

# Calcineurin-Crz1 Signaling in Lower Eukaryotes

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**Calcium ions are ubiquitous intracellular messengers. An increase in the cytosolic  $\text{Ca}^{2+}$  concentration activates many proteins, including calmodulin and the  $\text{Ca}^{2+}$ /calmodulin-dependent protein phosphatase calcineurin. The phosphatase is conserved from yeast to humans (except in plants), and many target proteins of calcineurin have been identified. The most prominent and best-investigated targets, however, are the transcription factors NFAT (nuclear factor of activated T cells) in mammals and Crz1 (calcineurin-responsive zinc finger 1) in yeast. In recent years, many orthologues of Crz1 have been identified and characterized in various species of fungi, amoebae, and other lower eukaryotes. It has been shown that the functions of calcineurin-Crz1 signaling, ranging from ion homeostasis through cell wall biogenesis to the building of filamentous structures, are conserved in the different organisms. Furthermore, frequency-modulated gene expression through Crz1 has been discovered as a striking new mechanism by which cells can coordinate their response to a signal. In this review, I focus on the latest findings concerning calcineurin-Crz1 signaling in fungi, amoebae and other lower eukaryotes. I discuss the potential of Crz1 and its orthologues as putative drug targets, and I also discuss possible parallels with calcineurin-NFAT signaling in mammals.**

Since its discovery more than 35 years ago, the  $\text{Ca}^{2+}$ /calmodulin-dependent phosphatase calcineurin has been investigated in detail mainly in mammalian cells and in the yeast *Saccharomyces cerevisiae* (reviewed in references 1 and 2). However, in the last decade calcineurin has also been investigated more extensively in filamentous fungi (reviewed in reference 3) as well as in other lower eukaryotes such as the social amoeba *Dictyostelium discoideum* (4, 5), the parasites *Plasmodium falciparum* (6), *Trypanosoma cruzi* (7, 8), *Trypanosoma rangeli* (9), *Schistosoma mansoni* (10), and *Leishmania major* (11), and in *Paramecium tetraurelia* (12). Furthermore, many target proteins of this phosphatase have been identified in different organisms (reviewed in reference 13). The best-investigated substrates, however, are the mammalian nuclear factor of activated T cells (NFAT) and the *S. cerevisiae* zinc finger transcription factor Crz1 (calcineurin-responsive zinc finger 1). In this review, I describe the latest findings concerning the role of Crz1 and its orthologues in yeast, filamentous fungi, and other lower eukaryotes. As Crz1 and its orthologues are involved in developmental aspects as well as in virulence of pathogenic fungi, I raise the question of whether Crz1 can serve as a potential drug target. Recent studies determined different  $\text{Ca}^{2+}$  signatures in a growth phase- and treatment-dependent manner of different organisms, and I discuss here the possibility that Crz1 or other transcription factors can decode these signatures. I also show that Crz1 signaling in lower eukaryotes has features in common with NFAT signaling in mammals, ranging from mechanistic parallels such as the nuclear localization pattern up to developmental aspects and cellular adaptation.

## THE CALCINEURIN-Crz1 SIGNALING CASCADE

Crz1 was first identified as a calcineurin target in the yeast *S. cerevisiae* (14). Since then, Crz1 orthologues have been identified in various lower eukaryotes, and different names have been assigned to the orthologues, such as Prz1 in *Schizosaccharomyces pombe* (15), CrzA in *Aspergillus fumigatus* and *Aspergillus nidulans* (16–18), and TacA in *D. discoideum* (19). Crz1 and all of its orthologues harbor C2H2 zinc finger DNA binding motifs. The number of zinc fingers can range from one to four (see below).

In different organisms, the calcineurin-Crz1 signaling cascade

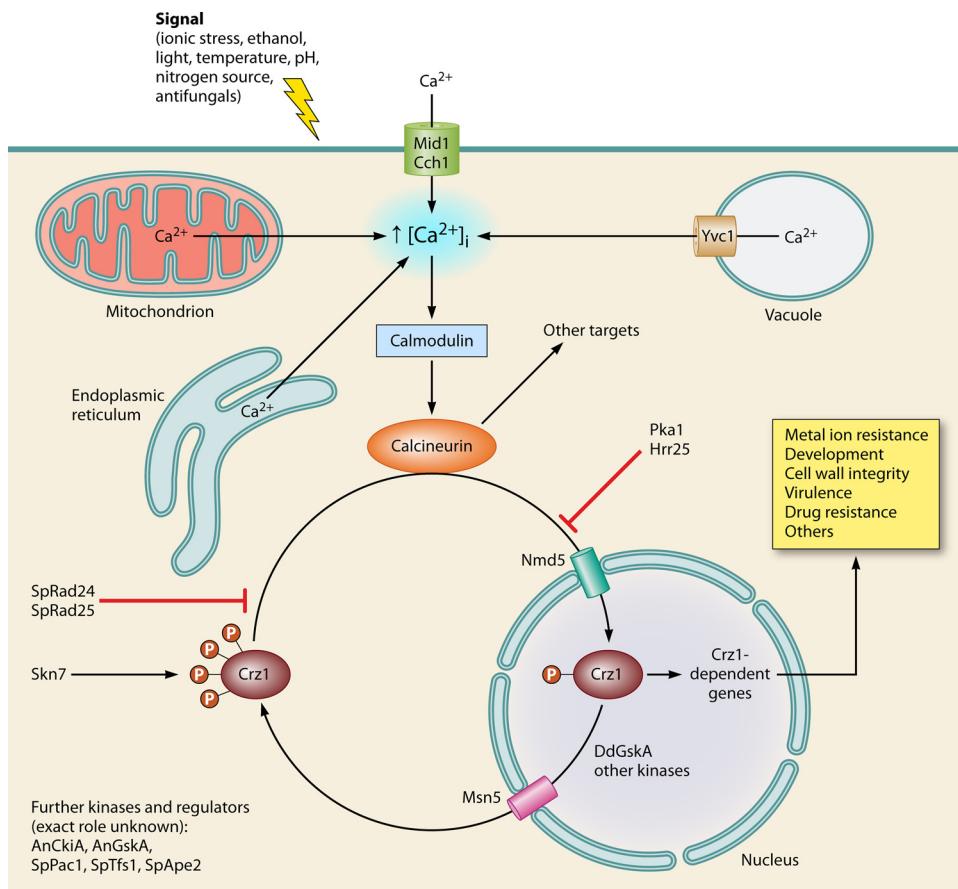
can be activated by different external stimuli (Fig. 1). Among those are cations such as  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Li}^+$ ,  $\text{K}^+$ , and  $\text{Na}^+$  (16–29), ethanol (30), caffeine (31), temperature (32), pH (33), blue light (34), the nature of the nitrogen source (35), and anti-fungal drugs (36). All of these signals increase the intracellular  $\text{Ca}^{2+}$  concentration. In *S. cerevisiae*, the  $\text{Ca}^{2+}$  ions can enter the cytoplasm either from the external environment through the plasma membrane  $\text{Ca}^{2+}$  channel complex Mid1/Cch1 or from intracellular calcium stores such as the endoplasmic reticulum, the vacuole via the cation channel Yvc1, or the mitochondria. The increase of the intracellular  $\text{Ca}^{2+}$  concentration leads to the activation of calmodulin, which in turn can activate calcineurin, which dephosphorylates its target proteins such as Crz1 (reviewed in references 1 and 2). The dephosphorylated transcription factor Crz1 enters the nucleus via the importin Nmd5 (37) and can bind to its target promoters. After phosphorylation, the transcription factor is exported from the nucleus via the exportin Msn5 (38, 39). It has recently been shown in *S. cerevisiae* that Crz1 alternates between the cytosol and the nucleus and that for the expression of downstream targets of Crz1 the frequency of nuclear localization is more important than the total abundance of the protein in the nucleus (40). After external calcium addition, the residence time of *S. cerevisiae* Crz1 in the nucleus is usually about 2 min and it has been shown that the calcium concentration controls the frequency, but not the duration, of nuclear localization of Crz1 (40). Such frequency-modulated gene expression has been discovered as a striking new mechanism by which cells can coordinate their response to a signal. However, induction of the calcineurin pathway by blue light leads to permanent nuclear localization of Crz1 after 10 min (34). This behavior resembles the nuclear localization of NFAT in mammals, where NFAT1 moves into the nucleus 3 to 9 min after stimulation with ionomycin and calcium without any

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**FIG 1** The calcineurin-Crz1 signaling pathway. When the cytosolic  $\text{Ca}^{2+}$  concentration increases, calmodulin activates calcineurin, which in turn dephosphorylates Crz1. Crz1 is then imported into the nucleus and induces or represses expression of its target genes. After phosphorylation (P), Crz1 is exported from the nucleus. Several regulators of this pathway have been identified. If not otherwise indicated, all denoted proteins were identified in *S. cerevisiae*. Sp = *Schizosaccharomyces pombe*; An = *Aspergillus nidulans*; Dd = *Dictyostelium discoideum*. For further explanation, see the main text.

oscillation (41). Nevertheless, the initial response of the yeast cells after Crz1 activation is proportional to the strength of the external signal ( $\text{Ca}^{2+}$  concentration and light intensity, respectively) (34, 40). In filamentous fungi, it has been shown that the cytoplasmic calcium concentration oscillates in a species-, growth phase-, and treatment-dependent manner (42, 43). Cell type-specific cytosolic calcium oscillations have also been observed in mammalian cells and during differentiation of *D. discoideum* (44, 45). Given these findings, one can speculate that different signals lead to different calcium signatures in *S. cerevisiae* as they do in filamentous fungi. This might then lead to different nuclear localization patterns of Crz1. However, this possibility will need to be addressed in future experiments.

A recent study aimed to identify regulators of *S. pombe* Prz1 (39). The authors found that the two 14-3-3 proteins Rad24 and Rad25 bind to phosphorylation sites of Prz1, thereby inhibiting their dephosphorylation by calcineurin. Other regulators identified in this study were *S. pombe* protein kinase A (Pka1), which, in contrast to the *S. cerevisiae* Pka1, does not phosphorylate Prz1 directly (39, 46), the exportin Msn5 (see above), and three novel regulators, the RNase Pac1, the predicted aminopeptidase Ape2, and the transcription elongation factor Tfs1. However, the role of these new regulators is not yet clear (39). A further regulator of Crz1 identified in *S. cerevisiae* is the eukaryotic response regulator

Skn7. It has been proposed that Skn7 protects Crz1 from degradation by binding to it and to calcineurin (47).

Several kinases that rephosphorylate mammalian NFAT have been identified. Among them are casein kinase I (48), glycogen synthase kinase 3 (GSK3) (49), dual-specificity tyrosine phosphorylation-regulated kinase (DYRK) (50), and the mitogen-activated protein (MAP) kinases p38 (51) and Jun N-terminal protein kinase (JNK) (52). In contrast, the kinases that phosphorylate Crz1 preceding its nuclear export have not been identified. It is known that the casein kinase I homologues Hrr25 and Pka1 phosphorylate Crz1 in *S. cerevisiae* (46, 53), but it is assumed that phosphorylation by Hrr25 and Pka1 inhibits the entry of the protein into the nucleus. That homologues of casein kinase I are involved in Crz1 phosphorylation without affecting the nucleocytoplasmic transport has also been shown recently for the interaction between the *A. nidulans* casein kinase I homologue CkiA and CrzA (54). In *S. pombe*, homologues of kinases that are known to phosphorylate mammalian NFAT were investigated in terms of phosphorylation of Prz1. Overexpression of the kinases could not rescue the Prz1 overexpression defects, suggesting that other kinases are involved or that phosphorylation of Prz1 requires multiple kinases (39). On the other hand, it has been shown in the social amoeba *Dictyostelium discoideum* that the GSK3 orthologue GskA is at least partially involved in the phosphorylation

TABLE 1 Number and functional categories of identified Crz1-dependent genes in different lower eukaryotes<sup>a</sup>

Organism	No. of Crz1-dependent genes	Functional categories of Crz1-dependent genes	Method(s) used for identification	Reference
<i>S. cerevisiae</i>	116	<b>Cell wall biogenesis, ion homeostasis, lipid and sterol metabolism, vesicle transport, degradative enzymes, signaling, glucose metabolism, others</b>	Microarray RT-PCR	55 33
<i>C. albicans</i>	65	<b>Cell wall biogenesis, ion homeostasis, stress response, signaling, others</b>	Microarray	22
<i>C. glabrata</i>	87	<b>Cell wall biogenesis, heat shock proteins, calcineurin regulation, others</b>	Microarray	32
<i>C. neoformans</i>	208	<b>Cell wall metabolism, signal transduction, protein metabolism, lipid metabolism, stress response, membrane transporters, carbohydrate metabolism, others</b>	Microarray	60
<i>B. cinerea</i>	45	<b>Cell wall organization, ion transport, protein degradation, carbohydrate metabolism, secondary metabolism, others</b>	Macroarray	23
<i>A. nidulans</i>	25	Transcription, <b>calcium metabolism</b> , membrane transport, others	Microarray	24
<i>A. fumigatus</i>	>400	<b>Ion transport, vesicle trafficking, secondary metabolism, lipid metabolism, amino acid metabolism, calcineurin, others</b>	Microarray qRT-PCR	61 18
<i>M. grisea</i>	140	<b>Cell wall related, signaling, ion transport, vesicle trafficking, degradative enzymes, lipid and sterol biosynthesis, others</b>	ChIP-chip, microarray	116
<i>D. discoideum</i>	5	Development, <b>calcium transport, others</b>	qRT-PCR	19

<sup>a</sup> Functional categories are specified according to the references cited. Common categories identified in three or more different organisms are shown in bold. RT-PCR, reverse transcription-PCR; qRT-PCR, quantitative reverse transcription-PCR.

preceding the nuclear export of the *D. discoideum* Crz1 orthologue TacA (19). In contrast, GskA of *A. nidulans* has been shown to be necessary for nuclear import of CrzA at alkaline pH but not under Ca<sup>2+</sup> stress conditions (54). These data show that the exact mechanism by which Crz1 and its orthologues are exported from the nucleus is not fully understood.

In the nucleus, Crz1 binds to 24-bp regions in target gene promoters named CDREs (calcineurin-dependent response elements) (14). The core consensus site for Crz1 binding was shown to be GNNGC(G/T)CA (55). For mammalian NFAT, it is known that diverse modes of DNA binding exist (reviewed in reference 56). NFAT can bind DNA as a monomer at cognate GGAA sites as well as as a homodimer at κB-like response elements. Furthermore, NFAT can form strong cooperative complexes with the basic region-leucine zipper (bZIP) transcription factor AP-1 (Fos-Jun dimers) and other transcription factors. Such complexes function as signal integrators; one of those signals must be Ca<sup>2+</sup>/calcineurin, while the second can be, e.g., a developmental signal (57). Accordingly, it has been shown in stimulated T cells that (at least) two distinct classes of NFAT target genes exist: one class controlled by NFAT with AP-1 and the other by NFAT without AP-1 (58).

Whether Crz1 binds as a monomer or as a (homo-/hetero-)dimer has not been investigated. The protein sequences of Crz1 and of most of its orthologues give no hint of a possible dimerization. However, the Crz1 orthologues of the social amoebae *Polyphondylium pallidum* and *Dictyostelium fasciculatum* contain putative LisH motifs, which might mediate dimerization (see below and reference 59). Studies are needed to see if those proteins really form dimers.

### GENES REGULATED BY Crz1

Several studies used genome-wide expression analyses to investigate which genes are regulated by Crz1 and its orthologues (Table 1). In 2002, Yoshimoto et al. identified 116 genes in *S. cerevisiae* whose regulation is Crz1 dependent (55). Promoter analyses revealed that the majority of the Crz1-dependent genes contain one to six copies of CDRE motifs in their promoter regions, indicating that these genes are likely to be direct transcriptional targets of

Crz1 (see above and reference 55). In other yeasts such as *Candida albicans* (22) and *Candida glabrata* (32), 65 and 87 Crz1-dependent genes, respectively, have been identified. In *C. albicans*, potential CDREs were also detected in the promoter regions of Crz1-dependent genes (22). In filamentous fungi, the expression profiles of Crz1/CrzA deletion strains have been analyzed under calcium stress conditions too. In the gray-mold fungus *Botrytis cinerea*, 45 Crz1-dependent genes were identified using a macroarray (23). Transcriptional profiling of a Δcrz1 strain in *Cryptococcus neoformans* under conditions of nutrient limitation revealed 208 downregulated genes compared to the wild type (60). In *Aspergillus nidulans*, 25 genes were induced under calcium stress conditions in a ΔcrzA strain (24), and in *A. fumigatus*, Soriani et al. identified between 400 and 3,200 genes (depending on the length of the Ca<sup>2+</sup> pulse) whose expression is CrzA dependent (61). In both *Aspergillus* studies, putative CDREs were identified in the promoter regions of CrzA-dependent genes. However, in contrast to the results seen with other fungi, CrzA seems not to regulate genes involved in cell wall biosynthesis in *A. nidulans* and *A. fumigatus*, although in other studies the ΔcrzA mutants were found to have cell wall defects (16, 18, 24). Interestingly, it has been shown that calcineurin regulates the transcription of chitin synthases in response to caspofungin treatment in *A. fumigatus* (62). However, CrzA seems to play only a minor role in this process: in the transcriptional profiles, only chitin synthase A (*chsA*) was misregulated in response to Ca<sup>2+</sup> treatment (61). Additionally, Cramer et al. showed that calcineurin but not CrzA is involved in the regulation of β-1,3-glucan biosynthesis genes in *A. fumigatus* (18). These data show that the imbalance between cell wall defects and regulation of cell wall biogenesis genes in ΔcrzA mutants needs further clarification.

A very elegant study to identify Crz1-dependent genes has been done in the rice blast fungus *Magnaporthe oryzae* (63). The authors used a combined approach of chromatin immunoprecipitation with microarray technology (ChIP-chip) and microarray analyses. From the ChIP-chip analysis, 346 putative Crz1 binding partners could be identified. In the corresponding microarray analysis, 140 genes were found to be Crz1 dependent and these were all also identified in the ChIP-chip assay (63). Furthermore,

the ChIP-chip and microarray analyses identified two main binding motifs of *M. oryzae* Crz1 [CAC(A/T)GCC and TTGNTTG], neither of which resembles the classical CDRE of *S. cerevisiae*. Also, in the promoter regions of TacA-dependent genes in *D. discoideum*, no classical CDREs could be identified (unpublished results). This indicates that different organisms use different Crz1 binding motifs.

Taken together, these expression analyses show that Crz1 regulates between ~30 and ~200 genes in the different species (the data for *A. fumigatus* are so far an exception). Common functional categories of Crz1-dependent genes range from signaling (including different transcription factors) through ion homeostasis, degradative enzymes, and cell wall biosynthesis to various genes involved in nutrient utilization of substrates. However, species-specific genes regulated by Crz1 have been identified in each study so far (Table 1). Crz1 can thereby act as an inducer (55) or repressor (19) of gene expression. It is noteworthy that not all Crz1-dependent genes have to be calcineurin dependent. It has been shown in *C. glabrata* that 38% of Crz1-dependent genes were calcineurin independent, indicating that proteins other than calcineurin might regulate Crz1 (32, 64). This has also been observed in other organisms such as *C. neoformans* (64).

### PHENOTYPIC CONSEQUENCES OF Crz1 DYSFUNCTION

As can be assumed from the variety of genes that are regulated by Crz1, the phenotypes of  $\Delta crz1$  mutants are quite diverse. The first phenotypes of  $\Delta crz1$  mutants were described in *S. cerevisiae* (reviewed in reference 2). Besides defects in cell cycle control,  $\Delta crz1$  mutants have defects mainly under conditions of  $\text{Ca}^{2+}$ - and  $\text{Na}^+$  stress in *S. cerevisiae*. Additional defects with respect to other cations such as  $\text{Mn}^{2+}$ ,  $\text{K}^+$ , and  $\text{Li}^+$  have also been observed to various extents in other yeasts such as *Torulaspora delbrueckii* (21), *S. pombe* (15), *C. albicans* (20, 22, 29), *Candida dubliniensis* (65), and *Candida lusitaniae* (66) and in filamentous fungi such as *A. nidulans* (17, 24), *A. fumigatus* (16), *Aspergillus parasiticus* (25), *Penicillium digitatum* (67), *Magnaporthe grisea* (26), and *M. oryzae* (27). In the social amoeba *D. discoideum*,  $\text{Na}^+$  sensitivity could be observed for the TacA-RNAi (TacA-RNA interference) mutant (19). Interestingly, for *T. delbrueckii* and *D. discoideum*, perturbation of Crz1 signaling leads to increased resistance to  $\text{Li}^+$  (19, 21). Together with the misregulation of genes involved in ion homeostasis (Table 1), these observations show that Crz1 is involved in resistance and sensitivity to different metal ions in a species-specific manner.

Other common effects of Crz1 perturbation are developmental defects, which preferentially accompany cell wall defects. Such defects (in hyphal morphology, conidiation, and/or the cell wall) have been described for different *Aspergillus* species (16–18, 25), *M. grisea* and *M. oryzae* (26, 27), *B. cinerea* (23), *C. neoformans* (60, 64, 68), and *P. digitatum* (67), as well as for different *Candida* species (20, 22, 28, 29, 32, 65, 66). Such defects are usually reflected in the transcriptional profiles of the different organisms in which there is misregulation of genes involved in cell wall biogenesis in  $\Delta crz1$  mutants (Table 1). Developmental defects can also be observed in fruiting bodies formed by *D. discoideum* TacA-RNAi mutants (19).

Further defects of  $\Delta crz1$  mutants have been found during growth at alkaline pH (16, 17) and at elevated temperature (16, 32), as well as in resistance to ethanol (30),  $\text{H}_2\text{O}_2$  (67), and caffeine (31). It has been shown in *C. neoformans* that Crz1 is involved in

survival under conditions of limited aeration and biofilm formation (68). Last but not least, Crz1 is also involved in the production of secondary metabolites such as aflatoxin in *A. parasiticus* (25).

In summary, Crz1 and its orthologues share involvement in metal ion resistance, development, and cell wall integrity (see also Fig. 1). However, the kind of metal ion to which Crz1 orthologues respond seems to be species specific. Further species-specific responses are evident in differences of stress stimuli and in the production of secondary metabolites. The species specificity can be explained if we assume that, although the  $\text{Ca}^{2+}$  signaling pathway is well conserved for executing organism-specific cellular and developmental responses to specific ecological niches, it has probably undergone evolutionary fine-tuning (43). The unique  $\text{Ca}^{2+}$  signatures of different species support this hypothesis (43).

### CALCINEURIN-Crz1 SIGNALING AND VIRULENCE

Studying pathogenic fungi (plant and human pathogens), it was determined that  $\Delta crz1$  mutants have reduced virulence (16, 18, 22, 23, 26–28, 32, 65–67). First investigations concerning Crz1 and virulence were conducted in the human-pathogenic fungus *C. albicans* (22, 69). It has been shown in virulence assays that Crz1 has a moderate effect on virulence whereas calcineurin is essential in a mouse model of infection (22). However, this discrepancy in the phenotypical outcome of mutations of calcineurin and its downstream targets has also been observed frequently in other studies (5, 14, 31, 70) and shows that calcineurin has other targets apart from Crz1. Crz1 is also involved in virulence in other *Candida* species. In the emerging fungal pathogen *C. lusitaniae*, calcineurin-Crz1 signaling controls virulence in a murine systemic infection model (66). Furthermore, the role for Crz1 in virulence has been investigated in *C. glabrata*, showing that calcineurin-Crz1 signaling controls virulence in a murine systemic infection model (28, 32). However, Chen et al. (32) showed that the requirement of calcineurin-Crz1 signaling for virulence depends on the specific host niche. Besides systemic infection, Crz1 is also important for murine ocular infection but does not control virulence in a murine urinary tract infection model (32). A host-niche-specific requirement for the calcineurin signaling pathway has also been shown for *C. albicans* calcineurin mutants (71). Last but not least, a niche-specific requirement for this particular signaling pathway has been shown for *C. dubliniensis* (65). Whereas in *C. dubliniensis* calcineurin is needed to establish a murine systemic and ocular infection, Crz1 is needed “only” for the systemic infection of mice. Interestingly, the authors of that study also found that  $\Delta crz1$  mutants show an aberrant thigmotrophic response that is calcineurin independent (65). This has also been shown for thigmotropism in *C. albicans* (72). In addition to its role in different *Candida* species, the involvement of Crz1 in virulence has been addressed in the opportunistic human pathogen *A. fumigatus* (16, 18) and in *C. neoformans* (60). The authors showed that the *A. fumigatus* Crz1 orthologue CrzA is necessary in a murine pulmonary infection model in a manner similar to that seen with calcineurin (16, 18, 73), whereas the *C. neoformans*  $\Delta crz1$  mutant exhibited attenuated virulence during systemic infection of mice (60).

The involvement of the calcineurin-Crz1 signaling pathway has also been investigated in plant-pathogenic fungi. Although Crz1 is necessary for full virulence in all tested fungi, the exact mechanism responsible for the involvement of this transcription factor in virulence is controversial. In the gray-mold fungus *Bot-*

*rytis cinerea*, Crz1 seems to be almost dispensable for the conidium-derived infection program on leaves or fruits whereas the transcription factor is essential for the penetration of hyphae into host tissue (23). In contrast, in *Penicillium digitatum*, the causal agent of green mold on postharvested citrus fruits,  $\Delta crz1$  mutants show reduced virulence after inoculation of fruits with conidial suspensions (67). Even more controversial is the role for Crz1 in the two closely related rice blast fungi *Magnaporthe grisea* and *M. oryzae* (26, 27). Both reports showed that  $\Delta crz1$  mutants have reduced virulence after inoculation of host plants with conidia. However, interpretations of the reason for the reduced virulence differ. Whereas Choi et al. showed that a failure of osmostability within the appressoria of *M. oryzae* might be the reason for the reduced virulence of  $\Delta crz1$  mutants (27), Zhang et al. indeed observed abnormal appressoria but with no difference in turgor pressure compared to the wild type (26).

These examples show that the exact mechanisms by which calcineurin-Crz1 signaling affects virulence differ between species and have still to be elucidated. The requirement of Crz1 and its orthologues for specific host niches seems to be common to all fungi, whereas calcineurin mutants usually have more-general defects in virulence. This raises the issue of how different host niches are sensed and whether different  $\text{Ca}^{2+}$  signatures are generated to activate Crz1 in a niche-specific manner.

### Crz1 AS A DRUG TARGET?

Many studies have investigated the role of Crz1 and its orthologues in resistance to antifungal agents. Although most of the studies were conducted in *C. albicans*, the antifungal agents tested in these studies are restricted to fluconazole, miconazole, and terbinafine, all interfering with ergosterol biosynthesis in fungi (20, 22, 29, 69, 74). Except for the study of Reedy et al. (29)  $\Delta crz1$  strains of *C. albicans* were found to be moderately hypersensitive to fluconazole and miconazole compared to the wild type. However, no difference could be detected when the cells were treated with terbinafine. In contrast, *S. cerevisiae*  $\Delta crz1$  strains are hypersensitive to fluconazole, miconazole, and terbinafine (36, 75). Hypersensitivity to fluconazole was also observed in *C. dubliniensis* (65). In that study, cell wall-perturbing agents such as the echinocandins caspofungin, micafungin, and anidulafungin were also tested; moderate hypersensitivity of  $\Delta crz1$  strains could be detected only for micafungin. The most comprehensive studies concerning Crz1 mutation and antifungal resistance were conducted in *C. glabrata* and *C. lusitaniae* (28, 32, 66). In contrast to *C. albicans* and *C. dubliniensis*,  $\Delta crz1$  strains were more resistant to azoles. It can be concluded that Crz1 negatively regulates genes responsible for membrane biogenesis in *C. glabrata* and *C. lusitaniae*. On the other hand,  $\Delta crz1$  strains of *C. glabrata* and *C. lusitaniae* are more susceptible than or show no difference from wild-type strains concerning echinocandins. At least for *C. glabrata*, these findings correlate well with the postulated role for Crz1 in cell wall biogenesis as identified also by gene expression profiling (32). Resistance to fluconazole has additionally been observed in a  $\Delta crz1$  strain of *C. neoformans* (68). In that study, it was shown that Crz1 is also involved in biofilm formation of *C. neoformans*. As increased resistance to antifungal drugs usually accompanies biofilm formation (76), this observation (decreased biofilm formation with increased susceptibility toward azoles) is of particular interest.

Data for filamentous fungi are more rare. Three studies showed

that  $\Delta crzA$  strains in *A. fumigatus* are hypersensitive to caspofungin and nikkomycin Z (16, 18, 62), and it has been shown for the plant pathogen *P. digitatum* that  $\Delta crz1$  strains are hypersensitive to treatment with the membrane-perturbing agents imazalil and difenoconazole (67). Interestingly, it has been shown in *A. fumigatus* that the “paradoxical effect” (attenuation of the antifungal activity of caspofungin at elevated concentrations) is dependent on calcineurin and CrzA (62).

The above-mentioned studies raise the issue of whether Crz1 could be a target for the development of new antifungal drugs. However, most of the studies showed that, compared to calcineurin, Crz1 is involved only partially in drug resistance (22, 28, 29, 32, 65, 66, 69) or is even a negative regulator of drug resistance (28, 32). Furthermore, the protein structures of Crz1 and its orthologues in other organisms are quite diverse except for the zinc finger binding domains (see below), making it difficult to find specific drugs targeting Crz1 without affecting zinc finger-containing proteins of the host. But as Crz1 and its orthologues are restricted to lower eukaryotes and cannot be found in higher eukaryotes (see below), it might be worth investigating this class of transcription factors in more detail to find structures that are common in those proteins and that could serve as potential drug targets. Such a strategy was successfully followed in investigating calcineurin of *A. fumigatus* (77). Calcineurin of *A. fumigatus* harbors a novel serine-proline-rich region that is conserved, unique to filamentous fungi, and completely absent in human calcineurin. This offers the potential to design a specific drug targeting this region (77).

It is further noteworthy that the calcineurin-Crz1 signal transduction pathway is (at least partially) involved in the evolution of drug resistance. It has been shown that calcineurin is a client protein of the highly conserved molecular chaperone heat shock protein 90 (Hsp90) (78, 79) and that Hsp90 potentiates the evolution of drug resistance in different fungi through calcineurin (80). Continuing studies revealed that Crz1 partially modulates the Hsp90-calcineurin-driven evolution of azole resistance in *S. cerevisiae* (75) and echinocandin resistance in *C. albicans* (81). This shows again that Crz1 and its orthologues might be promising drug target candidates if combined with other drugs that inhibit Hsp90 and/or calcineurin to increase the sensitivity to clinically important antifungal drugs (82, 83).

### CROSS TALK WITH OTHER SIGNALING PATHWAYS

Soon after the discovery of Crz1, it became evident that the calcineurin-Crz1 signaling pathway cross talks with other signaling pathways. The best-investigated pathways concerning a cross talk with calcineurin are the alkaline stress response pathway, the high osmolarity glycerol (HOG) pathway, and the cell wall integrity (CWI) pathway. It has been shown for the alkaline stress response pathway in *S. cerevisiae* and *C. albicans* that alkaline pH activates the Rim101 signal transduction pathway. In parallel, alkaline pH leads to an increase in the cytosolic  $\text{Ca}^{2+}$  concentration, activating the calcineurin-Crz1 signaling pathway (84–86). It was shown in those three studies that the Rim101 transcription factor regulates genes together with Crz1. However, both transcription factors also regulate specific genes under alkaline pH conditions (84, 85). In contrast, data concerning a possible cross talk between calcineurin-Crz1 and alkaline pH signaling (PacC signaling) in filamentous fungi are rare. It is known that CrzA of *A. nidulans* can be activated by alkaline pH (54). However, so far there are no data

indicating that calcineurin-Crz1 can act in parallel to the *Aspergillus* PacC signaling pathway. Rather, it has been shown in *Aspergillus giganteus* that the alkaline pH-induced upregulation of the *afp* gene encoding an antifungal protein is mediated not by PacC but by calcineurin (87).

The HOG pathway is one of five mitogen-activated protein kinase (MAPK) pathways in *S. cerevisiae* (88). Two studies evaluated the possible interaction between the HOG pathway and calcineurin-Crz1 in *S. cerevisiae* (89, 90). Direct activation of the calcineurin-Crz1 pathway in parallel to the HOG pathway has been observed (90), and it has been shown that calcineurin-Crz1 antagonizes the HOG pathway by downregulating it (89). However, the exact mechanism of the downregulation of the HOG pathway by calcineurin-Crz1 is not known.

The CWI pathway is the second of the five MAPK pathways in *S. cerevisiae*. It was shown in recent years that this pathway is highly complex and interacts with several other stress response pathways (reviewed in references 91 and 92). One cross talk is with the calcineurin-Crz1 pathway. It has been shown that Crz1 induces in parallel to other CWI pathways the *fks2* gene encoding a stress-induced beta-1,3-glucan synthase subunit (93, 94). Additionally, calcineurin-Crz1 induces other cell wall-related genes in different organisms (see above). It can therefore be concluded that Crz1 plays a major role, in addition to the CWI pathway, in maintaining cell wall integrity.

A putative cross talk between calcineurin-Crz1 and signaling pathways mediated via G proteins has been investigated only sparingly in lower eukaryotes. It is known that G proteins can activate phospholipase C (PLC), which in turn can generate inositol-1,4,5-trisphosphate (IP<sub>3</sub>), leading to calcium release from the endoplasmatic reticulum. Activation of calcineurin by an increase of cytosolic Ca<sup>2+</sup> mediated by G proteins and PLC has been observed in *B. cinerea* (95). In contrast, it has been shown for *C. neoformans* that PLC does not activate calcineurin signaling (96).

These data show that cross talk between calcineurin-Crz1 and other signaling pathways is common but has to be investigated further. A starting point for future investigations might be the fact that not all genes whose expression is Crz1 dependent also show calcineurin-dependent expression (32, 64). However, details of other pathways that can activate Crz1 in addition to calcineurin are mainly unknown.

### CALCINEURIN-Crz1 SIGNALING BEYOND THE FUNGAL KINGDOM

As stated above, in addition to the fungal kingdom, calcineurin has been investigated in other lower eukaryotes such as social amoebae (4, 5), various parasites (6–8, 11), and amoeboflagellates (97) and in *Paramecium* (12).

However, a Crz1 orthologue beyond the fungal kingdom has been investigated only in the social amoeba *D. discoideum* (19). On the other hand, orthologues are present in many lower eukaryotes such as *Acanthamoeba*, *Giardia*, *Leishmania*, *Plasmodium*, and others (Fig. 2). The number of zinc fingers in these putative transcription factors can vary between one and four, and the sizes of the protein differ from one organism to another. The zinc finger domains are the most conserved regions of the different proteins. In contrast, the N- and C-terminal parts of the proteins differ in size and sequence. Interestingly, based on the sequence of the zinc finger domains, the Crz1 orthologues of filamentous ascomycetes seem to be the most conserved ones.

This is also reflected in the similar sizes of the different proteins (Fig. 2). The protein sequences of *P. pallidum* and *D. fasciculatum* further contain putative LisH motifs in their N-terminal regions which might mediate dimerization (59). This raises again the issue of whether Crz1 or at least some of its orthologues can form dimers such as NFAT in mammals. Investigating Crz1 orthologues in different organisms in addition to fungi might resolve this issue.

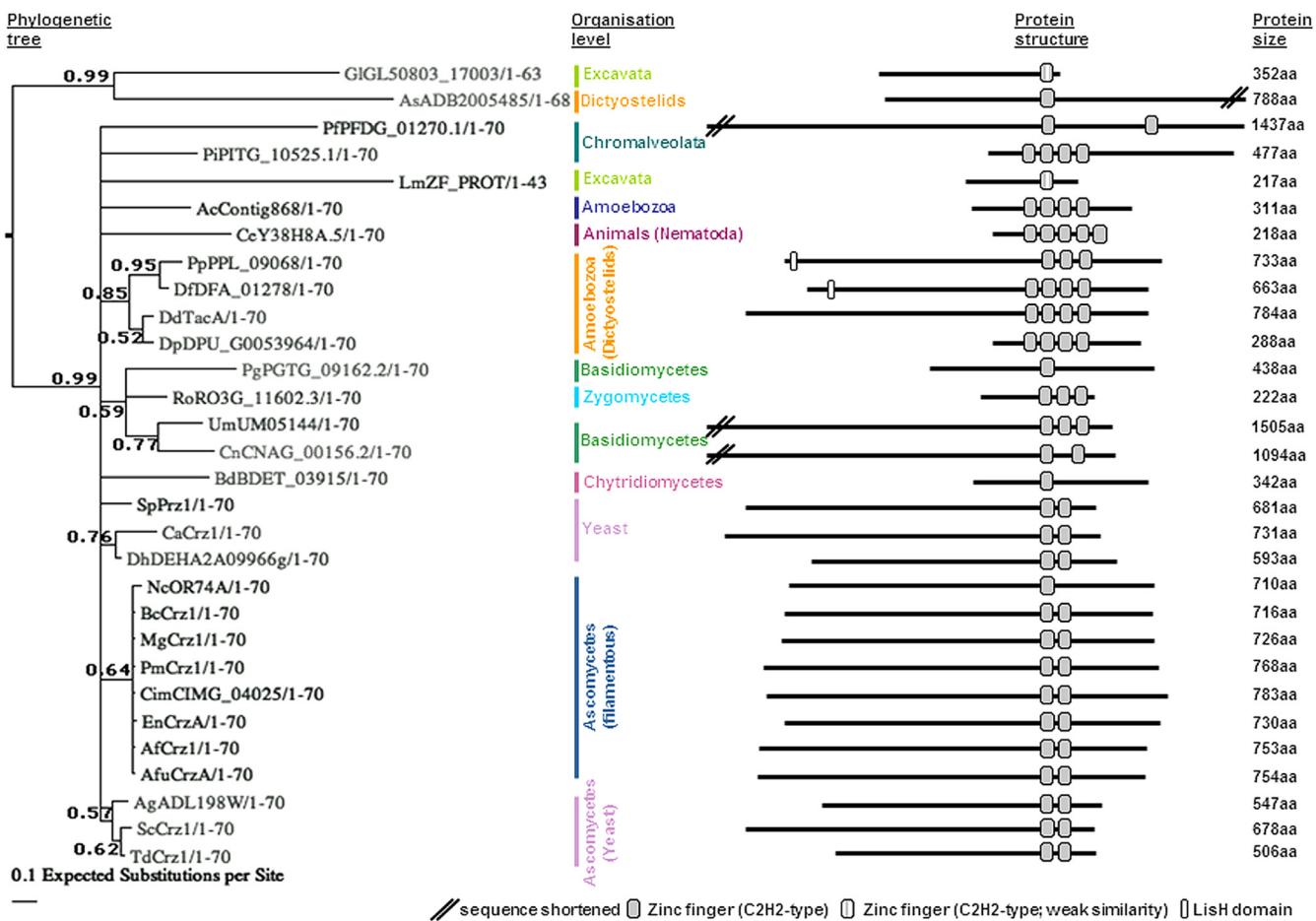
Interestingly, the genome of the nematode *Caenorhabditis elegans* harbors a Crz1 orthologue whereas no NFAT orthologue has been identified. All other animals investigated so far lack Crz1 orthologues in their genomes but have NFAT-like proteins instead (19). It is noteworthy that *C. elegans* seems to be the only organism investigated so far where the Crz1 orthologue has five zinc finger domains (Fig. 2). It is assumed that NFAT family proteins arose to suit the specialized needs of vertebrates (57). However, the finding that NFAT-like proteins are also present in invertebrates such as *Drosophila melanogaster* (98) and that *C. elegans* harbors a Crz1 orthologue instead of a NFAT orthologue raises the issue of when during evolution Crz1 proteins were lost or changed to NFAT-like proteins.

Thus, the role of Crz1 has still to be elucidated in many lower eukaryotes as well as in some fungi. Such studies will complete current knowledge concerning similarities and differences in calcineurin-Crz1 signaling among the lower eukaryotes and in comparison to mammalian NFAT.

### PARALLELS WITH, AND DIFFERENCES FROM, MAMMALIAN CALCINEURIN-NFAT SIGNALING

Shortly after the discovery of Crz1 in *S. cerevisiae* more than 15 years ago, it became evident that this transcription factor shares functional similarity with mammalian transcription factors of the NFAT family, although the two proteins have only limited sequence similarity (2). Since then, as Crz1 and its orthologues have been investigated in many different organisms, this functional similarity has become even more evident (Table 2). The mammalian NFAT family comprises five members (NFAT1 to -5), only four of which are regulated by intracellular Ca<sup>2+</sup> levels (NFAT1 to -4). All NFAT family members have a Rel homology region as a DNA binding domain and are classified as members of the extended NF-κB/Rel family (56). However, only members 1 to 4 possess calcineurin binding motifs in their regulatory domains. Initially, it was shown that NFAT1 to -4 regulate T cell activation and differentiation, but it is now clear that the members of this family of transcription factors also regulate the function of other immune cells such as dendritic cells, B cells, and megakaryocytes (reviewed in reference 99). Additionally, NFAT1 to -4 proteins are involved in many developmental processes, including those of the heart, bones, pancreas, skin, muscles, blood vessels, and neurons (reviewed in reference 99–101). NFAT5, the one member of the family that is not activated by calcineurin, is expressed in nearly all tissues and was found to be important for osmoregulation of cells (102–104).

Parallels between Crz1 and NFAT signaling are mainly in the regulation of “filamentous structures” such as axons, blood vessels, and hyphae. As stated above, perturbation of Crz1/CrzA in fungi leads to abnormal formation of hyphae and/or abnormal conidiation (16–18, 20, 22, 23, 25–29, 32, 64–67). It has been shown in mice that simultaneous mutation of NFAT2 and NFAT3 leads to general defects in blood vessel assembly and that the mice



**FIG 2** Molecular phylogeny of Crz1 orthologues. Using the *S. cerevisiae* Crz1 protein sequence as a query, orthologues were searched in the genomes of the indicated organisms. As a control, the human NFATc1 protein sequence was searched in the genomes but could not be identified in any of the organisms shown. All sequences were aligned using MUSCLE (117, 118) and Gblocks (119) with default settings, leading to a conserved core region of the proteins containing the zinc finger DNA binding domains (70 amino acids [1–70; for As, 1–68, and for Lm, 1–43]). The phylogenetic tree (including bootstrap values) of the aligned sequences on the left side was built using TOPALi v2 software (Bayesian Tree; MrBayes algorithm, 1,000,000 generations, 40% burn in) (120). Wherever possible, protein names are given (Crz1, CrzA, Prz1, TacA). For organisms where the protein has not been investigated so far, the corresponding gene identifiers or contigs containing the putative Crz1 orthologue are indicated. For better visualization, the organization levels of the different organisms are shown in level-specific color coding according to the calculated phylogeny of the Crz1 proteins. The protein structures and protein sizes given on the right side show that the different Crz1 orthologues have high sequence diversity outside the zinc finger domains. Even the number of zinc fingers can range between 1 and 5. aa, amino acids; Ac, *Acanthamoeba castellanii*; Af, *Aspergillus flavus*; Afu, *Aspergillus fumigatus*; Ag, *Ashbya gossypii*; As, *Acystostelium subglobosum*; Bc, *Botrytis cinerea*; Bd, *Batra-chochytrium dendrobatidis*; Ca, *Candida albicans*; Ce, *Caenorhabditis elegans*; Cim, *Coccidioides immitis*; Cn, *Cryptococcus neoformans* var. *grubii*; Dd, *Dictyostelium discoideum*; Df, *Dictyostelium fasciculatum*; Dh, *Debaromyces hansenii*; Dp, *Dictyostelium purpureum*; En, *Emericella nidulans*; Gl, *Giardia lamblia*; Lm, *Leishmania major*; Mg, *Magnaporthe grisea*; Nc, *Neurospora crassa*; Pf, *Plasmidium falciparum*; Pg, *Puccinia graminis* f. sp. *tritici*; Pi, *Phytophthora infestans*; Pm, *Penicillium marneffei*; Pp, *Polysphondylium pallidum*; Ro, *Rhizopus oryzae*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Td, *Torulospora del-brueckii*; Um, *Ustilago maydis*. (Modified from reference 19.)

die during embryogenesis (105). As the two single mutants had no obvious abnormalities, this suggests that the two proteins have redundant functions. The double mutant also had defects in axon outgrowth during embryogenesis in mice and *in vitro* which were even more pronounced in an NFAT2–4 triple mutant (106). Whereas the defects of  $\Delta$ crz1 mutants in hypha formation in fungi can be partially explained by defects in cell wall metabolism (see above), the underlying mechanism(s) of the defects of NFAT mutants in vascular assembly and axon outgrowth is not well understood. For example, most of the genes identified as regulators of vascular development are expressed at normal levels in NFAT3–NFAT4 double mutants (105). Concerning axonal outgrowth, it has been shown that NFAT is required for neurotrophin-dependent outgrowth (106). Recently, it was shown that NFAT3 is a tran-

scriptional repressor of growth-associated protein 43 (GAP-43), a neurotrophin-dependent membrane-bound phosphoprotein presumably promoting actin polymerization (107, 108). This suggests that the observed defects are due to a misregulation of actin polymerization. However, as the single mutations usually have no effect on vascular patterning or axon outgrowth, more research is needed to dissect the specific role(s) of each single NFAT protein. It is not known if the defects of  $\Delta$ crz1 mutants in hypha formation are also connected to defects in microfilament formation.

The similarities in the underlying signaling pathways for the formation of “filamentous structures” are even more pronounced at the level of calcineurin. It was recently shown that impairment of calcineurin function in mice leads to increased branching of dendrites (inhibition of calcineurin [109]) or to a decrease in den-

**TABLE 2** Common features of Crz1 and NFAT signaling in lower eukaryotes and vertebrates.

Feature	Crz1 signaling in lower eukaryotes (reference[s])	NFAT signaling in vertebrates (reference[s])
Development of “filamentous structures”	Hypha formation (18, 22, 23, 26, 27, 65, 66)	Vascular development (105) Axon outgrowth (106, 107)
Cellular adaptation	Response to blue-light (34) Glucose metabolism (23, 33, 60) Ion homeostasis/osmoregulation (14, 17, 19–24, 69)	Response to UV light (111) Glucagon and insulin expression (112, 113) Osmoregulation (102–104)
Nuclear localization	Constant after blue-light treatment (34)	Constant after ionomycin/Ca <sup>2+</sup> treatment (41)
Kinases for rephosphorylation	Glycogen synthase kinase GskA (19)	Glycogen synthase kinase GSK3 (49)

dritic branching (constitutively active calcineurin [110]). At least, the decrease in dendritic branching caused by constitutively active calcineurin is mediated via NFAT4 (110). In filamentous fungi, impairment of calcineurin also leads to either hyperbranching or lack of branching, and in the fruiting body formation of *D. discoideum*, calcineurin-RNAi mutants also show “branching” (ectopic tip formation) of the stalk (reference 5 and references therein).

Further parallels between Crz1 and NFAT signaling exist in the adaptation of cells to different environmental cues (Table 2). *S. cerevisiae* responds to blue light (450 to 490 nm) by activation of Crz1 (34). Similarly, epidermal cell lines and transgenic mice respond to UV radiation (100 to 380 nm) by activating NFAT (111). Both studies also showed that the activation of the transcription factors is calcineurin dependent. However, the detailed mechanism of light induction is not known for Crz1 or for NFAT. Other environmental features affecting Crz1/NFAT activation are extracellular ions and osmotic stress. As stated above, perturbation of Crz1 leads to an altered sensitivity to cations in a species-specific manner in most of the lower eukaryotes, and this is also reflected on the transcriptional level. In vertebrates, it has been shown that NFAT5 regulates the expression of proteins that transport osmolytes into the cell (102–104). Additionally, in invertebrates such as the fruit fly *D. melanogaster*, the NFAT orthologue is involved in salt stress tolerance (98). Since cations can also be osmotically active, one can compare these mechanisms. However, it has to be mentioned that NFAT5 is not activated via Ca<sup>2+</sup>/calcineurin, leaving unaddressed the issue of how Crz1 and NFAT proteins evolved to fulfill similar needs with different protein structures and activation modes.

Finally, Crz1 is responsible for the transcription of several genes involved in glucose metabolism in *S. cerevisiae*, *B. cinerea*, and *C. neoformans*, including genes encoding hexose transporters and enzymes of glycolysis, the citric acid cycle, and alcohol, glycogen, or trehalose metabolism (23, 33, 60). For NFAT2, it has been shown that it activates the expression of glucagon (112). Glucagon is a peptide-hormone responsible for the increase of the blood glucose level. The counterpart of glucagon, insulin (responsible for decrease of the blood glucose level), is also regulated by NFAT (113, 114). Beside these direct effects on glucagon and insulin expression, it became evident in recent years that calcineurin-NFAT signaling in general is crucial for glucose metabolism and for the onset of diseases such as diabetes (reviewed in reference 115). This shows us again that unicellular as well as multicellular organisms seem to have evolved functional similarities using different proteins. Understanding the basic principles underlying the glucose metabolism regulated by Crz1 in lower eukaryotes might therefore also help in the future to better understand human diseases such as diabetes.

Beside the described similarities, many differences between Crz1 and NFAT signaling exist. Apart from obvious species-specific differences such as activation of T cells and other immune cells via NFAT in vertebrates (99) or development of appressoria in pathogenic fungi via Crz1 (26, 27), most of the differences are at the mechanistic level of the signal transduction pathways. First of all, nuclear localization patterns differ between Crz1 and NFAT. Whereas it has been shown in *S. cerevisiae* that after calcium treatment the localization of Crz1 in the nucleus oscillates in a frequency-dependent manner (40), localization of NFAT in the nucleus seems to be stable after activation (41). However, different external cues seem to produce different nuclear localization patterns of Crz1, since blue light also leads to permanent accumulation of this transcription factor in the nucleus of *S. cerevisiae* (34). Other mechanistic differences are found among the kinases, which are needed for rephosphorylation preceding the nuclear export of Crz1 and NFAT, respectively. Whereas for NFAT several kinases such as casein kinase I, GSK3, DYRK, p38, and JNK have been described (48–52), attempts to identify kinases that rephosphorylate Crz1 in yeast and other fungi have failed so far. Only in *D. discoideum* has it been shown that the GSK3 orthologue GskA is at least partially responsible for the rephosphorylation of TacA (19). Therefore, more effort is needed to elucidate the details of Crz1 signaling in lower eukaryotes.

## CONCLUSIONS

Ca<sup>2+</sup>/calcineurin signaling is highly conserved within the eukaryotes from yeast to humans (excluding plants). Major targets of calcineurin in lower eukaryotes are Crz1 and its orthologues, and, although the different organisms have completely different life styles, the function of this transcription factor in relation to calcineurin signaling seems to be basically conserved. Major aspects of Crz1 signaling are cation homeostasis, development of filamentous structures, cell wall integrity, and virulence in pathogenic species. Crz1 signaling also has some similarity to NFAT signaling in mammals. However, investigation of calcineurin-Crz1 signaling has so far been largely restricted to yeasts and filamentous fungi. I anticipate that future studies will elucidate the role of Crz1 in other lower eukaryotes such as parasites. Furthermore, it will be of interest to see if Crz1 could be also a target for drugs against pathogenic fungi and parasites.

Recent studies in filamentous fungi have shown that the intracellular Ca<sup>2+</sup> concentration changes in a pulsatile manner and that each species exhibits a distinct Ca<sup>2+</sup> signature which is also growth dependent (43). Such Ca<sup>2+</sup> oscillations have already been known for a long time in *D. discoideum* (44) and in various cell lines (reviewed in reference 45). For mammalian cells, it has been shown that NFAT (together with other transcription factors) de-

codes such calcium oscillations in a specific frequency range (45). It will therefore be of great interest in the future to see if Crz1 and its orthologues can also decode different frequencies of calcium oscillations.

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