Aspergillus fumigatus hyphal branching mutant show enhanced susceptibility to host defenses and caspofungin *in vivo*

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Abstract

Purpose:

The efficacy of echinocandins for invasive aspergillosis treatment is marred by the ability of *Aspergillus* to mount compensatory alterations in cell wall integrity and stress response signaling. The transcription factor ZfpA regulates features of *A. fumigatus* growth reminiscent of echinocandin tolerance mechanisms, including hyphal branching, septa formation, and cell wall composition. However, the relevance of ZfpA-mediated changes to fungal growth and stress resistance during infection is unclear. We hypothesize that ZfpA regulation of fungal growth and critical stress response pathways shapes fungal interaction with immune cells and echinocandin tolerance *in vivo*, and therefore represents a valuable target for improving aspergillosis therapies.

Methods:

We coupled the optical transparency of larval zebrafish with RFP-expressing wild-type, ZfpA deletion (Δ*zfpA*), and overexpression (OE::*zfpA*) strains to track fungal development, immune cell recruitment, and host survival during *A. fumigatus* infection.

Results:

Previous *in vitro* analyses of ZfpA manipulation describe decreased hyphal branching and chitin content in *zfpA* deletion mutants, while *zfpA* overexpression increases both branching and chitin. Here, we demonstrate a novel role for ZfpA in disease progression and susceptibility to caspofungin *in vivo*. In a wild-type host, *zfpA* deletion does not alter germination or leukocyte recruitment but does reduce fungal burden and attenuate virulence of *A. fumigatus* in later stages of infection. Virulence of Δ*zfpA* is re-established in a neutropenic host, suggesting enhanced susceptibility of Δ*zfpA* to neutrophil killing. Overexpression of *zfpA* does not alter germination, fungal burden, leukocyte recruitment, or virulence in wild-type or neutropenic hosts. Concomitant with our *in vitro* analyses of capsofungin tolerance (see poster #45), caspofungin treatment improves survival of animals infected with Δ*zfpA* but has no effect on survival of OE::*zfpA*-infected animals, indicating enhanced caspofungin sensitivity of Δ*zfpA* and decreased sensitivity of OE::*zfpA in vivo*.

Conclusion:

Our study identifies ZfpA as a regulator of resistance to host defenses and caspofungin treatment during infection.

zfpA deletion attenuates virulence of *A. fumigatus* in wild-type, but not neutropenic, zebrafish larvae

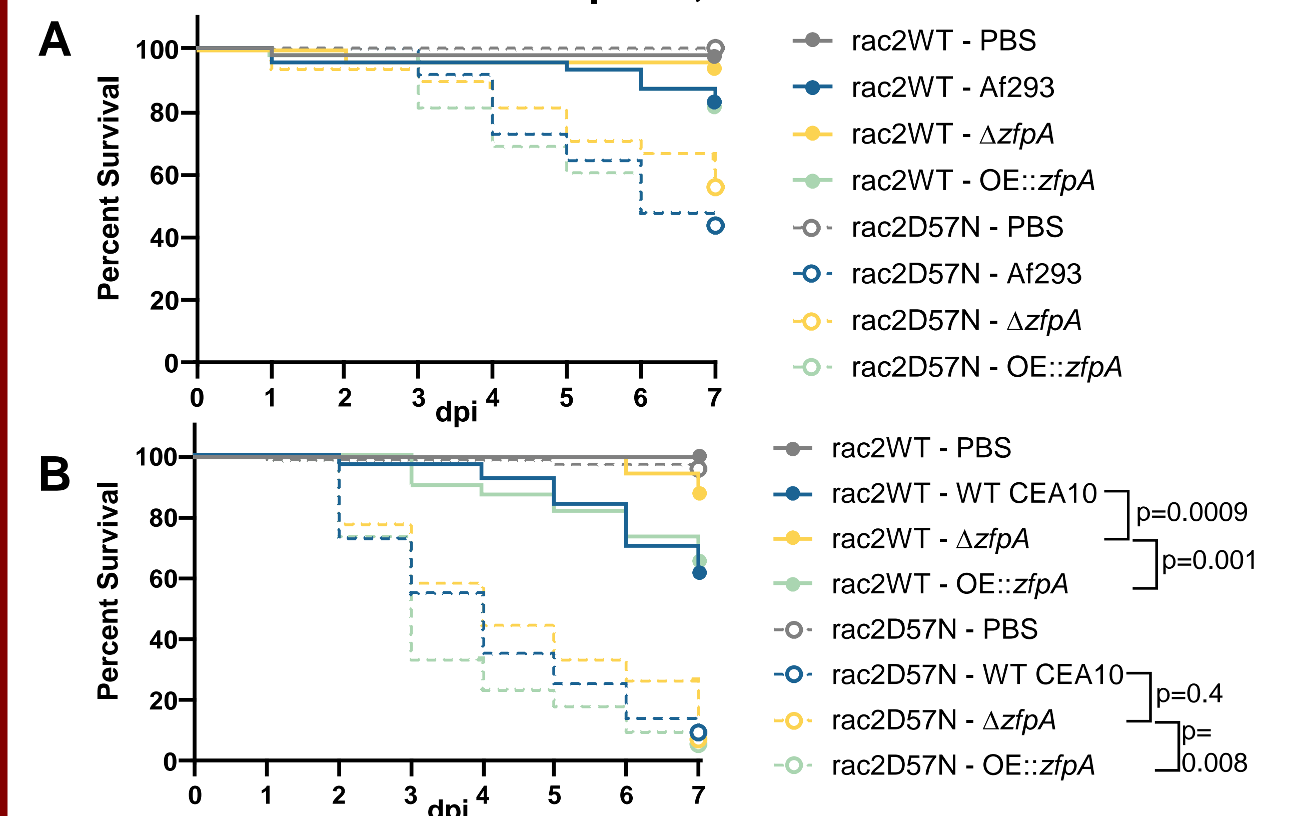
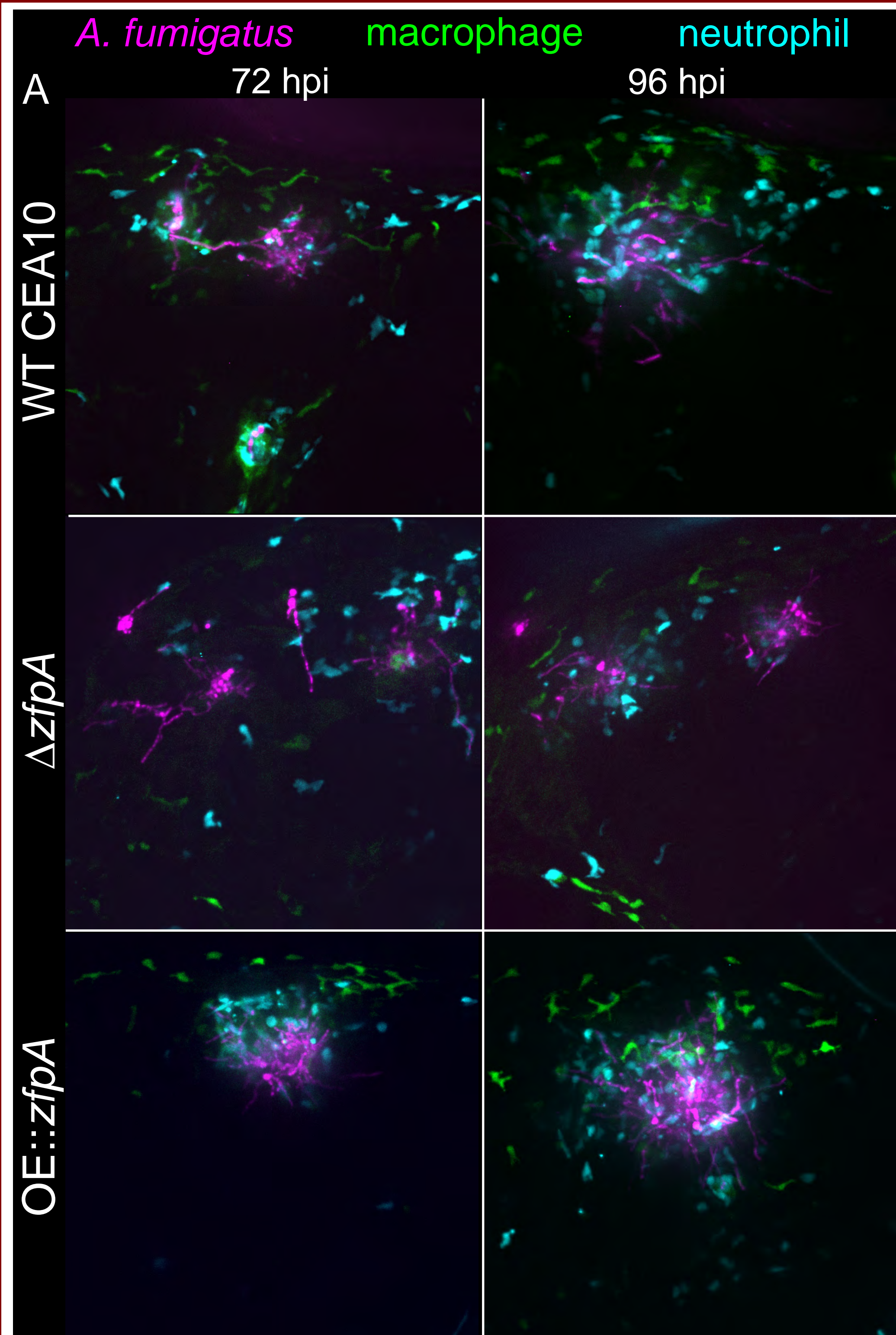
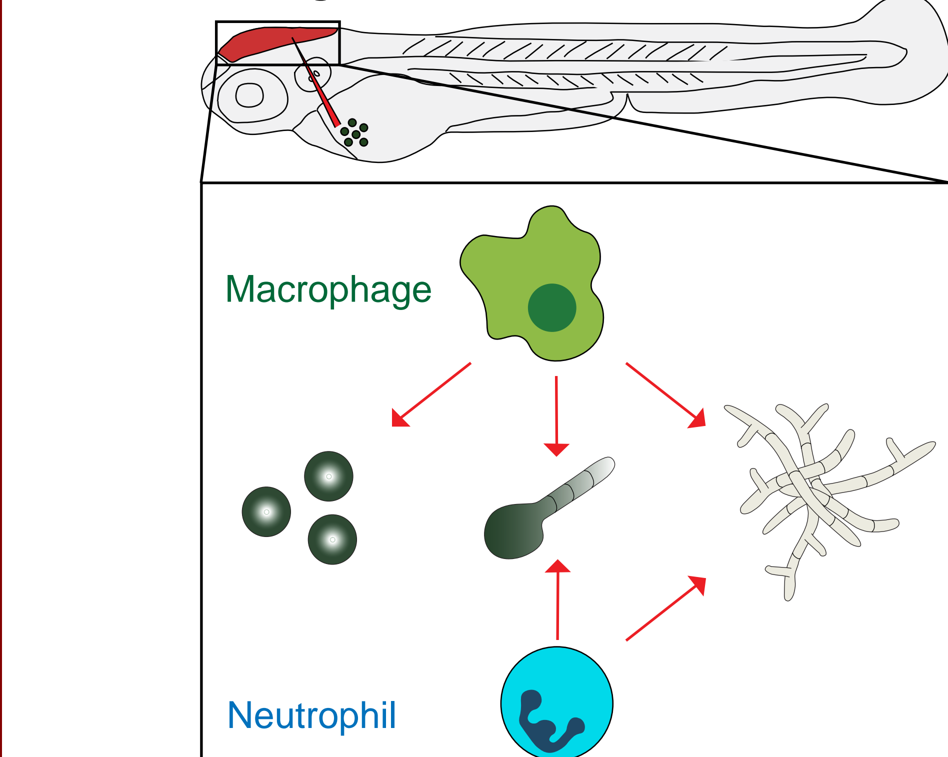


Figure 1 (A) Survival analysis of larvae with the dominant negative Rac2D57N mutation (model of human neutrophil deficiency) or wild-type siblings injected with PBS, wild-type Af293, Af293 Δ*zfpA*, or Af293 OE::*zfpA* strains. Results represent pooled data from 2 independent replicates. n=46-48 larvae per condition. (B) Rac2D57N or wild-type siblings injected with PBS, wild-type CEA10, CEA10 Δ*zfpA*, or CEA10 OE::*zfpA* strains. Results represent pooled data from 3 independent replicates. n = 71-72 larvae per condition. P values calculated by Cox proportional hazard regression analysis.



Tracking hyphal growth and immune cell recruitment during infection of larval zebrafish



zfpA deletion reduces fungal burden during infection but does not impact immune cell recruitment

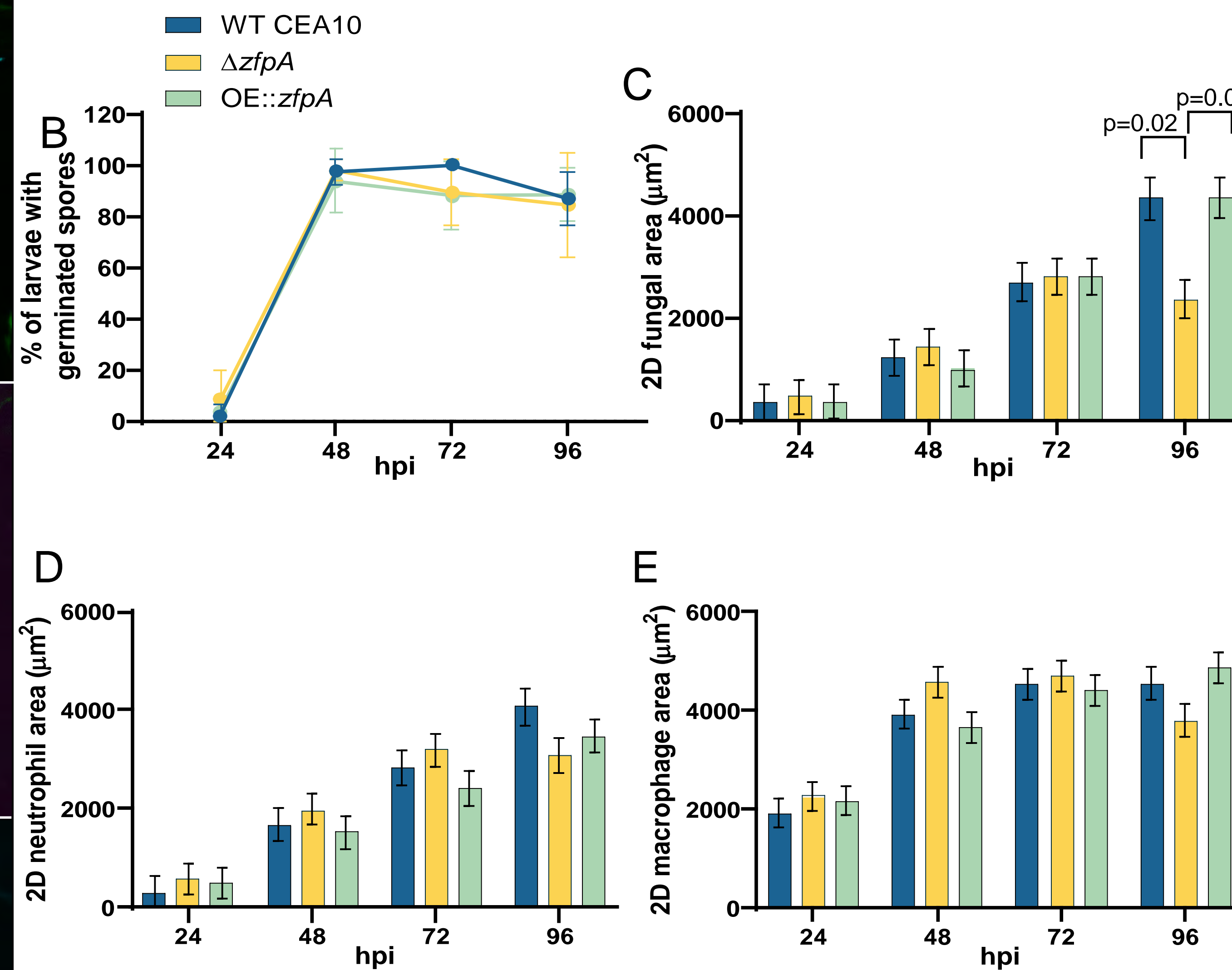
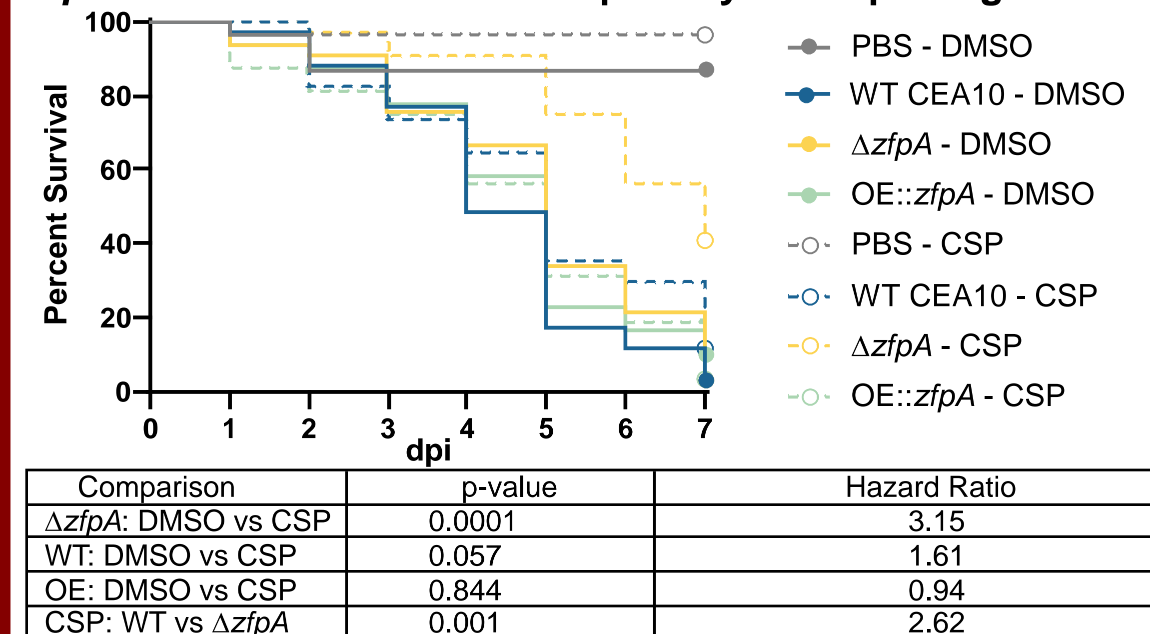


Figure 2 (A) 2 day post fertilization larvae with fluorescence labeled macrophages (GFP) and neutrophils (BFP) were infected with RFP-expressing wild-type CEA10, *zfpA*, or OE::*zfpA* strains and imaged with confocal microscopy up to 96 hours post infection. Images represent MIPs of z-stacks at 72 and 96 hpi. (B) Mean percentage of larvae containing germinated spores. (C-E) Bars represent lsmeans±SEM of fungal, neutrophil, and macrophage area pooled from 4 independent replicates. n = 45-48 larvae per condition. P values calculated by ANOVA with Tukey's multiple comparisons.

zfpA deletion enhances susceptibility to caspofungin in vivo



Comparison	p-value	Hazard Ratio
Δ*zfpA*: DMSO vs CSP	0.0001	3.15
WT: DMSO vs CSP	0.057	1.61
OE: DMSO vs CSP	0.844	0.94
CSP: WT vs Δ*zfpA*	0.001	2.62

Figure 3 Survival analysis of Rac2D57N larvae (neutropenic) infected with PBS, wild-type, Δ*zfpA*, or OE::*zfpA* strains and bathed in 1 μg/ml caspofungin or 0.01% DMSO. Results represent pooled data from 2 independent replicates. n = 30-35 larvae per condition. P values calculated by Cox proportional hazard regression analysis.

Reconstruction of *in vivo* hyphal branching patterns using neuronal tracing software

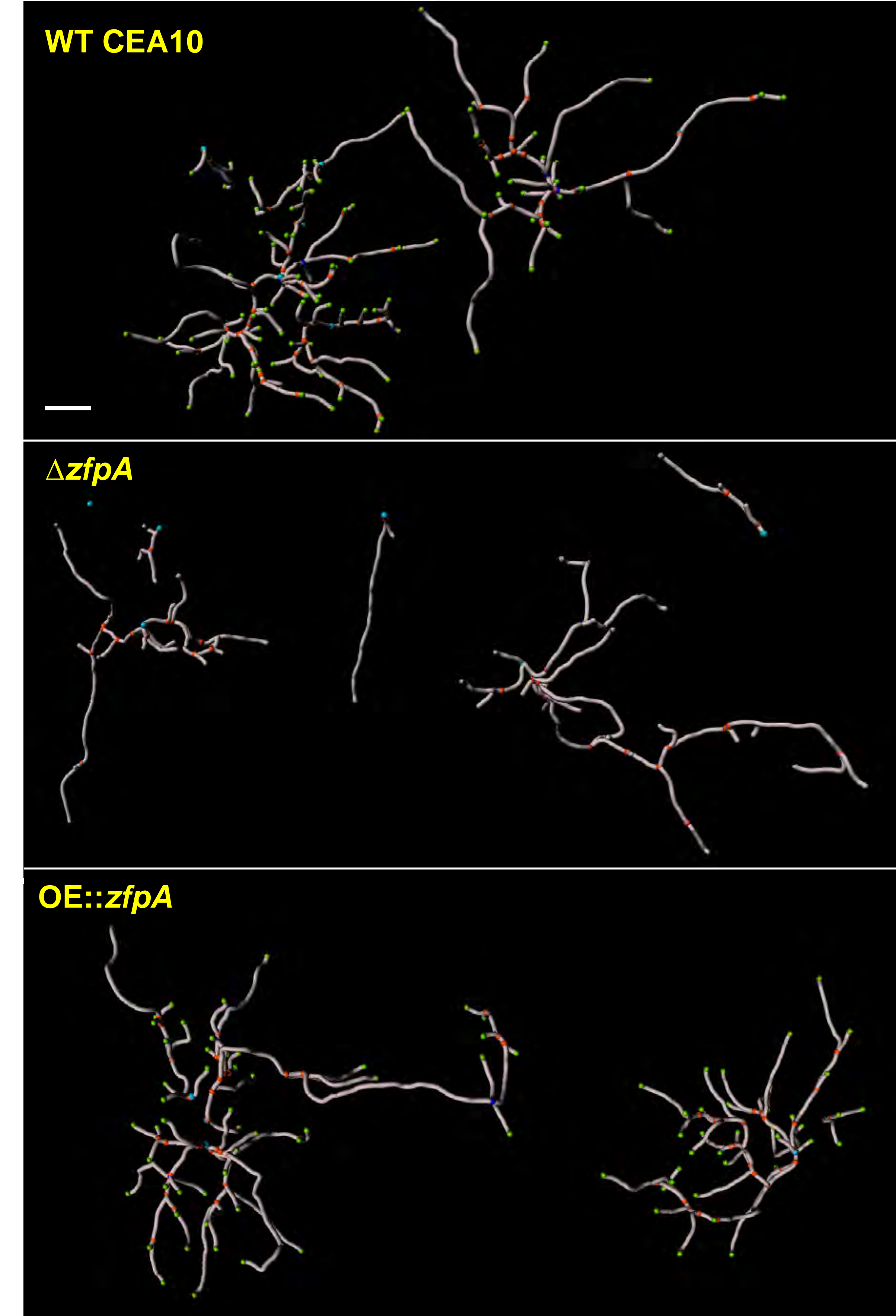


Figure 4: 3D reconstruction of hyphal organization in wild-type larvae infected with RFP-expressing CEA10 ZfpA strains. Images were created using Imaris Filament Tracer software from z-stacks acquired at 72 hours post infection. This technique will be used to assess changes in hyphal morphology of ZfpA strains *in vivo*. Scale bar = 20 μm.

Remaining questions:

1. Does ZfpA deletion enhance neutrophil killing?
2. How does ZfpA alter cell wall compositiion?
3. Is the enhanced antifungal susceptibility of Δ*zfpA* specific to echinocandins?
4. Is morphology/cell wall composition of Δ*zfpA* altered following caspofungin exposure?

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