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MYCOLOGY (M NOVERR, SECTION EDITOR)

The *Cryptococcus neoformans* Titan Cell: From In Vivo Phenomenon to In Vitro Model

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Abstract

Purpose of Review During infection, the human fungal pathogen *Cryptococcus neoformans* undergoes an unusual change in size, from small haploid yeast to large polyploid Titan cells. This transition is now well recognized as a virulence factor, but significant questions remain about how Titanisation is regulated and how it influences disease progression. Progress has been impeded by the lack of an in vitro model for the yeast-to-Titan transition, a challenge that was recently overcome by three independent groups.

Recent Findings Here, we review Titanization in the context of patient samples and animal models and set the stage for three new reports describing in vitro Titan cell induction assays. We compare and contrast key findings, place them in the broader research context, and identify areas of further interest.

Summary New in vitro models will allow pressing questions about molecular mechanisms driving the yeast-to-Titan transition and their influence on drug resistance and pathogenesis to be addressed.

Keywords *Cryptococcus* · Titan cell · Fungal morphogenesis · Antifungal resistance · Fungal pathogen

Introduction

Fungal infections are an underappreciated threat to global health, causing an estimated 1.5 million deaths each year [1]. Infection with *Cryptococcus* species remains a leading cause of morbidity and mortality among both immunocompromised and immunocompetent individuals [1, 2]. Disease occurs when desiccated yeast or spores are inhaled, proliferate in the lung, and disseminate to the central nervous system (CNS), causing life-threatening meningitis [3, 4]. A key determinate of disease progression is the *Cryptococcus* Titan cell, a striking fungal morphotype induced in the lung by host-relevant factors [5, 6••, 7••, 8, 9] (Fig. 1). This review addresses common questions surrounding this topic and highlights recent developments in our understanding of the cryptococcal yeast-to-Titan transition that reveal underlying mechanisms of pathogenesis, drug resistance, and immune evasion.

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Morphological transitions by fungi are hallmarks of pathogenicity that enable growth in the host microenvironment, mediate tissue damage, and influence immune evasion and immune cell recruitment [10–12]. Following inhalation of spores into the lung, or upon in vitro induction, members of the *Cryptococcus* species complex (*C. neoformans*, *C. deneoformans*, *C. gattii*) undergo a dramatic change in cell size from small (5–7 μm) yeast to large (> 10–100 μm) Titan cells, evading phagocytosis [6••, 7••, 8, 9, 13] (Fig. 1a, b). During this transition, yeasts grow isotropically and undergo repeated rounds of endo-reduplication to form large, highly polyploid Titan cells with distinct morphology and cell cycle (Fig. 1c). These large cells divide their DNA asymmetrically and produce disproportionately small haploid, diploid, or aneuploid daughter cells [6••, 14•] (Fig. 1d). The result is an overall population that is heterogeneous for both ploidy and size, and this heterogeneity has important implications for drug resistance and immune evasion [14•].

Despite their important roles in pathogenesis, Titan cells were unrecognized as a feature of cryptococcosis until 2010 and could not be reliably generated in vitro until 2018 [6••, 7••, 15, 16••, 17]. Our lab, together with two other groups, has recently demonstrated that the yeast-to-Titan transition can be reproducibly triggered by growth in minimal medium and exposure to an inducing condition: serum containing bacterial

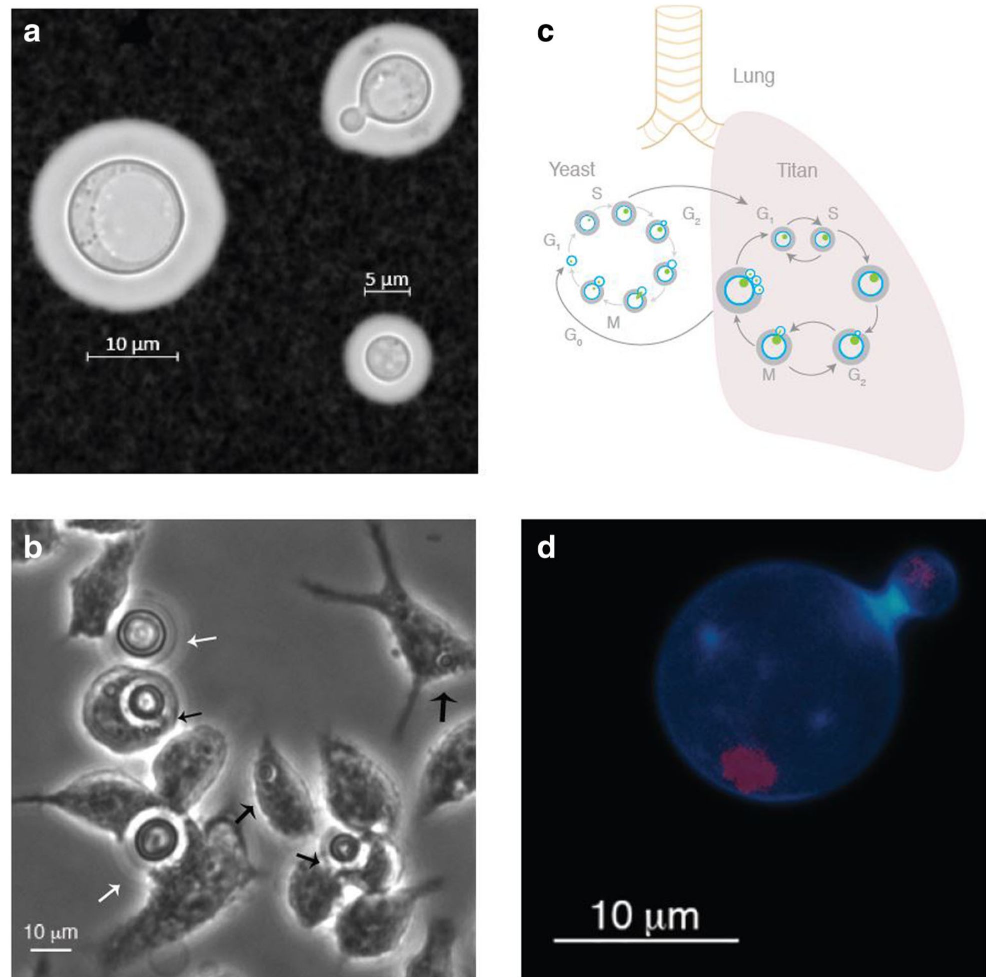

Fig. 1 *Cryptococcus neoformans* grows as a heterogeneous population of yeast and Titan cells. **a** In vitro-induced yeast and Titan cells counterstained with India ink to reveal capsule. **b** In vitro-induced yeast (black arrows) and Titan (white arrows) cells co-cultured with J77.4 macrophage-like cells. **c** A schematic showing yeast- and Titan-phase cell cycles in the environment and in the host lung. **d** A budding Titan mother with asymmetric DNA division (blue = calcofluor white, chitin; red = Cse4-mCherry, chromosomes)

cell wall, serum supplemented with sodium azide, or continuous mixing under hypoxia (Fig. 2) [6••, 7••, 16••]. The identification of in vitro inducing conditions, coupled with previous in vivo and in vitro work identifying regulatory signaling pathways, demonstrates that the yeast-to-Titan transition is a regulated developmental switch analogous to the yeast-to-hyphal transition of dimorphic fungi [6••, 18–21]. However, significant questions remain about the basic cell biology of this morphotype, and how it influences host-pathogen interactions.

How Frequently Are Titan Cells Observed in Patient Isolates?

Clinical reports of Titan cells can be found in the literature as early as 1973, when large encapsulated yeasts were isolated from the lung of a young African woman [22]. In 1985, Love et al. again reported unusually large, dimorphic cells in isolates directly from the brain of a young African man [23]. In each case, Titans were observed only immediately after isolation from the patient: large cells converted back to producing a typical homogenous yeast population upon in vitro culture on Sabouraud or brain heart infusion agar. When mice were infected with these strains, but not a laboratory control, atypical large cells were again observed [22, 23]. Morphologically, these large cells were reported to produce a distinct capsule and cell wall structure compared to their yeast-phase counterparts, consistent with formal descriptions of Titan cells [8, 9, 22]. However, these early isolates were dismissed as atypical until 2010 [8, 24].

Perhaps contributing to this underappreciated role, *C. neoformans* Titan cells have not been reported in patient cerebral spinal fluid (CSF), classically one of the most important sites for diagnosis of cryptococcosis. A 1985 report of serial CSF isolates from a patient with lupus notes that cryptococcal cells directly observed from the CSF were heterogeneous in cell size, and classified these cells as having either distinct or unstructured organelles following TEM [25]. While this is consistent with TEM of in vitro and in vivo Titan cells [6••, 9], the few available documented cells from these CSF isolates fail to reach the > 10-µm threshold [25]. A subsequent 2014 analysis of serial CSF samples from 134 patients likewise failed to identify cells > 10 µm

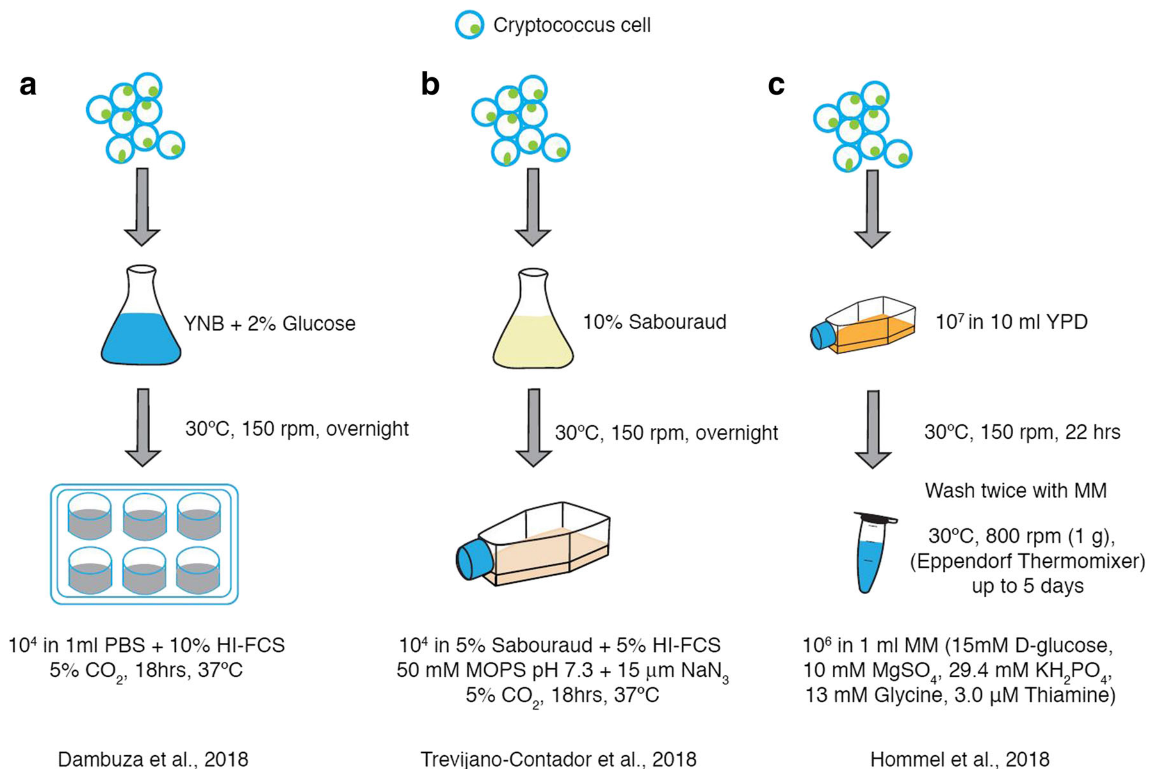

Fig. 2 Schematic showing three new in vitro Titan cell induction protocols. **a** Induction of Titan cells through pre-growth in minimal medium followed by exposure to 10% serum. **b** Induction of Titan-like cells through pre-growth in minimal medium followed by exposure to serum + sodium azide. **c** Induction of Titan cells through continuous shaking under hypoxia. Relevant references for each method are indicated

[26]. However, these authors did observe a subset of patients with a significantly larger overall median cell body size of 8.2 µm and reported cells > 30 µm including capsule. This highlights a key challenge when identifying Titan cells in patient samples: Current definitions use arbitrary size cutoffs and differ on whether to include capsule diameter, rather than relying on a complete analysis of key morphological features [12, 15, 27]. It remains to be seen whether cryptococcal cells in the CSF truly do not form Titan cells or whether they undergo the yeast-to-Titan transition in a limited way but do not cross the > 10-µm threshold. One intriguing data point to consider is that cells exposed to continuous mixing (800 rpm/~ 1 g) in minimal medium and hypoxia form Titan-like cells at a high rate in vitro (Fig. 2c) [7••]. This may be analogous to continuous mixing by CSF flow that cells are likely to be exposed to in the CNS, a nutritionally limited, hypoxic environment [28–30].

How Do We Define a Titan Cell?

As mentioned above, in vivo-derived Titan cells exhibit clear morphological and ploidy alterations relative to yeast [8, 18]. In vivo, Titans can reach up to 100 µm, while typical yeast cells are around 5 µm [8]. However, several authors have established conflicting definitions of the minimum size threshold, relying on arbitrary size cutoffs and differing on whether to include capsule diameter [12, 15, 27]. In addition to cell size, Titans exhibit increased cell wall thickness and maintain a characteristic single, large, intracellular vacuole that occupies the majority of the intracellular space [9]. Increased cell size is associated with increased DNA content: in vivo Titan cells are highly polyploid—often tetraploid or octoploid, but reaching as high as 64C—and they can produce diploid or aneuploid daughters with normal cell size [8, 9, 14•]. In addition, Titan cells exhibit changes in important virulence factors: they have highly compacted polysaccharide capsule and altered pathogen-associated molecular pattern (PAMP) exposure, including altered chitin and chitosan content in the cell wall [9, 31, 32]. Titan cells are also are less frequently phagocytosed and are more resistant to lung phagocyte killing and nitrosative and oxidative stress [8].

In vitro induction protocols have established a more parsimonious definition for Titans with four key criteria: (1) cell body size > 10 µm; (2) cell ploidy > 2C; (3) the presence of a single, large vacuole; and (4) altered cell wall and capsule [6••, 7••]. However, these criteria may require further refinement. For example, despite inclusion of the capsule in the Titan cell definition, capsule itself is not required for Titanization [6••]. In addition, definitions based on increased mother ploidy (> 2C) and cell size (> 10 µm) should also include asymmetric division of DNA and the production of disproportionately

small daughter cells. Large "Titan-like" cells (> 10 μm) divide DNA symmetrically and produce proportionate daughters, similar to yeast-phase budding [6••, 7••]. In some cases, these buds fail to fully divide, accumulating defects in cytokinesis [6••]. Finally, these definitions have relied on work with the *C. neoformans* H99 isolate, which may skew understanding of underlying diversity across the species complex [7••]. Future work should more fully define the yeast-to-Titan switch across clinical and environmental isolates. Despite these limitations, we will rely on these four criteria throughout this work.

How Do Titan Cells Influence Disease Outcome?

Typically, cryptococcosis patients are immunocompromised and present either with cryptococcal meningitis, diagnosed by visualization of yeast in CSF, or are *Cryptococcus* antigen (CrAg) positive upon diagnosis of HIV or during routine monitoring upon immune suppression [2, 33]. Cryptococcal meningitis arising from infection with *C. neoformans* accounts for 15% of HIV-related deaths [17, 34]. This represents an unacceptably high mortality rate: among HIV patients with low $CD4^+$ counts, only approximately 6% are positive for cryptococcal antigen. Overall, an estimated 278,000 cases of cryptococcal meningitis occur annually in HIV-positive patients, with 65% mortality [34]. While HIV status is the most frequent predisposing factor, cryptococcosis is also associated with solid organ transplant, particularly in patients receiving T cell depletion therapy or calcineurin inhibitors [33]. Among solid organ transplant patients, the overall incidence of cryptococcosis is 5%, and mortality attributed to cryptococcosis in transplant patients is 14% [33, 35]. Importantly, up to 20% of *C. neoformans*-infected patients have no identifiable underlying disease and this trend is consistent worldwide [36–38].

Cryptococcus infection begins in the lung, but cryptococcal pneumonia is often neglected in estimates of morbidity and mortality. In non-HIV/non-transplant (NHNT) patients, as well as in cyclosporin-treated transplant patients, there is an increased incidence of cryptococcal pneumonia in the absence of disseminated infection [27, 35, 37, 39]. While CNS involvement is predictive of poor outcome, pulmonary infection can also be fatal: Cryptococcal pneumonia was diagnosed upon autopsy in 7% of a cohort of patients who succumbed to respiratory disease and can be the cause of rapid respiratory failure upon introduction of antifungal therapy in patients with HIV [40, 41]. Finally, infection with the emerging pathogen *Cryptococcus gattii* is characterized by pulmonary cryptococcoma [42]. Taken together, there is growing evidence that cryptococcal pneumonia is an under-recognized challenge that is both a precursor to cryptococcal meningitis and a predictor of morbidity and mortality in its own right [43].

How might Titan cells contribute to these different disease processes? Dissemination from the lung occurs either via transcytosis or paracellular crossing of the lung epithelium or via phagocytosis and Trojan Horse-type dissemination (recently reviewed by [4]). In vivo and in vitro observations suggest that the morphological transition from yeast to Titan cell may contribute to pulmonary vs. disseminated disease [5, 6••]. In murine models of infection, Titan cells are associated with escape from the lung, crossing of the blood-brain barrier, and a non-protective immune response [5, 27, 32, 44]. Mice infected with low-Titanizing clinical isolates or mutants with Titanization defects exhibit differences in disease progression [5, 6••]. In these mice, dissemination to the brain is delayed, if not entirely prevented. However, it is not simply that Titans enable dissemination: Infection with cells that are hyper-Titanizing (*otc1Δ* or *usv101Δ*) prolongs the pulmonary phase and delays dissemination [5, 6••, 45].

How can these two observations be reconciled? First, Titans themselves are phagocytosed at low rates due to their large size (Fig. 1b) [8]. Thus, strains that produce more Titan cells are predicted to be less phagocytosed and disseminate more slowly (*otc1Δ*, *usv101Δ*). Moreover, daughters of Titan cells can themselves Titanize, further renewing the Titan population [6••]. In the case of the transcription factor *USV101*, a negative regulator of the yeast-to-Titan switch, we speculate that the progressive increase in the proportion of Titan cells relative to small cells generated by the *usv101Δ* mutant contributes to reduced dissemination to the brain [6••]. In addition, the presence of Titans reduces uptake of small cells through an unknown mechanism [9, 44]. However, under in vivo or in vitro Titan-inducing conditions, a heterogeneous population of daughter cells ranging from 2 to 7 μm is also observed [6••, 7••, 8, 9, 16••, 19]. These small cells simultaneously arise from normal yeast-phase budding and through asymmetric Titan budding and represent the majority of the infecting population [6••]. Additionally, Titan cells produce daughters at a more rapid rate (~ 60 min/bud) than yeast-phase cells [7••, 9]. Together, this increases the proportion of small daughter cells available to be phagocytosed and disseminated relative to a Titan-deficient strain. This may partially explain the association of Titans with dissemination, as both hypo-Titanizing and hyper-Titanizing mutants that exhibit reduced dissemination also show reduce lung CFUs compared to their wild-type parents [5, 45]. Perhaps the most significant finding is that, similar to Titans themselves, Titan daughters are more resistant to stress than their yeast-phase sisters, likely due to their altered ploidy [14•]. It remains to be seen whether Titan daughters undergo transcytosis or paracellular crossing at altered rates relative to yeast daughters, but it is clear that they are more resistant to killing by phagocytes and more likely to be drug resistant, thereby mediating pathogenesis.

Given the above differences in disease presentation in different populations, it is tempting to speculate that Titanizing

strains are more likely to be observed in clinical compared to environmental isolates. However, when we examined the Titanization capacity of environmental isolates and clinical isolates from an HIV patient cohort in Zambia, we found no association with clinical or environmental origin or clade [6••, 46]. Likewise, the capacity to form Titans has been observed in all three major species (*C. neoformans*, *C. deneoformans*, *C. gattii*) [7••]. This points to the yeast-to-Titan transition being a conserved and widely occurring mechanism to facilitate adaptation to and evasion of environmental stress [6••].

How Are Titan Cells Induced?

One of the biggest challenges impeding efforts to understand the contribution of Titan cells to disease has been the lack of an in vitro model for their induction. Thanks to the work of three major collaborations spanning the field of *Cryptococcus* research, we now have three robust induction protocols that identify three independent inducing signals: microbial, host-derived, and physical (Fig. 2) [6••, 7••, 16••]. Continuing the tradition established by the original Titan cell papers, these three methods were published back to back to back in a single journal. Although there are differences in inducing signal between the various methods, there are also common themes, including cell density, nutrient availability, and oxygen limitation.

First, cell density has a clear and reproducible impact on Titanization across in vivo and in vitro models. Mice infected with a low multiplicity of infection (MOI) (10^4/ml) or presenting with asymptomatic infection exhibit a higher rate of enlarged cells [8, 9]. This was recapitulated in all three reports of in vitro Titan and Titan-like cell induction, where increased cell density reduces Titanization frequency and cell size [6••, 7••, 16••]. Interestingly, there appears to be a lower limit to the MOI effect, at least in the physical induction protocol (Fig. 2c): the frequency of cells > 10 μm is reduced at lower MOI (37% at 10^6 vs. 10% at 10^4) when induced by continuous mixing under hypoxia [16••]. However, induction frequency and size increase progressively at MOI < 10^6 in the presence of serum alone or serum + sodium azide in minimal medium at 37 °C in 5% CO_2 (Fig. 2a, b) [6••, 16••].

One possible explanation for this is that density-dependent expression of secreted factors inhibits the yeast-to-Titan switch. *Cryptococcus neoformans* Qsp1 is a density-dependent secreted protein involved in protease expression and cell wall remodeling [47]. Loss of *QSP1* results in an increase in the size and frequency of Titan cells during hypoxic growth with continuous mixing [16••]. We likewise observed an increase in the frequency of Titan cells produced by the *qsp1Δ* mutant in our assay (unpublished results). However, while Qsp1 activity partially explains suppression of Titanization at high density, deletion of *qsp1Δ* does not cause constitutive Titanization in the absence of an inducing signal [16••].

Another density-dependent signal, pantothenic acid (PA), may positively regulate Titanization, with the frequency of Titan cells increasing upon exposure to physiologically relevant sub-micromolar concentrations [16••]. However, exposure to micromolar concentrations inhibits Titanization. PA was originally identified as a factor in conditioned medium from stationary cultures that was able to increase growth rate in a density-dependent manner [48]. It remains to be seen whether the impact of PA on growth rate is sufficient to explain its impact on Titan frequency, or how PA interacts with Qsp1.

Second, low density alone is not sufficient to prime cells for Titanization: nutrient availability both before and during induction influences outcome (Fig. 2). When cells grown in rich medium (YPD) were used as the inoculating culture at low density (5×10^6 or 1×10^4/ml), true Titan cells were not observed, even when cells were washed thoroughly in PBS to remove any secreted signal [6••]. Rather, large cells (> 10 μm) that produced proportionally sized daughters and accumulated cytokinesis defects could be observed at 10^4/ml. Although these cells exceed the 10-μm cutoff, we do not consider them to be true Titan cells because of these defects. In contrast, growth in minimal medium (YNB + 2% glucose) followed by low-density inoculation into 10% serum was sufficient to induce true Titan cells (Fig. 2a) [6••]. The impact of nutrients on induction was also reported by Hommell et al. and Trevijano-Contador et al., who note that pre-growth in either YPD or Sabouraud broth likewise inhibits induction of Titan-like cells [7••, 16••]. Interestingly, cells inoculated into a diluted minimal medium (5% Sabouraud buffered with MOPS and 5% FBS + 15 μM sodium azide) during induction produce proportionally sized daughter cells and also exhibit significant cytokinesis defects [7••]. Growth in Sabouraud has also been reported to inhibit the production of robust capsule, suggesting that nutrient availability is profoundly linked to the repression of virulence factor expression in this fungus [49].

Third, consistent with a role for nutrients and hypoxia in inducing Titanization, the cAMP/PKA pathway that mediates both of these signals is central to each of the in vitro protocols and is required for Titanization in vivo [6••, 7••, 16••, 18]. Elegant work from Choi et al. showed that perturbation of *PKA1*/*PKR1* regulation is sufficient to generate spontaneous Titans in vitro [19]. Patient isolates with *PKR1* truncations can be hyper-Titanizing in some instances, although the effect was dependent on genetic context [16••]. Despite this, activation of the cAMP/PKA pathway alone is not sufficient: *pkr1Δ* mutants do not constitutively form Titans, and the addition of exogenous dcAMP does not induce spontaneous Titanization [6••, 19].

How Does the cAMP Pathway Regulate Titanization?

The cAMP/PKA pathway is a central regulator of *C. neoformans* pathogenesis: cAMP/PKA regulates melanin

production and capsule structure, and mutants deficient in this pathway are avirulent [50–52]. cAMP-deficient mutants are rapidly cleared from the lung, complicating efforts to study its role in virulence. Despite this, the influence of cAMP/PKA on Titanization was first described in vivo, building from the initial observation that Titan cell production increases upon coinfection with cells of opposite mating type [8]. Later, it was reported that the ste3α pheromone receptor and the G protein-coupled receptor Gpr5 are required for Titanization [18]. Both receptors interact with Gα protein Gpa1, which signals via the adenylyl cyclase Cac1 to generate cyclic AMP and relieve Pkr1-mediated negative regulation of Pka1 [50, 53]. Gpr5, Cac1, and Pka1 are required for in vitro Titan induction, and targets of this pathway, including the Rim101 and Gat201 transcription factors, also regulate Titanization [6••, 16••, 18].

Previous work has demonstrated that CnCac1 adenylyl cyclase activity is stimulated by bicarbonate and the β-carbonic anhydrase Can2, which is essential for growth in ambient CO_2, but no activity has been previously attributed to *CAN1* [54]. Consistent with cAMP/PKA regulation, loss of *CAN1* increases the frequency of Titan-like cells [7••]. Although the specific mechanism underlying the *can1Δ* phenotype remains uncharacterized, oxygen tension influences Titanization in all three in vitro systems (Fig. 2) [6••, 7••, 16••].

This may speak to the intersection of cell cycle events and the yeast-to-Titan switch. It is well established that O_2 limitation causes *C. neoformans* to decouple DNA synthesis and bud emergence [55, 56]. During hypoxic growth in rich medium, cells arrest in G2, post synthesis but prior to budding. Release into fresh rich medium triggers synchronized bud emergence for a single cell cycle, although this synchrony quickly collapses. In contrast, it appears that hypoxic growth in minimal medium prompts G1-S cycling in the absence of bud emergence (Fig. 1c), leading to the accumulation of a high percentage of Titan mothers [7••]. Subsequent release into rich or minimal medium enables bud emergence. Consistent with this, loss of the cell cycle-responsive transcription factor *USV101* increases the frequency at which cells make the yeast-to-Titan switch [6••, 45]. We have recently reviewed progress on the interaction of the cell cycle with *C. neoformans* virulence and suggest that Titanization is similarly regulated at the level of the cell cycle [57]. A mechanistic understanding of how the above conditions influence these events is clearly needed.

Finally, accumulating evidence suggests a role for the mitochondrion in regulating fungal pathogenesis in general and the yeast-to-Titan switch in particular [58]. Perhaps most significant are the observations that perturbation of the mitochondrion, through the addition of the complex IV inhibitor sodium azide or through iron limitation, significantly increases the frequency of Titan-like cells [7••]. It is intriguing that the mitochondrion also influences capsule growth, another cAMP/PKA, and iron-regulated phenotype [59]. Moreover, transcription factors that influence capsule production (*USV101*, *RIM101*, *GAT201*) also influence Titanization [6••, 7••, 18, 45, 60]. Both *RIM101* and *GAT201* are negatively regulated by Usv101 and are targets of the cAMP/PKA pathway [45, 61]. In addition, the SAGA complex protein Ada2, which is a target of both cAMP and Gat201, is also required for both capsule and Titanization [7••, 60]. While the specific mechanisms by which these signals are integrated with mitochondrial function remain to be explored, these experiments are now within reach due to the development of in vitro Titanization assays.

How Do Host Dynamics Contribute to Titanization?

It is clear that the lung is particularly suited to Titan induction [6••, 8, 9]. From a microbial perspective, the lung is likely to be a hypoxic environment, particularly within fungal lesions [62]. However, host-relevant factors have also been shown to influence Titanization.

Host IgM levels have been implicated in in vivo Titan induction in the context of B cell inactivation [63]. T cell function has also been implicated: T_H2-tilted C57BL/6J mice exhibit increased Titanization compared to T_H1-tilted CD1 mice, and T_H2 responses are associated with poor outcome [27, 32, 64]. Although the mechanisms driving fungal morphogenesis in these models remain unexplored, increased Titanization was associated with significant accumulation of the T_H2-type cytokine IL4, increased eosinophilia, more abundant capsule IgM antibodies, and increased total IgE in sera [27].

Finally, the lung microbiome may influence Titanization: we have established that the bacterial cell wall component peptidoglycan is a key active component of serum sufficient for Titan cell induction [6••]. Healthy and diseased lungs are colonized by a robust bacterial microbiome [65, 66]. Upon inhalation of infectious spores or yeast, *C. neoformans* may sense this complex microbial environment and respond through a morphological transition from yeast to Titan form [6••]. The interaction of these microbial and host factors with fungal morphogenesis and drug resistance will have implications for disease progression and treatment.

How Do Titan Cells Contribute to Disease Progression?

Regardless of inducing condition, the yeast-to-Titan transition occurs within the first 24 h of exposure. In mouse models of infection, Titan cells can be observed in the lung 1 day post-infection and comprise up to 20% of fungal cells recovered through bronchio-alveolar lavage on day 3 [8]. When induced

by mixing, or exposure to host-relevant ligands, Titan cells can be observed within the first 24 h, and overall size increases through continued culture.

Infection directly with Titan cells leads to increased lung fungal burdens early in infection [5].

In vivo, the overall median cell size decreases from 30 to 10 μm over the course of infection [67]. A similar phenomenon is observed during in vitro induction, with the relative proportion of small cells increasing more rapidly than the number of Titan cells over time [6••, 7••]. In addition to changes in cell size, Titans exhibited altered cell wall: chitin levels increase, while chitosan and other PAMP levels decrease, and this promotes a non-protective T_H2 response [31, 32].

Up to 20% of patients may suffer from mixed infections with multiple serotypes, and there is evidence of the emergence of stable diploid serotype D or AD hybrid strains from patient isolates [17]. Sexual reproduction, through either bilateral or same-sex mating via the production of mono- or dikaryotic hyphae and the development of sporulating basidia, has never been observed in vivo for *C. neoformans* [68]. However, as mentioned above, Titan frequency increases during coinfection with strains of opposite mating type [8]. Both hybridization and Titanization lead to fundamental changes in cell ploidy that are likely to have significant implications for drug resistance. For example, 60% of patients will experience fluconazole resistance over the course of treatment [69]. Fluconazole resistance has been linked to the emergence of aneuploidy in chromosome 1, mediating increased gene dosage of the lanosterol 14α-demethylase *ERG11* and drug exporter *AFR1* [24]. While it is possible that exposure to fluconazole directly induces aneuploidy, as has been observed in vitro, the impact of Titanization on drug resistance in patients has not been investigated [70•]. However, it is clear that Titans exposed to fluconazole in vitro exhibit an increased frequency of aneuploidy and fluconazole resistance [14]. In addition, in vitro-induced Titan mothers produce aneuploid and diploid daughters at a high frequency even in the absence of chemical stress [6••]. Therefore, Titanization presents multiple possible mechanisms towards aneuploidy and drug resistance.

Concluding Remarks

With the development of robust in vitro induction protocols, the yeast-to-Titan transition has moved from an unusual host-specific virulence factor to a highly regulated morphological transition with profound implications for health and disease. Although many unanswered questions remain, we are now poised to investigate the basic biology of these cells to understand the molecular mechanisms underlying cryptococcal morphogenesis, drug resistance, and pathogenesis.

Compliance With Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- •• Of major importance

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