Protein Kinase C Overexpression Suppresses Calcineurin-Associated Defects in Aspergillus nidulans and Is Involved in Mitochondrial Function

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Abstract

In filamentous fungi, intracellular signaling pathways which are mediated by changing calcium levels and/or by activated protein kinase C (Pkc), control fungal adaptation to external stimuli. A rise in intracellular Ca²⁺ levels activates calcineurin subunit A (CnaA), which regulates cellular calcium homeostasis among other processes. Pkc is primarily involved in maintaining cell wall integrity (CWI) in response to different environmental stresses. Cross-talk between the Ca²⁺ and Pkc-mediated pathways has mainly been described in Saccharomyces cerevisiae and in a few other filamentous fungi. The presented study describes a genetic interaction between CnaA and PkcA in the filamentous fungus Aspergillus nidulans. Overexpression of pkcA partially rescues the phenotypes caused by a cnaA deletion. Furthermore, CnaA appears to affect the regulation of a mitogen-activated kinase, MpkA, involved in the CWI pathway. Reversely, PkcA is involved in controlling intracellular calcium homeostasis, as was confirmed by microarray analysis. Furthermore, overexpression of pkcA in a cnaA deletion background restores mitochondrial number and function. In conclusion, PkcA and CnaA-mediated signaling appear to share common targets, one of which appears to be MpkA of the CWI pathway. Both pathways also regulate components involved in mitochondrial biogenesis and function. This study describes targets for PkcA and CnaA-signaling pathways in an A. nidulans and identifies a novel interaction of both pathways in the regulation of cellular respiration.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All the microarray hybridization data is at: [GSE48535](http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE48535).

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Introduction

Cellular responses to environmental stimuli are often mediated through G-proteins, which consist of a G-protein coupled receptor (GPCR) and the associated heterotrimeric G-proteins [1]. One such G-protein is phospholipase C which produces the second messengers diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP₃) from the cell membrane phospholipid phosphatidylinositol 4,5-bisphosphate. These second messengers subsequently cause an increase in intracellular Ca²⁺ levels [2]. The concentration of intracellular calcium ions (Ca²⁺) serves as a signal for the regulation of many cellular processes and is constantly altered in response to environmental cues and physiological signals [3]. In mammalian cells, a rise in intracellular Ca²⁺ levels causes the activation of the calcineurin phosphatase and the protein kinase C (Pkc) pathways [2]. Protein kinases and phosphatases act as key regulators of signal transduction by adding or removing phosphate groups to their protein targets hence directing the activity, location and function of many proteins [4]. In the filamentous fungus *Aspergillus nidulans*, PkcA was shown to be fundamental to the cell wall integrity (CWI) pathway, is essential for viability, and has been shown to be involved in penicillin production, polarized growth, morphogenesis, septum formation and the apoptosis [5–9]. Crosstalk between Pkc and the unfolded protein response (UPR) or other MAP (mitogen-activated protein) kinase cascades has been observed [10,11].

The MAP-kinase (MAPK) cascades function via the sequential phosphorylation of three protein kinases (MAPKKK, MAPKK, MAPK) resulting in the activation of a multifunctional MAP kinase [12]. MAP kinase phosphorylation cascades are important for relaying, integrating and amplifying intracellular signals and are crucial signaling components involved in many cellular processes [12]. In *Saccharomyces cerevisiae*, MAP kinases control mating, the cellular response to high environmental osmolarity, pseudohyphal development, sporulation of diploid cells and the maintenance of CWI in response to stresses, such as heat stress and...
low osmolarity [13]. In *S. cerevisiae*, the CWI pathway is activated when Pkc1p phosphorylates Bck1p, a MAPKKK, which in turn activates the two MAPKKs, Mkk1p and Mkk2p, which then go on to phosphorylate the MAPK Slt2p. Mkk1p/2p and Slt2p regulate the expression of many downstream protein targets such as cell wall proteins and enzymes involved in cell wall biogenesis [14]. In *A. nidulans*, a similar mechanism operates in the activation of the CWI pathway. The orthologues of *S. cerevisiae* Bck1p and Slt2p, in *A. nidulans*, BcA (MAPKKK) and MpkA (MAPK), were shown to function downstream of PkcA and were involved in the suppression of apoptosis during heat stress [11].

*A. nidulans* PkcA contains a long conserved N-terminal regulatory region consisting of three subdomains (CN1, CN2 and CN3), which interact with cell membranes [15]. The CN3 subdomain has high similarity with the calcium-binding domain of mammalian PKCs, but the lack of an aspartate residue dramatically decreases the affinity for this ion [16]. In *S. cerevisiae*, the PkcA homologue, Pkc1p, is activated by the Ras-like GTPase Rom1p [17] which in turn is activated by the cell membrane stress sensors Wsc1p (cell wall integrity and stress response component) [18] and Mid2 (maturing induced death). These membrane sensors interact with the Rom1p guanine nucleotide exchange factor Rom2 [19]. In contrast to mammalian and *S. cerevisiae* cells, the mechanism of PkcA activation in *A. nidulans* remains unknown.

In filamentous fungi, intracellular Ca²⁺ levels are essential for the regulation of hyphal morphology (branching) and growth (orientation) [20–22]. The two major mediators of Ca²⁺-mediated signaling are the Ca²⁺-binding protein calmodulin (CaM) and the Ca²⁺/calmodulin-dependent calcineurin, a serine/threonine protein phosphatase [23]. Calcineurin consists of a catalytic subunit A and a regulatory subunit B, which through its association renders protein phosphatase [23]. Calcineurin consists of a catalytic subunit A and a regulatory subunit B, which through its association renders protein phosphatase [23]. Calcineurin consists of a catalytic subunit A and a regulatory subunit B, which through its association renders protein phosphatase [23]. Calcineurin consists of a catalytic subunit A and a regulatory subunit B, which through its association renders protein phosphatase [23]. Calcineurin consists of a catalytic subunit A and a regulatory subunit B, which through its association renders protein phosphatase [23]. Calcineurin consists of a catalytic subunit A and a regulatory subunit B, which through its association renders protein phosphatase [23]. Calcineurin consists of a catalytic subunit A and a regulatory subunit B, which through its association renders protein phosphatase [23].

Although an increase in intracellular Ca²⁺ levels does not directly activate protein kinase C in fungi, as is the case in mammalian cells, there is considerable crosstalk between Ca²⁺-signaling pathways and Pkc-mediated signaling. In *S. cerevisiae*, transcriptional regulation of FKS2, which encodes the catalytic subunit of glucan synthase, is governed by a combination of signals mediated by Pkc1p, calcineurin and the Kss1p MAPK pathway components Ste11p and Ste12p, in response to different environmental stimuli [32]. Furthermore, constitutively expressed calcineurin can partially suppress the lysing phenotypes caused by *PKC1* mutations [33]. Similarly, in *Candida albicans* and *Cryptococcus neoforans*, calcineurin was shown to induce FKS1, which encodes a β-1,3-glucan synthase subunit also regulated by the CWI pathway [33].

The presented study provides further evidence for a crosstalk between the calcium- and PkcA-mediated signaling pathways in *A. nidulans*. Overexpression of *pkcA* in a ΔcnaA background partially suppressed the phenotypic effects caused by the *cnaA* deletion. Furthermore, PkcA seemed to be involved in maintaining intracellular calcium homeostasis through controlling the expression of genes encoding mitochondrial components. This work clearly states the involvement of protein kinase C in various calcium-regulated processes in a filamentous fungus.

**Results**

**Genetic interaction between *pkcA* and *cnaA***

The deletion of the *A. nidulans* calcineurin phosphatase subunit A (*CnaA*) resulted in severe growth and conidiation defects, increased branching and septation [34], while both PkcA and CnaA are involved in maintaining cell wall integrity [32] [33,36]. Therefore, a connection between these two proteins may exist. Hence, the *alcA::pkcA* ΔcnaA strain was constructed by sexually crossing an *alcA::pkcA* strain (in which the *pkcA* gene was placed under the regulatory control of the *alcA* promoter) with a ΔcnaA strain. Transcription of *alcA* is repressed in the presence of glucose, derepressed in the presence of glycerol and induced to high levels in the presence of ethanol or L-threonine [37]. The *pkcA* mRNA accumulation is increased about 3 to 4-fold when *alcA::pkcA* and *alcA::pkcA* ΔcnaA growth in 2% glycerol+100 mM threonine was compared to glucose 2% for both, respectively (Figure 1). The *alcA::pkcA* ΔcnaA strain showed a severe growth defect in the presence of glucose when compared to the wild-type strain, and worse than the ΔcnaA strain (Figure 2A). Growth of the *alcA::pkcA* ΔcnaA strain on solid media was partially restored in the presence of glycerol and glycerol plus threonine, when compared to the ΔcnaA strain (Figure 2A). The observed phenotypes were confirmed by measuring fungal biomass (dry weight) in liquid media containing 2% glycerol plus 100 mM threonine for 12, 24 and 48 hours at 37°C (Figure 2B). Nevertheless, a relation between branch emergence and septum formation may exist, as increased branching was observed in both the ΔcnaA and *alcA::pkcA* ΔcnaA strains (Figure 2B). After 48 hours of growth in the presence of glycerol plus threonine, the dry weight of *alcA::pkcA* ΔcnaA strain was slightly higher than, while the ΔcnaA strain was less than half, of the wild-type strain (Figure 2C).

As previously observed [34], the ΔcnaA germlings contained a higher number of septa (5.9±0.88 septa in 100 germlings) than the wild-type and *alcA::pkcA* ΔcnaA (1.5±0.53 and 1.1±0.32 septa in 100 germlings) strains. The length between septa of the ΔcnaA was shorter than that of the wild-type (Figure 3). The length of the *alcA::pkcA* ΔcnaA strain was restored under the PkcA-overexpression condition, and the restored length was similar to that of the wild-type (Figure 3). Septa are fungal cell walls which divide the non-apical part of the hypha into symmetrical compartments from which new branches can emerge [38]. One hypothesis is that septa function as a spatial cue for branch emergence in some fungi and that localized spikes in calcium levels may also specify branching sites, although substantial evidence for both theories is lacking [39]. Overexpression of *pkcA* restores the number of septa similar to the wild-type (Figure 3). In *A. nidulans*, FITC (fluorescein isothiocyanate)-conjugated WGA (wheat germ agglutinin) can be used to detect chitin that is deposited into the cell wall of the growing hyphal tip [40]. FITC-WGA staining of actively growing hyphae revealed that in approximately half (46%, 100 germlings; Figure 4) of the ΔcnaA hyphae, cell wall extension at the apex was lost when compared to the wild-type strain (100%, 100 germlings, Figure 2B). On the other hand, in the *alcA::pkcA* ΔcnaA strain, cell wall deposition was the same as in the wild-type strain (100%, 100 germlings; Figure 4). These results support the hypothesis of a genetic interaction between the signaling pathways that involve PkcA and CnaA.
MpkA is targeted by calcineurin-mediated signaling

In S. cerevisiae, Pkc1p (the homologue of A. nidulans PkcA), is involved in the regulation of cell wall construction through indirectly phosphorylating the MAP kinase Mpk1p, which in turn activates the transcription factor Rlm1p. This transcription factor regulates the expression of genes whose products are important for cell wall biosynthesis [14]. The A. nidulans rlmA and pkcA are functional orthologs of S. cerevisiae RLM1 and MPK1 [41]. Cell wall stress, as experienced during hyphal extension and the addition of Congo red (CR) to liquid cultures, results in the phosphorylation of MpkA [10] [41]. Hence, the MpkA phosphorylation state in the ΔcnaA and alcA::pkcA ΔcnaA mutant strains was investigated. The wild-type strain contained a basal level of phosphorylated MpkA in the presence of 2% glycerol plus threonine, while the addition of CR resulted in a ∼4-fold increase of MpkA phosphorylation levels (Figures 5A and 5B). In the ΔcnaA strain, MpkA phosphorylation levels were roughly tenfold less than in the wild-type strain and addition of CR did not result in an increase in MpkA phosphorylation levels (Figures 5A and 5B). In agreement with previous results [10], pkcA overexpression increases MpkA phosphorylation levels by around 20%, when compared to the wild-type strain, at both the basal level and upon addition of CR (Figures 5A and 5B). In the alcA::pkcA ΔcnaA strain the amount of MpkA phosphorylation was higher (10- to 60-fold) than in the wild-type and ΔcnaA strains in all conditions tested (Figures 5A and 5B). These results confirm the role of pkcA in the CWI pathway in A. nidulans and demonstrate that its overexpression can partially suppress the growth defects associated with the ΔcnaA strain. In addition, these results also indicate that MpkA is targeted by calcineurin-mediated signaling.

PkcA overexpression maintains intracellular calcium homeostasis in the absence of CnaA

Intracellular calcium homeostasis is mediated through the products of a variety of genes, encoding proteins involved in calcium transport and metabolism, which are regulated by the transcription factor CrzA [29]. Appropriate calcium levels are vital for cell viability, as Ca2+ ions sequester phosphate molecules which can cause abnormal intracellular signaling [42]. Hence, to determine if pkcA overexpression could restore normal intracellular calcium levels in the absence of CnaA, the wild-type, alcA::pkcA, ΔcnaA and alcA::pkcA ΔcnaA strains were treated with different concentrations of CaCl2 for 10 minutes before Calcium Orange fluorophores (COF) were added for 30 min. Calcium orange chelates Ca2+ ions and upon binding exhibits increased fluorescence, allowing for the detection of the amount of calcium present (Life Technologies). The wild-type, alcA::pkcA and alcA::pkcA ΔcnaA strains had reduced intracellular calcium levels after 30 minutes of COF addition (Figure 6). In contrast, the ΔcnaA strain was unable to reduce Ca2+ levels after 30 minutes of COF addition and had approximately 2.5 times more intracellular Ca2+ than the wild-type, alcA::pkcA and alcA::pkcA ΔcnaA strains (Figure 6). These results indicate that pkcA overexpression can contribute to maintaining intracellular Ca2+ homeostasis in the absence of CnaA-associated signaling.

Induction of alcA::pkcA in the ΔcnaA background modulates transcription of calcium controlled processes

Microarray hybridizations were utilized to investigate in more detail which genes were being influenced by pkcA overexpression in the alcA::pkcA ΔcnaA strain, when compared to the ΔcnaA strain (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE48535). Both ΔcnaA and alcA::pkcA ΔcnaA strains were grown in minimal medium (MM) in the presence of glycerol plus threonine for 24 h and 48 h. The wild-type strain was used as a control. After COF addition and had approximately 2.5 times more intracellular Ca2+ than the wild-type, alcA::pkcA and alcA::pkcA ΔcnaA strains (Figure 6). These results indicate that pkcA overexpression can contribute to maintaining intracellular Ca2+ homeostasis in the absence of CnaA-associated signaling.

**Figure 1.** The alcA::pkcA ΔcnaA strain has increased pkcA mRNA accumulation. The wild-type, alcA::pkcA, ΔcnaA, and alcA::pkcA ΔcnaA strains were grown for 16 hours in minimal medium supplemented either with glucose 2% (G), glycerol 2% (Gly) or glycerol 2% plus 100 mM threonine (G+T), RNA extracted and RT-qPCR performed for pkcA.

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genes allowed determination of the functions of the proteins they encode and enabled the identification of overrepresented ($p<0.05$) gene ontologies (GO) (Tables S1, S2, S3). As shown in Table 1, the majority of these genes encoded proteins involved in cytoskeletal organization and trafficking, and, in particular, in mitochondria-related functions including oxidative phosphorylation. Genes encoding components of complex III of the respiratory chain and mitochondrial ribosomes were overrepresented within this dataset. The remaining 611 genes had increased expression levels in the $\Delta cnaA$ when compared to the $alcA::pkcA$ $\Delta cnaA$ strain (Figure 7, clusters 1, 3 and 4). However, upon FetGOat analysis, only two GO terms were overrepresented and both described proteasomal components (GO:0000502 and GO:0008540; Table S4).

Overexpression of $pkcA$ increases the mitochondria in the $\Delta cnaA$ genetic background

The microarray data showed a difference in the expression of genes which encoded for proteins involved in mitochondrial functions, between the $\Delta cnaA$ and $alcA::pkcA$ $\Delta cnaA$ strains. Subsequently, to validate these results, mitochondrial mass was evaluated. Protein extracts from the wild-type, $\Delta cnaA$, $alcA::pkcA$ and $alcA::pkcA$ $\Delta cnaA$ strains, when grown in MM supplemented with glycerol plus threonine, were incubated with anti-cytochrome $c$ antibody (Figure 8A). Cytochrome $c$ is a small heme protein found within the cristae of the inner mitochondrial membranes and is an essential component of the electron transport chain [43]. The abundance of cytochrome $c$ was less in the $\Delta cnaA$ strain than the wild-type and $alcA::pkcA$ strains (Figure 8B). Upon $pkcA$ overexpression, in the $alcA::pkcA$ $\Delta cnaA$ strain, cytochrome $c$ levels were approximately ten times that observed in the $\Delta cnaA$ strain and were also higher than those observed in the wild-type strain (Figure 8B). These results were in agreement with the fluorescent microscopy data and flow cytometric analyses (FCA), where hyphal mitochondria were stained with Nonyl Acridine Orange, observed under the microscope and subsequently counted, revealing a decrease in mitochondrial mass in $\Delta cnaA$ strain (Figures 9 and 10A). In addition, the rate of oxygen...
consumption was measured in all strains. The ΔcnaA strain consumed approximately 30% less oxygen than the wild-type strain, whereas pkeA overexpression in the alcA::pkeA ΔcnaA strain restored normal levels of oxygen consumption, similar to the wild-type strain (Figure 10B). These data indicate that pkeA

Figure 3. Overexpression of pkeA in a ΔcnaA background rescues aberrant septa formation in a ΔcnaA background. Wild-type, ΔcnaA and alcA::pkeA ΔcnaA strains were grown in minimal medium supplemented with 2% (w/v) glycerol plus 100 mM threonine for 16 hours at 30 °C before being fixed for 30 minutes at room temperature (RT) and stained with calcofluor white (CFW) for 5 minutes at RT. Mycelial fluorescence was then assessed under the microscope (scale bars indicate 5 μm).

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overexpression restores the number of mitochondria within the fungal cells, allowing normal levels of respiration to take place.

**Discussion**

Cellular signaling governs the cell’s responses to environmental stimuli, thus allowing an organism to adapt to different conditions. Cells have evolved a complex network of signaling pathways mediated by a great number of proteins, such as protein kinases.
Figure 5. Overexpression of pkcA in a ΔcnaA background restores the levels of phosphorylated MpkA. Western blots (A) of the protein crude extract from the wild-type, ΔcnaA, alcA::pkcA and alcA::pkcA ΔcnaA strains, when grown in 2% glycerol plus 100 mM threonine for 16 hours at 30° C before being treated with Congo Red (CR) for 1 hour. Phosphorylated MpkA (49 kDa band) was probed for with anti-MpkA antibodies (B) and
and phosphatases, which add or remove phosphate groups from target proteins, therefore directing their activity, location and function [4]. In mammalian cells, protein kinase C (Pkc) regulates gene transcription, membranes and cell growth, while being activated upon an increase in cellular Ca\(^{2+}\) levels [2]. In fungi, Pkc plays an important role in growth through activating the cell wall integrity (CWI) pathway which is mediated by a MAP (mitogen activated protein) kinase cascade [11] [14]. In S. cerevisiae, cell wall stress sensors activate the Rho GTPase Rho1p which in turn activates Pkc1p that then activates the CWI MAP pathway [17]. In the filamentous fungus A. nidulans, the mechanisms for PkcA activation remain unknown. An increase in intracellular Ca\(^{2+}\) levels activates the calcineurin phosphatase, whose catalytic subunit A, encoded by cnaA in A. nidulans, modulates the transcription of genes involved in maintaining cellular calcium homeostasis [29] [31]. Although crosstalk between the calcium- and Pkc-mediated pathways appears to take place in S. cerevisiae and other filamentous fungi [32,33], knowledge about the interactions of these signaling pathways is limited in Aspergillus species. The genus Aspergillus contains many species of medical and industrial importance, thus elucidating signaling pathways involved in growth in these species could be relevant for many biotechnological applications.

The presented study describes a genetic interaction between the CnaA- and PkcA-mediated signaling pathways in A. nidulans. Previous investigations showed that cnaA is not an essential gene in A. nidulans and A. fumigatus, but that its deletion caused severe defects in apical extension and polarized growth in both fungi [26] [34]. Accordingly, the A. nidulans ΔcnaA strain showed reduced growth in solid and liquid media supplemented with different carbon sources. Furthermore, the cnaA deletion strain had a different number of septa and aberrant apical growth when compared to the wild-type strain. The intracellular localization of Ca\(^{2+}\) gradients participates in the determination of the site of germ tube emergence and maintains the axis of polarized growth [44]. In A. fumigatus, calcineurin localizes to the septum and the catalytic activity of calcineurin subunit A is required for maintaining normal hyphal growth [45]. Deletion of cnaA in A. fumigatus also resulted in abnormal cell wall architecture with decreased β-glucan and increased chitin contents [45]. In other fungi, calcineurin and Pkc were shown to be involved in the regulation of the cell wall constructing enzyme β-glucan synthase.

![Graph](image_url)

**Figure 6.** Overexpression of pkcA in a ΔcnaA background strain enables the cell to return to normal intracellular calcium levels after exposure to high concentrations of CaCl\(_2\). The wild-type, alcA::pkcA, ΔcnaA and alcA::pkcA ΔcnaA strains were grown for 5 hours at 37°C before being exposed to 20 mM, 100 mM and 200 mM of CaCl\(_2\) for 30 minutes. The fluorophoric calcium chelator Calcium Orange was added then added for 30 minutes and fluorescence was assessed with a fluorometer at 579 nm.

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Similar roles for CnaA in cell wall integrity could therefore be considered in *A. nidulans*. Overexpressing *pkcA* in the ΔcnaA genetic background was able to partially rescue the observed growth ΔcnaA phenotypes. PkcA is involved in cell wall construction and maintenance through its involvement in the cell wall integrity pathway via MAPK signaling [11] [14]. In agreement, PkcA overexpression in the ΔcnaA strain restored MpkA (the CWI pathway MAPK) phosphorylation levels similar to the wild-type strain. Another explanation could be that both PkcA and calcineurin signaling affect cell cycle progression. In *S. cerevisiae*, a role for Pkc1p in G2/M cell cycle transition, separate from its role in the regulation of the MAPK pathway, has been previously suggested [46]. This could coordinate cell wall expansion with cell cycle progression. In *Schizosaccharomyces pombe*, calcineurin is activated by the cell cycle checkpoint kinase Cds1 and subsequently dephosphorylates Tip1, a protein involved in polarity factor transport, resulting in cell cycle progression delay [47]. All these results indicate that the CnaA- and PkcA-activated pathways share common targets, which could include MpkA, and in doing so coordinate the integrity of the cell wall during growth and in response to stress. This is not surprising as both proteins mediate signaling pathways involved in maintaining polarized hyphal growth.

Microarrays were utilized to provide an insight into the transcriptional impact of *pkcA* overexpression in the ΔcnaA strain. A comparison of the ΔcnaA and ΔcnaA *pkcA* overexpressing strains with the wild-type strain identified 1,643 genes as being differentially regulated between the two mutant strains, with 1,032 genes being down-regulated and 611 genes being up-regulated in the ΔcnaA strain when compared to *alcA*:Δ*pkcA* ΔcnaA strain. The majority of the up-regulated genes encoded proteasomal components, suggesting an increase in the degradation of misfolded proteins within the ΔcnaA strain. Scrimale *et al*. [48] showed that induction of the CWI pathway in *S. cerevisiae* activated the UPR.
Table 1. Number of genes and the associated functions of the proteins they encode that had restored or elevated expression in the *alcA::pkcA ΔcnaA* strain when compared to the *ΔcnaA* strain.

| GO term                                    | Annotation                                                                 | P-value | sP |
|--------------------------------------------|---------------------------------------------------------------------------|---------|----|
| Mitochondrial components, function and energy conversion |                                                                           |         |    |
| GO:0006122                                 | mitochondrial electron transport, ubiquinol to cytochrome c               | 1.12E-04| 6  |
| GO:0055114                                 | oxidation-reduction process                                               | 5.11E-05| 32 |
| GO:0042773                                 | ATP synthesis coupled electron transport                                  | 2.99E-05| 9  |
| GO:0006626                                 | protein targeting to mitochondrion                                        | 9.24E-07| 17 |
| GO:0032543                                 | mitochondrial translation                                                 | 7.89E-04| 10 |
| GO:0033617                                 | mitochondrial respiratory chain complex IV assembly                        | 3.85E-04| 6  |
| GO:0008535                                 | respiratory chain complex IV assembly                                     | 3.85E-04| 6  |
| GO:0007034                                 | mitochondrial respiratory chain complex IV biogenesis                     | 3.85E-04| 6  |
| GO:0007005                                 | mitochondrion organization                                                | 1.40E-20| 62 |
| GO:0033108                                 | mitochondrial respiratory chain complex assembly                          | 6.05E-07| 10 |
| GO:0007006                                 | mitochondrial membrane organization                                        | 2.93E-06| 13 |
| GO:0042775                                 | mitochondrial ATP synthesis coupled electron transport                     | 2.99E-05| 9  |
| GO:0022904                                 | respiratory electron transport chain                                      | 2.99E-05| 9  |
| GO:0007007                                 | inner mitochondrial membrane organization                                 | 2.99E-05| 9  |
| GO:0000002                                 | mitochondrial genome maintenance                                          | 6.75E-05| 13 |
| GO:0072655                                 | establishment of protein localization in mitochondrion                    | 4.87E-07| 18 |
| GO:0022900                                 | electron transport chain                                                  | 2.99E-05| 9  |
| GO:0006839                                 | mitochondrial transport                                                   | 6.64E-08| 22 |
| GO:0045039                                 | protein import into mitochondrial inner membrane                          | 1.80E-03| 5  |
| GO:0015980                                 | energy derivation by oxidation of organic compounds                       | 1.28E-07| 32 |
| GO:0070585                                 | protein localization in mitochondrion                                     | 4.87E-07| 18 |
| GO:0045333                                 | cellular respiration                                                      | 9.65E-12| 32 |
| GO:0009060                                 | aerobic respiration                                                       | 4.64E-12| 30 |
| GO:0030150                                 | protein import into mitochondrial matrix                                  | 1.72E-05| 10 |
| GO:0006119                                 | oxidative phosphorylation                                                 | 5.19E-04| 12 |
| GO:0006099                                 | tricarboxylic acid cycle                                                  | 7.89E-04| 10 |
| Cytoskeleton-related transport              |                                                                           |         |    |
| GO:0007018                                 | microtubule-based movement                                                | 9.65E-06| 11 |
| GO:0072384                                 | organelle transport along microtubule                                      | 3.99E-06| 11 |
| GO:0031109                                 | microtubule polymerization or depolymerization                            | 2.15E-03| 6  |
| GO:0030473                                 | nuclear migration along microtubule                                        | 7.16E-05| 9  |
| GO:0010970                                 | microtubule-based transport                                               | 3.99E-06| 11 |
| GO:0007017                                 | microtubule-based process                                                 | 2.82E-05| 22 |
| GO:0030705                                 | cytoskeleton-dependent intracellular transport                            | 9.03E-07| 12 |
| GO:0046907                                 | intracellular transport                                                   | 2.29E-07| 91 |
| GO:0017038                                 | protein import                                                            | 1.26E-07| 29 |
| GO:0006810                                 | transport                                                                 | 5.62E-04| 136|
| GO:0071806                                 | protein transmembrane transport                                           | 2.23E-05| 13 |
| GO:0065002                                 | intracellular protein transmembrane transport                             | 2.23E-05| 13 |
| GO:0006886                                 | intracellular protein transport                                           | 3.91E-04| 41 |
| GO:0015031                                 | protein transport                                                         | 8.09E-04| 41 |
| GO:0007097                                 | nuclear migration                                                         | 1.20E-05| 13 |
| Cellular organisation                      |                                                                           |         |    |
| GO:0051656                                 | establishment of organelle localization                                   | 2.15E-05| 23 |
| GO:0034613                                 | cellular protein localization                                             | 9.88E-04| 46 |
| GO:0006605                                 | protein targeting                                                         | 9.38E-04| 36 |
| GO:0006996                                 | organelle organization                                                    | 9.06E-08| 133|
| GO:0051647                                 | nucleus localization                                                      | 1.20E-05| 13 |
(unfolded protein response). The UPR is activated upon detection of misfolded proteins in the endoplasmic reticulum (ER), resulting from high throughput protein secretion causing cellular secretory stress [49]. Activation of the UPR results in the up-regulation of genes encoding proteins which control many cellular processes such as protein folding, cell wall architecture, protein trafficking, lipid biosynthesis and ERAD (ER-associated protein degradation) [48]. It appears that deletion of \textit{cnaA} causes an increase in cellular stresses which subsequently activate the UPR and increase protein degradation. Indeed, Colabardini \textit{et al.}, [10] have previously shown crosstalk between PkcA and the UPR in \textit{A. nidulans} in response to exposure to farnesol.

The majority of the down-regulated genes in the \textit{ΔcnaA} strain, when compared to the \textit{ΔcnaA pkcA}-overexpressing strain, encoded proteins involved in mitochondrial function and energy conversion, cytoskeleton-related transport, cellular organization and metabolism. Denis and Cyert [50] showed that the C2 domain of Pkc1p in \textit{S. cerevisiae} is responsible for targeting the protein to the mitotic spindle, whereas the HR1 and C1 regulatory motifs ensure localization of Pkc1p to the bud tip and cell periphery. \textit{A. nidulans} PkcA::GFP localizes to hyphal apices and growing septa, as well as to the conidigenous spicules of phialides, indicating a role for PkcA in polarized cell wall growth [5].

Calcineurin or calmodulin, activated through an increase in intracellular Ca$^{2+}$ levels, have been shown to localize at the apex, at the Spitzenko¨rper or at the septa during polar growth in \textit{A. nidulans} [51,52] and \textit{A. fumigatus} [45]. The Spitzenko¨rper is a sub-apical structure which coordinates polar growth that associates with and is surrounded by microtubules and actin filaments which shuttle vesicles carrying calcium (to ensure the tip-associated calcium gradient), signaling modules and cell wall components back and forth to theSpitzenko¨rper with the aid of motor proteins [53,54].

### Table 1. Cont.

| GO term                  | Annotation                                             | P-value | sP   |
|--------------------------|--------------------------------------------------------|---------|------|
| GO:0008104 GO:0051648    | protein localization                                    | 1.77E-03| 50   |
| GO:0051641               | establishment of protein localization                  | 6.37E-08| 108  |
| GO:0045184               | establishment of protein localization                  | 9.46E-04| 42   |
| GO:0040023               | establishment of nucleus localization                  | 1.20E-05| 13   |
| GO:0051234               | establishment of localization                          | 1.82E-04| 142  |
| GO:0051649               | establishment of localization in cell                  | 3.23E-08| 99   |
| GO:0303036               | macromolecule localization                             | 1.80E-03| 61   |
| GO:0051179               | localization                                           | 1.01E-04| 152  |
| GO:0051640               | organelle localization                                  | 3.10E-05| 26   |
| GO:0070727               | cellular macromolecule localization                   | 6.01E-04| 48   |
| GO:0071840               | cellular component organization or biogenesis          | 4.68E-07| 205  |
| GO:0071841               | cellular component organization at cellular level       | 1.49E-07| 192  |
| GO:0006364               | rRNA processing                                        | 2.48E-03| 35   |
| GO:0006091               | generation of precursor metabolites and energy         | 6.90E-06| 44   |
| GO:0044085               | cellular component biogenesis                          | 1.53E-03| 92   |
| GO:0016043               | cellular component organization                        | 2.78E-05| 165  |
| GO:0043623               | cellular protein complex assembly                      | 7.16E-05| 25   |
| GO:0071842               | cellular component organization at cellular level       | 9.03E-06| 146  |
| GO:0006744               | ubiquinone biosynthetic process                        | 9.95E-04| 6    |
| GO:0006084               | acetyl-CoA metabolic process                           | 9.86E-04| 11   |
| GO:0046356               | acetyl-CoA catabolic process                           | 7.89E-04| 10   |
| GO:0006743               | ubiquinone metabolic process                           | 9.95E-04| 6    |
| GO:0045426               | quinone cofactor biosynthetic process                  | 9.95E-04| 6    |
| GO:0042375               | quinone cofactor metabolic process                     | 9.95E-04| 6    |
| GO:0031884               | heterotrimeric G-protein complex cycle                 | 7.04E-04| 4    |

\textit{sP} is number of genes in each category. doi:10.1371/journal.pone.0104792.t001
Mitochondria sequester $\text{Ca}^{2+}$ ions, therefore playing an important role in decreasing intracellular $\text{Ca}^{2+}$ levels after it has carried out its signaling function [3]. $\text{Ca}^{2+}$ ions can diffuse readily through the outer membrane of the mitochondria and are then transported through the inner membrane into the mitochondrial cytoplasm through highly selective ion channels and transporters [55]. An increase in mitochondrial $\text{Ca}^{2+}$ levels boosts ATP production which as a consequence increases the concentration of cellular reactive oxygen species (ROS) which can lead to apoptosis [54]. In the presented study of $A. \text{nidulans}$, deletion of calcineurin caused an increase in cytosolic calcium levels, while previous work also showed that calcineurin regulates genes, encoding for proteins involved in calcium homeostasis, via the transcription factor CrzA [31]. It is therefore possible that increased cytosolic calcium levels in the $\Delta \text{cnaA}$ strain resulted in an increased uptake of $\text{Ca}^{2+}$ ions by the mitochondria, resulting in increased mitochondrial death. Upon overexpressing $\text{pkcA}$, mitochondrial number and function was restored. PkcA-mediated signaling is therefore somehow involved in maintaining the mitochondrial copy number. Indeed, overexpression of $\text{pkcA}$ upon exposure to farnesol, increases cell death through increasing metacaspase activity [10].

In summary, the presented study describes an interaction between calcium and protein kinase C signaling pathways.
uridine and uracil, or pyrodoxine (0.5 mg/ml) depending on the genetic background. Solid media was made by adding 2% (w/v) agar to minimal or complete media. Unless otherwise stated, all chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA).

**Mycelial staining and microscopy**

Coverslips were inoculated with the different strains (see results section) in 5 ml of minimal media at 30°C for 16 hours. Hyphae were then fixed [3.7% (v/v) formaldehyde, 50 mM sodium phosphate buffer pH 7.0 (NaH2PO4, Na2HPO4), 0.2% (v/v) Triton X-100] for 30 min at room temperature, rinsed with phosphate buffered saline (PBS: 140 mM NaCl, 2 mM KCl, 10 mM NaHPO4, 1.8 mM KH2PO4, pH 7.4) and incubated for 5 minutes in a 100 ng ml⁻¹ calcofluor white. Coverslips were again washed with PBS for 10 minutes at room temperature and rinsed with distilled water. FITC-WGA-staining was carried out by incubating coverslips in prewarmed (30°C) media containing 5 mg ml⁻¹ FITC-WGA for 5 minutes [56]. Hyphae were fixed and washed as previously described.

Germings were visualized on a Carl Zeiss (Jena, Germany) AxioObserver.Z1 fluorescent microscope equipped with a 100 W HBO mercury lamp, using a 100× magnification oil immersion objective (EC Plan-Neofluar, NA 1.3). Fluorescent and DIC (differential interference contrast) images were captured with an AxioCam camera (Carl Zeiss) and processed using the AxioVision software version 3.1. Further image processing was performed using Adobe Photoshop 7.0 (Adobe Systems Incorporated, CA).

**RNA extraction**

Mycelia were separated from the supernatant using Whatman filter paper. Fungal cell walls were broken through grinding the mycelia under liquid nitrogen and total RNA was extracted using RNeasy Plant Mini Kit (Qiagen) according to manufacturer’s instructions. RNA samples were run on a 2.2 M formaldehyde,

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**Figure 9. Overexpression of pkcA in a ΔcnaA background restores mitochondrial number.** Wild-type, ΔcnaA, alcA::pkcA and alcA::pkcA ΔcnaA germings were grown in minimal medium supplemented with 2% glycerol plus 100 mM threonine for 16 hours at 37°C and stained with nonyl acridine orange for 10 minutes at 37°C. Mycelial fluorescence was subsequently assessed under the microscope (scale bars indicate 5 μm). doi:10.1371/journal.pone.0104792.g009
1.2% (w/v) agarose gel to check RNA quality and quantified on the NanoDrop 2000 Thermo Scientific (Uniscience).

Microarray slides construction and gene expression analysis

Gene expression was compared between *A. nidulans* ΔcnaA and alcA::pkcA ΔcnaA strains when grown for 24 and 48 hours at 37°C in minimal media supplemented with 2% glycerol plus 100 mg/ml threonine, before RNA was extracted. Microarray slide design, sample labeling and hybridization were performed as described previously [56].

The extraction of data from the TIFF files, generated through scanning the microarray slides, was performed using the Agilent Feature Extraction (FE) Software version 9.5.3.1 (Agilent Technologies). This software used the linear Lowess algorithm to subtract the background noise and obtain normalized intensity values. Normalized values were uploaded into the software Express Converter (version 2.1, TM4 platform available at

Figure 10. Overexpression of *pkcA* in a ΔcnaA background restores oxygen consumption. (A) Wild-type, ΔcnaA, alcA::pkcA and alcA::pkcA ΔcnaA germlings were grown in minimal medium supplemented with 2% glycerol plus 100 mM threonine for 16 hours at 37°C and stained with nonyl acridine orange for 10 minutes at 37°C. Mycelial fluorescence was subsequently assessed under the microscope (scale bars indicate 5 μm). Flow cytometric analyses (FCA) is shown. The results are expressed as mean ± SD (standard deviation) of three independent biological replicates and were considered statistically different (*) when *p*<0.05. P-values were determined by a Student t test using GraphPad Prism software version 5 (GraphPad Software). DIC, differential interference contrast; FAU, fluorescent arbitrary units. (B) Oxygen consumption rates of the wild-type, ΔcnaA, alcA::pkcA and alcA::pkcA ΔcnaA strains when grown in minimal medium supplemented with 2% glycerol plus 100 mM threonine for 16 hours at 37°C. doi:10.1371/journal.pone.0104792.g010
Table 2. A. nidulans strains used in this work.

| Strain          | Genotype                              | Reference               |
|-----------------|---------------------------------------|-------------------------|
| TNO2A3 (wild-type) | pyrA4, pyrG89, argB2, ΔlnkUa-argB, cho1 | Nayak et al, 2006 (reference [58]) |
| alcA::pkcA      | pyrG89, pyroA4, alcA::pkcA::pyr4, choA1 | Colabardini et al, 2010 (reference [10]) |
| ΔcnaA           | pyrA4, pyrG89, ΔcnaA-pyro, wA3         | Soniani et al, 2008 (reference [34]) |
| alcA::pkcA ΔcnaA |                                      | This work               |

Mitochondrial mass measurements

Conidia from the different strains (1 × 10^8 ml^−1) were incubated in 50 ml liquid minimal media at 37°C on a reciprocal shaker for 6 hours. Subsequently, conidia were washed with PBS and incubated with 5 nM Nonyl Acridine Orange (NAO; Invitrogen) diluted in PBS plus 5% fetal bovine serum (FBS) for 10 minutes at 37°C. Stained conidia were washed with PBS, resuspended in PBS plus 5% FBS, and then analyzed by flow cytometry. Propidium iodide (0.5 μM) staining of the nucleus was utilized to exclude dead cells. Flow cytometry was analyzed by guava EasyCyte 8 HH (Millipore) using 10,000 acquisitions for each analysis. For cytochrome c measurements, 2 × 10^8 conidia/ml of each strain were inoculated in minimal media for 16 hours at 37°C. After this period, the cultures were filtered and the mycelia washed with 100 ml H_2O, immediately frozen in liquid nitrogen and grinded. For every 0.1 g of dry weight, 1.3 ml HB buffer (150 mM NaCl, 30 mM KCl, 10 mM Na_2HPO_4, pH 7.0) was added. The suspension was centrifuged at 21,000 g at 4°C for 30 minutes. The supernatant was removed and centrifuged again for 10 minutes under the same conditions. After determining protein concentration, samples were prepared for SDS-PAGE as described above. Thirty micrograms of total protein from each sample were loaded into each lane of a 15% SDS-PAGE gel. After separation of the proteins, the gel was blotted onto a pure nitrocellulose membrane (0.2 μm; Bio-Rad) and after being blocked in 5% (w/v) dried milk, the supernatant was loaded. The membranes were incubated with 5 nM Nonyl Acridine Orange (NAO; Invitrogen) for 1 hour at room temperature. The membrane was washed four times with TBS-Tween for 5 minutes and then incubated with a 1:5000 dilution of goat anti-rabbit IgG peroxidase-labeled (KPL) antibody for 1 hour. After being washed, the blot was developed by use of the SuperSignal Ultra chemiluminescence detection system (Pierce) and recorded by the use of Hyperfilm ECL (Amersham Biosciences).

Oxygen uptake measurements

Conidia (1×10^8) were inoculated in 15 ml of minimal medium supplemented with 2% glycerol plus 100 mM threonine for 5 hours at 37°C, 250 rpm. The germlings were harvested by centrifugation and washed twice with cold distilled water. Germlings were then washed three times with 0.7 mM sorbitol, 10 mM HEPES-KOH, pH 7.2 and subsequently kept on ice. Oxygen uptake was measured with a Clark-type electrode fitted to a Gilson oxygraph 1 (Gilson Medical Electronics, Inc.,2 Middleton, WI) in 1.8 ml of buffer containing 0.7 mM sorbitol, 10 mM HEPES-KOH, pH 7.2, 5 mM MgCl_2 and 0.5 mM EGTA at 30°C [57]. The initial solubility of oxygen in the reaction buffer was considered to be 445 ng of O_2 atoms/ml.

Determinant of intracellular calcium levels

To determine intracellular Ca^{2+} levels, wild-type, ΔcnaA, alcA::pkcA and alcA::pkcA ΔcnaA strains were first grown for 5 hours in minimal medium supplemented with 2% (w/v) glycerol plus 100 mM threonine at 37°C. Strains were then treated with 20 mM, 100 mM and 200 mM CaCl_2, for 10 minutes. Mycelia were then washed 3 times with PBS and incubated with 1 μM Calcium Orange fluorophore at 37°C for 30 minutes. Mycelia were washed again and fluorescence was measured using a fluorometer at 549 nm excitation and 579 nm emission, according to manufacturer’s instructions. The experiment was normalized by counting the number of cells in a Neubauer chamber.
Supporting Information

Table S1 FetGOat analysis for genes with reduced mRNA accumulation in A. nidulans ΔcnaA (similar to the wild-type strain) when compared to the Δalca::pkcA ΔcnaA strain.

Table S2 FetGOat analysis for genes with reduced mRNA accumulation in A. nidulans Δalca::pkcA ΔcnaA (similar to the wild-type strain) when compared to the ΔcnaA strain.

Table S3 FetGOat analysis for genes with reduced mRNA accumulation in A. nidulans Δalca::pkcA ΔcnaA (similar to the wild-type strain) when compared to the Δalca::pkcA ΔcnaA strain.

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Table S4 FetGOat analysis for genes with increased mRNA accumulation in A. nidulans ΔcnaA when compared to the Δalca::pkcA ΔcnaA strain.

(XLSX)

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Author Contributions

Conceived and designed the experiments: GHG. Performed the experiments: ACC MS TMD MRZ. Analyzed the data: NAB ACC TMD GHG. Contributed reagents/materials/analysis tools: ACC. Contributed to the writing of the manuscript: ACC LINAR NAB MHSG GHG.
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