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Calcineurin-Mediated Regulation of Hyphal Growth, Septation and Virulence in *Aspergillus fumigatus*

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Abstract

Calcineurin is a heterodimeric protein phosphatase complex composed of catalytic (CnaA) and regulatory (CnaB) subunits and plays diverse roles in regulating fungal stress responses, morphogenesis, and pathogenesis. Fungal pathogens utilize the calcineurin pathway to survive in the host environment and cause life-threatening infections. The immunosuppressive calcineurin inhibitors (FK506 and cyclosporine A) are active against fungi, making calcineurin a promising antifungal drug target. Here we review novel findings on calcineurin localization and functions in *A. fumigatus* hyphal growth and septum formation through regulation of proteins involved in cell wall biosynthesis. Extensive mutational analysis in the functional domains of *A. fumigatus* CnaA has led to an understanding of the relevance of these domains for the localization and function of CnaA at the hyphal septum. An evolutionarily conserved novel mode of calcineurin regulation by phosphorylation in filamentous fungi was found to be responsible for virulence in *A. fumigatus*. This finding of a filamentous fungal-specific mechanism controlling hyphal growth and virulence represents a potential target for antifungal therapy.

Keywords

calcineurin; *Aspergillus fumigatus*; hyphal growth; septum; virulence

Introduction

Invasive fungal infections are a leading cause of death in immunocompromised patients [1]. With a 40–60% mortality rate, invasive aspergillosis, caused by the filamentous fungus *Aspergillus fumigatus*, is the most frequent cause of death among mold infections [2]. The calcineurin pathway is an important signaling cascade in eukaryotes and calcineurin is a promising antifungal target due to the distinct mode of action from other antifungal classes,

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activity against drug resistant strains, and synergism with existing antifungals [3]. However, currently available calcineurin inhibitors lead to host immunosuppression and limit potential therapeutic effectiveness [4]. It is therefore important to identify targets that specifically inhibit fungal calcineurin, resulting in fungal killing without host immune suppression.

Calcineurin is a Ca^{2+}/calmodulin (CaM)-dependent protein phosphatase that is ubiquitous and conserved among the eukaryotes [5–8]. It is composed of a catalytic (CnaA) and a regulatory (CnaB) subunit. As a protein phosphatase, CnaA interacts with phosphorylated substrates through its amino-terminal catalytic domain. The other highly conserved domains in CnaA include a carboxy-terminal regulatory domain containing the CnaB binding helix (CnBBH), the CaM binding domain (CaMBD), and an autoinhibitory domain (AID) [9–11] (Fig.1A). After binding to its regulatory subunit, CnaB, which contains four EF hand Ca^{2+}-binding motifs (EF hand motif is a helix-loop-helix structural domain found in the family of calcium-binding proteins), CnaA is activated in the presence of Ca^{2+} and CaM [11]. In contrast to the calcineurin gene multiplicity observed in mammals [11], lower eukaryotes such as the budding yeast *Saccharomyces cerevisiae* contain two genes encoding the catalytic subunit (*CNA1* and *CNA2*) and a single gene for the regulatory subunit (*CNB1*) [12]. The fission yeast *Schizosaccharomyces pombe*, as well as filamentous fungal species, contain one gene encoding each calcineurin subunit [13].

Calcineurin plays a central role in the regulation of cation homeostasis, morphogenesis, cell-wall integrity, and pathogenesis in fungi [14, 8, 15]. It regulates growth at alkaline pH and at higher temperatures, membrane stress, mating and virulence in both *Candida albicans* and *Cryptococcus neoformans* [16–20]. In addition its role in morphogenesis, spindle body organization and membrane trafficking has been well described in *S. pombe* [13, 21, 22]. Previous reports in filamentous fungi have implicated calcineurin in cell cycle progression [23], hyphal branching [24], stress adaptation [25], sclerotial development [26], and appressorium formation [27]. Although calcineurin signaling is conserved among fungi, recent studies indicate important divergences in calcineurin-dependent functions among different human fungal pathogens. For example, while in the model yeast, *S. cerevisiae*, calcineurin (*CNA1*) null mutant was able to grow at higher temperature, the *C. neoformans CNA1* disruption strain was nonviable in host environment mimicking conditions (37° C, 5% CO_2 or alkaline pH) and was avirulent [28]. In contrast to *C. neoformans*, calcineurin was dispensable for survival of *C. albicans* at 37 or 42° C, and the *C. albicans cnb1* mutant strains had no defects in germination and filamentous growth [20, 29]. Host niche also seems to be an important factor for calcineurin control over virulence, as demonstrated in vaginal or pulmonary candidiasis models [30]. Therefore, critical understanding of the calcineurin pathway in *A. fumigatus* will pave the way for devising new drug targets for combating invasive aspergillosis. In this review we summarize recent results on the functional analysis of the calcineurin complex in *A. fumigatus* hyphal growth and septation.

Aspergillus fumigatus calcineurin mutants exhibit defects in germination, hyphal morphology and septum formation

Analysis of *cnaA* deletion mutant in *A. fumigatus* revealed the importance of calcineurin for growth and virulence [31]. To distinguish the relevance of the catalytic and regulatory

subunits of calcineurin for hyphal growth and septation in *A. fumigatus*, calcineurin single (Δ*cnaA*; Δ*cnaB*) and double deletion (Δ*cnaA*Δ*cnaB*) strains were generated. While the Δ*cnaB* strain showed a compact colony morphology indistinguishable from the Δ*cnaA* strain, revealing the absolute requirement of CnaB regulatory subunit for calcineurin function, the Δ*cnaA*Δ*cnaB* strain showed more delayed germination and a greater radial growth defect [32]. In contrast to the wild-type strain with fully-extended hyphae, the Δ*cnaA* strain showed a compact colony with blunt hyphae and irregular branching at the tips, while the Δ*cnaB* and the Δ*cnaA*Δ*cnaB* strains formed hyphae with fewer branches. Partial growth remediation of the Δ*cnaA* strain in presence of sorbitol indicated probable differences in the cell wall components of the individual Δ*cnaA* and Δ*cnaB* mutants or an osmotic defect in the Δ*cnaA* strain. Involvement of calcineurin in osmotic stress response pathways through the PKC and HOG pathways has previously been reported in fungi [33, 34]. These differing phenotypes resulting from deletion of individual calcineurin subunits and the entire complex suggested a previously unsuspected complexity in their individual functions.

Calcineurin complex coordinates hyphal cell wall organization

While both the Δ*cnaA* and Δ*cnaB* strains showed abnormal septa, the Δ*cnaA*Δ*cnaB* strain had curved or wavy septa, sometimes incomplete or even broken indicating a disorganization of β-glucan assembly at the septum upon deletion of both calcineurin subunits. Extracellular web-like material observed by scanning electron microscopy in all the calcineurin mutant strains further suggested the possibility of highly disordered cell wall architecture [32]. While the nature of the extracellular fibrous material is yet unknown, it might be a mixture of polysaccharides and mannoproteins that are improperly assembled due to defects in cell wall synthesis resulting from the deletion of the calcineurin genes.

Morphological analysis of the cell wall by transmission electron microscopy confirmed the requirement of calcineurin complex for proper cell wall architecture. While the cell wall in the wild-type strain was uniformly electron-dense, all of the calcineurin mutants displayed a thicker cell wall. The inner layer, which mostly consists of glucan, seemed enlarged, and the outer layer, which contains mannoproteins, was thicker. Septum formation in the Δ*cnaA*Δ*cnaB* strain was not coordinated properly from both sides of the hyphal wall, resulting in incomplete septum formation. The two sides of the septum were not formed at the same time, which resulted in improper co-ordination of septation from the two ends. While both the Δ*cnaA* and Δ*cnaB* strains showed abnormal septa, the Δ*cnaA* Δ*cnaB* strain had curved often wavy, incomplete or even broken septa. Aniline blue, which stains cell wall β-glucan, did not show septal staining in the Δ*cnaA* Δ*cnaB* strain [32]. These results indicated that, in comparison to the single deletion strains, deletion of both the subunits of calcineurin is more deleterious and results in greater abnormality of the cell wall and septa. The calcineurin complex may therefore be important for the correct deposition of new cell wall material at the septum and for normal cell wall structure. Collectively, these results indicated that the calcineurin mutants have an inherent defect in the composition of their cell walls.

The growth defect and septation abnormalities observed in the double mutant may be either due to the lack of proper synthesis of the major cell wall components, chitin and β-glucan, or

an improper assembly of these components. The β-glucan content in all the calcineurin mutants was reduced by ~40% when compared to the wild-type strain [32]. In contrast to the decreased β-glucan content of the calcineurin mutants, compensatory increase in the chitin levels was noted in all the mutants, with the Δ*cnaA*Δ*cnaB* strain showing an increase of ~40% and each Δ*cnaA* and Δ*cnaB* strain showing ~20% increase when compared to the wild-type strain. Evidence to clearly implicate calcineurin control of both β-glucan and chitin is not yet available but the increase in chitin content is a compensatory response to reduced β-glucan. Such compensatory increases in the chitin contents of strains treated with caspofungin, an inhibitor of β-1,3-glucan synthase, were also noted earlier [35]. Despite an increased growth defect in the Δ*cnaA*Δ*cnaB* strain compared to the single mutants, there were no statistically significant variations in the major cell wall components when comparing the single and double mutants.

While we do not have clear evidence on calcineurin impact on chitin levels, the β-glucan levels are controlled by calcineurin through the downregulation on *fksA* gene. Analysis of the transcriptional profiles of eight chitin synthase genes (*chsA, chsB, chsC, chsD, chsE, chsF, chsG* and *chsEb*) in the Δ*cnaB* and Δ*cnaA*Δ*cnaB* strains showed a down-regulation of all the chitin synthase genes, as was previously reported in the Δ*cnaA* strain [36]. The abnormality in the assembly of the cell wall components in the calcineurin mutants may result from the impaired incorporation of chitin in the cell wall due to the decreased proportion of β-glucan. Previous results have indicated an ~2-fold decrease in the transcription of *fksA*, encoding the catalytic subunit of β-1,3-glucan synthase, in the Δ*cnaA* strain [36], which coincides with decreased β- glucan levels in all the calcineurin mutants. Model depicting calcineurin control over cell wall biosynthetic genes and hyphal growth is shown in Fig. 2.

Localization of the calcineurin complex at the hyphal septum is required for regular septation and proper hyphal growth

CnaA localizes as punctate dot-like structures at the hyphal tips and in developing conidiophores [37]. CnaA also concentrates as a disc around the septal pore in both newly formed and mature septa. The Δ*cnaB* strain showed a similar growth phenotype as deletion of *cnaA* [31], indicating a cooperative regulation between the catalytic and regulatory subunits [32], and fluorescence microscopy revealed the co-localization of mcherry-CnaB and CnaA-EGFP at the septa. Time lapse microscopy of the calcineurin complex revealed that the dot-like structures initially present in the swollen conidium concentrated at the point of germ tube emergence and remained at the tip of the germling as hyphal extension occurred. Retrograde movement of the vesicular structures, containing the calcineurin subunits, from the hyphal tip towards the septation initiation sites and concentration at the center of the septum was evident during septum formation. The calcineurin complex was present throughout the process of septum formation. The presence of calcineurin during the initial germination phase and then during hyphal extension and septation indicated a diverse role for calcineurin in morphogenetic control. Treatment with FK506 or cyclosporine A did not affect localization of the calcineurin complex at the septum, although the treatment resulted in a phenotype that resembled a calcineurin subunit deletion. In the absence of

CnaA, CnaB remained in dot-like structures which were evenly distributed in the hyphal compartments, without septal-localization. However, CnaA localized to the hyphal septum even in the absence of CnaB. Although CnaA localizes at the septum independent of CnaB, the Δ*cnaB* phenotype could not be restored to that of the wild-type and showed septation defects similar to the Δ*cnaA* strain, indicating the absolute requirement of CnaA complexing with CnaB for normal calcineurin function at the hyphal septum. Furthermore, this indicated that CnaA may localize at the septum by binding to other as yet undefined proteins.

Important domains required for calcineurin function and septal localization

Complementation experiment involving the transformation of truncated *cnaA* that consisted of only the N-terminal catalytic domain into the Δ*cnaA* strain did not restore hyphal growth and septal localization which revealed that the N-terminal catalytic domain (1–347 aa) does not contain the determinants required for septal localization, however the inclusion of the CnBBH and CaMBD regions efficiently localized CnaA at the septum and restored proper hyphal growth [38]. Surprisingly, CaM, the well-known calcineurin interactor and activator, is not required for septal targeting of CnaA. It is possible that targeting CnaA to the hyphal septum occurs either independently or by binding to other unknown protein(s).

Binding studies with the human calcineurin previously revealed the PxIxIT motif as a common binding site for calcineurin on its substrates [39] (Fig. 1A). In *S. cerevisiae*, mutation of the calcineurin residues (N366 I367 R368) in contact with the PxIxIT motif resulted in defective substrate interaction [40]. Recent structural studies of Ca^{2+}/CaM bound to a 25-residue peptide spanning the CaMBD in the human calcineurin catalytic subunit also revealed that R408, V409, and F410 play a major role in rigidity and stabilization of the central helix of CaM bound to calcineurin [41]. In *A. fumigatus* transformation of the full length *cnaA* harboring the mutated PxIxIT-binding NIR residues (NIR-AAA) into the Δ*cnaA* strain to verify for complementation, only partially restored hyphal growth and completely mislocalized CnaA indicating that septal localization of CnaA occurs through binding to other protein(s). On the contrary, similar complementation experiment after mutation of the critical Ca^{2+}/CaM-binding RVF residues in the CaMBD to alanines (RVF-AAA) had partial hyphal growth restoration but did not affect CnaA septal localization (Fig. 1A; Table 1). Calmodulin is well known activator of calcineurin. Calmodulin binds to the Calmodulin-binding domain (CaMBD) in CnaA to displace the auto inhibitory domain (AID) and activates calcineurin. The observed growth defect with the RVFAAA mutation may be due to the inability of CaM to bind to CnaA, and as a result the AID remains bound to the regulatory domain, leading to continued inhibition of calcineurin activity (Table 1). Although CaM localizes at the hyphal tip and septum in *A. nidulans* [42], which was confirmed in *A. fumigatus*, these results, coupled with the truncational analyses, confirmed that CnaA localization at the septum is CaM-independent.

Critical regions controlling calcineurin function in *S. cerevisiae* have been identified by substitution of V385 with an aspartic acid that disrupted the interaction between the catalytic and the regulatory subunit, and also by random mutagenesis of three residues (S373, H375, and L379) that led to loss of calcineurin activity but did not disrupt calcineurin A binding to Ca^{2+}/CaM or to calcineurin B [43] (Fig. 1A). The importance of these domains for septal

localization in *A. fumigatus* was analyzed by mutation of V371 to aspartic acid (V371D) and the T359, H361, and L365 to proline, leucine and serine (THL-PLS), respectively. Both mutations had a significant effect on hyphal growth, calcineurin activity but neither affected CnaA septal localization (Table 1). The V371D mutation confirmed that although CnaB is not required for CnaA septal localization, it is required for CnaA function and growth. The THL-PLS mutation had an effect on the catalytic activity and therefore it is possible that although CnaA is localized at the hyphal septum it is catalytically inactive. The reduction in calcineurin activity due to these mutations and the lack of caspofungin-mediated paradoxical growth recovery (caspofungin at high concentrations reverses the growth inhibition of *Aspergillus fumigatus*, a process known as the "paradoxical effect") established that catalytic site residues and CnaB-binding activity of CnaA do not influence its septal localization, yet active calcineurin is required at the hyphal septum to direct proper hyphal growth.

The unique Serine-Proline Rich Region (SPRR) identified in *A. fumigatus* CnaA is phosphorylated and required for proper hyphal growth and virulence

By analyzing the conserved domains in CnaA, we identified a filamentous fungal-specific novel linker between the highly conserved CnBBH and the CaMBD [38] (Fig. 1A). Clustalw alignments confirmed the presence of the SPRR (404-PTSVSPSAPSPPLP-417) within the 23-residue linker that is completely absent in the human calcineurin α-catalytic subunit (Fig. 1B). Phylogenic analysis of this region clearly distinguished the filamentous fungal calcineurins from other organisms, indicating the evolutionarily importance of SPRR for filamentous hyphal growth. Phosphoproteomic analysis revealed the phosphorylation of all 4 clustered serines in the SPRR (S406, S408, S410 and S413) and two additional serine residues in the C-terminus at positions 537 and 542. Phosphorylation of CnaA was also examined in the presence of a specific inhibitor, FK506, to correlate phosphorylation versus activity. Two-fold decrease in the phosphorylation of S406 in the CnaA SPRR and a 1.2- and 1.8-fold increase in the phosphorylation of S537 and S542, respectively, was noted in the C-terminus compared to the untreated control [38]. These results suggested a previously unknown link between FK506-mediated inhibition of calcineurin activity and CnaA phosphorylation, including in the novel SPRR.

Heterologous expression of CnaA homologs from other closely related filamentous fungi, *N. crassa* and *M. grisea*, also revealed the phosphorylation of serine residues within the SPRR providing further evidence that filamentous fungal calcineurins have diverged from the yeasts and other organisms. Complementation experiments with *S.cerevisiae* calcineurin A (CNA1) did not restore/complement the *A. fumigatus cnaA* mutant (unpublished results) which indicated that the filamentous fungal calcineurins may have diverged. We postulate that conservation of this unique SPRR domain in CnaA among filamentous fungi is evolutionarily significant; filamentous fungi may have acquired this unique domain and that phosphorylation in this domain is another novel mode of calcineurin regulation. Based on these novel findings in the regulation of *A. fumigatus* calcineurin, we expect that calcineurin interacts with its substrates in a phosphorylated/dephosphorylated state to regulate different

cellular functions (Fig. 2). Furthermore *in vitro* phosphorylation assays revealed GSK3β, CK1, CDK1 and MAPK as potential kinases that might phosphorylate CnaA *in vivo*. Based on a recent report on the inactivation of GSK-3 by calcineurin inhibitors cyclosporine A and tacrolimus (FK506) in renal tubular cells [44], and our result demonstrating the phosphorylation of CnaA by GSK-3β and CK1 [38], it is possible that FK506 inhibits the activity of GSK-3β, resulting in its inability to phosphorylate CnaA. Post-translational modifications involving protein phosphorylation/dephosphorylation are important events regulating protein function *in vivo*, either by activation or inhibition of activity of the protein. Few studies have focused on phosphorylation of calcineurin and the *in vivo* consequence of mutations in its key domains but none of these residues are conserved in *A. fumigatus* and other filamentous fungi [45–49].

In comparison to the wild-type strain, the CnaA^{mt}-4SA strain, in which the 4 phosphorylated serine residues within the SPRR were mutated to alanine (S406A, S408A, S410A and S413A) to block phosphorylation, exhibited a significant growth defect but did not affect septal localization (Table 1). Supporting these observations, calcineurin activity was also decreased by ~70% in the CnaA^{mt}-4SA strain compared to the wild-type strain, indicating that phosphorylation plays an important role in the regulation of calcineurin activity. The mortality associated with CnaA^{mt}-4SA strain infection in a persistently neutropenic murine inhalational model of invasive aspergillosis was significantly lower (10%) in comparison to the wild-type strain (90%), indicating that phosphorylation in this novel SPRR is critical for calcineurin function and virulence. Analyzing phosphorylation-dependent interactants of calcineurin will help identify target proteins that can be exploited as additional fungal-specific therapeutic targets.

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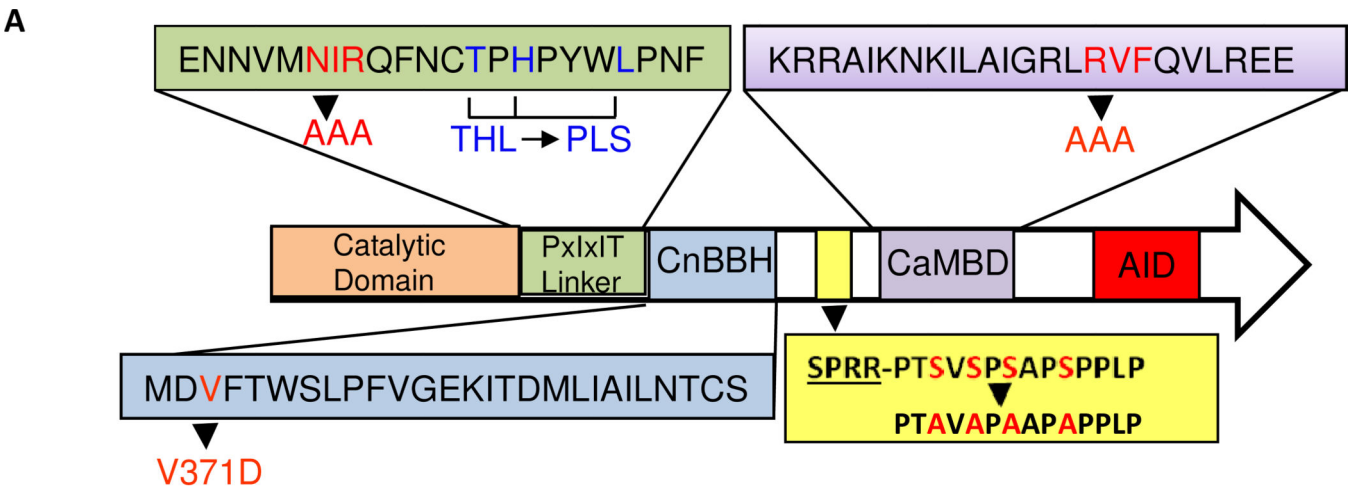

B

Serine-Proline Rich Region

A.fumigatus	PTSVSP--SAPSPPLP----MDVE-SSE
A.nidulans	PSTISP--AEPSPPMP----MDTV-DTE
A.oryzae	-TTVSP-TGPPSPPVP----MDVE-SSE
A.flavus	-TTVSP-TGPPSPPVP----MDVE-SSE
N.crassa	SATTSP--GSASPALPSAANQDPD-SIE
M.grisea	SSSTSP--GPQSPPQTPALPEAAG-DGE
C.neoformans	------EEFPLHAPEP----TDAE-SAA
S.cerevisiae	-ATK--ETGTPS---------DEKASSA
Human	----------FDGA-TAA

Figure 1. *A. fumigatus* CnaA domain organization and targeted mutations

(A) The various domains in CnaA and mutations in the important domains are shown. The PxIxIT linker region mutation (shown in green; 352NIR354 to alanines) affects substrate-binding, combined mutation of Thr359Pro (T359P), His361Leu (H361L) and Leu365Ser170 (L365S) close to the PxIxIT binding motif (THL-PLS) reduces CnaA enzyme activity, the V371D mutation in the Calcineurin B Binding Helix (CnBBH; shown in blue with the V371 residue mutated to Asp) blocks CnaB binding to CnaA. The 4 serine residues (S406, S408, S410 and S413) in the novel Serine-Proline Rich Region (SPRR; shown in yellow; 404PTSVSPSAPSPPLP417) were mutated to alanines to investigate the importance of CnaA phosphorylation for its function and activity. The key residues 442RVF444 in the Calmodulin Binding Domain (CaMBD; shown in purple) were mutated to alanines to block calmodulin binding. **(B)** Comparative sequence alignment of the unique Serine-Proline Rich Region is shown. This Serine-Proline Rich Region containing 14 amino acids is completely absent in the human calcineurin A and not conserved in the yeasts. The autoinhibitory domain (AID) is shown in red.

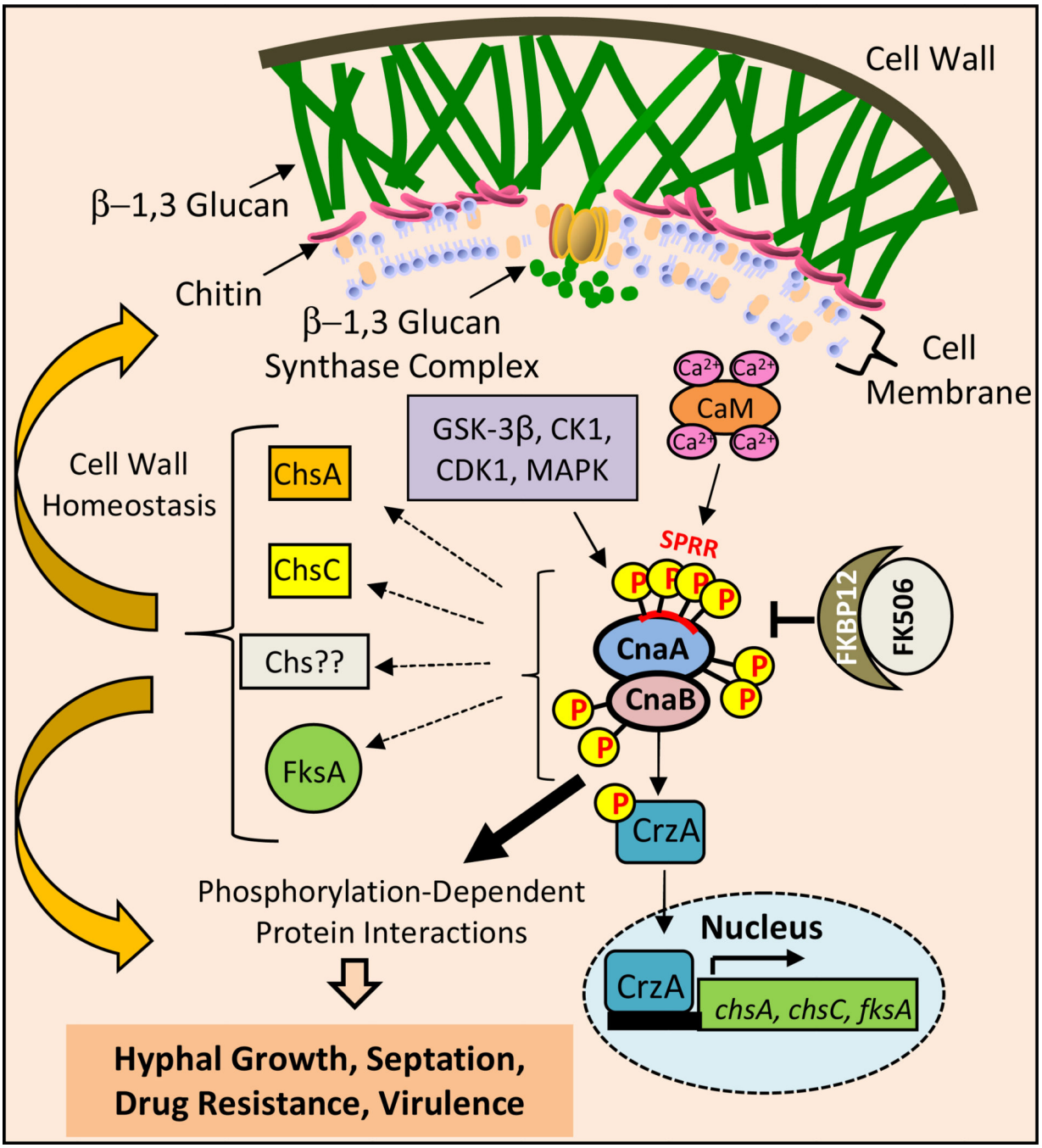

Figure 2. Model showing calcineurin-mediated regulation of hyphal growth, cell wall integrity and virulence in *A. fumigatus*

Calcineurin, comprising of the catalytic subunit (CnaA) and the regulatory subunit (CnaB), is activated by Ca^{2+}-calmodulin (CaM). CnaA is phosphorylated at four serine residues in the Serine Proline Rich Region (SPRR) and also at two serine residues in the C-terminus, and two serine residues in the N-terminus of CnaB by the activity of kinases (GSK-3β, CK1, CDK1, MAPK). Calcineurin is inhibited by the binding of the immunophilin-immunosuppresant complex (FK506-FKBP12). The phosphorylated calcineurin complex may dephosphorylate the transcription factor CrzA and translocate it into the nucleus to

activate the transcription of cell wall biosynthesis related genes (*chsA*, *chsC* and *fksA*). Similarly, the phosphorylated calcineurin complex may also interact with cell wall proteins directly in a phosphorylation-dependent manner to regulate their activity and cell wall homeostasis.

Table 1

Mutations affecting CnaA localization and function in *Aspergillus fumigatus*

CnaA Mutation	Domain	Localization	Reduction in Calcineurin Activity	Growth Defect
NIR-AAA	PxIxIT Motif	Cytoplasm	~70%	~57%
THL-PLS	PxIxIT Linker	Septum	~66%	~84%
V371D	CnBBH	Septum	~50%	~84%
RVF-AAA	CaMBD	Septum	~38%	~49%
$CnaA^{mt}$-4SA	SPRR	Septum	~70%	~49%