The vacuolar sorting protein Vps45 links iron uptake, mitochondrial function and virulence in the pathogenic fungus *Cryptococcus neoformans*.

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Objectives: *Cryptococcus neoformans* is the main causative agent of cryptococcal meningitis, a disease that is responsible for ~ 15% of AIDS-related deaths. In this regard, cryptococcosis is the second most common cause of mortality in people with HIV/AIDS. Unfortunately, very few antifungal drugs are available to treat cryptococcal infections. However, understanding the mechanisms of *C. neoformans* pathogenesis may lead to new therapeutic approaches. The competition for iron between invading microorganisms and mammalian hosts is a pivotal determinant of the outcome of infection, and *C. neoformans* employs multiple mechanisms to compete for iron during cryptococcosis. In this study, our goal was to examine the role of endocytic trafficking in iron uptake by characterizing a mutant defective in the Sec1/Munc18 (SM) protein Vps45. This protein is known to regulate the machinery for vesicle trafficking and fusion via interactions with SNARE proteins.

Vps45 is required for membrane trafficking to the vacuole.

Intracellular membrane staining with the lipophilic dye FM4-64. WT, *vps45* mutant and complemented strains were grown for 24h in YNB-BPS at 30°C (A) or 37°C (B). The *vps45* mutant complemented with a *VPS45*-GFP gene fusion was grown for 24h in YNB-BPS at 30°C (C) or 37°C (D). E) Growth of the WT, mutant and complemented strains in the presence of trafficking and glycosylation inhibitors.

Vps45 contributes to iron acquisition in a temperature dependent manner.

Growth of the WT, *vps45* mutants and complemented strains in iron chelated YNB-BPS media with organic and inorganic iron at 30°C (A) and 37°C (B). The optical density (600nm) was recorded over a period of 5 days. C) Inductively coupled plasma mass spectrometry (ICP-MS) measurements of iron content of pre-grown iron starved cells inoculated for 48h in YNB-BPS w/o 100μM FeCl₃ at 30°C and 37°C.

Vps45 contributes to endocytic transport of the ferroxidase Cfo1 for high-affinity iron uptake.

Localization of Cfo1-GFP in the absence of *VPS45*. Strains (WT, *vps45*Δ and complement) containing a *CFO1-GFP* construct were inoculated in YNB-BPS, stained with 20μM FM4-64 and incubated at 37°C for 3h (A) and at 24h (B). Images were taken with an Axio imager M.2.

Vps45 associates with mitochondria, and is required for mitochondrial function.

Association of Vps45-GFP with mitochondria. The mutant strain complemented with *VPS45-GFP* was cultured in YNB, YNB + 150μM BPS, or YNB-BPS + 100μM FeCl₃ for 24h at 30°C and 37°C (A). Cells were stained with 500nM MitoTracker Red CMXRos (mitotracker) and images were taken with an Axio imager M.2. B) Vps45 is involved in mitochondrial function, ROS sensitivity and calcium homeostasis. The WT, mutants and complemented strains were grown in the presence of inhibitors of the mitochondrial electron transport chain, hydrogen peroxide (H₂O₂), and calcium inhibitors (C).

Vps45 influences cell wall integrity and capsule formation in a temperature-dependent manner.

Cell wall composition and integrity are altered in *vps45* mutants. A) Spot assays of serial diluted WT, mutant and complemented strains grown in the presence of cell wall stressors. B) Flow cytometry measurement of differential fluorescence of cells stained with pontamine, concanavalin A and eosin Y. C) Capsule thickness, cell diameter measurements and GXM detection in supernatants of cell grown in defined limited iron media at 30°C (C) and 37°C (E). The capsule thickness and cell diameter of at least 40 cells of each strain were measured. Statistical significance relative to WT is indicated by an asterisk (ANOVA ** *P* < 0.0001). Shed polysaccharides were collected from culture supernatants of all strains incubated at 30°C (D) or 37°C (F).

Vps45 is involved in resistance to drugs that target the membrane and vacuole.

Loss of Vps45 causes increased sensitivity to chloroquine and azole drugs. The WT, mutants and complemented strains were grown in the presence of antimalarial and antifungal drugs.

Vps45 is required for virulence in a mouse model of cryptococcosis and for survival in phagocytic cells.

Vps45 is required for survival in macrophages and virulence in mice. A) WT, *vps45* mutants and complemented strains were incubated with the murine macrophage-like cell line J774A.1. B) 10 female BALB/c mice were inoculated intranasally with 2X10⁵ fungal cells and survival of the mice was monitored daily. Survival differences between groups of mice were evaluated by log-rank test against WT. (***) *P* < 0.0001.