Calcineurin Regulatory Subunit Calcium-Binding Domains Differentially Contribute to Calcineurin Signaling in *Saccharomyces cerevisiae*

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ABSTRACT The protein phosphatase calcineurin is central to Ca^{2+} signaling pathways from yeast to humans. Full activation of calcineurin requires Ca^{2+} binding to the regulatory subunit CNB, comprised of four Ca^{2+}-binding EF hand domains, and recruitment of Ca^{2+}-calmodulin. Here we report the consequences of disrupting Ca^{2+} binding to individual Cnb1 EF hand domains on calcineurin function in *Saccharomyces cerevisiae*. Calcineurin activity was monitored via quantitation of the calcineurin-dependent reporter gene, *CDRE*-lacZ, and calcineurin-dependent growth under conditions of environmental stress. Mutation of EF2 dramatically reduced *CDRE*-lacZ expression and failed to support calcineurin-dependent growth. In contrast, Ca^{2+} binding to EF4 was largely dispensable for calcineurin function. Mutation of EF1 and EF3 exerted intermediate phenotypes. Reduced activity of EF1, EF2, or EF3 mutant calcineurin was also observed in yeast lacking functional calmodulin and could not be rescued by expression of a truncated catalytic subunit lacking the C-terminal autoinhibitory domain either alone or in conjunction with the calmodulin binding and autoinhibitory segment domains. Ca^{2+} binding to EF1, EF2, and EF3 in response to intracellular Ca^{2+} signals therefore has functions in phosphatase activation beyond calmodulin recruitment and displacement of known autoinhibitory domains. Disruption of Ca^{2+} binding to EF1, EF2, or EF3 reduced Ca^{2+} responsiveness of calcineurin, but increased the sensitivity of calcineurin to immunophilin-immunosuppressant inhibition. Mutation of EF2 also increased the susceptibility of calcineurin to hydrogen peroxide inactivation. Our observations indicate that distinct Cnb1 EF hand domains differentially affect calcineurin function *in vivo*, and that EF4 is not essential despite conservation across taxa.

KEYWORDS calcineurin; calcium signaling; phosphatase; EF hand domain; Crz1

CALCINEURIN (formerly protein phosphatase 2B, now PPP3C) is a ubiquitously expressed Ca^{2+}-regulated serine/threonine protein phosphatase first isolated from the bovine brain, where it accounts for 1% of the total protein content (Klee *et al.* 1988). Calcineurin activation by intracellular Ca^{2+} signals orchestrates cell responses to developmental cues, environmental stimuli, and intracellular stress to affect cell proliferation, differentiation, and death (Shibasaki and McKeon 1995; Wang *et al.* 1999; Kahl and Means 2003; Zayzafoon 2006). Studies in diverse model organisms have shown that calcineurin regulates fundamental cell processes to control development, behavior, life span, and adaptive responses (Saneyoshi *et al.* 2002; Nishiyama *et al.* 2007; Dwivedi *et al.* 2009; Mair *et al.* 2011; Nakai *et al.* 2011; Lee *et al.* 2013; Kujawski *et al.* 2014; Deng *et al.* 2015). Calcineurin is also essential for the life cycle, host cell invasion, and virulence of human pathogens, including *Plasmodium falciparum*, *Toxoplasma gondii*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, the causative agents of malaria, toxoplasmosis, and cryptococcal meningitis, and invasive aspergillosis, respectively (Odom *et al.* 1997; Cruz *et al.* 2001; Juvvadi *et al.* 2013; Paul *et al.* 2015; Philip and Waters 2015; Chow *et al.* 2017).

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In mammals, calcineurin activation of transcription factor EB (TFEB) in response to lysosomal Ca^{2+} signals regulates autophagy initiated by oxidative stress or nutrient deprivation (Medina *et al.* 2015; Tong and Song 2015; Zhang *et al.* 2016; Tseng *et al.* 2017). Calcineurin activation of nuclear factor of activated T cells transcription (NFAT) factors promotes development and function of the immune, cardiovascular, nervous, and musculoskeletal systems (Crabtree and Olson 2002; Horsley and Pavlath 2002; Schulz and Yutzey 2004; Wu *et al.* 2007; Nguyen and Di Giovanni 2008; Musson *et al.* 2012; Patel *et al.* 2015; Peiris and Keating 2018). Alterations in calcineurin activity contribute to human diseases such as cardiac hypertrophy (Bueno *et al.* 2002; Wilkins and Molkentin 2004), cancer (Buchholz and Ellenrieder 2007; Peuker *et al.* 2016; Wang *et al.* 2017), neurodegeneration (Mukherjee *et al.* 2010; Mukherjee and Soto 2011; Qu *et al.* 2012; Luo *et al.* 2014; Aufschnaiter *et al.* 2017; Shah *et al.* 2017), and mental illness (Manji *et al.* 2003; Mathieu *et al.* 2008; Forero *et al.* 2016). Calcineurin inhibitors FK506 (tacrolimus) and cyclosporin A are widely used clinically to prevent allograft rejection in transplant patients and treat inflammatory skin diseases (Musson *et al.* 2012; Azzi *et al.* 2013; Nygaard *et al.* 2017).

Intracellular Ca^{2+} transients exhibit distinct spatial and temporal patterns, the amplitude, frequency, and duration of which are integrated by calcineurin to shape cellular responses (Dolmetsch *et al.* 1997; Berridge *et al.* 2003). Multiple mechanisms coordinately modulate calcineurin signaling in cells (Musson and Smit 2011). The requirement for two distinct EF hand Ca^{2+}-binding proteins, CNB and calmodulin, to activate calcineurin enables the activation and inactivation of calcineurin within the narrow window of intracellular Ca^{2+} concentrations. Interactions with additional cellular factors such as the immunophilins FKBP12 and cyclophilin A (Cardenas *et al.* 1994), Bcl-2 (Shibasaki *et al.* 1997; Erin *et al.* 2003), superoxide dismutase 1 (Wang *et al.* 1996; Agbas *et al.* 2007), heat shock proteins (Someren *et al.* 1999; Imai and Yahara 2000), RCANs (Kingsbury and Cunningham 2000; Rothermel *et al.* 2003; Hilioti *et al.* 2004), CIB1 (Heineke *et al.* 2010), KIF1Bβ (Li *et al.* 2016a), and AKAP79 (Li *et al.* 2011) impose additional layer of regulation via modulating phosphatase activity, conformation, localization, and ability to interact with either calmodulin or substrates. Post-translational modifications of calcineurin, including CNA phosphorylation, which can regulate calcineurin activity *in vitro*, presumably tune calcineurin function *in vivo* (Hashimoto and Soderling 1989; Martensen *et al.* 1989; Calalb *et al.* 1990; Juvvadi *et al.* 2013). CNB myristylation, conserved from yeast to humans, reduces Ca^{2+} responsiveness of calcineurin in yeast (Connolly and Kingsbury 2012). Reversible substrate docking and substrate competition further shape calcineurin signaling in cells (Roy *et al.* 2007; Roy and Cyert 2009; Li *et al.* 2011).

Calcineurin is highly conserved across taxa. Calcineurin is a heterodimer consisting of a ~60 kDa catalytic subunit (CNA) and a smaller ~19 kDa regulatory subunit (CNB), comprised of four EF hand Ca^{2+}-binding domains (Klee *et al.* 1998) (hereafter EF1, EF2, EF3, or EF4). *In vitro* analysis of recombinant mammalian calcineurin revealed that EF1 and EF2 have a lower affinity for Ca^{2+} than EF3 and EF4, suggesting Ca^{2+} binding to EF1 and EF2 mediates responsiveness to changes in intracellular Ca^{2+} (Feng and Stemmer 2001). EF3 and EF4 are predicted to constitutively bind Ca^{2+} within cells, consistent with a structural role for these sites. Maximal stimulation of calcineurin phosphatase activity requires Ca^{2+}, CNB, and Ca^{2+}/calmodulin (Klee *et al.* 1998). Ca^{2+} binding to EF1 and EF2 induces conformational changes in CNB that trigger conformational changes in CNA, leading to partial phosphatase activity. Binding of Ca^{2+}/calmodulin to CNA results in full enzyme activation via displacing the autoinhibitory segment (AIS) from the LxVP docking pocket and the C-terminal autoinhibitory domain (AID) from the catalytic site (Wang *et al.* 2008; Li *et al.* 2016b).

To begin to understand the requirements for calcineurin activation *in vivo*, we took advantage of the budding yeast *Saccharomyces cerevisiae*, in which CNB is encoded by a single gene (*CNB1*). Two CNA encoding genes are present: *CNA1* and *CNA2* (Cyert *et al.* 1991). Calcineurin is not essential in *S. cerevisiae*, except under conditions of environmental or cellular stress (Iida *et al.* 1990, 1994; Cyert *et al.* 1991; Cyert and Thorner 1992; Moser *et al.* 1996; Fischer *et al.* 1997; Paidhungat and Garrett 1997; Withee *et al.* 1997; Cyert 2003). Here, we investigated the consequences of introducing mutations into the Ca^{2+}-binding domains of Cnb1 to elucidate the requirement for specific EF hand domains in promoting calcineurin function in yeast. Our results demonstrate that EF4 is largely dispensable for calcineurin activity in response to intracellular Ca^{2+} signals, as previously reported for mammalian calcineurin *in vitro* (Feng and Stemmer 1999, 2001). In contrast, EF1, EF2, and EF3 each contribute to calcineurin activation, even in the absence of the bipartite CNA autoinhibitory elements and calmodulin binding. Our findings reveal an additional function of Ca^{2+} binding to CNB in the activation of calcineurin *in vivo*, beyond calmodulin recruitment: AIS and AID displacement.

Materials and Methods

Yeast culture

All yeast strains used in this study were derived from W303-1A (Table 1). Yeast strains were grown at 30° in standard rich media (YPD) or synthetic media (SD) (Clontech or Sigma, St. Louis, MO). Agar, amino acids and salts were purchased from Difco and Sigma. YPD medium was buffered to pH 5.5 by addition of 0.5 M succinic acid for experiments requiring the addition of extracellular $CaCl_2$. Yeast culture and transformations were conducted using standard techniques (Sherman 1991; Gietz *et al.* 1995).

Cloning and mutagenesis

QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA) was conducted according to manufacturer's instructions in pYDZ3 (Zhu *et al.* 1995). For EF1, EF2, and EF4

Table 1 Yeast strains used in this study

Yeast strain	Relevant genotype	Source
K603	*MAT**a** cnb1*::*LEU2*	Cunningham and Fink (1994)
TKY102	*MAT**a** cnb1*::*LEU2 cna1*Δ *cna2*::*HIS3*	This study
K687	*MAT**a** cnb1*::*LEU2 cmd1-3*	Cunningham laboratory
K650	*MAT**a** cnb1 vcx1*	Cunningham laboratory
DMY22	*MAT**a** cnb1 crz1 pmc1*	Cunningham laboratory

mutagenesis, the GAT encoding aspartic acid was mutated to GCT, to encode alanine. For EF3, GAC was mutated to GCC to convert the aspartic acid to alanine. *CNB1* alleles were cloned into pRS313 (Sikorski and Hieter 1989) (centromere (CEN)) or pYO323 (Qadota *et al.* 1992) (2 μm), using *Bam*HI-*Xho*I. Genomic DNA corresponding to *CNA1* was PCR amplified (forward: AGGATCCGTCTTCGTCAACAATTGGTTC; reverse: ACTCGAGCATTGGTTACAAGTCCCTAAC) and cloned into pRS314 *Bam*HI-*Xho*1. Truncated alleles were generated by PCR with CNA1 forward primer combined with either reverse GGACTGAAGGTTTGAATGAAACGCAACTGTGATAAATCTCA (Cna1ΔAID) or GCGATATTAGAAGATGAAACCCAACTGTGA TAAATCTCA (CNA1ΔCBD). All constructs were verified by DNA sequencing (Supplemental Material, Table S1).

Ion tolerance assays

Ion tolerance assays were performed as previously described (Cunningham and Fink 1996), with minor modifications. *cnb1*Δ yeast strains transformed with CEN-based *CNB1* alleles were precultured in SD-HIS medium. Ion tolerance assays were conducted in YPD supplemented with a range of NaCl, LiCl, or $MnCl_2$ concentrations. YPD (pH 5.5) medium was used for $CaCl_2$ assays to maintain salt solubility. Yeast were grown as 0.18 ml cultures in 96-well, flat-bottomed dishes in a 30° incubator without shaking for 36–48 hr. The OD_{600} was measured using a BioTek μQuant platereader.

β-Galactosidase assays

Yeast cultures were grown overnight in SD dropout media (–HIS –URA for *cnb1*Δ yeast or –HIS –URA –LEU –TRP for *cna1*Δ *cna2*Δ *cnb1*Δ yeast). Yeast strains were pelleted and resuspended in YPD (pH 5.5) before inoculation into media supplemented with $CaCl_2$, FK506, cyclosporine A (CsA), α-factor (Genscript), or H_2O_2 as indicated. For α-factor experiments yeast were diluted to 0.2 OD. Cells were incubated at 30° with shaking for 4 hr (3 hr for experiments with α-factor). After incubation, 0.9 ml of yeast was harvested by centrifugation and resuspended into 720 μl of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0) + 0.0075% SDS. Cells were lysed by addition of 50 μl of chloroform and vortexing for 10 sec. Reactions were initiated by addition of 180 μl of ONPG (4 mg/ml in Z buffer) and terminated by the addition of 450 μl 1 M Na_2CO_3. β-Galactosidase activity was quantitated by measuring the OD_{600} of the yeast culture used for the assay and the OD_{420} of the reaction product. β-Galactosidase activity was calculated as follows: $(1000 \times OD_{420})/OD_{600} \times$ time (min) $\times$ volume (ml) = units of β-galactosidase activity.

Protein analysis

Yeast were grown overnight in SD-HIS media, diluted into YPD, and grown an additional 4 hr before harvesting for protein. Cells were pelleted and washed once in PBS. Cell pellets were lysed with lithium acetate and NaOH as previously described (Zhang *et al.* 2011), except that 1 M LiOAc was used. Pellets were resuspended in 2× Laemmli buffer, boiled for 5 min, and cleared by centrifugation before separation by PAGE using Novex Wedgewell 16% Tris-Glycine gels run in 1× SDS Tris-Glycine buffer (Invitrogen, Carlsbad, CA). When indicated, protein lysates were for incubated for 10 min with either 1 mM $CaCl_2$ or 2 mM EGTA before gel loading. Western blotting was conducted using anti-Cnb1 (Zhu *et al.* 1995) at 1:5000 in TBS-Tween as primary antibody and HRP-conjugated anti-rabbit at 1:10,000 as secondary antibody (Jackson ImmunoResearch Laboratories). Anti-PGK at 1:2000 (Invitrogen) was used as a loading control.

Data availability

All yeast strains used in this study are available upon request. Table S1 contains list of plasmids utilized in this study. All plasmids generated in this study will be deposited at Addgene for distribution. Data necessary to confirm findings of this article are present within article, figures, and tables. Supplemental material available at Figshare: https://doi.org/10.25386/genetics.6229040.

Results

Generation of CNB1 mutant alleles

The Ca^{2+}-binding regulatory subunit calcineurin B is encoded by a single gene *CNB1* in *S. cerevisiae* (Cyert and Thorner 1992). To investigate the contribution of specific Cnb1 EF hand domains to calcineurin function (Figure 1A), we generated a series of *CNB1* alleles expressed from low-copy CEN-based vectors in which an individual EF hand domain harbored a single amino acid mutation designed to disrupt Ca^{2+} binding. EF hand domains are comprised of two α-helices separated by a short loop that binds Ca^{2+}. Within the Ca^{2+}-binding loop, amino acids 1, 3 5, 7, 9, and 12 (X, Y, Z, −Y, −X, and −Z, respectively) coordinate Ca^{2+} (Nakayama and Kretsinger 1994). Using site-directed mutagenesis, the aspartic acid located at position X was mutated to alanine (Figure 1B) in EF1, EF2, EF3, or EF4. EGTA depletion of Ca^{2+} from EF3 and EF4, but not EF1 or EF2, reduces the electrophoretic mobility of mammalian CNB in high-percentage SDS-PAGE (Feng and Stemmer 1999). We observed that treatment of yeast cell lysates treated with EGTA similarly reduced Cnb1 electrophoretic mobility in high-percentage SDS-PAGE (Figure 1C). In the presence of Ca^{2+}, multiple species of Cnb1 were observed in high-percentage gels. Treatment with EGTA resulted in the loss of the faster

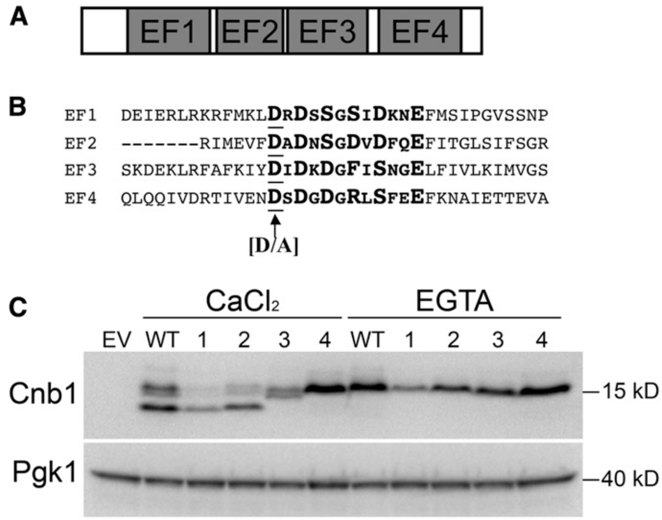

Figure 1 Site-directed mutagenesis of yeast calcineurin B EF hand domains. (A) Schematic of Cnb1. (B) Sequence of the four EF hand domains of Cnb1. The Ca^{2+}-binding loop is denoted in bold lettering. Amino acids that bind Ca^{2+} are enlarged. The aspartate residue (D) mutated to alanine in each EF hand is indicated by an arrow. (C) Electrophoretic mobility of Cnb1 mutant proteins. Protein lysates from *cnb1*Δ yeast expressing the wild type (WT), Cnb1 EF hand mutants *vs.* empty vector (EV) were treated with 1 mM CaCl2 or 2 mM EGTA before SDS-PAGE and Western blot analysis using anti-Cnb1 antibody (Zhu *et al.* 1995).

migrating species. In the presence of Ca^{2+}, Cnb1 harboring mutations in either EF3 (Cnb1mutEF3) or EF4 (Cnb1mutEF4) lacked the faster migrating species, consistent with the D/A mutations disrupting Ca^{2+} binding. In contrast, the mobility of Cnb1mutEF1 and Cnb1mutEF2 was comparable to the fast migrating species observed for wild-type Cnb1, suggesting that mutation of EF1 or EF2 neither altered Cnb1 mobility nor disrupted Ca^{2+} binding to EF3 or EF4.

Disruption of EF1, EF2, and EF3 reduces calcineurin function: Calcineurin mediates yeast responses to Ca^{2+} signals triggered by extracellular stimuli and intracellular stress. Intracellular Ca^{2+} signals stimulate calcineurin, which dephosphorylates the transcription factor Crz1. Crz1 then translocates to the nucleus to mediate calcineurin-dependent changes in gene expression (Cyert 2003; Thewes 2014). Calcineurin activity can be readily quantitated by measuring the expression of the calcineurin-dependent reporter gene *CDRE*-lacZ (calcineurin-dependent response element), composed of four copies of the Crz1-binding site (Stathopoulos and Cyert 1997). In the absence of calcineurin, *CDRE*-lacZ expression is not stimulated by Ca^{2+} signaling. Wild-type *vs.* mutant *CNB1* alleles were transformed into *cnb1*Δ yeast (Y203) harboring the *CDRE*-lacZ reporter gene. Yeast transformed with empty vector were used as negative controls. To stimulate Ca^{2+} signaling, yeast were treated with 100 mM extracellular $CaCl_2$ for 4 hr before harvesting for quantitative ortho-nitrophenyl-beta-galactoside (ONPG) assays to measure β-galactosidase activity. Yeast expressing wild-type Cnb1 exhibited a robust activation of *CDRE*-lacZ activity in response to 100 mM $CaCl_2$ (Figure 2A). Yeast expressing Cnb1mutEF4 were most similar, with ~80% of the stimulated *CDRE*-lacZ activity observed in yeast expressing wild-type Cnb1. In contrast, yeast expressing Cnb1mutEF2 had *CDRE*-lacZ activity levels similar to yeast transformed with empty vector. Yeast expressing Cnb1mutEF1 and Cnb1mutEF3 exhibited intermediate levels of *CDRE*-lacZ activity. Overexpression of Cnb1 mutants from high-copy 2 μm expression constructs (Figure S1) was unable to rescue the reduced *CDRE*-lacZ activity levels observed upon mutation of EF1, EF2, or EF3 (Figure 2A). In addition, increased expression of wild-type Cnb1 did not significantly increase *CDRE*-lacZ levels following stimulation with 100 mM $CaCl_2$, indicating Cnb1 is not limiting for Crz1 activation. A similar pattern of *CDRE*-lacZ activity was observed in yeast lacking the calcineurin-dependent vacuolar Ca^{2+} transporters Vcx1 and Pmc1, indicating that reduced ability of calcineurin to stimulate Crz1 was not dependent on altered function of these transporters (Figure 1, B and C), although transporter deletion enhanced our ability to detect Cnb1mutEF2-dependent *CDRE*-lacZ activity above background levels, presumably due to increased cytosolic Ca^{2+} levels (Cunningham and Fink 1994; Cui *et al.* 2009).

Mutation of EF1, EF2, or EF3 reduces Ca^{2+} responsiveness of calcineurin: Our observation that the absence of Vcx1 enhanced our ability to detect Cnb1mutEF2 activity above background suggested that mutant Cnb1 had reduced Ca^{2+} responsiveness. We therefore assayed *CDRE*-lacZ activity across a range of extracellular $CaCl_2$ from 0 to 100 mM to determine the relative Ca^{2+} sensitivity of the EF hand mutants. Assays were conducted in the *cnb1*Δ *vcx1*Δ (Y250) background, where we could consistently detect Ca^{2+} stimulated Cnb1mutEF2-dependent *CDRE*-lacZ activity, albeit at very low levels (~5% wild type). Differences in the ability of Ca^{2+} to activate the mutant calcineurin would be observed as a shift in the Ca^{2+} dose-response curve. For each mutant, the *CDRE*-lacZ activity measured at 100 mM $CaCl_2$ was set to 100% activity, and *CDRE*-lacZ activity at the remaining $CaCl_2$ concentrations was plotted as a percentage of the activity at 100 mM $CaCl_2$. As shown in Figure 2D, calcineurin comprised of Cnb1mutEF1, Cnb1mutEF2, or Cnb1mutEF3 required higher levels of Ca^{2+} for stimulation of *CDRE*-lacZ expression than wild-type Cnb1 or Cnb1mutEF4. Thus, the Cnb1mutEF1, Cnb1mutEF2, or Cnb1mutEF3 mutants have reduced sensitivity to elevated intracellular Ca^{2+}.

EF1, EF2, and EF3 mutants disrupt calcineurin responses to physiologic stimuli

Calcineurin mediates yeast responses to environmental stimuli, including extracellular stress and mating pheromone. Calcineurin activation of Crz1 is required for yeast to grow in the presence of increasing levels of extracellular Mn^{2+}, Li^+, and Na^+. We therefore tested the ability of *cnb1*Δ yeast (Y203) transformed with wild-type *vs.* mutant *CNB1* alleles to grow in the presence of high levels of $MnCl_2$, LiCl, or NaCl (Figure 3, A–C). *cnb1*Δ yeast transformed with wild-type

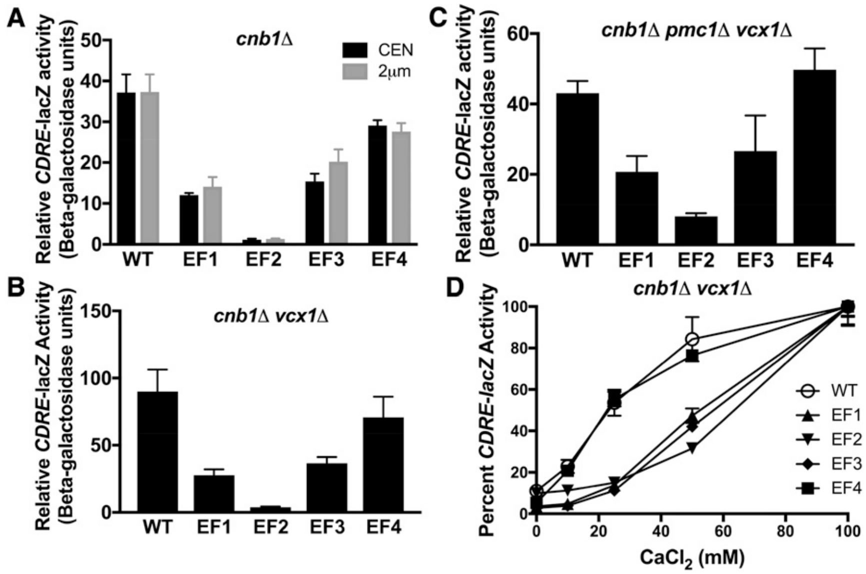

Figure 2 Cnb1 EF hand mutants reduce calcineurin-stimulated reporter gene expression. (A–C) The *CDRE*-lacZ reporter gene was introduced into *cnb1*Δ (A), *cnb1*Δ *vcx1*Δ (B), or *cnb1*Δ *pmc1*Δ *vcx1*Δ (C) yeast expressing either wild-type or mutant Cnb1, expressed from low-copy centromere-based plasmids (CEN) or high-copy 2 μm plasmids (2 μm). β-Galactosidase activity was quantitated after 4 hr stimulation in YPD (pH 5.5) supplemented with 100 mM $CaCl_2$. Data are normalized to the activity measured in yeast transformed with empty vector in the absence of stimulation. (D) *CDRE*-lacZ activity was measured following 4 hr stimulation at 0, 10, 25, 50, and 100 mM added extracellular $CaCl_2$ in *cnb1*Δ *vcx1*Δ yeast. For each Cnb1 mutant construct, the *CDRE*-lacZ activity at each $CaCl_2$ concentration is plotted as a percentage of the activity obtained at 100 mM $CaCl_2$ (100%). For all assays, data plotted are the average of four independent yeast transformants ± SD.

CNB1 or empty vector were used as positive and negative controls, respectively. Yeast expressing Cnb1mutEF4 exhibited a similar level of ion resistant growth as yeast expressing wild-type Cnb1 in the presence of Mn^{2+}, Li^{+}, and Na^{+}. In contrast, yeast expressing Cnb1mutEF1, Cnb1mutEF2, or Cnb1mutEF3 exhibited reduced ion tolerance, becoming growth inhibited at lower concentrations of extracellular $MnCl_2$, LiCl, or NaCl than yeast expressing wild-type Cnb1. In each case, Cnb1mutEF2 expressing yeast were the most sensitive to growth inhibition. In the presence of NaCl, Cnb1mutEF2 expressing yeast were indistinguishable from *cnb1*Δ yeast transformed with empty vector. These results demonstrate that EF1, EF2, and EF3 are required for calcineurin activity in response to environmental stress due to high levels of Mn^{2+}, Li^{+}, and Na^{+}.

We next tested whether Cnb1 mutants exhibited reduced calcineurin activity in response to mating pheromone. Haploid yeast cells of opposite mating type sense each other through plasma membrane receptors, grow toward each other, and ultimately fuse at the tips of extended cytoplasmic projections. Exposure of *a* mating type haploid yeast to α-factor mating pheromone triggers Ca^{2+} signals and calcineurin-mediated stimulation of Crz1 (Matheos *et al.* 1997; Stathopoulos and Cyert 1997). Wild-type *vs.* mutant *CNB1* alleles were transformed into *a cnb1*Δ (Y203) yeast harboring the *CDRE*-lacZ reporter gene. Yeast transformed with empty vector were used as negative controls. Ca^{2+} signals were induced by treating yeast with α-factor mating pheromone for 3 hr before harvesting for quantitative ONPG assays to measure β-galactosidase activity. Yeast expressing either wild-type Cnb1 or Cnb1mutEF4 stimulated *CDRE*-lacZ expression ~19-fold in response to α-factor (Figure 3D). In contrast, yeast expressing Cnb1mutEF2 were similar to yeast transformed with empty vector. Yeast expressing Cnb1mutEF1 and Cnb1mutEF3 had an intermediate response, exhibiting ~fivefold stimulation of *CDRE*-lacZ activity following α-factor treatment. As observed following stimulation with high $CaCl_2$, overexpression of wild-type Cnb1 or Cnb1mutEF4 from high-copy 2 μm vectors did not increase *CDRE*-lacZ activity, and overexpression of Cnb1EF1, Cnb1mutEF2, or Cnb1mutEF3 did not rescue *CDRE*-lacZ activity.

EF1, EF2, and EF3 function beyond autoinhibitory domain displacement

The carboxyl terminal domain of CNA contains an AID that binds to the active site of calcineurin. Ca^{2+} binding to CNB increases the interaction of calcineurin with Ca^{2+}/calmodulin, which leads to displacement of the AID during phosphatase activation (Yang and Klee 2000) (Figure 4A). Deletion of the Cna1 AID (Cna1-ΔAID) generates a constitutively active calcineurin that can be robustly stimulated by Ca^{2+} signaling (Figure 4C). We tested whether deletion of the AID could restore calcineurin activity in Cnb1 mutants. *cnb1*Δ *cna1*Δ *cna2*Δ (TKY102) yeast harboring the *CDRE*-lacZ reporter gene and expressing Cna1-ΔAID were transformed with wild-type *vs.* mutant *CNB1* alleles. Calcineurin stimulation of *CDRE*-lacZ activity was tested following 4 hr in the presence of 100 mM extracellular $CaCl_2$ before harvesting for ONPG assays. In the absence of stimulation, only yeast expressing wild-type Cnb1 or Cnb1mutEF4 exhibited the expected increase in basal *CDRE*-lacZ activity (Figure 4C). As observed in the presence of wild-type endogenous Cna1 and Cna2, Cnb1mutEF1, Cnb1mutEF2, and Cnb1mutEF3 exhibited reduced *CDRE*-lacZ activity compared to Cnb1mutEF4 and wild-type Cnb1. Thus, the reduced calcineurin activity observed upon mutation of either EF1, EF2, or EF3 is not solely due to a defect in displacement of the AID in response to Cnb1 Ca^{2+} binding.

Mutation of EF1, EF2, or EF3 impairs calmodulin-independent calcineurin function

Full activation of calcineurin requires Ca^{2+} binding to Cnb1 and recruitment of Ca^{2+}/calmodulin. In the absence of the

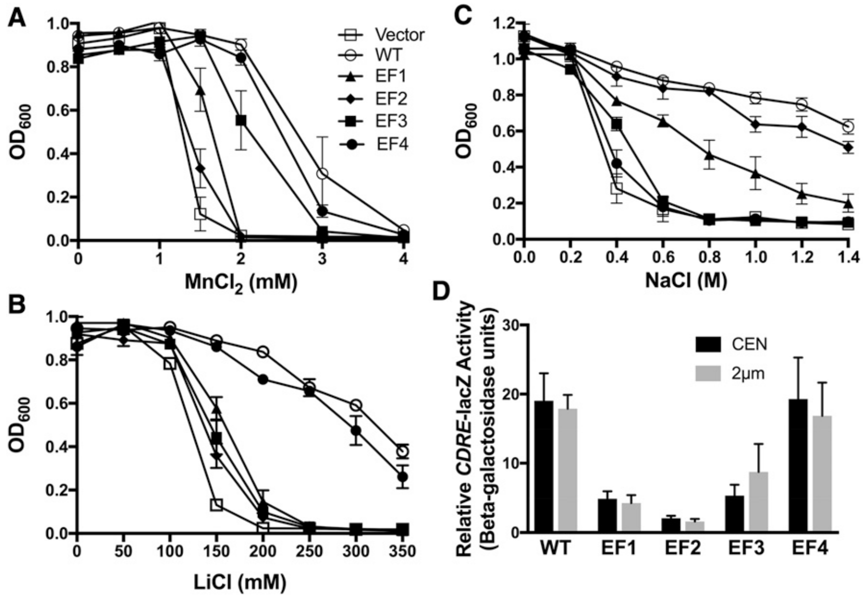

Figure 3 Mutation of EF1, EF2, and EF3 reduces calcineurin responses to physiologic stimuli. (A–C) Wild-type Cnb1, Cnb1mutEF1, Cnb1mutEF2, Cnb1mutEF3, or Cnb1mutEF4 were expressed in *cnb1*Δ yeast to assess the ability of the mutants to promote ion-resistant growth. Yeast strains were grown in YPD supplemented with increasing concentrations of $MnCl_2$ (A), LiCl (B), or NaCl (C). Following incubation for 2 days at 30°, the optical density of cultures was measured at 600 nm and plotted as a function of ion concentration. Data plotted are the average of four independent yeast transformants ± SE. (D) *CDRE*-lacZ activity was measured following 3 hr stimulation with 20 μM α-factor in a *cnb1*Δ yeast. Cnb1 was expressed from either low-copy centromere-based plasmids (CEN) or high-copy 2 μm plasmids (2 μm). For all assays, data plotted are the average of four independent yeast transformants ± SD.

Ca^{2+}/calmodulin interaction, Crz1 activation in response to Ca^{2+} signals is dramatically reduced. To determine whether Cnb1 EF hand mutants alter calmodulin-independent calcineurin activity in cells, *cnb1*Δ *cmd1-3* (K687) yeast harboring the *CDRE-lacZ* reporter were transformed with wild-type *vs.* mutant *CNB1* alleles. *cmd1-3* yeast express calmodulin that cannot bind to Ca^{2+} and fails to stimulate calcineurin activity (Geiser *et al.* 1991). Stimulation of *cmd1-3* yeast expressing wild-type Cnb1 with 100 mM extracellular $CaCl_2$ for 4 hr resulted in less than fourfold stimulation of *CDRE*-lacZ expression (Figure 4D). Cnb1EFmut4 resulted in twofold stimulation in *CDRE*-lacZ activity following Ca^{2+} stimulation in *cmd1-3* background, whereas Cnb1mutEF1, Cnb1mutEF2, or Cnb1mutEF3 were unable to stimulate *CDRE*-lacZ expression in the absence of Ca^{2+}/calmodulin.

Calmodulin-independent calcineurin function was further tested in *cnb1*Δ *cna1*Δ *cna2*Δ (TKY102) yeast expressing Cna1 protein truncated before the calmodulin-binding domain (Cna1-ΔCBD). Truncation of the catalytic subunit upstream of the calmodulin-binding domain also eliminates the AIS and the AID of Cna1, resulting in a constitutively active enzyme that can be further stimulated by Ca^{2+} binding to Cnb1. Stimulation of yeast expressing Cna1-ΔCBD with either wild-type Cnb1 or Cnb1mutEF4 by addition of 100 mM $CaCl_2$ to the media resulted in similar levels of *CDRE*-lacZ activity (Figure 4E). Yeast expressing Cnb1mutEF1 or Cnb1mutEF3 had lower levels of both basal and stimulated *CDRE*-lacZ activity, consistent with impaired calmodulin-independent calcineurin function. Yeast expressing Cnb1mutEF2 resembled the empty vector negative control in the presence and absence of stimulation.

Cnb1 mutants increase sensitivity to immunosuppressant inhibition

Calcineurin is the target of the immunosuppressants FK506 and CsA. Interaction of calcineurin with immunophilin drug complexes has previously been shown to require Ca^{2+} and CNB (Li and Handschumacher 1993; Milan *et al.* 1994). We therefore tested the ability of FK506 and CsA to inhibit calcineurin activity in yeast expressing the Cnb1 EF mutants. Yeast were stimulated with 100 mM $CaCl_2$ in the presence or absence of increasing doses of FK506 or CsA for 4 hr before harvesting to assay *CDRE*-lacZ activity. As shown in Figure 5, yeast expressing Cnb1mutEF1, Cnb1mutEF2, or Cnb1mutEF3 were more sensitive to inhibition by FK506 (Figure 5A) and CsA (Figure 5B) than yeast expressing wild-type Cnb1. Cnb1mutEF4 expressing yeast resembled wild type in the presence of FK506, but exhibited a small increase in sensitivity to inhibition by CsA. Estimation of the concentration of drug at which *CDRE*-lacZ stimulation was reduced by 50%, suggested that Cnb1mutEF1, Cnb1mutEF2 and Cnb1mutEF3 expressing yeast were 2.7-, 6.4-, and 4.0-fold more sensitive to FK506 inhibition than wild-type Cnb1 expressing yeast, respectively. In the presence of CsA, EF1, EF2, EF3, and EF4 mutant calcineurin was 3.0, 13, 4.5, and 1.8 times as sensitive as wild-type calcineurin, respectively.

Cnb1 mutants exhibit increased sensitivity to inactivation by oxidative stress

Calcineurin activity is sensitive to oxidative inactivation (Wang *et al.* 1996; Namgaladze *et al.* 2002; Musson and Smit 2011). Since the reduced activity we observe in the EF mutants could reflect increased inactivation, we investigated the sensitivity of calcineurin to hydrogen peroxide in yeast expressing wild-type *vs.* mutant Cnb1. *Cnb1*Δ *vcx1*Δ yeast harboring the indicated *CNB1* alleles and the *CDRE*-lacZ reporter gene were stimulated with 100 mM $CaCl_2$ for 4 hr in either the absence or presence of 2 mM H_2O_2 before harvesting for ONPG assays. H_2O_2 had no effect on the levels of *CDRE*-lacZ β-galactosidase activity in the absence of added exogenous $CaCl_2$ (data not shown). In the presence of

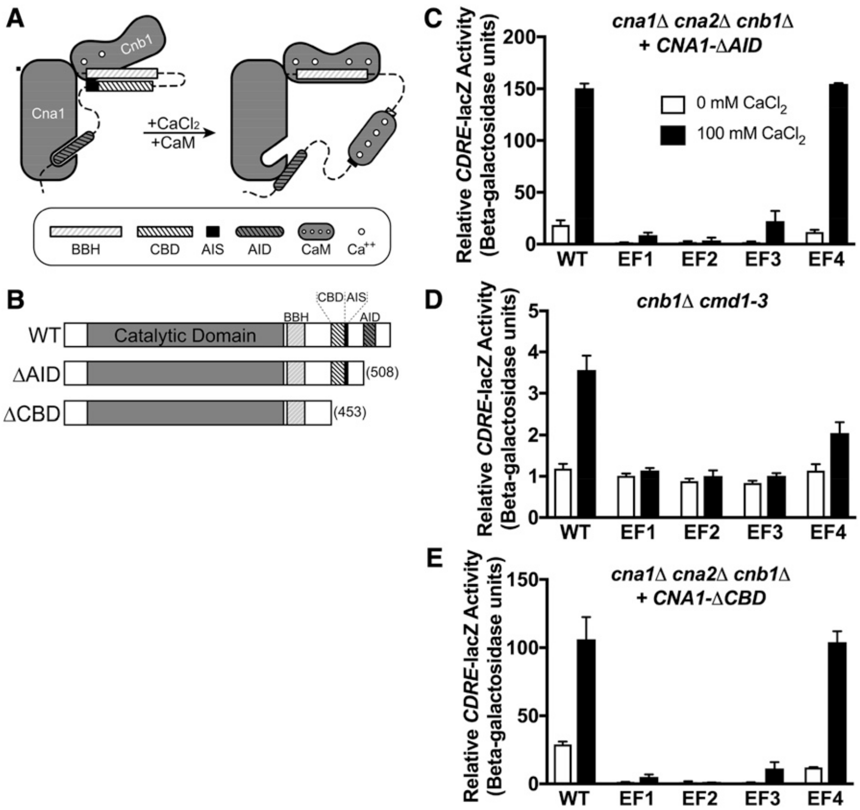

Figure 4 Removal of Cna1 autoinhibitory domains or calmodulin interaction does not bypass the requirement for Cnb1 EF1, EF2, or EF3. (A) Cartoon model of calcineurin activation by Ca^{2+} and calmodulin. (B) Schematic of wild type (WT) and Cna1 truncations. (C–E) *cnb1*Δ *cna1*Δ *cna2*Δ yeast harboring the *CDRE*-lacZ reporter expressing either Cna1-ΔAID (C), *cnb1*Δ *cmd1-3* (D), or Cna1-ΔCBD (E) were transformed with WT *vs.* mutant Cnb1 constructs. Each strain was grown to log phase and exposed for 4 hr to 100 mM $CaCl_2$ in YPD medium (pH 5.5) before β-galactosidase activity measurement. Error bars represent the SD of four independent transformants. BBH, CNB binding helix.

100 mM $CaCl_2$, calcineurin composed of wild-type Cnb1 or Cnb1mutEF4 was inhibited only 10–15% by inclusion of H_2O_2 in the media (Figure 6). In contrast, yeast expressing Cnb1mutEF1, Cnb1mutEF2, or Cnb1mutEF3 were inhibited ~45, 72, and 35%, respectively, by the presence of H_2O_2. Thus, mutation of EF1, EF2, or EF3 increased the sensitivity of calcineurin to inhibition by H_2O_2, with mutation of EF2 being the most dramatically affected. Although our data reveal that EF1, EF2, and EF3 mutant calcineurin are more sensitive to oxidative stress, we were unable to reverse reduced activity of mutants by inclusion of reduced glutathione in the media (S Connolly and TJ Kingsbury unpublished data), suggesting that increased oxidation of calcineurin in the absence of induced oxidative stress does not account for the reduced activity observed in these mutants.

Cnb1 EF1, EF2, and EF3 mutants impair Crz1-independent calcineurin function

To determine whether the reduced function of Cnb1mutEF1, Cnb1mutEF2, or Cnb1mutEF3 calcineurin extends to additional phosphatase substrates, we tested the ability of the Cnb1 mutants to inhibit the Ca^{2+}/H^+ exchanger, Vcx1. In wild-type yeast, the vacuolar ATPase Pmc1 maintains Ca^{2+} homeostasis and enables growth in the presence of high extracellular $CaCl_2$. In the absence of Pmc1, disrupting calcineurin function increases Ca^{2+} tolerance due to loss of calcineurin-mediated inhibition of Vcx1 (Cunningham and Fink 1996). Calcineurin inhibition of Vcx1 is independent of Crz1. To test the ability of Cnb1 EF hand mutants to inhibit Vcx1 activity, *cnb1 pmc1*Δ *crz1*Δ (Figure 7) were transformed with wild-type *vs*. mutant Cnb1 expression constructs and growth quantitated after 2 days culture in the presence of increasing concentrations of $CaCl_2$. Yeast expressing either Cnb1mutEF1 or Cnb1mutEF2 exhibited increased Ca^{2+}-resistant growth similar to that observed in yeast lacking Cnb1, suggesting an impaired ability to inhibit Vcx1. Cnb1mutEF3 displayed an intermediate level of Ca^{2+}-resistant growth, while Cnb1mutEF4 resembled yeast expressing wild-type Cnb1. These results show that the reduced activity of EF1, EF2, or EF3 mutant Cnb1 calcineurin is not limited to a single physiologic substrate.

Discussion

CNB interaction with CNA is essential for calcineurin activation *in vitro* and function *in vivo* (Cyert and Thorner 1992; Nakamura *et al.* 1993; Kawamura and Su 1995; Tokoyoda *et al.* 2000). Yeast in which the *CNB1* has been disrupted exhibit calcineurin-deficient phenotypes such as failure to recover from mating pheromone and ion sensitivity (Cyert 2003). To begin to understand the requirements for Ca^{2+} binding to CNB in promoting calcineurin function *in vivo*, we investigated the functional consequences of individually mutating each of the Ca^{2+}-binding domains of Cnb1. Here we show that mutation of Cnb1 Ca^{2+}-binding domains EF1, EF2, and EF3 reduces calcineurin function in yeast. Using a

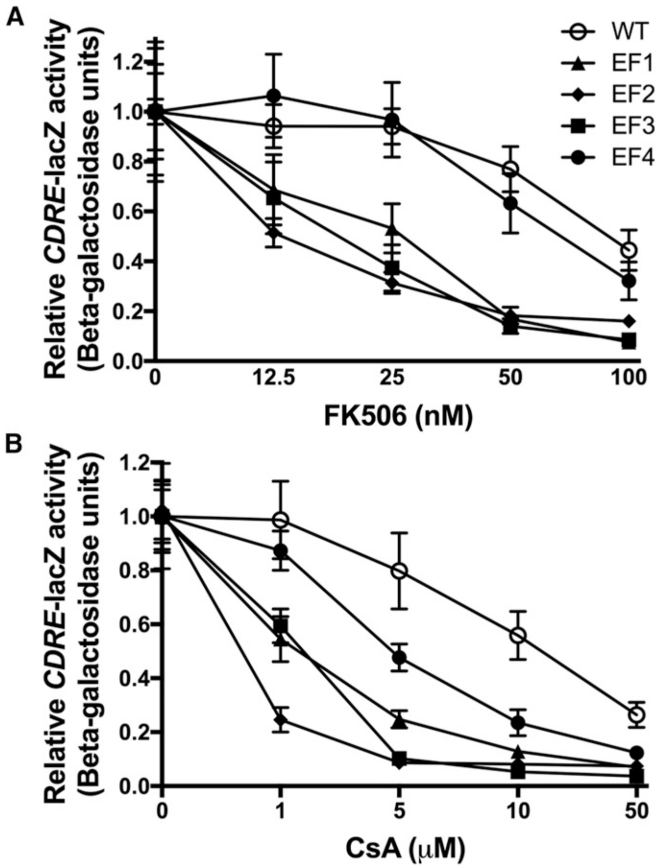

Figure 5 EF hand mutants increase calcineurin immunophilin-immunosuppressant sensitivity. The ability of immunosuppressants to inhibit calcineurin-dependent stimulation of *CDRE*-lacZ activity was assayed across a range of (A) FK506 (nM) or (B) CsA (μM) concentrations as indicated. *cnb1*Δ *vcx1*Δ yeast expressing wild-type (WT) or mutant cnb1 were stimulated for 4 hr in YPD supplemented with 100 mM $CaCl_2$ in the presence or absence of FK506 or CsA. Ca^{2+}-stimulated *CDRE*-lacZ activity measured in the absence of immunosuppressant drugs for each strain containing a *cnb1* construct was set to 100%, and the β-galactosidase activity observed in the presence of drugs was plotted as a corresponding percentage. Data plotted represent the average of four independent yeast transformants ± SD.

calcineurin-dependent reporter gene, we show that mutation of EF1, EF2, or EF3 decreased total calcineurin activity, with mutation of EF2 exhibiting the most dramatic reduction in response to Ca^{2+} signaling stimulated by either high extracellular Ca^{2+} levels or α-factor stimulation. Consistent with reduced calcineurin stimulation of Crz1 activity, Cnb1 EF1, EF2, and EF3 mutants exhibited reduced calcineurin-mediated ion-tolerant growth, which is primarily but not exclusively mediated by Crz1. Thus, multiple distinct sources of Ca^{2+} signaling each revealed reduced calcineurin function upon mutation of EF1, EF2, or EF3. Mutation of EF1, EF2, or EF3 also impaired the ability of calcineurin to inhibit the vacuolar Ca^{2+}/H^+ exchanger Vcx1, a Crz1-independent calcineurin function, demonstrating that the observed defect in calcineurin was not specific to Crz1 substrate. In contrast, EF4 was found to be largely dispensable for calcineurin function in wild-type yeast. Our findings are consistent with previous *in vitro* analysis of recombinant mammalian calcineurin (Feng and Stemmer 1999, 2001) despite the dynamic complexity of intracellular Ca^{2+} signals, which are in part shaped by calcineurin-dependent processes, and additional calcineurin regulatory interactions presumably occurring within the context of a cell. To our knowledge, our data are the first demonstration of the functional role of EF1, EF2, and EF3 in conferring calcineurin responsiveness to changes in intracellular Ca^{2+} levels.

Consistent with impaired calcineurin function observed with Cnb1 EF1, EF2, or EF3 mutants, we found reduced sensitivity to activation across a range of Ca^{2+} signals, indicating that these EF hand domains determine calcineurin responsiveness to increases in intracellular Ca^{2+}. The reduced Ca^{2+} sensitivity of Cnb1 EF1 and EF2 mutant calcineurin is consistent with their proposed role in binding Ca^{2+} in response to intracellular Ca^{2+} transients based on their lower affinity for Ca^{2+} *in vitro* (Feng and Stemmer 1999, 2001; Yang and Klee 2000; Gallagher *et al.* 2001). In contrast, EF3 and EF4 are higher affinity Ca^{2+}-binding sites predicted to be constitutively bound to Ca^{2+} in cells. The reduced Ca^{2+} sensitivity of Cnb1 EF3 mutant calcineurin reveals an essential role for EF3 in mediating calcineurin responses to changes in intracellular Ca^{2+}. It is unclear how reduced Ca^{2+} affinity of EF3 causes reduced Ca^{2+} sensitivity of calcineurin, although it is possible that mutation of EF3 lowers the affinity of EF1 or EF2 for Ca^{2+} due to communication between the sites (Gallagher *et al.* 2001). Mutation of EF4 did not alter phosphatase Ca^{2+} sensitivity.

Full activation of calcineurin during Ca^{2+} signaling requires both Ca^{2+} binding to Cnb1 and the recruitment of Ca^{2+}/calmodulin (Klee *et al.* 1998). In the inactive state, the CBD interacts with the CNB binding helix and the AID acts as a pseudosubstrate blocking the catalytic site. Recent crystal structure of the full-length β-isoform of mammalian CNA has revealed an additional inhibitory domain, called the autoinhibitory segment (AIS), located at the carboxy terminus of the CBD, which interacts with a hydrophobic domain previously shown to mediate substrate and immunophilin interaction (Li *et al.* 2016b). Existence of an additional AID was previously implicated from CNA mutant analysis (Perrino *et al.* 1995; Wang *et al.* 2008). *In vitro* functional analysis further suggested that AID and AIS cooperate to inhibit calcineurin and suggested an updated model for calcineurin activation (Li *et al.* 2016b). In this model, Ca^{2+} binding to CNB EF1 and EF2 induces a conformational change in CNB, which leads to movement of a large domain of CNA and subsequent stimulation of basal activity. Recruitment of Ca^{2+}/calmodulin causes dissociation of AIS from the substrate docking domain and displacement of the AID from the catalytic site, likely via a reorientation rather than displacement, allowing full enzyme activation.

The reduced *in vitro* activity of recombinant mammalian calcineurin comprised of mutant EF1, EF2, or EF3 CNB also reduced calcineurin activity in the absence of calmodulin. Using the *cmd1-3* mutant yeast background, which expresses

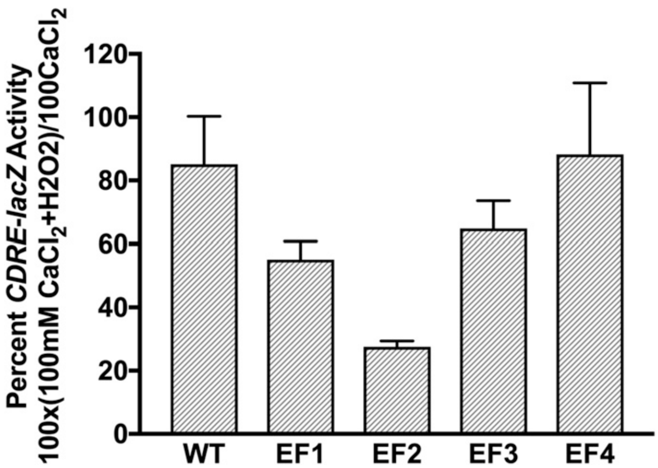

Figure 6 Ca^{2+}-binding domains modulate the hydrogen peroxide sensitivity of calcineurin. The *CDRE*-lacZ plasmid was transformed into *cnb1*Δ *vcx1*Δ yeast expressing either wild-type (WT) or mutant cnb1 as indicated. β-Galactosidase assays were conducted after 4 hr stimulation of yeast strains in YPD (pH 5.5) supplemented with 100 mM $CaCl_2$ in the presence or absence of 2 mM H_2O_2. Data are plotted as the percentage Ca^{2+}-stimulated activity observed in the presence of H^2O_2 relative to activity observed in cultures lacking H_2O_2. Data plotted are the average of four independent yeast transformants ± SD.

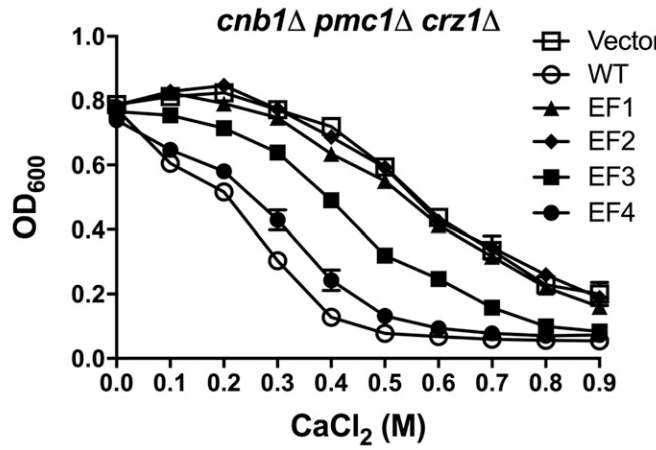

Figure 7 Cnb1 mutants impede calcineurin-mediated inhibition of the vacuolar H^+/Ca^{2+} exchanger Vcx1. *cnb1*Δ *pmc1*Δ *crz1*Δ yeast expressing wild-type (WT) *vs.* mutant Cnb1 were grown for 2 days in YPD (pH 5.5) in the presence of the increasing concentrations of extracellular $CaCl_2$ at 30°. Optical density (OD_{600}) was measured for each culture and plotted as four individual transformants ± SD.

a form of calmodulin that cannot bind Ca^{2+} and therefore does not stimulate calcineurin (Moser *et al.* 1996), we found that EF1, EF2, or EF3 Cnb1 mutant calcineurin failed to stimulate calmodulin-independent calcineurin activity. Since binding of Ca^{2+}/calmodulin is necessary to displace AID and AIS (Li *et al.* 2016b), we further tested whether removal of the Cna1 C-terminal regulatory CBD, AIS or AID could bypass the requirement for EF1, EF2, or EF3. Removal of the Cna1 AID alone or in combination with the CBD and AIS was not able to restore wild-type levels of calcineurin activity. Further, Cna1 truncation to additionally remove the CBD and AIS (Cna1-ΔCBD) revealed that mutation of EF1, EF2, or EF3 impairs transmission of an activation signal from Cnb1 to Cna1 that is independent of conformational changes involving the CBD, AIS, or AIDs of Cna1. Although our findings suggest an additional role, they do not rule out the possibility that reduced calmodulin interaction or ineffective displacement of the bipartite Cna1 AIDs contribute to the reduced activity of Cnb1 EF1, EF2, or EF3 mutant calcineurin in the context of full-length Cna1 and functional calmodulin. Indeed, mutation of EF2 has previously been reported to reduce the interaction of recombinant mammalian calcineurin with Ca^{2+}/calmodulin *in vitro* (Feng and Stemmer 2001).

Interaction of calcineurin with immunophilin-immunosuppressant complexes requires Ca^{2+} and CNB (Cardenas *et al.* 1994; Milan *et al.* 1994; Rodríguez *et al.* 2009). Cnb EF1, EF2, and EF3 mutant calcineurin had increased sensitivity to inhibition by immunophilin-immunosuppressant complexes despite their reduced activity and Ca^{2+} responsiveness. These findings suggest that mutation of specific EF hands alters the conformation of calcineurin leading to enhanced interaction with immunophilin-immunosuppressant complexes. Since these complexes share an overlapping binding site with substrates (Rodríguez *et al.* 2009), our observations suggest that substrate interactions may be altered in EF1, EF2, and EF3 Cnb1 mutants, contributing to either reduced binding or ineffective dephosphorylation due to inappropriate positioning of phosphorylated residues with respect to the catalytic site. Altered conformation and increased accessibility of the active site is also suggested by the increased sensitivity of EF1, EF2, and EF3 mutant calcineurin to oxidative inactivation. *In vitro* analysis of mammalian CNB has demonstrated that Ca^{2+} binding to CNB reduces the Michaelis-Menten constant (K_m) for substrate binding (Perrino *et al.* 1995).

In summary, our findings reveal striking conservation in the roles of Cnb1 EF hand domains in regulating calcineurin function between yeast *in vivo* and mammalian calcineurin *in vitro*, and extend those findings to reveal that EF1, EF2, and EF3 stimulate phosphatase activity independent of known conformational changes induced upon Ca^{2+} binding that lead to calmodulin binding and relief of bipartite autoinhibition mediated by C-terminus of CNA. Further analysis of these mutants may shed light on how CNB stimulates calcineurin activity following Ca^{2+} binding within the context of interacting cellular factors that may function to shape calcineurin activity downstream of Ca^{2+} signaling, or development of novel strategies to inhibit calcineurin activity in cells.

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