1 **Spatiotemporal dynamics of the calcineurin target CrzA**

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21 8 Running Head: CrzA dynamics in *Aspergillus nidulans*

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Highlights

- Signaling of CrzA occurs in a basipetal, tip to nucleus, manner and dependent on the apical localization of calmodulin and calcineurin together with actin cytoskeleton.
- CrzA is the only element of its signaling pathway that changes in localization after calcium or alkaline pH stress.
- Calcineurin is the key factor in the spatiotemporal dynamics of CrzA in the fungal cell.
- Timing of nuclear localization of CrzA depends on the strength and nature of ambient stimulus.

Abstract

The response of *Aspergilli* to elevated concentrations of extracellular calcium and manganese, or environmental alkalinization is mediated by CrzA, a calcineurin-responsive transcription factor (TF). CrzA is the effector of a signaling pathway which includes the apical proteins calmodulin and calcineurin, and the protein kinases GskA and CkiA. Preferentially located in the cytoplasm, CrzA is the only element of the pathway modifying its localization under those stress conditions, being imported into nuclei. Remarkably, there is a direct relationship between the nature/intensity of the stimulus and the pace of nuclear import and time of nuclear permanence of CrzA. Alkalinity caused a transient nuclear accumulation of CrzA while high Ca^{2+} and Mn^{2+} concentrations generated a long-lasting accumulation. Furthermore, Ca^{2+} concentrations (below 5mM) that are non-toxic for a *crzA* Δ mutant promoted full signaling of CrzA. However, micromolar concentrations or a mutation disrupting the interaction of CrzA with the phosphatase complex calcineurin, permitted the visualization of a transient and polarized nuclear accumulation of the TF in a tip-to-base gradient. Overall, these results support a model in which nucleo-cytoplasmic dynamics and transcriptional activity of CrzA are driven

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40 by apical signals transmitted by calmodulin and calcineurin. This communication is essential to
41 understand Ca^{+2} -induced stress response in fungi.
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43 Keywords: Filamentous fungi, calcineurin, calcium signaling, nucleo-cytoplasmic trafficking,
44 transcription factor
45

1. Introduction

Calcium, Ca^{2+} , is an essential secondary component in transduction of many environmental signals but also it is an important source of stress because inappropriate management of intracellular stores may lead to cell death [1]. Intracellular Ca^{2+} concentrations change in response to environmental and physiological signals, which are followed by the adaptation of the organisms to the new conditions. A ubiquitous and essential Ca^{2+} -signaling mediator protein in eukaryotic cells is calmodulin (CaM) [2]. Under conditions causing high cytosolic Ca^{2+} concentrations, CaM binds four Ca^{2+} atoms inducing structural rearrangements and promoting the interaction of CaM with its effectors [3, 4]. One of those effectors is the serine/threonine protein phosphatase calcineurin (CN) [5]. CN is ubiquitously expressed in eukaryotes and is composed of a catalytic A subunit (CNA) and a regulatory B subunit (CNB) [6]. CN activity requires the recognition of its substrates through a Calcineurin Docking Domain (CDD) composed mainly of non-polar residues following the consensus sequences PxIxIT or LxVP [7, 8].

Although both CaM and CN are highly conserved proteins in lower and higher eukaryotes, the downstream effectors of this signaling pathway are not as well conserved. Among CN substrates is the Nuclear Factor of Activated T cells (NFAT) in higher eukaryotes and the Calcineurin-Responsive-Zinc-fingers (CRZ) in the fungal kingdom [9, 10, 11]. NFATs are a conserved family of transcription factors (TF) that play important roles in the immune response and in the cardiovascular and nervous systems [12, 13], whereas CRZ proteins comprise a family of TFs playing key roles in fungal tolerance to abiotic stresses and in pathogenicity [11, 14]. The phosphorylation state of these TFs regulates their cellular localization and activity. Phosphorylation is associated with cytoplasmic localization [11, 12] while their CN-induced dephosphorylation under increased Ca^{2+} levels promotes nuclear import [11, 12]. Phosphorylation of fungal CRZs is carried out by different kinases. CK1, Hrr25p and PKA are responsible for phosphorylation of Crz1p in *Saccharomyces cerevisiae* while a CK1

homologue CkiA and the GSK3 β homologue GskA participate in phosphorylation of CrzA in the filamentous fungus *Aspergillus nidulans* [15, 16, 17].

The activity of the CaM-CN-CrzA pathway in filamentous fungi has been adapted to fit in with their growth-mechanism. The characteristic cell-type is the vegetative hypha, which continuously elongate through directed deposition of cell wall and plasmatic membrane materials at one of the apices of the cell [18]. CaM and CN are apical [19, 20, 21], while the proteins kinase CkiA is nuclear [22] and GskA is cytoplasmic (this work). All are key elements in the control of cell-growth and this process is associated with the presence of an elevated Ca²⁺ concentration at the hyphal tip [23]. Thus, the study of this pathway is important to understand how filamentous fungi can grow and thrive under changing environments. Furthermore, the largeness of the apical compartment of a hypha (the region between the growing-apex and the closest nucleus) is a morphological characteristic that facilitates studies of long-distance signaling in eukaryotes. Both features made us use *A. nidulans* as a model organism to understand long-distance propagation of the calcium signaling pathway. Our results indicate that CrzA is the only effector of the pathway capable of modifying its subcellular localization in response to abiotic signals such as high concentrations of Ca²⁺ or Mn²⁺ and alkalinity [15]. Nuclear import of CrzA occurs in a tip-to-base gradient, depends on the nature/intensity of the stimulus and is modulated by the additional members of the pathway, being dephosphorylation a major regulatory step. Results suggest that the nuclear import and transcriptional activity of CrzA is induced by signals originating at the tip and transmitted by calmodulin and calcineurin.

2. Material and methods

2.1 Strains and media conditions

Media, *Aspergillus* minimal medium (AMM) and complete medium (ACM), and general techniques for transformation, genetic and molecular analyses of *A. nidulans* strains were used as previously described in [15] and references therein. The *A. nidulans* strains used are listed in

Table 1. Transformation DNA cassettes were generated following standardized fusion PCR procedures [24] and using the oligonucleotides listed in Table 2. *Aspergillus fumigatus* *pyrG^{Af}* or *pyroA^{Af}* genes were used as prototrophic selection markers. MAD2446 was the recipient strain for the *gskA::gfp::pyrG^{Af}* transformation cassette and the *crzA::mCherry-pyroA^{Af}* cassette was transformed into recipient strain MAD4366. Integration events were in all cases verified by Southern blotting.

Phenotypic analyses were made by inoculating conidiospores onto solid MMA supplemented with different concentrations of CaCl₂ as indicated in the text or with 0.1 M Na₂HPO₄ to generate alkaline pH (pH 8), and incubated at 37° C for 2 days.

2.2 Protein extraction and Western blot analyses

The expression of tagged proteins was analyzed by Western blotting following the VFAL method [15]. For total protein extractions, strains were cultivated in supplemented fermentation media for 18 h at 37° C, and then CaCl₂ (1 mM or 100 mM) was added to the culture and further incubated for time periods ranging from 1 to 30 min, as indicated in the text. Then, mycelia was vacuum filtered using 0.45-µm-pore-size nitrocellulose membranes (Scharlau), frozen in dry ice and lyophilized for 16 h. Protein extraction was carried out by alkaline-lysis (AL) extraction procedure as described in [15]. Samples were resolved in 4–15% Mini-PROTEAN® TGX™ precast polyacrylamide gels (Bio-Rad) before transfer to nitrocellulose filters using TransBlot® Turbo™ Transfer System (Bio-Rad). GFP-tagged proteins were detected using a polyclonal mouse anti-GFP (1/5000; Roche). Peroxidase-conjugated goat anti-mouse IgG immunoglobulin (Jackson Immuno Research Laboratories) was used as a secondary antibody at a 1/4000 dilution. Peroxidase activity was assayed with the Amersham Biosciences ECL kit and luminescence detected and recorded using a LAS3000 imager system (Fuji). Images were analyzed using the Multi Gauge V3.0 software (Fuji)

2.3 Microscopy

A. nidulans cells were cultured in appropriately supplemented watch minimal medium (WMM) [25] and incubated at 25° C for 18 hours. After this period, the medium was replaced with fresh WMM adjusted to alkaline pH with Na₂HPO₄ (using 0.1 M and 0.01 M concentrations) or Tris-HCl pH8 buffer at the concentration of 10 mM. Media were also supplemented with cations, calcium as CaCl₂ salt at final concentrations of 0.01 mM, 1 mM and 50 mM, and manganese, as MnCl₂ salt, at 10 mM or 1 mM concentration. When indicated, cytoskeleton depolymerizing agents Latrunculin B (100 μM) and Benomyl (4.8 μg/ml) were added to the replacing medium. In all cases, image acquisition was started immediately after the shift. For all shift experiments a single cell was imaged and after the period of incubation with the stress other cells were visualized to confirm the homogeneity of localization for the fusion protein. These shift experiments were repeated for at least 10 times to ensure a correct description for the localization of each fluorescent protein.

For all microscopy experiments, conidia were germinated on uncoated μ-Slide 8 well (Ibidi GmbH, Germany) for a direct observation of cultures using a Leica DMI-6000b inverted microscope equipped with Normarski and epifluorescence optics. Images were acquired with a Hamamatsu ORCA ER digital camera driven by Metamorph software (Universal Imaging Corporation), using Semrock Brightline GFP-3035B and TXRED-4040B (mCherry) filter sets. Images were processed to a minimum using Metamorph (Universal Imaging Corporation) or ImageJ (<http://rsb.info.nih.gov/ij/index.html>) software.

3. Results

3.1. Cellular distribution of components of the CrzA signaling machinery.

In order to deepen in the mechanism of signal transmission to CrzA in a filamentous fungus, we first focused on the analysis of the cellular distribution of its signaling components. The catalytic subunit of the protein phosphatase calcineurin, CnaA, and protein kinases CkiA and

GskA were tagged a their respective C-terminus with the green fluorescent protein, GFP. Calmodulin (CaM) localization was also included in this study given its role in calcineurin activation [2, 6]. With the sole exception of the strain expressing CaM-GFP, which displayed compact morphology and reduced radial extension, the resulting tagged strains showed normal colony growth and conidiation on AMM, indicating that these fluorescent chimaeras were functional (Fig. S1).

As expected, in the absence of stress, CrzA-GFP fusion located dispersed along the cytoplasm and excluded from nuclei (Fig. 1, [15]). The CaM-GFP fusion preferentially located at the tip of the cell (asterisk at Fig. 1), at septa (white arrowheads, Fig. 1) and in dot-like accumulations along the cytoplasm (black arrowheads, Fig. 1, [21]). The GFP-tagged CnaA fusion showed a similar localization to CaM (Fig. 1). However, CnaA-GFP accumulated at both sides of the septum, being this distribution in *A. nidulans* similar to that described for calcineurin in the pathogen *A. fumigatus* [26]. In contrast to the calcineurin/CaM system, tagged kinases CkiA-GFP and GskA-GFP did not accumulate at the cell apex (Fig. 1). As previously noted by Apostolaky *et al* [22], CkiA-GFP was observed in nuclei and along the cytoplasm but we also observed accumulation of this kinase in septa (Fig. 1). Finally, GskA-GFP was evenly distributed through the cytoplasm and excluded from nuclei and septa (Fig. 1). Results evidenced the distinctive cellular localization patterns of signaling elements of CrzA protein in resting cells, showing a highly polarized localization of calmodulin and calcineurin in contrast to a widespread distribution for CkiA and GskA kinases.

3.2. *CrzA* is the only member of the pathway modifying its subcellular localization under calcium and alkaline pH stresses.

A further objective was the analysis of the cellular distribution of CaM, CnaA, CkiA and GskA under high calcium concentration or alkaline pH stress. None of the tagged strains presented sensitivity to these stimuli, thus an appropriate functional response was expected (Fig. S1).

Figure 2A shows a co-culture of cells expressing CaM-GFP (MAD4310) along with cells expressing CrzA-GFP (MAD3021) which served as control for the response of CrzA to these stresses. Calmodulin distribution did not change either in response to 50 mM calcium or to alkaline pH stress remaining accumulated at the tip of the cell, meanwhile, as expected, CrzA responded to both stimuli accumulating in nuclei. Next we focused in the dynamics of calcineurin and a strain co-expressing CnaA-GFP and a monomeric red fluorescence Cherry (mCh) tagged CrzA version (MAD4522) was constructed, allowing a simultaneous localization of both fluorescent proteins under stressing conditions. After calcium and alkaline-stress induction the CrzA-mCh fusion accumulated in nuclei as visualized before for the GFP-tagged CrzA. In contrast to CrzA nuclear import, addition of calcium did not modify CnaA cellular distribution, but reduction in the apical accumulation was observed after alkalinization (Fig. 2B). Notably, nuclear accumulation of CnaA was not observed, indicating that the predicted calcineurin-CrzA complex required for proper signaling of this TF may be generated in the cytoplasm but not imported into nuclei. Finally we analyzed the effects of Ca^{2+} and alkalinity in the distribution of the protein kinases CkiA and GskA. Both protein kinases maintained their original location under calcium or alkaline pH stress conditions that is mainly nuclear for CkiA and cytoplasmic for GskA (Fig. 2C and D). Thus, CrzA the only component of the cascade analyzed here which undergoes a major change of cellular localization under calcium or alkaline pH stress.

3.3. Toxic and non-toxic levels of calcium for a null crzA mutant cause similar effects in the post-translational modification pattern and the cellular distribution of CrzA

CrzA function is expected to be dependent on its nuclear accumulation. To correlate functionality and nuclear import of this TF, we evaluated a range of calcium concentrations for toxicity of the null *crzA* strain and its effect on the localization and post-translational modifications on CrzA.

A comparative phenotypic analysis was carried out between CrzA-GFP and null *crzA* strains in order to determine the differences in tolerance to a range of Ca^{2+} concentrations. Figure 3A shows that the null *crzA* strain was sensitive to a calcium concentration above 50 mM while below 5 mM grew normally. We then investigated how different concentrations of calcium modulate the pattern of post-translational modifications of CrzA. The electrophoretic mobility of this TF was studied in crude extracts of a strain expressing the CrzA-GFP fusion protein. We previously demonstrated that calcium induced an increase in the electrophoretic mobility of CrzA compared to that observed in resting cells, as a result of CrzA dephosphorylation after calcineurin activation [15]. As shown in Figure 3B, addition of a non-toxic, 1 mM, or a highly toxic, 100 mM, concentration of CaCl_2 promoted an immediate increment in the electrophoretic mobility of CrzA from the first minute after addition of the cation. Gradually, low mobility CrzA forms, interpreted as phosphorylated forms (P-CrzA, Fig. 3B, see also [15]), were newly observed along the analyzed times. Thus, results suggest that low and high concentrations of extracellular calcium promoted similar post-translational modifications of CrzA.

Since phosphorylation/dephosphorylation of CrzA is directly associated with its cytoplasmic/nuclear localization, the re-appearance of a phosphorylated form after the described increase in the electrophoretic mobility suggests that the nuclear accumulation of the TF may be transient. To confirm this, dependence of CrzA cellular localization on the ambient calcium concentration was examined. As expected, the toxic Ca^{2+} concentration of 50 mM to a null *crzA* mutant induced the nuclear accumulation of CrzA at 1 minute and remained in nuclei after 30 min (Fig. 3C). As a control, and in agreement with previous results, addition of 10 mM MnCl_2 , also induced a similar effect in the localization of CrzA-GFP (Fig 3C, see also [15]). When cells expressing CrzA-GFP were treated with 1 mM CaCl_2 , nuclear transport of CrzA was observed within the first minute and this localization was maintained for, at least, 30 minutes as it was visualized before for a higher calcium concentration (Fig. 3D). Thus, both toxic (50 mM) and non-toxic (1 mM) levels of extracellular Ca^{2+} led to an immediate and long-lasting response of CrzA. A ten times reduction in manganese concentration also induced CrzA nuclear localization

but had a temporal effect (Fig. 3E) becoming nuclei empty of fluorescence after 30 min of exposure to this cation. Taken together these results demonstrate that activity of CrzA is not essential for cell survival at low levels of calcium but its molecular and cellular signaling follow a similar pattern of post-translational modifications to that observed when using higher Ca^{2+} concentrations. Additionally, calcium is a stronger signal for than manganese for maintaining nuclear localization of CrzA.

3.4. Alkalinity induces a transient nuclear localization of CrzA.

Alkaline pH constitutes another major stimulus for the nuclear accumulation of CrzA [15]. In view of the long lasting nuclear accumulation of CrzA in cells treated with millimolar concentrations of calcium (1 mM) or manganese (10 mM), we analyzed the timing of nuclear accumulation under pH stress. As reported before, an immediate import of CrzA in nuclei was visualized after medium alkalization, which was induced by adding 100 mM Na_2HPO_4 buffer (Fig. 4A). However this effect on CrzA localization was transient and nuclei were depleted of fluorescence after 10 min of treatment (Fig. 4A). To verify that this transient effect was not exclusive of the phosphate buffering conditions used we assayed the effect of using 10mM Tris-HCl pH 8 as buffering agent. Figure 4B shows that the dynamics of CrzA nuclear accumulation using Tris-HCl buffer essentially followed those described for 100 mM Na_2HPO_4 buffer, but CrzA-GFP fluorescence was first accumulated in the apical nucleus. Next we determined whether using a lower concentration Na_2HPO_4 buffer (10 mM) could cause a similar effect. Interestingly, 10 mM Na_2HPO_4 generated a brief moderate accumulation of CrzA in nuclei (Fig. 4C). These results indicated a differential effect of ambient stimuli in the activation of CrzA nuclear import machinery, probably relying in differential transmission of ambient pH signal by calcineurin to CrzA.

3.5. Deletion of the calcineurin docking domain 2 reveals a polarized nuclear distribution of

CrzA

Calcineurin directly interacts with CrzA through the CDD2 and this motif was found to be required for proper signaling of this TF in response to calcium and alkaline pH ([15] and Fig S2). In agreement with this previous observation, the fluorescent mutant version of CrzA lacking CDD2, CrzA^{ΔCDD2}-GFP, remained cytoplasmic after medium alkalinization or calcium addition meanwhile a wild type CrzA-GFP fusion was immediately internalized in the nucleus. However, a CrzA^{ΔCDD2} strain (MAD4234) still displayed tolerance to calcium stress which suggested a residual response from this mutant form of CrzA [15]. To test this hypothesis we analyzed the cellular distribution of CrzA^{ΔCDD2}-GFP after longer periods of incubation of cells at stressing conditions. While nuclear exclusion of the TF was maintained for 30 minutes in an alkaline medium as previous observations (results not shown), a remarkable signaling response was observed after prolonged calcium exposure using a 50 mM concentration of CaCl₂. Remarkably, nuclear accumulation of CrzA^{ΔCDD2} was found to be dependent on the distance of each nucleus from the cell apex. This phenomenon is illustrated in the cell shown in Figure 5. The most apical nucleus was the first to accumulate fluorescence after 8 minutes while rest of nuclei remained empty. Ten minutes later, all nuclei accumulated CrzA^{ΔCDD2}. After 15 minutes we observed nuclear transport in two ways: an increment in nuclear fluorescence was detected in basal nuclei meanwhile nuclear export of CrzA started in apical nuclei. Further incubation allowed the visualization of a progressive nuclear exit of CrzA to the cytoplasm, leading to nuclear exclusion from all nuclei after 30 min of treatment. Therefore, in the absence of a CDD2 site CrzA is still signalized in the presence of elevated concentrations of extracellular calcium exhibiting a strongly polarized and transient distribution pattern of nuclear accumulation.

3.6. Spatiotemporal nuclear accumulation of CrzA is visualized under micromolar concentrations of extracellular calcium.

The fact that a strain expressing CrzA^{ΔCDD2} revealed a non-synchronized CrzA nuclear import at high calcium concentration levels could be indicating an attenuated signaling of the TF. We aimed to obtain this attenuated response in a wild type CrzA-GFP strain by exposing cells to a minimum calcium stress. A reduction to a 10 μM concentration of extracellular calcium allowed the observation of a transient and tip-to-base nuclear accumulation of CrzA, similar to that previously detected when using 10 mM Tris-HCl pH8 buffer. This effect is exemplified in the cell shown in Figure 6 in which CrzA-GFP fluorescence was followed in three nuclei, numbered 1, 3 and 5 starting from the tip to the basal region of the cell. Initially all nuclei were empty of fluorescence (time 0 min, Fig 6). One minute after calcium addition nuclear accumulation of CrzA was visible in the first nucleus, meanwhile other nuclei were in different stages of CrzA nuclear import. At minute 6 all nuclei accumulated CrzA. From minute 8 onwards, CrzA nuclear export started in the apical nucleus and it was propagated in the same order than nuclear import was previously observed. Fluorescence distribution along the hypha was homogenous after 30 minutes, most probably indicating the cease of the calcium signaling.

Consequently, a non-toxic micromolar concentration of calcium constituted an extracellular signal for the activation of CrzA response and induced a cell tip-dependent nuclear accumulation of CrzA. This low-calcium response along with the previous findings on CrzA^{ΔCDD2} signaling suggests that calcium signaling is originated at the tip of the cell.

3.7. Calcium and alkaline pH activation of CrzA is independent of microtubule cytoskeleton.

In filamentous fungi owing to their elongated cell shape, microtubule-dependent transport ensures long-range transport of cargos [27]. To test whether the transport of CrzA requires the integrity of microtubule cytoskeleton during signaling, we examined the cellular localization of CrzA after tubulin depolymerization. Treatment of *A. nidulans* with the microtubule-disorganizing agent benomyl causes microtubule disassembly in less than ten minutes and the effects on cell morphology are mainly detected as swellings at the tip of cells [28]. A strain

expressing a CrzA-GFP protein was incubated with benomyl and the localization of CrzA was followed for fifteen minutes. Despite morphological defects observed at the tip, demonstrating the efficacy of the drug treatment, CrzA cellular localization was not altered remaining at the cytoplasm (Fig. 7A). We then investigated the response of CrzA after stress induction in cells previously treated with benomyl. As shown in Figure 7A, CrzA nuclear accumulation was completed after 1 minute of incubation with 50 mM calcium and this localization continued, at least, for 20 minutes. Also microtubule disruption neither affected nuclear import nor nuclear export of CrzA after medium alkalization (Fig. 7A). These results strongly suggest that nucleo-cytoplasmic trafficking of CrzA is not a microtubule-based transport mechanism.

3.8. Disorganization of actin cytoskeleton induces nuclear entry of CrzA in a calcineurin-dependent manner

We further analyzed the role of tubulin and actin cytoskeletons in the cellular localization of CrzA and the signaling elements CnaA and CaM. Figure 7B shows that CaM-GFP maintains its apical localization in the presence of benomyl when cell tips swells and after recovery of polar growth. In contrast, the apical accumulation of CnaA-GFP was partially lost during tip swelling (Fig 7B, left) and some accretions were still visible, which rapidly aggregated at the tip when polarized growth was recovered (Fig. 7B. right). Treatment with actin depolymerizing agents as latrunculin B or cytochalasin A resulted in disassembling of F-actin and the arrest of polar growth [28]. A strain expressing a fluorescent tagged version of the actin binding protein AbpA (AbpA-mRFP, MAD1399) was used as a control of efficacy for these drug treatments. Cells treated with latrunculin B detain apical extension and tip started to swell and, following these morphological alterations, the accumulation of AbpA-mRFP in the tips was lost and an even distribution of fluorescence along the cytoplasm was observed [29] and Fig. 7C). Localization of CaM-GFP at a hyphal tip is disrupted by latrunculin B as shown in Figure 7C. Previous reports showed that the disruption of actin cytoskeleton using CytA altered the normal pattern

of CaM localization at hyphal tips in *A. nidulans* [21]. We also observed that actin cytoskeleton participates in maintenance of CnaA at the apex without affecting the cytoplasmic dot-like accumulations (Fig. 7C). Treatment of cells with actin depolymerizing agents as latrunculin B or cytochalasin D resulted in the induction of transport of CrzA to nuclei (Fig. 7C, S3). Interestingly, cytochalasin had a similar effect to low levels of extracellular calcium and polarized nuclear entry of CrzA was observed (Fig. S3). These results indicate that actin depolymerization promotes signaling of CrzA in the absence of stress stimuli. Furthermore, calcineurin activity is required for the nuclear recruitment of CrzA after actin disruption. Figure 7C shows that in the mutant *cnaAΔ* the nuclear import of CrzA was lost after latrunculin B treatment.

Finally in order to exclude a potential contribution of the microtubule cytoskeleton in nuclear recruitment of CrzA after disruption of actin cytoskeleton, we treated cells with both cytoskeleton depolymerizing drugs, benomyl and latrunculin B. Cells were first treated for 20 minutes with benomyl to disrupt microtubule cytoskeleton and subsequently with latrunculin B. As indicated above, CrzA-GFP did not modify its distribution in presence of benomyl (Fig. 7D). After addition of latrunculin B to the same culture the TF was then recruited to nuclei (Fig. 7D). These observations suggest that actin filaments and not the might be necessary for maintaining CrzA excluded from the nuclei. In fact, microtubule cytoskeleton is most probably not involved in nuclear import nor export of CrzA.

4. Discussion

In eukaryotes, a general mechanism to regulate the activity of transcription factors is to control the access to their target sequences at the genome ([30] and references therein). Among the numerous regulatory pathways subjected to this control mechanism are the Ca^{2+} -CN activated TFs, as fungal CRZs and mammalian NFATs [12]. Despite the obvious differences between fungi and mammals most regulatory elements and the signaling process of these TFs are extraordinarily conserved. Here we use the filamentous fungus *A. nidulans* and CrzA as models to understand how a CN-dependent TF is dynamically regulated in a polarized cell. We gained evidence for the role of the tip of a multinucleated filamentous fungal cell as a sensing hub for environmental conditions allowing the transmission of signals from tip to nuclei in an ordered mode in response to calcium and alkaline pH stresses. These findings are summarized in the molecular and cellular models shown in Figure 8.

Several key steps play a part in modulating the function and cellular localization of CrzA (Fig. 7A). A rapid nuclear accumulation of CrzA occurs under specific ambient conditions including medium alkalization, elevated concentration of manganese and a range of concentrations for Ca^{2+} (this work and [15]). For all conditions nuclear accumulation of CrzA is observed within the first minute after addition of the stress. However, permanence of CrzA in nuclei is not uniform and depends on the stimulus. Medium alkalization had a short effect compared to that caused by an elevated concentration of either Ca^{2+} or Mn^{2+} . Traffic of CrzA to nucleus is a consequence of a direct effect of CN activity [15]. There are no experimental evidences of direct effects of alkalinity or Mn^{2+} on promoting the activity of CN in vivo, however biochemical studies showed for Mn^{2+} and other metal cations a cofactor role in activation of CN [31]. In *Saccharomyces cerevisiae* the stress produced by high levels of Mn^{2+} or alkalinity promotes an increase in intracellular Ca^{2+} levels which leads to signaling of Crz1p [32]. In *Aspergillus*, a reduction of Ca^{2+} concentration had a direct effect on the timing of nuclear accumulation and alkaline pH signal is dependent on CN activity (this work and [15]). Therefore, a conversion of alkalinity and high Mn^{2+} concentration in an intracellular Ca^{2+} signal is expected in this fungus.

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380 This Ca^{2+} signal can be originated either from a release of internal stores or by increasing the
381 influx of Ca^{2+} from the extracellular medium (Fig. 8B). In *Candida albicans*, alkaline pH
382 enhances the influx of extracellular Ca^{2+} , rising intracellular Ca^{2+} levels which activate Crz1p
383 signaling [33].

384 Nuclear CrzA certainly constitutes the functional version of this TF and its timely permanence
385 in the nucleus must guarantee a correct transcriptional regulation of genes under its control (Fig.
386 8A and [34, 35]). Colonial growth of the *crzAA* strain is not affected at Ca^{2+} concentrations
387 below 5 mM CaCl_2 indicating that CrzA activity is not required at those extracellular Ca^{2+} levels
388 for colony survival. However, both high and low external Ca^{2+} concentrations induce
389 posttranslational modifications and nuclear import of CrzA suggesting a possible scenario in
390 which CrzA could play different transcriptional roles at high and low calcium stress conditions.
391 To date, the majority of calcium/Crz-dependent transcriptional profiles have been performed
392 with high levels of calcium (50-200mM, see [34, 36, 37, 38]). Consequently, transcriptomic
393 analyses should consider that gene expression under CrzA regulation may differ when the
394 CN/Crz system is activated at stressing or non-stressing calcium conditions. This should answer
395 the question whether a transcriptome can be induced by treatment with high and low external
396 calcium concentration but the magnitude of global gene expression be different.

397 CrzA is the only element belonging to this pathway with major changes in cellular localization
398 in response to stress (Fig. 8B) but the localization of signaling components may play an
399 important role in regulating CrzA activity. A competition between CN and protein kinases
400 activities becomes determinant to modify CrzA when cytoplasmic Ca^{2+} drops from a given
401 concentration (Fig 8A). The presence of casein kinase I, CkiA, in the nucleus points to this
402 kinase as responsible for phosphorylation of CrzA in this organelle and of importance for the
403 recognition of CrzA by the nuclear export machinery. CkiA remains nuclear after alkalinization
404 or high Ca^{2+} concentrations, and there is no evidence of a Ca^{2+} -modulation of CkiA activity in
405 the nucleus which could suggest differential capacity of this kinase to phosphorylate CrzA
406 under stress. In the cytoplasm, the activity of the GskA kinase may produce different

phosphorylation states in CrzA, which have been detected in this and previous studies (Fig. 8A and [15]). As noted for GSK-3 isoforms on activating NFATc nuclear export [39], the activity of GskA on CrzA may oppose to that of CN at the cytoplasm, contributing to improvement of nuclear exclusion of this TF in the absence of stress or after a stimulation period. As other glycogen synthase kinase homologues, GskA may also be involved in positively regulating the activity of the CN regulator RCAN (RcnA in *A. nidulans*), with a expected direct consequence in reducing CN activity [38, 40]. In summary, the activities of both kinases may coordinately ensure the exit of CrzA from nuclei and preclude nuclear transport leading to cytoplasmic accumulation for this TF and its subsequent inactivation.

CaM and CN preferentially locate at the tip of the cell and at septa, and this localization has been associated with their roles in polarized growth or cell wall integrity pathways [19, 20, 21]. Addition of a high extracellular concentration of Ca^{2+} did not modify the localization of CaM and CN, although these proteins display dynamic movement along the hypha (not shown). Interestingly the formation of the predicted complex between active CN and CrzA was not observed. Besides, the absence of nuclear accumulation of CN and CaM suggests that after activation of CrzA this heterocomplex must disassemble and consequently CrzA migrate alone to the nucleus.

Integrity of actin but not tubulin cytoskeleton is required for proper regulation of CrzA localization. Disruption of tubulin cytoskeleton had no effect on the nuclear export mechanism of CrzA in resting cells neither affected nuclear import, since Ca^{2+} induced the rapid entry of CrzA in nuclei of treated cells. However, disruption of actin cytoskeleton had a major impact on CrzA signaling, inducing its nuclear accumulation in resting cells. The fact that F-actin disassembly on CrzA nuclear localization is CN dependent, suggests that mislocalization of both CaM and CN by actin polymerization agents would be triggering the activation of the signaling pathway. Nevertheless the presence of cytoplasmic Ca^{2+} would be required in these conditions. Supporting this idea is the finding that in mammalian cells disruption of the actin cytoskeleton results in elevation of intracellular Ca^{2+} levels leading to a prolonged accumulation

of NFAT in the nucleus [41]. Also in neurons, actin filaments can modify Ca^{2+} -influx through Ca^{2+} channels at the plasma membrane as well as Ca^{2+} -release from internal stores [42]. This interaction between the actin cytoskeleton and intracellular Ca^{2+} levels could therefore be considered in *A. nidulans* as the responsible for nuclear recruitment of CrzA following the disruption of the actin cytoskeleton.

Finally, the analysis of CrzA signaling at low Ca^{2+} concentration revealed the spatiotemporal dynamics of CrzA signaling. A final concentration of 10 μM calcium induced a CrzA nuclear recruitment following a tip-to-base pattern. Recently, a new mechanism of communication in the asexual development pathway in *A. nidulans* has been described in which the TF FlbB shows a tip-to-nucleus migration [43]. That work provided evidence for the strict control in the transmission of the information sensed from the tip to the first apical nucleus. The polarized morphology that hyphae show is therefore essential for apical dominance during vegetative growth, required for asexual development and, as our results suggest, important for the adequate stress response. Our work shows that the intensity of the response correlates with intensity of the signal. Only when low levels of calcium are present, the tip-to-base nuclear accumulation of CrzA was observed. The polarized distribution of the Ca^{2+} signaling elements upstream in the cascade, CaM and CN, strongly correlates with this polarized response of CrzA (see Fig.7B). The pool of CaM localized in the apex of the hyphae could initiate intracellular Ca^{2+} cascade leading to nuclear recruitment of CrzA in the apical nucleus. Ca^{2+} spikes and waves would facilitate the propagation of the signal to distal regions, where CaM and CN are also present. The mutant version of CrzA lacking a CN docking domain $\text{CrzA}^{\Delta\text{CDD2}}$ displays the same but delayed polarized response at 50 mM calcium. The absence of CDD2 highly reduced but did not completely abolish CrzA binding to CN ([15] and Fig. S2), which would debilitate the intensity of the response as observed at a low dose of Ca^{2+} stress. Again this finding would be consistent with the tip-to-base dependence of CN-mediated signaling of CrzA. We propose the contribution of the pool of CaM and CN proteins in the apex to the tip-to-base signaling of CrzA (Fig. 8B).

461

462 **5. Conclusions**

463 Due to the polar mode of growth of filamentous fungi during substrate colonization, hyphal tips
464 are the first region of a mycelium in penetrating a new environment. Consequently, besides
465 sustaining growth, hyphal tips must sense the ambiance to lead to a fast adaptive response.
466 Recent publications support a sensorial role for the hyphal tip and demonstrate its importance in
467 coupling ambient signals with the transcriptional control of pathogenicity and reproduction [43,
468 44, 45]. Here we describe a novel aspect of dynamics of CrzA nucleo-cytoplasmic transport,
469 revealing a fine tune signaling of the TF in relation to the stimulus encountered and consistent
470 with a model in which tip-to-base gradient of upstream components generates a polarized
471 signaling of this transcription factor. Apical localization of the calmodulin/calcineurin module
472 maintained by the actin cytoskeleton orchestrates the adequate signaling of CrzA to high or low
473 levels of calcium stress and to ambient alkaline pH resulting in longer or shorter periods of
474 nuclear accumulation. The differential localizations of GskA and CkiA protein kinases in the
475 fungal cell is probably important for maintaining the correct localization of CrzA, most
476 probably through the modulation of the functionality of the nuclear localization signals in CrzA.
477 These observations increase our understanding of how CrzA mediates its function and how
478 calcium and alkaline pH signaling occurs in a multinucleated cell of a filamentous fungus.

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481 **Conflict of interest**

482 The authors disclose no potential conflicts of interest. Both authors approved the final article.

483

484 **Author contributions**

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485 P.H-O. conducted the experiments, analyzed the data and wrote the manuscript. E.A.E. designed
486 the study, supervised the research and wrote the manuscript.

487
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Figure Legends

Fig. 1. Cellular localization of components of the calcium signaling machinery. Fluorescence images of cellular localization of GFP-tagged proteins CrzA, CaM, CnaA, CkiA and GskA. Insets and magnified images show in detail the apex (marked with asterisk), septa (s, white arrowhead) (also Normarski images) and nuclei (n, arrow) of the cells. Also indicated is the cytoplasmic accretion of CaM-GFP, black arrowhead. Strains used in this experiment are, top to bottom: MAD3021, MAD4310, MAD4366, VIE172 and MAD4643. Scale bar, 5 μ m.

Fig. 2. Analysis of cellular localization of CaM, CnaA, CkiA and GskA in response to stress. (A) Conidiospores from the *crzA-gfp* strain (MAD3021) and the *cam-gfp* strain (MAD4310) were inoculated in the same chamber and incubated in WMM for 18 h at 25°C. Inset image shows that non-overexposed fluorescence of CaM-GFP did not modify its localization in response to stress compared to CrzA that migrated from cytoplasm (CrzA_{cyt}) to the nuclei (CrzA_{nuc}). (B) Visualization of cells from strain MAD4522 co-expressing CnaA-GFP and CrzA-mCherry. Addition of calcium or medium alkalization (pH 8) promoted the accumulation of CrzA in nuclei, CrzA_{nuc}, while did not induce major changes in the cellular location of CnaA. Inset images display fluorescence of CnaA-GFP along the hyphae (left) and at the apex (right). (C) Strain VIE172 did not exhibit any change in the cellular distribution of CkiA-GFP after stress induction. (D) Cell from strain MAD4643 shows that GskA-GFP (green) did not co-localize with the nuclear marker histone H1, HhoA (red), and maintained this distribution in the presence of stress. For all experiments in this figure: RC, resting conditions; Ca²⁺, 50 mM CaCl₂; pH8, 100 mM Na₂HPO₄. Scale bar, 5 μ m.

Fig. 3. Effects of different concentrations of calcium and manganese in the signaling of CrzA. (A) Growth tests of CrzA-GFP (MAD3021) and null *crzA* (MAD3709) strains. Strains were inoculated on standard AMM (-) and AMM supplemented with increasing concentrations (mM) of Ca²⁺ (CaCl₂). Images of colonies were taken after 2 days of incubation at 37°C. (B)

Electrophoretic mobility of CrzA-GFP in protein extracts from strain MAD3021 after addition of calcium (Ca^{2+}). The concentration of CaCl_2 used in each assay is indicated in mM on top of the image and mycelium samples of each condition were taken at the indicated times (min). (C, D and E) Conidiospores from strain MAD4641 were grown in WMM at 25°C for 18 h and observed by fluorescence microscopy under different stress conditions. (C) CrzA was nuclear for at least 30 min in cells treated with 50 mM Ca^{2+} or 10 mM Mn^{2+} . (D) Initially (RC) CrzA is excluded from nuclei. After addition of 1 mM CaCl_2 , CrzA accumulated in nuclei from minute 1 and remained in this location for at least 30 minutes. (E) Addition of 1 mM MnCl_2 caused a temporal accumulation of CrzA in nuclei. Scale bars, 5 μm

Fig. 4. Effects of medium alkalisation in the signaling of CrzA. (A, B and C) Conidiospores from strain MAD4641 were grown in WMM at 25°C for 18 h and observed by fluorescence microscopy after addition of different buffering agents. (A) 100 mM Na_2HPO_4 generates a transient nuclear accumulation of CrzA. (B) 10 mM Tris-HCl pH 8 caused a similar effect to that observed with Na_2HPO_4 buffer. (C) A tenfold reduction in Na_2HPO_4 concentration, still maintaining the buffering effect at pH 8 in WMM, caused a short and partial pulse of nuclear accumulation of CrzA. RC, resting conditions.

Fig. 5. Differential tip-to-base nuclear accumulation of CrzA ^{ΔCDD2} . Conidiospores from the strain MAD4234 expressing a GFP-tagged version of CrzA lacking the CDD2 were cultured in WMM for 18 h at 25°C. In resting cells (RC), basal conditions, CrzA remained in the cytoplasm. In cells shifted to fresh WMM supplemented with Ca^{2+} (50 mM CaCl_2), the recruitment of CrzA to the nucleus most proximal to the apex (white arrowhead) was visible after 5 minutes of stress induction. Subsequently, nuclear recruitment of CrzA spread towards the base of cell compartment. The white arrow points to a nucleus distant to the cell tip. Insets show the dynamics of CrzA transport in the apical and basal nuclei. The white star indicates the tip of a cell. Scale bar, 5 μm

Fig. 6. Low calcium concentration promotes a spatiotemporal nuclear recruitment of CrzA. Cells of the *crzA-gfp* strain MAD3021 were grown in WMM for 18 h at 25°C. In the absence of stimulus (0 min), CrzA is distributed along the cytoplasm. 1 minute after addition of 10 μ M CaCl₂, CrzA-GFP fluorescence accumulated in the apical nucleus. Nuclear recruitment of CrzA was propagated to the rest of nuclei from the tip towards the base of the cell. The white star indicates the position of the tip. Arrowheads point to nuclei, numbered 1, 3 and 5 for their first, third and fifth position relative to the tip. On the right, magnification of nuclei 1, 3 and 5 organized from minute 0 to 30. Scale bar, 5 μ m

Fig. 7. Effects of disruption of microtubule and actin cytoskeletons in the signaling of CrzA. (A) Conidiospores of the *crzA-gfp* strain MAD3021 were grown in WMM for 18 h at 25°C. Cells were incubated in the presence of 4.8 μ g/ml benomyl (Ben) during 20 minutes in order to ensure microtubule polymerization disruption, as it is observed as swelling in the tips and subsequent stop of apical growth. After this initial treatment, cells were imaged after addition of 50 mM CaCl₂ (Ca²⁺) or 100 mM Na₂HPO₄ (pH8). Presence of the drug did not alter the nuclear entry of CrzA-GFP after stress induction. The white star indicates the tip. (B) Strains MAD4310 (CaM-GFP) and MAD4366 (CnaA-GFP) were grown and treated with benomyl as in (A). Black arrows indicate the localization of fluorescent CaM and CnaA fusions in each strains in cells showing tip swelling and recovery of polar growth. Grey arrows point to the swelling area of each cell generated by benomyl treatment. (C) Conidiospores from strains MAD4310 (CaM-GFP), MAD4366 (CnaA-GFP), MAD3021 (CrzA-GFP) and MAD3222 (CrzA-GFP *cnaAΔ*) were inoculated along with conidiospores from the strain MAD1399 (AbpA-mRFP) in the same chamber containing WMM and incubated for 18 h at 25°C. Images were taken before and after treatment with 100 μ M Latrunculin B (LatB) for 20 minutes. The white star indicates the tip at the cell expressing AbpA-mRFP. (C) Conidiospores from the *crzA-gfp* strain (MAD3021) and the *abpA-mrpf* strain (MAD1399) were co-inoculated in WMM and incubated for 18 h at 25°C.

After the initial period of growth in resting conditions (RC), cells were treated with 4.8 $\mu\text{g/ml}$ benomyl for 20 minutes (+Ben). Subsequently, 100 μM Latrunculin B was added to the same culture and incubated for 20 minutes. The white star indicates the tip at the cell expressing AbpA-mRFP. Scale bars, 5 μm .

Fig. 8. Tentative models of CrzA regulation and nuclear transport in *A. nidulans*. (A) Model combining posttranslational modifications in CrzA and nuclear transport. Differential localization of casein kinase I, CkiA, and glycogen synthase kinase 3 β , GskA, predict for CkiA a role in nucleus for phosphorylation of CrzA and promoting nuclear export, and for GskA in regulating the different levels of CrzA phosphorylation at the cytoplasm (Activation/inactivation step, labeled with number 1). Low level or non-phosphorylated CrzA forms, generated by the CN phosphatase activity, are transported in the nucleus. In contrast, phosphorylated forms, hypothetically generated by CkiA activity, are preferentially exported to the cytoplasm. The identity of exportins (exp) and importins (imp) involved remain unknown in *A. nidulans* (regulation of nuclear transport step, labeled with number 2). Nuclear CrzA regulates transcription of genes required for the response to calcium, manganese and alkaline pH stresses, among other stimuli (transcriptional regulation step, labeled with number 3) (B) A schematic representation of part of an apical compartment in a vegetative cell. Vesicles accumulate at the tip due to a continuous feed of exocytic vesicles, some of which are originated at the endocytic ring where AbpA protein locates. The Spitzenkörper (SPK) is formed at the apex by a continuous supplementation of vesicles, being structurally supported by the actin cytoskeleton and other associated protein complexes. Calcineurin (CN) and calmodulin (CaM) accumulate at the SPK. CRZ factors become activated by CN, possibly after a rise of cytoplasmic calcium levels. Calcium may enter the cell by an excess of extracellular concentration through channels or transporters located at the plasma membrane (PM). Alkalinity and Mn^{2+} may activate those PM Ca^{2+} -transporters or cause the release of Ca^{2+} from

internal stores. Numbers indicate the three predicted steps for activation and functional role of CRZ proteins in most fungi as indicated in panel A.

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TABLE 1. List of strains used in this work

Strain	Relevant genotype	Source
MAD1399	<i>abpA-mrfp::pyrG^{Af}</i>	(3)
MAD2666	<i>wild type</i>	(36)
MAD2446	<i>hhoA::mCh::pyroA^{Af}</i>	(15)
MAD3021	<i>crzA::gfp::pyrG^{Af}</i>	(23)
MAD3222	<i>cnaAΔ::pyroA^{Af} ; crzA::gfp::pyrG^{Af}</i>	(23)
MAD3709	<i>crzAΔ::riboB^{Af}</i>	(23)
MAD4234	<i>crzA(ΔM356-A372)::gfp::pyrG^{Af}</i>	(23)
MAD4310	<i>caM::gfp::pyrG^{Af}</i>	Etxebeste, O. Univ Basque Country
MAD4366	<i>cnaA::gfp::pyrG^{Af}</i>	(23)
MAD4522	<i>cnaA::gfp::pyrG^{Af} ; crzA::mCh::pyroA^{Af}</i>	This work
MAD4641	<i>crzA::gfp::pyrG^{Af} ; hhoA::mCh::pyroA^{Af}</i>	(23)
MAD4643	<i>gskA::gfp::pyrG^{Af} ; hhoA::mCh::pyroA^{Af}</i>	This work
VIE172	<i>ckiA::gfp::pyrG^{Af}</i>	(1)

TABLE 2. List of oligonucleotides used in this work

Primer	Sequence 5' 3'	Purpose
CrzA(3)	CTTTGCTACCTATTTCTCTGTTTCAGTAGGG	Generation of <i>crzA::mCh-pyroA^{Af}</i> cassette
CrzA(4)	CAACCCGCACCCCTACACTTCTCTACATCG	
CrzA(5)	GACTCCTCCAACAGAAGGCTATCCGAATG G	
CrzA(6)	TTTCCCCCAGTTCGGATTTGCGTAGCCAGC	
CrzA(6')	GCTGGCTACGCAAATCCGAAGTGGGGGAA AGGAGCTGGTGCAGGCGCTGGAGCC	
CrzA(3')	CCCTACTGAACAGAGAAATAGGTAGCAAA GCTGTCTGAGAGGAGGCACTGATG	
GskA(3)	GTCAACGTCATAGTGCTG	Generation of <i>gskA::gfp::pyrG^{Af}</i> cassette
GskA(4)	CGGTGATTGAGTGAAGTGTCC	
GskA(5)	GACTCCTTCCCCGATCTTC	
GskA(6)	GTCAAGGTGTGCCATCATTTTC	
GskA(6')	GAAATGATGGCACACCTTGACGGAGCTGG TGCAGGCGCTGGAGCCGGTGC	
GskA(3')	CAGCACTATGACGTTGACCTGTCTGAGAG GAGGCACTGATG	

Supplementary Material Fig S1

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Supplementary Material Fig S2

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Supplementary Material Fig S3

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