Phospho-regulation and nucleocytoplasmic trafficking of CrzA in response to calcium and alkaline-pH stress in *Aspergillus nidulans*.

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2 **calcium and alkaline-pH stress in *Aspergillus nidulans***

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20

Abstract

Tolerance to abiotic stresses by microorganisms require of appropriate signaling and regulatory pathways. Calcineurin phosphatases mediate calcium-dependent signaling pathways which are widely distributed among phylogeny. In *Saccharomyces cerevisiae*, calcineurin mediates the post-translational modification of downstream effectors, most of them transcription factors, being the best characterized the calcineurin-regulated zinc-finger factor 1, Crz1p. Here we study the signaling process of CrzA, a filamentous fungal Crz orthologue, in response to calcium and ambient-pH alkalinization. In *Aspergillus nidulans* resting cells CrzA locates in the cytoplasm being excluded from nuclei. CrzA is a phospho-protein and upon calcium, manganese or alkaline-pH stresses, accumulates in nuclei in a calcineurin-dependent manner. Functional analysis of CrzA defined the presence of a nuclear-export and two nuclear-localization signals as well as a PSINVE sequence that constitutes the major calcineurin-docking-domain. First 450 amino acids of CrzA contain these functional motifs and in this region is where phosphorylated residues locate. Different phosphorylation steps are identified in CrzA and activities of casein kinase 1 homologue, CkiA, and of glycogen synthase kinase-3 β , identified for the first time here as GskA, are involved. The phospho-signaling process and nucleocytoplasmic trafficking of CrzA shows similarities to those described in yeast for Crz1p homologues and of NFATs in mammals.

1 Introduction.

2
3 Calcium (Ca^{2+}) signaling plays a major role in the regulation of diverse physiological responses
4 in either prokaryotic or eukaryotic organisms, although the regulatory machinery is being
5 mainly studied in eukaryotic cells. Ca^{2+} is an ubiquitous secondary messenger participating in
6 many vital biological processes such as cell cycle, differentiation or ion homeostasis but
7 intracellular Ca^{2+} levels must be carefully regulated because excessive concentrations can lead
8 to cell death (reviewed in (Clapham, 2007)). An essential Ca^{2+} -signaling mediator is calmodulin
9 (CaM). Ca^{2+} binds to CaM triggering structural rearrangements which allow the interaction of
10 CaM with its effectors. A key target of CaM is the serine-threonine protein phosphatase type B
11 (PP2B) calcineurin (Chin and Means, 2000). Calcineurin, Cn, is a heterodimer composed of a
12 catalytic A subunit (CnA) and a regulatory B subunit (CnB) (Guerini, 1997).

13 Activation of phosphatase calcineurin requires binding of Ca^{2+} to the regulatory CnB subunit
14 and of the Ca^{2+} -CaM complex to the CaM-binding domain present in CnA (Guerini, 1997).
15 Efficient dephosphorylation by calcineurin implies targeting and interaction with an specific
16 sequence in its substrates, named the calcineurin-docking domain (CDD). Two classes of
17 docking sites for calcineurin are identified, the consensus sequence PxIxIT and the LxVP motif
18 (reviewed in (Li *et al.*, 2011)).

19 In mammalian cells, among calcineurin substrates is the nuclear factor of activated T cells
20 (NFAT). Dephosphorylation by calcineurin promotes NFAT translocation to the nucleus, where
21 these factors become transcriptionally active (Crabtree and Olson, 2002). Phosphorylation
22 maintains NFATs inactive in resting cells and this post-translational process is accomplished by
23 several constitutive kinases including casein kinase 1 (CK1), glycogen synthase kinase-3 β
24 (GSK3 β) and the dual specificity tyrosine phosphorylation regulated kinase (DYRK) (Beals *et al.*,
25 1997;Gwack *et al.*, 2006;Zhu *et al.*, 1998). The final cellular localization of NFATs is the
26 result of an unbalanced phosphorylation/dephosphorylation process of nuclear localization
27 (NLS) and nuclear export (NES) sequences present in these factors (Okamura *et al.*, 2000).

28 In fungi, the model yeast *Saccharomyces cerevisiae* has been extensively used to understand
29 calcium signaling. The best characterized target of calcineurin is the transcription factor (TF)
30 Crz1p, a protein unrelated to NFATs but sharing common regulatory mechanisms. In response
31 to stress, the Ca^{2+} /calmodulin/calcineurin pathway is activated leading to the dephosphorylation
32 of Crz1p and therefore its translocation to the nucleus to modulate gene expression. As in
33 NFATs signaling, the nucleocytoplasmic trafficking of Crz1p depends on the phosphorylation
34 state of its NLS and NES (reviewed in (Cyert, 2003)).

35 A CDD of the PxIxIT class mediates the interaction of Crz1p with calcineurin (Boustany and
36 Cyert, 2002;Roy *et al.*, 2007). Although calcineurin is the only phosphatase involved in the
37 signaling of Crz1p, phosphorylation is carried out by different kinases. Casein kinase I, Hrr25p,

1 and cAMP-dependent protein kinase A activities are responsible for preventing the activity and
2 nuclear accumulation of Crz1p (Kafadar *et al.*, 2003; Kafadar and Cyert, 2004).

3 Calcineurin and Crz1p are required for cell growth under physiological stresses such as high
4 salinity, alkaline pH and cell wall damage (Cyert, 2003). Many studies have focused on
5 calcineurin function and regulation as well as the molecular mechanism that govern the activity
6 of the phosphatase in fungal models. However, a complete catalog of calcineurin downstream
7 effectors remains to be determined (Fox and Heitman, 2002; Stie and Fox, 2008). The
8 understanding of the physiological role of calcineurin signaling components is being addressed
9 by several investigations in yeasts and filamentous fungi ((Risipail *et al.*, 2009) and references
10 therein). Particularly the work done in *Aspergillus* species showed that, CrzA, the orthologue of
11 Crz1p in *A. nidulans*, mediates tolerance to Ca^{2+} , Mn^{2+} and alkalinity as well as it is required for
12 the maintenance of cell wall integrity (Hagiwara *et al.*, 2008; Spielvogel *et al.*, 2008). The ΔcrzA
13 mutant of *A. fumigatus* shows altered temperature, pH and ion tolerance, defects in germination,
14 polarized growth and asexual development and loss of virulence (Cramer, Jr. *et al.*,
15 2008; Soriani *et al.*, 2008), and, finally, in *A. parasiticus* CrzA is required for growth and
16 aflatoxin biosynthesis under calcium stress conditions (Chang, 2008).

17 To better understand calcium signaling in filamentous fungi, we further investigated *cis* and
18 *trans*-regulatory elements acting on CrzA from *A. nidulans*. Functional, biochemical and
19 cellular localization studies have provided a detailed model of phospho-regulation and
20 nucleocytoplasmic transport of CrzA. We show that both calcium and alkalinity trigger CrzA
21 nuclear localization and activation probably based on a phosphorylation/dephosphorylation
22 process. Like in other fungal models, *A. nidulans* CrzA is a target for the phosphatase
23 calcineurin. Furthermore, CrzA-inactivation mechanism shares components of the mammalian
24 NFAT and yeast Crz1p pathways, since *A. nidulans* casein kinase I CkiA and *A. nidulans* Gsk3 β
25 homologue are involved in CrzA phosphorylation.

28 Results

30 *Calcium, manganese or alkaline ambient pH stress induce nuclear accumulation of CrzA*

32 We demonstrated the role of CrzA for tolerance of *Aspergillus nidulans* to both alkalinity and
33 an elevated extracellular concentration of calcium (Spielvogel *et al.*, 2008), and others also
34 showed this for tolerance to manganese cation (Hagiwara *et al.*, 2008). Calcium, as shown for
35 Crz1p (Cyert, 2003), induces the nuclear accumulation of CrzA ((Spielvogel *et al.*, 2008) and
36 Fig. 1). To determine whether nuclear transport of CrzA is a major regulatory step we analyzed
37 the preferential localization of CrzA in cells challenged to elevated concentrations of a number

of cations and different ambient pH values. Cells of a strain expressing a CrzA-GFP fusion were grown in standard minimal medium without cation or pH stress, hereafter designated as resting cells, and, as expected, CrzA remained cytoplasmic and excluded from nuclei ((Spielvogel *et al.*, 2008) and Fig. 1). Nuclei were identified using the specific nuclear marker Histone H1 tagged with monomeric-cherry fluorescent protein, H1-mCh. Addition of calcium induced nuclear accumulation of CrzA (50 mM CaCl_2 shown in Fig. 1) and this effect was also observed when the medium was alkalinized using 100 mM Na_2HPO_4 as buffering agent (pH 8). Manganese, Mn^{2+} , is a toxic cation for *Aspergillus* at low concentrations (7.5 mM, (Hagiwara *et al.*, 2008)) and addition of 10 mM MnCl_2 also induced the nuclear accumulation of CrzA (Fig. 1).

However, CrzA remained preferentially cytoplasmic, as in resting cells, after addition of the divalent cation Mg^{2+} (200 mM MgCl_2) to the medium or after its acidification (using 100 mM NaH_2PO_4 ; pH 5.2). A similar effect was observed when an elevated concentration of sodium (1 M NaCl) or potassium (1 M KCl) was added to the culture. In these later conditions, CrzA-GFP fluorescence aggregated at the cytoplasm but nuclei remained empty, as showed by DAPI staining (Fig. 1). These results strongly support a model in which nuclear transport is a key regulatory point for modulating CrzA transcriptional function. Nuclear accumulation of CrzA is observed in those stress conditions in which the function of this TF is required.

Delimiting functional regions in CrzA.

A basic deletion analysis has provided with valuable information on the signaling and functional mechanisms acting upon CrzA. Sequence conservation among yeast, Crz1p, and filamentous fungal CrzA homologues is poor and practically concentrates at the DNA binding region (DBD) which comprises three zinc-fingers, two of which belong to the C_2H_2 class (Spielvogel *et al.*, 2008). A major difference between Crz1p and CrzA-like proteins is the presence of a conserved C-terminal region after the DBD in CrzA homologues (Supplementary Fig. 1A). Using a expression system based the *gpdA^{mini}* promoter, a constitutive but low-expression *gpdA* promoter (Pantazopoulou and Peñalva, 2009), the functionality of truncated CrzA proteins was analyzed in wild-type and null *crzA* genetic backgrounds. Expression of GFP-CrzA(1-730), the full-tagged version, complemented calcium and alkaline-pH sensitive phenotypes caused by the null *crzA* allele (Fig. 2A). Deletion of residues 613-730, the conserved C-terminal region, resulted in a functional truncated CrzA protein. The GFP-CrzA(1-612) protein complemented the null *crzA* allele for resistance to calcium and alkaline pH (Fig. 2A). Interestingly, this phenotype was also observed in a strain expressing a truncated CrzA lacking the first 444 amino acids, GFP-CrzA(445-730) (Fig. 2A). In fact, colonies expressing GFP-CrzA(1-612) fusion

grew less healthy than those expressing the full length construct or a truncated GFP-CrzA(445-730) fusion, indicating a functional role for this conserved C-terminal region.

As expected fluorescent chimeras lacking the DBD, GFP-CrzA(1-448) and GFP-CrzA(608-730), were not functional (Fig. 2A). None of the previous fusion proteins caused any major distortion in a wild-type *crzA* background, however, expression of the zinc-finger region alone reduced colony growth and when in presence of calcium generated multiple sectors emerging from the inoculation point (Fig. 2A, see Ca^{2+}/wt background sector). As a possible source of loss-of-function mutations in the DBD, we analyzed some of these sectors finding in all cases that either the construct was not expressed or had been deleted from the site of integration within the genome, suggesting a deleterious effect due to constitutive over-expression of this domain (data not shown).

To extend the functional analysis of CrzA(1-612) and CrzA(445-730), these forms were expressed as unique source of CrzA from its endogenous promoter as C-terminally tagged GFP fusion proteins. As shown above for the N-terminal GFP-tagged CrzA, the CrzA(1-730)-GFP fusion protein is functional (see also (Spielvogel *et al.*, 2008)) and colonial growth of a strain expressing this fusion is indistinguishable of a wild type (Fig. 2B). The *crzA*^{prom}-driven expression of CrzA(1-612) also resulted in a functional truncated version but a strain expressing CrzA(445-730) showed some sensitivity to alkaline pH and no growth under calcium stress conditions (Fig. 2B). Tolerance of *A. nidulans* to calcium was also tested and a concentration higher than 0.5 M affected colonial growth. The strain expressing the CrzA(1-730)-GFP showed a wild-type phenotype but that expressing CrzA(1-612)-GFP displayed strong sensitivity to concentrations of CaCl_2 above 0.5 M (Fig. 2B).

Analysis of the cellular distribution of these GFP-chimeras shed light on the location of putative nuclear transport sequences in CrzA (Fig. 2C). Distribution of CrzA(1-612) was comparable to CrzA(1-730), both located mainly at the cytoplasm and excluded from nuclei in resting cells and nuclear under Ca^{2+} or pH 8 stresses. CrzA(1-448) holds regulatory regions required to mediate correct nucleocytoplasmic trafficking, although nuclear import was apparently reduced under cation or alkaline-pH stresses. However, CrzA(445-730) was always located in nuclei, in both resting cells and stress conditions. CrzA(445-612), essentially the DBD, also showed nuclear localization independently of ambient conditions. Notably, nuclear accumulation of CrzA(445-612) was increased compared to CrzA(445-730), suggesting that C-terminal region might modulate nuclear import. Nevertheless, CrzA(608-730) seems not to carry any nuclear localization or cytoplasmic retention signal as it showed a dispersed distribution along the cell under any condition assayed. Therefore, calcium and alkaline signaling requires the N-terminal region of CrzA, and this involves the regulation of nucleocytoplasmic transport signals.

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37

Nuclear accumulation of CrzA induced by calcium and alkalinity depends on calcineurin activity.

A $\Delta cnaA$ strain displays impaired colonial growth with severe morphological defects and sensitivity to an elevated concentration of calcium or to alkalinity (see (Soriani *et al.*, 2008) and Fig. 3A). We focused to determine whether the catalytic subunit of the calcineurin, CnaA, is needed for nucleocytoplasmic transport of CrzA in response to stress. Strain MAD3222 expresses the CrzA-GFP chimera as unique source of this TF in the absence of calcineurin activity ($\Delta cnaA$). In the absence of stress, CrzA-GFP fluorescence was excluded from nuclei and dispersed through the cytoplasm in both wild-type and null *cnaA* genetic backgrounds (Fig. 3B). These results suggested that the nuclear export system acting on CrzA was not affected. Nuclear accumulation of CrzA in the wild-type *cnaA* strain was completed in 5-10 minutes after calcium addition or medium alkalization whereas in the null *cnaA* strain the localization of CrzA-GFP remained unaltered after 30 minutes of treatment with either stimulus (Fig. 3B). Thus, the calcineurin activity is responsible for the nuclear accumulation of CrzA in response to Ca^{2+} and alkaline stress in *A. nidulans*.

CrzA is a target of calcineurin and different phosphorylated forms are detected under stressing and non-stressing conditions

Regulation of *S. cerevisiae* Crz1p implies the existence of phosphorylated forms which are detected by their differences in electrophoretic mobility (Stathopoulos-Gerontides *et al.*, 1999). The positive role of calcineurin phosphatase in the nuclear import of CrzA prompted us to investigate the phosphorylation status of this TF under stress and in different mutant backgrounds. Various culture conditions and protein isolation procedures were developed to preserve post-translational modified states of CrzA (see Supplementary Text, and Fig. S2A and S2B). Firstly, the electrophoretic mobility of a CrzA-GFP fusion was followed in protein extracts from resting cells of a wild type and a null *cnaA* mutant. Fig. 4A shows the reduced mobility of this tagged form of CrzA when using protein extracts from the $\Delta cnaA$ MAD3222 strain compared with the mobility of CrzA-GFP from the wild-type *cnaA* strain (MAD3021). Low mobility of CrzA was indicative of presence of phosphorylated forms and this was confirmed by inhibiting the activity of calcineurin, using cyclosporine A (CsA), and by performing *in vitro* dephosphorylation assays. A reduction of CrzA-GFP mobility was observed in protein extracts of a wild type, MAD3021, treated with increasing amounts of CsA (Fig. 4A). Notably, this CsA effect on CrzA mobility was never observed to be as strong as the effect of lacking calcineurin activity (see also Fig. 4B).

1 When evaluating the *in vitro* activity of lambda protein phosphatase (λ PP) in protein extracts of
2 a wild type we found that mobility of CrzA-GFP did not change after treatment (Fig. 4B, left).
3 This result and the need of using a specific protein-extraction protocol for λ PP assays led us to
4 suppose that those were not the ideal conditions to observe phosphorylated forms of CrzA (see
5 Experimental Procedures and Supplementary Text). The use of CsA would prevent calcineurin
6 activity and, consistently with this inhibitor being added to cultures, mobility of CrzA-GFP was
7 significantly reduced. A λ PP assay using protein extracts of a wild type grown with CsA
8 clearly showed an increase in the mobility of CrzA fusion, similar to that observed before
9 without CsA treatment (Fig. 4B, right). The mobility of CrzA-GFP was similar in protein
10 extracts from mycelia of null *cnaA* strain MAD3222 treated with or without CsA, demonstrating
11 that the effect of CsA in CrzA mobility was in fact through inhibiting the activity of calcineurin
12 (Fig. 4C). In addition, these experiments corroborated the existence of phosphorylated forms of
13 CrzA.

14 To verify that CrzA was a substrate of calcineurin we performed an *in vitro* dephosphorylation
15 assay using human calcineurin. This experiment also showed an increase in mobility of CrzA
16 similar to that observed for the protein phosphatase of λ phage (Fig. 4D). The overall data
17 demonstrates that CrzA is a phospho-protein and a target for calcineurin phosphatase activity.

18 Once demonstrated the presence of phosphorylated forms of CrzA in a wild type background,
19 we analyzed the effect of Ca^{2+} addition or alkalization in CrzA mobility (Fig. 4E). Calcium
20 had an immediate effect on increasing the mobility of CrzA (1 min time point, Fig. 4E).
21 Because these protein extracts were obtained in a procedure which preserves CrzA
22 phosphorylation states, it is possible to detect differences in the mobility of CrzA after stress
23 treatment (compare 0 and 1 min time points, Fig. 4E). However, this calcium-derived effect was
24 transient and low-mobility forms of CrzA were detected newly at 5 and 15 min after calcium
25 addition. This effect on CrzA mobility was consistent with the signaling model for other Crz
26 factors in which dephosphorylation induce their nuclear entry (Karababa *et al.*,
27 2006; Stathopoulos-Gerontides *et al.*, 1999).

28 Surprisingly, alkalization had a different effect although increase in the mobility of CrzA was
29 also detected (Fig. 4E). At 1 minute after alkalization it was possible to detect a mixture of
30 bands rather similar to that observed with calcium at 15 minutes. This pattern of bands varied
31 with time and 30 min after induction of pH 8 stress it was possible to observe mainly the highest
32 mobility of CrzA (see Fig. 4E and Fig. S2B). Meanwhile nuclear accumulation of CrzA-GFP is
33 observed with both stimuli, calcium and alkalinity (Fig. 1), it is not obvious that a complete
34 dephosphorylated state of CrzA is responsible for its preferential nuclear import, and this has
35 been already noted in other calcineurin signaling and nuclear transport systems (Okamura *et al.*,
36 2000; Holmberg *et al.*, 2002).

Calcineurin-calcium and alkaline stress response elements in CrzA locate at the N-terminal region.

The CrzA(1-612) truncated protein holds the regulatory regions required to mediate nucleocytoplasmic trafficking in response to calcium stress and alkalinity (Fig. 2C). The need of calcineurin signaling in this transport process was evaluated by expressing the GFP-CrzA(1-612) fusion, driven by the *gpdA^{mini}*-promoter, in the Δ *cnaA* mutant. In the absence of calcineurin activity, the addition of calcium or medium alkalization did not promote nuclear import of GFP-CrzA(1-612), and this was also observed for the, *gpdA^{mini}*-driven, full GFP-CrzA fusion (Fig. 5A). Accordingly with the non-regulated nuclear localization shown by the GFP-CrzA(445-730) truncated form, the absence of calcineurin did not alter its nuclear accumulation at any growth condition (Fig. 5A). These results suggested that residues lying within 1-612 region might be under regulation by phosphorylation-dephosphorylation. λ PP assays confirmed that CrzA(1-612) was phosphorylated in a null *cnaA* mutant, whereas CrzA(445-730) was not (Fig. 5B). Thus calcineurin regulates signaling (nucleocytoplasmic trafficking) of CrzA in response to Ca^{2+} and pH 8 and this must require, at least, a docking domain located into the first 445 amino acids of this TF. In addition, constitutive expression of these full or truncated-CrzA forms did not suppress phenotypes derived of Δ *cnaA* allele such as sensitivity to calcium or alkaline-pH stress, and defects in the colony morphology (Fig. 5C).

Identification of a CnaA docking domain in CrzA

The catalytic subunit of calcineurin recognizes in its substrates a short motif, denoted as calcineurin-docking domain (CDD), which conforms the consensus sequence PxIxIT defined in NFAT transcription factors (Fig. 6A). A possible dual calcineurin-docking site was predicted between amino acids Q294 and H316 (Spielvogel *et al.*, 2008) (Fig. 6B). To study the role of this putative CDD a mutant version of GFP-tagged CrzA lacking this region, designated as CDD1 (Fig. 6B), was generated by gene replacement at the *crzA* locus. Transformants expressing CrzA ^{Δ CDD1}-GFP as unique source of CrzA displayed a similar phenotype to that observed for the *crzA* wild-type strain with respect to calcium or alkaline-pH tolerance (Fig. 6C). In agreement with this observation, CrzA ^{Δ CDD1}-GFP accumulated in nuclei after calcium or alkaline-pH stress (Fig. 6D), indicating that signaling of this mutant protein occurs as in the native CrzA protein.

An alternative CDD of PxIxIT type was found between amino acids M356 and A371, a PSINVE motif designated as CDD2, sharing strong similarity to the low-affinity Crz1p

1 calcineurin-binding sequence PVIADV (Roy *et al.*, 2007). This PSINVE motif is conserved
2 among CrzA homologues of filamentous fungi (Fig. 5A and B). A strain expressing a mutant
3 version of CrzA lacking CDD2, CrzA^{ΔCDD2}-GFP, was obtained as above for site CDD1. In
4 contrast to the wild type-like localization of CrzA^{ΔCDD1}-GFP, the mutant CrzA^{ΔCDD2}-GFP
5 remained cytoplasmic after medium alkalization or calcium addition (Fig. 5D). The
6 localization of mutant CrzA^{ΔCDD2}-GFP resembled that of CrzA-GFP in the absence of
7 calcineurin activity.

8 However, a strain expressing CrzA^{ΔCDD2}-GFP protein still displayed mild sensitivity to elevated
9 levels of calcium or alkalinity. To study a possible synergic effect of CDD1 site in this stress
10 tolerance a mutant strain expressing a CrzA-GFP fusion protein lacking both CDD sites was
11 generated, CrzA^{ΔCDD1+2}-GFP. Localization of CrzA^{ΔCDD1+2}-GFP was indistinguishable of
12 CrzA^{ΔCDD2}-GFP protein and both ΔCDD2- and ΔCDD1+2-CrzA-mutant strains displayed
13 similar tolerances to calcium and alkalinity (Fig. 6C). An explanation for this contradictory
14 phenotype was obtained when analyzing the electrophoretic mobility of mutant ΔCDD1+2-
15 CrzA. Addition of calcineurin inhibitor CsA reduced the mobility of CrzA^{ΔCDD1+2}-GFP (Fig. 6E)
16 and λ PP assays of protein extracts from mycelia treated or not with CsA showed CrzA^{ΔCDD1+2}-
17 GFP was phosphorylated differently in both conditions (Fig. 6F). Calcium but not medium
18 alkalization increased the mobility of this mutant CrzA protein as observed before for the full
19 CrzA-GFP fusion, although a delay in this response was observed (Fig. 6E). These results
20 indicated that this mutant CrzA protein could be subjected to a
21 phosphorylation/dephosphorylation process mediated by calcineurin by recognizing alternative
22 sites such as LxVP motif (Rodriguez *et al.*, 2009). However, a scanning of this CnaA binding
23 motif in CrzA sequence was unsuccessful suggesting either the presence of novel CDD in CrzA
24 or a direct action of this phosphatase without docking.

25 Finally to confirm that CDD2 is a key element on CrzA-calcineurin interaction a pull down
26 experiment was performed using bacterially expressed GST-CrzA fusions either in its wild-type
27 sequence or lacking the CDD2 motif, and protein extracts from strain MAD4366 expressing a
28 CnaA-GFP fusion. A CrzA-CnaA interaction was detected when using the wild-type version of
29 GST-CrzA but not with the CrzA^{ΔCDD2} (Fig. 6G), thus we propose CDD2 as the most important
30 site for calcineurin signaling in CrzA.

31
32 ***Mutations in casein kinase I, CkiA, affect CrzA phosphorylation but not nucleocytoplasmic***
33 ***transport.***

34
35 Hrr25p mediates phosphorylation of Crz1p in *S. cerevisiae* (Kafadar *et al.*, 2003). Hrr25p is an
36 essential kinase as its homologue in *A. nidulans*, the casein kinase I CkiA (Apostolaki *et al.*,

2012). Three partial loss-of-function mutants in *ckiA*, alleles *ckiA2*, *ckiA1919* and *ckiA102*, and a conditional, thiamine-dependent, null allele *thiA^P::ckiA* (kindly provided by Prof. Scazzocchio and Dr. Sotiris), were analyzed for their potential effects on CrzA phosphorylation.

Figure 7A and Fig. S3 show the colonial growth of *ckiA* mutant strains expressing the CrzA-GFP chimera, as the unique source of CrzA, on different media conditions. There are no visible effects of excess of calcium or alkalinity in the colonial growth of these *ckiA* mutants (Fig. 7A and Fig. S3) but they showed the expected sensitivity or reduced growth due to defects in amino acid transport (shown for arginine in Fig. 7A, and see also (Apostolaki *et al.*, 2012)). A triple mutant *ckiA2*, null *cnaA*, *crzA-gfp* strain was also constructed and displayed a similar phenotype to that of $\Delta cnaA$, *crzA-gfp*, strain except for reduced growth in medium containing arginine as main nitrogen source due to the presence of *ckiA2* mutation.

We analyzed the localization of CrzA-GFP in these partial loss-of-function *ckiA* backgrounds and it was similar to that described above for this fusion protein in control strain MAD3021, displaying nuclear exclusion in the absence of stress and nuclear accumulation after alkalization or calcium addition (Fig. 7B). Importantly, CrzA-GFP also showed the requirement of calcineurin activity to be imported in the nucleus in the *ckiA2* background (Fig. 7B). These results suggested that CrzA was still subjected to phosphorylation and to verify this hypothesis protein extracts *ckiA* mutant strains were analyzed. The mobility of CrzA-GFP in each of the *ckiA* partial loss-of-function mutants was higher compared to that found in extracts from the wild-type *ckiA* strain (Fig. 7C). In particular, *ckiA102* and *ckiA2* showed higher mobility of CrzA in contrast to *ckiA1919* which is a moderate loss-of-function allele (Apostolaki *et al.*, 2012). Mobility of CrzA was reduced in these mutant backgrounds when CsA was added, however this mobility was not exactly that found for the CrzA form described under the same conditions in a wild type (compare Fig. 4 and 7C), suggesting a reduction on the phosphorylation levels of CrzA in the absence of full CkiA activity. When analyzing the conditional null *ckiA* strain we found that mobility of CrzA was similar to that observed in a *ckiA2*, the strongest loss-of-function mutant, either in the absence or presence of calcineurin inhibitor (Fig. S3). This effect was also observed in a *ckiA2*, $\Delta cnaA$ mutant background where the electrophoretic mobility of CrzA was higher than that of CrzA in a null *cnaA* background (Fig. 7C). These results evidenced a role of CkiA casein-kinase in the phosphorylation level of CrzA, but functionally, this might have a minor impact because it is not affecting either the regulation of nuclear import and/or export mechanisms for CrzA, or an adequate response to calcium and alkaline-pH stress by this transcription factor.

AnGSK3 activity is required for nuclear accumulation of CrzA under alkaline growth conditions.

1
2 Glycogen-synthase kinase 3- β (GSK3 β) phosphorylates NFAT factors (Beals *et al.*, 1997),
3 being an alternative mechanism to casein kinase I for CrzA phosphorylation. A motif scan of
4 the CrzA sequence, using NetPhosK 1.0 on-line resources
5 (www.cbs.dtu.dk/services/NetPhosK/), revealed the presence of numerous putative target sites
6 for a GSK3 β -like kinase activity, mostly located at the N-terminal region with respect to the
7 DBD (Fig. 8A).

8 We identified the locus coding for *A. nidulans* GSK3 β homologue, AN6508, using BlastN and
9 BlastP searches and generated a null *An6508-GSK3 β , Δ gskA* strain. This putative kinase, in
10 contrast to *ckiA*, is not essential but the mutant *gskA* strain displays a severe defect of colonial
11 growth and poor conidiation (Fig. 8B, these results are consistent with those presented recently
12 in a systematic functional analysis of *A. nidulans* kinome (De Souza *et al.*, 2013)). Despite the
13 strong morphological defects, cellular localization of CrzA-GFP was not altered and exhibited a
14 wild type-like distribution in the absence of Ca²⁺ or pH 8 stresses (Fig. 8C). Nuclei of *Δ gskA*
15 resting cells were altered in their cellular distribution and size, but this did not affect the
16 preferential cytoplasmic localization of CrzA-GFP (Fig. 8C). Calcium induced nuclear entry of
17 the fusion protein similarly to other Ca²⁺-stimulation experiments. However alkaline-pH stress
18 did not cause the same effect in the localization of CrzA. After alkalinization nuclear exclusion
19 of CrzA-GFP was lost. Although nucleoplasm became fluorescent, accumulation of CrzA-GFP
20 was not immediately observed (see magnification of a selected nucleus, Fig. 8C), or even after
21 30 min treatment (not shown), indicating that GskA activity is a requisite to activate the nuclear
22 import mechanism of CrzA under alkaline-pH stress.

23 Phosphorylation levels of CrzA are reduced in the *Δ gskA* mutant. Mobility of CrzA in *Δ gskA*
24 was higher than in the wild type RC (Fig. 8D), being this effect shared with mutants in *CkiA*
25 activity (see above, Fig. 7C and Fig. S2C). Treatment of *Δ gskA* cells with CsA reduced CrzA
26 mobility but not to the level found in the wild type (Fig. 8D) or *ckiA* mutants (Fig. S2C),
27 suggesting a very low level of phosphorylation of CrzA in the *Δ gskA*, which was confirmed
28 by λ PP assays (Fig. 8E). In fact, the mobility of CrzA in the null *gskA* background was similar
29 to that of a completely dephosphorylated CrzA form (Fig. 8E and Fig. S2C). Nevertheless,
30 CrzA is susceptible to be phosphorylated in the absence of GskA activity as showed the
31 reduction in CrzA mobility after CsA treatment and further dephosphorylation assays using
32 λ PP (Fig. 8E). Interestingly, a reduction in electrophoretic mobility of CrzA in the *Δ gskA*
33 mutant was also detected after addition of calcium or medium alkalinization, indicating that
34 both stimuli are inducing a modification in this TF in the absence of GskA kinase (Fig. 8F). The
35 overall results clearly support a fundamental role of GSK3 β homologue in proper signaling of
36 CrzA.

Discussion

Signaling of calcineurin-responsive zinc-finger (Crz) proteins has been studied in detail only in *S. cerevisiae* (Cyert, 2003). Here we demonstrate that signaling mechanisms acting on a filamentous fungal Crz, CrzA of *Aspergillus nidulans*, are conserved with yeast Crz1p and mammalian NFATc transcription factors. CrzA is a phospho-protein that is dephosphorylated in response to a limited number of ambient stress factors through the calcineurin phosphatase. Essentially, calcineurin activity is required for CrzA to migrate into nuclei (see regulatory model in Fig. 9). Activities of at least two kinases, the casein kinase I homologue, CkiA, and glycogen-synthase-3 β kinase homologue, GskA, are involved in CrzA phosphorylation. We visualized a wide range of electrophoretic mobility for CrzA, however it was not possible to correlate phosphorylation level with cellular localization. In fact, activity of GskA is required for nuclear accumulation of CrzA at ambient alkaline pH but not for calcium stress. Thus a precise balance of phosphorylation/dephosphorylation must be required to modulate nucleocytoplasmic trafficking of CrzA in response to a limited number of extracellular signals.

Nuclear entry of CrzA, a major regulatory step in its signaling.

A null *crzA* strain displays sensitivity to elevated concentrations of extracellular calcium (above 10 mM), manganese (above 5 mM) and alkaline pH (above pH 7.5), suggesting that the transcriptional function of CrzA is required for tolerance to these abiotic stresses (Hagiwara *et al.*, 2008; Spielvogel *et al.*, 2008). In agreement with this hypothesis we observed that CrzA accumulated in nuclei when cells are exposed to those stresses. In resting cells, CrzA as other homologues in fungi exhibits a cytoplasmic distribution being excluded from nuclei (Karababa *et al.*, 2006; Lev *et al.*, 2012; Stathopoulos-Gerontides *et al.*, 1999), and here we show that this localization of CrzA is maintained in those stress conditions which do not require its function (Fig. 1). An excess of monovalent cations such as sodium or potassium and the divalent cation magnesium do not induce nuclear entry of CrzA. Interestingly, whereas magnesium or medium acidification do not alter the cytoplasmic distribution of CrzA, addition of elevated concentrations of Na⁺ or K⁺ cations induce the presence of fluorescent aggregates in the cytoplasm as those recently observed in *Cryptococcus neoformans* upon salt-stress (Lev *et al.*, 2012). The nature of those aggregates has not been studied yet in *A. nidulans*, but its presence indicates the existence of a common fungal mechanism acting on CrzA homologues to prevent nuclear entry under salt stress.

Thus, in a specific way, calcium, manganese and alkalinity must stimulate the nuclear import system. Our functional analysis of CrzA indicates the presence of a NES within the first 445

1 amino acids and an NLS in its proximities as shows the localization of CrzA(1-448) truncated
2 form. However, we detect a second NLS into CrzA(445-612) fragment, most probably the
3 immediacy or into the DBD. A similar number and distribution of nuclear transport signals were
4 described in Crz1p (Boustany and Cyert, 2002; Polizotto and Cyert, 2001), but functional
5 conservation extends to their regulation by stress. As in Crz1p, a CrzA fragment containing both
6 N-terminal NLS and NES, CrzA(1-448), is regulated by calcium or alkalinity, and a fragment
7 containing essentially the DBD, CrzA(445-612), is permanently located in the nucleus
8 independently of calcineurin signaling. The absence of sequence conservation between Crz1p
9 and CrzA prevented the identification of both NES and NLS in the N-terminal region, however
10 the strong sequence conservation at the zinc-finger region clearly identifies Crz1p-NLS2,
11 ⁶¹²RKR⁶¹⁵, into CrzA DBD. Finally, it was shown that a null *nmd5* strain displayed the same
12 phenotype as a null *crz1* strain demonstrating the role of this karyopherin in nuclear import of
13 Crz1p (Polizotto and Cyert, 2001). During our analysis of nuclear transport pathways in *A.*
14 *nidulans* was stated the absence of conservation of such phenotype for a null *kapD* strain, being
15 KapD the putative homologue for Nmd5, and for any other viable null mutant in the transport
16 system (Markina-Inarrairaegui *et al.*, 2011). Currently we are identifying and analyzing these
17 transport signals and possible transporters involved.

19 ***Calcineurin activity and post-translationally modified states of CrzA.***

20 In this work we demonstrate for the first time in filamentous fungi a role of calcineurin in
21 calcium and alkaline-pH signaling of CrzA. Calcineurin promotes CrzA dephosphorylation and
22 subsequently this induces its nuclear accumulation. As observed for Crz1p (Stathopoulos-
23 Gerontides *et al.*, 1999), the absence of calcineurin activity restricts CrzA localization to the
24 cytoplasm and the highest level of phosphorylation is observed as the lowest electrophoretic
25 mobility of CrzA. It is interesting to note that such low mobility of CrzA in a Δ *cnaA*
26 background was not modified by any external stimulus demonstrating the essential role of
27 calcineurin in this regulatory system of *A. nidulans*.

28 In a previous work (Spielvogel *et al.*, 2008), we predicted a double calcineurin docking domain
29 in CrzA, CDD1, not conforming the PxIxIT consensus but similar to the PIISIQ motif identified
30 in Crz1p (Matheos *et al.*, 1997). However our functional study of double-site CDD1 showed
31 that, although conserved among CrzA homologues, removal of these putative calcineurin
32 binding sequences did not affect signaling of CrzA. In contrast, an alternative site, PSINVE,
33 was found downstream of CDD1 and to be necessary for nuclear translocation of CrzA in
34 response to both calcium and alkaline pH. This conserved putative calcineurin site at CDD2 is
35 similar to a low affinity site for calcineurin studied in Crz1p, PVI⁶¹²AVN (Roy *et al.*, 2007). An *in*
36 *vitro* interaction experiment has proven the important role of CDD2 in the interaction between
37 CrzA and CnaA. However, deletion of either CDD2 alone or in combination with CDD1 did not

1 prevent growth of the mutant strain in the presence of calcium or alkaline pH. This phenotype
2 was anticipated from a loss-of-signaling transduction between active calcineurin and CrzA. The
3 effect of calcineurin-inhibitor CsA on CrzA^{ΔCDD1+2} mobility suggested the presence of either
4 residual calcineurin activity or additional, alternative, CDDs. Absence of LxVP motifs
5 (Rodriguez *et al.*, 2009) suggests that if alternative CDDs are present in CrzA, these might be
6 completely unrelated to those defined previously. Detailed experimental work will be required
7 to determine the exact number of CDD sites, however we propose CDD2 as the most important
8 site for calcineurin signaling in CrzA.

9 Demonstration of different phosphorylation levels in CrzA has been possible due to an
10 alternative protocol for protein isolation which prevented, most probably, unspecific activation
11 by calcineurin during the process by reducing manipulation time. In these conditions we
12 visualized five different phosphorylation stages, ranging from forms detected in the absence of
13 calcineurin activity, highest phosphorylation level, to those found after calcium treatment, the
14 lowest (see Fig. S2B and Supplementary Text). In *Candida albicans*, different phosphorylation
15 steps of CRZ1 have been described after mutant, drug treatments and calcium-addition studies
16 (Karababa *et al.*, 2006). Under non-stressing conditions, mobility of CrzA suggests that is
17 partially phosphorylated and truncated forms indicate that most of target residues locate in the
18 N-terminal region 1-445. The presence of a conserved serine-rich region (SRR) is found
19 between residues 233 and 352 in CrzA, some of which overlap with CDD1 (Fig. 6). Although
20 amino acid sequence is not highly conserved amongst yeast and filamentous fungal Crz1p/CrzA
21 orthologues, residues targeted for phosphorylation, SRR, locate in the proximities of nuclear
22 transport signals and calcineurin binding sites (Karababa *et al.*, 2006; Stathopoulos-Gerontides
23 *et al.*, 1999).

25 ***Kinases involved in CrzA phosphorylation***

26 Here we identify two phosphorylation mechanisms acting on CrzA. The essential casein kinase
27 I, CkiA (Apostolaki *et al.*, 2012), regulates the phosphorylation state of CrzA, as does Hrr25p in
28 *S. cerevisiae* for Crz1 (Kafadar *et al.*, 2003). All three available partial loss-of-function
29 mutations in CkiA and its conditional null allele modify CrzA phosphorylation levels. In mutant
30 *ckiA* resting cells CrzA remains cytoplasmic and calcineurin activity was still essential for
31 calcium or pH 8-induced nuclear import suggesting a minor role of CkiA in CrzA signaling. A
32 predicted CK1 phosphorylation site was found at serine 173, however this and flanking residues
33 are not conserved among CrzA homologues (Spielvogel *et al.*, 2008).

34 Gsk3β kinases recognize a short motif in their substrates, S/TxxxS/T, where the last residue is
35 already phosphorylated (Fiol *et al.*, 1987). The presence of eighteen putative target sites in CrzA
36 for a Gsk3β-like kinase prompted us to study its homologue in *A. nidulans*. This kinase has not

1 been studied in-depth in filamentous fungi. Gsk3 β homologue in *S. cerevisiae* was designated as
2 RIM11, which also relates this kinase with the RIM101/PacC pathway (Mitchell and Bowdish,
3 1992). Gsk3 β homologue in *A. nidulans* is coded by locus *An6508* designated here as *gskA*. We
4 show that this is not an essential kinase, however its absence cause major morphological defects
5 which are greater in minimal than in complete medium (see also (De Souza *et al.*, 2013)). In the
6 absence of GskA activity CrzA is found in a low phosphorylation state, even when the
7 calcineurin inhibitor was added, indicating a major role of this kinase in CrzA post-translational
8 modification. However, CrzA is excluded from nuclei in null *gskA* resting cells, thus GskA
9 activity is not required for the nuclear export of CrzA. In the null *gskA* background, calcium
10 induces nuclear accumulation of CrzA but alkalinity seems preferentially affect the nuclear
11 export system as nuclear exclusion of CrzA is lost. These changes in localization of CrzA are in
12 contrast with an effect of calcium or alkaline pH in reducing its electrophoretic mobility,
13 indicating the existence of a phosphorylation step in those conditions. CrzA mobility is different
14 between null *gskA* or *ckiA* mutants, providing evidence of dissimilar modes of signaling. The
15 overall of these results indicate that GskA is a principal component of CrzA signaling but state
16 that other kinases, possibly activated by calcium or alkalinity, might be acting on CrzA.
17 Participation of stress-induced activated kinases in Crz or NFAT signaling processes have been
18 proposed. In this respect, would be interesting to determine whether cAMP-dependent protein
19 kinase A, PKA, plays a role in post-translational modification CrzA because, Crz1p and NFAT
20 are phosphorylated by PKA, being in the latter that a primary modification by PKA anticipates
21 GSK3 β -dependent modifications (Kafadar and Cyert, 2004; Sheridan *et al.*, 2002) and a
22 coordinated regulatory mechanism between PKA and RIM11 has been shown for Ime1, the key
23 transcriptional activator of meiosis-specific genes in *S. cerevisiae* (Rubin-Bejerano *et al.*, 2004).

25 ***Integration of calcium and alkaline pH signals***

26 A regulatory model for CrzA is pictured in Fig. 9. In resting cells equilibrium between modified
27 forms of CrzA is predicted. Antagonistic activities of calcineurin and kinases, at least CkiA and
28 GskA, may regulate the final state of CrzA phosphorylation, and this is a common mechanism
29 among all calcineurin-dependent regulatory systems, including now a filamentous fungus.
30 When no stimulus is present, it is expected that kinase activities predominate on CrzA. CkiA,
31 GskA and other kinases, as those cited above, might be acting alone or in a concerted manner to
32 regulate CrzA.

33 An elevation of cytoplasmic calcium due to an elevated extracellular concentration of this cation
34 activates the calmodulin/calcineurin signaling pathway in a similar way to what has been
35 described in other organisms, and we show here that CrzA is one of its final targets in *A.*
36 *nidulans*. In *S. cerevisiae* calcineurin acts on different effectors and subsequent pathways (see
37 (Roy *et al.*, 2007) and references therein). Pleiotropic effects of lacking calcineurin are also

1 visible in *A. nidulans*, as noted before (Soriani *et al.*, 2008), $\Delta crzA$ and $\Delta cnaA$ strains do not
 2 display identical sensitive phenotypes to either calcium or alkaline pH (see Fig. 6) and a $\Delta cnaA$
 3 strain exhibits strong morphological defects not displayed by the $\Delta crzA$ mutant.
 4 Alkaline pH signaling remains still obscure. Elevation of intracellular calcium levels upon stress
 5 seems to be a general physiological response for yeasts and mammals (for a review see
 6 (Aramburu *et al.*, 2000)), and recently, an alkaline pH-dependent burst of intracellular calcium
 7 has been reported in *C. albicans* (Wang *et al.*, 2011). Hypothetically, alkaline pH would cause a
 8 release of internal calcium stores and induce a similar response to that proposed for calcium
 9 stress. Additionally, through other mechanisms alkalinity could act on the phosphorylation step.
 10 It is noticeably that alkalization do not promote a strong dephosphorylation effect in CrzA,
 11 although nuclear transport seems to be equally activated.
 12 In *A. nidulans* tolerance to alkalinity requires the activity of at least three major regulators,
 13 CrzA, SltA and PacC. Here we show that signaling of CrzA to alkaline pH or calcium does not
 14 require a proteolytic processing step as for PacC. The presence of a conserved YPxL motif, as
 15 those recognized by PalA in PacC (Vincent *et al.*, 2003), at the C-terminal region of CrzA-like
 16 factors suggested a possible relationship of CrzA with the Pal pathway. However there is no
 17 evidence of such a Pal/CrzA crosstalk since any interaction was detected between CrzA and
 18 PalA (Fig. S1B). We have not found any direct relationship, at this stage, between calcineurin
 19 and PacC or SltA. Thus alkaline pH adaptation may occur through parallel signaling pathways
 20 but common target genes regulated by these transcription factors, as has been suggested before
 21 in (Spielvogel *et al.*, 2008), or in *C. albicans* for CRZ1/2 and RIM101 (Kullas *et al.*, 2007).

23 Experimental Procedures

25 *Strains and media*

26 *Aspergillus nidulans* strains used in the present study are listed in Table 1. *Escherichia coli*
 27 strain DH5 α (GibcoBRL) was used for standard DNA cloning, plasmid manipulation or
 28 propagation, and GST fusion expression. General molecular techniques followed (Sambrook *et*
 29 *al.*, 1989). *A. nidulans* strains were cultivated in appropriately supplemented standard minimal
 30 medium (MMA) and complete medium (CMA) for *Aspergillus* as described previously (Cove,
 31 1966). Colony growth was studied by inoculating conidiospores on solid MMA supplemented
 32 with 50 mM CaCl₂ or 0.1 M Na₂HPO₄ to generate alkaline pH (pH 8) and incubated at 37°C for
 33 2 days.
 34 For total protein extractions, strains were cultivated in fermentation media for 18 hours at 37°C
 35 under agitation, essentially as described in (Orejas *et al.*, 1995). When indicated, calcium or
 36 alkaline stress was induced by adding 0.1 M CaCl₂ or 0.1 M Na₂HPO₄, respectively, to the

growing culture and further incubated at 37°C for time periods from 1 to 30 minutes, as indicated in the text, before mycelium collection. Calcineurin activity was inhibited in the presence of the immunosuppressant Cyclosporin A (CsA). CsA (Sigma-Aldrich) stock was prepared in dimethyl sulfoxide and added to the fermentation culture at final concentration of 50 μ M and incubated for 30 minutes at 37°C, otherwise indicated.

Construction of strains

Gene replacements, favored by the presence of null *nkuA* mutations, followed protocols described in (Markina-Inarrairaegui *et al.*, 2011; Nayak *et al.*, 2006). Strains obtained by transformation using either linear-DNA fragments or plasmids followed the protocol described in (Tilburn *et al.*, 1983). As selectable markers different genes from *Aspergillus fumigatus* were used, such as *pyrG*, *riboB* or *pyroA*. Pyrimidine, riboflavin or pyridoxine prototrophs were selected and homokaryotic transformants carrying single-copy integration events were identified by Southern blotting (Sambrook *et al.*, 1989). Mutant strains carrying GFP-tagged CrzA cassettes with either Δ *cnaA* and/or *ckiA* mutant alleles were obtained by meiotic recombination following standard genetic procedures in this fungus. Tagging of CnaA with GFP followed the procedures indicated in (Markina-Inarrairaegui *et al.*, 2011; Nayak *et al.*, 2006). A transformation cassette was generated by fusion PCR procedures using oligonucleotides listed in Table S1, and transformed into recipient strain MAD1739. Among *pyrG*⁺ transformants those showing a double recombination event at *cnaA* locus were selected and tested for expression of the CnaA-GFP chimera, MAD4366 was used in this work.

Deletion, GFP-tagging and directed mutagenesis of *crzA* locus

Two different null *crzA* strains were used in this work, Δ *crzA::pyr4*^{Nc} and Δ *crzA::riboB*^{Af} (MAD2449 and MAD3709). Strain MAD2449 was constructed by replacing the *crzA* coding sequence with the *pyr4* gene from *Neurospora crassa* as described for strain BER02 (see (Spielvogel *et al.*, 2008)). For the construction of MAD3709 strain the wild-type *crzA* locus was replaced by the *A. fumigatus riboB*, *riboB*^{Af}, following the procedure described in (Markina-Inarrairaegui *et al.*, 2011). A replacement cassette was generated by fusion to the *riboB*^{Af} gene of 1.5 kb PCR-amplified fragments corresponding to the 5' and 3' UTRs of AN5726 locus, using oligonucleotides pairs CrzA(1)/CrzA(2) and CrzA(3)/CrzA(4), respectively. The *A. fumigatus riboB* cassette was amplified from plasmid p1830, which contains a 2.2 kb insert of the genomic sequence corresponding to locus Afu1g13300, using specific primers (CrzA(2')/CrzA(3')). Oligonucleotides for this and subsequent sections are listed in Table S1.

A strain expressing a C-terminally GFP-tagged CrzA was generated by (Spielvogel *et al.*, 2008). Using primer pair CrzA(1) and CrzA(4) a fragment comprising 5'UTR::*crzA*::*gfp::pyrG*^{Af}::3'UTR cassette was amplified using high fidelity DNA polymerase

1 PrimeStar (Takara) and cloned into plasmid pGEM-T-Easy (Promega) to give plasmid pGEM-
 2 T-CrzA-GFP. The absence of mutations in the *crzA::gfp/pyrG^{Δf}* construct was verified by
 3 sequencing and subsequently used as template in further PCR amplifications. Strain MAD4641
 4 co-expressing CrzA-GFP and histone H1 tagged with monomeric cherry fluorescent protein was
 5 obtained after transformation of MAD2446 with the tagged *crzA*-transformation cassette.
 6 Strains expressing truncated forms of the C-terminally GFP-tagged CrzA, CrzA(1-612) and
 7 CrzA(445-730), were generated by replacement of $\Delta crzA::riboB^{\Delta f}$ allele in MAD3709 strain,
 8 using 5'UTR::*crzA(1-612)::gfp/pyrG^{Δf}*::3'UTR and 5'UTR::*crzA(445-730)::gfp/pyrG^{Δf}*::3'UTR
 9 cassettes. Both DNA cassettes were generated in a two-way PCR by fusion of fragments
 10 amplified from pGEM-T-CrzA-GFP using primer pairs CrzA(1)/CrzA(1-612)rv and CrzA(1-
 11 612)fw/CrzA(4) for the *crzA(1-612)::gfp*, construct, and primer pairs CrzA(1)/CrzA(2) and
 12 CrzA(445-730)fw/CrzA(4) for the *crzA(445-730)::gfp*, construct.
 13 Strains expressing CrzA^{ΔCDD1}-GFP, CrzA^{ΔCDD2}-GFP or CrzA^{ΔCDD1+2}-GFP mutant versions were
 14 generated by replacement of $\Delta crzA::riboB^{\Delta f}$ locus using PCR-mutagenized
 15 5'UTR::*crzA::gfp::pyrG^{Δf}*::3'UTR cassettes. The whole pGEM-T-CrzA-GFP plasmid was PCR-
 16 amplified using mutant primer pairs to specifically delete either CDD1 or CDD2 site. CDD1 site
 17 was deleted using primer pair $\Delta CDD1$ -fw / $\Delta CDD1$ -rv. CDD2 site was deleted using primer
 18 pair $\Delta CDD2$ -fw / $\Delta CDD2$ -rv. The PCR product was digested with *DpnI* before transformation
 19 in *E. coli* DH5 α to select mutagenized plasmids. Plasmid carrying the double deletion of CDD1
 20 and CDD2, pGEM-T- CrzA^{ΔCDD1+2}-GFP, was generated as described above using pGEM-T-
 21 CrzA^{ΔCDD1}-GFP as template and mutant primers $\Delta CDD2$ -fw and $\Delta CDD2$ -rv. In each case
 22 sequencing confirmed the proper deletion of the CCDs before amplification and use in
 23 transformation. Recipient strain for these constructs was MAD3709.
 24 When required, the *riboB2* allele was replaced by the wild-type *riboB* by transformation with a
 25 1.3 kb PCR fragment amplified from a wild-type strain using primers RiboB^{AN0670}_{up} and
 26 RiboB^{AN0670}_{down}, and further selection of transformants for riboflavin prototrophy.

28 *Expression of GFP-CrzA fusion driven by the gpdA-mini promoter*

29 Plasmid p1660 is the recipient for all constructs and holds the *gpdA^{mini}* promoter and a truncated
 30 *pyroA* used as selection marker for directed integration at *pyroA4* mutant locus (Pantazoupoulou
 31 and Penalva, 2009). Fragments or complete CDS of CrzA were obtained by PCR amplification
 32 of *crzA* cDNA cloned in plasmid pGEM-T-CrzAcDNA (Spielvogel *et al.*, 2008). Specific
 33 primer pairs carrying *NcoI* or *EcoRI* sites were used for amplification of different CrzA regions
 34 studied in this work. These fragments were cloned into plasmid pGEM-T-Easy (Promega),
 35 sequenced to avoid undesirable mutations due to PCR polymerase and excised using *NcoI* and
 36 *EcoRI* restriction enzymes and ligated to p1660 digested with the same enzymes. To introduce

the fragment coding for GFP, each derivative p1660-CrzA plasmid was digested with *NcoI* and an *NcoI-NcoI* fragment from plasmid p1155 (Mingot *et al.*, 2001) was inserted providing with *gpdA^{mini}::GFP::CrzA*-complete/truncated plasmid collection. Fidelity and the correct orientation of the inserts were verified by DNA sequencing. Plasmids were targeted in single copy to the *pyroA* locus as described previously (Pantazopoulou and Peñalva, 2009) using strains MAD1425 and MAD2449.

Expression of GST-CrzA fusions in bacteria and pull down assays.

Two recombinant vectors of original pGEX-2T (GE Healthcare) expressing either wild type or Δ CDD2 CrzA versions fused to Glutathione S-transferase (GST) were constructed. Firstly plasmid pGEX-CrzA was generated by introducing the cDNA of *crzA* coding region in frame with GST coding gene. CrzA coding fragment was generated by PCR and *NcoI* and *EcoRI* restriction sites were included at each end of the fragment using specific oligonucleotides (see Table S1). This fragment, after *NcoI* and *EcoRI* digestion, was inserted into an already modified version of pGEX-2T containing an *NcoI* between *BamHI* and *EcoRI* at the polycloning site of the original plasmid. pGEX-CrzA ^{Δ CDD2} was generated by mutagenic PCR as indicated above for the generation of the CrzA ^{Δ CDD2}-GFP coding mutant allele, using as template plasmid pGEX-CrzA.

CrzA or PalA glutathione S-transferase fusions were expressed using standard protocols using IPTG as inducer mainly following manufacturer instructions. Pull-down experiments were done essentially as described in (Vincent *et al.*, 2003).

Deletion of GSK3 β homologue in A. nidulans

The gene encoding for the putative homologue of glycerol synthase kinase 3 β , named here *gskA*, was identified in the *A. nidulans* genome database, <http://www.aspgd.org/>. BlastP and tBlastN searches using human GSK3 β (P49841) and *Saccharomyces cerevisiae* RIM11 (DAA10036) identified AN6508 as the GSK3 β homologue, showing 70% and 59.7% amino acid sequence conservation, respectively. Deletion of *gskA* CDS was completed following our standardized protocol using *riboB^{Δf}* as the selectable marker (Markina-Inarriraegui *et al.*, 2011). Briefly, approximately 1.5 kb of 5'- and 3'- UTR of AN6508 were amplified by PCR using genomic DNA and the primer pairs GskA(1)/ GskA(2) and GskA(3)/ GskA(4), respectively. *riboB^{Δf}*, was obtained by PCR using primers GskA(2') and GskA(3') from plasmid p1830 (see above). The deletion construct was generated in a three-way PCR by fusion of the upstream and downstream regions of AN6508 together with the *riboB^{Δf}* selection marker. The deletion DNA cassette was used for transformation in MAD3020. After selection of

homokaryons, several transformants were analyzed by Southern blot and specific radioactive probes (Sambrook *et al.*, 1989) and MAD3844 was selected for this study.

Protein extraction and analysis procedures

Mycelia, cultured under the different growth conditions indicated, were collected by two different methods: 1) filtered through Miracloth (Calbiochem) (Miracloth/filtration procedure, MF), squeezed to dry and frozen in dry ice or 2) vacuum filtered (vacuum/filtration procedure, VF) using 0.45 μ m pore size nitrocellulose membranes (Scharlau) and frozen in dry ice. In both cases, frozen samples were lyophilized for 16 hours before protein extraction.

Two different protocols were followed for obtaining total protein extracts from lyophilized samples. To prevent protein degradation, the alkaline lysis (AL) extraction procedure was used as described in (Hervás-Aguilar and Peñalva, 2010). Essentially, 6 mg of pulverized mycelium was resuspended in 1ml buffer (0.2 M NaOH, 0.2% β -mercaptoethanol) and usually 5 μ L of each sample were loaded on polyacrylamide gels for protein-content evaluation and estimation of concentration. For those samples requiring further analyses, proteins were extracted as described by Fernández-Martínez *et al.* (2003) using A50 buffer (25 mM Hepes pH 7.5, 0.1 M EDTA, 50 mM KCl, 5 mM MgCl₂, 10% glycerol, 0.5 mM DTT) supplemented with 1 mM Pefabloc (Roche), 1 μ M Pepstatin and 0.6 μ M Leupeptin as proteinase inhibitors. Total protein concentration was determined by Bradford assay (Bradford, 1976) using Bio-Safe™ Coomassie G-250 Stain from BioRad and following manufacturer's instructions.

Combinations of mycelium harvest and protein isolation were as follows: MFA50: Miracloth/filtration procedure followed by protein extraction using A50 buffer, MFAL: Miracloth/filtration procedure followed by alkaline lysis protein extraction, VFA50: Vacuum/filtration procedure followed by protein extraction using A50 buffer and VFAL: Vacuum/filtration procedure followed by alkaline lysis protein extraction.

For Western blotting pProteins were resolved in either 10% SDS-polyacrylamide gels or 4–15% Mini-PROTEAN® TGX™ precast polyacrylamide gels (Bio-Rad) and subsequently transferred to nitrocellulose filters using TransBlot® Turbo™ Transfer System (Bio-Rad). GFP tagged proteins were detected using mouse anti-GFP (1/5,000; Roche). MYC3-PacC fusion was detected using mouse anti-MYC (1/10,000; Sigma). Actin, detected with mouse anti- γ actin antibody (1/50,000; ICN Biomedicals), was used as loading control. Peroxidase conjugated goat anti-mouse IgG immunoglobulin (Jackson ImmunoResearch Laboratories) at 1/4,000 was used as a secondary antibody. Peroxidase activity was detected with Amersham Biosciences ECL kit.

Phosphatase treatments

The phosphorylation state of the protein samples was mainly analyzed using the procedure by Fernández-Martínez et al (2003). 400 U of λ Protein Phosphatase (λ PP) (NE BioLabs Inc) were added to reaction mixtures containing 100 μ g of total protein extract in NEBuffer for PMP (50 mM HEPES pH 7.5, 0.1 M NaCl, 2 mM DTT and 0.01% Brij 35) supplemented with 1 mM $MnCl_2$. Appropriate controls were set to show that electrophoretic mobility changes were specifically due to phosphatase activity in which no enzyme was added to the reaction or it was inhibited adding 10 mM sodium ortho-vanadate to the reaction mixture. The reactions were incubated for 20 min at 30°C and then stopped with the addition of 10% trichloroacetic acid and further incubation on ice for 10 minutes. Precipitated proteins were collected by centrifugation and washed twice with ethanol:ether solutions 1:1 and 1:3. Protein pellets were resuspended in loading buffer (62.5 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate, 5% β -mercaptoethanol, 6 M urea, 0.05% bromophenol blue) and denatured by heating to 100°C for 5 min. For each condition or strain analyzed, 25 μ g of precipitated proteins were loaded onto polyacrylamide gels.

Phosphatase assays using human calcineurin were done following (Heath *et al.*, 2004). For each reaction, 100 μ g of total protein extract from mycelia of MAD3222 strain were treated with 500 U of recombinant human calcineurin (Calbiochem) together with 2,500 U of recombinant bovine calmodulin (Sigma) in CP buffer (50 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 1 mM DTT), supplemented with 1 mM Pefablock (Roche), 1 μ M Pepstatin and 0.6 μ M Leupeptin as proteinase inhibitors. When indicated 50 μ M of cyclosporin A was used per assay to inhibit calcineurin activity. Reactions were incubated, stopped and processed for PAGE as indicated above for λ PP assays.

Microscopy

A. nidulans cells were cultured in appropriately supplemented watch minimal medium (WMM) (Peñalva, 2005) using uncoated μ -Slide 8 well (Ibidi GmbH, Germany) for a direct observation of cultures using a Leica DMI-6000b inverted microscope. For all microscopy experiments, germlings were incubated at 25°C for 18 hours except for strain MAD3844 which was incubated at 37°C for 20 hours.

After this period, the medium was replaced with fresh WMM containing a range of ambient stresses i.e. supplemented with $CaCl_2$ (50 mM), $MnCl_2$ (10 mM), $MgCl_2$ (0.1 M), NaCl (0.1 M), KCl (0.1 M), NaH_2PO_4 (0.1 M) or Na_2HPO_4 (0.1 M) for adjusting media pH to 5.2 or 8.0 respectively for the last two stimuli.

Fluorescence images were acquired using a Leica DMI6000b inverted microscope equipped with a GFP-specific filter (λ_{ex} , 470 nm and λ_{em} , 525 nm), and recorded with an Orca-ER camera (Hamamatsu) driven by the MetaMorph imaging system (Universal Imaging

1 Corporation). Nuclei were visualized using either following fluorescence of H1-mCh fusion
2 with a TxRed filter or DAPI (4'-6-diamidino-2-phenylindole) staining by replacing the medium
3 with a solution of 60 ng ml⁻¹ of DAPI in fresh WMM also containing 0.01% Triton to induce
4 entry of the dye in living cells.

5

6

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1 Table 1. List of strains used in this work

Strain	Relevant genotype	Source
MAD1425	<i>pyrG89</i>	(Markina-Inarrairaegui <i>et al.</i> , 2011)
MAD1739	<i>pyrG89, pyroA4</i>	(Findon <i>et al.</i> , 2010)
CNA1	$\Delta cnaA::pyroA^{Af}$	(Soriani <i>et al.</i> , 2008)
VIE050	<i>thiA^P::ckiA</i>	(Apostolaki <i>et al.</i> , 2012)
MAD1775	$\Delta palB::pyroA^{Af}$; <i>pacC900</i>	(Rodriguez-Galan <i>et al.</i> , 2009)
MAD2446	<i>hhoA::mCh::pyroA^{Af}</i> , <i>pyrG89</i>	(Etxebeste <i>et al.</i> , 2009)
MAD2449	$\Delta crzA::pyr4^{Nc}$, <i>pyroA4</i>	This work
MAD3709	$\Delta crzA::riboB^{Af}$	This work
MAD3020	<i>crzA::gfp::pyrG^{Af}</i> , <i>riboB2</i>	This work
MAD3021	<i>crzA::gfp::pyrG^{Af}</i>	This work
MAD4366	<i>cnaA::gfp::pyrG^{Af}</i>	This work
MAD3222	$\Delta cnaA::pyroA$; <i>crzA::gfp::pyrG^{Af}</i>	This work
MAD4641	<i>hhoA::mCh::pyroA^{Af}</i> ; <i>crzA::gfp::pyrG^{Af}</i>	This work
MAD2166	<i>pyroA4</i> [<i>pyroA</i> *:: <i>gfp::crzA</i> (M1-K730)]	This work
MAD3493	<i>pyroA4</i> [<i>pyroA</i> *:: <i>gfp::crzA</i> (M1-L612)]	This work
MAD2168	<i>pyroA4</i> [<i>pyroA</i> *:: <i>gfp::crzA</i> (M1-R448)]	This work
MAD2169	<i>pyroA4</i> [<i>pyroA</i> *:: <i>gfp::crzA</i> (MD+P445-K730)]	This work
MAD3648	<i>pyroA4</i> [<i>pyroA</i> *:: <i>gfp::crzA</i> (MD+P445-L612)]	This work
MAD2170	<i>pyroA4</i> [<i>pyroA</i> *:: <i>gfp::crzA</i> (ME+Q608-K730)]	This work
MAD2171	<i>pyroA4</i> [<i>pyroA</i> *:: <i>gfp::crzA</i> (M1-K730)]; $\Delta crzA::pyr4^{Nc}$	This work
MAD3494	<i>pyroA4</i> [<i>pyroA</i> *:: <i>gfp::crzA</i> (M1-L612)]; $\Delta crzA::pyr4^{Nc}$	This work
MAD2172	<i>pyroA4</i> [<i>pyroA</i> *:: <i>gfp::crzA</i> (M1-R448)]; $\Delta crzA::pyr4^{Nc}$	This work
MAD3386	<i>pyroA4</i> [<i>pyroA</i> *:: <i>gfp::crzA</i> (MD+P445-K730)]; $\Delta crzA::pyr4^{Nc}$	This work
MAD3649	<i>pyroA4</i> [<i>pyroA</i> *:: <i>gfp::crzA</i> (MD+P445-L612)]; $\Delta crzA::pyr4^{Nc}$	This work
MAD3388	<i>pyroA4</i> [<i>pyroA</i> *:: <i>gfp::crzA</i> (ME+Q608-K730)]; $\Delta crzA::pyr4^{Nc}$	This work
MAD3842	<i>crzA</i> (M1-L612):: <i>gfp::pyrG^{Af}</i>	This work
MAD3843	<i>crzA</i> (MD+P445-K730):: <i>gfp::pyrG^{Af}</i>	This work
MAD3806	<i>crzA</i> (Δ Q294-H316):: <i>gfp::pyrG^{Af}</i>	This work
MAD4234	<i>crzA</i> (Δ M356-A372):: <i>gfp::pyrG^{Af}</i>	This work
MAD4390	<i>crzA</i> (Δ Q294-H316; Δ M356-A372):: <i>gfp::pyrG^{Af}</i>	This work
MAD3555	$\Delta cnaA::pyroA^{Af}$, <i>pyroA4</i> [<i>pyroA</i> *:: <i>gfp::crzA</i> (M1-K730)]; $\Delta crzA::pyr4^{Nc}$	This work
MAD3559	$\Delta cnaA::pyroA^{Af}$, <i>pyroA4</i> [<i>pyroA</i> *:: <i>gfp::crzA</i> (M1-L612)]; $\Delta crzA::pyr4^{Nc}$	This work
MAD3527	$\Delta cnaA::pyroA^{Af}$, <i>pyroA4</i> [<i>pyroA</i> *:: <i>gfp::crzA</i> (MD+P445-K730)]; $\Delta crzA::pyr4^{Nc}$	This work

MAD2946	<i>ckiA102; crzA::gfp::pyrG^{Af}</i>	This work
MAD3045	<i>ckiA1919; crzA::gfp::pyrG^{Af}</i>	This work
MAD3044	<i>ckiA2; crzA::gfp::pyrG^{Af}</i>	This work
MAD3524	<i>ckiA2, ΔcnaA::pyroA^{Af}; crzA::gfp::pyrG^{Af}</i>	This work
MAD4675	<i>thiA^p:: ckiA; crzA::gfp::pyrG^{Af}</i>	This work
MAD3844	<i>ΔgskA::riboB^{Af}; crzA::gfp::pyrG^{Af}</i>	This work

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For Peer Review

Figure Legends

Figure 1. Cellular localization of CrzA under different abiotic stress growth conditions.

Strains MAD3021 and MAD4641, both expressing a CrzA-GFP, were grown in WMM for 18 h at 25°C and observed by fluorescence microscopy under different stress inducing stimuli. Prior to stress treatments (RC, resting cells), CrzA-GFP is mainly localized in the cytoplasm and excluded of nuclei, as shown by the localization of the nuclear marker histone H1 tagged with monomeric-cherry fluorescent protein (H1-mCh, strain MAD4641). Medium alkalinization (pH 8) promoted the accumulation of fluorescence in nuclei while acidification (pH 5.2) did not induce changes in the location of CrzA. Supplementation with either Ca^{2+} or Mn^{2+} induced nuclear accumulation of CrzA, while after the addition of Mg^{2+} CrzA remained cytoplasmic. Elevated concentrations of Na^+ and K^+ in the medium caused a distortion of green fluorescence in the cytoplasm but did not stimulate the translocation of CrzA to the nucleus. Nuclei of cells from strain MAD3021 were visualized by DAPI staining after addition of Na^+ or K^+ . Scale bar, 5 μm .

Figure 2. Phenotypic analysis of truncated versions of CrzA. (A) On the left, a diagram

showing a comparison of the protein structure of *S. cerevisiae* Crz1p and *A. nidulans* CrzA followed by a schematic representation of the CrzA truncated forms generated in this work. Initial and final amino acids for each construct are indicated. DBD, orange box, indicates the DNA binding domain. The green box labels the GFP tag. On the right, growth tests of mutant strains expressing the different CrzA truncated forms under the control of the *gpdA* promoter either in a wild-type or null *crzA* genetic background. Strains were inoculated on standard MMA (-), MMA supplemented with calcium (Ca^{2+}) or buffered to alkaline pH (pH 8). Images of colonies were taken after 2 days of incubation at 37°C. Strains used in this study were MAD2166, MAD3493, MAD2169, MAD2168, MAD3648, MAD2170, MAD1425, MAD2171, MAD3494, MAD3386, MAD3386, MAD2172, MAD3649, MAD3388, MAD2449. (B) On the left, a schematic representation of the construction of the GFP-tagged (green box) truncated forms expressed under the *crzA* native promoter (square-filled box). On the right, growth tests of strains using MMA (-), alkaline medium (pH 8) or supplemented with increasing molar concentrations of Ca^{2+} . Images of colonies were taken after 2 days of incubation at 37°C. Strains used in these experiments were: MAD1425, MAD3709, MAD3021, MAD3842, MAD3843. (C) Comparison of the cellular distribution of the GFP-tagged CrzA mutant proteins under stressing and non stressing conditions. Conidiospores were cultured in WMM for 18 h at 25°C and photographed at basal conditions, RC (resting cells), after a shift to fresh WMM supplemented with Ca^{2+} (50 mM CaCl_2) or WMM buffered at alkaline pH (pH 8). Strains used, from left to right: MAD2166, MAD3493, MAD2169, MAD2168, MAD3648, MAD2170. Scale bar, 5 μm .

1

2 **Figure 3. Calcineurin-dependent signaling of CrzA.** (A) Growth tests of GFP-tagged and
3 untagged-CrzA strains under calcium or alkaline pH stress. Media and culture conditions are as
4 in Fig. 2. Strains used here were MAD1425, CNA1, MAD3021, MAD3222 and MAD3709.
5 (B) Calcineurin is required for the nuclear accumulation of CrzA under stressing growth
6 conditions. Microscopy observations were conducted as in Fig. 2C. Strains used: MAD3021 and
7 MAD3222. Scale bar, 5 μ m.

8

9 **Figure 4. Changes in the electrophoretic mobility of CrzA due to calcineurin activity.** (A)
10 Western blot showing changes in CrzA-GFP in a null *cnaA* background or by inhibiting
11 calcineurin using cyclosporine-A (CsA). The concentration of CsA used in each assay is
12 indicated in μ M on top of the image. Detection of CrzA in protein extracts of a wild type is
13 shown as control of CrzA-GFP mobility. (B) λ -Protein phosphatase (λ PP) treatment of protein
14 extracts from a wild-type *cnaA* strain expressing CrzA-GFP with or without addition of CsA. As
15 control ortho-vanadate (o-vanadate) was used to specifically inhibit λ PP reaction. (C) λ PP
16 assays showing the change in mobility of CrzA-GFP in a null *cnaA* background with or without
17 CsA treatment. As control, 10 mM o-vanadate was used to inhibit λ PP reaction. (D) Western
18 blot showing the mobility of CrzA-GFP fusion in extracts from a null *cnaA* strain after
19 phosphatase assays with either recombinant human calcineurin (h CN) or λ PP. CsA and o-
20 vanadate were used to inhibit h CN and λ PP reactions, respectively (labeled as inhibitor). (E)
21 Electrophoretic mobility of CrzA in protein extracts from a wild-type *cnaA* strain after addition
22 of calcium (Ca^{2+}) or medium alkalization (pH 8). Mycelium samples of each condition were
23 taken at the indicated times (min). For all Western blots, detection of CrzA-GFP from wild-type
24 and null *cnaA* strains were used as control of CrzA mobility. CrzA-GFP fusions were detected
25 using a mouse-monoclonal GFP antibodies mixture. As loading control actin was detected using
26 a commercial polyclonal antibody. Total protein extracts were from mycelia of strains:
27 MAD3021 and MAD3222. Protein extracts for panels A and E were obtained using VFAL
28 protocol (see Supplementary Text and Experimental Procedures section). For panels B, C and D
29 protein extracts were obtained using VFA50 protocol.

30

31 **Figure 5. Calcineurin regulates nuclear localization and phosphorylated state of CrzA**
32 **through its N-terminal region.** (A) Comparison of the cellular localization of the truncated
33 forms of CrzA in the absence and presence of calcineurin function. The wild-type and null *cnaA*
34 strains expressing the full-length or the CrzA(1-612) form are indistinguishable under basal and
35 stressing conditions. The CrzA(445-730) form remains nuclear at any growth condition or
36 genetic background. At the top of each block of insets is shown an schematic representation of

1 the CrzA construct under study, including amino acid coordinates. The orange box labels the
 2 DBD. RC, resting cells. Scale bar, 5µm. (B) λ PP assays of functional truncated versions of
 3 CrzA. Phosphorylated CrzA(1-612) protein was detected in a null *cnaA* mutant. No changes in
 4 electrophoretic mobility after λ PP treatment were detected in the CrzA(445-730) form. o-
 5 vanadate was used to specifically inhibit λ PP reaction. GFP-CrzA fusions were detected using
 6 a monoclonal antibody against GFP. Protein extracts were obtained using VFA50 protocol. (C)
 7 Constitutive expression of full or truncated functional CrzA forms does not complement null
 8 *cnaA* growth defects. Strains were grown for 2 days at 37°C on MMA (-) and on MMA
 9 supplemented with Ca²⁺ or buffered at pH 8. Strains used in A, B and C panels are: MAD2171,
 10 MAD3555, MAD3494, MAD3559, MAD3386 and MAD3527.

11

12 Figure 6. Functional analyses of identified calcineurin docking domains (CDDs) in CrzA

13 (A) Diagram of the CDD truncated forms of CrzA studied here. Removal of the violet and light
 14 blue rectangles represents the deletion of the CDD1 (sites 1 and 2) and CDD2 (site 3),
 15 respectively. DBD, DNA binding domain. On the right it is shown a multiple alignment
 16 comprising the three CDD predicted sites in CrzA together with the functional CDD motif
 17 present in Crz1p, the alternative low and high affinity CDD sequences also analyzed in Crz1p
 18 (Roy *et al.*, 2007), and the consensus sequence of CDDs defined in mammalian NFAT
 19 (Aramburu *et al.*, 1998). (B) Multiple sequence alignment of CrzA homologues from
 20 filamentous fungi showing the region containing putative CDDs. Dark blue rectangles above the
 21 alignment indicate putative CDD sites 1, 2 and 3 identified in *A. nidulans* CrzA ((Spielvogel *et al.*,
 22 2008), and this work). Violet and light blue rectangles below the alignment illustrate the
 23 limits of the region deleted in the mutant CrzA proteins generated. Proteins alignments of the
 24 CrzA homologues from Anid: *A. nidulans* (BAE94327), Aory: *Aspergillus oryzae* (BAE57003),
 25 Afum: *Aspergillus fumigatus* (EAL88401), Afla: *Aspergillus flavus* (EED48569), Ncra:
 26 *Neurospora crassa* (EAA32849), Cglo: *Chaetomium globosum* (EAQ88414) and Gzea:
 27 *Gibberella zeae* (XP_381517) were performed using in-site ClustalX software (Thompson *et al.*,
 28 1997). Shading follows Blosum32 for similarities in residue composition, white <60%, light
 29 grey 60%, dark grey 80% and black 100% conservation. (C) Growth test of mutant *crzA* strains
 30 lacking the putative CDDs. Strains were inoculated on MMA, alkaline medium or supplemented
 31 with increasing molar concentrations of Ca²⁺ and incubated for 2 days at 37°C. Deletion of the
 32 predicted CDDs did not affect to the survival of the strains. Strains shown from top to bottom:
 33 MAD1425, MAD3709, MAD3021, MAD3806, MAD4234, MAD4390 and MAD3222. (D)
 34 Localization analysis of the hypothetical CDD defective forms of CrzA. Conidia from strains
 35 expressing mutant forms of CrzA were grown in WMM at 25°C for 18h and observed by
 36 fluorescence microscopy before and after stress inducing conditions. In the ΔCCD1 mutant

1 strain (MAD3806), CrzA localizes to nuclei in response to stimuli. Lack of CDD2 (strain
 2 MAD4234) prevents CrzA from nuclear accumulation at any condition. RC, resting cells. Scale
 3 bar, 5 μ m. (E) Western analysis showing the changes in mobility of Δ CDD1+2 CrzA-GFP
 4 mutant version. Lanes on the left show the mobility shift due to the deletion of CDD1 and
 5 CDD2 sites compared with the wild-type CrzA-GFP fusion, and the effect of CsA treatment
 6 reducing the mobility of both wild-type and mutant chimaeras. Lanes on the right show the
 7 mobility of Δ CDD1+2 CrzA-GFP under stressing conditions. Mobility shifts are observed when
 8 the culture was incubated with CsA or Ca^{2+} at the incubation times (min) indicated above the
 9 panel but no changes were detected with alkalinity. Detection of CrzA-GFP from Δ *cnaA* strain
 10 MAD3222 was used as control of low mobility of CrzA. Protein extracts were obtained using
 11 VFAL protocol. (F) λ PP treatment of protein extracts of strain MAD4390 expressing
 12 Δ CDD1+2 CrzA-GFP. Mycelia were treated with or without CsA prior to protein extraction. As
 13 control o-vanadate was used to specifically inhibit λ PP reaction. The Western-blot image is
 14 over-exposed to show the diffuse band of CrzA-GFP fusion detected with the GFP antibody in
 15 the absence of CsA treatment. Below it is shown a reduced exposure for detection of Δ CDD1+2
 16 CrzA-GFP in protein extracts after CsA treatment. Protein extracts were obtained using VFA50
 17 protocol. (G) Pull-down experiment using bacterially expressed GST-CrzA and GST-CrzA ^{Δ CDD2}
 18 forms as baits and CnaA-GFP expressed in *A. nidulans* (strain MAD4366) as prey. The CDD2
 19 motif is necessary for interaction between CnaA and CrzA. I= input of total protein extract from
 20 *A. nidulans*. B= bound protein fraction. For all Western blots, detection of CrzA-GFP and
 21 CnaA-GFP fusions were done using a monoclonal antibody against GFP.

22
 23 **Figure 7. *ckiA* mutations alters the mobility pattern but not the cellular localization of**
 24 **CrzA.** (A) Phenotypic analysis of *ckiA* mutant strains expressing CrzA-GFP. The *ckiA* defective
 25 strains are not able to grow in MMA containing arginine (Arg, 5mM) as main nitrogen source
 26 but are resistant to elevated concentrations of extracellular Ca^{2+} and alkalinity (pH 8). Triple
 27 mutant MAD3524 displayed an additive phenotype as a result of the effect of single mutations.
 28 (B) Localization of CrzA-GFP in *ckiA* mutants. Conidiospores from the indicated strains were
 29 cultured in WMM at 25°C for 18 h and shifted to fresh medium supplemented with Ca^{2+} or
 30 buffered at pH 8. None of the strains carrying *ckiA* mutations exhibit changes in the cellular
 31 distribution of CrzA when compared to a *ckiA* wild-type background at any condition assayed.
 32 Scale bar, 5 μ m. (C) Western blot showing differences in the mobility of CrzA in *ckiA* defective
 33 mutants compared with either wild-type or Δ *cnaA* genetic backgrounds, and the effect of the
 34 calcineurin inhibitor, CsA. Protein extracts were obtained using VFAL protocol. CrzA detection
 35 and electrophoresis conditions as those mentioned in legend of Fig. 4. Strains used in

experiments shown in this figure are MAD1425, MAD3709, MAD3021, MAD2946, MAD3045, MAD3044, MAD3524 and MAD3222.

Figure 8. CrzA requires the activity of GSK3 β , GskA, for a proper stimulus response.

(A) Schematic representation and distribution of potential Gsk3 β phosphorylation sites (*) along the CrzA amino acid sequence. DBD, orange box, indicates the location of the DNA binding domain. (B) Comparison of colonial growth phenotype of wild-type, null *crzA* and null *gskA* strains. The null *gskA* strain (MAD3844) shows strong morphological defects on MMA that can be slightly overcome using CMA. The addition of Ca²⁺ or alkalization of CMA plates has no major effects on the already poor colonial growth of the null *gskA* strain. (C) In the absence of GskA, CrzA does not accumulate in nuclei at alkaline pH. Conidiospores of the strain MAD3844 were cultured in WMM at 37°C for 20 h and afterwards medium was replaced with fresh WMM supplemented with a stress-inducing agent. The addition of Ca²⁺ promoted the accumulation of CrzA in the nucleus (each white arrow point to a nucleus before and after calcium stimulation). Alkaline pH did not enhance the nuclear accumulation of fluorescence. Magnification of a nucleus along media shift shows how the fluorescence was excluded from nucleus in resting cells (RC) and alkalization induced nuclear entry of fluorescence but not its accumulation. Scale bar, 5 μ m. (D) Western blot analysis showing high mobility bands of the CrzA-GFP chimera from a null *gskA* strain. A lower mobility band was observed by treatment of the culture with CsA, but it does not reach the mobility of a non-treated wild type extract. Strains used: MAD3844 and MAD3021. (E) λ PP assay of CrzA-GFP in a null *gskA* background. In the absence of CsA no shifts of mobility of CrzA are detected after λ PP. When calcineurin activity is inhibited by incubation with CsA in the absence of *gskA* expression, CrzA was dephosphorylated *in vitro* by λ PP. O-vanadate was used to inhibit λ PP reaction. Mobility of CrzA-GFP from a wild type strains after λ PP treatment is shown as control of a low level of phosphorylation of CrzA. Strains used were MAD3844 and MAD3021. (F) Western blot analysis of CrzA-GFP from the *gskA* mutant under stress inducing conditions. Incubation of the culture (MAD3844) with Ca²⁺ or alkaline conditions promotes a delay on the mobility of CrzA. For all western blots, CrzA-GFP fusions were detected using a mouse-monoclonal GFP antibodies mixture. For panels D and F protein extracts were obtained using VFAL protocol, and for panel E with VFA50 protocol.

Figure 9. Models for the signaling and nuclear transport regulation of CrzA in *A. nidulans*.

Figure 1. Colour figure
154x168mm (300 x 300 DPI)

Figure 2, Colour figure
194x253mm (300 x 300 DPI)

Figure 3. B/W Figure, Colour online
169x91mm (300 x 300 DPI)

Figure 4. B/W Figure
188x129mm (300 x 300 DPI)

Figure 5. B/W Figure, Colour online
197x156mm (300 x 300 DPI)

Figure 6, B/W Figure, Colour online
196x262mm (300 x 300 DPI)

Figure 7. B/W Figure, Colour online
194x142mm (300 x 300 DPI)

Figure 8. B/W Figure, Colour online
169x161mm (300 x 300 DPI)

Figure 9. B/W Figure
114x123mm (300 x 300 DPI)