The *CRZ1/SP1*-like gene links survival under limited aeration, cell integrity and biofilm formation in the pathogenic yeast *Cryptococcus neoformans*

Zuzana Moranova^{a,b}, Eric Virtudazo^b, Kristyna Hricova^a, Misako Ohkusu^b, Susumu Kawamoto^b, Vendula Husickova^a, Vladislav Raclavsky^a

Aims. Limited aeration has been demonstrated to cause slowdown in proliferation and delayed budding, resulting eventually in a unique unbudded G2-arrest in the obligate aerobic pathogenic yeast *Cryptococcus neoformans*. Also, the ability to adapt to decreased oxygen levels during pathogenesis has been identified as a virulence factor in *C. neoformans*. The aim of this study was to identify and characterize genes that are necessary for the proliferation slowdown and G2-arrest caused by limited aeration.

Methods. Random mutants were prepared and screened for lack of typical slowdown of proliferation under limited aeration. The CNAG_00156.2 gene coding for a zinc-finger transcription factor was identified in mutants showing most distinctive phenotype. Targeted deletion strain and reconstituted strain were prepared to characterize and confirm the gene functions. This gene was also identified in a parallel studies as homologous both to calcineurin responsive (Crz1) and PKC1-dependent (SP1-like) transcription factors.

Results. We have confirmed the role of the cryptococcal homologue of *CRZ1/SP1*-like transcription factor in cell integrity, and newly demonstrated its role in slowdown of proliferation and survival under reduced aeration, in biofilm formation and in susceptibility to fluconazole.

Conclusions. Our data demonstrate a tight molecular link between slowdown of proliferation during hypoxic adaptation and maintenance of cell integrity in *C. neoformans* and present a new role for the *CRZ1* family of transcription factors in fungi. The exact positioning of this protein in cryptococcal signalling cascades remains to be clarified.

Key words: *Cryptococcus*, hypoxia, G2 arrest, CRZ1, SP1, cell integrity, capsule, biofilm, fluconazole, dormant infection

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^aDepartment of Microbiology, Faculty of Medicine and Dentistry, Palacky University Olomouc, Czech Republic

^bDivision of Molecular Biology, Medical Mycology Research Center, Chiba University, Chiba, Japan

Corresponding author: Vladislav Raclavsky, e-mail: vladislav.raclavsky@upol.cz

INTRODUCTION

For *Cryptococcus neoformans* to cause disease, it must adapt to the host environment, which is very different from its natural habitat. *C. neoformans* has three well established virulence factors: a capsule, melanin and the ability to grow at human body temperature¹. Recently, the ability to adapt to decreased oxygen levels during pathogenesis has emerged as another virulence factor in this obligate aerobic yeast species. It is generally accepted that well ventilated lungs are the primary site of cryptococcal infection, typically followed by spread to central nervous system (CNS). Other organs are affected by cryptococcal infection rather rarely. Oxygen was demonstrated to be a growth-limiting nutrient for *C. neoformans*² and deficit in oxygen under limited aeration was demonstrated to delay cryptococcal budding resulting eventually in a unique unbudded G2-arrest³⁻⁵.

When searching for genes important for hypoxic adaptation, Chun et al. isolated hypoxia sensitive mutants harbouring mutations in three homologs of proteins that act in the mammalian SREBP pathway, namely *cryptococ-*

cal SREBP homologue Sre1, Site-2-protease homologue Stp1, SCAP homologue Scp1, and also Tco1, a member of a highly conserved family of fungal-specific hybrid histidine kinases⁶. These mutants exhibit defect in growth under hypoxic conditions, but not under normoxic conditions. In parallel with the work of Chun et al., Chang et al. confirmed these observations in *sre1* and *scp1* deletion mutants⁷ and described several new members of this pathway⁸. Mutants in either the SREBP pathway or the Tco1 pathway display defects in their ability to proliferate in host tissues and to cause disease in infected mice, linking hypoxic adaptation and pathogenesis. For recent reviews on this topics see also ref.⁹⁻¹².

Here we report that the cryptococcal *CRZ1/SP1*-like gene plays an important role not only in cell wall integrity, but also in delay of cell cycle progression, survival under limited aeration, and in biofilm formation in the pathogenic yeast *C. neoformans*. This represents novel biological roles for this conserved protein and also opens a new field in the study of hypoxia sensing and signalling in *C. neoformans*.

MATERIALS AND METHODS

Strains and cultivation

Cryptococcus neoformans var. *grubii* (serotype A) strain IFM49144 showing typical unbudded G2-arrest⁴ was from Culture Collection of the Medical Mycology Research Center, Chiba University, Chiba, Japan. In-house prepared YPG medium (10 g/L of each yeast extract, peptone, and glucose) was used for liquid cultivation, YPG plates with appropriate supplements were used for phenotypical analysis. Rice extract agar and Sabouraud agar were from Trios (Prague, Czech Republic) and Caffecic Acid Ferric Citrate (CAFC) Agar was from Himedia Laboratories (Mumbai, India). For controlled hypoxic conditions plates were incubated in a 1% O₂, 10% CO₂, 9.4% H₂, and 79.3% N₂ atmosphere using an Anoxomat system (Mart Microbiology, Drachten, Netherlands).

Agrobacterium mediated random insertional mutagenesis

Random insertional mutagenesis of *C. neoformans* was performed by transformation with *A. tumefaciens* harbouring kanamycin resistance plasmid pYCC716. The cocultivation experiments were performed as described earlier¹³. Briefly, *C. neoformans* cells were co-cultivated with *A. tumefaciens* for 3 days at 24 °C on co-cultivation medium (CCM) at the 1C:10A ratio. The bacteria/yeast mixture was scratched from CCM and resuspended in sterile water. Aliquots were plated on YPG agar containing 200 µg/mL G418 (Sigma Aldrich) and 200 mM cefotaxime (Alchemica). After 2-day cultivation 665 transformants were obtained.

Screening for mutants showing hypoxia-specific phenotypes

Transformants were screened according to hypoxia response with regard to cell cycle progression to identify components of hypoxia-induced G2 arrest on molecular level. Previously optimized high-throughput arrangement of experiments was used to induce typical unbudded G2-arrest under moderate hypoxia compared to unrestricted normoxic cell cycle progression¹⁴. Briefly, cultivation

was performed in 24-well cell culture plates sealed with SecureSeal adhesive film (Simport) which is less than 0.01% oxygen permeable and filled with 300 µL of YPG media to secure good aeration for normoxic conditions and 600 µL for reduced aeration resulting in moderately hypoxic conditions. The plates were shaken reciprocally at 200 rpm at 30 °C for 48 h and cell count was measured using a Multisizer 3 Coulter Counter (Beckman Coulter, Brea, California, USA). To select those insertional mutants that lacked typical response, degree of proliferation slowdown in response to reduced aeration was calculated as the ratio of the number of cells grown at moderately reduced aeration (600 µL) to the number of cells grown at good aeration (300 µL). Thus, higher ratio indicated lower degree of slowdown in response to reduced aeration. The insertional mutant showing highest ratio was selected for identification of the insertional locus.

Identification of the insertional locus

Thermal asymmetric interlaced-PCR (TAIL-PCR) (ref.¹⁵) was performed to gain genomic sequences adjacent to the inserted DNA. Amplicons were sorted in 2% (w/v) agarose gel, purified using QIAquick gel extraction Kit (Qiagen) and sequenced using a BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems) with an ABI Prism 3130xl Genetic Analyzer apparatus (Applied Biosystems) in accordance with manufacturer's instructions. Acquired sequences were searched for homology using the BLAST algorithm in *C. neoformans* var. *grubii* H99 genomic sequence (www.broadinstitute.org) to identify the insertional locus.

Targeted deletion strategy

Overlap extension PCR was used to create overlap fragment for biolistic transformation as already described¹⁶. The primer pairs TF1.1F/TF2R and TF3F/TF4.1R were designed and used with genomic DNA to obtain the left (1 kb upstream of the CNAG_00156.2 gene) and right (1 kb downstream of the same gene) arms for the disruption construct. The selectable marker NEO was amplified using the primers M13F and M13R from the plasmid

Table 1. List of primers used in the study.

Name	Sequence
KT8.1F	GGGTTTAAGACGAAAGAAAG
KT8.1R	GATTGCCTTTCCCGACTTTG
KT8.2R	CAGCAGGATTGTAATCTGTG
M13F	GTAAAACGACGGCCAG
M13R	CAGGAAACAGCTATGAC
NAT-F	TGGATCAACAGGGACTTCTC
NAT-R	ACTCGCCCAACATGTCTATG
TF1.1F	CAACTGCCGCATCATTTGCA
TF1.3F	GAGCAGATTGTGTATCTTGC
TF1.3F LP	CGTGAGTAACTTTTCCTTCG
TF2R	GTCATAGCTGTTTCCTGTTTTTGTGGATTATAGGGGTGA
TF3F	CTGGCCGTCGTTTTACTCGCCCGATGGTCATAGGGCGCTG
TF3R+M13R	gtcatagctgttctctgTTAATCCTCTTCACTCGTTT
TF4.1R	GAAGTCGAGGCGCAGTGATT

pJAF1, which contains the neomycin resistance marker cassette for *C. neoformans*. Primers TF1.1F and TF4.1R were used to create an overlap fragment (1 µL of each amplified fragment); see Table 1 for list of primers. All PCR reactions were performed with KOD Plus (Toyobo) DNA polymerase (0.1 U/µL) using 0.5 µM primers, 0.2 mM dNTPS, 1.5 mM Mg²⁺ and amplified using an iCycler Thermal Cycler (BioRad) with initial denaturation at 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 68 °C for 6 min, with final extension at 68 °C for 10 min. To confirm overlap reaction, the PCR product was run on 1% (w/v) agarose gel and overlap fragment was cut out of the gel and purified using QIAquick gel extraction Kit (Qiagen). The resulting 4-kb overlap PCR product was used to transform the IFM49144 strain by biolistic transformation as described below. Three independent transformants were screened for proper targeted deletion by colony PCR using primers KT8.1F and KT8.1R which were designed outside of overlap sequence resulting in a 5978 bp fragment in control strain harbouring the CNAG_00156.2 (*CRZI/SPI*-like) gene and in a 4367 bp fragment in the targeted deletion strain with neomycin resistance gene. Proper deletion was also confirmed by Southern blot analysis described below.

Reconstitution of CNAG_00156.2 (*CRZI/SPI*-like) gene targeted deletion strain

Primers TF1.3F and TF3R+M13R were used to amplify 1 kb region upstream of the CNAG_00156.2 (*CRZI/SPI*-like) gene stop codon, and primers TF3F+M13F and TF4.1R to amplify 1 kb region downstream of the same stop codon from IFM49144 strain genomic DNA. These two fragments were PCR overlapped with the NAT marker (M13R-M13F direction) and the overlap product (4042 bp) was used to transform the IFM49144 wild type strain. Primers TF1.3F LP and KT8.1R were used to screen for correct transformants that have the NAT marker inserted downstream of the CNAG_00156.2 gene (4184 bp in NAT transformants compared to 2269 bp in wild type). The genomic copy of the CNAG_00156.2 (*CRZI/SPI*-like) gene amplified by TF1.1F and KT8.1R primers was fused with the NAT marker (7807 bp). Purified PCR product was used to transform the CNAG_00156.2del::NEO targeted deletion strain. Primers KT8.1F and KT8.2R were used to screen for three independent correct transformants (7979 bp in CNAG_00156.2-NAT reconstituted transformants compared to 4466 bp in the CNAG_00156.2del::NEO targeted deletion strain and 6064 bp in the wild type strain) and also paired KT8.1F with NAT-R and KT8.2R with NAT-F to have an internal PCR check with NAT marker. See Table 1 for list of primers.

Biolistic transformations

The procedure described earlier¹⁷ was modified and optimized empirically to achieve best transformation rate in the IFM 49144 strain. Briefly, 1 mL of an overnight culture in YPG was pelleted and resuspended in 0.5 mL of fresh YPG. Approximately 200 µL of the cell suspension were spread per each minimal medium plate with

1 M sorbitol and plates were air-dried. DNA samples were prepared by combining 600 µg of gold microcarrier beads (0.6 µm in diameter, Bio-Rad) with 1-4 µg of the overlap DNA fragment, 10 µL of 2.5 M CaCl₂, and 2 µL of 1 M spermidine. This mixture was incubated for 2-3 min at room temperature while vortexing vigorously, followed by 10 min incubation without agitation. Beads were centrifuged, washed with 30 µL of 70% ethanol followed by 30 µL of 100% ethanol, and then resuspended in 12 µL of 100% ethanol. The solution was placed on macrocarrier disks (Bio-Rad) and YPG plates supplemented with 1 M sorbitol (without antibiotics) were then bombarded with the DNA-bound gold microcarrier beads in a PDS-1000 biolistic transformation apparatus (Bio-Rad) at a helium gas pressure of approximately 1350 psi under a vacuum of 29 in of Hg. After the transformation plates were incubated for 4 h at 30 °C, the cells were harvested and resuspended in 200 µL of PBS and spread on plates supplemented with 200 µg/mL of G418, followed by further cultivation at 30 °C for 3-5 days.

Southern Blot Analysis

Southern blot analysis for verification of single targeted deletion and reconstitution was performed as follows: *C. neoformans* genomic DNA (~10 µg) was digested with restriction enzyme BglII, fractionated by electrophoresis in 1% agarose gel, transferred to a nylon membrane, and fixed by UV crosslinking. The membrane was hybridized overnight with 1 kb DNA fragment prepared with PCR DIG Probe Synthesis Kit as probe and the membranes were washed, dried and exposed to film.

Cell cycle and cell death assays

Cells were fixed by ethanol and stained by PicoGreen fluorescent dye (Invitrogen) diluted 1:500. PicoGreen shows high affinity to dsDNA, making the RNase treatment unnecessary compared to conventional PI staining, and it also surpasses performance of the dsDNA selective dye Hoechst 33258 (ref.¹⁸). This is particularly useful in *C. neoformans*, where the cell cycle analysis based on DNA content measurement can be tedious because of the less permeable cell wall and variable background fluorescence. PicoGreen has already been used for cell cycle assays in bacteria; however, this is its first use for such purpose in yeasts to our knowledge. After washing with PBS, proper staining of nuclei in contrast to low cytoplasmic background was checked under fluorescence microscope. If needed, dilution of PicoGreen dye was adjusted to reduce background or increase fluorescence of nuclei for proper DNA content monitoring. A Cytomics FC 500 flow cytometer (Beckmann-Coulter) was used for measurement and cell cycle analysis was performed manually in plots of PicoGreen fluorescence vs. forward scatter based on cell size and fluorescence intensity. A cell death rate assay already described in *C. albicans*¹⁹ was modified as follows: cells were suspended in 0.01% Eosin Y in 1 M sorbitol, observed under fluorescence microscope, photographed and percentage of dead cells was counted in at least 50 cells per sample.

Biofilm formation

C. neoformans was grown on Sabouraud glucose agar (SGA) plates for 48 h, a suspension in Sabouraud broth was prepared ($\sim 10^7$ cells/mL), 200 μ L were aliquoted into wells of a microtiter plate and incubated at 30 °C for 48 h. Nonadherent cells were then washed gently by tap water and adherent cells were fixed by 200 μ L of 99% methanol per well for 15 min. After removing the fixative and air drying, adherent cells were stained by 160 μ L of aqueous crystal violet solution per well for 10 min. After gentle washing by tap water, 160 μ L of 33% acetic acid was added, shaken and colorimetric reading of the destaining solution was performed at 570 nm. Four wells were processed in parallel in each strain and in negative control.

Antifungal susceptibility testing

Minimum inhibitory concentration of fluconazole was determined using E-test strips (bioMérieux, Marcy l'Etoile, France) on the Antimycotic Sensitivity Test Agar (Trios, Prague, Czech Republic) in accordance with manufacturer's instructions.

RESULTS

The *CRZI/SPI*-like gene was disrupted in the random mutant that lacked slowdown of cell cycle progression under reduced aeration

Altogether, 665 *C. neoformans* random mutants were prepared by *Agrobacterium* mediated random insertional mutagenesis. Slowdown of proliferation in response to reduced aeration is the hallmark of cryptococcal response to moderate hypoxia that results in a typical unbudded G2-arrest eventually. Altogether, 18 random insertional mutants were selected for further analysis. Sequencing revealed insertion in 12 unique locuses, one of the insertion mutants contained 2 inserts in 2 different locuses. We selected the random insertional mutant showing lowest degree of proliferation slowdown in response to moderately reduced aeration for further study. The targeted locus was present in two other random mutants and was identified as the CNAG_00156.2 gene based on homology search in *C. neoformans* var. *grubii* H99 genomic DNA database using BLAST (www.broadinstitute.org). When a translated protein sequence of this gene was used to search for homology in *S. cerevisiae*, the *CRZI* gene coding for a zinc-finger transcription factor was identified as a potential homologue. Reversed search for a *CRZI* homologue in cryptococcal genome revealed the same gene, as already published independently when identification of a cryptococcal homologue of *ACE2* transcription factor was sought²⁰. However, a report refuting CNAG_00156.2 to be a functional homologue of *CRZI* and collecting evidence for its homology to the Sp family of transcription factors was published during finishing our study²¹. Another study that was published during revision of our manuscript demonstrated its product to be a *bona fide* target of calcineurin under non-starvation conditions and to be

the true ortholog of fungal Crz1 proteins²². Therefore, we will further refer to the CNAG_00156.2 gene as to cryptococcal *CRZI/SPI*-like gene.

Cn *CRZI/SPI*-like gene is required for survival under limited aeration and for slowdown of proliferation

The *CRZI/SPI*-like gene was removed in our IFM49144 strain by targeted deletion strategy and a reconstituted strain was also prepared as described in Material and methods. Two different volumes of culture medium (500 and 1000 μ L) were used to expose the culture of the targeted deletion strain to different degrees of aeration (moderately and severely reduced) in contrast to well-aerated normoxic control (100 μ L), all in shaken liquid cultures in 24-well cell culture plates as described earlier¹⁴. Death rate was evaluated using Eosin Y staining and fluorescence microscopy. A convincing detrimental effect of the targeted *CRZI/SPI*-like gene deletion on survival was observed already after 24 h, with 18% of dead cells under moderately limited aeration and 58% of dead cells under severely limited aeration (2× or 7× more compared to 8% of dead cells in well aerated control, respectively). This effect was almost completely reversed in reconstituted strain (Fig. 1). To verify the role of hypoxia in this effect, serial dilutions of cells were also incubated on YPG plates under 1% oxygen atmosphere in Anoxomat, resulting in reduced growth of the targeted deletion strain compared to both wild type and reconstitution strain after 2 weeks of incubation (Fig. 2). Furthermore, a decrease in G2 fraction in response to moderately limited aeration was observed as a result of the targeted deletion, in contrast to an increase in G2 fraction at the same conditions observed in wild type, indicating an essential role of intact

Fig. 1. Death rate under variable aeration.

Death rate after 24 h of cultivation in liquid shaken culture at 30°C under variable aeration – well aerated culture in 100 μ L volume (normoxic conditions), moderately limited aeration in 500 μ L (mild hypoxic conditions) and culture severely limited in aeration in 1000 μ L (profound hypoxic conditions); WT – wild-type strain, del = CNAG_00156.2 (*CRZI/SPI*-like) gene targeted deletion strain, rev = reconstituted strain.

Fig. 2. Viability under reduced oxygen tension.

Aliquots of 10-fold serial dilutions were plated onto YPG plates supplemented as indicated and cultured at 30 or 37 °C in a 1% O₂, 10% CO₂, 9.4% H₂, and 79.3% N₂ atmosphere for 2 weeks. WT - wild-type strain, del = CNAG_00156.2 (*CRZ1*/*SP1*-like) gene targeted deletion strain, rev = reconstituted strain.

Fig. 3. Cell cycle analysis based on proliferation and DNA content assays.

Number of cells was monitored using Multisizer 3 Coulter Counter and DNA content was monitored in PicoGreen stained cells using a Cytomics FC 500 flow cytometer after 24 h of growth in well aerated (100 μL) and moderately aerated (500 μL) shaken liquid culture at 30 °C. WT - wild-type, del = CNAG_00156.2 (*CRZ1*/*SP1*-like) gene targeted deletion strain, rev = reconstituted strain.

Fig. 4. Effect of cell integrity disturbing agents and calcium ions.

Aliquots of 10-fold serial dilutions were plated onto YPG plates supplemented as indicated and cultured at 30 or 37 °C for 3, 6, or 8 days, depending on the particular plate. All spot assays were repeated at least three times, and representative experiments are shown. WT – wild-type, del = CNAG_00156.2 (*CRZ1/SPI*-like) gene targeted deletion strain, rev = reconstituted strain.

CRZ1/SPI-like gene in the hypoxia-induced G2 arrest in *C. neoformans* (Fig. 3). Accordingly, the cell number was reduced significantly under moderately reduced aeration compared to normoxic conditions in the wild type strain and reconstituted strain ($P=0.05$), but not in the targeted deletion strain (Fig. 3). Because of the high death rate, conclusive interpretation of the effect of targeted *CRZ1/SPI*-like gene deletion on proliferation under severely reduced aeration was not possible. It was also impossible to evaluate the DNA content under severely reduced aeration, because of inconsistent staining and dead cells background. Further, reversion of the decreased G2 content under moderately reduced aeration was lacking in the reconstituted strain, most likely because the delicate

cell cycle machinery was still somehow disturbed after reconstitution.

Cn *CRZ1/SPI*-like gene is required for cell wall damage repair and for biofilm formation

Whereas no differences in growth were observed at elevated temperature (37 °C) on YPG plates, increased susceptibility of the targeted *CRZ1/SPI*-like gene deletion strain to cell wall damaging agents Congo Red, Rylux BSU (a Calcofluor White related compound, see ref.²³ for details), and SDS were observed, both at 30 and 37 °C, as already described²¹ (Fig. 4). Increased susceptibility of the targeted deletion strain to calcium ions, but not to LiCl (150 mM) or NaCl (1.5 M), was not apparent

Fig. 5. Biofilm assay in polystyrene microtiter plates. Cells were incubated in polystyrene microtiter plates at 30 °C for 2 days. Then, biofilm formation was detected by crystal violet staining after washing out nonadherent cells. Stained biofilm was quantified by measuring absorbance at 570 nm. WT – wild-type, del = CNAG_00156.2 (*CRZ1/SPI*-like) gene targeted deletion strain, rev = reconstituted strain.

at 30 °C, but was unveiled at 37 °C (Fig. 4). In addition to the role in cell wall damage repair, we also observed significantly reduced ability of the targeted deletion strain to form biofilm compared to wild type and reconstituted strain ($P=0.05$, Fig. 5).

Cn *CRZ1/SPI*-like gene deletion results in stronger capsule induction and increased resistance to fluconazole

Whereas no apparent difference in melanin formation was observed on CAFC medium, significantly stronger induction of capsule formation was observed in the targeted *CRZ1/SPI*-like gene deletion strain compared to wild type and reconstituted strain ($P=0.01$) when grown on Rice extract agar under 5% CO₂ at 37 °C for 3 days. At the same time, no significant differences in cell diameter were observed (Fig. 6). Surprisingly, the targeted deletion strain also showed increased resistance to fluconazole (6 µg/mL) compared to wild type (1.5 µg/mL) and reconstituted strain (3 µg/mL) when minimum inhibitory concentration of fluconazole was determined using E-test strips.

DISCUSSION

We have shown that the cryptococcal homologue of the *CRZ1/SPI*-like gene is required for slowdown of cryptococcal proliferation under reduced aeration, and its deletion increases susceptibility to cell wall stress and calcium stress. It remains unclear whether one or both of these defects cause the reduced fitness of the targeted deletion strain under hypoxic conditions. We believe in combined participation, because slowdown is beneficial

Fig. 6. Capsule induction assay on Rice extract agar. Cells were grown on nutrient-poor Rice extract agar at 37 °C under 5% CO₂ atmosphere for 3 days and capsules were visualized by India ink staining. Capsule thickness and cell diameter were measured on representative photographs. WT – wild-type, del = CNAG_00156.2 (*CRZ1/SPI*-like) gene targeted deletion strain, rev = reconstituted strain.

to reduce oxygen consumption and thus to keep it in balance with reduced oxygen availability, while maintenance of cell integrity is energy demanding. Therefore, any cell integrity challenge, either by intrinsically impaired cell wall synthesis or by extrinsic damaging agents, should increase oxygen demand. Although unchallenged cells of the targeted deletion strain did not show any apparent phenotype, their colonies appeared glossy-shiny (data not shown), as already described²¹, indicating somehow impaired cell wall or capsule formation. In addition, the increased induction of capsule formation observed in the targeted deletion strain also indicates possible activation of compensatory mechanisms because of some latent cell wall damage caused by the *CRZ1/SPI*-like gene deletion. Furthermore, the reduced ability of biofilm formation observed by us can be related to impaired cell wall construction as well, although the decreased ability to adapt to hypoxic conditions inside of biofilm body can also play its role. The latter link is further supported by the substantial overlap between the hypoxic and biofilm transcriptomes found in *C. parapsilosis* and *C. albicans*^{24,25} and other findings that demonstrate adaptation to hypoxia to be an integral component of biofilm formation in *C. albicans*²⁶. We also have already suggested a possible link between biofilm-like adaptation to hypoxic environment in human tissue and the dormant state of cryptococcal infection earlier¹⁰.

Surprisingly, we observed increased resistance to fluconazole as a result of targeted deletion of the *CRZ1/SPI*-like gene. We can only speculate about the underlying mechanisms. In a recent study, *C. albicans* cells with increased chitin level that was induced by activation of the Ca^{2+} /calcineurin and *PKC1*-pathways were demonstrated to be less susceptible to caspofungin²⁷. Therefore, it is rather conceivable that a latent cell wall damage resulting from the *CRZ1/SPI*-like gene deletion activates compensatory response that results in increased resistance to fluconazole.

CONCLUSIONS

Taken together, our data clearly indicate that the cryptococcal homologue of the *CRZ1/SPI*-like gene plays important role in survival under limited aeration, resistance to cell wall damaging agents, susceptibility to fluconazole, and biofilm formation, thus linking hypoxic adaptation, cell integrity and biofilm production in the pathogenic yeast *C. neoformans*. However, the exact positioning of the protein in cryptococcal signalling cascades is still puzzling. It also remains to be clarified what its primary role in cellular processes is, i.e. whether proper *CRZ1/SPI*-regulated cell wall synthesis is the key to hypoxic adaptation, or rather an adequate *CRZ1/SPI*-mediated slowdown of proliferation is a prerequisite to successful survival under hypoxia, including proper cell wall construction. In our opinion, slowed proliferation should enable the cells to keep their oxygen consumption in balance with its availability and thus also helps them to keep their integrity under hypoxic conditions.

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CONFLICT OF INTEREST STATEMENT

The authors state that there are no conflicts of interest regarding the publication of this article.

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