

RESEARCH PAPER



## The putative flavin carrier family FlcA-C is important for *Aspergillus fumigatus* virulence

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### ABSTRACT

*Aspergillus fumigatus* is an opportunistic fungal pathogen and the most important species causing pulmonary fungal infections. The signaling by calcium is very important for *A. fumigatus* pathogenicity and it is regulated by the transcription factor CrzA. We have previously used ChIP-seq (Chromatin Immunoprecipitation DNA sequencing) aiming to identify gene targets regulated by CrzA. We have identified among several genes regulated by calcium stress, the putative flavin transporter, *flcA*. This transporter belongs to a small protein family composed of FlcA, B, and C. The  $\Delta flcA$  null mutant showed several phenotypes, such as morphological defects, increased sensitivity to calcium chelating-agent ethylene glycol tetraacetic acid (EGTA), cell wall or oxidative damaging agents and metals, representative of deficiencies in calcium signaling and iron homeostasis. Increasing calcium concentrations improved significantly the  $\Delta flcA$  growth and conidiation, indicating that  $\Delta flcA$  mutant has calcium insufficiency. Finally,  $\Delta flcA-C$  mutants showed reduced flavin adenine dinucleotide (FAD) and were avirulent in a low dose murine infection model.

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### Introduction


Calcium signaling is very important for fungal morphology and metabolism.<sup>1–5</sup> The calcineurin phosphatase and the transcription factor Crz1p1/CrzA are essential for fungal calcium signaling.<sup>1,2</sup> In *Saccharomyces cerevisiae*, several environmental stresses, for instance osmotic, extreme pH, high temperature, ER stress and prolonged incubation with mating pheromone  $\alpha$ -factor, are regulated by calcineurin.<sup>5,6</sup> Calcineurin also connects many stress response signaling pathways.<sup>7</sup> Cyclosporin A, an immunosuppressant, reduces calcineurin activity and causes morphological changes and growth reduction in *A. nidulans*, *A. oryzae*, *Magnaporthe oryzae* and *Neurospora crassa*.<sup>8–12</sup> Calcineurin activates the transcription factor Crz1 (Calcineurin Responsive Zinc Finger 1) transcription factor by dephosphorylating it when there is an increase in cytosolic calcium and permitting its translocation to the nuclei.<sup>1,13</sup> Crz1 has a C<sub>2</sub>H<sub>2</sub> zinc finger motif responsible for the binding to a CDRE (calcineurin-dependent response element) sequence in the promoter regions of the Crz1-regulated genes.<sup>1,14</sup>

Fungal infections are becoming very important considering that presently there is a larger number of people dying from fungal infections than malaria and tuberculosis, what is due to the increase of the number of patients with immunosuppression.<sup>15</sup> The signaling by calcium is very important for fungal virulence and drug resistance.<sup>1,2</sup> Calcineurin has been demonstrated to be required for virulence in human fungal pathogens, such as *Cryptococcus* spp, *Candida* spp, *Paracoccidioides brasiliensis*, and *Aspergillus fumigatus*,<sup>16–23</sup> and fungal plant pathogens, such as *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Magnaporthe oryzae*, and *Ustilago* spp.<sup>24–28</sup> In all these fungal pathogens, calcineurin is important for growth, morphology, state transitions, cation homeostasis, stress responses, and cell membrane and cell wall integrity pathways.<sup>1,2,29–33</sup>

*Aspergillus fumigatus* is an opportunistic fungal pathogen and the most important species causing pulmonary fungal infections.<sup>15,34–38</sup> Although *A. fumigatus* is able to cause many clinical forms, the most important is the invasive pulmonary aspergillosis

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(IPA), that has mortality rates as high as 80% in immunosuppressed patients.<sup>15,36-38</sup> We have used ChIP-seq (Chromatin Immunoprecipitation DNA sequencing) aiming to identify gene targets regulated by CrzA. We have identified among several genes regulated by calcium stress, *flcA*, encoding the putative flavin adenine dinucleotide (FAD) transmembrane transporter (the predicted homolog of the *S. cerevisiae* flavin carrier *FLC2*).<sup>39,40</sup> The *Candida albicans* homolog of this gene was identified when expressed in *S. cerevisiae* because it increased the heme transport. Since *S. cerevisiae* cannot grow very well on media with heme as a single iron source, the overexpression of the *C. albicans* homolog, *CaFLC1*, improved considerably its growth on heme iron as single iron source.<sup>40</sup> In *S. cerevisiae* Flc mutants have deficiencies in the cell wall, are resident in the endoplasmic reticulum, and cannot transport FAD into the lumen of the endoplasmic reticulum.<sup>40</sup> Here, we characterized in more detail FlcA, which belongs to a small protein family of putative flavin transporters composed of 3 members, FlcA, B, and C. The *flcA* null mutant had several important phenotypes, such as morphogenetic defects, sensitivity to calcium, cell wall and oxidative damaging agents, and metals. Finally,  $\Delta flcA-C$  mutants were avirulent in a low dose murine infection model.

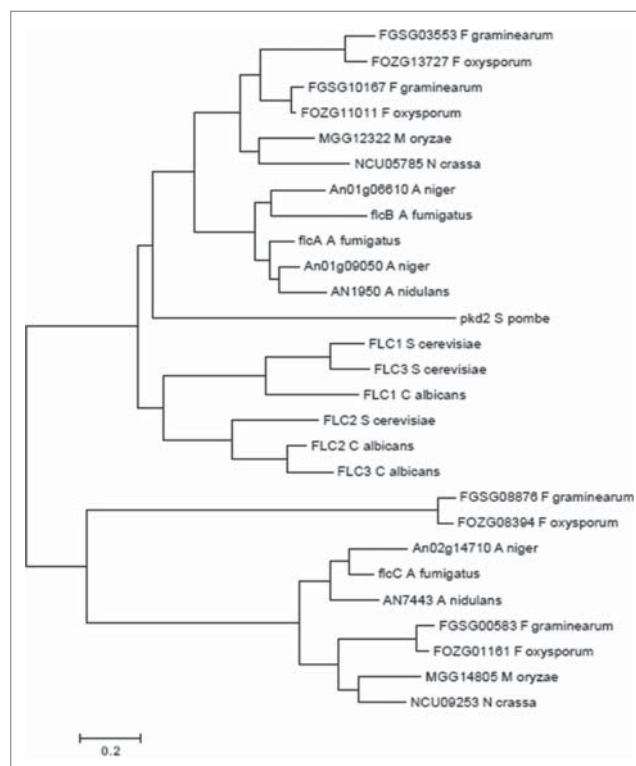
## Results

### *FlcA is a member of a small protein family*

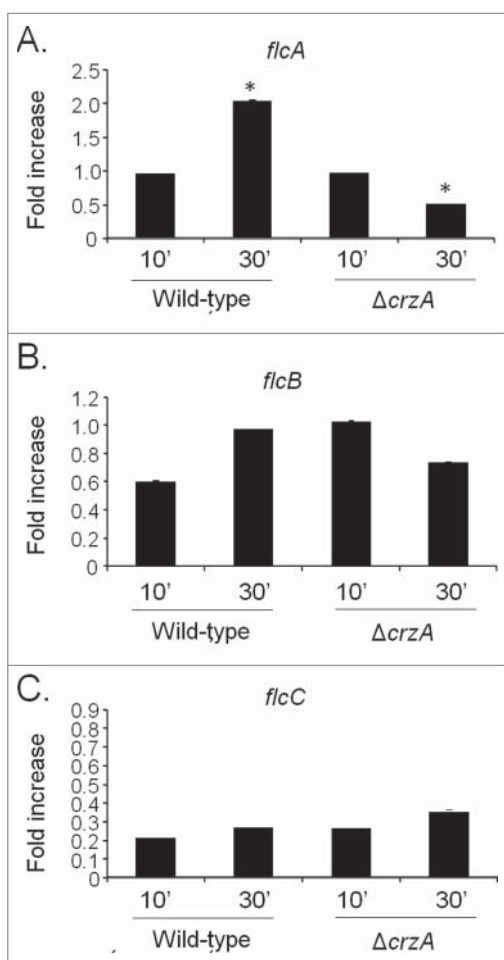
By using ChIP-seq to reveal CrzA targets in *A. fumigatus*, we identified the *A. fumigatus* homolog of the *S. cerevisiae* FAD transmembrane transporter *FLC2* (Afu4g13340, *flcA*). BLASTp analysis revealed 2 putative paralogues of FlcA, FlcB (Afu2g17650, 61.1 % identity, 77.3 % similarity, e-value 0.0) and FlcC (Afu2g06100, 28.9 % identity, 46.8 % similarity, e-value  $7e-59$ ). The *flcA-C* gene models are supported by RNA-seq data (available at [www.aspgd.org](http://www.aspgd.org)). The hypothetical proteins encoded by *flcA-C* were predicted to be 721, 612, and 723 amino acids in length and possessed a mass of 78.6, 66.3, and 79.4 kDa, respectively. We have compared the protein structures and organizations between FlcA-C by using the SMART interface (<http://smart.embl-heidelberg.de/>). The organization of the protein FlcA-C domains was conserved with 2 important Pfam domains (Fig. S1): (i) a TRP\_N (PF14558) that might be involved in lipid binding and (ii) A TRP (PF06011) that represents a family of transient receptor protein channel-like proteins, which may be responsible for FAD transport into the endoplasmic reticulum lumen where it is

important for oxidative protein folding. The predicted FlcA-C amino acid sequences have between 9 and 7 hydrophobic transmembrane domains, respectively (Fig. S2). FlcA-C contained N-terminal signal peptides putatively assigned by several prediction programs as endoplasmic reticulum resident proteins (SignalP, [www.cbs.dtu.dk/services/SignalP](http://www.cbs.dtu.dk/services/SignalP); Euk-mPLoc 2.0, <http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/>; Predotar, <https://urgi.versailles.inra.fr/predotar/predotar.html>). The FlcA-C have several homologues in other filamentous fungi (protein identity greater than 70%) (Fig. 1). However, the phylogenetic analyses clearly demonstrated 2 different branches from yeasts and molds, and that FlcA and FlcB homologues are much closer than FlcC homologues (Fig. 1).

Next step, we compared *flcA-C* mRNA accumulation in the wild-type and  $\Delta crzA$  strains in response to a short exposure (10 or 30 min) to calcium (200 mM  $\text{CaCl}_2$ ) by using qRT-PCR (Fig. 2). These conditions are absolutely identical to the experimental conditions used for identifying *flcA* by ChIP-seq (de Castro *et al.*, 2014). The *flcA* showed a twice increase in mRNA accumulation post calcium exposure in the wild-type strain, while its mRNA accumulation was decreased also twice after 30 minutes in the post calcium exposure  $\Delta crzA$  mutant



**Figure 1.** Phylogeny of *A. fumigatus* FlcA-C. The optimal tree for the *A. fumigatus* is represented. The tree was inferred using the Neighbor-Joining Method. Sequences were aligned with ClustalW and the tree was constructed by using MEGA6.



**Figure 2.** The *A. fumigatus flcA* expression is dependent on CrzA. The qRT-PCR for the *A. fumigatus* (A) *flcA*, (B) *flcB*, and (C) *flcC* genes. The strains were grown for 16 hours at 37 °C and transferred to 200 mM CaCl<sub>2</sub> for 10 and 30 min. The results are expressed as fold increase of the control (in the absence of CaCl<sub>2</sub>) and the results were normalized with the *βtub* expression (\*,  $p < 0.001$ ).

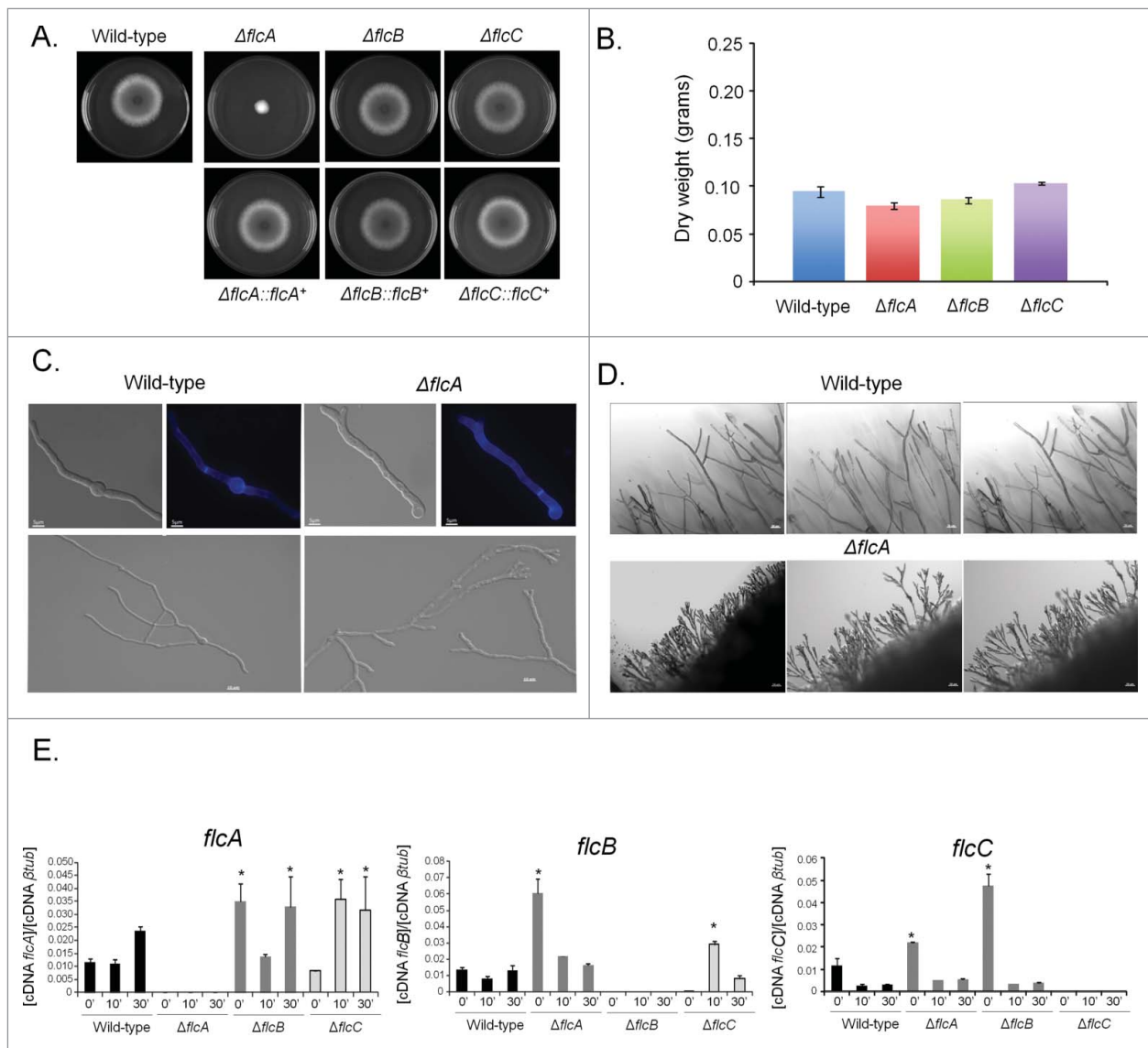
(Fig. 2A). The *flcB* and *flcC* do not show significant modulation post calcium exposure in the wild-type and  $\Delta crzA$  strains (Figs. 2B and C). These results suggest that upon calcium exposure, CrzA influences *flcA* mRNA accumulation.

### Phenotypic characterization of an *A. fumigatus* putative flavin flcA-C transporter family

To a better understanding of the function of FlcA-C in *A. fumigatus*, the *flcA-C* genes were deleted with the *pyrG* marker (Fig. S3A to C). To exclude the possibility that undesired mutations throughout the creation of the deletion strains contributed to the phenotypes observed (Fig. S4), we have complemented the null mutants with the equivalent wild-type genes. The promoters of the *flcA*, *B*, and *C* genes are not affecting the *pyrG* promoter because

the deletion strains grow to the same extent on minimal medium and minimal medium supplemented with uridine and uracil (Figs. S4). That provided strong evidence that the lack of uridine and uracil into the host is not affecting the *pyrG* promoter and consequently the reduced viability of these strains into the host is not due to a marker effect. The  $\Delta flcA-C$  mutants were exhaustively investigated for phenotypes that could be affected by their absence (Figs. S6–8). However, we were only able to identify phenotypes for  $\Delta flcA$  as described here. The  $\Delta flcA$  mutant showed a dramatic reduction in radial growth in solid minimal and complete media when compared to the wild-type and  $\Delta flcB-C$  strains, but surprisingly exhibited about the same dry weight in liquid minimal media (Fig. 3A and B and Fig. 4B, lower panel, left row). The viability of the  $\Delta flcA-C$  conidia is similar to the wild-type strain (Fig. S9). Accordingly, conidial germination of the  $\Delta flcA$  and wild-type strains in liquid minimal media showed the same germination and nuclear kinetics, while the apical tip of the  $\Delta flcA$  strain showed bipolar elongation (Fig. 3C). The compact morphology and reduced radial growth of the  $\Delta flcA$  mutant on solid media (Fig. 3A) was attributed to an increase in apical branching in comparison to the wild-type strain (Figs. 3C and D). We also investigated a possible transcriptional compensatory mechanism for the absence of each *flc* gene by measuring the *flcA-C* mRNA accumulation in  $\Delta flcA-C$  mutant strains in response to a short pulse (10 or 30 min) of calcium (200 mM CaCl<sub>2</sub>) via qRT-PCR (Fig. 3E). There is a significant increased *flcA* expression in  $\Delta flcB$  and  $\Delta flcC$  (about 3-fold at 0 and 30 and 10 and 30 min post calcium exposure, respectively; Fig. 3E, left graph). In  $\Delta flcC$  and  $\Delta flcA$  mutant strains, there are significant increases of about 6- and 3-fold in the *flcB* mRNA accumulation at 0 and 10 min, respectively (Fig. 3E, middle graph). There is significant increase in the *flcC* expression (about twice and 5-fold) at time 0 for both  $\Delta flcA$  and  $\Delta flcB$  mutant strains (Fig. 3E; right graph). These results suggest that there are compensatory transcriptional mechanisms affecting increased *flcA-C* mRNA accumulation in the  $\Delta flcA-C$  mutant strains.

The  $\Delta flcA$  mutant was more sensitive than the wild-type strain to the calcium chelating-agent ethylene glycol tetracetic acid (EGTA), calcofluor white (CFW), congo red (CR), *t*-butyl hydroperoxide, and paraquat (Fig. 4A). The increased sensitivity of  $\Delta flcA$  to EGTA suggests that this mutant has a calcium shortage. Increasing CaCl<sub>2</sub> concentrations in YAG medium improved significantly the  $\Delta flcA$  growth and conidiation (Fig. 4B), indicating that  $\Delta flcA$  mutant has calcium insufficiency. The  $\Delta flcA$  mutant was also more sensitive to metals, such as lithium, manganese and iron, but not to iron starvation (Fig. 5A–C).

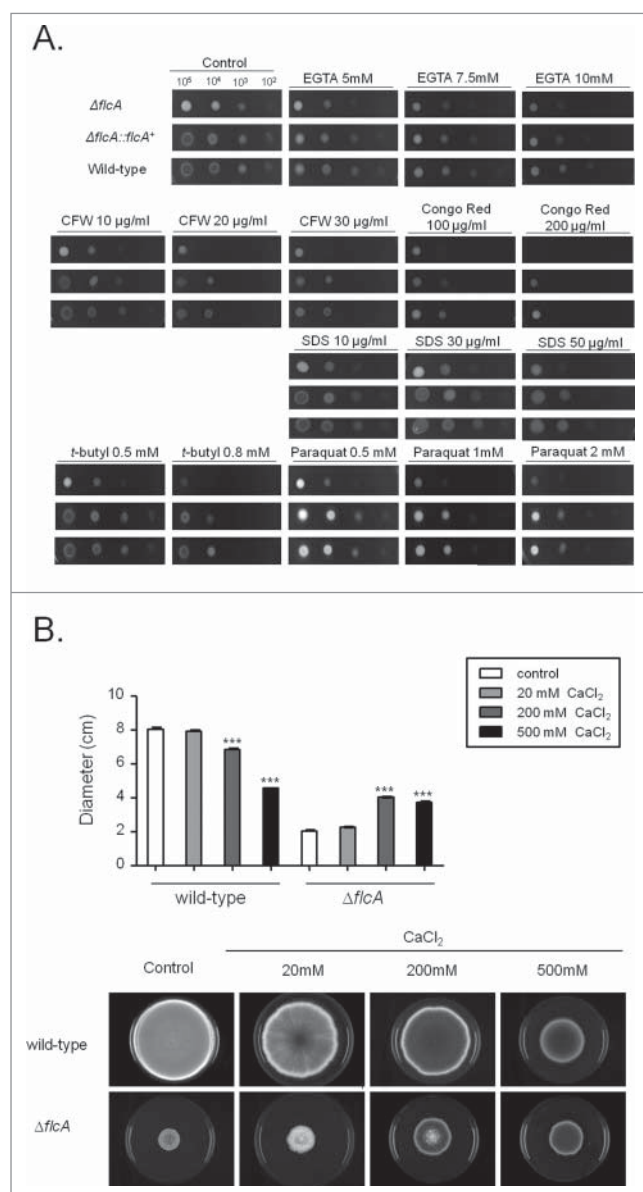


**Figure 3.** The *A. fumigatus*  $\Delta flcA$  has morphogenetic defects. The wild-type,  $\Delta flcA$ -C, and their corresponding complementing strains were grown for 48 h at 37 °C on solid (A) or liquid MM (B). *A. fumigatus* wild-type and  $\Delta flcA$  germlings were grown in liquid MM for 12 h and stained or not with calcofluor white (C, top panels, bars 5  $\mu$ M) or for 20 h at 30 °C (C, lower panels, bars, 10  $\mu$ M). (D) The edge of the colonies represented in the plates of (A). Bars, 50  $\mu$ M. (E) The qRT-PCR for the *A. fumigatus* *flcA*-C genes in the wild-type,  $\Delta flcA$ ,  $\Delta flcB$ , and  $\Delta flcC$  strain. The strains were grown for 16 hours at 37 °C (time 0) and transferred to 200 mM  $CaCl_2$  for 10 and 30 min. The results are expressed as the number of cDNA copies of a specific *flc* gene divided by the number of copies of the cDNA of the normalizer  $\beta tub$  ( $p < 0.001$ ).

To verify FlcA-C cellular localization, we generated FlcA-C::GFP strains which behaved identical to the wild-type strain (data not shown). Very low fluorescence was observed for FlcB::GFP and FlcC::GFP, not allowing us to determine its subcellular location (data not shown). In contrast, we were able to observe FlcA::GFP expressed as a single band of 103.6 kDa (Fig. S10) and when the FlcA::GFP strain was grown in minimal media for 16 hours at 30 °C, a weak and diffuse fluorescent signal was distributed along the germlings in the cytosol and in some structures resembling vesicles, as confirmed by vacuolar staining with CMAC (in about 100 % of the germlings; Fig. S11). In

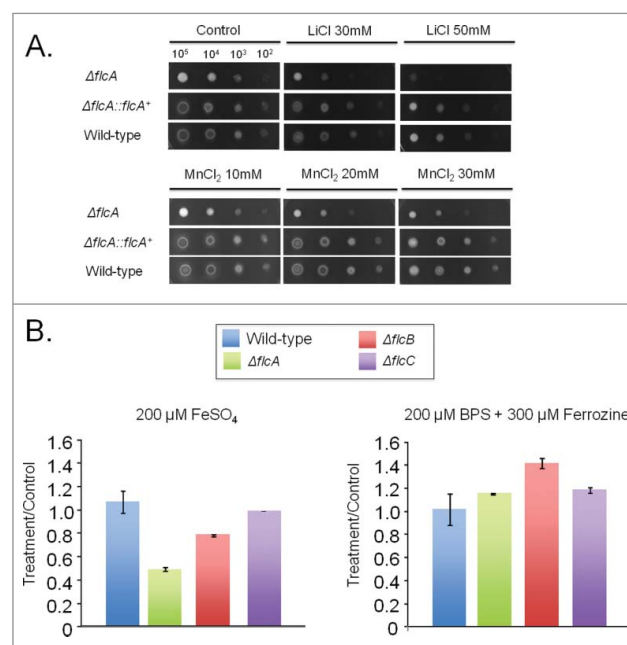
addition, strong staining was visible in the apical tip (about 50 % of the germlings, Fig. 6). The sub-cellular localization or intensity of the signal was not altered when the germlings were either exposed to iron excess or starvation for 1 or 2 hours (Fig. 6) or paraquat (Fig. S11). Furthermore, we have not observed any difference in the FlcA::GFP localization in the presence of high calcium chloride concentrations (Fig. S11). The same results were observed at 37 °C (Fig. S11).

We also measured the retention of FAD into microsomes by using wild-type and  $\Delta flcA$ -C protoplasts, and measured retained FAD by fluorescence spectrophotometry. We



**Figure 4.** The  $\Delta flcA$  mutant strain is more sensitive to calcium stress, cell wall damaging and oxidative stressing agents. (A) The wild type, the mutant, and the complemented strains were grown on YAG medium with increasing concentrations of EGTA, cyclosporin, calcofluor white (CFW), congo red (CR), Sodium Dodecyl Sulfate (SDS), *t*-butyl hydroperoxide, and paraquat for 48 h at 37 °C. (B) Radial growth of the wild-type and  $\Delta flcA$  mutant strains grown in YAG medium for 120 hours at 37 °C in the absence and presence of increasing  $CaCl_2$  concentrations. The results are the average  $\pm$  standard deviation of 3 repetitions. Statistical analysis was performed by using One-way Anova with post test Dunnett (\*\*\*)  $p < 0.05$ .

observed that wild-type protoplasts showed a vigorous accumulation of FAD while the  $\Delta flcA-C$  protoplasts exhibited very low accumulation of FAD (Fig. 7). Taken together these results suggest that FlcA is important for morphogenetic development, and sensitivity to calcium, cell wall damaging agents, ions and oxidative stress. Additionally, all 3 genes are important for FAD accumulation.

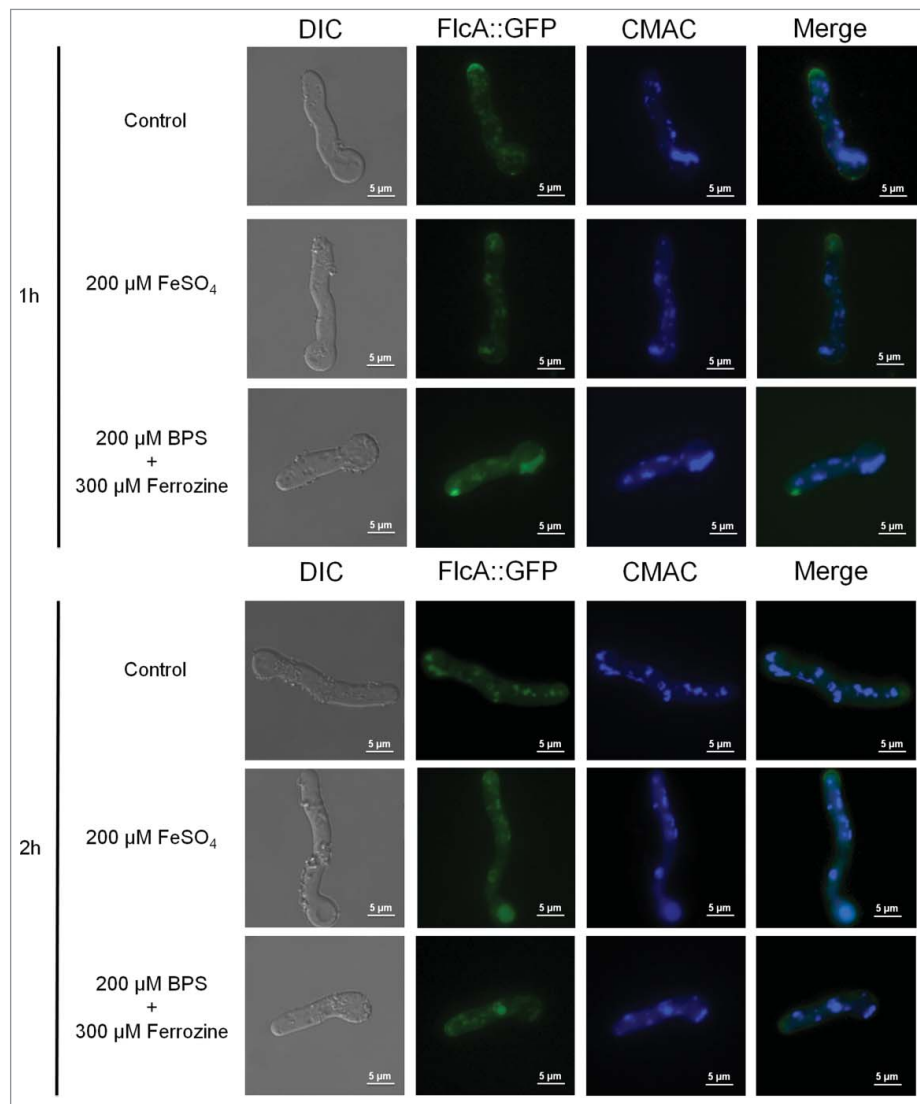


**Figure 5.** The *A. fumigatus*  $\Delta flcA$  is more sensitive to metals. (A) The wild type, the mutant, and the complemented strains were grown on YAG medium with increasing concentrations of LiCl and MnCl<sub>2</sub>. (B) The wild type and the mutant strains were grown for 48 h at 37 °C in MM+200  $\mu$ M  $FeSO_4$  or AMM+ferrozine+BPS. The data were normalized by dividing the dry weight of the treatments by the dry weight of the corresponding strains grown for 48 h at 37 °C in MM.

### The flcA-C are important for virulence in a neutropenic murine model of invasive pulmonary aspergillosis

We investigated the involvement of FlcA-C in *A. fumigatus* pathogenicity by using a neutropenic murine model of invasive pulmonary aspergillosis (Fig. 8). The infection with the wild-type strains produced 100 % mortality 12 to 15 d post-infection, but the infection with  $\Delta flcA$ ,  $\Delta flcB$ , and  $\Delta flcC$  yielded a significantly reduced mortality rate, at approximately 20 to 30% 15 d post-infection (Fig. 7A, C, and E, no statistical differences between the mutants and PBS negative control,  $p > 0.1951$  to  $0.2024$  Log-rank (Mantel-Cox) test and  $p > 0.1967$  to  $0.2024$  Gehan-Breslow-Wilcoxon test). The complemented strains (single ectopic re-integrations of the wild-type *flcA-C* loci) have not shown statistical difference with the wild-type strain (Fig. 8A, C, and E,  $p > 0.2099$  to  $0.5886$  and  $p > 0.3029$  to  $0.7557$  for the comparison between the wild-type and the complemented strains, Log-rank, Mantel-Cox and Gehan-Breslow-Wilcoxon tests, respectively).

As an additional measurement of the fungal growth into the lungs, we have used fungal burden estimation by qPCR (Fig. 8). The  $\Delta flcA-C$  strains were not able to grow within the lungs as well as the wild-type and the



**Figure 6.** FlcA::GFP accumulates in the apical tip, cytoplasm and in vesicles. The FlcA::GFP strain was grown for 12 h at 30 °C in MM and transferred to either MM+FeSO<sub>4</sub> or AMM+ferrozine+BPS for 1 or 2 h. Bars, 5 μm.

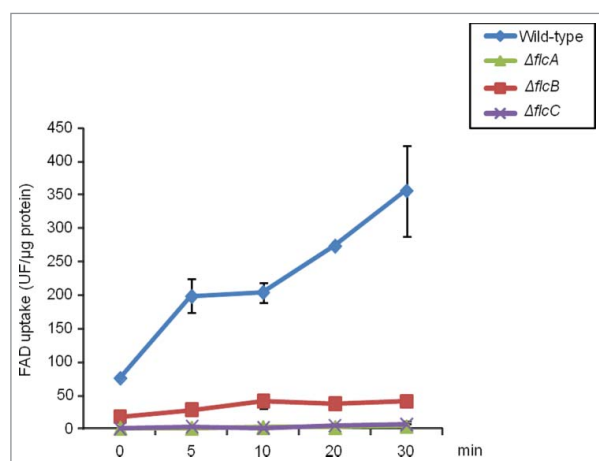
complemented strains (Fig. 8B, D, and F,  $p < 0.0001$  for the comparison between the wild-type and the deletion mutant, and  $p > 0.05$  between the wild-type and the complemented strain). Taken together, our results strongly indicate that FlcA-C are important for *A. fumigatus* virulence.

## Discussion

In *A. fumigatus* FlcA belongs to a small protein family composed by 3 members, FlcA-C. In *S. cerevisiae* and *C. albicans*, the 3 homologues, FLC1-3, were required for the uptake of FAD into endoplasmic reticulum and heme iron uptake. These proteins were detected in the endoplasmic reticulum. Here, we constructed functional GFP fusions for all 3 *A. fumigatus* FlcA-C proteins, using their endogenous promoters. However, we were only able to observe FlcA::

GFP localization, because the expression of the other fusions was very low (data not shown). Bioinformatic predictions of FlcA-C localization suggested they were resident endoplasmic reticulum proteins. However, FlcA::GFP localized to the apical tip and in vesicles that resemble the sorting multivesicular bodies (MVB) of the endocytic pathway.<sup>41,42</sup> We were also able to show that FlcA-C were important for FAD accumulation, which suggests that this function is conserved among different fungi.

All three genes *flcA-C* were important for *A. fumigatus* virulence. The *flcB* gene (but not *flcA* and *flcC*) is more expressed *in vivo* during initiation of murine infection<sup>43</sup>; however, we have not observed increased *flcA*, *-B* and *-C* mRNA accumulation induced either by human platelets *in vitro* or during airway epithelial cells interacting with *A. fumigatus* conidia.<sup>44,45</sup> This represents the first evidence that a FLC homolog to be involved in



**Figure 7.** There is a decreased FAD transport in the  $\Delta flcA$ -C mutants. FAD transport was observed in *A. fumigatus* wild-type and  $\Delta flcA$ -C protoplasts.

virulence, since the impact of the *C. albicans* FLC null mutants on virulence was not evaluated. It is surprising that  $\Delta flcB$  and  $\Delta flcC$  mutant strains are avirulent since the single phenotypes observed *in vitro* for these strains are: (i) a decrease in FAD accumulation and (ii) more specifically in the  $\Delta flcC$  a transcriptional compensatory mechanism upon calcium exposure that increases the *flcA* and *flcC* mRNA accumulation. However, both phenotypes do not cause any *in vitro* growth and conidiation defects. It is tempting to speculate that *flb* and *flc* genes are essential for *in vivo* growth but dispensable for *in vitro* growth. It is very intriguing how the dramatic reduction of FAD accumulation in these 2 strains that do not affect *in vitro* growth could impact *in vivo* growth. It is possible that FlcB and FlcC are collaborating during *in vivo* growth and this could help to explain the lack of virulence in the corresponding mutant strains. We were not able to construct a double  $\Delta flcB \Delta flcC$  mutant (data not shown), what could suggest FlcB and FlcC are interacting redundantly *in vitro*. However, all these possibilities remain to be investigated.

The *A. fumigatus*  $\Delta flcA$  mutant had several phenotypes that phenocopy calcium deficiency, such as those observed in calcineurin and calcium transport or channel mutants.<sup>46-48</sup> For example, similar to the  $\Delta cnaA$  (catalytic subunit of the calcineurin) mutant, we found that FlcA was required for *A. fumigatus* colony extension and influenced hyphal branching. The  $\Delta flcA$  mutant was more sensitive to calcium and cyclosporine, excess of metals, and cell wall and oxidative stressing agents. Similar to  $\Delta cnaA$ , the  $\Delta flcA$  can also grow better in liquid than in solid medium, what could be due to the effect of defective apical branching of these strains on fungal growth on solid medium. In addition, the  $\Delta flcA$  growth is improved in the presence of high calcium concentrations. All these different phenotypes suggest that

FlcA was involved in calcium release and/or modulation. Actually, Rigamonti *et al.*<sup>49</sup> proposed that the *S. cerevisiae* hypertonic stress response, which was mediated by calcium release, involved *FLC2*. These authors performed extensive bioinformatics analysis and suggested that the 3 *S. cerevisiae* homologues, the *Schizosaccharomyces pombe* *pkd2* and *Neurospora crassa* calcium-related spray protein are members of the fungal branch of TRP-like ion transporters.<sup>49-51</sup> Taken together, this evidence suggests that FlcA may also be involved in calcium transport.

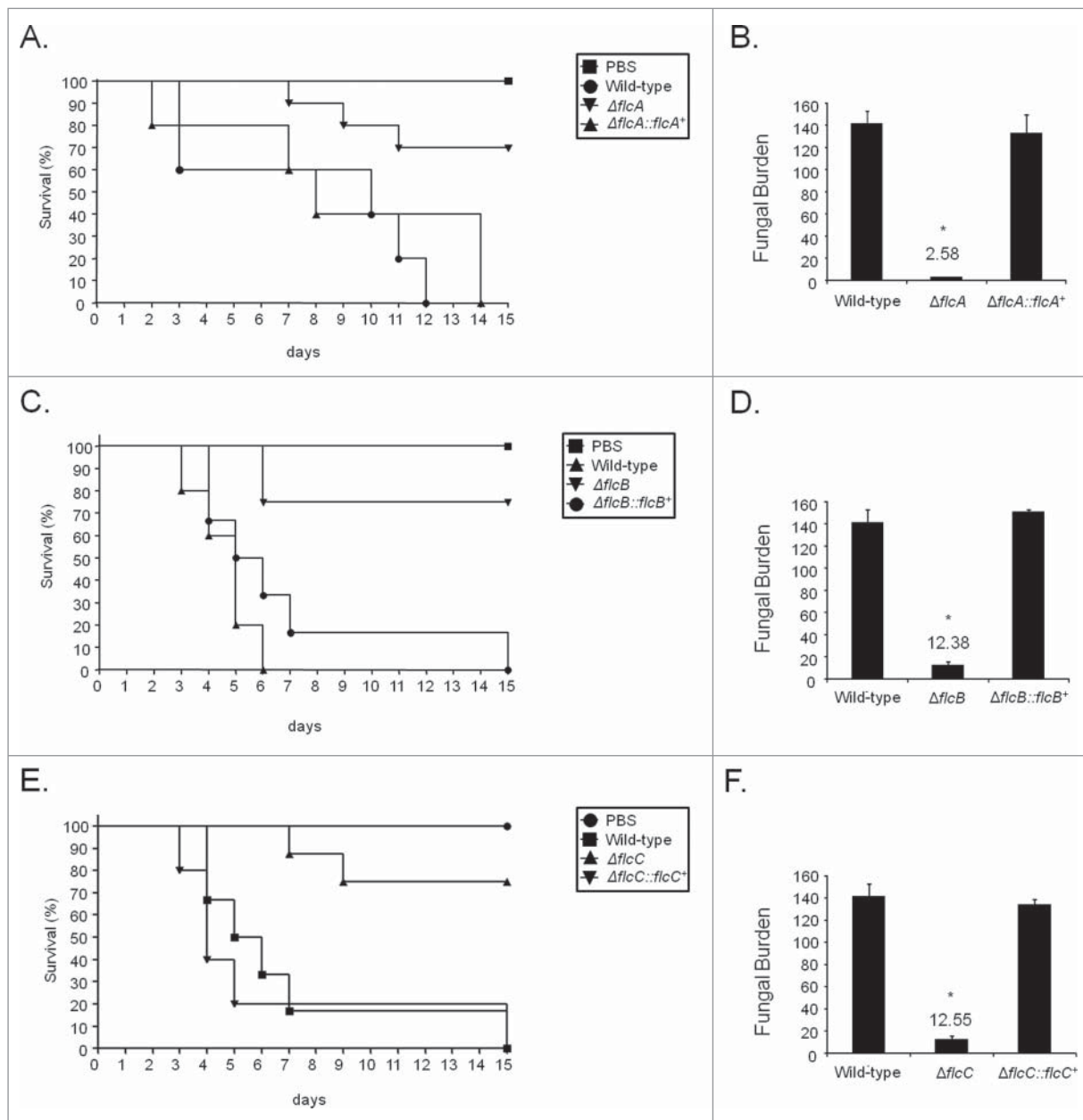
In conclusion, this study identified a novel protein family related to calcium metabolism and virulence in *A. fumigatus*. FlcA was identified as regulated by CrzA upon calcium stress and it is important for FAD metabolism. It is important now to understand the connections among these pathways during the *A. fumigatus* pathogenicity. It remains to be determined the precise *in vivo* role played by FlcB and FlcC during *A. fumigatus* virulence and if these 2 putative transporters are interacting.

## Materials and methods

### Strains, media and culture methods

The *A. fumigatus* strains used in this study were CEA17 (*pyrG*<sup>+</sup> and *pyrG*<sup>-</sup>),  $\Delta crzA$ ,  $\Delta flcA$ ,  $\Delta flcB$ ,  $\Delta flcC$ ,  $\Delta flcA::flcA$ <sup>+</sup>,  $\Delta flcB::flcB$ <sup>+</sup>, and  $\Delta flcC::flcC$ <sup>+</sup>.<sup>52,53</sup> All the comparisons with the deletion strains were performed with the CEA17 *pyrG*<sup>+</sup>. The media used were: complete medium composed for 2% w/v glucose, 0.5% w/v yeast extract, 2% w/v agar, trace elements (YAG) or YUU [YAG supplemented with 1.2 g (each) of uracil and uridine], and liquid YG or YG + UU medium with the same composition (but without agar). The minimal medium (MM) consist of 1% glucose, trace elements (22.0 g/l ZnSO<sub>4</sub>, 11 g/l boric acid, 5 g/l MnCl<sub>2</sub>, 5 g/l FeSO<sub>4</sub>, 1.6 g/l CoCl<sub>2</sub>, 1.6 g/l CuSO<sub>4</sub>, 1.1 g/l (NH<sub>4</sub>)<sub>2</sub>MoO<sub>4</sub>, 50 g/l ethylenediaminetetraacetic acid (EDTA)) and adjusted to pH 6.5 with NaOH ) and salt solution 20x, 2% agar, pH 6.5.<sup>54</sup> Strains were grown at 37 °C or at 30 °C for microscopy experiments.

For the iron starvation experiments, the strains were grown in MM for 24 hours at 37 °C and transferred the mycelia to modified MM [consist of 1% glucose, trace elements without iron and salt solution 20x (NaNO<sub>3</sub> 272 g/l; KCl 10.4 g/l, KH<sub>2</sub>PO<sub>4</sub> 30.4 g/l, MgO<sub>4</sub>·7H<sub>2</sub>O 10.4 g/l, and 50 ml of this solution are added to 1 l of MM) plus BPS 200 μM [Bathophenanthrolinedisulfonic acid (4,7-diphenyl-1,10-phenanthrolinedisulfonic acid) and 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (ferrozine)] 300 μM for 1 or 2 hours at 37 °C. For the iron excess experiments, the strains were grown in MM for 24 hours and then FeSO<sub>4</sub>·7H<sub>2</sub>O 200 μM or FeCl<sub>3</sub> 200 μM were added for 1 or 2 hours at 37 °C.



**Figure 8.** *A. fumigatus*  $\Delta flcA$ - $C$  mutants are avirulent. (A) Comparative analysis of wild type, mutant, and complemented strains in a neutropenic murine model of pulmonary aspergillosis. Mice in groups of 10 per strain were infected intranasally with a 20  $\mu$ l suspension of conidia at a dose of  $10^5$ . Fungal burden was determined 48 h post-infection by real-time qPCR based on 18 S rRNA gene of *A. fumigatus* and an intronic region of the mouse GAPDH gene. Fungal and mouse DNA quantities were obtained from the Ct values from an appropriate standard curve. Fungal burden was determined through the ratio between ng of fungal DNA and mg of mouse DNA. The results are the means ( $\pm$  standard deviation) of 5 lungs for each treatment. Statistical analysis was performed by using *t*-test. (A) The  $\Delta flcA$  mutant compared to the wild-type and  $\Delta flcA::flcA^+$  strains. (B) Fungal burden for  $\Delta flcA$  mutant, wild-type and  $\Delta flcA::flcA^+$  strains. (C) The  $\Delta flcB$  mutant compared to the wild type and  $\Delta flcB::flcB^+$  strains. (D) Fungal burden for  $\Delta flcB$  mutant, wild-type and  $\Delta flcB::flcB^+$  strains. (E) The  $\Delta flcC$  mutant compared to the wild type and  $\Delta flcC::flcC^+$  strains. (F) Fungal burden for  $\Delta flcC$  mutant, wild-type and  $\Delta flcC::flcC^+$  strains. PBS = phosphate Buffer Saline.

### Construction of the *A. fumigatus* mutants

We have used the ‘*in vivo*’ recombination method in *S. cerevisiae* as previously described by Colot *et al.*<sup>55</sup> for the construction of gene replacement cassettes. Thus, about 1.0 kb from the 5’-UTR and 3’-UTR flanking region of

the targeted ORF regions were used for designing primers. The primers 5F and 3R also contains a short homolog sequence to the MCS of the plasmid pRS426. Both fragments, 5- and 3-UTR, were PCR-amplified from *A. fumigatus* genomic DNA (gDNA). The *pyrG* used in the *A. fumigatus* cassette for generating the



mutant strains were used as marker for prototrophy and was amplified from pCDA21 plasmid.<sup>56</sup> The DNA fragments together with plasmid linearized pRS426 *Bam*HI/*Eco*RI were transformed into *S. cerevisiae* strain SC9721 (FGSC) by the lithium acetate method<sup>57</sup> and DNA of the transformants extracted as previously described<sup>58</sup> TaKaRa Ex Taq<sup>TM</sup> DNA Polymerase (Clontech Takara Bio) was used for DNA amplification and Southern blot analyses demonstrated that the transformation cassettes had integrated homologously at the targeted *A. fumigatus* loci. *A. fumigatus* transformation was performed as described by de Castro *et al.*<sup>48</sup>

The complementing strains were constructed by first isolating from the corresponding deletion strain, a *pyrG*<sup>-</sup> auxotroph sector resistant to 0.75 mg/ml of 5-FOA (5-Fluoroorotic acid, Sigma-Aldrich), a fluorinated derivative of the pyrimidine precursor orotic acid. This analog was used to select for the absence of a functional *pyrG*<sup>+</sup> gene, which encodes the enzyme for the decarboxylation of 5-fluoroorotic acid to 5-fluorouracil, a toxic metabolite. The *flcA-C* gene deletions were confirmed in these strains and were complemented by co-transforming a DNA fragment (approximately 1 kb from each 5' and 3'-flanking regions plus the ORF) together with the PCDA21 vector and selecting for the ability to grow in medium without uridine and uracil. Homologous recombination and gene replacement were confirmed by PCR or Southern blot analyses (Fig. S3).

To FlcA-C::GFP strains were constructed by cloning the *flcA-C* ORF in frame with the green fluorescent protein (GFP) gene. We linked GFP to the FlcA-C C terminus and separated them by 4 additional codons that, after translation, produce a 4-amino-acid linker (glycine-threonine-arginine-glycine).<sup>59</sup> The *S. cerevisiae* *in vivo* recombination system was used for production of the transformation cassette. First, the *flcA-C* ORF and approximately 500 pb its 5'-UTR flanking region were amplified from gDNA of the wild-type strain by the use of the primers FlcA-C pRS426 5 Fw and FlcA SPACER GFP Rv. The stop codon of the *flcA-C* gene was omitted in this construction. The GFP ORF was amplified from the pMCB17apx plasmid (provided by Vladimir P. Efimov) by the use of the primers Spacer GFP Fw and GFP VE3' AF. The selective marker *pyrG* fragment was PCR amplified from the pCDA21 plasmid and the primers used for this PCR amplification were GFP *pyrG* Fw and *pyrG* Rv. The amplification of the 3'-UTR (approximately 600pb) was done with the Afu *flcA-C* 3 Fw and Afu FlcA-C 3 Rv primers. The PCR-amplified cassette was transformed into the *A. fumigatus* wild-type strain. The primers used are described in Table S1.

## DNA manipulation

We have used Southern blot analysis to prove the cassettes had integrated homologously at the targeted *A. fumigatus flcA-C* loci. Genomic DNA was extracted as previously described.<sup>60</sup> Standard techniques for manipulation of DNA and Southern blot analyses were carried out as described.<sup>61</sup> Southern blot schemes are shown in Figure S3.

## Microscopy

For microscopy, we have grown FlcA::GFP conidiospores on coverslips in 4 ml of MM media for 16 h at 30°C. After incubation, the coverslips with adherent germlings were left untreated or treated with iron starvation or excess. Subsequently, the coverslips were rinsed with phosphate-buffered saline (PBS; 140 mM NaCl, 2 mM KCl, 10 mM NaHPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH7.4) and mounted for examination. Slides were visualized on an Observer Z1 fluorescence microscope using a 100x objective oil immersion lens for GFP, filter set 38-high efficiency [HE], excitation wavelength of 450 to 490 nm, and emission wavelength of 500 to 550 nm. DIC (differential interference contrast) images and fluorescent images were captured with an AxioCam camera (Carl Zeiss) and processed using AxioVision software (version 4.8).

## RNA extraction and real-time PCR reactions

RNA extraction and real-time PCR experiments RNase free DNase I treatment were performed as previously described by Semighini *et al.*<sup>62</sup> All the PCR reactions were performed using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, USA) and Taq-Man<sup>TM</sup> Universal PCR Master Mix kit (Applied Biosystems, USA). The reactions and calculations were performed according to Semighini *et al.*<sup>62</sup> The primers and fluorescent probes (TaqMan<sup>®</sup>, Applied Biosystems) used in this work are described in Table S1.

## Murine model of pulmonary aspergillosis, lung histopathology and fungal burden

We have housed outbred female mice (BALB/c strain; body weight, 20 to 22 g) in vented cages with 5 animals each. Mice immunosuppression was performed with cyclophosphamide (150 mg per kg of body weight), administered intraperitoneally on days -4, -1, and 2 before and after infection. Hydrocortisonacetate (200mg/ kg body weight) was injected subcutaneously on day -3. *A. fumigatus* strains were grown on YAG for 3 d prior to infection. Fresh conidia were harvested in

PBS and filtered through a Miracloth (Calbiochem). Conidial suspensions were spun for 5 min at  $3,000 \times g$ , washed 3 times with PBS, counted using a hemocytometer, and resuspended at a concentration of  $5.0 \times 10^6$  conidia/ml. The viability of the administered inoculum was determined by incubating a serial dilution of the conidia on YAG medium, at  $37^\circ\text{C}$ . Mice were anesthetized by halothane inhalation and infected by intranasal instillation of  $1.0 \times 10^5$  conidia in  $20\mu\text{l}$  of PBS. As a negative control, a group of 5 mice received PBS only. Mice were weighed every 24 h from the day of infection and visually inspected twice daily. The statistical significance of the comparative survival values was calculated using log rank analysis and the Prism statistical analysis package.

Fungal burden was investigated in murine lungs, mice were immunosuppressed with cyclophosphamide (150 mg/kg of body weight), which was administered intraperitoneally on days  $-4$  and  $-1$ , while hydrocortisone acetate was injected subcutaneously (200 mg/kg) on day-3. Five mice per group were intranasally inoculated with  $1 \times 10^6$  conidia/  $20\mu\text{l}$  of suspension. A higher inoculum, in comparison to the survival experiments, was used to increase fungal DNA detection. Animals were sacrificed 72 h post-infection, and the lungs were harvested and immediately frozen in liquid nitrogen. Samples were ground to a fine powder under liquid  $\text{N}_2$  and DNA was extracted via the phenol-chloroform method. DNA quantity and quality were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific). At least  $500\mu\text{g}$  of total DNA from each sample was used for quantitative real-time PCRs. PCR reactions were performed using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, USA) and SYBR Green detection. SYBR<sup>®</sup> Green PCR Master Mix (Applied Biosystems, USA) was used for reaction mixture preparation. The primer sets for the analyses were used to amplify the 18 S rRNA region of *A. fumigatus* (18 S rRNA Afu sybr FW, 5'-GACCTCGGCCCTTAAATAGC-3' and 18 S rRNA Afu sybr Rv, 5'-CTCGGCCAAGGTGATGTA-3') and an intronic region of mouse GAPDH, encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH mouse sybr FW, 5'-GAGGGACTTGGAGGACACAG-3' and GAPDH mouse sybr Rv, 5'-ACATCACCCCATCACTCAT-3'). Six-point standard curves were calculated using serial dilutions of gDNA from all the *A. fumigatus* strains used and the uninfected mouse lung. Fungal and mouse DNA quantities were obtained from the threshold cycle ( $C_T$ ) values from an appropriate standard curve. Fungal burden was determined as the ratio between picograms of fungal and micrograms of mouse DNA. Virulence survival and fungal burden were repeated at least twice.

### FAD transport assay

Protoplasts were produced for all the strains as previously described.<sup>48</sup> Protoplasts were resuspended in 1 ml of reaction buffer (20 mM HEPES pH 6.8, 150 mM potassium acetate, 250 mM sorbitol, 5 mM magnesium acetate). Protoplasts ( $1.25 \times 10^6$ ) were washed 3 times with reaction buffer at  $4^\circ\text{C}$  and resuspended in the same buffer. Protoplasts were incubated with 1 mM of flavin adenine dinucleotide (Sigma, USA) in the absence of light for 0, 5, 10, 20, and 30 minutes at  $30^\circ\text{C}$ . The reaction was stopped by adding 1 ml of reaction buffer at  $4^\circ\text{C}$ , and washed 3 times with the same buffer. Finally, the protoplasts were lysed in 2 % Triton X-100, and the fluorescence of the supernatant was evaluated at 450 nm excitation and 530 nm emission.

### Immunoblot analysis

To detect FlcA::GFP fusion, fresh harvested conidia ( $1 \times 10^7$ ) of the wild-type and mutant strains were inoculated in 50 ml liquid MM at  $37^\circ\text{C}$  for 20 h (160 rpm) and after this period, the mycelia were treated with  $\text{CaCl}_2$  (200mM) for 10 and 30 min. The mycelia was frozen and ground in liquid nitrogen. For protein extraction, 0.5 ml lysis buffer<sup>63</sup> containing 10% (v/v) glycerol, 50 mM Tris-HCl pH 7.5, 1% (v/v) Triton X-100, 150 mM NaCl, 0.1% (w/v) SDS, 5 mM EDTA, 5 mM sodium orthovanadate, 1 mM PMSF, and 1X Complete Mini-protease inhibitor (Roche Applied Science) was added to the ground mycelium. Extracts were centrifuged at 20,000 g for 40 minutes at  $4^\circ\text{C}$ . The supernatants were collected and the protein concentrations were determined using the Bradford method<sup>64</sup> (BioRad).  $50\mu\text{g}$  of protein from each sample were resolved in a 12% (w/v) SDS-PAGE and transferred polyvinylidene difluoride (PVDF) membranes using the iBlot<sup>®</sup> 2 Dry Blotting System (Thermo Scientific<sup>™</sup>). The flc::GFP fusion was detected by the anti GFP antibody (Sigma G1544) at a 1:1,000 dilution in TBST containing. Incubation was performed at  $4^\circ\text{C}$  for 16 hours. The primary antibody was detected with an HRP-conjugated secondary antibody raised in rabbits (A0545; Sigma) in TBST buffer for an one-hour incubation, at room temperature. Chemoluminescent detection was performed by using an ECL Prime Western Blot detection kit (GE HealthCare). Images were generated by exposing the PVDF membranes to the ChemiDoc XRS gel imaging system (BioRad).

### Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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