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Review

Molecular genetic analysis of the calcineurin signaling pathways

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Abstract. Calcineurin is a Ca^{2+}- and calmodulin-regulated protein phosphatase that is important in Ca^{2+}-mediated signal transduction. Recent application of the powerful techniques of molecular genetics has demonstrated that calcineurin is involved in the regulation of critical biological processes such as T cell activation, muscle hypertrophy, memory development, glucan synthesis, ion homeostasis, and cell cycle control. Notably, specific transcription factors have been shown to play a key role in regulating these functions, and their calcineurin-mediated dephosphorylation and nuclear translocation appear to be a central event in the signal transduction pathways. This review focuses on recent progress in these areas and discusses the evidence for cross-talk between calcineurin and other signaling pathways.

Key words. Calcineurin; protein phosphatase; cross-talk; transcription factor; MAP kinase.

Introduction

Ca^{2+}/calmodulin-dependent protein phosphatase, calcineurin, corresponds to protein phosphatase type 2B, one of four principal types of serine/threonine-specific protein phosphatases present in mammalian tissues [1]. Calcineurin is a target of the immunosuppressant drug-receptor complexes, cyclosporin A (CsA)-cyclophilin and tacrolimus (FK506)-FKBP [2]. It was originally discovered as a neural tissue-enriched calmodulin-binding protein [3–5]. The phosphatase is a heterodimer of the catalytic (calcineurin A) and regulatory (calcineurin B) subunits [3, 4] (fig. 1). Molecular cloning studies by our laboratory and others have identified three distinct genes encoding the α, β and γ isoforms of calcineurin A in mammalian tissues [6–8]. Transcripts of the α and β isoforms were ubiquitously detected in different mammalian tissues, with very prominent expression in neural tissues [6, 7]. Each isoform has a distinct regional distribution in the brain, strengthening the suggestion that the α and β isoforms serve different roles in neuronal signaling [9, 10]. In contrast, the γ isoform is specifically expressed in the testis. Similarly, there are two mammalian isoforms of calcineurin B, the broadly distributed α and the testis-specific β isoforms [11, 12].

Calcineurin is conserved from yeast to human. There are two genes encoding calcineurin A [13, 14] and one for calcineurin B [10, 15] in budding yeast. Functional homologues of calcineurin A have been reported in fission yeast and three other fungal microorganisms [16–19]. These genetically amenable eukaryotic microorganisms have allowed an efficient structural and

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functional analysis of calcineurin signaling. Due to the evolutionary conservation, potential insights into calcineurin signaling in these model systems can be transferred to higher eukaryotes. Recently, genetically engineered mice have become an invaluable biological tool for better understanding of physiological and pathological processes in many fields of biomedical research. The transgenic and gene-targeting technologies allow researchers to carry out specific genetic manipulation in all cells of a laboratory animal, and make it possible to dissect gene function in a living organism. These animals have also shed much light on the basic mechanisms of calcineurin function.

Notably, in both yeast and mammals, specific transcription factors have been shown to play a key role in the calcineurin signaling pathway. Their calcineurin-mediated dephosphorylation and nuclear translocation appear to be a central event in signal transduction, suggesting an evolutionarily conserved mechanism by which calcineurin regulates gene expression in response to Ca^{2+}-mobilizing stimuli. This review focuses on the recent progress in these molecular genetic studies of calcineurin and discusses the evidence for cross-talk with other signaling pathways. Biochemical characteristics and regulation of calcineurin have been extensively reviewed elsewhere [20, 21] and will not be discussed in great detail here.

Calcineurin function in mammals

In mammalian cell signaling, calcineurin is involved in the regulation of critical biological processes such as T cell activation, muscle function, and memory development.

T cell activation

The immunosuppressants CsA and FK506 have been widely used to prevent and treat graft rejection after human organ and tissue transplantations. One of the pathways they suppress is the T cell receptor (TCR)-activated signal transduction pathway. Antigen recognition by the TCR causes an increase in intracellular Ca^{2+}, which activates calmodulin and calcineurin B to bind to Ca^{2+}. Activated calmodulin binding to calcineurin leads to a conformational change which allows the autoinhibitory domain of calcineurin A to move away from the catalytic active site of calcineurin, and

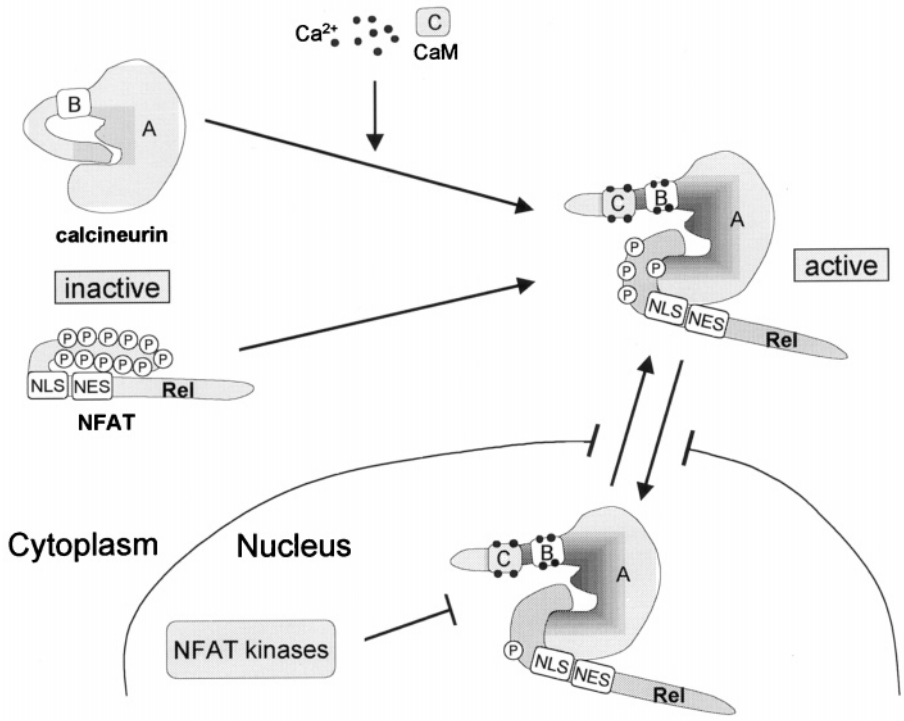

Figure 1. Calcineurin activation leads to dephosphorylation of NFAT transcription factor, formation of calcineurin/NFAT complex, and nuclear translocation of the complex. A, catalytic (A) subunit of calcineurin; B, regulatory (B) subunit of calcineurin; C, calmodulin; P, phosphorylation; NES, nuclear export signal; NLS, nuclear localization signal.

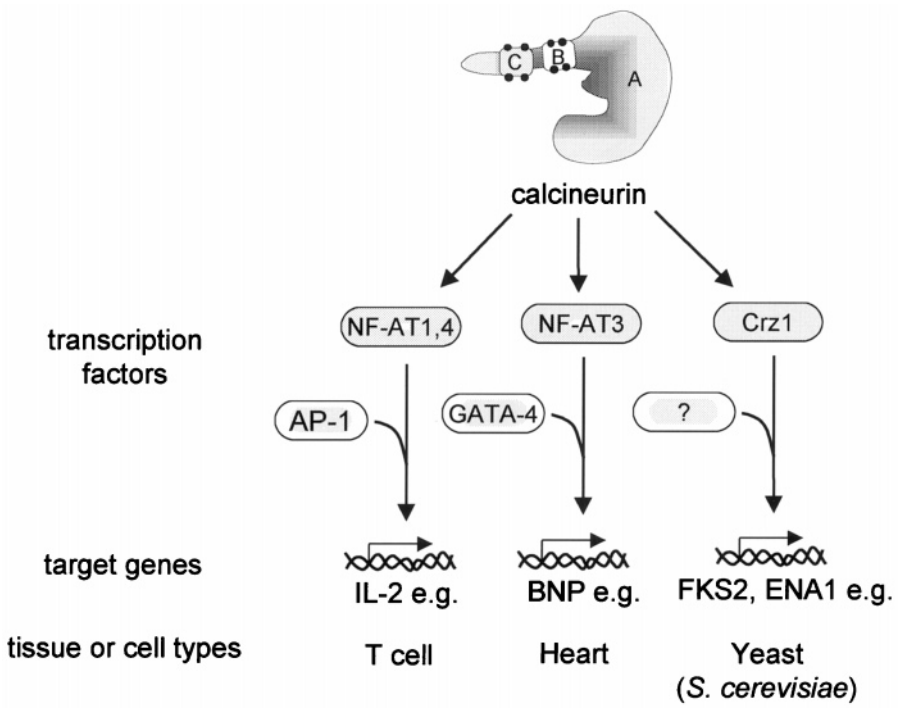

Figure 2. Evolutionarily conserved calcineurin signaling pathway mediated by various transcription factors.

calcineurin is activated. Calcineurin dephosphorylates members of the nuclear factor of activated T cells (NF-AT) family of transcription factors [22, 23]. NF-AT transcription factors contain the Rel homology domain that is sufficient for DNA recognition and cooperative binding interactions with other transcription factor such as activator protein-1 (AP-1) (fig. 2). In resting cells, NF-AT proteins are phosphorylated and retained in the cytoplasm. Activated calcineurin dephosphorylates conserved serine residues in the amino terminus of NF-AT proteins which, after removal of phosphate, are translocated into the nucleus to serve as subunits of transcription factor complexes (fig. 1). T cell activation leads to enhanced transcription of the T cell gene encoding interleukin (IL)-2. The immunosuppressants CsA and FK506 readily diffuse into the cell cytoplasm. CsA is bound to a cytoplasmic receptor protein, cyclophilin, while FK506 is bound to FKBP. The complexes bind to inhibit calcineurin, thereby preventing the dephosphorylation, nuclear translocation, and activation of NF-AT which is required for IL-2 gene expression and T cell activation, hence, the suppression of the TCR-activated signal transduction pathway by CsA and FK506 (fig. 3).

It should be noted that the inhibition of calcineurin pathways by these immunosuppressants is seen in all organisms tested, including budding and fission yeasts. Along with these drugs, the truncated calcineurin is a powerful tool in molecular genetic experiments. The truncated calcineurin which is used in many studies is calmodulin independent, has full enzymatic activity, and is active at intracellular Ca^{2+} levels found in resting cells [20, 21].

In addition to the dephosphorylation of NF-AT, calcium signaling induces an association between NF-AT and calcineurin. These molecules are transported as a complex to the nucleus, where calcineurin continues to dephosphorylate NF-AT [24]. The nuclear complex of NF-AT and calcineurin may maintain calcium signaling by counteracting nuclear NF-AT kinases (fig. 1).

There are several candidates for the NF-AT kinase. The c-Jun amino-terminal kinase (JNK) has been reported to phosphorylate NF-AT4 on two sites. Mutational removal of the JNK phosphorylation sites caused constitutive nuclear localization of NF-AT4. In contrast, JNK activation in calcineurin-stimulated cells caused nuclear exclusion of NF-AT4. These findings indicate that the nuclear accumulation of NF-AT4 promoted by calcineurin is opposed by the JNK signal transduction pathway [25]. Casein kinase Iα (CKIα) directly binds and phosphorylates NF-AT4, resulting in inhibition of NF-AT4 nuclear translocation. Mitogen-activated

protein kinase/extracellular signal-regulated kinase kinase 1 (MEKK1) indirectly suppresses NF-AT4 nuclear import by stabilizing the interaction between NF-AT4 and CKIα. CKIα thus acts to establish an intramolecular masking of the nuclear location signal on NF-AT4, while MEKK1 augments this mechanism [26]. Glycogen synthase kinase-3 (GSK-3) is also suggested to be involved in the phosphorylation and translocation of NF-AT [27]. Thus, nuclear import of the NF-AT transcription factors during T cell activation requires calcineurin, which unmasks nuclear location signals on NF-AT. In addition, calcineurin masks nuclear export signals on NF-AT targeted by the exportin protein Crm1 by a non-catalytic mechanism, which is also required for NF-AT activation [28].

Muscle hypertrophy

Cardiac hypertrophy is an increase in the mass of the heart. It is a major risk factor for the development of myocardial infarction and congestive heart failure, diseases that afflict millions of patients worldwide. In response to numerous pathologic stimuli or growth factors, such as brain natriuretic peptide (BNP), the myocardium undergoes a hypertrophic response characterized by increased myocardial cell size and activation of fetal cardiac genes. Molkentin et al. [29] showed that cardiac hypertrophy is induced by calcineurin, which dephosphorylates the transcription factor NF-AT3, enabling it to translocate to the nucleus. NF-AT3 interacts with the cardiac zinc finger transcription factor GATA-4, resulting in synergistic activation of cardiac transcription (fig. 2). Transgenic mice that express constitutively active forms of calcineurin or NF-AT3 in the heart develop cardiac hypertrophy and heart failure that mimic human heart disease. The immunosuppressants blocked this hypertrophy in vivo and in vitro. Furthermore, administration of the immunosuppressants prevented disease in mice that were genetically predisposed to develop hypertrophic cardiomyopathy as a result of aberrant expression of tropomodulin, myosin light chain-2, or fetal β-tropomyosin in the heart. CsA had a similar effect in a rat model of pressure-overload hypertrophy [30]. These results suggest the presence of a novel hypertrophic signaling pathway in myocardium that is very similar to that of the T cell activation pathway described above, and suggest pharmacologic approaches to prevent cardiac hypertrophy and heart failure. However, the therapeutic efficacy of immunosuppressants for pressure-overload left ventricular hypertrophy is still controversial. Several studies showed that pressure overload induces severe hypertrophy in mice or rats treated with CsA or FK506 and suggested that pressure-overload hypertrophy can arise through calcineurin-independent pathways [31–33].

The molecular pathways underlying the hypertrophic response of skeletal muscle are similar to those responsible for cardiac hypertrophy [34–36]. Treatment with insulin-like growth factor (IGF)-1 mobilizes intracellular calcium, activates calcineurin, and induces the nuclear translocation of the transcription factor NF-AT2 (NFATc1). Expression of activated calcineurin mimics the effects of IGF-1, whereas expression of a dominant-negative calcineurin mutant or addition of immunosuppresants represses myocyte differentiation and hypertrophy. Either IGF-1 or activated calcineurin induces expression of the transcription factor GATA-2, which accumulates in a subset of myocyte nuclei, where it associates with calcineurin and a dephosphorylated NF-AT2 [34].

Very recently, calcineurin-dependent gene regulation in skeletal myocytes was shown to be mediated also by

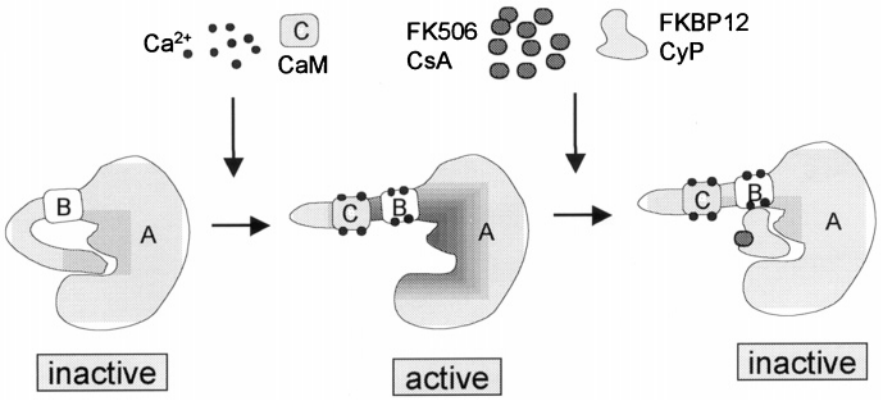

Figure 3. Inhibition of calcineurin by immunosuppressant/immunophilin complex. CyP, cyclophilin.

MEF2 transcription factors. In skeletal muscles of transgenic mice, both NF-AT and MEF2 binding sites are necessary for properly regulated function of a slow fiber-specific enhancer, and either forced expression of activated calcineurin or motor nerve stimulation upregulates an MEF2-dependent reporter gene [37].

Synaptic plasticity and memory development

The role of calcineurin in memory development was investigated using transgenic mice overexpressing a truncated form of calcineurin under the control of the calmodulin-dependent protein kinase IIα promoter. Mice expressing this transgene show increased calcineurin activity in the hippocampus. Physiological studies of these mice and parallel pharmacological experiments in wild-type mice revealed that induced overexpression impairs both an intermediate form of long-term potentiation (LTP) in the hippocampus and the storage of spatial memory [38]. These mice have normal short-term memory but defective long-term memory evident in both a spatial and a visual recognition task. The study provided genetic evidence for the role of the rodent hippocampus calcineurin in spatial and non-spatial memory. The defect in long-term memory could be fully rescued by increasing the number of training trials, suggesting the mice had the capacity for long-term memory. These results suggest that calcineurin negatively regulates memory formation and has a role in the transition from short- to long-term memory, which correlates with a novel intermediate phase of LTP [39].

Mice lacking the predominant calcineurin isoform in the central nervous system, A$\alpha -/-$, have also been used to investigate the role of calcineurin in synaptic plasticity. In the knockout mouse, depotentiation was abolished completely, whereas neither long-term depression (LTD) nor LTP were affected. These studies provide genetic evidence that the Aα isoform of calcineurin is important for the reversal of LTP in the hippocampus and indicate that depotentiation and LTD operate through somewhat different molecular mechanisms [40].

Calcium entry through L-type voltage-gated calcium channels activates calcineurin and induces nuclear translocation of the transcription factor NF-AT3 (NF-ATc4) in hippocampal neurons. GSK-3 can phosphorylate NF-AT3, promoting its export from the nucleus and antagonizing NF-AT3-dependent transcription. Interestingly, induction of inositol 1,4,5-trisphosphate receptor type 1 is controlled by the calcineurin/NF-AT3 pathway. Thus, NF-AT-mediated gene expression may be involved in the induction of hippocampal synaptic plasticity and memory formation [41].

Calcineurin function in eukaryotic microorganisms

Calcineurin, which regulates cellular processes in mammalian cells, is also involved in the regulation of glucan synthesis, ion homeostasis, and cell cycle control in eukaryotic organisms.

Regulation of glucan synthesis

In budding yeast, the *fks1* mutant shows hypersensitivity to the immunosuppressants FK506 and CsA [42]. It also exhibits a slow-growth phenotype that can be partially alleviated by exogenously added Ca^{2+}. *FKS1* encodes a transmembrane catalytic subunit of 1,3-β-D-glucan synthase, which is responsible for synthesizing 1,3-β-glucan chains, the major structural polymer of the *Saccharomyces cerevisiae* cell wall. Genomic disruption experiments indicate that *FKS1* encodes a non-essential function. *FKS1* disrupted cells exhibit the same growth and recessive drug-hypersensitive phenotypes observed in the original *fks1* mutants. Simultaneous disruption of the two genes (*CNA1* and *CNA2*) encoding the alternative forms of the catalytic A subunit of calcineurin, or of the gene (*CNB1*) encoding the regulatory B subunit, is lethal in an *fks1* mutant [43]. These data suggest that Fks1 provides a unique cellular function which, when absent, increases FK506 and CsA sensitivity by making the calcineurin function essential. *FKS2*, a homologue of *FKS1*, has been cloned and Fks1 and Fks2 were shown to be alternative catalytic subunits of the glucan synthase complex [44]. Transcription of *FKS1* is regulated in the cell cycle and predominates during growth on glucose, while *FKS2* is expressed in the absence of glucose. *FKS2* is essential for sporulation, a process which occurs during nutritional starvation. *FKS2* is induced by the addition of Ca^{2+} to the growth medium, and this induction is completely dependent on calcineurin. Thus, the sensitivity of *FKS1* mutants to immunosuppressants can be explained by the calcineurin-dependent transcription of *FKS2*. A 24-bp region of the *FKS2* promoter was defined as sufficient to confer calcineurin-dependent transcriptional induction on a minimal promoter in response to Ca^{2+} and was named CDRE (for calcineurin-dependent response element). The product of *CRZ1/TCN1* was identified as an activator of CDRE-driven transcription [45]. Crz1 contains zinc finger motifs and binds specifically to the CDRE [46]. Genetic analysis revealed that *CRZ1* disrupted cells exhibit several phenotypes similar to those of calcineurin mutants, and that overexpression of *CRZ1* in calcineurin mutants suppressed these phenotypes. Moreover, the calcineurin-dependent transcriptional induction of *FKS2* in response to Ca^{2+}, α factor, and Na^{+} was found to require *CRZ1*. Calcineurin dephosphorylates Crz1 resulting in the translocation of

Crz1 to the nucleus. A region of Crz1 required for calcineurin-dependent regulation has significant sequence similarity to a portion of NF-AT, a family of mammalian transcription factors whose localization is also regulated by calcineurin as described above [47]. These results suggest an evolutionarily conserved mechanism by which calcineurin regulates gene expression in response to Ca^{2+}-mobilizing stimuli (fig. 2).

Regulation of ion homeostasis

In budding yeast, calcineurin is dispensable for growth under normal conditions; however, calcineurin deletion causes growth inhibition under certain stress circumstances. The growth of calcineurin disrupted cells was inhibited by NaCl and LiCl, but not by KCl, $CaCl_2$, $MgCl_2$, or non-specific osmotic stresses. Upon shift to high-NaCl medium, intracellular Na^+ levels of both wild-type yeast and the mutants initially increased at a comparable rate. However, internal Na^+ in wild type cells started to decline more rapidly than in the mutant cells during cultivation in high-NaCl medium, indicating that calcineurin is important in maintaining an Na^+ gradient across the membrane. In the presence of FK506, the growth behavior and intracellular Na^+ of wild-type cells in high-NaCl medium became very similar to those of the calcineurin-deficient mutant in a manner dependent on the presence of the FK506 binding protein [48]. Cation efflux in *S. cerevisiae* is mainly mediated by the P-type ATPase encoded by the *ENA1/PMR2* gene, a putative plasma membrane Na^+ pump whose expression is salt induced. Calcineurin mediates high salt-induced expression of the *ENA1* gene [49]. Crz1/Tcn1 was identified as a transcription factor in yeast required for the calcineurin-dependent induction of *ENA1*, as well as *PMC1*, *PMR1*, and *FKS2*, which confer tolerance to high Na^+, Ca^{2+}, Mn^{2+}, and cell wall damage, respectively [45, 46]. Interestingly, transcription of *ENA1* and *PMC1* was activated by only a subset of the treatments that activated *FKS2* transcription. Thus, in response to multiple signals, calcineurin may act through the Crz1 transcription factor to differentially regulate the expression of several target genes in yeast. Crz1 was not required for other calcineurin-dependent processes, such as inhibition of a vacuolar H^+/Ca^{2+} exchanger and inhibition of a pheromone-stimulated Ca^{2+} uptake system, suggesting that Crz1 functions downstream of calcineurin on a branch of the calcium signaling pathway leading to gene expression [46]. Interestingly, calcineurin controls the expression of isoforms of the plasma membrane Ca^{2+} pump [50] and the Na^+/Ca^{2+} exchanger [51] in mammalian neurons, suggesting conserved mechanisms.

In fission yeast, disruption of the *ppb1*$^+$ gene, encoding a catalytic subunit of calcineuirin, resulted in severe sensitivity to NaCl. However, further study revealed that calcineurin deletion confers sensitivity to Cl^- but not to Na^+ [52]. These data suggest distinct mechanisms underlying ion homeostasis in these two yeast species. The precise mechanisms of Cl^- homeostasis in fission yeast and the role of calcineurin need further studies.

Cell cycle control

The onset of mitosis is determined by activation of a complex of the cyclin-dependent protein kinase (Cdc2/Cdc28) and a cyclin protein that is specific to the G2 phase of the cell cycle. In budding yeast, Swe1 tyrosine kinase inhibits Cdc28 by phosphorylating it, and is needed to determine the length of the G2 phase. In the presence of high calcium levels, cells lacking Zds1/Oss1/Hst1, a repressor of Swe1 transcription in the G2 phase, are delayed in entering mitosis. Calcineurin and Mpk1 have been shown to regulate Swel activation at the transcriptional and posttranslational levels, respectively, and both are required for the calcium-induced delay in the G2 phase [53]. These results suggest that calcineurin is involved in control of the onset of mitosis by regulating Swel.

The budding yeast mating pheromones, a and α factors, bind to specific G protein-coupled receptors in haploid cells and bring about both growth arrest in the early G1 phase of the cell cycle and differentiation into cells capable of mating. This induces an increase in Ca^{2+} influx leading to elevated intracellular calcium concentrations, which have been shown to be essential for subsequent downstream events and the mating process itself. As was observed for *MAT*a *CNA1 CNA2* double mutants, *MAT*a *CNB1* mutants were defective in their ability to recover from α factor-induced growth arrest [10, 14]. In addition, calcineurin mutants lose viability when incubated with mating pheromone, and overproduction of constitutively active calcineurin improves the viability of wild-type cells exposed to pheromone in Ca^{2+}-deficient medium [54]. Thus, one essential consequence of the pheromone-induced rise in cytosolic Ca^{2+} is activation of calcineurin, which is also mediated by Crz1 [47].

The gene encoding the homologue of the catalytic subunit of the Ca^{2+}/calmodulin-regulated protein phosphatase 2B (calcineurin A) has been isolated from *Aspergillus nidulans*. This gene, *cnaA*$^+$, is essential for viability in this fungal system. Analysis of growth-arrested cells following gene disruption by homologous recombination reveals that they are blocked early in the cell cycle. The *cnaA*$^+$ gene encodes a 2.5-kb mRNA which varies in a cell cycle-dependent manner with maximal levels found early in G1 and considerably before the G1/S boundary. As calmodulin is also essen-

tial for *A. nidulans* cell cycle progression and levels rise before the G1/S boundary, this suggests that calcineurin may represent a primary target for calmodulin at this cell cycle transition point.

In contrast, fission yeast calcineurin, *ppb1*$^+$, mRNA levels vary slightly during the cell cycle with maximum levels observed coincident with each S phase [55]. In addition, expression of *ppb1*$^+$ mRNA is induced by nitrogen starvation, a condition that favors mating in *S. pombe*. The *ppb1*$^+$ gene promoter contains a *cis*-acting element for the Ste11 transcription factor, and induction of the *ppb1*$^+$ mRNA during nitrogen starvation was shown to be dependent on the *ste11*$^+$ gene product. Since disruption of the *ppb1*$^+$ gene in *S. pombe* results in sterility, the *ppb1*$^+$ gene seems to play a role in the gene expression cascade that is essential for mating and sporulation in *S. pombe*.

These studies suggest the involvement of calcineurin at multiple points in cell cycle regulation, but details of its mechanism of action are unknown and the general role of calcineurin in cell cycle control remains unclarified.

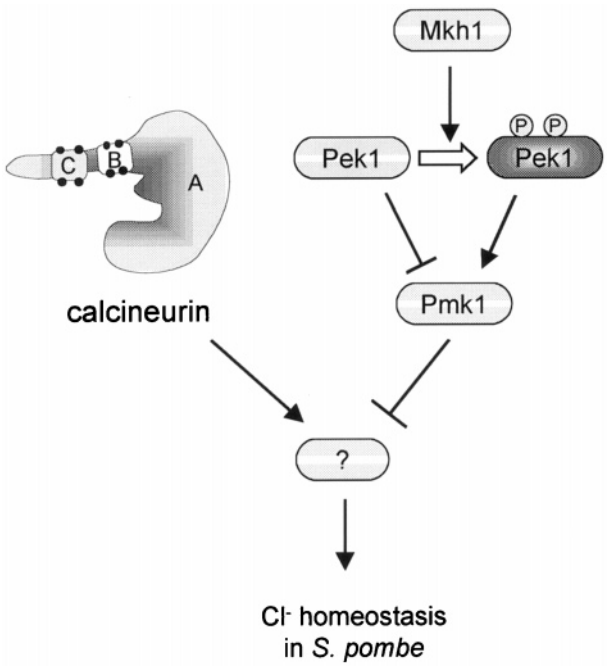

Figure 4. Ppb1 calcineurin signaling and Pmk1 MAP kinase signaling pathways play counteractive roles in the regulation of chloride ion homeostasis in fission yeast.

Cross-talk with the MAP kinase and other signaling pathways

Disruption of the *ppb1*$^+$ gene of fission yeast, encoding a catalytic A subunit of calcineurin, results in greatly delayed cytokinesis and a large number of multi-septated cells at low temperature. Cell polarity control is also impaired, causing branched cells [16]. In addition to these phenotypes, we discovered that calcineurin deletion confers hypersensitivity to Cl^- [52]. Interestingly, wild-type cells display similar defects in septation, polarity and ion homeostasis when they were treated with FK506 or CsA [16, 52]. To isolate genes that function in the calcineurin-mediated pathway, we screened an *S. pombe* genomic library for genes that, when overexpressed, could suppress the Cl^--sensitive growth defect of calcineurin deletion. One of the genes identified was *pmp1*$^+$, which encodes a dual-specificity phosphatase for Pmk1 MAP kinase [52, 56]. The genes for protein kinase C (fission yeast Pck2 or budding yeast Pkc1) and a MAP kinase (fission yeast Pmk1 or budding yeast Mpk1/Slt2) in yeasts seem to be involved in a signaling pathway required to maintain a normal cell wall and cell integrity [56, 57]. Further analysis revealed that inhibition of Pmk1 MAP kinase signaling suppresses effects of the calcineurin deletion. Surprisingly, overexpression of a MAP kinase kinase (MAPKK) for Pmk1, *pek1*$^+$, also rescued the Cl^- sensitivity of Ppb1 calcineurin deletion [58]. This contradiction suggests the possibility that Pek1 MAPKK has an inhibitory, in addition to an activating function. Consistent with this, in vitro biochemical experiments showed that unphosphorylated Pek1 has a potent inhibitory effect on Pmk1 kinase activity. These results suggest that Pek1 MAPKK has dual stimulatory and inhibitory functions, thereby playing a key role as a robust molecular switch in Pmk1 MAPK signaling [58]. Thus, Ppb1 calcineurin signaling and Pmk1 MAP kinase signaling pathways play counteractive roles in the regulation of chloride ion homeostasis in fission yeast. Although the biochemical mechanism accounting for this functional interaction is not known, calcineurin may dephosphorylate a MAPK substrate, possibly a transcription factor, involved in regulation of chloride ion transport (fig. 4).

In contrast to fission yeast, in budding yeast, calcineurin and Mpk1 MAP kinase synergistically regulate Swe1 activation at the transcriptional and posttranslational levels, respectively, and both are required for the calcium-induced delay in the G2 phase [53], suggesting that they share an essential function in budding yeast. Consistent with this notion, loss-of-function mutations in calcineurin and Mpk1 MAP kinase caused a synthetic lethal phenotype [59, 60]. As described above, disruption of *ppb1*$^+$ resulted in severe sensitivity to Cl^- in fission yeast; on the other hand, inhibition of budding yeast calcineurin activity by immunosuppressants or disruption of calcineurin genes resulted in Na^+ sensitivity [48]. This may indicate some difference between

budding and fission yeast that could be pertinent to an understanding of the physiological role of calcineurin. Toda and colleagues isolated a series of fission yeast *sts* mutants that show super-sensitivity to staurosporine, a protein kinase C inhibitor, and showed that they are also sensitive to immunosuppressants [16]. The *ppb1*$^+$ calcineurin gene showed positive genetic interaction with *sts1*$^+$, encoding a polypeptide containing the membrane-spanning domain and resembling the chicken lamin B receptor [61]. Similarly, the double mutant *ppb1-sts5* was lethal, indicating that the *ppb1*$^+$ gene shared an essential function with the gene *sts5*$^+$ [62]. Recently, the Rrp44/Dis3 protein that is related to Sts5 has been shown to have 3′-to-5′ exonuclease activity for RNA and to be a component of exosomes [63]. It would be of great interest to examine whether Ppb1 calcineurin is involved in exosome regulation.

To identify gene products sharing an essential function with calcineurin, we have developed a genetic screen using the immunosuppressive drug FK506 for mutants that depend on calcineurin for growth (*its* mutants for immunosuppressant and temperature sensitive). Characterization of these *its* mutants may identify new components in the calcineurin signaling pathway

Conclusions

Depending upon cell type, calcineurin employs multiple strategies in mediating calcium-dependent signal transduction (table 1, fig. 5). Many of these involve interaction between calcineurin and transcription factors, followed by transcriptional regulation of various functional proteins. In some cases, calcineurin itself forms part of a signaling complex, exemplified by the formation between calcineurin and NF-AT4 in the T cell, which plays a key role in the nuclear shuttling of transcription factor NF-AT4. Transcription factors such as NF-ATs, Elk1 [64], and MEF2 [65] are also localized at the interface between calcineurin and other signal transduction systems and mediate the communication between them. In addition to the transcriptional regulation, calcineurin may modulate cell function by direct dephosphorylation of various functional proteins or by cross-talk with other signal transduction pathways (fig. 5). Very recently, a novel class of conserved calcineurin-binding proteins, such as myocyte-enriched calcineurin-interacting proteins (MCIPs) encoded by *DSCR1*, a gene located in the Down syndrome critical region [66], CBP1 in *Cryptococcus neoformans* [67], and RCN1 in *S. cerevisiae* [67], has been described, although

Table 1. Targets, effects, mediators and mechanisms of calcineurin signaling pathways.

Target/process	Effect	Mediator	Mechanism	References
T-cell activation				
E.g., IL-2, 3, 4, GM-CSF, TNF-α	stimulation	NF-AT	transcriptional activation	22–24
Vesicle trafficking				
Dynamin 1, amphiphysin, synaptojanin	stimulation		direct dephosphorylation	68, 69
Cell growth				
Genes acitivated by MAP kinase signaling	inhibition	Elk1	transcriptional inhibition	64, 70
Apoptosis				
Fas ligand	stimulation	NF-AT	transcriptional activation	71
BAD	stimulation		direct phosphorylation	72
Nur77	stimulation	MEF2	transcriptional activation	65
Bcl2	inhibition	NF-AT	sequestration	73
Neuron depotentiation				40
IP_3 receptor type 1	stimulation	NF-AT3	transcriptional activation	41, 74
Ca^{2+} pump	inhibition		transcriptional inhibition	50
Na^+/Ca^{2+} exchanger	inhibition		transcriptional inhibition	51
Muscle development				
E.g., fetal cardiac genes, etc.	stimulation	NF-AT3	transcriptional activation	29, 30
Skeletal muscle	stimulation	NF-AT2	transcriptional activation	34, 35
	stimulation	MEF2	transcriptional activation	37
Cardiac valve formation	stimulation	NF-AT2	transcriptional activation	75, 76
Budding yeast				
Cell wall synthesis Fks2	stimulation	Crz1/Tcn1	transcriptional activation	45, 46
Ion homeostasis Ena1, Pmc1, Pmr1	stimulation	Crz1/Tcn1	transcriptional activation	45, 46

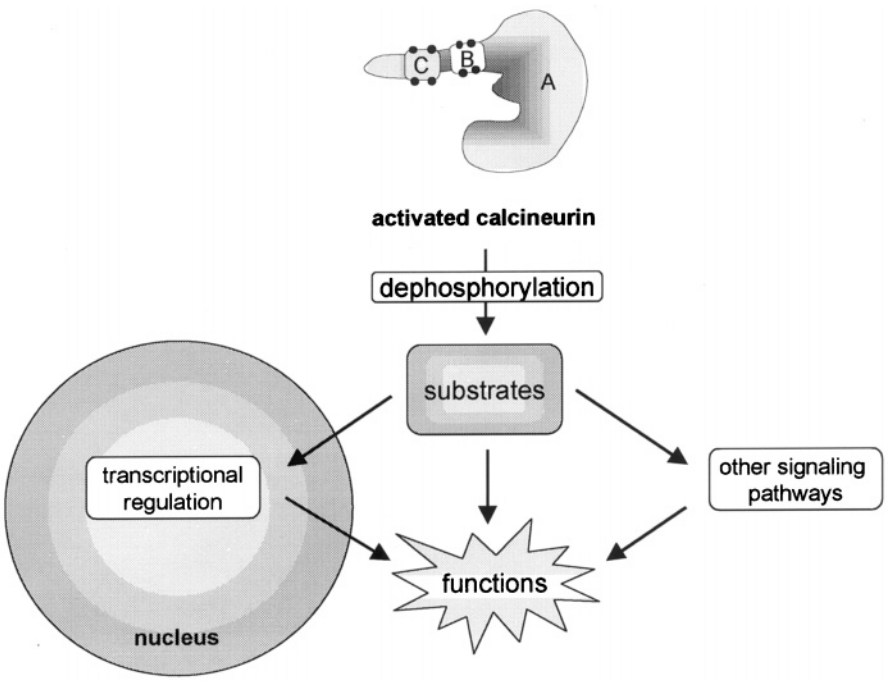

Figure 5. Calcineurin employs multiple strategies in mediating signal transduction.

their functions remain undefined. Thus, calcineurin signaling is part of a complex and interdependent cellular network. Further elucidation of this molecular and cellular communication system and its physiological as well as clinical significance will be of primary interest in the future.

1 Cohen P. (1989) The structure and regulation of protein phosphatases. Annu. Rev. Biochem. **58:** 453–508
2 Liu J., Farmer J. D., Lane W. S., Friedman J., Weissman I. and Schreiber S. L. (1991) Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell **66:** 807–815
3 Klee C. B. and Krinks M. H. (1978) Purification of cyclic 3′,5′-nucleotide phosphodiesterase inhibitory protein by affinity chromatography on activator protein coupled to Sepharose. Biochemistry **17:** 120–126
4 Wang J. H. and Desai R. (1977) Modulator binding protein: bovine brain protein exhibiting the Ca^{2+}-dependent association with the protein modulator of cyclic nucleotide phosphodiesterase. J. Biol. Chem. **252:** 4175–4184
5 Klee C. B., Crouch T. H. and Krinks M. H. (1979) Calcineurin: a calcium- and calmodulin-binding protein of the nervous system. Proc. Natl. Acad. Sci. USA **76:** 6270–6273
6 Ito A., Hashimoto T., Hirai M., Takeda T., Shuntoh H., Kuno T. et al. (1989) The complete primary structure of calcineurin A, a calmodulin binding protein homologous with protein phosphatases 1 and 2A. Biochem. Biophys. Res. Commun. **163:** 1492–1497
7 Kuno T., Takeda T., Hirai M., Ito A., Mukai H. and Tanaka C. (1989) Evidence for a second isoform of the catalytic subunit of calmodulin-dependent protein phosphatase (calcineurin A). Biochem. Biophys. Res. Commun. **165:** 1352–1358
8 Muramatsu T., Giri P. R., Higuchi S. and Kincaid R. L. (1992) Molecular cloning of a calmodulin-dependent phosphatase from murine testis: identification of a developmentally expressed nonneural isoenzyme. Proc. Natl. Acad. Sci. USA **89:** 529–533
9 Takaishi T., Saito N., Kuno T. and Tanaka C. (1991) Differential distribution of the mRNA encoding two isoforms of the catalytic subunit of calcineurin in the rat brain. Biochem. Biophys. Res. Commun. **174:** 393–398
10 Cyert M. S. and Thorner J. (1992) Regulatory subunit (*CNB1* gene product) of yeast Ca^{2+}/calmodulin-dependent phosphoprotein phosphatases is required for adaptation to pheromone. Mol. Cell. Biol. **12:** 3460–3469
11 Chang C. D., Mukai H., Kuno T. and Tanaka C. (1994) cDNA cloning of an alternatively spliced isoform of the regulatory subunit of Ca^{2+}/calmodulin-dependent protein phosphatase (calcineurin Bα2). Biochim. Biophys. Acta **1217:** 174–180
12 Mukai H., Chang C. D., Tanaka H., Ito A., Kuno T. and Tanaka C. (1991) cDNA cloning of a novel testis-specific calcineurin B-like protein. Biochem. Biophys. Res. Commun. **179:** 1325–1330
13 Liu Y., Ishii S., Tokai M., Tsutsumi H., Ohki O., Akada R. et al. (1991) The *Saccharomyces cerevisiae* genes (*CMP1* and *CMP2*) encoding calmodulin-binding proteins homologous to the catalytic subunit of mammalian protein phosphatase 2B. Mol. Gen. Genet. **227:** 52–59
14 Cyert M. S., Kunisawa R., Kaim D. and Thorner J. (1991) Yeast has homologs (*CNA1* and *CNA2* gene products) of mammalian calcineurin, a calmodulin-regulated phosphoprotein phosphatase. Proc. Natl. Acad. Sci. USA **88:** 7376–7380
15 Kuno T., Tanaka H., Mukai H., Chang C. D., Hiraga K., Miyakawa T. et al. (1991) cDNA cloning of a calcineurin B homolog in *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. **180:** 1159–1163

16 Yoshida T., Toda T. and Yanagida M. (1994) A calcineurin-like gene *ppb1*$^+$ in fission yeast: mutant defects in cytokinesis, cell polarity, mating and spindle pole body positioning. J. Cell Sci. **107:** 1725–1735
17 Higuchi S., Tamura J., Giri P. R., Polli J. W. and Kincaid R. L. (1991) Calmodulin-dependent protein phosphatase from *Neurospora crassa*: molecular cloning and expression of recombinant catalytic subunit. J. Biol. Chem. **266:** 18104–18112
18 Rasmussen C., Garen C., Brining S., Kincaid R. L., Means R. L. and Means A. R. (1994) The calmodulin-dependent protein phosphatase catalytic subunit (calcineurin A) is an essential gene in *Aspergillus nidulans*. EMBO J. **13:** 3917–3924
19 Odom A., Muir S., Lim E., Toffaletti D. L., Perfect J. and Heitman J. (1997) Calcineurin is required for virulence of *Cryptococcus neoformans*. EMBO J. **16:** 2576–2589
20 Klee C. B., Ren H. and Wang X. (1998) Regulation of the calmodulin-stimulated protein phosphatase, calcineurin. J. Biol. Chem. **273:** 13367–13370
21 Hemenway C. S. and Heitman J. (1999) Calcineurin: structure, function, and inhibition. Cell Biochem. Biophys. **30:** 115–151
22 O'Keefe S. J., Tamura J., Kincaid R. L., Tocci M. J. and O'Neill E. A. (1992) FK-506- and CsA-sensitive activation of the interleukin-2 promoter by calcineurin. Nature **357:** 692–694
23 Jain J., McCaffrey P. G., Miner Z., Kerppola T. K., Lambert J. N., Verdine G. L. et al. (1993) The T-cell transcription factor NFATp is a substrate for calcineurin and interacts with Fos and Jun. Nature **365:** 352–355
24 Shibasaki F., Price E. R., Milan D. and McKeon F. (1996) Role of kinases and the phosphatase calcineurin in the nuclear shuttling of transcription factor NF-AT4. Nature **382:** 370–373
25 Clipstone N. A. and Crabtree G. R. (1992) Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. Nature **357:** 695–697
26 Zhu J., Shibasaki F., Price R., Guillemot J. C., Yano T., Dotsch V. et al. (1998) Intramolecular masking of nuclear import signal on NF-AT4 by casein kinase I and MEKK1. Cell **93:** 851–861
27 Beals C. R., Sheridan C. M., Turck C. W., Gardner P. and Crabtree G. R. (1997) Nuclear export of NF-ATc enhanced by glycogen synthase kinase-3. Science **275:** 1930–1934
28 Zhu J. and McKeon F. (1999) NF-AT activation requires suppression of Crm1-dependent export by calcineurin. Nature **398:** 256–260
29 Molkentin J. D., Lu J. R., Antos C. L., Markham B., Richardson J., Robbins J. et al. (1998) A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. Cell **93:** 215–228
30 Sussman M. A., Lim H. W., Gude N., Taigen T., Olson E. N., Robbins J. et al. (1998) Prevention of cardiac hypertrophy in mice by calcineurin inhibition. Science **281:** 1690–1693
31 Meguro T., Hong C., Asai K., Takagi G., McKinsey T. A., Olson E. N. et al. (1999) Cyclosporine attenuates pressure-overload hypertrophy in mice while enhancing susceptibility to decompensation and heart failure. Circ. Res. **84:** 735–740
32 Ding B., Price R. L., Borg T. K., Weinberg E. O., Halloran P. F. and Lorell B. H. (1999) Pressure overload induces severe hypertrophy in mice treated with cyclosporine, an inhibitor of calcineurin. Circ. Res. **84:** 729–734
33 Zhang W., Kowal R. C., Rusnak F., Sikkink R. A., Olson E. N. and Victor R. G. (1999) Failure of calcineurin inhibitors to prevent pressure-overload left ventricular hypertrophy in rats. Circ. Res. **84:** 722–728
34 Musaro A., McCullagh K. J., Naya F. J., Olson E. N. and Rosenthal N. (1999) IGF-1 induces skeletal myocyte hypertrophy through calcineurin in association with GATA-2 and NF-ATc1. Nature **400:** 581–585
35 Semsarian C., Wu M. J., Ju Y. K., Marciniec T., Yeoh T., Allen D. G. et al. (1999) Skeletal muscle hypertrophy is mediated by a Ca^{2+}-dependent calcineurin signalling pathway. Nature **400:** 576–581
36 Dunn S. E., Burns J. L. and Michel R. N. (1999) Calcineurin is required for skeletal muscle hypertrophy. J. Biol. Chem. **274:** 21908–21912
37 Wu H., Naya F. J., McKinsey T. A., Mercer B., Shelton J. M., Chin E. R. et al. (2000) MEF2 responds to multiple calcium-regulated signals in the control of skeletal muscle fiber type. EMBO J. **19:** 1963–1973
38 Winder D. G., Mansuy I. M., Osman M., Moallem T. M. and Kandel E. R. (1998) Genetic and pharmacological evidence for a novel, intermediate phase of long-term potentiation suppressed by calcineurin. Cell **92:** 25–37
39 Mansuy I. M., Mayford M., Jacob B., Kandel E. R. and Bach M. E. (1998) Restricted and regulated overexpression reveals calcineurin as a key component in the transition from short-term to long-term memory. Cell **92:** 39–49
40 Zhuo M., Zhang W., Son H., Mansuy I., Sobel R. A., Seidman J. et al. (1999) A selective role of calcineurin Aα in synaptic depotentiation in hippocampus. Proc. Natl. Acad. Sci. USA **96:** 4650–4655
41 Graef I. A., Mermelstein P. G., Stankunas K., Neilson J. R., Deisseroth K., Tsien R. W. et al. (1999) L-type calcium channels and GSK-3 regulate the activity of NF-ATc4 in hippocampal neurons. Nature **401:** 703–708
42 Parent S. A., Nielsen J. B., Morin N., Chrebet G., Ramadan N., Dahl A. M. et al. (1993) Calcineurin-dependent growth of an FK506- and CsA-hypersensitive mutant of *Saccharomyces cerevisiae*. J. Gen. Microbiol. **139:** 2973–2984
43 Douglas C. M., Foor F., Marrinan J. A., Morin N., Nielsen J. B., Dahl A. M. et al. (1994) The *Saccharomyces cerevisiae FKS1* (*ETG1*) gene encodes an integral membrane protein which is a subunit of 1,3-beta-D-glucan synthase. Proc. Natl. Acad. Sci. USA **91:** 12907–12911
44 Mazur P., Morin N., Baginsky W., El-Sherbeini M., Clemas J. A., Nielsen J. B. et al. (1995) Differential expression and function of two homologous subunits of yeast 1,3-beta-D-glucan synthase. Mol. Cell. Biol. **15:** 5671–5681
45 Stathopoulos A. M. and Cyert M. S. (1997) Calcineurin acts through the *CRZ1*/*TCN1*-encoded transcription factor to regulate gene expression in yeast. Genes Dev. **11:** 3432–3444
46 Matheos D. P., Kingsbury T. J., Ahsan U. S. and Cunningham K. W. (1997) Tcn1p/Crz1p, a calcineurin-dependent transcription factor that differentially regulates gene expression in *Saccharomyces cerevisiae*. Genes Dev. **11:** 3445–3458
47 Stathopoulos G. A., Guo J. J. and Cyert M. S. (1999) Yeast calcineurin regulates nuclear localization of the Crz1p transcription factor through dephosphorylation. Genes Dev. **13:** 798–803
48 Nakamura T., Liu Y., Hirata D., Namba H., Harada S., Hirokawa T. et al. (1993) Protein phosphatase type 2B (calcineurin)-mediated, FK506-sensitive regulation of intracellular ions in yeast is an important determinant for adaptation to high salt stress conditions. EMBO J. **12:** 4063–4071
49 Mendoza I., Rubio F., Rodriguez N. A. and Pardo J. M. (1994) The protein phosphatase calcineurin is essential for NaCl tolerance of *Saccharomyces cerevisiae*. J. Biol. Chem. **269:** 8792–8796
50 Guerini D., Garcia-Martin E., Gerber A., Volbracht C., Leist M., Merino C. G. et al. (1999) The expression of plasma membrane Ca^{2+} pump isoforms in cerebellar granule neurins is modulated by Ca^{2+}. J. Biol. Chem. **274:** 1667–1676
51 Li L., Guerini D. and Carafoli E. (2000) Calcineurin controls the transcription of Na^+/Ca^{2+} exchanger isoforms in developing cerebellar neurons. J. Biol. Chem. **275:** 20903–20910
52 Sugiura R., Toda T., Shuntoh H., Yanagida M. and Kuno T. (1998) *pmp1*$^+$, a suppressor of calcineurin deficiency, encodes a novel MAP kinase phosphatase in fission yeast. EMBO J. **17:** 140–148
53 Mizunuma M., Hirata D., Miyahara K., Tsuchiya E. and Miyakawa T. (1998) Role of calcineurin and Mpk1 in regulating the onset of mitosis in budding yeast. Nature **392:** 303–306
54 Withee J. L., Mulholland J., Jeng R. and Cyert M. S. (1997) An essential role of the yeast pheromone-induced Ca^{2+} signal is to activate calcineurin. Mol. Biol. Cell **8:** 263–277

55 Plochocka Z. D., Rasmussen G. and Rasmussen C. (1995) Regulation of calcineurin gene expression in *Schizosaccharomyces pombe*: dependence on the ste11 transcription factor. J. Biol. Chem. **270:** 24794–24799
56 Toda T., Dhut S., Superti F. G., Gotoh Y., Nishida E., Sugiura R. and Kuno T. (1996) The fission yeast *pmk1*$^+$ gene encodes a novel mitogen-activated protein kinase homolog which regulates cell integrity and functions coordinately with the protein kinase C pathway. Mol. Cell. Biol. **16:** 6752–6764
57 Lee K. S., Irie K., Gotoh Y., Watanabe Y., Araki H., Nishida E. et al. (1993) A yeast mitogen-activated protein kinase homolog (Mpk1p) mediates signalling by protein kinase C. Mol. Cell. Biol. **13:** 3067–3075
58 Sugiura R., Toda T., Dhut S., Shuntoh H. and Kuno T. (1999) The MAPK kinase Pek1 acts as a phosphorylation-dependent molecular switch. Nature **399:** 479–483
59 Nakamura T., Ohmoto T., Hirata D., Tsuchiya E. and Miyakawa T. (1996) Genetic evidence for the functional redundancy of the calcineurin- and Mpk1-mediated pathways in the regulation of cellular events important for growth in *Saccharomyces cerevisiae*. Mol. Gen. Genet. **251:** 211–219
60 Garrett E. P., Moilanen B. and Cyert M. S. (1995) Calcineurin, the Ca^{2+}/calmodulin-dependent protein phosphatase, is essential in yeast mutants with cell integrity defects and in mutants that lack a functional vacuolar H^+-ATPase. Mol. Cell Biol. **15:** 4103–4114
61 Shimanuki M., Goebl M., Yanagida M. and Toda T. (1992) Fission yeast *sts1*$^+$ gene encodes a protein similar to the chicken lamin B receptor and is implicated in pleiotropic drug-sensitivity, divalent cation-sensitivity, and osmoregulation. Mol. Biol. Cell **3:** 263–273
62 Toda T., Niwa H., Nemoto T., Dhut S., Eddison M., Matsusaka T. et al. (1996) The fission yeast *sts5*$^+$ gene is required for maintenance of growth polarity and functionally interacts with protein kinase C and an osmosensing MAP-kinase pathway. J. Cell Sci. **109:** 2331–2342
63 Mitchell P., Petfalski E., Shevchenko A., Mann M. and Tollervey D. (1997) The exosome: a conserved eukaryotic RNA processing complex containing multiple $3' \rightarrow 5'$ exoribonucleases. Cell **91:** 457–466
64 Tian J. and Karin M. (1999) Stimulation of Elk1 transcriptional activity by mitogen-activated protein kinases is negatively regulated by protein phosphatase 2B (calcineurin). J. Biol. Chem. **274:** 15173–15180
65 Youn H. D., Sun L., Prywes R. and Liu J. O. (1999) Apoptosis of T cells mediated by Ca^{2+}-induced release of the transcription factor MEF2. Science **286:** 790–793
66 Rothermel B., Vega R. B., Yang J., Wu H., Bassel-Duby R. and Williams R. S. (2000) A protein encoded within the Down syndrome critical region is enriched in striated muscles and inhibits calcineurin signaling. J. Biol. Chem. **275:** 8719–8725
67 Gorlach J., Fox D. S., Cutler N. S., Cox G. M., Perfect J. R. and Heitman J. (2000) Identification and characterization of a highly conserved calcineurin binding protein, CBP1/calcipressin, in *Cryptococcus neoformans*. EMBO J. **19:** 3618–3629
68 Lai M. M., Hong J. J., Ruggiero A. M., Burnett P. E., Slepnev V. I., De Camilli P. et al. (1999) The calcineurin-dynamin 1 complex as a calcium sensor for synaptic vesicle endocytosis. J. Biol. Chem. **274:** 25963–25966
69 Bauerfeind R., Takei K. and De Camilli P. (1997) Amphiphysin I is associated with coated endocytic intermediates and undergoes stimulation-dependent dephosphorylation in nerve terminals. J. Biol. Chem. **272:** 30984–30992
70 Sugimoto T., Stewart S. and Guan K. L. (1997) The calcium/calmodulin-dependent protein phosphatase calcineurin is the major Elk-1 phosphatase. J. Biol. Chem. **272:** 29415–29418
71 Beals C. R., Clipstone N. A., Ho S. N. and Crabtree G. R. (1997) Nuclear localization of NF-ATc by a calcineurin-dependent, cyclosporin-sensitive intramolecular interaction. Genes Dev. **11:** 824–834
72 Wang H. G., Pathan N., Ethell I. M., Krajewski S., Yamaguchi Y., Shibasaki F. et al. (1999) Ca^{2+}-induced apoptosis through calcineurin dephosphorylation of BAD. Science **284:** 339–343
73 Shibasaki F., Kondo E., Akagi T. and McKeon F. (1997) Suppression of signalling through transcription factor NF-AT by interactions between calcineurin and Bcl-2. Nature **386:** 728–731
74 Genazzani A. A., Carafoli E. and Guerini D. (1999) Calcineurin controls inositol 1,4,5-trisphosphate type 1 receptor expression in neurons. Proc. Natl. Acad. Sci. USA **96:** 5797–5801
75 Ranger A. M., Grusby M. J., Hodge M. R., Gravallese E. M., Brousse F. C. de la, Hoey T. et al. (1998) The transcription factor NF-ATc is essential for cardiac valve formation. Nature **392:** 186–190
76 Pompa J. de la, Timmerman L. A., Takimoto H., Yoshida H., Elia A. J., Samper E. et al. (1998) Role of the NF-ATc transcription factor in morphogenesis of cardiac valves and septum. Nature **392:** 182–186