reporter gene assays and calcineurin-mediated ion-resistant growth assays. *A*, *Cnb1*Δ yeast harboring the *CDRE-lacZ* reporter gene were transformed with centromere-based plasmids expressing wild type Cnb1, Cnb1-S6A (*S6A*) or Cnb1-S6D (*S6D*). Yeast were subsequently grown in YPD (pH 5.5) in the presence or absence of added extracellular $CaCl_2$ (0, 10, 25, or 100 mM). Four hours after $CaCl_2$ stimulation, yeast were harvested for quantitative β-galactosidase assays. Data plotted are average of four independent yeast transformants ± S.D. *B*, *Cnb1*Δ yeast expressing the indicated *CNB1* alleles were grown in YPD supplemented with increasing concentrations of LiCl (in mM) at 30 °C for 2 days. Growth was quantitated by measuring A_{600}. Yeast transformed with empty vector were included as a negative control. Data points are average of four independent yeast transformants assayed in parallel ± standard error.

(Fig. 1*A*). Interestingly, yeast expressing Cnb1-S6A or Cnb1-S6D exhibited 2–3-fold increased *CDRE-lacZ* activity relative to wild type Cnb1 in response to either 0 mM or 10 mM added extracellular $CaCl_2$. In contrast, no significant difference in *CDRE-lacZ* activity was observed between yeast expressing wild type Cnb1, Cnb1-S6A, or Cnb1-S6D in response to a strong Ca^{2+} signaling stimulus of 100 mM extracellular $CaCl_2$. Thus, mutation of Ser-6 specifically enhanced calcineurin activity at low intracellular Ca^{2+} levels, resulting in constitutive phosphatase activation.

Because the primary role of calcineurin signaling in yeast is to mediate stress responses, we tested whether the enhanced calcineurin activity observed in yeast expressing Cnb1-S6A or Cnb1-S6D could confer a selective advantage in response to environmental stress. The ability of yeast to grow in the presence of high extracellular Li^+ is dependent upon calcineurin signaling (29). We therefore compared the ability of *cnb1*Δ yeast transformed with Cnb1, Cnb1-S6A, or Cnb1-S6D to grow in the presence of a range of extracellular Li^+. Yeast transformed with empty vector, and therefore lacking functional calcineurin, were included as negative control. Li^+-resistant growth was assayed by measuring the A_{600} 2 days after inoculation of yeast into medium supplemented with increasing concentrations of LiCl (Fig. 1*B*). As expected, growth of *cnb1*Δ yeast transformed with empty vector was inhibited by the presence of extracellular LiCl, exhibiting 75% inhibition of growth at 150 mM LiCl. In contrast, the growth of yeast transformed with wild type Cnb1 was resistant to LiCl, retaining greater than 50% growth in the presence of 300 mM extracellular LiCl. Consistent with the increased calcineurin activity observed in *CDRE-lacZ* reporter gene assays, yeast expressing Cnb1-S6A or Cnb1-S6D exhibited even more resistance to extracellular Li^+ than yeast with wild type calcineurin. Cnb1-G2A- and Cnb1-G2E-expressing yeast maintained greater than 90% cell growth at 350 mM LiCl, the highest concentration of ion tested. These observations suggest that Ser-6 limits calcineurin activity in response to endogenous intracellular Ca^{2+} signaling triggered by environmental stress.

Although our initial mutations were designed to generate phosphoryl-defective and phosphoryl-mimic versions of Cnb1, our observation that both Cnb1-S6A and Cnb1-S6D enhance calcineurin-dependent phenotypes is not consistent with Ser-6 modulating calcineurin activation via a phosphorylation-dependent switch. Closer examination of the Cnb1 sequence revealed that Ser-6 (underlined) is located within the Cnb1 myristoylation consensus site Met-Gly-*X*-*X*-*X*-Ser-*X*-*X*-*X*. *N*-Myristoylation refers to the covalent attachment of myristate, a short 14-carbon fatty acid, to the amino-terminal glycine residue following removal of the initiating methionine residue during protein translation (30, 31). Myristoylation of CNB is conserved from yeast to humans (28, 32), but a functional consequence of this modification had not previously been reported.

To test the possibility that the increased calcineurin activity observed in yeast expressing Cnb1-S6A and Cnb1-S6D was due to the loss of Cnb1 myristoylation, we conducted site-directed mutagenesis to mutate the myristoylated glycine residue to alanine (Cnb1-G2A). The G2A mutation has previously been shown to disrupt Cnb1 myristoylation in yeast (28). Plasmids expressing either wild type Cnb1 or Cnb1-G2A were transformed into *cnb1*Δ yeast harboring the *CDRE-lacZ* reporter gene. The functional consequence of the Cnb1-G2A mutation on calcineurin activity was assayed by quantitating *CDRE-lacZ* activity following stimulation by the addition of a range of extracellular $CaCl_2$. Similar to our observations for Cnb1-S6A and Cnb1-S6D, expression of Cnb1-G2A increased *CDRE-lacZ* activity in unstimulated (0 mM $CaCl_2$) and submaximally (10 and 25 mM $CaCl_2$) stimulated yeast (Fig. 2*A*). In contrast, similar levels of *CDRE-lacZ* activity were observed for wild type Cnb1 and Cnb1-G2A following the addition of 100 mM extracellular $CaCl_2$. Thus, disruption of Cnb1 myristoylation by the G2A mutation stimulates calcineurin activity in yeast under submaximal Ca^{2+} signaling conditions.

Examination of the dbSNP database revealed an SNP (rs61757747) in which the myristoylated glycine of CNB is mutated to glutamic acid. The CNB SNP was reported from a large-scale analysis of cancer patients,[3] but the functional sig-

[3] Cancer Genome Project, The Wellcome Trust, Sanger Institute.

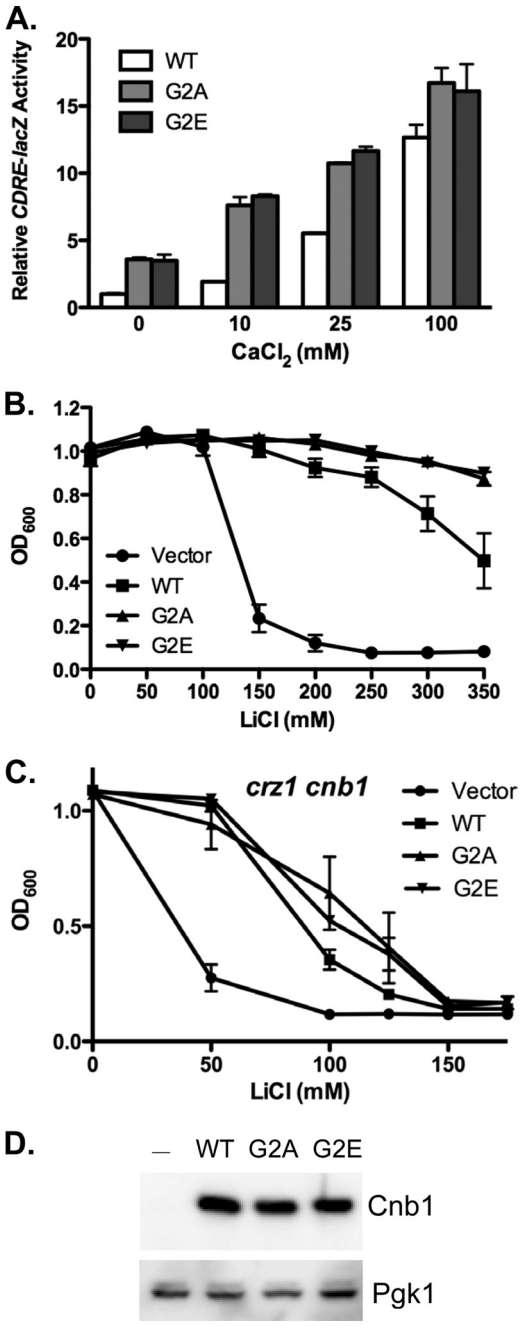

FIGURE 2. **Cnb1 myristoylation antagonizes calcineurin activation.** The myristoylated glycine residue of Cnb1 was mutated to either alanine (Cnb1-G2A) or glutamic acid (Cnb1-G2E). Mutants were then tested for their ability to restore calcineurin-mediated gene expression and ion-resistant growth to *cnb1*Δ yeast. *A*, *Cnb1*Δ yeast harboring the *CDRE-lacZ* reporter gene were transformed with plasmids expressing Cnb1, Cnb1-G2A, or Cnb1-G2E. Yeast growing in YPD (pH 5.5) were stimulated for 4 h by the addition of extracellular $CaCl_2$ prior to harvesting for quantitative β-galactosidase assays. Data plotted are average of four independent yeast transformants ± S.D. *B* and *C*, ion tolerance assays. *cnb1*Δ (*B*) or *cnb1*Δ *crz1*Δ (*C*) yeast were transformed with Cnb1, Cnb1-G2A, Cnb1-G2E, or empty vector as indicated. Yeast were inoculated into YPD containing increasing concentrations of LiCl (in mM) and grown at 30 °C for 2 days. Growth was quantitated by measuring the A_{600}. Data points are average of three independent yeast transformants assayed in parallel ± S.E. *D*, anti-Cnb1 Western blot analysis of protein extracts prepared from *cnb1*Δ yeast transformed with wild type Cnb1, Cnb1-G2A, Cnb1-G2E, or empty vector. Anti-Pgk1 was used for loading control comparison.

nificance or clinical relevance of the SNP is unknown. To determine whether the glycine to glutamic acid mutation would similarly stimulate calcineurin activity, we performed site-directed mutagenesis to generate a *CNB1-G2E* allele. As observed for Cnb1-G2A, expression of Cnb1-G2E resulted in constitutive expression of *CDRE-lacZ* and enhanced *CDRE-lacZ* expression in response to submaximal Ca^{2+} signaling.

To test the ability of Cnb1-G2A and Cnb1-G2E to enhance calcineurin activity in response to endogenous Ca^{2+} signaling, we conducted ion tolerance growth assays. As observed for yeast expressing Cnb1 Ser-6 mutants, yeast expressing Cnb1-G2A or Cnb1-G2E exhibited increased resistance to extracellular Li^+, retaining 90% growth even at 350 mM LiCl (Fig. 2*B*). A similar enhancement of calcineurin-mediated Mn^{2+} resistance was also observed in yeast expressing Cnb1-G2A or Cnb1-G2E as compared with wild type Cnb1 (supplemental Fig. 1). Thus, introduction of the G2A or G2E mutation into Cnb1 increases calcineurin-dependent ion-tolerant growth in yeast. Comparison of Cnb1, Cnb1-G2A, and Cnb1-G2E protein by Western blot revealed similar levels of expression (Fig. 2*C*), indicating that the increased calcineurin activity observed in yeast expressing Cnb1-G2A or Cnb1-G2E was not simply due to increased Cnb1 expression. These observations suggest that disruption of Cnb1 myristoylation leads to increased calcineurin activity in yeast in response to endogenous Ca^{2+} signaling.

Dephosphorylation and activation of Crz1 are a critical component of calcineurin-mediated stress responses. To determine whether calcineurin activity toward additional targets is also enhanced in the absence of Cnb1 myristoylation, *crz1*Δ *cnb1*Δ yeast were transformed with Cnb1, Cnb1-G2A, or Cnb1-G2E and assayed for Li^+-resistant growth. In the absence of Crz1, calcineurin promotes a modest level of Li^+ resistance (Fig. 2*C*). *Crz1*Δ yeast transformed with empty vector exhibit 50% growth inhibition at 40 mM LiCl. In contrast, *crz1*Δ yeast expressing wild type Cnb1 did not exhibit 50% growth inhibition until ~90 mM LiCl. Yeast expressing either Cnb1-G2A or Cnb1-G2E had slightly higher Li^+ resistance, retaining 50% growth at 110 mM LiCl. Thus, the enhanced calcineurin activity observed for Cnb1-G2A and Cnb1-G2E is not unique to Crz1.

N-Myristoylation of proteins is mediated by *N*-myristoyltransferase (33, 34). If the enhanced calcineurin activity we observe in Cnb1-G2A- and Cnb1-G2E-expressing yeast is due to the loss of myristoylation, then reduced *N*-myristoyltransferase activity should similarly lead to enhanced basal calcineurin activity. *N*-Myristoyltransferase is encoded by the *NMT1* gene in *Saccharomyces cerevisiae* (35). Although deletion of the *NMT1* gene leads to the loss of viability in yeast, a temperature-sensitive allele (*nmt1–181*) with reduced activity has been reported (36). The *nmt1–181* mutant and isogenic wild type yeast were transformed with the *CDRE-lacZ* reporter gene. Calcineurin activity was subsequently assayed by quantitating *CDRE-lacZ* activity at a semi-permissive temperature of 30 °C in medium supplemented with a range of $CaCl_2$ from 0 to 100 mM. As shown in Fig. 3, *nmt1–181* mutant yeast exhibited increased *CDRE-lacZ* activity in unstimulated or submaximally stimulated yeast as compared with wild type yeast. *CDRE-lacZ* activity was similar in wild type and *nmt1–181* at 100 mM

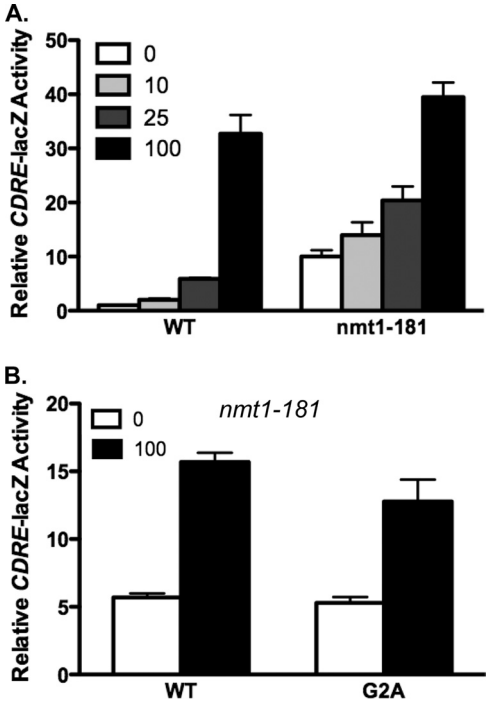

FIGURE 3. ***N*-Myristoyltransferase antagonizes calcineurin activity.** *A*, the *CDRE-lacZ* reporter gene was transformed into *nmt1–181* or isogenic wild type yeast. Quantitative β-galactosidase assays were conducted following overnight growth at 30 °C in YPD (pH 5.5) supplemented with $CaCl_2$ (in mM) as indicated. *B*, *nmt1–181 cnb1*Δ yeast harboring the *CDRE-lacZ* reporter gene were transformed with plasmids expressing wild type Cnb1, Cnb1-G2A, or Cnb1-G2E. Yeast were grown in YPD (pH 5.5) in the presence or absence of 100 mM added extracellular $CaCl_2$ for 4 h prior to harvesting for quantitative b-galactosidase assays. Data shown are average of quadruplicates ± S.D.

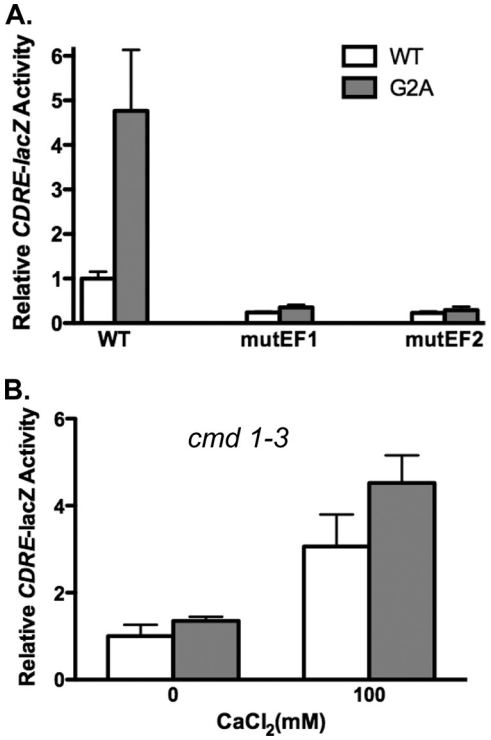

FIGURE 4. **Ca^{2+} and calmodulin requirements of myristoylation-defective calcineurin.** *A*, site-directed mutagenesis of wild type Cnb1 and Cnb1-G2A was conducted to mutate the first or second EF hand Ca^{2+} binding domain as indicated. Plasmids expressing either wild type or mutant Cnb1 were transformed into *cnb1*Δ yeast harboring the *CDRE-lacZ* reporter gene. Quantitative β-galactosidase assays were conducted on yeast grown in YPD (pH 5.5) in the absence of added extracellular $CaCl_2$ to measure basal *CDRE-lacZ* activity. *B*, Cnb1 or Cnb1-G2A expression plasmids were transformed into *cmd1–3 cnb1*Δ yeast harboring the *CDRE-lacZ* reporter gene. *CDRE-lacZ* activity was measured after 4 h of growth in YPD (pH 5.5) in the presence or absence of 100 mM $CaCl_2$. Data plotted are average of quadruplicates ± S.D.

$CaCl_2$. We subsequently generated *nmt1–181 cnb1*Δ yeast harboring the *CDRE-lacZ* reporter gene to test whether the increased basal activity observed for Cnb1-G2A calcineurin as compared with wild type Cnb1 could be explained solely by disruption of myristoylation. Following transformation of *nmt1–181 cnb1*Δ yeast with plasmids expressing either wild type Cnb1 or Cnb1-G2A, we quantitated *CDRE-lacZ* expression. Basal *CDRE-lacZ* activity was indistinguishable between *nmt1–181 cnb1*Δ expressing either wild type Cnb1 or Cnb1-G2A, indicating that the loss of myristoylation was responsible for the increased calcineurin activity associated with Cnb1-G2A.

Activation of calcineurin requires Ca^{2+} binding to Cnb1 and recruitment of Ca^{2+}/calmodulin. To determine whether the increased basal activity of Cnb1-G2A calcineurin reflected either enhanced Ca^{2+}-dependent or enhanced Ca^{2+}-independent phosphatase activation, we tested the consequences of mutating the EF hand Ca^{2+} binding domains of either Cnb1 or calmodulin. Cnb1, like its mammalian counterpart CNB, contains four EF hand Ca^{2+} binding domains. Mutation of either the first or the second Ca^{2+} binding EF hand domain of mammalian CNB inhibits calcineurin activation *in vitro* (37). We have recently obtained similar results when EF hand mutations are introduced into yeast Cnb1.[4] Site-directed mutagenesis was conducted to independently mutate the first or second EF hand calcium binding domain of Cnb1 and Cnb1-S6A to generate Cnb1mutEF1, Cnb1mutEF2, Cnb1-G2AmutEF1, and Cnb1-G2AmutEF2. If disruption of myristoylation stimulates calcineurin activity by increasing the sensitivity of calcineurin to Ca^{2+}, then mutation of the EF hand domains will prevent the enhanced phosphatase activity observed in Cnb1-G2A relative to Cnb1. Plasmids expressing either mutant or control Cnb1 or Cnb1-G2A were transformed into *cnb1*Δ yeast harboring the *CDRE-lacZ* reporter gene, and basal *CDRE-lacZ* activity was measured. As shown in Fig. 4*A*, introduction of the EF hand mutations reduces basal *CDRE-lacZ* expression. Comparison of yeast expressing Cnb1mutEF1 *versus* Cnb1-G2AmutEF1 or Cnb1mutEF2 *versus* Cnb1-G2AmutEF2 revealed that the presence of the EF hand mutations blocked the ability of the G2A mutation to stimulate basal calcineurin activity. Cnb1 and Cnb1-G2A EF hand mutant-expressing yeast exhibited similar levels of *CDRE-lacZ* activity. Thus, the increased phosphatase activity observed for Cnb1-G2A calcineurin does not bypass the requirement for Ca^{2+} binding. Enhanced Ca^{2+}-dependent calcineurin activation upon the loss of Cnb1 myristoylation is consistent with our observation that the elevated basal activity of Cnb1-G2A calcineurin is inhibited by FK506 (supplemental Fig. 2*A*).

To further test the Ca^{2+} requirements of Cnb1-G2A basal activity, we tested the consequence of expressing Cnb1-G2A in

[4] S. Connolly and T. Kingsbury, manuscript in preparation.

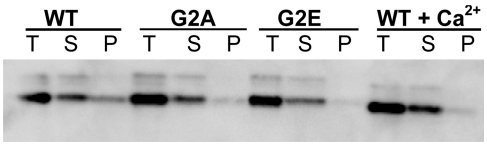

FIGURE 5. **Myristoylation promotes calcineurin membrane association.** *Cnb1*Δ yeast expressing wild type Cnb1 (*WT*), Cnb1-G2A (*G2A*), or Cnb1-G2E (*G2E*) were grown in YPD (pH 5.5) for 4 h prior to lysis and ultracentrifugation to yield supernatant *versus* pellet fractions. Wildtype yeast were stimulated by the addition of 100 mM $CaCl_2$ to media 15 min prior to harvesting as indicated ($+Ca^{2+}$). Protein samples were separated by SDS-PAGE and probed with anti-Cnb1. *T*, total protein; *S*, supernatant; *P*, pellet.

cmd1–3 mutant yeast, which expresses a mutant form of calmodulin that cannot bind Ca^{2+} or stimulate calcineurin activity (38). If the enhanced basal calcineurin activity observed in Cnb1-G2A-expressing yeast reflects increased Ca^{2+}-dependent phosphatase activation, then the loss of calmodulin function should disrupt the increase in basal activity. Comparison of basal *CDRE-lacZ* activity between Cnb1 and Cnb1-G2A calcineurin in *cnb1*Δ *cmd1–3* yeast revealed similar levels of calcineurin activity (Fig. 4*B*). Thus, Ca^{2+}/calmodulin is required to promote the increased basal calcineurin activity observed for Cnb1-G2A calcineurin. Taken together, our findings suggest that Cnb1 myristoylation limits Ca^{2+}- and Ca^{2+}/calmodulin-mediated activation of cellular calcineurin.

Because myristoylation of proteins can promote interaction with membranes, we next tested the consequences of disrupting Cnb1 myristoylation on calcineurin membrane association. High-speed ultracentrifugation of protein extracts prepared from yeast expressing wild type Cnb1, Cnb1G2A, or Cnb1-G2E was conducted to separate membrane-bound *versus* cytosolic calcineurin as described previously (28). As shown in Fig. 5, Cnb1-G2A and Cnb1-G2E have reduced membrane association as compared with wild type Cnb1. Quantification of the ratio of calcineurin observed in the pellet *versus* supernatant (membrane fraction *versus* soluble) revealed a 2.5- and 2.8-fold decrease for Cnb1-G2A and Cnb1-G2E calcineurin as compared with wild type enzyme. Cnb1 myristoylation therefore appears to promote membrane association of a fraction of calcineurin in yeast. Because disruption of Cnb1 myristoylation also increased phosphatase activation, we tested the possibility that Ca^{2+} signaling might modulate calcineurin membrane association. Yeast expressing wild type Cnb1 were stimulated with 100 mM $CaCl_2$ for 15 min prior to harvesting extracts for ultracentrifugation. As observed for the myristoylation mutants, Ca^{2+} signaling reduced membrane association of calcineurin (Fig. 5). Quantification of Cnb1 revealed a 3-fold decrease in the ratio of calcineurin in the pellet *versus* supernatant following stimulation of Ca^{2+} signaling as compared with unstimulated yeast.

DISCUSSION

Myristoylation of the calcineurin regulatory subunit is conserved from yeast to humans (4). In this study, we used a combination of Cnb1 mutational analysis and mutant yeast strains to demonstrate that Cnb1 myristoylation reduces calcineurin activity in response to submaximal Ca^{2+} signals. Disruption of Cnb1 myristoylation via mutation of the critical glycine residue or inhibition of *N*-myristoyltransferase activity resulted in constitutive phosphatase activity. Our findings are the first demonstration of a functional role of Cnb1 myristoylation in modulating calcineurin activity. Previous studies, conducted in yeast and *in vitro* using recombinant mammalian calcineurin, had demonstrated that myristoylation was not essential for phosphatase activity (28, 39). These studies had focused on testing the requirement for myristoylation in promoting calcineurin activity and were not designed to detect enhanced activity.

The increased basal activity observed in yeast expressing nonmyristoylated calcineurin was blocked when the Ca^{2+} binding domains of either Cnb1 or calmodulin were mutated. Thus, disruption of Cnb1 myristoylation appears to stimulate calcineurin activity by enhancing the Ca^{2+} sensitivity of phosphatase activation rather than promoting Ca^{2+}-independent enzyme activation. The enhanced Ca^{2+} sensitivity of nonmyristoylated calcineurin could reflect increased affinity for Ca^{2+}. Such a mechanism would be consistent with our observation that Cnb1-G2A specifically increased calcineurin activity in response to submaximal Ca^{2+} signals. *N*-Myristoylation of the EF hand proteins recoverin, neuronal calcium sensor (NCS-1), and guanylyl cyclase-activating protein (GCAP1) has previously been shown to modulate Ca^{2+} binding (40–42). Myristoylation of recoverin alters the conformation of the protein, causing an apparent reduction in Ca^{2+} affinity (40). *N*-Myristoylation of NCS-1 and GCAP1 increases Ca^{2+} affinity (41, 42). Interestingly, myristoylation of GCAP1 also increases the affinity of GCAP1 for retinal guanylyl cyclase (RetGC) and modulates the Ca^{2+}-induced conformational changes in GCAP1 critical for regulating RetGC activation (42, 43). Myristoylated GCAP1 consequently stimulates RetGC activation to a higher maximal level than nonmyristoylated GCAP1. Myristate-mediated conformational changes in calcineurin activation may similarly be important for triggering catalytic subunit activation upon Cnb1 Ca^{2+} binding. Unfortunately, available calcineurin crystal structures were obtained using recombinant proteins lacking the amino terminus of CNB and therefore do not address the localization of the myristate group. Determining whether the myristate interacts with other domains within calcineurin will be critical in developing molecular models of how regulatory subunit myristoylation regulates enzyme activation. Structural details may reveal an alternative role of the Cnb1 myristoylation in reducing enzyme activation, such as binding to a hydrophobic pocket near the catalytic domain to prevent enzyme activation, as has been demonstrated for c-Abl tyrosine kinase 1b isoform (44).

Protein myristoylation is now recognized as a key determinant of signaling pathway function via regulation of protein-protein and protein-membrane interactions (45, 46). Myristoylation of the neuronal calcium sensor proteins influences their interactions with cellular factors and targets (47). *N*-Myristoylation of neurocalcin δ is required for Ca^{2+}-dependent interaction with clathrin heavy chain, tubulin, and actin (48). Increased proteasome-substrate interaction in the absence of the Rpt2 orthologue myristoylation has been proposed as a potential mechanism to explain the observation that disruption of *N*-myristoyltransferase leads to enhanced proteasome activity in *Aspergillus nidulans* (49). Increased substrate affinity may contribute to the elevated basal activity of nonmyristoylated

calcineurin. Yeast expressing Crz1 with increased affinity for calcineurin have previously been shown to exhibit enhanced Crz1 dephosphorylation and transcriptional activation in the absence of Ca signaling, similar to our findings in yeast expressing Cnb1-G2A (18). Because changes in calcineurin complex composition and protein interactions may differentially affect the interaction of calcineurin with specific substrates, myristoylation may ultimately help shape the output of calcineurin signaling via influencing the hierarchy of substrate preference. Consistent with altered calcineurin conformation and protein interactions, we have observed that Cnb1-G2A calcineurin exhibited increased sensitivity to the calcineurin inhibitor FK506 (supplemental Fig. 2*B*).

We also observed that nonmyristoylated calcineurin exhibited reduced membrane association. The finding that reduced membrane association correlated with increased calcineurin activation suggests that sequestration of the phosphatase at the membrane may contribute to the regulation of calcineurin signaling in yeast. Preincubation of purified bovine brain calcineurin with phospholipids has previously been shown to inhibit phosphatase activity *in vitro* (50). We further demonstrated that membrane association of wild type calcineurin was reduced by Ca^{2+} transients. Thus, in yeast, Ca^{2+} transients not only activate calcineurin, but also alter the subcellular localization of a fraction of the phosphatase. The development of new tools to monitor the local activation of calcineurin signaling in real time will be required to determine whether dynamic membrane interactions are important in the regulation of cellular phosphatase activation and to determine whether distinct phosphatase pools can be activated by spatially restricted Ca^{2+} transients. Despite the correlation between reduced membrane association and increased phosphatase activity, the majority of calcineurin localized to the soluble fraction even in the absence of Ca^{2+} signaling. Thus, additional mechanisms must also regulate cellular phosphatase activity.

Our findings demonstrate that Cnb1 myristoylation functions to limit calcineurin signaling in yeast and implicate cellular *N*-myristoyltransferase activity levels as a critical determinant of the Ca^{2+} responsiveness of phosphatase activation. Myristoylation may regulate calcineurin activity via multiple mechanisms, which are not mutually exclusive. It will be important to determine whether myristoylation of CNB similarly antagonizes calcineurin activity in mammalian cells. If the role of myristoylation in regulating calcineurin is conserved, differences in *N*-myristoyltransferase activity could contribute to cell-specific or developmental stage-specific regulation of calcineurin activity. Changes in *N*-myristoyltransferase activity would provide a novel mechanism by which calcineurin activity could be altered in disease development and progression.

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