Calcineurin-dependent regulation of Crz1p nuclear export requires Msn5p and a conserved calcineurin docking site

Leila M. Boustany and Martha S. Cyert[1]

Department of Biological Sciences, Stanford University, Stanford, California 94305-5020, USA

Calcineurin, a conserved Ca²⁺/calmodulin-regulated protein phosphatase, plays a crucial role in Ca²⁺ signaling in a wide variety of cell types. In *Saccharomyces cerevisiae*, calcineurin positively regulates transcription in response to stress by dephosphorylating the transcription factor Crz1p/Tcn1p. Dephosphorylation promotes Crz1p nuclear localization in part by increasing the efficiency of its nuclear import. In this work, we show that calcineurin-dependent dephosphorylation of Crz1p also down-regulates its nuclear export. Using a genetic approach, we identify Msn5p as the exportin for Crz1p. In addition, we define the Crz1p nuclear export signal (NES) and show that it interacts with Msn5p in a phosphorylation-dependent manner. This indicates that calcineurin regulates Crz1p nuclear export by dephosphorylating and inactivating its NES. Finally, we define a motif in Crz1p, PIISIQ, similar to the PxIxIT docking site for calcineurin on the mammalian transcription factor NFAT, that mediates the in vivo interaction between calcineurin and Crz1p and is required for calcineurin-dependent regulation of Crz1p nuclear export and activity. Therefore, in yeast as in mammals, a docking site is required to target calcineurin to its substrate such that it can dephosphorylate it efficiently.

Ca²⁺-dependent signal transduction pathways elicit diverse physiological responses. One way that Ca²⁺ exerts an effect is through activation of the Ca²⁺-binding protein calmodulin, which, in turn, activates the serine/ threonine protein phosphatase calcineurin. In mammalian cells, calcineurin regulates the subcellular localization of the transcription factor NFAT (<u>n</u>uclear <u>f</u>actor of <u>a</u>ctivated <u>T</u>-cells) that is required for T-cell activation in response to antigen. Dephosphorylation of NFAT by calcineurin promotes its nuclear localization and, as a result, gene transcription and T-cell proliferation (Flanagan et al. 1991; Jain et al. 1993; Shaw et al. 1995). Activation of NFAT is blocked, however, when calcineurin phosphatase activity is inhibited by the immunosuppressive drugs FK506/FK520 and cyclosporin A (Liu et al. 1991a; Clipstone and Crabtree 1992; O'Keefe et al. 1992). Calcineurin interacts with NFAT through a defined consensus binding site, PxIxIT; when this site is mutated, dephosphorylation and nuclear translocation of NFAT do not occur (Aramburu et al. 1998). Calcineurin similarly regulates NFAT family members in a variety of cell types to play a role in other processes including cardiac and skeletal muscle development and angiogenesis (Chin et al. 1998; de la Pompa et al. 1998; Molkentin et al. 1998; Ranger et al. 1998; Graef et al. 2001).

In the budding yeast *Saccharomyces cerevisiae*, calcineurin is activated by extracellular stresses. Calcineurin mutants deficient for either the catalytic subunits (*CNA1/CNA2*; Cyert et al. 1991; Liu et al. 1991b) or the regulatory subunit (*CNB1*; Kuno et al. 1991; Cyert and Thorner 1992) are viable under standard growth conditions but are sensitive to high concentrations of ions such as Na⁺, Li⁺, Mn⁺, and OH⁻ (Nakamura et al. 1993; Mendoza et al. 1994; Farcasanu et al. 1995; Pozos et al. 1996). They also lose viability during sustained treatment with mating pheromone (Moser et al. 1996; Withee et al. 1997). Under these conditions calcineurin is required to activate a number of genes, including *PMC1*, *PMR1*, and *PMR2*, which encode ion pumps (Rudolph et al. 1989; Haro et al. 1991; Cunningham and Fink 1994, 1996; Mendoza et al. 1994), and *FKS2*, which encodes a major cell wall biosynthetic enzyme (Mazur et al. 1995). Calcineurin regulates transcription by activating Crz1p/ Tcn1p, which, in turn, binds a 24-bp promoter element termed the CDRE (<u>c</u>alcineurin-<u>d</u>ependent <u>r</u>esponse <u>e</u>lement; Matheos et al. 1997; Stathopoulos and Cyert 1997) to activate transcription of its target genes.

Crz1p is regulated by calcineurin in a remarkably similar manner to NFAT; upon Ca²⁺ addition to the media, Crz1p rapidly translocates from the cytosol to the nucleus in a calcineurin-dependent fashion (Stathopoulos-Gerontides et al. 1999). Previous studies established that when Crz1p is dephosphorylated, its nuclear import rate increases because Crz1p binds to its importin

Nmd5p more efficiently (Polizotto and Cyert 2001). Crz1p and NFAT are not homologous proteins but both do contain an SRR (serine-rich region) domain (Beals et al. 1997; Stathopoulos-Gerontides et al. 1999). In Crz1p mutants lacking the SRR, calcineurin-independent nuclear localization is observed, indicating that the SRR is important for regulation of Crz1p nuclear transport (Stathopoulos-Gerontides et al. 1999).

In this study we further characterize the calcineurin-dependent regulation of Crz1p nuclear transport. We establish that calcineurin negatively regulates Crz1p nuclear export and identify Msn5p as the required exportin. Furthermore, we define the Crz1p NES and show that its phosphorylation state is critical for function. Finally, we show that calcineurin interacts with Crz1p through a site, PIISIQ, that is similar to the calcineurin-binding site in NFAT; this motif is required for proper regulation of Crz1p nuclear transport by calcineurin and, thus, for wild-type Crz1p function.

Results

Crz1p nuclear export is regulated by calcineurin

Ca^{2+}/calcineurin-dependent dephosphorylation of Crz1p up-regulates nuclear import and causes rapid translocation of Crz1p from the cytosol to the nucleus of the cell (Polizotto and Cyert 2001). We wished to determine whether nuclear export of Crz1p was also regulated by calcineurin. To address this question, we used a strain deleted for the importin *NMD5* such that we could observe Crz1p localization in the absence of calcineurin-regulated nuclear import. Because Crz1p does not localize to the nucleus with Ca^{2+} addition in this strain (Polizotto and Cyert 2001), to facilitate Crz1p nuclear import we used an exogenous nuclear localization signal (NLS) from the SV40 large T antigen (Kalderon et al. 1984a,b). The SV40 NLS targeted GFP to the nucleus; this targeting was unaffected by Ca^{2+} or FK506 addition to the media (Fig. 1), and thus is not regulated by calcineurin. The GFP–SV40NLS–Crz1p fusion localized to the cytosol of untreated cells, showing that Crz1p contains an NES and suggesting that under these conditions the rate of Crz1p nuclear export is greater than the rate of import directed by the SV40 NLS. However, when Ca^{2+} was added, GFP–SV40NLS–Crz1p localized to the nucleus of *nmd5Δ* cells (Fig. 1). This change did not occur when calcineurin was inhibited by FK506. Because the SV40 NLS provides constant nuclear import, we concluded that the Ca^{2+}-induced nuclear localization of GFP–SV40NLS–Crz1p was owing to decreased export. Thus, dephosphorylation of Crz1p by calcineurin leads to decreased nuclear export.

A genetic screen identifies MSN5 as the Crz1p exportin

To establish the mechanism of Crz1p nuclear export, we devised a genetic screen to identify the Crz1p exportin. Previous studies revealed that expression of the
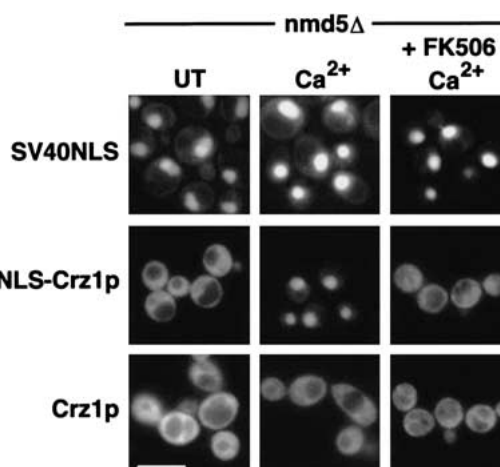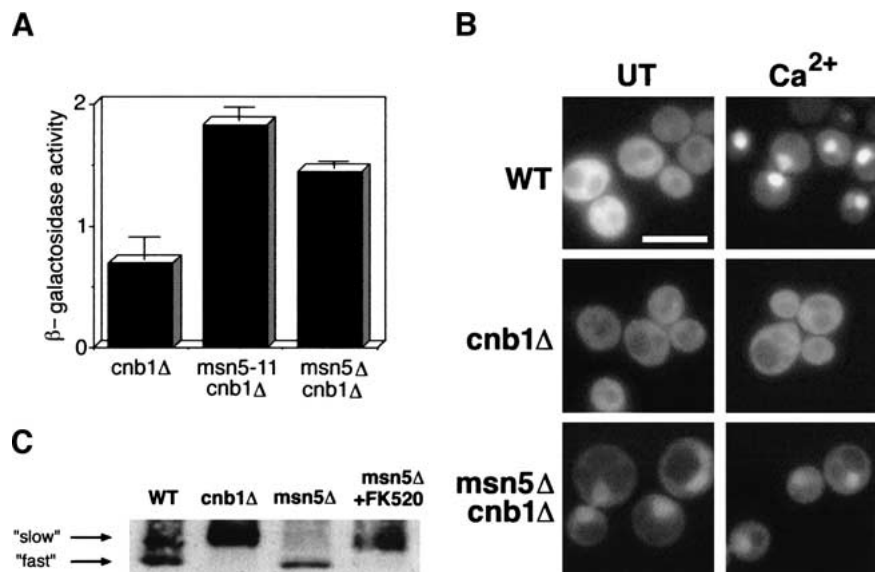

Figure 1. Crz1p nuclear export is regulated by calcineurin. *nmd5Δ* (HFY133) cells expressing 3xGFP–SV40NLS (LMB134), 3xGFP–SV40NLS–Crz1p (LMB148), or 3xGFP–Crz1p (LMB127) were grown to log phase at 21°C. Cells were treated with 200 mM CaCl₂ or pretreated with FK506 (2 μg/mL) for 30 min before CaCl₂ addition and visualized using fluorescence microscopy. Bar, 20 μm.

CDRE::lacZ reporter element is dependent on both calcineurin and Crz1p (Stathopoulos and Cyert 1997). We reasoned that a mutation in the exportin for Crz1p might result in constitutive nuclear localization of Crz1p and consequent activation of the *CDRE::lacZ* reporter in a calcineurin mutant background (*cnb1Δ*). We identified 40 recessive mutants that expressed *CDRE::lacZ* in the absence of calcineurin activity, and a single complementation group containing 5 strains was chosen for characterization (see Materials and Methods). These strains were complemented by plasmids containing *MSN5*. Furthermore, an *msn5Δ cnb1Δ* strain failed to complement the mutants, confirming that each contained a mutation in *MSN5*. The *msn5Δ cnb1Δ* strain also had the same phenotype as the mutant isolated from our screen (*msn5-11cnb1Δ*). In a quantitative β-galactosidase assay, both strains showed a small but reproducible increase in basal *CDRE::lacZ* activity over the *cnb1Δ* strain (Fig. 2A). Therefore, we used the null allele of *MSN5* for all further experiments.

Mutations in MSN5 affect Crz1p cellular localization

Because Msn5p had been previously characterized as a nuclear transport factor (Kaffman et al. 1998; Blondel et al. 1999; DeVit and Johnston 1999), we tested whether mutations in *MSN5* affected Crz1p localization. GFP–Crz1p localizes to the cytosol of untreated cells, but translocates to the nucleus after Ca^{2+} addition to the media (Fig. 2B; Stathopoulos-Gerontides et al. 1999). This change in localization is completely dependent on calcineurin as GFP–Crz1p remains cytosolic in a *cnb1Δ* strain. In contrast, GFP–Crz1p was partially localized to the nucleus of *msn5Δ cnb1Δ* cells (Fig. 2B). Ca^{2+} addition had no further effect on GFP–Crz1p localization in this strain, as would be expected in the absence of calcineu-

Figure 2. *msn5* mutants activate Crz1p-dependent transcription in a *cnb1Δ* background and alter Crz1p subcellular localization. (*A*) Strains harboring an integrated *CDRE::lacZ* reporter were grown to log phase at 30°C and harvested. β-Galactosidase activity was measured for *cnb1Δ* (DD12), *msn5-11cnb1Δ* (ASY11), and *msn5Δ cnb1Δ* (LBY196) strains. For each strain two cell extracts were prepared and assayed in triplicate. The standard deviation is the error between the samples. (*B*) WT (ASY472), *cnb1Δ* (ASY475), and *msn5Δ cnb1Δ* (LBY172) cells expressing GFP–Crz1p (AMS463) were grown to log phase at 21°C. Cells were treated with 200 mM CaCl$_2$, and GFP–Crz1p was visualized using fluorescence microscopy. Bar, 20 μm. (*C*) Msn5p does not affect Crz1p phosphorylation state. Whole cell extracts from wild-type (YPH499), *cnb1Δ* (DD12), and *msn5Δ* (ASY788) strains containing HA–Crz1p (AMS446) were treated with or without 2 μg/mL FK520 and analyzed by Western blot using anti-HA antibody.

rin activity. The nuclear accumulation of Crz1p observed in untreated *msn5Δ cnb1Δ* cells is consistent with a defect in Crz1p nuclear export.

Crz1p subcellular localization is regulated by its phosphorylation state (Stathopoulos-Gerontides et al. 1999; Polizotto and Cyert 2001); therefore, we examined whether Msn5p affects Crz1p phosphorylation. Crz1p shows a faster electrophoretic mobility in wild-type strains than it does in *cnb1Δ* strains (Fig. 2C; Stathopoulos-Gerontides et al. 1999). This difference is due to phosphorylation because treatment of purified Crz1p with calcineurin in vitro eliminates the mobility shift (Stathopoulos-Gerontides et al. 1999). We determined that the electrophoretic mobility of HA–Crz1p also showed a calcineurin-dependent shift in *msn5Δ* strains. When *msn5Δ* cells were incubated with the calcineurin inhibitor FK520, Crz1p showed reduced mobility (Fig. 2C). Thus, in the absence of Msn5p and calcineurin activity, Crz1p is phosphorylated, yet it accumulates in the nucleus.

Msn5p is required for Crz1p nuclear export

We next sought to determine whether Msn5p is required for Crz1p export from the nucleus. To address this question, wild-type or *msn5Δ* cells expressing GFP–Crz1p were first treated with the protein synthesis inhibitor cycloheximide to ensure that we followed the same pool of GFP–Crz1p throughout the experiment. Ca^{2+} was added to promote nuclear localization of GFP–Crz1p, and cells were subsequently incubated with FK506 for 30 min to inhibit calcineurin activity. In wild-type cells, FK506 treatment caused GFP–Crz1p to return to the cytosol (Fig. 3), indicating that continual dephosphorylation of Crz1p by calcineurin is required to maintain its

nuclear localization. However, in *msn5Δ* cells, GFP–Crz1p remained nuclear even after treatment with FK506 (Fig. 3), suggesting that Msn5p is required for Crz1p to exit the nucleus. Notably, the addition of Ca^{2+} to *msn5Δ* cells led to increased nuclear localization of GFP–Crz1p (Fig. 3). Because export is blocked in these cells, this change in localization is likely caused by increased nuclear import of Crz1p, and provides further evidence for the role of Ca^{2+}/calcineurin in regulating this process (Polizotto and Cyert 2001).

Identification of the Crz1p NES

To further explore the mechanism of Crz1p nuclear export, we identified the Crz1p NES. We tested the ability of different portions of Crz1p to drive export of the constitutively nuclear GFP–SV40NLS fusion. Using this ap-
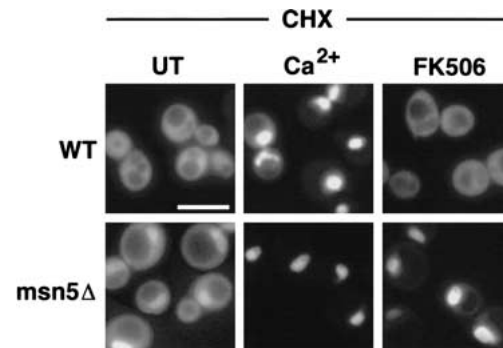

Figure 3. Msn5p is required for Crz1p nuclear export. WT (ASY472) and *msn5Δ* (ASY788) cells expressing GFP–Crz1p (AMS463) were grown to log phase at 21°C. Cells were treated with cycloheximide (10 μg/mL) for 20 min. CaCl$_2$ (200 mM) was added for 10 min followed by FK506 (5 μg/mL). Images were captured 30 min after FK506 treatment. Bar, 20 μm.

proach, we defined two distinct domains. One domain was required for calcineurin-dependent regulation of Crz1p nuclear export and is described in detail below. The other domain, amino acids 186–279, was sufficient to cause cytosolic localization of GFP–SV40NLS in the presence and absence of Ca^{2+} (Fig. 4A) as well as in a $cnb1\Delta$ strain (data not shown). In an $msn5\Delta$ background, however, GFP–SV40NLS–Crz1p$_{186-279}$ is nuclear (data not shown). Thus, amino acids 186–279 of Crz1p contain an Msn5p-dependent NES. Residues 186–279 define the smallest region sufficient for nuclear export as GFP–SV40NLS–Crz1p$_{186-250}$ and GFP–SV40NLS–Crz1p$_{201-279}$ both failed to be exported (Fig. 4A,B). It is likely that this is the only NES in Crz1p because constructs encompassing the N terminus (amino acids 1–186) or the C terminus (amino acids 433–679) of the protein failed to be exported (Fig. 4B). Interestingly, the NES includes the SRR domain, which contains many putative Crz1p phosphorylation sites. The SRR is not sufficient for export, but it is necessary; when this region was deleted (Δ186–233), export activity was lost (Fig. 4B). In addition, when all serines and two threonines in the SRR domain were mutated to alanine (mSRR), export activity was again

disrupted, suggesting that phosphorylation of the NES is required for its function (Fig. 4A, B). Mutating different subsets of serines in the SRR resulted in less severe export defects (data not shown); thus, elimination of multiple sites is required to disrupt the NES.

Msn5p interacts with the Crz1p NES

We examined the interaction between the Crz1p NES and Msn5p using a directed yeast two-hybrid approach. Amino acids 186–279 of Crz1p fused to the Gal4p activation domain (AD–CRZ1$_{186-279}$) interacted with Msn5p fused to the Gal4p DNA-binding domain (BD–MSN5; Fig. 4C; DeVit and Johnston 1999). Yeast containing a GAL4 UAS-driven HIS3 reporter showed robust growth on media lacking histidine when both fusions were expressed, whereas yeast expressing either construct alone showed little or no growth. Therefore, Msn5p physically interacts with the Crz1p NES in vivo. However, Msn5p failed to interact with AD–mSRR$_{186-279}$ containing serine/threonine to alanine changes (Fig. 4C), suggesting that phosphorylation is required for binding between Crz1p and Msn5p and, thus, for nuclear export.
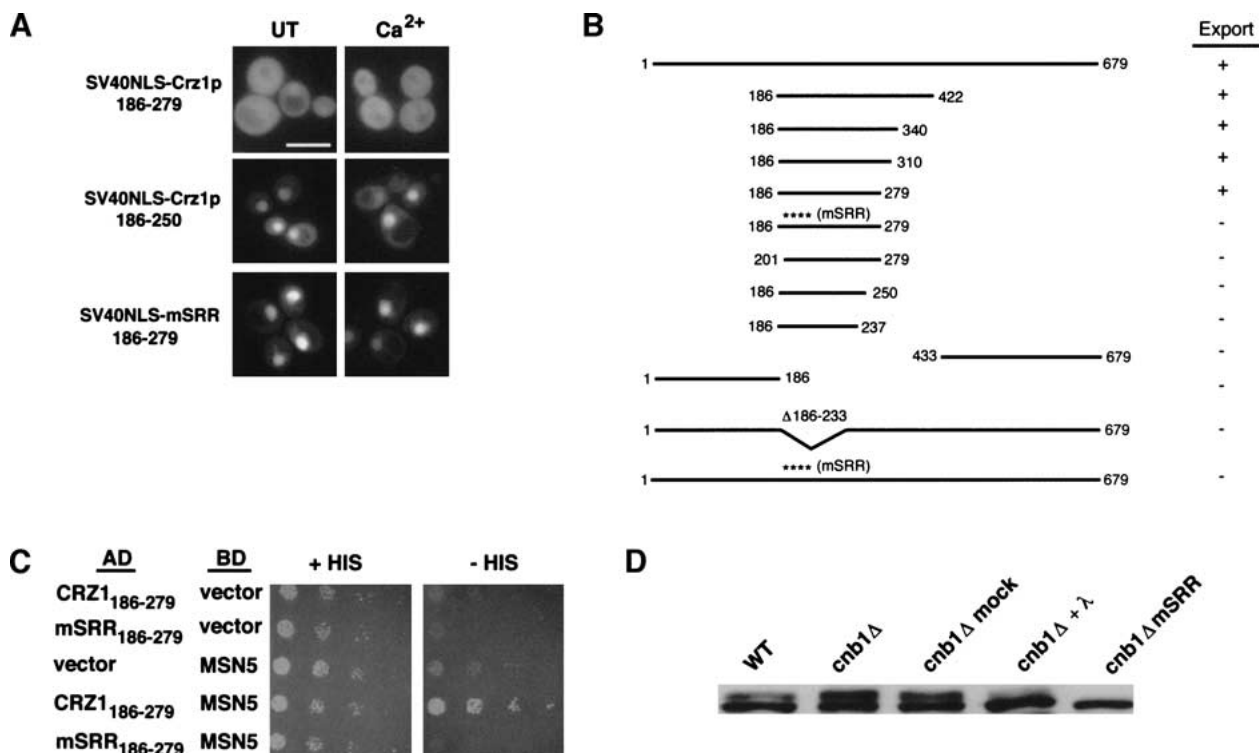

Figure 4. Identification of the Crz1p NES. (A) WT (ASY472) cells expressing 3xGFP–SV40NLS–Crz1p$_{186-279}$ (LMB162), 3xGFP–SV40NLS–Crz1p$_{186-250}$ (LMB207), or 3xGFP–SV40NLS–mSRR$_{186-279}$ (LMB 211) were grown to log phase at 21°C. Cells were treated with 200 mM CaCl$_2$ and visualized using fluorescence microscopy. Bar, 20 μm. (B) Summary of export data for 3xGFP–SV40NLS–Crz1p constructs. (+) Cytosolic localization (export); (–) nuclear localization (no export). (C) The Crz1p NES interacts with Msn5p in vivo. A two-hybrid strain containing a HIS3 reporter (PJ69-4A) expressing AD–CRZ1$_{186-279}$ (LMB193), AD–mSRR$_{186-279}$ (LMB218), or GAL4BD–MSN5 (BM3694) alone or in combination was spotted onto media with or without histidine and grown at 21°C for 5 d. Cells were spotted using fivefold serial dilutions beginning at an OD$_{600}$ of 0.05. (D) The Crz1p NES is similarly phosphorylated in WT and $cnb1\Delta$ cells. Whole cell extracts from WT (ASY472) or $cnb1\Delta$ (ASY475) strains containing LMB162 or LMB211 were analyzed by Western blot using anti-GFP antibody. Where indicated, extracts were treated with or without (mock) 200 units of λ phosphatase and incubated at 30° for 30 min.

Our data indicate that phosphorylation within the SRR domain is required for NES activity; therefore, we investigated the phosphorylation state of the NES under different conditions. We examined GFP–SV40NLS–Crz1p$_{186-279}$ protein isolated from wild-type and $cnb1\Delta$ backgrounds. Unlike full-length Crz1p (Fig. 2C), the NES showed similar electrophoretic mobility in wild-type and $cnb1\Delta$ cells (Fig. 4D). An upper band was evident in both samples that was missing in the GFP–SV40NLS–mSRR$_{186-279}$ sample, where all putative phosphorylation sites had been mutated. When dephosphorylated in vitro, the protein collapsed to a single band, showing that the upper band was, indeed, a result of phosphorylation (Fig. 4D). Therefore, in this context, the NES directs export in wild-type and $cnb1\Delta$ cells and shows similar phosphorylation in both strains. These observations are consistent with our hypothesis that phosphorylation of the Crz1p NES is required for interaction with Msn5p.

Identification of the Crz1p export regulatory region

Full-length Crz1p shows calcineurin-dependent regulation of nuclear export, but the NES defined above, amino acids 186–279, is exported independently of calcineurin activity. To explore this difference, we used additional GFP–SV40NLS–Crz1p fusions to examine the mechanism by which calcineurin regulates Crz1p nuclear export. GFP–SV40NLS–Crz1p$_{186-340}$ was the smallest fusion that behaved like full-length Crz1p. It localized to the nucleus of wild-type cells upon Ca^{2+} addition but remained cytosolic in $cnb1\Delta$ cells (Fig. 5A). A smaller fusion, GFP–SV40NLS–CRZ1$_{186-310}$, was cytosolic in both wild-type and $cnb1\Delta$ backgrounds with or without Ca^{2+} addition (Fig. 5A). Therefore, amino acids 311–340 are required for calcineurin-dependent regulation of Crz1p export.

To further investigate the effect of calcineurin activity on Crz1p nuclear export, we examined the localization of GFP–SV40NLS–Crz1p constructs in strains overexpressing a constitutively active, truncated form of calcineurin, *CNA2* trunc (Withee et al. 1997). GFP–SV40NLS–Crz1p$_{186-340}$ was nuclear in this background even in the absence of Ca^{2+} (Fig. 5B). This construct lacks a calcineurin-dependent NLS, hence, its nuclear localization must result from down-regulated export. In contrast, expression of activated calcineurin
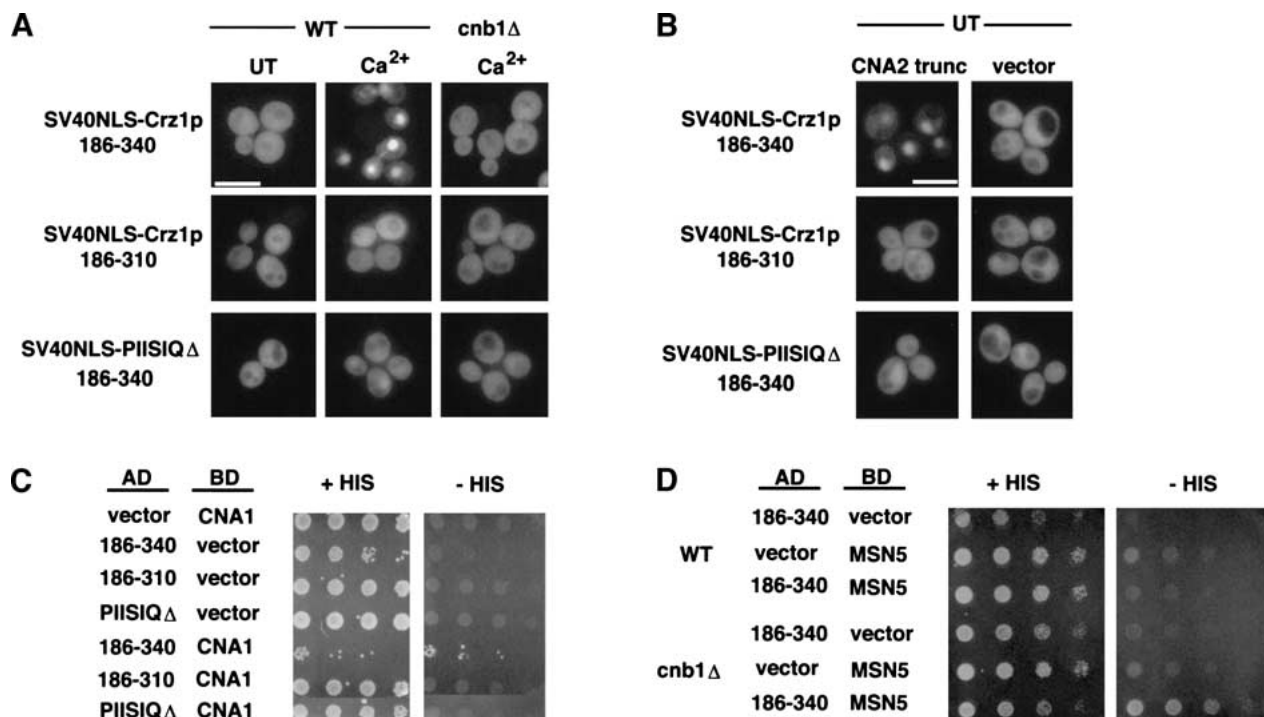

Figure 5. Identification of the export regulatory region. (*A*) WT (ASY472) or $cnb1\Delta$ (ASY475) cells expressing 3xGFP–SV40NLS–Crz1p$_{186-340}$ (LMB160), 3xGFP–SV40NLS–Crz1p$_{186-310}$ (LMB180), or 3xGFP–SV40NLS–PIISIQΔ$_{186-340}$ (LMB228) were grown to log phase at 21°C. Cells were treated with 200 mM CaCl$_2$ and visualized using fluorescence microscopy. Bar, 20 µm. (*B*) Overexpression of activated calcineurin affects the localization of GFP–SV40NLS–Crz1p$_{186-340}$. ASY472 cells expressing *CNA2* trunc or vector (pVT100-L) and LMB160, LMB180, or LMB228 were grown to log phase at 21°C and visualized using fluorescence microscopy. Bar, 20 µm. (*C*) Constructs containing the Crz1p export regulatory region interact with calcineurin in vivo. A two-hybrid strain (PJ69-4A) containing a *HIS3* reporter and expressing GAL4AD–Crz1p fusions LMB189, LMB226, or LMB224 and GAL4BD–CNA1 (BJP2014) alone or in combination were spotted on plates with or without histidine plus 100 mM CaCl$_2$ and grown at 30°C for 2–3 d. Cells were spotted using fivefold serial dilutions beginning at an OD$_{600}$ of 1.0. (*D*) The Crz1p/Msn5p interaction is dependent on Crz1p phosphorylation state. WT and $cnb1\Delta$ two-hybrid strains (PJ69-4A and ASY3) containing a *HIS3* reporter and expressing LMB189 and GAL4BD–MSN5 (BM3694) alone or in combination were spotted on media with or without histidine plus 100 mM CaCl$_2$ and grown at 21°C for 5 d. Cells were spotted using fivefold serial dilutions beginning at an OD$_{600}$ of 1.0.

had no effect on the cytosolic localization of GFP–SV40NLS–Crz1p$_{186-310}$ (Fig. 5B).

We hypothesized that residues 311–340 of Crz1p are required for its association with calcineurin. Using a directed yeast two-hybrid approach, we looked for an interaction between calcineurin (Cna1p) and Crz1p. AD–CRZ1$_{186-340}$, the region that shows regulated nuclear localization, interacted with BD–CNA1 (Jiang and Cyert 1999), but AD–CRZ1$_{186-310}$ did not (Fig. 5C). In addition, an examination of phosphorylation state revealed that, like amino acids 186–279, residues 186–310 were phosphorylated in both wild-type and $cnb1\Delta$ backgrounds (data not shown). These findings support the idea that residues 311–340 of Crz1p are required for its interaction with and efficient dephosphorylation by calcineurin.

A consensus binding site for calcineurin in NFAT family members has been defined, PxIxIT (Aramburu et al. 1998) We identified a similar site, PIISIQ (PxIxIQ), within amino acids 311–340 of Crz1p and examined its role in calcineurin-dependent regulation of Crz1p. First, GFP–SV40NLS–Crz1p$_{186-340}$ lacking the PIISIQ motif (GFP–SV40NLS–PIISIQ$\Delta_{186-340}$) no longer localized to the nucleus with Ca^{2+} addition (Fig. 5A). Next, we examined the effect of activated calcineurin on GFP–SV40NLS–PIISIQ$\Delta_{186-340}$ localization and found that it remained cytosolic (Fig. 5B). Finally, we tested whether these residues were necessary for Crz1p/calcineurin interaction and found by two-hybrid analysis that AD–PIISIQ$\Delta_{186-340}$ failed to interact with BD–CNA1 (Fig. 5C). These data suggest that the PIISIQ motif is a docking site for calcineurin on Crz1p and is required for calcineurin-dependent regulation of Crz1p nuclear export. We hypothesized that Crz1p fragments containing the PIISIQ motif are dephosphorylated by calcineurin, and thus, they show calcineurin-regulated nuclear export. In contrast, smaller pieces of Crz1p that lack the PIISIQ motif,

such as 186–279 and 186–310, are not efficiently dephosphorylated by calcineurin, and therefore show unregulated nuclear export. If this model is correct, then Crz1p$_{186-340}$ but not Crz1p$_{186-279}$ should show a calcineurin-sensitive interaction with the exportin Msn5p. We confirmed this prediction using two-hybrid analysis. Amino acids 186–340 of Crz1p interacted more strongly with Msn5p in a $cnb1\Delta$ background than in a wild-type strain (Fig. 5D). However, we found that residues 186–279 (NES) interacted similarly with Msn5p in both wild-type and $cnb1\Delta$ strains (data not shown).

Deleting the PIISIQ motif affects Crz1p function

We established that amino acids 311–340 are required for calcineurin-dependent regulation of protein fusions containing small regions of Crz1p. To determine the function of this region in full-length Crz1p, we examined the localization of GFP–Crz1p constructs with in-frame deletions of either residues 311–340 or 331–336 (PIISIQ). These constructs lack the SV40NLS, relying instead on the endogenous Crz1p NLS for Nmd5p-mediated nuclear import. As previously shown, full-length GFP–Crz1p localized to the nucleus within 10 min of Ca^{2+} addition to the media (Fig. 6A). GFP–Crz1p$_{311-340\Delta}$ and GFP–Crz1p$_{PIISIQ\Delta}$, however, remained cytosolic under these conditions (Fig. 6A). At longer times, partial nuclear localization of GFP–Crz1p$_{311-340\Delta}$ and GFP–Crz1p$_{PIISIQ\Delta}$ was observed (data not shown). Therefore, the PIISIQ motif is required for efficient nuclear localization of Crz1p in response to Ca^{2+}. Next, we tested the ability of these constructs to complement the Li$^+$ sensitivity of $crz1\Delta$ strains. When plated on 350 mM LiCl$_2$, GFP–Crz1p fully complemented the $crz1\Delta$, whereas GFP–Crz1p$_{311-340\Delta}$ and GFP–Crz1p$_{PIISIQ\Delta}$ did not (Fig. 6B). Finally, we analyzed the transcriptional activity of the de-
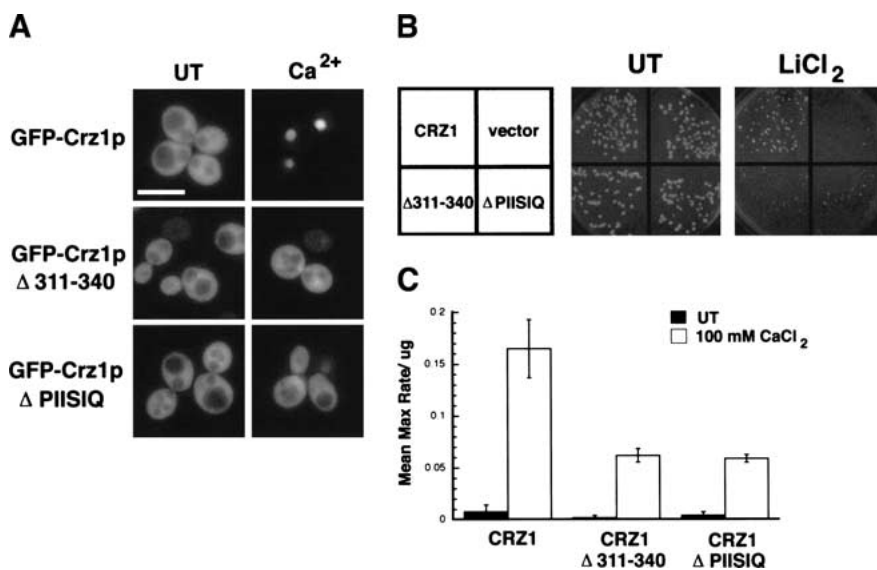

Figure 6. Deletion of the PIISIQ motif affects Crz1p localization. (*A*) WT (ASY472) cells expressing 3xGFP–Crz1p (LMB127), 3xGFP–Crz1p$_{\Delta311-340}$ (LMB230), 3xGFP–Crz1p$_{\Delta PIISIQ}$ (LMB231), or GFP vector were grown to log phase at 21°C. Cells were treated with 200 mM CaCl$_2$ for 10 min and visualized with fluorescence microscopy. Bar, 20 µm. (*B*) The PIISIQ motif is required for complementation of a $crz1\Delta$ strain. ASY472 cells expressing LMB127, LMB230, LMB231, or GFP vector were plated on selective media with or without 350 mM LiCl$_2$ and grown at 30°C for 3 d. (*C*) Deletion of the PIISIQ motif affects Crz1p activity. β-Galactosidase activity was measured in $crz1\Delta$ cells harboring an integrated *CDRE::lacZ* reporter (ASY834). Strains expressed LMB127, LMB230, or LMB231 and were treated with (open bars) or without (black bars) 100 mM CaCl$_2$. The numbers are the result of two replicates analyzed in triplicate, and the standard deviation is the error between samples.

letion constructs. Full-length GFP–Crz1p activated transcription of the *CDRE::lacZ* reporter in response to Ca^{2+} (Fig. 6C), whereas *crz1Δ* strains expressing vector alone showed no Ca^{2+} induction (data not shown). The ability of the deletion constructs to drive transcription of *CDRE::lacZ* was reduced (Fig. 6C), although all three fusion constructs were expressed at equivalent levels (data not shown). Together, these data indicate that the PIISIQ motif is required in vivo for maximal activation of Crz1p.

Discussion

Calcineurin regulates nuclear export of Crz1p

In vivo, activation of yeast calcineurin causes rapid translocation of the Crz1p transcription factor from the cytosol to the nucleus, where it turns on transcription of specific target genes. Studies from this laboratory have characterized the nuclear transport of Crz1p to elucidate the mechanisms by which this change in localization occurs. In this report we examined export of Crz1p from the nucleus and showed that calcineurin down-regulates Crz1p export. To understand how calcineurin regulates this process, we identified the key elements of Crz1p nuclear export: its exportin and its NES.

Msn5p is the exportin for Crz1p

We have determined that Msn5p is the exportin for Crz1p. Crz1p is partially nuclear in *msn5Δ* cells even when calcineurin is inactive, and this allows calcineurin-independent expression of the *CDRE::lacZ* reporter. Furthermore, once Crz1p is in the nucleus, Msn5p is required for its return to the cytosol. Although we were unable to show direct binding between Crz1p and Msn5p in vitro, perhaps because it is a weak interaction or other stabilizing proteins are necessary, we do show that Msn5p interacts with the Crz1p NES by two-hybrid analysis. Msn5p has been identified as the exportin for several other proteins including Pho4p, Mig1p, Far1p, and Ste5p (Kaffman et al. 1998; Blondel et al. 1999; DeVit and Johnston 1999; Mahanty et al. 1999; Kunzler et al. 2001) and appears to also function in some cases as an importin (Yoshida and Blobel 2001), providing the first example of a karyopherin that functions bidirectionally.

Interestingly, *MSN5* was isolated both in a screen for mutants that activate a calcineurin/Crz1p-dependent reporter (this work) and in a screen for mutants that fail to do so (Matheos et al. 1997). In *msn5* mutants, we find that Crz1p accumulates in the nucleus and allows calcineurin-independent expression of *CDRE::lacZ*. However, *msn5* cells fail to induce expression of the Ca^{2+}-activated calcineurin/Crz1p-dependent *PMC1::lacZ* reporter gene (Matheos et al. 1997). This apparent contradiction may result from differences between the two reporters: *CDRE::lacZ* consists of four tandem repeats of a 24-bp sequence that binds Crz1p (Stathopoulos and Cyert 1997), whereas *PMC1::lacZ* contains 585 bp of DNA upstream of the *PMC1* start site (Cunningham and Fink 1996). Thus, although the CDRE reporter specifi-

cally reflects Crz1p activity, the *PMC1* promoter could contain binding sites for numerous proteins. Msn5p is pleiotropic and affects localization of several proteins (Kaffman et al. 1998; Alepuz et al. 1999; Blondel et al. 1999; DeVit and Johnston 1999; Mahanty et al. 1999). Therefore, in *msn5* cells, a protein that represses transcription of *PMC1::lacZ* may also accumulate in the nucleus and prevent its expression despite Crz1p nuclear localization.

The Crz1p NES

The Crz1p NES does not share the same characteristics as the classical NES. As defined in the protein kinase A inhibitor (PKI) and HIV-1 Rev, the classical NES is a short leucine-rich sequence in which the key residues are all hydrophobic (Fischer et al. 1995; Wen et al. 1995), and is recognized by the exportin Crm1p (Fornerod et al. 1997; Ossareh-Nazari et al. 1997; Stade et al. 1997). In Crz1p, we have instead defined a much larger 93-amino-acid region that is sufficient to drive nuclear export. This length is consistent with the sequences identified in other Msn5p cargoes (Blondel et al. 1999; DeVit and Johnston 1999). It is not yet understood why such a large NES is required for Msn5p-mediated export. Perhaps there are multiple NES sequences within the large region that work cooperatively. Alternatively, NES secondary structure rather than a specific amino acid sequence may be important for recognition by Msn5p. Interestingly, the Crz1p NES (amino acids 186–279) does contain one classical leucine-rich sequence, LDDLLSL (amino acids 257–263) that is necessary for its activity. The Crz1p NES also contains the SRR, a domain that is conserved between NFAT and Crz1p (see below). Although in NFAT the SRR was shown to be involved in regulating nuclear import (Beals et al. 1997; Okamura et al. 2000), our data show that it plays a key role in nuclear export, which is a novel function for the Crz1p SRR.

Mechanism of calcineurin-regulated Crz1p nuclear export

Calcineurin must interact with a conserved docking site in Crz1p to regulate its nuclear export. Crz1p fragments lacking the PIISIQ motif are constitutively exported and are unaffected by the overexpression of activated calcineurin. We propose that this motif is required for Crz1p to interact with and be efficiently dephosphorylated by calcineurin. In support of this model, we show using two-hybrid analysis that Crz1p$_{186–340}$ interacts with calcineurin, but that it fails to do so when the PIISIQ motif is deleted. Also, fusion proteins containing residues 186–310 or 186–279, both missing the PIISIQ motif, are similarly phosphorylated in extracts of wild-type and calcineurin mutant cells, indicating that they are not dephosphorylated in vivo.

Several results suggest that the Crz1p NES must be phosphorylated to function. First, in the two-hybrid assay, Msn5p preferentially interacts with phosphorylated Crz1p$_{186–340}$. Second, mutating potential phosphoryla-

tion sites within the Crz1p NES disrupts its interaction with Msn5p and nuclear export. Although specific phosphorylation sites in Crz1p have not been identified, mutating serines and threonines in the SRR reduces the calcineurin-dependent electrophoretic mobility shift (R. Polizotto, unpubl.), indicating that this region is phosphorylated. Combining these observations we propose that the PIISIQ motif, which is distinct from the NES, mediates a critical association between calcineurin and Crz1p that is required for calcineurin-dependent dephosphorylation of the NES. This dephosphorylation inhibits the interaction between the Crz1p NES and Msn5p, thereby decreasing Crz1p nuclear export. Similarly, Msn5p-mediated export of other cargoes, Pho4p and Mig1p, also requires their phosphorylation, and it has been suggested that Msn5p specifically exports phosphoproteins (Kaffman et al. 1998; DeVit and Johnston 1999). However, Far1p binding to Msn5p does not depend on phosphorylation state (Blondel et al. 1999).

Calcineurin-dependent regulation of Crz1p activity

In unstimulated cells, Crz1p resides in the cytosol because the rate of its nuclear export is greater than the rate of its nuclear import. When calcineurin-dependent signaling is activated, either in response to specific environmental stresses or by addition of Ca^{2+} to the media, Crz1p rapidly translocates to the nucleus and activates gene expression. Dephosphorylation of Crz1p increases its nuclear import, by causing a conformational change that exposes its nuclear localization signal (NLS) and promotes its interaction with the importin Nmd5p (Polizotto and Cyert 2001). At the same time, dephosphorylation of the Crz1p NES inhibits its interaction with Msn5p, thereby decreasing nuclear export. Thus, dephosphorylation of Crz1p by calcineurin performs two distinct functions: it both increases the rate of Crz1p nuclear import and decreases the rate of its nuclear export. These two effects combine to cause rapid and efficient nuclear accumulation of Crz1p in response to calcineurin activation. Continual dephosphorylation by calcineurin is required to maintain Crz1p nuclear localization. When signaling terminates or calcineurin is inhibited with FK506, Crz1p quickly returns to the cytosol because of its rephosphorylation. The kinases that phosphorylate Crz1p are as yet unidentified, although the rapidity with which Crz1p localization changes suggests that they may reside in the nucleus. Crz1p phosphorylation state oppositely affects its nuclear import and export; thus, the balance between calcineurin and kinase activity tightly regulates Crz1p localization and leads to efficient and reversible transcriptional activation in response to stress.

Although the accessibility of Crz1p to its target genes is clearly regulated through its subcellular localization, nuclear localization alone is apparently not sufficient to fully activate Crz1p. The increase in Crz1p-dependent transcription observed in *msn5Δ cnb1Δ* cells is low despite considerable nuclear accumulation of Crz1p in these cells. Therefore, calcineurin may regulate additional aspects of Crz1p function. Calcineurin activity modulates NFAT binding to DNA (Park et al. 1995; Shaw et al. 1995); however, the ability of Crz1p to bind DNA is not calcineurin-dependent; Crz1p purified from *cnb1Δ* cells associates well with the CDRE (Stathopoulos 1998). Alternatively, dephosphorylation of Crz1p by calcineurin may increase its ability to function as a transcriptional activator. Future investigations will address this possibility.

Parallels between calcineurin-regulated pathways in mammals and yeast

Calcineurin activity induces gene expression in both mammalian cells and yeast by regulating the subcellular localization of a transcription factor; both NFAT and Crz1p show calcineurin-regulated nuclear import and export (Beals et al. 1997; Zhu and McKeon 1999; Okamura et al. 2000; Polizotto and Cyert 2001). Despite their similar regulatory mechanisms, Crz1p and NFAT bind DNA through distinct motifs (Jain et al. 1995; Matheos et al. 1997; Stathopoulos and Cyert 1997) and share little sequence homology. However, two critical domains are conserved in these proteins, the SRR and a calcineurin docking site, both of which are required for calcineurin-dependent regulation. The SRR, or serine-rich region, contains multiple phosphorylated residues and is required in both proteins for calcineurin-dependent regulation of nuclear transport. Phosphorylation sites in the NFAT SRR contribute to regulation of its nuclear import (Beals et al. 1997; Okamura et al. 2000), whereas phosphorylation of the Crz1p SRR is required for NES activity. Also, both proteins contain a conserved calcineurin docking site. A consensus calcineurin-binding site, PxIxIT, has been defined for NFAT family members (Aramburu et al. 1998). Here we describe a similar motif in Crz1p, PIISIQ, which is required for its interaction with calcineurin in vivo. Crz1p lacking the calcineurin docking site, Crz1p$_{PIISIQΔ}$, shows defects in Ca^{2+}-induced nuclear accumulation and transcriptional activation. Therefore, the PIISIQ interaction motif is required for efficient dephosphorylation of Crz1p by calcineurin in vivo. Crz1p$_{PIISIQΔ}$ retains some activity, suggesting that other regions of Crz1p may also promote its interaction with calcineurin. Calcineurin forms stable associations with several other substrates, including the IP$_3$ and ryanodine receptors as well as dynamin 1 (Cameron et al. 1995; Lai et al. 1999). In each case, when this interaction is perturbed, calcineurin-dependent regulation of the substrate is compromised. Our work shows that in yeast, as in mammalian cells, this interaction occurs at a site on the substrate that is distinct from the phosphorylated region, and is required to target calcineurin to its substrate.

Materials and methods

Yeast media and general methods

Culture conditions and yeast media were essentially as described (Sherman et al. 1986), except that in synthetic media the

nutritional supplements were added to twice the indicated levels. When Ca^{2+} was added to synthetic media, 3.5 g of ammonium chloride was substituted for ammonium sulfate. Buffered YPD media was made with 40 mM succinate, and the pH was adjusted to 5.5 with KOH.

All recombinant DNA procedures were carried out according to standard protocols (Ausubel et al. 1987). Yeast cells were transformed using the lithium acetate method, and bacterial cells were transformed by electroporation (Ausubel et al. 1987). DNA templates for sequencing were prepared according to the manufacturer's instructions (Wizard Miniprep kit, Promega). Sequencing was also carried out according to the manufacturer's instructions (Sequenase, USB) using [α-^{35}S]dATP (Amersham).

Yeast strains

The yeast strains used in this study are described in Table 1. ASY788 was created by homologous recombination using an *msn5::loxP-kanMX-loxP* disruption cassette generated by PCR (Guldener et al. 1996). LBY172 was made by crossing ASY788 to MCY3-1D. The resulting diploid was sporulated and the double mutant selected. pAMS367 containing the *CDRE::lacZ* reporter was integrated at the *URA3* locus of LBY196, ASY456, and ASY569.

Plasmids

The plasmids used in this study are described in Table 2. pLMB134 was created by first annealing complementary oligonucleotides encoding the SV40 large T antigen NLS (Kalderon et al. 1984a,b) flanked by *Spe*I and *Hin*dIII restriction sites. The annealed oligonucleotides were then ligated into pOM4, a 3xGFP vector (Polizotto and Cyert 2001), digested with *Spe*I and *Hin*dIII. A series of in-frame 3xGFP–SV40NLS–Crz1p fusions were constructed in the following manner: PCR was used to amplify the indicated regions of *CRZ1* and to introduce *Hin*dIII and *Sal*I restriction sites flanking the PCR product. The PCR product was first ligated into a bacterial vector, PCR2.1TOPO, using the TOPO-TA kit (Invitrogen), and then shuttled into pLMB134 predigested with *Hin*dIII and *Sal*I. pLMB184 and pLMB186 were constructed as above except *Hin*dIII and *Cla*I restriction sites were used. pLMB189, pLMB193, pLMB218,

pLMB224, and pLMB 226 were constructed using PCR to amplify the specified regions of *CRZ1* with *Nco*I and *Bam*HI sites flanking the PCR product. PCR products were then ligated into pACTII to create an in-frame fusion with the Gal4p activation domain. pLMB230 was constructed by a three-way ligation of base pairs 1–930 of *CRZ1* flanked by *Hin*dIII and *Cla*I sites and base pairs 1021–2037 of *CRZ1* flanked by *Cla*I and *Sal*I sites into pOM4 digested with *Hin*dIII and *Sal*I. pLMB231 was constructed similarly but using base pairs 1–990 and 1009–2037 of *CRZ1*.

β-Galactosidase assays

Quantitative assay Exponentially growing yeast cells in selective synthetic media were diluted to an OD$_{600}$ of 0.2 in buffered YPD and grown at 30°C for 7 h. Cells were harvested and washed once, and the cell pellets were frozen. Cells were broken using glass bead lysis (Withee et al. 1997) in Breaking Buffer (100 mM Tris at pH 8, 20% glycerol, 1 mM DTT) plus protease inhibitors (1 mM PMSF, 1 mM benzamidine, 2 µg/mL leupeptin, 2 µg/mL aprotinin). Protein concentrations of the resulting cell extracts were determined using the Bio-Rad protein assay. β-Galactosidase activity was measured at 30°C in a microtiter plate using 75 µg of total protein, 100 µL of Z-buffer (100 mM Na$_2$HPO$_4$, 40 mM NaH$_2$PO$_4$, 10 mM KCl, 1 mM MgSO$_4$, 0.027% β-mercaptoethanol), and 40 µL of 4 mg/mL ONPG (*O*-nitrophenyl-β-D-galactopyranoside, Sigma). Values result from the average of two independent extracts each measured in triplicate. Alternatively, cells were grown for 5 h in buffered YPD with or without 100 mM CaCl$_2$ and measured at room temperature in a microtiter plate using 90 µL of Z buffer and 20 µL of ONPG.

Qualitative assay Yeast colonies were scored for β-galactosidase activity as described (Stathopoulos and Cyert 1997). Blue colonies were selected after overnight incubation with 0.2 mg/mL X-gal (Sigma).

Genetic screening

Calcineurin mutant strains containing the *CDRE::lacZ* reporter gene (ASY456 or ASY569) were grown to log phase

Table 1. *Yeast strains used in this study*

Strain	Relevant genotype	Source
YPH499	MAT**a** *ura3-52 lys2-801 ade2-101 trp-Δ63 his3-Δ200 leu2-Δ1*	Sikorski and Hieter 1989
YPH500	MATα *ura3-52 lys2-801 ade2-101 trp-Δ63 his3-Δ200 leu2-Δ1*	Sikorski and Hieter 1989
DD12	Same as YPH499 except *cnb1::URA::hisG*	Cyert and Thorner 1992
MCY3-ID	Same as YPH500 except *cnb1::LEU2*	Cyert and Thorner 1992
PJ69-4A	MAT**a** *trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ*	James et al. 1996
ASY3	Same as PJ69-4A except *cnb1::URA3::hisg*	A. Stathopoulos-Gerontides
ASY456	Same as YPH499 except *ura3-52*::URA3-4xCDRE	A. Stathopoulos-Gerontides
ASY472	Same as YPH499 except *crzl::loxP-kanMX-loxP*	Stathopoulos and Cyert 1997
ASY475	Same as DD12 except *crzl::loxP-kanMX-loxP*	Stathopoulos and Cyert 1997
ASY569	Same as MCY3-1D except *ura3-52*::URA3-4xCDRE	A. Stathopoulos-Gerontides
ASY788	Same as YPH499 except *msn5::loxP-kanMX-loxP*	A. Stathopoulos-Gerontides
ASY834	Same as ASY472 except *ura3-52*::TRP1-4xCDRE	A. Stathopoulos-Gerontides
ASY835	Same as ASY475 except *ura3-52*::TRP1-4xCDRE	A. Stathopoulos-Gerontides
LBY172	Same as ASY788 except *cnb1::LEU2*	This study
LBY196	Same as LBY172 except *ura3-52*::URA3-4xCDRE	This study
HFY133	*ura3-1 ade2-1 trp1-1 his3-11,15 leu2-3,11 can1-100 nmd5::HIS3*	A. Jacobson

Table 2. *Plasmids used in this study*

Plasmid	Description	Reference
AMS367	URA3-4xCDRE integrating plasmid	Stathopoulos and Cyert 1997
AMS446	HA-CRZ1	Stathopoulos and Cyert 1997
AMS463	GFP-CRZ1	Stathopoulos-Gerontides et al. 1999
pVT CNA2	CNA2 trunc	Withee et al. 1997
pOM4	3xGFP vector	Polizotto and Cyert 2001
LMB127	3xGFP-CRZ1	This study
LMB134	3xGFP-SV40NLS vector	This study
LMB148	CRZ1 in LMB134	This study
LMB158	CRZ1 (aa 186–422) in LMB134	This study
LMB160	CRZ1 (aa 186–340) in LMB134	This study
LMB162	CRZ1 (aa 186–279) in LMB134	This study
LMB163	CRZ1 (aa 186–237) in LMB134	This study
LMB165	CRZ1 (aa 433–679) in LMB134	This study
LMB178	CRZ1 (aa 1–186) in LMB134	This study
LMB180	CRZ1 (aa 186–310) in LMB134	This study
LMB184	CRZ1 (aa Δ186–233) in LMB 134	This study
LMB186	CRZ1 (mSRR) in LMB 134	This study
LMB199	CRZ1 (aa 201–279) in LMB134	This study
LMB207	CRZ1 (aa 186–250) in LMB134	This study
LMB211	mSRR (aa 186–279) in LMB134	This study
LMB228	PIISIQΔ (aa 186–340) in LMB134	This study
LMB189	GAL4AD-CRZ1 (aa 186–340)	This study
LMB193	GAL4AD-CRZ1 (aa 186–279)	This study
LMB218	GAL4AD-mSRR (aa 186–279)	This study
LMB224	GAL4AD-PIISIQΔ (aa 186–340)	This study
LMB226	GAL4AD-CRZ1 (aa 186–310)	This study
LMB230	CRZ1 (aa Δ311–340) in pOM4	This study
LMB231	CRZ1 (aa Δ331–336) in pOM4	This study
BM3694	GAL4BD-MSN5	DeVit and Johnston 1999
BJP2014	GAL4BD-CNA1	Jiang and Cyert 1999

(OD$_{600}$ = 0.8–1.0) and plated onto selective agar plates. Cells were subjected to UV mutagenesis to ~50% killing in a UV Stratalinker (Stratagene). β-Galactosidase filter lifts were performed and blue colonies were selected as described (Stathopoulos and Cyert 1997). There were 40 mutants identified from 128,000 colonies screened. The mutants were tested for dominance or recessiveness by mating to a *cnb1Δ* strain and checking *CDRE::lacZ* reporter activation in the diploid. MAT**a** and MATα mutants were crossed to each other to place them into complementation groups. The majority of mutants did not fall into groups, but one complementation group of five members was defined. Mutants were then tested for specificity by selecting those that failed to activate a mutant version of the CDRE reporter. In addition, a requirement for *CRZ1* was established by deleting *CRZ1* in the mutant strains and assaying *CDRE::lacZ* reporter activation. Mutants were cloned by complementation using the YPH1 genomic library (ATCC #77162); complementing plasmids were rescued, and the ends of the insert were sequenced. The resulting sequence was compared to the genome using BLAST to determine the ORFs contained on the plasmids. The sequence shared by both complementing plasmids contained 4 ORFs on Chromosome IV: YDR334w, *MSN5*, YDR336w, and *MRPS28*. Allelic analysis with an *msn5Δ* strain confirmed the mutation was in *MSN5*.

Immunoblot analysis

Yeast cultures were grown to log phase at 21°C, harvested, and the cell pellets were frozen. FK520 (Merck), in 90% ethanol, 10% Tween-20, was added to 2 µg/mL where indicated. Protein extracts were made in Buffer 88 (20 mM HEPES at pH 6.8, 150 mM KOAc, 250 mM Sorbitol, 2 mM MgOAc, 2 mM DTT, protease inhibitors) using glass bead lysis as described above. Samples (10 µg of total protein in wild-type and *cnb1Δ* strains, or 50 µg of total protein in *msn5Δ* strains) were resolved on a 7% reducing gel. Where indicated, extracts were treated with 200 units of λ phosphatase (NEB) at 30° for 30 min. HA–Crz1p was detected using monoclonal anti-HA 12CA5 antiserum (Roche Molecular Biochemicals) and anti-mouse IgG-coupled HRP secondary antibody (Amersham). GFP–Crz1p was detected using a monoclonal GFP antibody (Covance) and anti-mouse IgG coupled HRP secondary antibody (Amersham). Immunoblots were developed using ECL (Amersham).

Fluorescence microscopy

Living cells expressing green fluorescent protein (GFP) were visualized as described (Stathopoulos-Gerontides et al. 1999) but using an Eclipse E600 microscope (Nikon) with fluorescence optics and an HB100 mercury lamp. Fluorescein filter sets (Chroma) were used to visualize GFP, and digital images were captured with a CCD 4742-95 camera (Hammamatsu) and QED software (QED Imaging). Cells were treated with 10 µg/mL cycloheximide for 20 min to inhibit protein synthesis where noted. Also, CaCl$_2$ was added to 200 mM, and FK506 (Fugisawa) in 90% ethanol, 10% Tween-20 was added to 5 µg/mL as indicated.

Two-hybrid analysis

For the Crz1p/Msn5p interaction, strains containing the two-hybrid constructs to be tested were spotted onto selective agar media using fivefold serial dilutions beginning at an OD_{600} of 0.05. Plates were incubated at 21°C for 5 d. The interaction was scored by assessing growth on media lacking histidine. For the Crz1p/calcineurin interaction, yeast were spotted onto selective agar media containing 100 mM $CaCl_2$. Fivefold serial dilutions beginning at an OD_{600} of 1.0 were used, and plates were incubated at 30°C for 2–3 d.

Acknowledgments

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