When and why an essential popular mineral—calcium—is an effective antifungal adjuvant against the human pathogen?

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12 **Abstract**

13 In eukaryotes, calcium not only is an essential mineral nutrient but also serves as an intracellular
 14 second messenger that is necessary for many physiological processes. Here, we show that
 15 exogenous calcium is toxic when fungal cells lack functional calcineurin, a calcium-dependent
 16 protein phosphatase that acts as the central regulator of the calcium signaling pathway. By
 17 monitoring intracellular calcium, particularly by tracking vacuolar calcium dynamics in living cells
 18 through a novel procedure using modified aequorin, we found that calcineurin dysfunction
 19 perturbed calcium homeostasis in intracellular compartments including the cytosol, mitochondria,
 20 and vacuole, leading to drastic autophagy global organelle fragmentation, and even lastly resulting
 21 in cell death upon an extracellular calcium stimulus. Notably, the defective phenotypes seen with
 22 calcineurin mutants can be significantly suppressed by alleviating a cytosolic calcium overload or
 23 increasing vacuolar calcium storage capacity, suggesting toxicity of exogenous calcium to
 24 calcineurin mutants is tightly associated with abnormal cytosolic calcium accumulation and
 25 vacuolar calcium storage capacity deficiency. Our findings provide insights into how calcineurin
 26 regulates intracellular calcium homeostasis for cell survival and may have important implications
 27 for antifungal therapy and clinical drug administration.

28 Introduction

29 Calcium (Ca^{2+}), as an intracellular second messenger in eukaryotic cells, plays a critical role in the
 30 regulation of various cellular processes including synaptic transmission, secretion, and cytokinesis
 31 (*Islam, 2020; Rosendo-Pineda, Moreno, & Vaca, 2020*). The intracellular Ca^{2+} concentration is
 32 strictly and precisely controlled by a sophisticated calcium homeostasis system, which is
 33 composed of various calcium channels, calcium pumps, and calcium antiporters (*Carafoli, 1987;*
 34 *Cyert & Philpott, 2013; Rosendo-Pineda et al., 2020*). Overwhelming evidence demonstrates that
 35 calcineurin, the conserved Ca^{2+} -calmodulin (CaM) activated protein phosphatase, is a master
 36 regulator of intracellular calcium homeostasis in fungi (*Blatzer & Latge, 2017*). Calcineurin is
 37 required for the regulation of cation homeostasis, morphogenesis, cell-wall integrity, and
 38 pathogenesis (*H. S. Park, Lee, Cardenas, & Heitman, 2019*). However, the mechanism by which
 39 calcineurin regulates the optimal Ca^{2+} concentrations in the cytosol and in intracellular
 40 compartments such as the vacuole and mitochondria remain elusive.

41 When fungal cells encounter environmental stresses, a transient elevation of cytosolic calcium is
 42 induced primarily through calcium influx systems in the plasma membrane such as the high-affinity
 43 calcium uptake Cch1-Mid1 channel complex system (*Cyert & Philpott, 2013; Thewes, 2014*).
 44 Calcium-activated calcineurin then dephosphorylates the transcription factor Crz1, which induces
 45 its translocation to the nucleus to regulate gene expression. It has been shown that the free
 46 cytosolic calcium concentration ($[\text{Ca}^{2+}]_c$) must be maintained at a low, steady level within a narrow
 47 physiological range (50-200 nM) after a sudden increase, peaking within seconds and then sharply
 48 decreasing within 30 seconds as the excess cytosolic calcium is transiently transferred to calcium
 49 stores including the endoplasmic reticulum (ER), vacuoles, and mitochondria by various calcium

pumps and calcium antiporters (*Cunningham, 2011; Cyert & Philpott, 2013; Miseta, Fu, Kellermayer, Buckley, & Bedwell, 1999; Miseta, Kellermayer, Aiello, Fu, & Bedwell, 1999; Munoz et al., 2014*). In mammals, the ER, mitochondria, and lysosomes are physically and/or functionally linked (*Dolgin, 2019*). The role of Ca^{2+} signaling in mitochondria is to determine cellular survival by controlling basal mitochondrial bioenergetics and by regulating apoptosis. Moreover, the lysosomes can act as a Ca^{2+} store for Ca^{2+} release into the cytosol, thereby influencing Ca^{2+} homeostasis. Ca^{2+} signaling in lysosomes may regulate autophagy to respond to cell damage and cell stress (*La Rovere, Roest, Bultynck, & Parys, 2016*). As a topological equivalent, the vacuole is the primary Ca^{2+} storage organelle in fungi, containing more than 90% of total cellular Ca^{2+} (*Dunn, Gable, & Beeler, 1994*). Notably, until now there was no reliable system to probe or detect the calcium concentration in fungal vacuole, probably due to its low internal pH. Vacuolar Ca^{2+} sequestration is performed by the collaboration of $\text{Ca}^{2+}/\text{H}^{+}$ exchangers in the Vcx family and the P-type ATPase Pmc family (*Forster & Kane, 2000; Kmetzsch et al., 2010a*). In contrast, vacuolar Ca^{2+} can be released into the cytosol through the mechanosensitive channel Yvc1 and/or transient receptor protein (TRP) channels (*Chang, Schlenstedt, Flockerzi, & Beck, 2010; Denis & Cyert, 2002; Hamamoto, Mori, Yabe, & Uozumi, 2018; Lange et al., 2009; Thewes, 2014; Yu et al., 2014*). Several lines of evidence in yeast-like and filamentous fungi indicate that the transcription of Pmc family members is dependent on an activated calcineurin-Crz1/CrzA complex, whereas Vcx1 activity is negatively regulated by the calcineurin complex (*Cunningham & Fink, 1996; Dinamarco et al., 2012; Ferreira et al., 2012; Kmetzsch et al., 2010b; Thewes, 2014*).

In addition to being a calcium store, the fungal vacuole is responsible for the last step of autophagy. Autophagy is a highly conserved catabolic process that mediates the self-degradation

of intracellular material, such as proteins, lipids, or even entire organelles. In the conserved autophagy pathway, the double-membrane autophagosome (AP) engulfs cellular components to be delivered for degradation in the lysosome/vacuole (*Klionsky & Emr, 2000; Nakatogawa, 2020*). Autophagy exists at a basal level in all living cells, but it can be induced in response to various factors such as nitrogen starvation, oxidative stress, ER stress, and rapamycin drug treatment (*Liu et al., 2014; Y. Wang & Zhang, 2019; Yi, Tong, & Yu, 2018*). In most of these situations, autophagy has both beneficial and harmful effects. It can function as a protective mechanism to promote cell survival in nutritional starvation and other stresses, but excessive autophagy also leads to damage to organelles and even cell death (*Scarlati, Granata, Meijer, & Codogno, 2009; White & DiPaola, 2009*).

Aspergillus fumigatus is an opportunistic filamentous fungus that causes a wide spectrum of life-threatening diseases with poor treatment outcomes in immunocompromised individuals (*Denning & Bromley, 2015*). Calcineurin is considered a potential drug target due to its essential roles in fungal growth and virulence (*Juvvadi, Lee, Heitman, & Steinbach, 2017; Steinbach et al., 2006; Steinbach et al., 2007*). In this study, we observed the phenomenon that a relatively low concentration of calcium is toxic to calcineurin-mutant fungi. By monitoring the calcium dynamics in the cytosol and intracellular calcium stores with modified forms of aequorin as a calcium reporter, we found that the calcineurin mutant exhibited abnormal calcium homeostasis and triggered autophagy and global fragmentation of the nucleus and organelles under a calcium stimulus. Additionally, the defective phenotypes of the calcineurin mutant can be significantly suppressed by alleviating cytosolic calcium overload through deletion of the Ca^{2+} channel *cchA* or by increasing vacuolar calcium storage capacity with overexpressed vacuolar P-type Ca^{2+} -ATPase *PmcA*,

94 highlighting the feedback relationship between calcineurin and CchA and the critical role of
 95 vacuoles in the detoxification of excess calcium for fungal survival. Our findings shed light on the
 96 underlying mechanism by which calcineurin regulates calcium homeostasis for cell survival, and
 97 these results provide a rationale for combination therapy of calcium and calcineurin inhibitors to
 98 combat human fungal pathogens. Additionally, the potential toxicity to mammalian cells of
 99 combined calcium supplements and calcineurin inhibitors should be taken into consideration when
 100 administering immunosuppressive drugs to patients in clinical settings.

Results

Extracellular calcium is toxic to *A. fumigatus* calcineurin mutants

Calcineurin is a highly conserved Ca^{2+} -calmodulin-dependent protein phosphatase that plays a central role in morphological development and cation homeostasis in fungi (Fox & Heitman, 2002; H. S. Park et al., 2016). To further characterize the functions of calcineurin in Ca^{2+} signaling transduction, we constructed deletion mutants of the catalytic subunit CnaA and the regulatory subunit CnaB by CRISPR-Cas9 technique in *A. fumigatus* (Figure S1). Morphological analysis in solid and liquid minimal media showed that the ΔcnaA mutant displayed defective morphological phenotypes including reduced hyphal growth and abnormal branching (Figure 1A). Notably, the abnormal hyphal phenotypes of the ΔcnaA mutant were exacerbated in the presence of 10 mM CaCl_2 , i.e., swollen hyphae tips, whereas no obvious differences were observed in the cnaA^R complementary strain. Moreover, the ΔcnaA mutant showed a significant decrease in mycelial production compared to that of the wild-type at indicated time-points when cultured in media with or without 10 mM CaCl_2 (Figure 1B). Interestingly, the calcineurin mutants ceased growth when incubated in the presence of calcium after 72 h, suggesting that the addition of calcium is toxic to the ΔcnaA mutant (Figure 1C). To confirm this toxicity, we cultured the wild-type and ΔcnaA strains in liquid minimal media (MM) with or without 10 mM CaCl_2 for 96 h. Subsequently, the homogenized mycelia were inoculated into fresh liquid MM for another 48 h. The wild-type strains pretreated with or without calcium grew relatively well after transferring to fresh liquid MM. In contrast, the growth of the ΔcnaA mutant pretreated with calcium was dramatically decreased compared to that of the untreated ΔcnaA mutant (Figure 1D and 1E), confirming that the relatively low levels of extracellular calcium significantly inhibited the growth of the calcineurin mutant.

Moreover, the $\Delta cnaB$ mutant exhibited identical phenotypes to those of the $\Delta cnaA$ mutant under the conditions we tested (Figure S2). Taken together, these data suggest that extracellular calcium is toxic to *A. fumigatus* calcineurin mutants and that calcineurin is essential for the survival of *A. fumigatus* in response to calcium stimuli.

$\Delta cnaA$ mutant exhibits increased cytosolic and vacuolar calcium accumulation in response to extracellular calcium stimuli

Given that calcineurin is critical for regulating *A. fumigatus* survival in response to extracellular calcium, to further explore the mechanisms by which calcineurin regulates calcium homeostasis in *A. fumigatus*, we constructed wild-type and calcineurin mutants expressing codon-optimized aequorin to perform real-time monitoring of the dynamics of free Ca^{2+} concentration $[Ca^{2+}]_c$ in living hyphal cells (Nelson *et al.*, 2004). Upon treatment with 10 mM $CaCl_2$, the $[Ca^{2+}]_c$ (the free Ca^{2+} concentration in cytosol) in the wild-type strain transiently increased from a basal resting level of approximately 0.1 μM to a peak concentration of 0.63 μM and then gradually returned to a stable resting level (Figure 2A); thus, the amplitude of $[Ca^{2+}]_c$ between the peak and the resting status was 0.53 μM . In comparison, $\Delta cnaA$ showed a two-fold increased basal resting level of $[Ca^{2+}]_c$ (0.2 $\mu M \pm 0.04$) and 26% increased amplitude of $[Ca^{2+}]_c$ (0.94 $\mu M \pm 0.21$) under the same conditions, suggesting that cytosolic calcium homeostasis was affected by the deletion of *cnaA*. Next, we wondered whether the calcium equilibrium in mitochondria would be affected by calcineurin mutants. To test this possibility, we compared the $[Ca^{2+}]_{mt}$ (the free Ca^{2+} concentration in mitochondria) using a modified aequorin (Mt-Aeq) protein fused with a mitochondrial signal peptide. As shown in Figure 2B, the $\Delta cnaA$ mutant displayed a 20% increase in the basal resting level and

a similar amplitude response of $[Ca^{2+}]_{mt}$ compared to the wild-type and *cnaA* complementing strains, suggesting that the deletion of *cnaA* only has a minor effect on mitochondrial calcium homeostasis. Next, we wondered in fungi, the vacuole might be a major intracellular Ca^{2+} store that plays a role in the regulation of Ca^{2+} homeostasis. However, until now, no reliable detection approach has been available for the real-time monitoring of the vacuolar free Ca^{2+} concentration ($[Ca^{2+}]_{vac}$) in fungi. To set up this system, we fused a guide sequence encoding the vacuolar lumen marker protein carboxypeptidase Y (CpyA) at the N-terminus of aequorin to generate the fusion protein CpyA-Aeq (Kikuma, Ohneda, Arioka, & Kitamoto, 2006). To verify the localization of the guided protein CpyA in *A. fumigatus*, the CpyA-GFP/RFP strain was generated as control. As shown in Figure 2C, the localization of the N-terminal CpyA-GFP fusion protein shows a vacuolar pattern. Further colocalization analysis showed that the previously verified vacuole membrane-localized protein, PmcA (the homolog of yeast P-type Ca^{2+} -ATPase Pmc1), surrounded the CpyA-RFP (Figure 2C), demonstrating that CpyA acts as a typical vacuole lumen protein. Next, to further confirm the feasibility of the $[Ca^{2+}]_{vac}$ measurement system, we generated the overexpression strains of the vacuolar P-ATPases PmcA and PmcB, which are responsible for calcium influx into the vacuole by consuming ATP, and assessed the calcium levels in the vacuole. As shown in Figure S3, overexpressing *pmcA/B* remarkably elevated the basal resting level of $[Ca^{2+}]_{vac}$ and enhanced calcium influx into the vacuole compared with the wild-type strain, confirming the reliability of this vacuolar calcium measurement method. Thus, we employed the CpyA-Aeq system to monitor the free Ca^{2+} concentration of vacuoles. Interestingly, the basal resting $[Ca^{2+}]$ level in vacuoles (0.65 μ M) prior to stimuli was significantly greater than that in the cytosol and mitochondria (Figure 2D), confirming that the vacuole is a major intracellular calcium

store in *A. fumigatus*. Notably, in the $\Delta cnaA$ cells, the basal resting level of $[Ca^{2+}]_{vac}$ increased by 23% (0.8 μM), and the $[Ca^{2+}]_{vac}$ amplitude sharply increased to a 4.6-fold higher value (4.6 μM) compared to that of in wild-type cells upon exposure to 10 mM Ca^{2+} (Figure 2D). Collectively, these data suggested that the $\Delta cnaA$ mutant exhibits a dramatic increase in vacuolar Ca^{2+} influxes and induces excessive vacuolar Ca^{2+} accumulation.

Extracellular calcium induces vacuolar fragmentation and autophagy in the $\Delta cnaA$ mutant

Next, we wondered whether the excessive vacuolar Ca^{2+} accumulation in the $\Delta cnaA$ mutant would cause any changes of vacuolar morphology. As shown in Figure 3A, fluorescence microscopy analysis showed that wild-type and $\Delta cnaA$ cells had similar oval-shaped mature vacuoles as indicated by CpyA-GFP when cultured in liquid minimal media for 36 h with or without the addition of calcium. Notably, when incubation time was prolonged to 60 h, the $\Delta cnaA$ mutant showed highly fragmented fluorescence patterns of vacuoles in the presence of 2.5 or 10 mM calcium (Figure 3A). In comparison, in the absence of calcium treatment, the vacuolar morphologies of $\Delta cnaA$ mutants showed no detectable differences compared to the parental strain. These data indicate that significant vacuolar fragmentation occurs in the $\Delta cnaA$ mutant when grown in liquid minimal media containing extra calcium. Because the vacuole is the major site for macromolecular degradation through autophagy, to examine whether the deletion of *cnaA* would trigger autophagy under extracellular calcium condition, we generated double-labeled strains with the autophagy marker Atg8 tagged at its N-terminus with GFP and the vacuole marker CpyA fused with RFP and monitored these in the $\Delta cnaA$ and wild-type backgrounds. As shown in Figure 3B, GFP-Atg8 fusion proteins show a punctate pattern that is proximal to the CpyA-RFP-labeled vacuoles in the

189 wild-type and $\Delta cnaA$ strains when incubated for 36 h in the presence or absence of calcium.
190 However, when cultures were stimulated by the addition of CaCl_2 for 60 h, the green fluorescence
191 patterns of GFP-Atg8 overlapped with the CpyA-RFP-labeled vacuoles (Figure 3B). Furthermore,
192 Western blotting showed that the $\Delta cnaA$ strain displayed increased cleavage of GFP from the
193 GFP-Atg8 fusion protein compared to the wild-type cells when cultured for 60 h under the control
194 of the constitutive *gpdA* promoter (Figure 3C) or the native *atg8* promoter (Figure S4A-S4B) in the
195 presence of calcium. In addition, cleavage of GFP was not significantly exacerbated when media
196 were supplemented with other divalent metal cations including magnesium, zinc, iron, and copper
197 (Figure S4C), suggesting that the autophagy was specifically induced by calcium. Next, to
198 examine the contribution of autophagy to Ca^{2+} toxicity in the $\Delta cnaA$ mutant, we deleted the classic
199 autophagy-dependent gene *atg2*, which encodes a peripheral membrane protein for autophagic
200 vesicle formation (Kotani, Kirisako, Koizumi, Ohsumi, & Nakatogawa, 2018; Tang, Takahashi, &
201 Wang, 2019), in the $\Delta cnaA$ background. As shown in Figure 3D, GFP-Atg8 consistently exhibited
202 a punctate pattern and did not co-localize with the vacuolar marker CpyA-RFP in the $\Delta cnaA\Delta atg2$
203 mutant. Furthermore, Western blotting showed that no (or an undetectable level of) GFP was
204 cleaved from GFP-Atg8 fusion protein in the $\Delta cnaA\Delta atg2$ double mutant compared with the $\Delta cnaA$
205 mutant (Figure 3E), suggesting that autophagy was suppressed in the $\Delta cnaA\Delta atg2$ mutant.
206 Notably, as shown in Figure 3F, the $\Delta cnaA\Delta atg2$ mutant displayed a delay in growth cessation (at
207 84 h) compared to that of the $\Delta cnaA$ mutant (at 72 h) under calcium treatment conditions (Figure
208 3F). However, the $\Delta cnaA\Delta atg2$ mutant pretreated with or without calcium showed a comparable
209 biomass production compared to that of the $\Delta cnaA$ mutant after homogenization and transfer to
210 fresh liquid MM, suggesting that the deletion of *atg2* did not prevent the cell death induced by a

211 lack of *cnaA* after culture (Figure 3G), indicating that Atg2 is required for autophagy but not Ca^{2+}
 212 toxicity in the calcineurin-deficient mutant upon calcium stimuli. Taken together, these data
 213 suggest that a lack of CnaA results in specific calcium-induced autophagy in *A. fumigatus*.

214
 215 **Lack of the calcium channel CchA alleviates the calcium toxicity-related phenotypes in the**
 216 ***cnaA* null mutant**

217 Our previous studies in a model fungus *A. nidulans* indicated that calcineurin negatively regulates
 218 CchA (the voltage-gated Ca^{2+} channel component of high-affinity calcium influx system) upon
 219 calcium uptake in response to extracellular calcium; the deletion of *cchA* suppressed the
 220 hypersensitivity to external calcium in calcineurin mutants (S. Wang, Liu, Qian, Zhang, & Lu, 2016).
 221 We hypothesized that a similar situation would occur in *A. fumigatus*. As expected, *cchA* deletion
 222 partially suppressed the colony radial growth defects and hyperbranching polarity growths in *cnaA*
 223 deletion mutants with or without calcium treatment (Figure S5). Notably, a lack of CchA
 224 significantly rescued the defective hyphal growth of the *cnaA* mutant in calcium treatment
 225 conditions (Figure 4A and 4B). The detection of calcium dynamics in living cells showed that the
 226 $[\text{Ca}^{2+}]_{\text{vac}}$ amplitude and the basal resting $[\text{Ca}^{2+}]_{\text{c}}$ of the $\Delta\text{cnaA}\Delta\text{cchA}$ mutant were approximately
 227 53% and 20% lower, respectively, than those in the ΔcnaA mutant (Figure 4C and 4D), suggesting
 228 that calcineurin mediates cytosolic and vacuolar calcium homeostasis by negatively regulating
 229 CchA. Accordingly, the colocalization of GFP-Atg8 and CpyA-RFP (vacuolar marker) and the
 230 vacuolar fragmentation were not observed in the $\Delta\text{cnaA}\Delta\text{cchA}$ mutant (Figure 4E). Moreover,
 231 Western blots showed that the $\Delta\text{cnaA}\Delta\text{cchA}$ mutant exhibited an autophagy level similar to that of
 232 the wild-type strain (Figure 4F), suggesting that autophagy in response to external calcium is

inhibited when *cchA* is deleted in the $\Delta cnaA$ mutant. These data suggest that a lack of the calcium channel CchA alleviates the calcium toxicity-related phenotypes in the *cnaA* null mutant.

Lack of CchA partially restores the virulence of the $\Delta cnaA$ mutant in a *Galleria mellonella* infection model but not in a mouse infection model.

The aforementioned data demonstrated that the absence of CchA in the $\Delta cnaA$ mutant partially suppressed the growth defects of *A. fumigatus* *in vitro*. Next, we further tested whether the $\Delta cnaA\Delta cchA$ mutant would affect virulence in *in vivo* animal models. As shown in Figure 5A, in the insect infection model of the wax moth *Galleria mellonella*, the $\Delta cnaA\Delta cchA$ mutant had a significantly lower survival rate (40%) than that of the $\Delta cnaA$ mutant (90%), suggesting that a lack of *cchA* partially restored the virulence of the $\Delta cnaA$ mutant. Furthermore, the virulence of the $\Delta cnaA\Delta cchA$ mutant was tested in a mouse model. Immunocompromised ICR mice were infected with fresh conidia, homogenized hyphae, or luciferase-probed related strains by an intratracheal route. After two weeks, the mice infected with wild-type *A. fumigatus* display high mortality, whereas both the $\Delta cnaA\Delta cchA$ and $\Delta cnaA$ mutants caused low mortality (Figure 5A). Statistical analysis showed that there was no significant difference between $\Delta cnaA\Delta cchA$ and $\Delta cnaA$ in survival rates for either the conidia or the homogenized hyphal infection model. Histopathological examinations and live animal imaging showed that the lungs from mice inoculated with the wild-type fungus displayed aggressive fungal growth and luminescence signals around the lungs (Figure 5B and 5C). In contrast, there was no detectable fungal growth or luminescence signals in the lungs of mice infected with the $\Delta cnaA\Delta cchA$ or $\Delta cnaA$ mutants, implying that the immune system of mice might be able to eliminate inoculated conidia or hyphae.

255 These data suggest that a lack of CnaA results in the loss of virulence in immunocompromised
256 mice, and the deletion of *cchA* partially restored virulence in wax moths but not in the mouse
257 model.

258

259 **Overexpressing P-type Ca^{2+} -ATPase PmcA significantly rescued the calcium** 260 **toxicity-related phenotypes in the ΔcnaA mutant**

261 In fungi, the vacuole serves as the major Ca^{2+} store and plays a critical role in the detoxification of
262 cytoplasmic Ca^{2+} . Vacuolar calcium equilibrium is maintained by the P-type Ca^{2+} -ATPase Pmc
263 family and the $\text{H}^+/\text{Ca}^{2+}$ exchanger Vcx family, which are regulated by calcineurin (Cyert & Philpott,
264 2013). In line with this information, the ΔcnaA mutant exhibited lower expression levels of *pmcA*
265 and *pmcC* compared to those of the wild-type, whereas the transcript level of vacuolar $\text{H}^+/\text{Ca}^{2+}$
266 exchanger family members, especially *vcxB*, *vcxD*, and *vcxE*, was upregulated in *A. fumigatus*
267 (Figure S6), demonstrating that calcineurin positively regulates the P-type Ca^{2+} -ATPase Pmc
268 family while negatively regulating the $\text{H}^+/\text{Ca}^{2+}$ exchanger Vcx family. Given the calcium
269 toxicity-related phenotypes observed in the ΔcnaA mutant, we hypothesized that the upregulation
270 of $\text{H}^+/\text{Ca}^{2+}$ exchanger Vcx family members in the ΔcnaA mutant would be insufficient to detoxify
271 excess cytoplasmic calcium in response to external calcium, and thus a $\Delta\text{cnaA}^{\text{OE}::\text{pmcA}}$ strain
272 overexpressing PmcA, the yeast P-type Ca^{2+} -ATPase Pmc1 homolog in *A. fumigatus*, under the
273 control of the strong constitutive promoter P_{gpdA} was used in the ΔcnaA background to test whether
274 the defective phenotypes in the ΔcnaA mutant would be suppressed. qRT-PCR analysis confirmed
275 that *pmcA* was highly overexpressed in the ΔcnaA mutant (Figure S3). Further live-cell detection
276 of $[\text{Ca}^{2+}]_{\text{vac}}$ showed that the $\Delta\text{cnaA}^{\text{OE}::\text{pmcA}}$ strain displayed an elevated basal resting level of

[Ca²⁺]_{vac} and had an enhanced [Ca²⁺]_{vac} amplitude compared to that of the $\Delta cnaA$ mutant (Figure 6A), indicating that calcium was effectively transported from the cytoplasm to vacuoles by PmcA. Additionally, autophagy assays showed that the $\Delta cnaA^{OE::pmcA}$ strain displayed reduced autophagy compared to that of the $\Delta cnaA$ mutant in response to external calcium stimuli (Figure 6B and 6C). Moreover, the overexpression of *pmcA* in the *cnaA* mutant markedly suppressed the growth cessation in calcium treatment conditions (Figure 6D). The $\Delta cnaA^{OE::pmcA}$ strain pretreated with or without calcium (96 h) showed dramatically increased biomass production compared to that of the $\Delta cnaA$ mutant after homogenization and transfer to fresh liquid MM (Figure 6E). Thus, these data suggested that overexpressing P-type Ca²⁺-ATPase PmcA partially rescues calcium toxicity-related phenotypes in the $\Delta cnaA$ mutant.

The $\Delta cnaA$ mutant exhibits global fragmentation of nuclei and organelles upon calcium stimuli

To further dissect the molecular mechanisms operating in the *A. fumigatus* $\Delta cnaA$ mutant upon *in vitro* calcium stimuli, we performed proteomic comparison analysis using tandem mass tags (TMTs) and liquid chromatography tandem MS (LC/MS-MS) and compared wild-type and $\Delta cnaA$ strains grown in the presence of extracellular calcium. Proteins with change ratios significantly different from general protein variation ($1.50 < \Delta cnaA/\text{wild-type} < 0.50$) were selected for gene ontology (GO) annotation and enrichment analysis. As shown in Table S3, the expression levels of 618 and 218 proteins were increased and decreased, respectively, in the $\Delta cnaA$ mutant compared to the wild-type strain. Most upregulated proteins were involved in metabolic processes of biomolecules, especially in catabolism and proteolysis (Figure S7A). In contrast, most of the

299 downregulated proteins were associated with biosynthesis and/or the transport of biomolecules
300 and inorganic ions (Figure S7B). Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation
301 revealed that the upregulated proteins were primarily associated with various amino acid
302 metabolism pathways, especially tyrosine, beta-alanine, valine, and leucine (Figure 7A), whereas
303 the downregulated proteins mainly focused on biosynthesis-related pathways including ribosome
304 (protein synthesis), thiamine metabolism, and steroid biosynthesis. Of note, the upregulated
305 enriched group in the $\Delta cnaA$ mutant contained 23 peptidases, 18 hydrolases, 4 nucleases, 3
306 proteases, 3 autophagy-related proteins, and 1 metacaspase (Figure 7A), which may lead to
307 global protein degradation and cause apoptosis/apoptotic-like programmed and/or autophagic cell
308 death in $\Delta cnaA$ cells under calcium treatment. Thus, we hypothesized that CnaA-involved cellular
309 calcium equilibrium may regulate whole-cell organelle networks. To further assess whether a lack
310 of CnaA would induce abnormal organelle and nuclear morphology with calcium treatment, we
311 probed the nucleus (RFP-H2A), endoplasmic reticulum (Erg11A-GFP), Golgi apparatus
312 (RFP-PH^{OSBP}), and mitochondria (MrsA-RFP). As predicted, fluorescence observation showed that
313 these organelles displayed fragmentation that was comparable to that of the vacuole in the $\Delta cnaA$
314 mutant after calcium treatment for 60 h (Figure 7C); apoptosis/apoptotic-like and/or autophagic cell
315 death may occur. A similar phenomenon was observed in *A. fumigatus* cells treated with the
316 calcineurin inhibitor FK506 (Figure 7D). Taken together, these data suggested that global nuclear
317 and organelle fragmentation may contribute to calcium toxicity in mutants lacking functional
318 calcineurin.

Discussion

The critical role of calcineurin in calcium dysregulation-related human diseases has been recognized for decades (Y. J. Park, Yoo, Kim, & Kim, 2020). In fungi, calcineurin has been considered an attractive antifungal target because it is essential for stress survival, development, drug resistance, and virulence (Juvvadi et al., 2017). Previous studies have reported that calcineurin protects *Candida albicans* from toxicity caused by the endogenous levels of calcium present in serum (Blankenship & Heitman, 2005). However, the underlying mechanism by which calcineurin orchestrates intracellular calcium dynamics remains elusive. Through the successful development of an intracellular calcium dynamics monitoring method in this study, we demonstrated that calcineurin dysfunction disturbed calcium homeostasis in the cytosol, vacuole, and mitochondria, leading to a dramatic induction of autophagy and organelle fragmentation and ultimately resulting in significant cell growth inhibition and cell death in the presence of calcium stimuli.

Typically, the resting cytosolic Ca^{2+} concentration in *A. fumigatus* ranges from 0.05 to 0.1 μM . In the ΔcnaA mutant, the cytosolic resting Ca^{2+} level increased by two-fold compared to that of the wild-type prior to calcium stimuli, which is in line with our previous reports on *A. nidulans*. Moreover, when cells were challenged with 10 mM Ca^{2+} , the ΔcnaA mutant displayed a 33% increase in $[\text{Ca}^{2+}]_c$ amplitude compared to that of the wild-type. These data support the evolutionarily conserved role of calcineurin in maintaining cytosolic calcium homeostasis across fungal species. The sustained increased cytosolic calcium concentration led us to question whether calcium homeostasis in the intracellular calcium stores such as mitochondria and vacuole would be affected by a lack of calcineurin. We established a novel vacuolar Ca^{2+} detection

approach by fusing the vacuole-targeted protein carboxypeptidase Y (CpyA) to the N-terminus of aequorin, and we found that in the presence of extracellular calcium stimuli, the basal resting level of $[Ca^{2+}]_{vac}$ and the transient $[Ca^{2+}]_{vac}$ in the $\Delta cnaA$ mutant were significantly increased, whereas the calcium signature in mitochondria was not significantly affected, implying that vacuoles may be the major site for the detoxification of excess cytosolic calcium in *A. fumigatus*. Collectively, these data demonstrated that calcineurin not only regulates the resting cytosolic calcium but also affects the vacuolar calcium transient response. Importantly, the increased cytosolic resting $[Ca^{2+}]_c$ level and the $[Ca^{2+}]_{vac}$ amplitude seen in the $\Delta cnaA$ mutant can be suppressed by deleting the calcium channel CchA, suggesting that calcineurin negatively regulates CchA when cells are exposed to calcium. Accordingly, the feedback relationship between calcineurin and the calcium channel CchA has been described in yeast and *A. nidulans* (Ma et al., 2011; S. Wang et al., 2016). In yeast, it has been proposed that Cch1 is dephosphorylated by calcineurin *in vitro* (Bonilla & Cunningham, 2003), leading to the inhibition of calcium channel activity, which is in accord with our phosphoproteomic analysis showing that Serine 55 in CchA could be directly dephosphorylated by calcineurin in *A. fumigatus* (Fig. S8).

In addition to the disordered intracellular calcium homeostasis, the $\Delta cnaA$ mutant exhibited the global fragmentation of organelles including the vacuole, the nucleus, ER, and mitochondria, and aggressive autophagy was observed when the $\Delta cnaA$ mutant was grown in the presence of 10 mM calcium. This autophagy resulted in the cessation of growth and cell death with prolonged incubation time, indicating that the relatively low concentration of calcium is toxic to calcineurin-deficient mutant *A. fumigatus*. It has been well established that the vacuole plays a key role in detoxifying the excess calcium when cells are exposed to external calcium. Thus, we hypothesized

that the cytoplasmic calcium excess combined with deficient vacuolar detoxification capacity would give rise to the calcium-toxicity related phenotypes observed in the $\Delta cnaA$ mutant. This hypothesis was supported by the fact that alleviating cytosolic calcium overload by the deletion of *cchA* or increasing vacuolar calcium storage capacity by overexpressing of the P-type Ca^{2+} -ATPase PmcA can significantly suppress the defective phenotypes of the $\Delta cnaA$ mutant. These results highlighted the important roles of calcineurin in mediating calcium homeostasis for fungal survival and the detoxification functions of the vacuoles. Of note, blocking CchA or the overexpression of PmcA is unable to completely suppress the vacuolar fragmentation, aggressive autophagy, and growth cessation in the $\Delta cnaA$ mutant in the presence of calcium stimuli, implying that there are other calcium pumps or transporters may also be co-operated with them and involved in the regulation of calcium homeostasis. Probably, examples include the mitochondrial Ca^{2+} uniporter McuA, the Golgi-localized P-type $\text{Ca}^{2+}/\text{Mn}^{2+}$ -ATPase PmrA, and the ER-localized P-type ATPase SpfA (Pinchai et al., 2010; Song, Liu, Zhai, Huang, & Lu, 2016; Yu et al., 2012), all of which have been demonstrated to be required for mediating calcium homeostasis in response to environmental stresses. In addition, although our results suggested that autophagy triggered by the dysregulation of intracellular calcium homeostasis in the $\Delta cnaA$ mutant correlates with cell death because blocking autophagy by *atg2* deletion could slow the cell death progress, we cannot eliminate the possibility that other “toxic factors” affect the viability of *A. fumigatus* calcineurin mutants in the presence of calcium. Proteomic analysis revealed that various peptidases, nucleases, metacaspases, and autophagy-related proteins were upregulated in the $\Delta cnaA$ mutant compared to the wild-type strain in calcium-treated conditions, implying that apoptosis/apoptotic-like and/or autophagic cell death may occur.

385 Although cyclosporine A and FK506 (tacrolimus), calcineurin inhibitors, have been shown to
 386 possess *in vitro* antifungal activity against fungal pathogens including *A. fumigatus*, *C. albicans*,
 387 and *C. neoformans*, the clinical use of calcineurin inhibitors for antifungal therapy remains limited
 388 due to their immunosuppressive side effects (Fox & Heitman, 2002; Groll, De Lucca, & Walsh,
 389 1998; Hamamoto *et al.*, 2018). Because calcineurin inhibitors exhibit synergistic fungicidal activity
 390 with antifungal drugs, research on analogues of calcineurin inhibitors with antifungal activity but
 391 low immunosuppressive properties may be a promising direction. Encouragingly, APX879, the
 392 FK506 analogue, has been shown to exhibit reduced immunosuppressive activity while
 393 maintaining limited antifungal activity against pathogens in a murine infection model (Juvvadi *et al.*,
 394 2019). Our findings showing that a relatively low concentration of calcium is sufficient to
 395 significantly inhibit the growth of calcineurin mutants highlights a possibility that the combination of
 396 calcium and nonimmunosuppressive calcineurin inhibitors could enhance therapeutic efficacy
 397 against human pathogens, and the addition of calcium may reduce the dosage of
 398 immunosuppressive agents needed, thus minimizing side effects.

Materials and Methods

Strains, media, and culture conditions

was listed in the supplementary data Table S1. Strains were routinely grown in the following media: minimal media (MM) containing 1% glucose, 2% agar, 1 ml/L trace elements and 50 ml/L 20 × salt solution, as described previously (*Zhang & Lu, 2017*). The pH was adjusted to 6.0 ; MM supplemented with 5 mM uridine and 10 mM uracil were used for uracil and uridine auxotroph strains. The recipe for liquid glucose minimal media is identical to that for MM, except without agar. All strains were cultured at 37°C.

Quantification of biomass production

10⁵ conidia were incubated in 100 ml of MM with or without 10 mM CaCl₂ at 37°C in a rotary shaker at 200 rpm for 60, 72, 84, and 96 h. Biomass curves were drawn after mycelial pellets were collected, dried, and weighed.

Assessment of hyphal survival

The related strains were grown in liquid MM with or without 10 mM CaCl₂ with 220 rpm shaking at 37°C for 96 h. Subsequently, the mycelium pellets of each strain were crushed and homogenized into hyphae fragments using tissue grinding and filtered with lens-cleaning paper in sterile ddH₂O. The optical density at 600 nm (OD₆₀₀) of each group was finally adjusted to 0.2. Then, 100 µL of suspension containing hyphae fragments was inoculated into liquid MM for 48 h via shake cultivation, and the biomass was weighed and compared.

Constructions for plasmids

For the construction of complementary *cnaA/cnaB*, p-zero-hph-cnaA plasmid was generated as follows: the selectable marker *hph* from PAN7-1 was amplified by PCR using the primers hph-F and hph-R and then cloned into the pEASY-Blunt vector (TransGen Biotech), generating the plasmid p-zero-hph. The primers *cnaA/cnaB-notI-F* and *cnaA/cnaB-notI-R* were used to generate a fragment that includes the promoter sequence, the complete ORF, and the 3'UTR of *cnaA/cnaB*. This fragment was then cloned into the *NotI* site of p-zero-hph to generate the plasmid p-zero-hph-cnaA/cnaB.

For measuring the calcium level in the cytosol and mitochondria, the plasmids pAMA1- P_{gpdA} -Aeq and pAMA1- P_{gpdA} -mt-Aeq were generated as follows: PCR was performed using the primers Ama1-BamHI-gpd-F and Ama1-BamHI-trpC-R to amplify P_{gpdA} -Aeq- T_{trp} and P_{gpdA} -mt-Aeq- T_{trpC} fragments from the existing plasmids pAEQS1-15 and pAEQ_m (Greene, Cao, Schanne, & Bartelt, 2002; Nelson et al., 2004; Song, Liu, et al., 2016). The fragments were subcloned into the *BamHI* site of the plasmid prg3-AMAI-*NotI* to generate pAMA1- P_{gpdA} -Aeq and pAMA1- P_{gpdA} -mt-Aeq, respectively.

For monitoring the calcium level in the vacuole, the plasmid pAMA1- P_{gpdA} -CpyA-Aeq was generated as follows: PCR was performed using the primers *Clal*-cpyA-R and *Clal*-cpyA-F to generate a *cpyA*' ORF fragment. The fragment was subcloned into the *Clal* site of pBARGPE, which has a P_{gpdA} promoter in front of its *Clal* site, to generate the plasmid pBARGPE- P_{gpdA} -cpyA. The P_{gpdA} -cpyA fragment was amplified from the pBARGPE- P_{gpdA} -cpyA with Ama1-BamHI-gpd-F and CpyA-linker-R primers and then fused with Aeq-TrpC fragment generated using Linker-Aeq-F

442 and Ama1-BamHI-trpC-R to yield fusion fragment P_{gpdA}-cpyA-Aeq. The pAMA1-P_{gpdA}-CpyA-Aeq
443 plasmid was generated by ligating P_{gpdA}-cpyA-Aeq into prg3-AMAI-NotI.

444 pAMA1-P_{gpdA}-CpyA-GFP/RFP was generated as follows: Fusion PCR using primers
445 Ama1-BamHI-gpd-F and Ama1-BamHI-GFP/RFP-R, was performed to fuse the P_{gpdA}-cpyA with
446 GFP/RFP fragment amplified by primers Linker-GFP/RFP-F and Ama1-BamHI-GFP/RFP-R. Then,
447 the fusion fragment was ligated into the *Bam*HI site of the plasmid prg3-AMAI-NotI, generating
448 pAMA1-P_{gpdA}-CpyA-GFP/RFP.

449 For indicating the vacuole, the plasmid pAMA1-P_{gpdA}-CpyA-GFP/RFP was generated as follows:
450 Fusion PCR using primers Ama1-BamHI-gpd-F and Ama1-BamHI-GFP/RFP-R, was performed to
451 fuse the P_{gpdA}-cpyA with GFP/RFP fragment amplified by primers Linker-GFP/RFP-F and
452 Ama1-BamHI-GFP/RFP-R. Then, the fusion fragment was ligated into the *Bam*HI site of the
453 plasmid prg3-AMAI-NotI to generate pAMA1-P_{gpdA}-CpyA-GFP/RFP.

454 For visualization of cell nucleus, the pAMA1-P_{gpdA}-RFP-H2A was generated as follows: The
455 P_{gpdA}-RFP-H2A fragment was amplified from the pBARGPE-P_{gpdA}-RFP-H2A with primers
456 Ama1-BamHI-gpd-F and Ama1-BamHI-trpC-R and then was subcloned into the *Bam*HI site of the
457 plasmid prg3-AMAI-NotI to generate pAMA1-P_{gpdA}-RFP-H2A.

458 For visualization of ER, the plasmid pAMA1-P_{gpdA}-erg11A-GFP was generated as follows: using
459 primers Ama1-BamHI-gpd-F and Erg11A-R, the P_{gpdA}-erg11A fragment was amplified from the
460 CX23 (Song, Zhai, et al., 2016). Using primers Erg11A-GFP-F and Ama1-BamHI-GFP-R, the GFP
461 was amplified. Then, P_{gpdA}-erg11A and GFP were fused together with primers Ama1-BamHI-gpd-F
462 and Ama1-BamHI-GFP-R. Subsequently, the fusion fragment was subcloned into the *Bam*HI site
463 of the plasmid prg3-AMAI-NotI to generate pAMA1-P_{gpdA}-erg11A-GFP.

For visualization of mitochondria, the plasmid pAMA1-MrsA-RFP was generated as follows:
Using primers Ama1-BamHI-MrsA-F and MrsA-R, the MrsA fragment containing *mrsA* promoter and ORF without stop codon was amplified from the *A. fumigatus* genomic DNA (gDNA). The RFP was amplified with primers MrsA-RFP-F and Ama1-BamHI-RFP-R. Then, MrsA fragment and RFP were fused together with primers Ama1-BamHI-MrsA-F and Ama1-BamHI-RFP-R. Subsequently, the fusion fragment was subcloned into the *Bam*HI site of the plasmid prg3-AMAI-NotI to generate pAMA1-MrsA-RFP.

For visualization of Golgi apparatus pAMA1-P_{gpdA}-mRFP-PH^{OSBP} was generated as follows: The pleckstrin homology domain of the human oxysterol binding protein (PH^{OSBP}) was PCR-amplified with primers gpdA-RFP-F and BamHI-PH^{OSBP}-R and then fused with P_{gpdA} promoter (PCR-amplified with Ama1-BamHI-gpd-F and Gpd-R) using primers Ama1-BamHI-gpd-F and BamHI-PH^{OSBP}-R. The fusion fragment was subcloned into *Bam*HI site of the plasmid prg3-AMAI-NotI to pAMA1-P_{gpdA}-mRFP-PH^{OSBP}.

For measuring the level of autophagy, plasmid pAMA1-P_{gpdA}-GFP-Atg8 overexpressing GFP-Atg8 was generated as follows: PCR, using the joint primers Ama1-BamHI-gpd-F/Gpd-GFP-F/Atg8-F and Gpd-R/Atg8-GFP-R/Ama1-BamHI-Atg8-R, were performed to generate P_{gpdA} promoter/GFP/Atg8(ORF) fragments. Next, the fusion fragments P_{gpdA}-GFP-Atg8 were generated by fusion PCR with primers Ama1-BamHI-gpd-F and Ama1-BamHI-Atg8-R. The fusion fragments were subcloned into the *Bam*HI site of the plasmid prg3-AMAI-NotI to generate pAMA1-P_{gpdA}-GFP-Atg8. The plasmid pAMA1-P_{atg8}-GFP-Atg8 expressing GFP-Atg8 under *atg8* native promoter was generated as follows: PCR, using the joint primers Ama1-Bam1-atg8(p)-F/Atg8(p)-GFP-F/ and Atg8-promoter-R/Ama1-BamHI-Atg8-R, was

used to generate *atg8* promoter/GFP-Atg8 fragments. Next, the two fragments were fused together with primers with primers Ama1-Bam1-atg8(p)-F and Ama1-BamHI-Atg8-R. The fusion fragment was subcloned into the *Bam*HI site of prg3-AMAI-NotI to generate pAMA1-P_{atg8}-GFP-Atg8.

For assessing the co-localization of CpyA-RFP and GFP-Atg8, the plasmid pAMA1-P_{gpdA}-CpyA-RFP-P_{gpdA}-GFP-Atg8 was generated as follows: The fragment P_{gpdA}-CpyA-RFP/P_{gpdA}-GFP-Atg8 was amplified with primers pairs Ama1-BamHI-gpd-F and TAA-RFP-R/RFP-gpdA-F and Ama1-BamHI-Atg8-R. Next, the two fragments were subcloned into prg3-AMAI-NotI with the One Step Cloning Kit (Vazyme, C113) forming pAMA1-P_{gpdA}-CpyA-RFP-P_{gpdA}-GFP-Atg8.

For overexpressing *pmcA/pmcB*, plasmid pBARGPE-hph-P_{gpdA}-pmcA/pmcB overexpressing *pmcA/pmcB* were generated as follows: PCR, using the joint primers Clal-pmcA/pmcB-F and Clal-pmcA/pmcB-R, were used to generate pmcA/pmcB fragment that include the complete ORF and 3'UTR of the indicated genes. Next, the three fragments were, respectively, subcloned into the *Clal* site of the plasmid to generate overexpression plasmids pBARGPE-hph-P_{gpdA}-pmcA/pmcB, which contain a selective marker gene *hph* to resist hygromycin.

For amplifying the repair template gfp-hph, the plasmid p-zero-gfp-hph was generated as follows: the selectable marker *hph* was amplified by PCR using the primers HPH-F and HPH-R, and then cloned into the pEASY-Blunt vector, generating a plasmid p-zero-hph. Primers NOTI-gfp-F and NOTI-gfp-R were used to generate a GFP fragment. This fragment was then cloned into the *NotI* site of p-zero-hph to generate p-zero-gfp-hph.

507 For amplifying the repair template $\text{ptrA-P}_{\text{gpdA}}/\text{ptrA-P}_{\text{gpdA}}\text{-gfp}$, the plasmid $\text{p-zero-ptrA-P}_{\text{gpdA}}\text{-gfp}$
 508 was generated as follows: the selectable marker *ptrA* was amplified by PCR using the primers
 509 *ptrA-F* and *ptrA-R*, and then cloned into the pEASY-Blunt vector, generating a plasmid p-zero-ptrA .
 510 Next, the plasmid was linearized with primer *zero-F* and *zero-R*. PCR, using primers
 511 $\text{zero-P}_{\text{gpdA}}\text{-F}/\text{P}_{\text{gpdA}}\text{-GFP-F}$ and $\text{P}_{\text{gpdA}}\text{-R}/\text{zero-GFP-R}$, were used to amplify P_{gpdA} promoter/gfp
 512 fragments. The two fragments were fused with primers *zero-P_{gpdA}-F* and *zero-GFP-R*, generating
 513 $\text{P}_{\text{gpdA}}\text{-gfp}$ fragment. Finally, the fusion fragment was subcloned into the linearizing p-zero-ptrA ,
 514 yielding the plasmid $\text{p-zero-ptrA-P}_{\text{gpdA}}\text{-gfp}$.

515 For amplifying the repair template $\text{hph-P}_{\text{gpdA}}$, the plasmid $\text{p-hph-P}_{\text{gpdA}}$ was generated as
 516 follows: PCR, using primer *NotI-P_{gpdA}-F* and *NotI-TtrpC-R*, was used to amplify *gpdA* promoter
 517 (P_{gpdA}). Then, the *gpdA* promoter was ligated into the *NotI* site of p-zero-HPH , generating
 518 $\text{p-hph-P}_{\text{gpdA}}$.

519 For expressing the N-terminus (300 aa) of CchA, the plasmid $\text{pAMA1-P}_{\text{gpdA}}\text{-GFP-CchA}^{\text{N300}}$ was
 520 generated as follows: The $\text{P}_{\text{gpdA}}\text{-GFP}$ fragment was obtained by PCR with primers,
 521 *Ama1-BamHI-gpd-F* and *CchA-GFP-R* from $\text{p-zero-ptrA-P}_{\text{gpdA}}\text{-gfp}$; The $\text{CchA}^{\text{N300}}$ fragment
 522 containing 900 bp of *cchA* ORF 5' was amplified by PCR with primer *CchA-F* and
 523 *Ama1-BamHI-CchA^{N300}-R*. Then, the two fragments were subcloned into *prg3-AMAI-NotI* with the
 524 One Step Cloning Kit (Vazyme, C113), generating the plasmid $\text{pAMA1-P}_{\text{gpdA}}\text{-GFP-CchA}^{\text{N300}}$.

525 For live animal bioluminescence imaging, the plasmid $\text{pBARGPE-pyr4-P}_{\text{gpdA}}\text{-Luc}$ expressing
 526 Luciferase was generated as follows: PCR, using the joint primers *Clal-Luc-F* and *Clal-Luc-R*,
 527 were used to generate *luc* (Luciferase gene) ORF. Next, the *Luc* fragments was subcloned into the
 528 *Clal* site of the plasmid pBARGPE-pyr4 , yielding $\text{pBARGPE-pyr4-P}_{\text{gpdA}}\text{-Luc}$.

All the primers and primer annotations were given in supplementary data Table S2.

Gene-editing with MMEJ-CRISPR in *A. fumigatus*, transformation and strain verification.

For editing the *A. fumigatus* gene, the MMEJ-CRISPR system was used as described in our previous published papers (Zhang & Lu, 2017; Zhang, Meng, Wei, & Lu, 2016). sgRNA targeted to the appropriate site of the target gene was synthesized *in vitro* using the MEGAscript T7 Kit (Life Technologies, AM1333). The corresponding repair template (DNA) with microhomology arms was amplified by PCR. Then, the repair template fragments and sgRNA were cotransformed into a Cas9-expressing *A. fumigatus* recipient strain. The primers and annotations for sgRNAs and repair templates are listed in Table S2. Transformation procedures were performed as previously described. Transformants were selected in medium lacking uridine or uracil or in the presence of 150 µg/ml hygromycin B (Sangon) or 0.1 µg/ml pyrithiamine (Sigma). For the recycling usage of the selectable marker *pyr4*, 1 mg/mL 5-FOA was used for screening recipient strains. All primers used are listed in the supplementary data Table S2. All transformant isolates were verified by diagnostic PCR analysis using mycelia as the source of DNA. Primers were designed to probe upstream and downstream of the expected cleavage sites as labeled in Figure S1D.

Microscopy observation

For microscopic observation of hyphae morphology, fresh conidia were inoculated onto sterile glass coverslips overlaid with 1 mL of liquid glucose media with or without 10 mM CaCl₂. Strains were cultivated on the coverslips at 37°C for 14 h before observation. The coverslips with hyphae were gently washed with PBS three times. Differential interference contrast (DIC) and green/red

551 fluorescent images of the cells were collected with a Zeiss Axio Imager A1 microscope (Zeiss,
552 Jena, Germany). To observe the fluorescence localization of GFP/RFP-tagged proteins, strains
553 were grown in liquid medium at 37°C with shaking at 200 rpm for indicated time, and then the
554 mycelium pellet sandwiched between the microscope slide and coverslips was observed directly
555 by fluorescence microscopy.

556

557 **Measurement of the free Ca²⁺ concentration ([Ca²⁺])**

558 Measurement of the free Ca²⁺ concentration was performed as described previously with some
559 modifications (*Nelson et al., 2004; Y. Zhang et al., 2016*). The strains expressing
560 Aeq/Mt-Aeq/CpyA-Aeq were cultured for 2 days to form fresh spores. The spores were filtered
561 through nylon cloth and washed 10 times in distilled deionized water. One million (10⁶) spores in
562 100 µL liquid MM were inoculated into each well of a 96-well microtiter plate (Thermo Fischer). The
563 plate was incubated at 37°C for 24 h. The medium was then removed gently with a pipette, and the
564 cells in each well were washed twice with 150 µL PGM (20 mM PIPES pH 6.7, 50 mM glucose, 1
565 mM MgCl₂). Aequorin was reconstituted by incubating mycelia in 100 µL PGM containing native
566 coelenterazine (2.5 µM) (Sigma-Aldrich, C-7001) at 4°C for 4 h in the dark. Then, mycelia were
567 washed twice with 150 µL PGM and allowed to recover to room temperature for 1 h.
568 Luminescence was measured with an LB 960 Microplate Luminometer (Berthold Technologies,
569 Germany), which was controlled by a dedicated computer running the MikroWin 2000 software. At
570 the 20-s time point of luminescence reading, 10 mM CaCl₂ was applied as a stimulant. At the end
571 of each experiment, the active aequorin was completely discharged by permeabilizing the cells
572 with 20% (vol/vol) ethanol in the presence of an excess of calcium (3 M CaCl₂) to determine the

total aequorin luminescence of each culture. The conversion of luminescence (relative light units [RLU]) into $[Ca^{2+}]$ was performed with using Excel 2019 software (Microsoft). Input data were converted using the following empirically derived calibration formula: $pCa = 0.332588 (-\log k) + 5.5593$, where k is luminescence (in RLU) s^{-1} /total luminescence (in RLU).

***In vitro* phosphorylation analysis.**

GFP-CchA^{N300}-expressing *A. fumigatus* were grown in liquid MM for 36 with shaking at 200 rpm. Then, the mild buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.01% Triton X-100, 1 mM DTT, 1 mM PMSF, and 1:100 protease inhibitor cocktail) was used to extract the total proteins. The GFP-CchA were enriched with a GFP-Trap kit (ChromoTek). After enrichment, samples were separated on 10% SDS-PAGE. The gel bands corresponding to the targeted protein GFP-CchA were excised from the gel. The gel pieces were destained 3 times with 125 mM NH_4HCO_3 /50% acetonitrile. Then, proteins were reduced with 10 mM DTT/125 mM NH_4HCO_3 at 56°C for 30 min and alkylated with 55 mM iodoacetamide/125 mM NH_4HCO_3 for 30 min in the dark. After washing twice with 50% acetonitrile, the gel pieces were dried in a vacuum centrifuge. Then, 200 ng trypsin in 25 mM NH_4HCO_3 was added, and the digestion was maintained at 37°C overnight. After digestion, 0.5% formic acid/50% acetonitrile was added to extract peptides from the gel pieces. The extraction procedure was repeated, and the three extracts were combined and dried by vacuum centrifugation. The peptide samples were dissolved in 2% acetonitrile/0.1% formic acid and analyzed using a TripleTOF 5600+ mass spectrometer coupled with the Eksigent nanoLC System (SCIEX, USA). Peptide was loaded onto a C18 trap column (5 μ m, 100 μ m×20 mm) and eluted at 300 nL/min onto a C18 analytical column (3 μ m, 75 μ m×150 mm) over a 60-min

gradient. The two mobile phases were buffer A (2% acetonitrile/0.1% formic acid/98% H₂O) and buffer B (98% acetonitrile/0.1% formic acid/2% H₂O). For information dependent acquisition (IDA), survey scans were acquired in 250 ms, and 40 product ion scans were collected in 50 ms/per scan. MS1 spectra were collected in the range 350-1500 m/z, and MS2 spectra were collected in the range of 100-1500 m/z. Precursor ions were excluded from reselection for 15 s. Phosphorylation site identification was achieved by searching the obtained MS spectra files using Mascot Daemon software.

Proteomics

The Proteomics experiment was performed at Hangzhou PTM Biolabs Co., Ltd. as a commercial service. In brief, lysis buffer (8 M urea, 1% Triton-100, 10 mM dithiothreitol, and 1% Protease Inhibitor Cocktail (Calbiochem)) was used to extract the total protein. Proteins were digested with trypsin (Promega) to be peptides and then processed according to the manufacturer's protocol for TMT kit. The labeled tryptic peptides were fractionated by HPLC using a Thermo Betasil C18 column (5 μm particles, 10 mm ID, 250 mm length). After fractioning, the tryptic peptides were analyzed with LC-MS/MS system. The MS/MS data processing were performed using Maxquant search engine (v.1.5.2.8). Tandem mass spectra were searched against *Aspergillus* UniProt database concatenated with reverse decoy database. Proteins with change ratios significantly different from general protein variation ($1.50 < \Delta cnaA/wild\text{-}type < 0.50$) were analyzed for GO terms and KEGG pathway analysis using the UniProt-GOA database (<http://www.ebi.ac.uk/GOA/>) and the KEGG database, respectively.

Virulence Assay

For *G. mellonella* infection model, *G. mellonella* larva, in groups of 10, were injected through one of the hind pro-legs with 10 μ l of PBS containing 8×10^7 conidia of the respective strain. Untreated larvae and larvae injected with 10 μ l of PBS served as controls. Larvae were incubated at 37°C in the dark and monitored daily for up to 10 days. The significance of survival data was evaluated using Kaplan-Meier survival curves and analyzed with the log-rank (Mantel Cox) test on GraphPad Prism software. Differences were considered significant at p values < 0.05 .

For mouse infection model challenged with *A. fumigatus* conidia, ICR mice (6–8 weeks old, male, 25 ± 5 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The mice were randomized into four groups ($n = 10$ each). Mice were immunosuppressed on day -3 and -1 with cyclophosphamide (150 mg/kg) and on day -1 with hydrocortisone acetate (40 mg/kg). On day 0, mice were anesthetized with 1% pentobarbital sodium (10 ml/kg) and infected intratracheally with a 50- μ L slurry containing 5×10^6 conidia or 50 μ L of PBS as the control according to previously described methods (REF Li et al. (2014) and Zhang et al. (2015)). Cyclophosphamide (150 mg/kg) was injected every 3 days after infection to maintain immunosuppression. Survival was monitored two weeks after inoculation. Lung tissue was processed for histology, and Grocott's methenamine silver stain and periodic acid-Schiff (PAS) staining were performed. For mouse infection model challenged with *A. fumigatus* hyphae, the mycelia of each strain were homogenized into hyphae fragments using tissue grinding and filtered with lens-cleaning paper in sterile normal saline. The optical density at 600 nm of the 10-fold dilutions of each group were finally adjusted to 0.5. The ICR mice described as above (4 groups, $n=10$ each) were immunosuppressed on day -3 and -1 with cyclophosphamide (150 mg/kg). On

day 0, mice were anesthetized with pentobarbital sodium and then infected intratracheally with a 50- μ l hyphal suspension. Cyclophosphamide (150 mg/kg) was injected every 3 days to maintain immunosuppression. Survival was monitored 10 days after inoculation. To directly monitor the infection status in the hyphal infection model, luciferase-expressing *A. fumigatus* strains were used to infect mice, and live mouse bioluminescence imaging was performed. The mice (4 groups, n=5 each) were immunosuppressed and infected as in the above description. The infected mice were introduced intraperitoneally with 100 μ l D-luciferin in PBS (33.3 mg/ml) 10 min before bioluminescence imaging. Mice were anesthetized using 2.5% isoflurane with a constant flow and then placed in an IVIS Lumina XR system chamber. Scanning was performed on day 4. The region was cropped in the chest area from each animal, and the total bioluminescence signal intensity of the lung region was extracted and analyzed using Living Image 4.2 (Caliper Life Sciences).

Statistics

Data were given as means $\pm$ SD. The SD was from at least three biological replicates. Statistical significance was estimated with Graphpad Prism 7 using Student's *t*-test or log-rank test. P-values less than 0.05 were considered statistically significant.

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665 **Author contributions**

666 C.Z., Y.Z. and L.L., conceived the study; C.Z., Y.R., H.G., and L.G. performed the experiments;
 667 and C.Z., Y.Z. and L.L. analyzed and interpreted the data and wrote the manuscript with input from
 668 all authors.

670 **Conflict of interest**

671 The authors declare that they have no conflict of interests.

672 **Figures**

673

674 **Figure 1. Calcium is toxic to calcineurin mutants.**

675 **A.** Differential interference contrast (DIC) images of hyphae grown in liquid MM with or without 10
676 mM CaCl_2 at 37°C in stationary culture for 14 h. Scale bar represents 10 μm .

677 **B.** The morphology of mycelial pellets of the indicated strains grown in MM with or without 10 mM
678 CaCl_2 at 37°C for 60 h.

679 C. Quantification of biomass production for the wild-type, $\Delta cnaA$, and $cnaA^R$ complementary
680 strains grown in MM with or without 10 mM CaCl_2 at different time points (60, 72, 84, and 96 h).

681 D. Schematic diagram for determination of calcium toxicity to *A. fumigatus* cells. The wild-type
682 and $\Delta cnaA$ strains were cultured in liquid MM with or without 10 mM CaCl_2 at 37°C for 96 h. Then,
683 the harvested mycelia were homogenized and inoculated into fresh liquid MM for another 48 h at
684 37°C.

685 E. Quantification of biomass production for the wild-type and $\Delta cnaA$ strains pretreated with or
686 without 10 mM CaCl_2 grown in liquid MM at 37°C for 48 h. Statistical significance was determined
687 by Student's *t*-test. ** $p < 0.01$.

Figure 2. Deletion of *cnaA* affects intracellular calcium accumulation in response to extracellular calcium stimuli.

A, B, and D. The linear graphs indicate the real-time $[Ca^{2+}]_c$, $[Ca^{2+}]_{mt}$, and $[Ca^{2+}]_{vac}$ changes in response to calcium stimuli. $[Ca^{2+}]_c$, the free Ca^{2+} concentration in cytosol; $[Ca^{2+}]_{mt}$, the free Ca^{2+}

693 concentration in mitochondria; $[Ca^{2+}]_{vac}$, the free Ca^{2+} concentration in vacuoles. Basal $[Ca^{2+}]$, the
 694 resting level prior to extracellular calcium stimulus, and $[Ca^{2+}]$ amplitude, the difference between
 695 the basal $[Ca^{2+}]$ level and the poststimulatory peak value, are presented. Data are the average of
 696 at least six experiments. Error bars show the standard deviation. Statistical significance was
 697 determined by Student's *t*-test. **p* < 0.05; ***p* < 0.01; ns, not significant.

698 C. The localization of CpyA. The letter V in the DIC image indicates vacuole (Left). The localization
 699 analysis (Middle) and schematic illustration (Right) of PmcA and CpyA. Scale bar represents 5 μm .

Figure 3. The loss of CnaA causes vacuolar fragmentation and *atg2*-related autophagy in the presence of excess calcium.

A. Vacuolar morphology was monitored by fluorescence microscopy using CypA-GFP as vacuolar lumen marker. The indicated strains were grown in liquid MM with or without calcium CaCl₂ for 36 and 60 h. Scale bar represents 5 μ m.

706 B and D. The localization of CpyA-RFP and GFP-Atg8 was used to monitor the autophagy process.
 707 When autophagy is induced, the outer membrane of the autophagosome was fused with the
 708 membrane vacuole, whereas the inner membranes and the cargo were delivered into the vacuolar
 709 lumen for degradation. In this process, the Atg8 protein transferred from the autophagosome to the
 710 vacuole and was cleaved. The GFP/RFP signals in the mycelium pellets were observed after the
 711 indicated strains were cultured in a rotary shaker for 36 or 60 h. Scale bars represent 5 μ m.

712 C and E. Western blotting showed the GFP-Atg8 cleavage of the related strains in MM with or
 713 without 10 mM CaCl_2 for 36 or 60 h. The extent of autophagy was estimated by calculating the
 714 amount of free GFP compared to the total amount of GFP-Atg8 and free GFP. Coomassie blue
 715 staining served as a loading control.

716 F. Quantification of biomass production for the $\Delta cnaA$ and $\Delta cnaA\Delta atg2$ strains grown in MM with
 717 or without 10 mM CaCl_2 at different time points (60, 72, 84, and 96 h).

718 G. Quantification of biomass production for the $\Delta cnaA$ and $\Delta cnaA\Delta atg2$ strains pretreated with or
 719 without 10 mM CaCl_2 grown in liquid MM at 37°C for 48 h. Statistical significance was determined
 720 by Student's *t*-test. ns, not significant.

Figure 4. Deletion of *cchA* alleviates the calcium toxicity-related phenotypes in the $\Delta cnaA$ mutant.

A. Quantification of biomass production for the $\Delta cnaA$ and $\Delta cnaA\Delta cchA$ strains grown in MM with or without 10 mM CaCl_2 at different time points (60, 72, 84 and 96 h).

B. Quantification of biomass production for the $\Delta cnaA$ and $\Delta cnaA\Delta cchA$ strains pretreated with or without 10 mM CaCl_2 grown in liquid MM at 37°C for 48 h. Statistical significance was determined by Student's *t*-test. $**p < 0.01$.

C and D. The comparison of $[\text{Ca}^{2+}]_c$ and $[\text{Ca}^{2+}]_{vac}$ of WT, $\Delta cnaA$ and $\Delta cnaA\Delta cchA$ in resting and dynamic level. $*p < 0.05$; $**p < 0.01$; ns, not significant.

E and F. The co-localization analysis of CpyA-RFP and GFP-Atg8 and Western blotting showed the localization and cleavage of GFP-Atg8 of the related strains in MM with or without 10 mM CaCl_2 for 60 h. Scale bars represent 5 μm .

Figure 5. Deletion of *cchA* in the $\Delta cnaA$ mutant partially restores the virulence in a *Galleria mellonella* infection model but not in a mouse infection model.

A. Survival curves of *G. mellonella* larvae infected with WT, $\Delta cnaA$, and $\Delta cnaA\Delta cchA$. Larvae were infected with 8×10^5 conidia, and PBS-injected larvae served as controls (left panel). Survival curve of mice infected with conidia or hyphae (middle and right panels) of WT, $\Delta cnaA$, and $\Delta cnaA\Delta cchA$ *A. fumigatus*. Statistical analysis between groups were using the log-rank test. * $p < 0.05$; ** $p < 0.01$; ns, not significant.

B. Histopathological sections of lung tissue from sacrificed mice infected with conidia of each strain. Periodic acid-Schiff stain was used to visualize fungal growth. Scale bar represents 50 μm .

744 C. Luciferase signals in mice infected with hyphae were measured with a live animal imaging
745 system 4 days after infection.

Figure 6. Overexpressing P-type Ca^{2+} -ATPase PmcA in the $\Delta cnaA$ mutant significantly rescues calcium toxicity-related phenotypes.

A. The comparison of $[Ca^{2+}]_{vac}$ of $\Delta cnaA$ and $\Delta cnaA^{OE::pmcA}$ in resting and dynamic level.

B and C. Western blots analysis and co-localization analysis of CypA-RFP and GFP-Atg8 were used to estimate the autophagy level in $\Delta cnaA$ and $\Delta cnaA^{OE::pmcA}$ strains. Scale bar represents 5 μm .

D. Quantification of biomass production for the $\Delta cnaA$ and $\Delta cnaA^{OE::pmcA}$ strains grown in the indicated conditions at different time points (60, 72, 84 and 96 h).

755 E. Quantification of biomass production for the $\Delta cnaA$ and $\Delta cnaA^{OE::pmcA}$ strains pretreated with or
 756 without 10 mM CaCl_2 grown in liquid MM at 37°C for 48 h. Statistical significance was determined
 757 by Student's *t*-test. ***p* < 0.01.

Figure 7. *ΔcnaA* mutant shows global fragmentation of nuclei and organelles upon calcium stimuli.

761 A. KEGG pathways enriched in proteins with more than 1.5-fold changes between WT and $\Delta cnaA$.
762 Red and green items indicate upregulated and downregulated pathways, respectively. The KEGG
763 online service tool KAAS (http://www.genome.jp/kaas-bin/kaas_main) was used to annotate
764 protein KEGG database descriptions.

765 B. Selected enzymes related to material degradation in the upregulated set of proteins are
766 presented.

767 C. The organelle morphologies of the wild-type and $\Delta cnaA$ strains grown in liquid MM with or
768 without calcium for 60 h. Scale bar represents 5 μm .

769 D. The organelle morphologies of the wild-type strain grown in liquid MM supplemented with
770 FK506 in the presence or absence of 2.5 mM CaCl_2 for 60 h. A 50 μL sample of 2 mM FK506 was
771 added into 100 ml liquid MM at 0 and 48 h during culture. Scale bar represents 5 μm .

772 E. Quantification of biomass production for the wild-type strain pretreated with or without 10 mM
773 CaCl_2 grown in liquid MM supplemented with FK506 at 37°C for 48 h. Statistical significance was
774 determined by Student's *t*-test. $**p < 0.01$.

775 **Supplement Figures**

777 **Figure S1. Constructions of *cnaA* and *cnaB* deletion mutants in *A. fumigatus* via CRISPR-Cas9**
778 **system.**

779 **A. Schematic illustration of gRNA location and repair template for deleting genes via the**
780 **CRISPR-Cas9 system.**

781 **B. Details of gRNA and the repair template for *cnaA* and *cnaB* deletion.**

C. The colony morphologies of $\Delta cnaA$, $\Delta cnaB$, and wild-type *A. fumigatus*.

D. Diagnostic PCR for *ptrA* integration in the corresponding gene's ORF. Each pair of diagnostic primers was complementary to upstream and downstream sequences flanking the deletion fragment.

Figure S2. Phenotypic characterization of the $\Delta cnaB$ mutant.

A. Differential interference contrast (DIC) images of hyphae grown in liquid MM with or without 10 mM CaCl₂ at 37°C in stationary culture for 14 h. Scale bar represents 10 μ m.

B. The morphology of mycelial pellets of the indicated strains grown in MM with or without 10 mM CaCl₂ at 37°C for 60 h.

C. Quantification of biomass production for the wild-type, $\Delta cnaB$, and $cnaB^R$ complementary strains grown in MM with or without 10 mM CaCl₂ at different time points (60, 72, 84, and 96 h).

Figure S3. mRNA expression analysis of *pmcA* and *pmcB* in the wild-type and $\Delta cnaA$ background strains and calcium-induced $[Ca^{2+}]_{vac}$ transients in the wild-type, OE::*pmcA*, and OE::*pmcB* strains.

A. Transcript levels of *pmcA/B* in WT and OE::*pmcA/B* (*pmcA/B*-overexpressed strains) and in $\Delta cnaA$ and $\Delta cnaA^{OE::pmcA}$ were determined by qRT-PCR. The indicated strains of *A. fumigatus* were incubated in MM for 36 h at 37°C. ** $p < 0.01$.

B. Comparison of resting and dynamic $[Ca^{2+}]_{vac}$ levels in the wild-type and OE::*pmcA/B* strains. Statistical significance was determined by Student's *t*-test. * $p < 0.05$; ** $p < 0.01$.

Figure S4. Fluorescence microscope observation and Western blots were used to examine the cleavage and localization of GFP-Atg8 under the control of the native *atg8* promoter (A and B) or the *gpdA* promoter (C). WT and $\Delta cnaA$ were cultured in MM with or without various divalent ions (10 mM CaCl_2 , 10 mM MgCl_2 , 10 mM ZnCl_2 , 2 mM FeCl_2 , or 0.2 mM CuCl_2) for 60 h. Scale bar represents 5 μm .

Figure S5. Growth morphologies of the $\Delta cnaA\Delta cchA$ mutant.

A. DIC images of hyphae grown in liquid MM with or without 10 mM CaCl_2 at 37°C for stationary culture 14 h. Scale bar represents 10 μm .

B. Colony morphologies of WT, $\Delta cnaA$ and $\Delta cnaA\Delta cchA$ on solid MM or MM plus 10 mM CaCl_2 .

The 1×10^4 conidia were inoculated onto solid media for 48 h at 37°C. Statistical significance was determined by Student's *t*-test. Statistical significance was determined by Student's *t*-test. $**p < 0.01$.

Figure S6. The transcript levels of *pmcA/B/C*, *vcxA/B/C/D/E*, and *yvcA* in the wild-type and $\Delta cnaA$ strains grown in MM for 36 h. Statistical significance was determined by Student's *t*-test. * $p < 0.05$; ** $p < 0.01$; ns, not significant.

Figure S7. The enriched GO terms in the upregulated (A) and downregulated (B) proteins with greater than 1.5-fold changes in the $\Delta cnaA$ mutant compared to the wild-type. GO annotation was derived from the UniProt-GOA database (<http://www.ebi.ac.uk/GOA/>).

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