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Molecular Characterization of Adenylyl Cyclase Complex Proteins Using Versatile Protein-Tagging Plasmid Systems in *Cryptococcus neoformans*

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In this study, we aimed to generate a series of versatile tagging plasmids that can be used in diverse molecular biological studies of the fungal pathogen *Cryptococcus neoformans*. We constructed 12 plasmids that can be used to tag a protein of interest with a GFP, mCherry, 4×FLAG, or 6×HA, along with nourseothricin-, neomycin-, or hygromycin-resistant selection markers. Using this tagging plasmid set, we explored the adenylyl cyclase complex (ACC), consisting of adenylyl cyclase (Cac1) and its associated protein Aca1, in the cAMP-signaling pathway, which is critical for the pathogenicity of *C. neoformans*. We found that Cac1-mCherry and Aca1-GFP were mainly colocalized as punctate forms in the cell membrane and non-nuclear cellular organelles. We also demonstrated that Cac1 and Aca1 interacted in vivo by co-immunoprecipitation, using Cac1-6×HA and Aca1-4×FLAG tagging strains. Bimolecular fluorescence complementation further confirmed the in vivo interaction of Cac1 and Aca1 in live cells. Finally, protein pull-down experiments using *aca1*Δ*::ACA1-GFP* and *aca1*Δ*::ACA1-GFP cac1*Δ strains and comparative mass spectrometry analysis identified Cac1 and a number of other novel ACC-interacting proteins. Thus, this versatile tagging plasmid system will facilitate diverse mechanistic studies in *C. neoformans* and further our understanding of its biology.

Keywords: Cyclic AMP, adenylyl cyclase-associated protein, Cac1, Aca1

Introduction

Cryptococcus neoformans is a major etiological agent of fatal fungal meningoencephalitis in humans, which causes more than 1,000,000 infections and 600,000 deaths annually [1]. Despite its clinical importance, the therapeutic options for *Cryptococcus* infection (cryptococcosis) are currently limited [2]. To understand the biology and pathogenicity of *C. neoformans*, a number of molecular and genetic tools have been developed in recent decades, including gene disruption by overlap or split marker/double joint PCR [3, 4] and biolistic transformation [5], a conditional gene expression system [6], *Agrobacterium*-mediated random insertion mutagenesis [7], signature-tag mutagenesis for large-scale virulence studies [8], and so on [9]. However, the protein-tagging systems for proteins and the mechanistic studies currently available for *C. neoformans* are far less advanced than those for non-pathogenic model yeasts, mainly because a stable plasmid with an autonomously replicating sequence has not been developed for *C. neoformans*.

To facilitate diverse molecular biological studies in *C. neoformans*, we constructed 12 different tagging plasmids, each of which allows a protein of interest to be tagged with an epitope (GFP, mCherry, 4×FLAG, or 6×HA) and contains one of three dominant selection markers, nourseothricin (*NAT*), neomycin (*NEO*), or hygromycin (*HYG*) resistance genes. These plasmids will allow us to perform mutant complementation studies with epitope-tagged proteins, protein localization and colocalization studies, co-immunoprecipitation and immunoblot assays for studying in vivo protein-protein

interactions, and pull-down experiments followed by mass spectrometry to identify interacting proteins.

To validate the usefulness of this plasmid system, we explored the adenylyl cyclase complex (ACC) in the cAMP-signaling pathway of *C. neoformans*, which plays pivotal roles in differentiation, stress responses, and virulence (for a review see [10]). The ACC consists of the adenylyl cyclase Cac1 [11] and its associated protein Aca1 [12]. Cac1 is required for both basal and induced cAMP levels [11], whereas Aca1 is only required for induction of cAMP [12]. Nevertheless, how Cac1 and Aca1 functionally cooperate and whether Cac1 interacts directly with Aca1 in vivo are not yet known, although the two proteins have been shown to interact in a yeast two-hybrid assay [12]. Furthermore, Aca1 also has cAMP-independent functions, such as the salt stress response and maintenance of cell wall integrity [13], indicating that Aca1 may interact with other proteins. In this study, using our versatile tagging plasmid system, we investigated the cellular location and in vivo interaction of Cac1 and Aca1 and identified additional ACC-interacting proteins. Furthermore, we performed a bimolecular fluorescence complementation (BiFC) assay to visualize the interaction between Cac1 and Aca1 in living cells. Our study provides further insight into our understanding of the cAMP-signaling pathway and a useful tool for the *Cryptococcus* research community.

Materials and Methods

Construction of Versatile Tagging Plasmids

To construct versatile tagging plasmid systems, four tags (*GFP*, *mCherry*, 4×*FLAG* and 6×*HA*) and the *HOG1* terminator (*HOG1t*) were amplified by PCR with the primers listed in Table S1. Each amplified tag was fused to *HOG1t* by overlap PCR and then cloned into pTOP-V2 (Enzynomics, Korea) to construct four tagging plasmids, pTOP-GFPht, pTOP-mCherryht, pTOP-4×FLAGht, and pTOP-6×HAht. Three selection markers, *NAT*, *NEO*, and *HYG*, were amplified by PCR and then cloned into the pTOP-V2 vector to construct pNAT, pNEO, and pHYG, respectively. pNAT or pHYG was digested with HindIII to release a *NAT* or *HYG* fragment, respectively, and the resistance genes were subcloned to the four tagging plasmids. The *NEO* fragment was released from pNEO by BamHI digestion for subcloning into pTOP-GFPht and pTOP-mCherryht, and by HindIII digestion for subcloning into pTOP-4×FLAGht and pTOP-6×HAht. Therefore, the final tagging plasmid set consisted of 12 plasmids (Fig. 1).

Construction of *CAC1-mCherry*, *aca1Δ::ACA1-GFP*, *aca1Δ::ACA1-GFP cac1Δ*, *CAC1-6×HA*, and *ACA1-4×FLAG* strains

All strains were constructed in a *C. neoformans* serotype A H99S strain background using our tagging plasmids described above (Table S2 and Fig. S1). To construct the *aca1Δ::ACA1-GFP* strain, the promoter and open reading frame (ORF) of *ACA1* were amplified by PCR with the primer pair B5908/B5909, which contain NotI sites, and cloned into the pNEO-GFPht plasmid

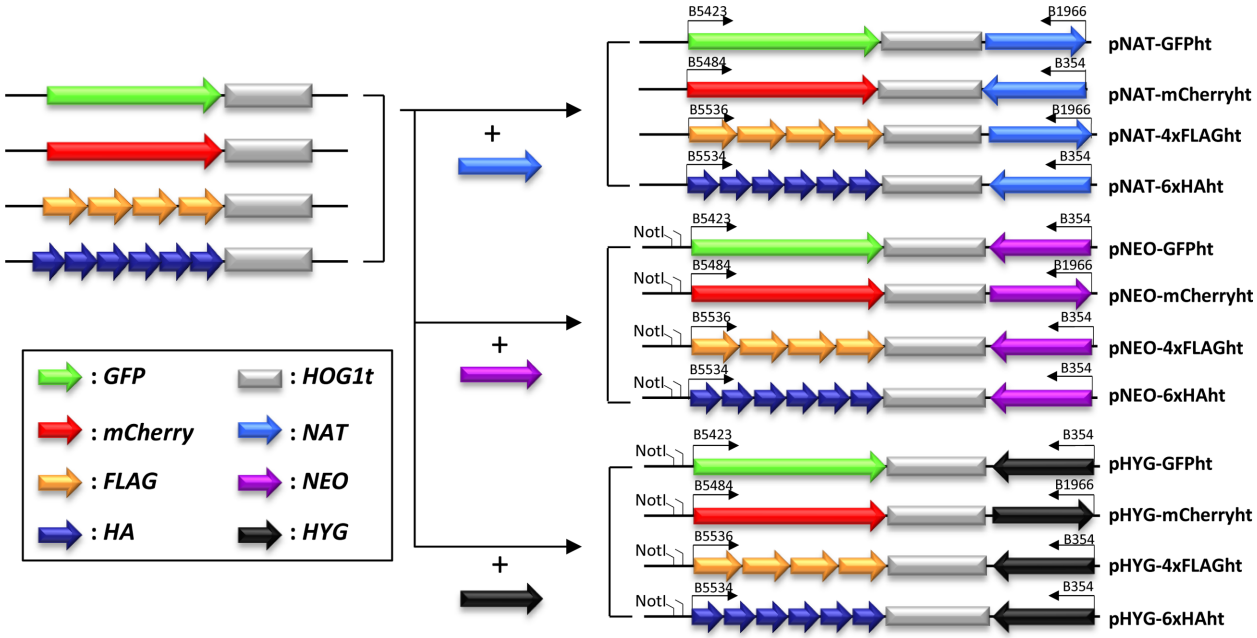

Fig. 1. Schematic diagram of the *Cryptococcus neoformans* versatile tagging plasmid set.
The versatile tagging plasmid set consists of 12 plasmids, which contain a GFP, mCherry, 4×FLAG, or 6×HA tag along with one of three dominant selection markers, nourseothricin (*NAT*), neomycin (*NEO*), or hygromycin (*HYG*) resistance genes.

(Fig. 1) to construct pNEO-ACA1-GFPht. pNEO-ACA1-GFPht was linearized by SalI digestion and introduced into the *aca1*Δ mutant (YSB6) by biolistic transformation. Targeted integration of the *ACA1-GFP* gene into the *ACA1* locus was confirmed by diagnostic PCR and phenotypic analysis (Fig. S2). The *CAC1-mCherry*, *CAC1-6×HA*, and *ACA1-4×FLAG* cassettes for chromosomal C-terminal tagging were generated (using the primers and plasmids listed in Tables S1 and S3, respectively) by a split marker/double-joint PCR strategy that has been reported previously [4]. The tagging cassettes were delivered into the H99S strain by biolistic transformation. To construct the *aca1*Δ::*ACA1-GFP cac1*Δ strain, a *CAC1* gene disruption cassette was constructed using the *HYG* marker via a split marker/double-joint PCR strategy (with the primers listed in Table S1) and introduced into the *aca1*Δ::*ACA1-GFP* strain (YSB4402) by biolistic transformation. Proper construction of each mutant or tagged strain was confirmed by Southern blot and phenotypic analyses (Figs. S1 and S2).

Construction of the *ACA1-Vc* and *CAC1-Vn* Strains

To construct strains containing *ACA1-Vc* and *CAC1-Vn*, seven PCR products (the 3′-ORF regions of *ACA1* and *CAC1*, N-terminal (*Vn*) and C-terminal (*Vc*) regions of the Venus protein, *HOG1*t, and 3′-flanking regions of *ACA1* and *CAC1*) were separately amplified by PCR with the primers listed in Table S1. The *ACA1-Vc-HOG1t* fragment was generated via overlap PCR by combining the 3′-ORF region of *ACA1*, *Vc*, and *HOG1t* as templates and primers B4584 and B4589 and then cloning into pTOP-V2 to generate pTOP-ACA1-Vc-HOG1t. The 3′-flanking regions of *ACA1* were cloned into pTOP-V2 to generate pTOP-ACA1-3UTR. The BamHI-digested *ACA1-Vc-HOG1t* fragment from pTOP-ACA1-Vc-HOG1t was subcloned into pNAT to generate pNAT-ACA1-Vc-HOG1t. pTOP-ACA1-3UTR was digested with PstI, and the *ACA1_3UTR* insert was cloned into pNAT-ACA1-Vc-HOG1t to generate pNAT-ACA1-Vc. Likewise, the amplified *CAC1-Vn-HOG1t* fragment and 3′-flanking region of *CAC1* were cloned into pTOP-V2 to generate pTOP-CAC1-Vn-HOG1t and pTOP-CAC1-3UTR, respectively. The cloned *CAC1-Vn-HOG1t* fragment was subcloned into pNEO to produce pNEO-CAC1-Vn-HOG1t. pTOP-CAC1-3UTR was digested with NotI, and the *CAC1-3UTR* insert was cloned into pNEO-CAC1-Vn-HOG1t to generate pNEO-CAC1-Vn. pNAT-ACA1-Vc was digested with SacI and XhoI, and pNEO-CAC1-Vn was digested with KpnI and XhoI. Then, the digested products were introduced into the H99S strain by biolistic transformation. The tagged strains were confirmed by Southern blot and phenotypic analyses (Figs. S1 and S2).

Imaging of the Cellular Localization of GFP-, mCherry-, and Venus-Tagged Proteins

Strains harboring Aca1-GFP, Cac1-mCherry, Aca1-Vc, Cac1-Vn, or Aca1-Vc Cac1-Vn were incubated in YPD liquid medium overnight at 30°C. Cells from each sample were fixed as previously described [14] and then incubated with Hoechst dye (Hoechst 33342 solution; Thermo Fisher Scientific, USA) in the dark for 1 h to stain the nucleus. After incubation, samples were mixed with 10 μl of mounting solution (Biomeda, USA) and then visualized with a Nikon Eclipse Ti microscope.

Co-Immunoprecipitation and Immunoblotting

The *ACA1-4×FLAG*, *CAC1-6×HA*, and *ACA1-4×FLAG CAC1-6×HA* strains were incubated in YPD liquid medium overnight at 30°C. The overnight culture was inoculated into 100 ml of fresh YPD liquid medium and then incubated at 30°C until the optical density at 600 nm (OD_{600}) reached approximately 0.8. Whole-cell lysates were prepared with lysis buffer containing 50 mM Tris-Cl (pH 7.5), 1% sodium deoxycholate, 5 mM sodium pyrophosphate, 0.2 mM sodium orthovanadate, 50 mM NaF, 0.1% SDS, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, and 2.5 × protease inhibitor cocktail solution (Calbiochem, USA). After adding an anti-FLAG antibody (Sigma-Aldrich, USA), the lysates were rotated overnight at 4°C. Next, Sepharose protein G beads (GE Healthcare Life Sciences, USA) were added to the whole-cell lysates and rotated for 6 h at 4°C. To remove the unbound proteins, the mixture was centrifuged, and the pellet was washed six times with lysis buffer. The proteins bound to the beads were eluted with SDS sample buffer (50 mM Tris-Cl, 2% SDS, 10% glycerol, and 0.01% β-mercaptoethanol) and detected by immunoblotting with anti-FLAG (Santa Cruz Biotechnology, USA) and anti-HA (Roche, USA) antibodies.

Protein Pull-Down Assays and Mass Spectrometry

A pull-down experiment was performed with the GFP-trap kit (ChromoTek, Germany). Whole-cell lysates of the *aca1*Δ::*ACA1-GFP* and *aca1*Δ::*ACA1-GFP cac1*Δ strains were prepared according to the method described above. To precipitate GFP-tagged Aca1, GFP-trap agarose beads were mixed with whole-cell lysates of *aca1*Δ::*ACA1-GFP* and *aca1*Δ::*ACA1-GFP cac1*Δ on a rotator at 4°C for 2 h and then washed six times with lysis buffer. After washing, proteins were eluted with SDS sample buffer. The samples were separated by 10% SDS-PAGE. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 and then cut into five slices according to Coomassie Blue-stained band intensity. Each gel slice was washed, reduced, alkylated, and subjected to in-gel trypsin digestion (sequencing grade trypsin; Promega, USA). The tryptic peptides were extracted with 0.02% (v/v) formic acid in 0.5% (v/v) acetic acid and lyophilized for liquid chromatography-tandem mass spectrometry (LC-MS/MS). Mass spectrometric analyses were performed using a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometry (Thermo Scientific, USA) according to the previously described protocol [15]. For protein identification, MS/MS spectra were searched against the Universal Protein Resource (http://uniprot.org) using MASCOT (ver. 1.4; Thermo Scientific). Methionine oxidation and cysteine carbamidomethylation were set as variable and fixed modifications, respectively. Two missed cleavages were allowed in the peptide identification.

Results and Discussion

Construction of the Versatile Tagging Plasmid Set for *C. neoformans*

To perform molecular biological studies in *C. neoformans*, we generated a series of versatile tagging plasmids as described above. This system consists of 12 tagging plasmids, in which a GFP, mCherry, 4×FLAG, or 6×HA tag was cloned in combination with a *NAT*, *NEO*, or *HYG* selection marker (Fig. 1). These tagging plasmids can be used for C-terminal tagging of a protein of interest. The plasmids containing *NEO* or *HYG* can be used for both chromosomal tagging by overlap PCR and ORF cloning using the NotI site in the tagging plasmids. The plasmids containing *NAT* can only be used for chromosomal tagging by overlap PCR. Using this versatile tagging plasmid set, three distinct tags can be incorporated into three different genes in a single strain.

Cellular Localization of the ACC Proteins, Cac1 and Aca1

To validate the usefulness of this tagging plasmid system, we chose the ACC, which consists of Cac1 and Aca1, for the following reasons. First, the basic cellular functions of Cac1 and Aca1 in *C. neoformans* have been well characterized. Therefore, the functionality of a tagged version of these proteins can be easily evaluated. Second, the interaction between the C-terminal domain of Cac1 and Aca1 has been examined in vitro by using a yeast two-hybrid system [12], although their in vivo interaction and cellular localization remain unknown. Third, Aca1 appears to have both Cac1-dependent and -independent roles in *C. neoformans*, and yet its interacting proteins have not been identified and characterized.

To examine the cellular localization of Aca1 and Cac1, we constructed *aca1*Δ::*ACA1-GFP* and *CAC1-mCherry* strains, in which the C-termini of Aca1 and Cac1 were tagged with GFP and mCherry fluorescent proteins, respectively. The *ACA1-GFP* gene was integrated into the *ACA1* locus in the *aca1*Δ mutant, and we confirmed that complementation with Aca1-GFP successfully restored the normal phenotype to the *aca1*Δ mutant strain (Fig. S2A), suggesting that Aca1-GFP is functional. We also confirmed that the mCherry tag

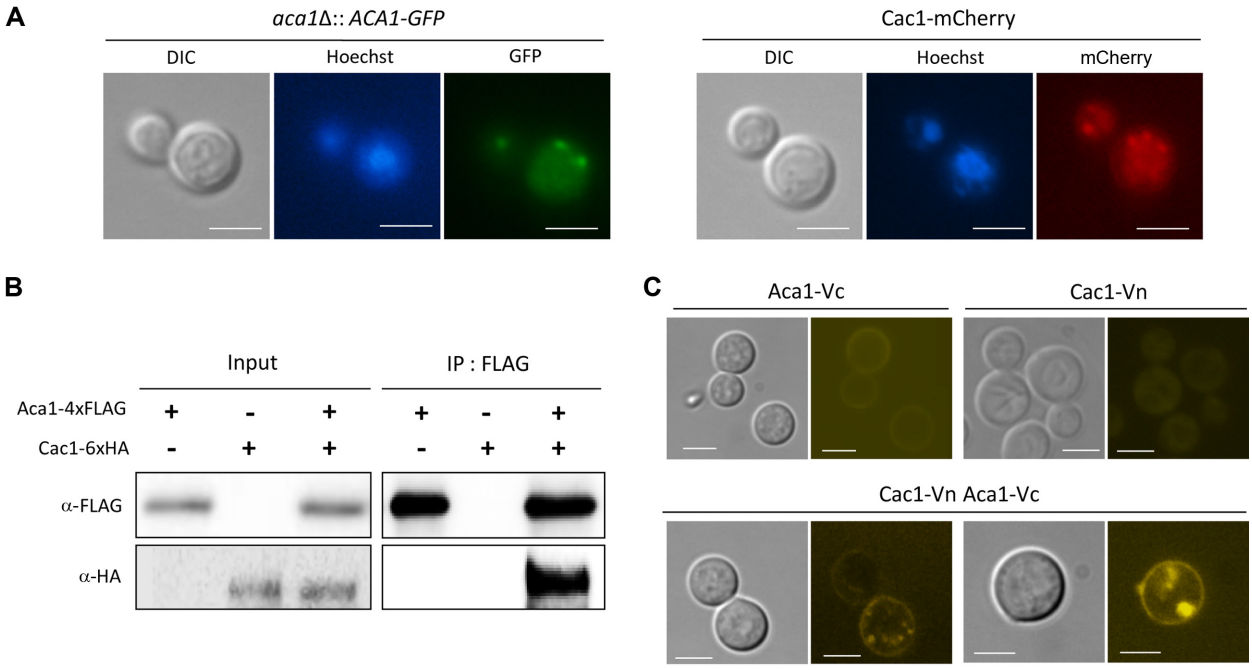

Fig. 2. Fluorescent microscopic cellular localization and in vivo interaction of adenylyl cyclase (Cac1) and adenylyl cyclase-associated protein (Aca1) in *Cryptococcus neoformans*.

(**A**) The *aca1*Δ::*ACA1-GFP* and *CAC1-mCherry* strains were incubated in liquid YPD medium at 30°C for 16 h. Then, the cells were fixed and stained with Hoechst dye to visualize the nucleus. Scale bar: 10 μm. (**B**) Co-immunoprecipitation showing that Aca1 interacts with Cac1 in vivo. The Aca1-4×FLAG protein was immunoprecipitated with an anti-FLAG antibody (IP: α-FLAG), and the Cac1-6×HA protein was detected by immunoblotting with an anti-HA antibody (IB: α-HA). (**C**) Bimolecular fluorescence complementation assay showing that Aca1 interacts with Cac1 in living cells. The *ACA1-Vc*, *CAC1-Vn*, and *ACA1-Vc CAC1-Vn* strains were incubated in liquid YPD medium at 30°C for 16 h. The cells were fixed and visualized by fluorescence microscopy. Scale bar: 10 μm.

at the C-terminus of Cac1 did not affect its normal cellular functions, as the *CAC1-mCherry* strain exhibited wild-type levels of melanin and capsule production (Fig. S2B), which are regulated by the cAMP signaling pathway. Both Aca1-GFP and Cac1-mCherry were observed as punctate forms at the cell periphery of *C. neoformans* (Fig. 2A). These observations are consistent with the finding that adenylate cyclase and its-associated protein 1 (Cap1/Srv2), an Aca1 ortholog, are also enriched as punctate forms at the cell periphery of *S. cerevisiae* [16].

Aca1 Physically Interacts with Cac1 In Vivo

To elucidate the in vivo interaction between Cac1 and Aca1, we constructed the following strains: *ACA1-4×FLAG*, *CAC1-6×HA*, and *ACA1-4×FLAG CAC1-6×HA*. Similar to the *ACA1-GFP* and *CAC1-mCherry* strains, the C-terminal 4×FLAG and 6×HA tags did not affect the normal cellular functions of Aca1 and Cac1, respectively (Figs. S2D and S2E). We used an anti-FLAG antibody to immunoprecipitate Aca1-4×FLAG and its interacting proteins and detected the Cac1-6×HA protein by western blotting using an anti-HA antibody (Fig. 2B). The results clearly showed that Aca1 interacts with Cac1 in vivo.

The main limitation of the co-immunoprecipitation assay is that in vivo interaction between two proteins cannot be monitored in live cells and the cellular localization cannot be detected. Therefore, we performed BiFC assays by constructing *ACA1-Vc*, *CAC1-Vn*, and *ACA1-Vc CAC1-Vn* strains, in which the truncated N-terminal (2–173 aa; Vn) and C-terminal (155–239 aa; Vc) fragments of the Venus fluorescent protein [17] are fused in-frame to the C-termini of Cac1 and Aca1, respectively. The truncated Vn and Vc fragments are not individually fluorescent but become fluorescent upon interaction between fused proteins [17]. We only observed yellow fluorescence in the *ACA1-Vc CAC1-Vn* strain, but not in the *ACA1-Vc* or *CAC1-Vn* strain, indicating that Cac1 interacts with Aca1 in vivo (Fig. 2C). Furthermore, supporting this interaction data, cellular localization studies of Cac1 and Aca1 (Fig. 2A) showed that both colocalized as punctate forms at the cell periphery of *C. neoformans* (Fig. 2C).

Identification of Aca1-Interacting Proteins in *C. neoformans*

In addition to the adenylyl-cyclase-binding domain (RLEXXXXRLE), Aca1 contains several other protein-binding domains, including a proline-rich region and actin-binding domain (Fig. 3A), which may be involved in its Cac1-independent functions. To identify Aca1-interacting proteins, we used the *aca1*Δ::*ACA1-GFP* strain for a protein pull-down experiment. We also constructed an *aca1*Δ::*ACA1-GFP cac1*Δ strain by deleting *CAC1* in the *aca1*Δ::*ACA1-GFP* strain to determine whether any proteins that were pulled down with the Aca1-GFP protein interacted directly with Aca1 or indirectly through Cac1. By using an anti-GFP antibody coupled to agarose beads, we immunoprecipitated Aca1-GFP and its interacting proteins and identified them by gel electrophoresis and mass spectrometry. The list of Aca1-interacting proteins identified in the *aca1*Δ::*ACA1-GFP* and *aca1*Δ::*ACA1-GFP cac1*Δ strains are listed in Table S4. The Aca1-interacting proteins were arranged from the largest to smallest number of peptide spectrum matches (# PSMs). The # PSMs indicates the total number of identified peptide sequences for the protein, including redundant sequences.

As expected, the Aca1-GFP protein (80 kDa) was the most enriched protein in the immunoprecipitation with the anti-GFP antibody in both the *aca1*Δ::*ACA1-GFP* and *aca1*Δ::*ACA1-GFP cac1*Δ strains. Cac1 was identified in the *aca1*Δ::*ACA1-GFP* strain but not in the *aca1*Δ::*ACA1-GFP cac1*Δ strain (Fig. 3B), corroborating the quality of our analysis. We identified a number of Aca1-interacting proteins, and 143 were detected in both strains (Fig. 3C), suggesting that they interact with Aca1 regardless of the presence of Cac1. As expected, we detected actin and actin-binding proteins. The proline-rich domain is known to interact with Src homology 3 (SH3) domains [18], which are present in a variety of proteins that associate with the cytoskeleton and membrane [19]. We also identified CNAG_00334 (heat shock protein), CNAG_04738 (*SAC6*, fimbrin), and CNAG_01537 (*VAD1*, virulence-associated ATP-dependent mRNA helicase), which have been reported to physically interact with Cap1/Srv2 in *S. cerevisiae* [20–24]. Sac6 is also an actin-binding protein that is involved in growth under low oxygen conditions [25]. It was demonstrated that Vad1 regulates virulence-related genes in *C. neoformans* [26]. Similar to the *aca1*Δ or *cac1*Δ mutants, *vad1*Δ mutants showed a marked reduction in laccase activity and virulence in a murine model [12, 26]. Therefore, it is possible that Vad1 may regulate the cAMP signaling pathway through interaction with Aca1.

Furthermore, we identified several novel Aca1-interacting proteins, including CNAG_01137 (aconitase), CNAG_06400 (plasma membrane H^+-ATPase), CNAG_05750 (*ATP1*, ATPase α subunit), CNAG_05918 (F0F1 ATP synthase subunit β), and CNAG_06101 (ADP/ATP carrier) (Table S4). Interestingly, all these proteins are related to ATP synthesis or hydrolysis. Aconitase is a mitochondrial enzyme involved in the citric acid cycle. The reason why Aca1 interacts with

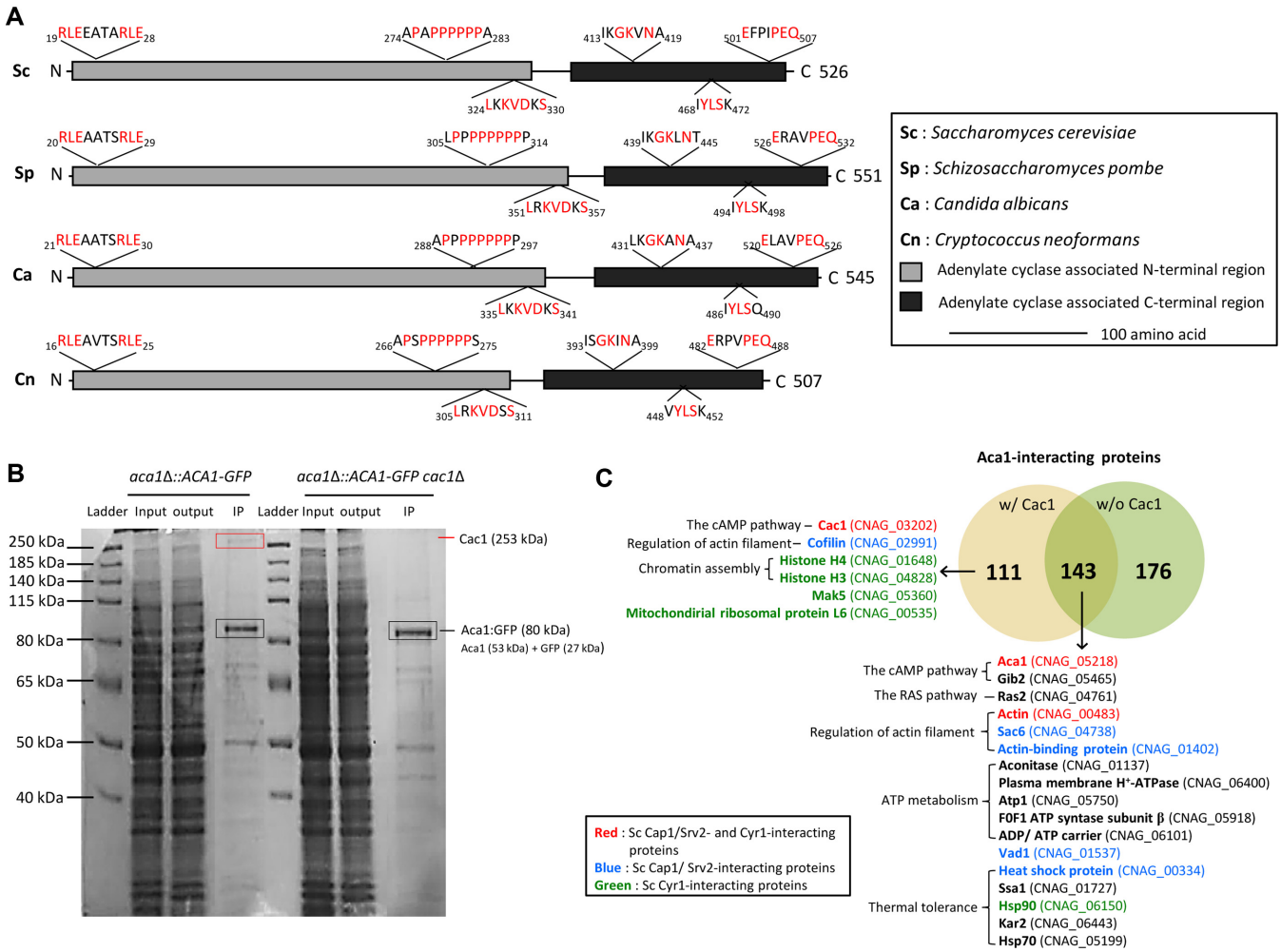

Fig. 3. Schematic outline of adenylate cyclase-associated proteins in fungi and identification of adenylyl cyclase complex-interacting proteins in *Cryptococcus neoformans*.

(**A**) The functional domains of each adenylate cyclase-associated protein were analyzed by Pfam 30.0 (http://pfam.xfam.org/). The adenylyl cyclase-binding domain contains a conserved RLEXXXXRLE motif. The proline-rich region contains a conserved XPXPPPPPPX motif. The verprolin homology domain contains a conserved LXKVDXS sequence. The actin-binding domain contains three conserved sequences, XXGKXNX, XYLSX, and EXXXPEQ. The numbers indicate the locations of the amino acids in the sequence. X indicates a nonconserved amino acid. (**B**–**C**) Adenylyl cyclase complex (Cac1 and Aca1)-interacting proteins were identified in *aca1*Δ::*ACA1-GFP* and *aca1*Δ::*ACA1-GFP cac1*Δ strains. The Venn diagram shows the number of Aca1- and/or Cac1-interacting proteins in the two strains. Colored words indicate Cap1/Srv2 (Aca1 ortholog, blue)- or Cyr1 (Cac1 ortholog, green)-interacting proteins, or co-interacting proteins (red) with Cap1/Srv2 and Cyr1 in *S. cerevisiae*.

these proteins may be that mitochondria-generated ATP is required for actin polymerization and Aca1 regulates actin dynamics by enhancing the exchange of actin-bound ADP for ATP [27]. Furthermore, Aca1 also appeared to indirectly interact with other proteins, including CNAG_01648 (histone H4), CNAG_04828 (histone H3), CNAG_05360 (*MAK5*, ATP-dependent RNA helicase), CNAG_00535 (mitochondrial ribosomal protein subunit L16), and CNAG_02991 (cofilin), which have been reported to be Cyr1 (Cac1 ortholog)-interacting proteins in *S. cerevisiae* (Table S4) [21, 28, 29]. Cofilin is a Cap1/Srv2 and Cyr1-interacting protein in *S. cerevisiae;* however, it was not detected in the *C. neoformans aca1*Δ::*ACA1-GFP cac1*Δ strain. These proteins, including cofilin, were detected in the *aca1*Δ::*ACA1-GFP* strain, but not in the *aca1*Δ::*ACA1-GFP cac1*Δ strain, suggesting that these proteins interact with Cac1, and not directly with Aca1, in *C. neoformans*.

Notably, for the first time, we identified Ras2 and Gib2 as Aca1-interacting proteins in *C. neoformans*. In *S. cerevisiae*, Cyr1 plays a crucial role in the Ras signaling pathway and

physically interacts with Ras2 [30]. However, whether adenylate cyclase-associated proteins directly interact with Ras proteins in *S. cerevisiae* or *C. neoformans* has not been reported. The Ras signaling pathway and Gib2 are known to regulate cAMP levels through interactions between Gib2 and Ras1, and Cac1 and Ras1, in *C. neoformans* [31]. It was demonstrated that Gib2 functions as a scaffolding adapter protein. However, no interaction between Cac1 and Gib2 was detected in *C. neoformans* [31]. Our results showed that Gib2 interacts with Aca1 even in the absence of Cac1. Therefore, Ras2 and Gib2 seem to have crucial roles in the cAMP pathway by interacting with Aca1 in *C. neoformans*.

In addition, we also identified several heat shock proteins as Aca1-interacting proteins, including CNAG_00334 (heat shock protein), CNAG_01727 (*SSA1*), CNAG_06150 (*HSP90*), CNAG_06443 (*KAR2*), and CNAG_05199 (*HSP70*), in both *aca1*Δ::*ACA1-GFP* and *aca1*Δ::*ACA1-GFP cac1*Δ. These heat shock proteins function as molecular charperones and thereby play a central role in tolerance against a variety of environmental stresses that lead to unfolded or misfolded proteins. This result is in agreement with our previous finding showing that Aca1 plays significant roles in stress responses and adaptation in *C. neoformans* [13].

In conclusion, we have provided useful genetic tools for molecular biological studies in *C. neoformans*, which will facilitate mechanistic studies of the genes and proteins in complex signaling pathways.

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