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Marcio L. Rodrigues *Editor*

Fungal Physiology and Immunopathogenesis

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Marcio L. Rodrigues
Editor

Fungal Physiology and Immunopathogenesis

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Preface

Fungal diseases have been neglected for decades, which has negatively impacted the expansion of Medical Mycology and the rate of knowledge generation in this field. However, an unacceptably high number of deaths due to fungal infections (1.5 million people every year) has stimulated an appreciable expansion of Medical Mycology. Regrettably, the unquestionable progress achieved during the last two decades is still insufficient to place fungal infections at the level of knowledge generation and innovation that is observed for other infectious diseases. For instance, there are no licensed antifungal vaccines. Treatment of fungal diseases is unaffordable for millions of patients living under socio-economical restrictions. When available, antifungal treatment is expensive and associated with many undesirable side effects. The most recent antifungal drug introduced into clinics is now 18 years old. We are now facing the unexpected emergence of multidrug-resistant fungal pathogens and the alternatives to fighting this problem are very limited. This complex scenario reveals our unpreparedness to deal with clinical conditions deriving from fungal infections. These problems are likely a consequence of reduced funding for fungal research, which is much lower than that available for diseases of similar impact to human health. In summary, even with the expansion of Mycology and great scientific contributions in the last decades, it is clear that the field of fungal diseases demands more research and consequently accelerated the generation of knowledge and innovation.

The great scientific advances resulting in more sophisticated and comprehensive methods for the analysis of biological questions related to human health has positively impacted Medical Mycology research. Unquestionably, the methodological advances developed in Genetics, Immunology, Systems Biology, and Cell Biology have improved our understanding on how fungal pathogens interact with the host, resulting in the generation of damage to host tissues or the control of fungal infections. In this context, this volume efficiently illustrates the progress of

knowledge generation in Medical Mycology. We invite the reader to visit the recent findings showing how fungal cells dynamically respond to different stimuli to cause damage to host cells or to adapt to different microenvironments related to disease progress or control.

Curitiba, Brazil Marcio L. Rodrigues

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The Role of Melanin in Fungal Pathogenesis for Animal Hosts

Daniel F. Q. Smith and Arturo Casadevall

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Abstract Melanins are a class of pigments that are ubiquitous throughout biology. They play incredibly diverse and important roles ranging from radiation protection to immune defense, camouflage, and virulence. Fungi have evolved to use melanin to be able to persist in the environment and within organisms. Fungal melanins are often located within the cell well and are able to neutralize reactive oxygen species and other radicals, defend against UV radiation, bind and sequester non-specific peptides and compounds, and produce a physical barrier that defends the cell. For this reason, melanized fungi are often well-suited to be human pathogens—melanin allows fungi to neutralize the microbicidal oxidative bursts of our innate immune system, bind and inactivate to antimicrobial peptides and enzymes, sequester antifungal pharmaceuticals, and create a shield to block immune recognition of the fungus. Due to the importance and pervasiveness of melanin in fungal virulence,

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mammalian immune systems have evolved antifungal strategies that involve directly detecting and binding to fungal melanins. Such strategies include the use of melanin-specific antibody responses and C-type lectins like the newly discovered melanin-specific MelLec receptor.

1 Diversity of Fungal Melanins

Melanins are found in all biological kingdoms where they perform fundamental roles in the survival of organisms. In the microbial world, melanin pigments have been associated with numerous functions including energy harvesting and survival in stressful environments (Casadevall et al. 2017; Gessler et al. 2014). Broadly speaking, melanins are a class of black, brown, red, and yellow pigments that have similar characteristics such as: resistance to acid hydrolysis, amorphous structures, form through a catecholic, phenolic, or indolic precursor, insolubility in water, negative charges have broad monotonic absorbance spectra, and a stable free radical structure. Despite these similar characteristics, melanins are a highly heterogenous lot, with regards to structure, composition, and functional use.

There are several different classes of melanins, each produced through different biosynthetic pathways including: black–brown eumelanin produced by catecholamine oxidation and reactive quinone intermediates, and the polymerization of subsequently produced indoles, red–yellow pheomelanin produced by the reaction of oxidized catecholamines with cysteine-containing amino acids, light-brown pyomelanin produced through tyrosine catabolism and polymerization of homogentisic acid, and black–brown DHN melanin produced from acetyl-CoA and the polyketide synthesis pathway. The predominant melanins found in the fungal kingdom are DOPA melanins and DHN melanins. In addition to these common fungal melanins, there is evidence that some fungi produce pyomelanins, resulting from the breakdown of aromatic amino acids, particularly tyrosine. A partial list of melanized fungi, the type of melanin(s) they produce, and the enzymatic pathway(s) are summarized in Table 1.

In this essay, we focus on the role of melanin on fungal pathogenesis in animals. We note that melanin also has important in fungal pathogenesis for plants, particularly in fungal invasion and penetration into the plant through turgor pressure buildup. Interested readers may consult other reviews and papers such as (Chen et al. 2004; Howard and Valent 1996; Martin-Urdiroz et al. 2016; Ryder and Talbot 2015). Further, we note that there have been previous reviews on the role of melanin on fungal pathogenesis (Chowdhary et al. 2014; Gómez and Nosanchuk 2003; Jacobson 2000; Nosanchuk and Casadevall 2003; Revankar and Sutton 2010; Seyedmousavi et al. 2014) which provide additional perspectives.

Table 1 Melanin and melanin biosynthesis of selected fungi

Species	Fungal form	Melanin type	Enzymes and pathways	References
Aspergillus flavus	Conidia	DOPA	Tyrosinase	Inamdar et al. (2014), Pal et al. (2014)
Aspergillus fumigatus	Conidia	DOPA, DHN, Pyo	Laccase, PKS (*alb1, arp1, arp2*), TDP (*hhpD, hmgA*)	Schmaler-Ripcke et al. (2009), Tsai et al. (1999), Youngchim et al. (2004)
Aspergillus nidulans	Conidia	DOPA	Tyrosinase, Laccase	Bull (1970a, b), Bull and Carter (1973), Gonçalves et al. (2012), Kurtz and Champe (1982)
Aspergillus niger	Conidia	DOPA, DHN	Unknown, PKS (*albA, aygA*)	Chiang et al. (2011), Pal et al. (2014)
Candida albicans	Yeast	DOPA	Unknown/ Laccase	Morris-Jones et al. (2005)
Cryptococcus neoformans	Yeast	DOPA, Pyo	Laccase (*CNLAC1, CNLAC2*)	Frases et al. (2007), Shaw and Kapica (1972), Williamson (1994)
Cryptococcus gattii	Yeast	DOPA	Laccase	Chan and Tay (2010)
Exophiala (Wangiella) dermatitidis	Yeast Hyphae	DOPA, DHN DHN	Laccase; PKS (*WdPKS1*) PKS (*WdPKS1*)	Dixon et al. (1992), Geis, et al. (1984), Feng et al. (2001), Paolo et al. (2006), Wheeler et al. (2008)
Fonsecaea pedrosoi	Conidia Hyphae	DHN DHN	PKS PKS	Cunha et al. (2005)
Histoplasma capsulatum	Yeast Condia	DOPA, Pyo DOPA, DHN	Laccase, TDP PKS? Laccase?	Almeida-Paes et al. (2018), Nosanchuk et al. (2002)
Lomentospora prolificans	Conidia	DHN	PKS (*PKS1, 4HNR, SCD1*)	Al-Laaeiby et al. (2016)
Paracoccidioides brasiliensis	Yeast Conidia	DOPA DHN?	Laccase PKS?	Gómez et al. (2001)
Sporothrix schenckii	Yeast Conidia Hyphae	DOPA, Pyo DHN, DOPA, Pyo DOPA, Pyo	Laccase, TDP PKS, Laccase, TDP Laccase, TDP	Almeida-Paes et al. (2009), Almeida-Paes et al. (2012), Morris-Jones et al. (2003), Teixeira et al. (2010)
Talaromyces (Penicillium) marneffei	Yeast Conidia/ Hyphae	DOPA, DHN, Pyo DHN	Laccase, PKS (*alb1, arp1, arp2*), TDP (*hpdA, hmgR*) PKS (*alb1, arp1, arp2*)	Boyce et al. (2015), Kaewmalakul et al. (2014), Liu et al. (2014), Youngchim et al. (2005), Woo et al. (2010)

Abbreviations DOPA melanin (DOPA), DHN Melanin (DHN), Pyomelanin (Pyo), Polyketide Synthase Pathway (PKS), Tyrosine Degradation Pathway (TDP)

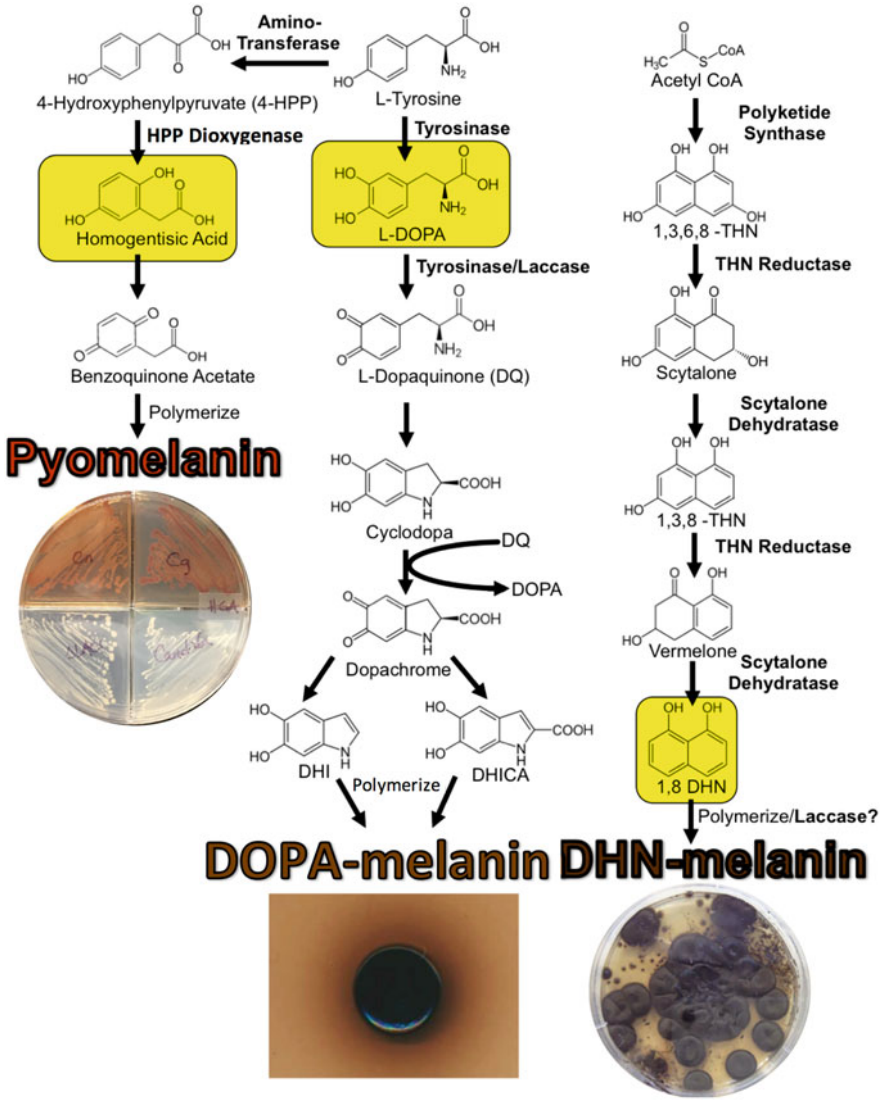
1.1 Characteristics and Synthesis of DOPA-Melanin

DOPA melanin is a highly common black–brown form of melanin that is typically produced from the hydroxylation of tyrosine, and the oxidation of catecholamines, such as L-DOPA (3,4-dihydroxyphenylalanine) and dopamine, into quinones (García-Borrón and Sánchez 2011). The quinones are highly reactive and spontaneously cyclize, undergo further oxidation, and inevitably form indoles such as dihydroxyindole (DHI) and dihydroxyindole carboxylic acid (DHICA). DHI and DHICA polymerize into eumelanin, forming amorphous, hydrophobic, black and brown polymers (Fig. 1). The Raper-Mason pathway of melanin biosynthesis best typifies this process, uncovered in the first half of the twentieth century (Mason 1948; Raper 1927).

While the oxidation of catecholamines, resulting in inevitable formation of dark pigments, can occur spontaneously at room temperature and following light exposure, organisms typically use enzymes to catalyze and control this reaction. Two classes of enzymes, laccases (EC 1.10.3.2) and phenol oxidases (EC. 1.14.18.1 and EC. 1.10.3.1) are typically responsible for the formation of eumelanin.

Laccases are multi-copper metalloenzymes that contain four copper ions in their catalytic core. These enzymes have broad classes of substrates, which perform single electron oxidations of hydroxyl groups, mostly attached to aromatic groups (Jones and Solomon 2015; Riva 2006; Yaropolov et al. 1994). In the environment, organisms such as fungi use laccases to oxidize dead material and decompose it; these enzymes are responsible for delignification and decomposition of wood and lacquer formation (Christopher et al. 2014). In the context of melanin production, laccases oxidize the hydroxyl groups on catechols and catecholamines to create unstable phenoxyl radicals. The radicals are unstable and rapidly cause the oxygen to form a double bond with the aromatic ring, forming a quinone. As previously mentioned, the quinone is highly reactive and subject to continued cyclization, oxidation, and polymerization without the need for further enzymatic catalysis. Laccases are important enzymes for melanin production in many fungi, bacteria, and insects. Notably, many disease-causing fungi use laccases to produce melanin,

Fig. 1 Biosynthetic Pathways of Fungal Melanin Production. In fungi, melanin is produced predominately through three pathways: Pyomelanin through the polymerization of homogentisic acid (HGA) produced through the Tyrosine Degradation Pathway, DOPA-melanin through the polymerization of dihydroxyindole (DHI) and dihydroxyindole carboxylic acid (DHICA) produced through tyrosine and L-DOPA oxidation, and the polymerization of 1,8-dihydroxynaphthalene (DHN) produced through the polyketide synthase pathway. "Namesake" molecules are highlighted in yellow. Enzymes responsible or aiding in melanogenesis are bolded. Photograph examples of pyomelanin, courtesy of Dr. Emma Camacho are *Cryptococcus neoformans* (top left) and *C. gattii* (top right) cultures treated with homogentisic acid. Non-pyomelanized controls are seen in the laccase mutant *C. neoformans* (bottom left) and *C. albicans* (bottom right). DOPA-melanin example is H99 strain of *C. neoformans* grown in L-DOPA minimal media. DHN-melanin example is a culture of *Fonsecaea pedrosoi* from (Adibelli et al. 2016) ▶



such as the genus of human pathogenic yeast, *Cryptococcus* spp. (Williamson 1994; Zhu and Williamson 2004). Interestingly, *Cryptococcus* spp. cannot produce melanin from tyrosine and cannot produce their own melanin precursors. They thus require exogeneous catecholamines for laccase substrates (Nurudeen and Ahearn 1979). The fact that *C. neoformans* requires exogenous precursors for the synthesis of melanins means that it is possible to control melanin composition through the substrate provided and this has been exploited to explore the biochemistry of melanin (Chatterjee et al. 2018).

Another class of melanin-producing enzymes is phenol oxidase. Phenol oxidase is a broad umbrella term describing metalloenzymes with two copper ions in the catalytic core, a generally conserved active site, and is able to oxidize phenols and catechols into quinones (Casella et al. 1996; Gerdemann et al. 2002; Monzani et al. 1998; Réglier et al. 1990). Phenol oxidases are divided into two sub-classes of enzymes: tyrosinases and catechol oxidases. Tyrosinases have two distinct enzymatic roles, whereas catechol oxidases only have one of these enzymatic functions. First, tyrosinases have a monooxygenase/monophenolase role, in which a monophenol such as tyrosine is hydroxylated to form an -*o*-diphenol, typically a catechol such as L-DOPA. The second enzymatic activity of the tyrosinase (and the only enzymatic activity of the catechol oxidases) is a diphenolase activity (Ramsden and Riley 2014). This reaction oxidizes catechols such as DOPA into *o*-quinones such as dopaquinone. Similar to the laccase-mediated reaction, the quinone formed from tyrosinase activity is highly reactive and prone to rapid and spontaneous cyclization, oxidation, and polymerization into eumelanin as per the canonical Raper-Mason pathway. Some organisms have additional enzymes to assist in the progression of melanin production post-tyrosinase. These enzymes such as dopachrome tautomerase (Dct) and tyrosinase-related protein 1 (Trp1) help catalyze the process of melanin formation. Dct catalyzes the conversion of the melanin intermediate dopachrome into DHICA, resulting in increased formation of DHICA-based eumelanin compared to the normally more abundant DHI-based eumelanin (Tsukamoto et al. 1992). In mice but not humans, Trp1 oxidizes DHICA (Boissy et al. 1998). Fungi, protozoa, insects, mammals, and birds commonly have tyrosinases, while plants typically have catechol oxidases, sometimes called polyphenol oxidases.

1.2 Characteristics and Synthesis of DHN Melanin

DHN melanin is common among many fungal species including the conidia spores of many filamentous fungi including some *Aspergillus* spp. and *Lomentaspora prolificans* (Al-Laaeiby et al. 2016; Langfelder et al. 1998; Pal et al. 2014; Tsai et al. 1999). DHN melanin arises from the polyketide (pentaketide) synthesis pathway (Fig. 1). Typically, five molecules of the precursor acetyl-CoA or malonyl-CoA are joined together by polyketide synthase enzymes (EC 2.3.1.233) to form a two-ringed molecule called 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) (Austin et al. 2004; Izumikawa et al. 2003; Takano et al. 1995). Subsequently, the molecules undergo two enzymatic reduction and two enzymatic dehydration steps. 1,3,6,8-THN is reduced through a THN reductase (EC 1.1.1.252) into scytalone (Thompson et al. 2000; Wang and Breuil 2002). The scytalone is dehydrated by a scytalone dehydratase (EC 4.2.1.94) to form 1,3,8-THN (Kubo et al. 1983, 1996), which is further reduced by THN reductase into vermelone (Wang and Breuil 2002). This vermelone can be enzymatically dehydrated into 1,8-DHN through scytalone dehydratase (Basarab et al. 1999). The 1,8-DHN is then oxidized to form

polymers of DHN melanin. Some species have laccases that are able to catalyze the polymerization of 1,8-DHN (Sapmak et al. 2015; Sugareva et al. 2006).

The process of DHN melanin formation differs from DOPA melanin production is several ways. One such way is the heavy reliance on enzymes to catalyze the reactions and polymerizations. DOPA melanin synthesis is typically dependent upon one enzyme and predominately occurs through spontaneous and continuous oxidation-reduction cycling. DHN melanin uses an enzyme to essentially synthesize each intermediate. If activity of one of these enzymes is blocked, the intermediate accumulates or spontaneously oxidizes to form different metabolites or pigments (Lee et al. 2003), which is a feature that also makes DHN melanin synthesis able to be inhibited at several different synthesis pathway steps (Wheeler and Klich 1995). An additional difference is that DOPA melanin may at times require exogenous addition of the melanin precursor, as is the case in *C. neoformans* melanization, whereas DHN melanin is produced predominately through abundant metabolites in the cell such as acetyl-CoA and could occur in non-supplemented conditions. Like all melanins, DHN melanin has a dark black color, is acid resistant, and has a stable radical structure.

Many filamentous fungi, such as *Aspergillus* spp. complex produce non-melanin green, yellow, and brown pigments, through polyketide synthesis pathways similar to the pathway that produces DHN melanin, while perhaps not producing DHN melanin at all, like *A. nidulans* (Mayorga and Timberlake 1992). The synthesis of these additional pigments may use the same enzymes, similar precursors, and even produce intermediate products that overlap with the synthetic pathway of DHN melanin (Cary et al. 2014; Wheeler et al. 2008). For instance, *T. marneffei* uses the PKS pathway to produce several pigments and mycotoxins besides DHN melanin (Tam et al. 2015), and *Aspergillus* spp. that use PKS for a myriad of products as reviewed in (Bhetariya et al. 2011). The non-melanin pigments synthesized by PKS may result in the mistaken understanding that a fungus only known to produce DOPA melanin—such as *A. nidulans*—is actually a DHN-melaninized fungus. Some articles base the existence of DHN melanin merely on PKS genes present. This can lead to discrepancies in literature as to what type(s) of melanin(s) is present in a fungus. There are reports that *A. niger* produces DHN melanin through the PKS pathway, while other reports indicate that *A. niger* exclusively produces DOPA melanin (Chiang et al. 2011; Pal et al. 2014). That being said, we did our best in trying to sort out which melanins are produced by each fungus in Table 1, and we recognize that there could be future discrepancies as more biochemical and biophysical studies are done on the precise nature of the melanins.

1.3 Characteristics and Synthesis of Pyomelanin

Pyomelanin, also known as homogentisic acid (HGA) melanin, is a light-brown pigment that is produced by some bacteria including *Pseudomonas* spp. (Serre et al. 1999; Yabuuchi and Ohyama 1972), and fungi including *Aspergillus fumigatus,*

Histoplasma capsulatum, Sporothrix schenckii, and *Penicillium* (Almeida-Paes et al. 2012, 2018; Schmaler-Ripcke et al. 2009; Vasanthakumar et al. 2015). Similar to many DOPA melanins, pyomelanin may be derived from tyrosine, specifically the degradation pathway of tyrosine (Turick et al. 2010). Tyrosine is deanimated through an aminotransferase to form 4-hydroxyphenylpyruvate (4-HPP) (EC 2.6.1.5). 4-HPP is converted into homogentisic acid by a dioxygenase enzyme (EC 1.13.11.27) (Singh et al. 2018). Homogentisic acid spontaneously oxidizes into benzoquinone acetate (Schmaler-Ripcke et al. 2009), which polymerizes to form pyomelanin (Fig. 1). Alternatively, pyomelanin can be produced directly from the oxidation of HGA, as seen in *C. neoformans* which produces a pigment when it is grown with homogentisic acid. This is presumably pyomelanin produced via direct laccase oxidation (Frases et al. 2007). Pyomelanin is acid resistant, however, unlike DOPA melanin or DHN melanin, it is highly soluble in water. Pyomelanin was first described in *P. aeruginosa* as a water-soluble brown pigment (Yabuuchi and Ohyama 1972).

2 Melanin Localization, Structure, and Remodeling

Melanin and melanin intermediates appear toxic and may induce oxidative stress for cells through production of free radicals, a property of melanin that insects use to their benefit in their innate immune response. By encapsulating pathogens with melanin, insects are able to use the oxidative stress to kill the invader (Graham et al. 1978; Zhao et al. 2011). The cytotoxicity of melanin is one of the reasons that melanin localization tends to be highly organized and compartmentalized throughout kingdoms of life. In mammals, melanin and melanogenesis are confined to a membrane-bound organelle called a melanosome, which it is hypothesized to help to retain the toxic compounds and prevents oxidative stress (Denat et al. 2014). There is evidence that fungi also have membrane-bound melanosomes within their cytoplasm. Intracellular melanin-containing organelles have been reported in *Fonsecaea pedrosoi*, a mold responsible for chromoblastomycosis in humans (Franzen et al. 2008). On an ultrastructural level, these organelles are similar to mammalian melanosomes. Additionally, fungal melanosomes have recently been reported in *C. neoformans* (Camacho et al. 2019). The cryptococcal melanosomes described here are produced in the cytoplasm and exported to the cell wall inside of vesicles and multivesicular bodies (MVBs), where they form dense concentric shells of deposited melanin, and correlate to duration of cell melanization (Eisenman et al. 2005) (Fig. 2b).

This extracellular localization of melanin is common throughout the fungal kingdom, as many melanotic fungi deposit melanin in or around their cell walls, a reaction thought to be mediated by binding between melanin and the cell wall chitin and chitosan or another scaffolding molecule. In *C. neoformans,* mutants that lack functional chitin synthase 3 (*CHS3*) exhibit a "leaky" melanin phenotype (Walton et al. 2005). This phenotype is when melanin leaks from *C. neoformans* colonies, leaving the media pigmented and the colonies and cells white. *CHS3* is necessary

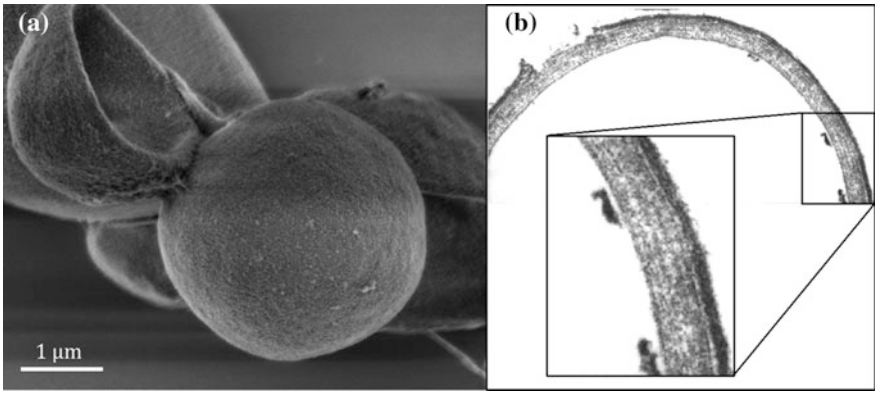


Fig. 2 Structure of Melanin in *Cryptococcus neoformans*. **a** An SEM image of a *C. neoformans* "melanin ghost" from a culture grown with L-DOPA. Image is courtesy of Dr. Emma Camacho. Scale bar indicates 1 µM. **b** A TEM of a melanin ghost from *C. neoformans* with an inset showing the concentric rings of melanin granules formed in the cryptococcal cell wall. Image courtesy of Dr. Helene Eisenman

for proper cell wall integrity, normal growth, chitin production, and chitosan production (Baker et al. 2007; Banks et al. 2005). The inability of *CHS3* mutants to retain their melanin is consistent with the notion that melanin localization and adherence to the cell wall is dependent upon properly formed chitin and chitosan cell wall structures. Similarly, dyes that bind to the cell wall also prevent melanin deposition, resulting in a phenotype similar to that of the leaky mutants, in which melanin is present in high levels in the media (Perez-Dulzaides et al. 2018). Melanin has been reported in the cell walls of *Candida albicans* cultures grown with L-DOPA, as well as during in vivo *Candida* infections (Morris-Jones et al. 2005). When the substrate for chitin synthesis, N-acetylglucosamine, is added to the media, melanin deposition in the cell wall increases (Walker et al. 2010). Conversely, when chitin synthase genes were knocked out, there were major changes in *C. albicans* melanin deposition in the cell wall and externalization (Walker et al. 2010). The *CSH2* mutant did not secrete melanin into the extracellular space, and melanin remained within the cell. Other chitin synthase mutants, such as the *CSH3* mutant, secreted "melanosomes" that remained embedded in the cell wall rather than fully secreted into extracellular space.

Externalization of melanin could provide an extracellular physical and chemical shield against stressors or factors within the host or environment. The external layers of filamentous fungal conidia cell walls are often melanized, such as *Aspergillus* spp., *L. prolifcans, F. pedrosoi.* This external localization of conidial melanin could help prevent physical, chemical, and biological degradation (Gessler et al. 2014), as well as prevent the desiccation of the spore and promote survival in harsh conditions (Tudor et al. 2012).

While the cell wall localization of fungal melanin is practical as per its function, it also provides a logistical issue for the fungi as it grows and divides. Melanized *C.*

neoformans must be able to remodel its thick layer of cell wall melanin before undergoing budding in order to allow mitosis and cytoplasmic contents to be conferred to the daughter cell. Melanin remodeling is not completely understood after decades of investigation. However, it is theorized to be a process mediated by reconfiguration of melanin granules in the cell wall, which have been described as small 50–80 nm (Eisenman et al. 2005) up to 200 nm (Camacho et al. 2019) spherical units of melanin associated with the cell wall. These larger melanin granules are in turn composed of smaller "fungal melanosomes," roughly 30 nm in diameter (Camacho et al. 2019). Through electron microscopy and atomic force microscopy, the "melanin ghost"—a melanin shell remaining intact after the rest of cell material is enzymatically and chemically decomposed (Fig. 2a)—of *C. neoformans* appears to be composed of concentric layers of these melanin granules (Fig. 2b, c) (Eisenman et al. 2005). These layers are presumably added as the cell ages, with the innermost portion of the cell wall melanizing first followed by the more external cell wall regions. Newly formed daughter buds have fewer of these concentric layers of melanin (Eisenman et al. 2005). The melanin granules are tightly associated but become dissociated with increased duration of acid hydrolysis, suggesting that they are separate units tethered together to form a "bead on a string" structure (Camacho et al. 2019; Eisenman et al. 2005). These beaded string structures have also been reported for melanin granules in the cell wall of *P. marneffei* (Liu et al. 2014). This provides a potential mechanism by which cryptococcal melanin is remodeled to allow for cell division and cell growth, where the individual melanin granules are dynamic and rearranged and restructured at will, akin to rearranging bricks in a wall to create a passageway.

In recent years, *C. neoformans* was found to secrete extracellular vesicles or exosomes. These are membrane-bound structures up to 100 nm in diameter that are somehow able to be secreted past the cell wall and capsule (Wolf et al. 2014). Although the capsule was thought to be a rigid structure that would not allow transport of such large structures as exosomes, recent evidence shows that its viscoelastic properties permit vesicular transit (Walker et al. 2018). These exosomes contain a wide variety of virulence factors such as urease and secreted polysaccharide (Rodrigues et al. 2008). For this reason, the cryptococcal extracellular vesicles are often termed "virulence bags". In addition to these other virulence factors, the exosomes contain laccase, the enzyme necessary for *Cryptococcus* to produce melanin. In some instances, the exosomes appear to contain melanin pigment (Rodrigues et al. 2008). Additionally, preparations of exosomes have laccase enzymatic activity (Rodrigues et al. 2008). This has implications for *Cryptococcus* pathogenesis, as these secreted vesicles are capable of making melanin and are theoretically able to disseminate throughout the host in concentrated packages. Throughout the body and in the area of infections, these exosomes can have impact on the host via the same properties of cell-bound melanin. Given that all microbes that have been investigated produce exosomes (Deatherage and Cookson 2012), the propensity for virulence factors to be packed inside exosomes (Schorey et al. 2015), and the common trend of melanin as a fungal virulence factor (Polak 1990), it stands to reason that melanin and melanin-producing enzymes may

be common in fungal exosomes. Additionally, it is possible that melanotic exosomes can play a significant role in microbial pathogenesis through interaction with the host, including eliciting an anti-melanin antibody response or acting as a sponge for microbicidal agents.

3 Roles of Melanin in Fungal Pathogenesis

Melanin is commonly found in many fungi, indicative of its important role in fungal survival. Many—if not all—human pathogenic fungi produce melanins. The melanization of fungal pathogens is diverse and includes DOPA melanins, DHN melanins, and pyomelanins. Fungal melanin characteristics and its relation to pathogenesis are summarized in Tables 1 and 2, respectively, which includes common human pathogens such as *Cryptococcus* spp., *Aspergillus* spp., *Candida albicans, Sporothrix schenckii,* and *Histoplasma capsulatum.*

There is often a direct association between degree of melanization and the virulence of fungal pathogens. For example, a mutant strain of *C. neoformans* with a deletion in the region of *CNLAC1*, the primary cryptococcal gene that encodes laccase, is less virulent in both mouse and *Galleria mellonella* infection models (Zhu and Williamson 2004). Further, when a melanin inhibitor is used to treat mice infected with a wild-type strain of *C. neoformans*, they have prolonged survival compared to the survival of control-treated groups. This is seen in the case of treatment of infected mice with glyphosate, a melanin inhibitor in fungi (Nosanchuk et al. 2001). Similarly, *E. dermatitidis* strains that did not produce melanin were less pathogenic than normally melanized strains (Dixon et al. 1992). Clinical isolates of *Sporothrix* spp. associated with higher melanin content were also found to be more virulent, with more dissemination to internal organs (Almeida-Paes et al. 2015).

3.1 Oxidative Stress Protection

One of the most prominent uses of melanin in biology is based on its role as an antioxidant and shield against free radicals from fungi to mammalian retina and skin (Brenner and Hearing 2008; Wang et al. 2006). While melanin and its intermediates are often toxic and highly oxidative due to the free radicals they produce, it is simultaneously well established to be a strong antioxidant with a stable free radical structure that is able to neutralize other free radicals (Jacobson and Tinnell 1993; Różanowska et al. 1999). Melanin is able to quench free radicals and reduce the oxidative stressors of the environment around it. This could be useful in the environment to help microbes manage oxidative stress (Gessler et al. 2007; Jacobson and Tinnell 1993).

Table 2 Roles of fungal melanins in pathogenesis

Species	Disease	Virulence	Oxidation protection	Antifungal resistance	AMP resistance	Enzyme inhibition	Antibody response	Effect on phagocytosis	References
Aspergillus fumigatus	Aspergillosis	✓	✓	✓	ND	ND	✓	+	Jahn et al. (1997), van de Sande et al. (2007), Volling et al. (2011), Youngchim et al. (2004)
Aspergillus nidulans	Aspergillosis	ND	✓	ND	ND	✓	ND	ND	Gonçalves and Pombeiro-Sponchiado (2005), Kuo and Alexander (1967)
Candida albicans	Candidiasis	✖	ND	ND	ND	ND	ND	ND	Walker et al. (2010)
Cryptococcus neoformans	Cryptococcosis	✓	✓	✓	✓	✓	✓	–	Doering et al. (1999), Duin et al. (2002), Nosanchuk et al. (1998), Rosas and Casadevall (2001), Wang et al. (1995)
Exophiala dermatitidis	Phaeohyphomycosis	✓	✓	✓	✓	✓	ND	None	Paolo et al. (2006), Schnitzler et al. (1999)
Fonsecaea pedrosoi	Chromoblastomycosis	✓	✓	ND	ND	ND	✓	+ or −	Alviano et al. (2004), Cunha et al. (2005), Cunha et al. (2010)
Histoplasma capsulatum	Histoplasmosis	ND	ND	✓	ND	ND	✓	ND	Duin et al. (2002), Nosanchuk et al. (2002)
Lomentospora prolificans	Lomentosporiosis	ND	✓	✖	ND	ND	ND	ND	Al-Laaeiby et al. (2016)
Paracoccidioides brasiliensis	Paracoccidioidomycosis	✓	✓	✓	ND	ND	✓	–	da Silva et al. (2006), Urán et al. (2011)

(continued)

Table 2 (continued)

Species	Disease	Virulence	Oxidation protection	Antifungal resistance	AMP resistance	Enzyme inhibition	Antibody response	Effect on phagocytosis	References
Sporothrix schenckii	Sporotrichosis	✓	✓	✓	ND	ND	✓	–	Almeida-Paes et al. (2016), Morris-Jones et al. (2003), Romero-Martinez et al. (2000)
Talaromyces marneffei	Penicilliosis	✓	✓	✓	ND	ND	✓	–	Liu et al. (2014), Kaewmalakul et al. (2014), Woo et al. (2010), Youngchim et al. (2005)

Key: Fungus' melanin is does (✓) or does not (✖) play role, *ND* indicate not determined to play role, + indicates increased phagocytosis, - indicates decreased phagocytosis.
Abbreviations Antimicrobial peptide (AMP)

This property of melanin is also useful within host tissues upon infection and encountering the oxidative defenses of the immune system. When a microbe is recognized by the host defenses, it is targeted by oxidative stress produced by the innate immune system. Macrophages, monocytes, and neutrophils release reactive oxygen species (ROS) including superoxide and peroxide ions, as well as nitric oxide (Babior 1978; Marcinkiewicz 1997; Nathan and Hibbs 1991). These chemicals are highly cytotoxic and are typically effective in killing foreign cells, however, melanin is capable of neutralizing the oxidative bombardment. As a result, many melanized fungal pathogens are resistant to oxidative/respiratory bursts produced by the innate immune system, including *C. neoformans* (Wang and Casadevall 1994) and *A. nidulans* (Ruiz-Díez and Martínez-Suárez 2003). The oxidative respiratory bombardment continues within the phagosome (Bedard and Krause 2007; Kotsias et al. 2012; Winterbourn et al. 2016). The antioxidant property of melanin, along with other virulence factors such as urease, may allow the melanized cell to survive once engulfed and residing within the phagosome.

While melanin production creates superoxide molecules (Komarov et al. 2005), formed melanins are able to quench and detoxify superoxide ions (Tada et al. 2010). This signifies that melanin has some redundant roles with superoxide dismutase (SOD), a common class of antioxidant enzymes and known virulence factors in *C. neoformans* and *C. albicans* (Hwang et al. 2002; Jacobson et al. 1994). Melanin and SOD can work together in microbial pathogens in order to evade host-produced oxidative bursts, as they may not be localized together—melanin is often extracellular and located in the cell wall of fungi, and SOD is often intracellular within in the mitochondria or cytosol (Broxton and Culotta 2016), although some species of fungi have SOD reported to presented on the cell surface or within extracellular vesicles to some degree (Gleason et al. 2014; Rodrigues et al. 2008; Youseff et al. 2012).

One interesting potential use of this redundancy is in instances of high-temperature environments. Under such conditions, the melanin is heat stable and continues to quench superoxide and oxygen radicals, while the SOD's enzymatic activity is diminished and unable to perform its protective functions at high temperatures. Conversely, in *C. neoformans,* levels of melanization are reported to be lower at cultures grown at higher temperatures, whereas levels of SOD are higher at 37 °C than at lower temperatures (Jacobson et al. 1994). This inverse relationship implies a possible compensatory mechanism. Similarly, but outside of the fungal kingdom, melanin and SOD have been reported to have complementary and compensatory roles in reducing oxidative stress in amphibian livers. In the liver of amphibians, there is an inverse relationship between melanin and SOD expression (Schiel et al. 1987). The lower the melanin content of the liver is, the more SOD is expressed. High melanin can quench a majority of the superoxide ions and cause the low SOD levels effect due to a feedback loop between superoxide concentration and SOD expression.

3.2 Antifungal Drug Sequestration

Melanin is able to sequester some antifungal drugs. The external localization of the melanin is important for this role in virulence; being located in the cell wall keeps antifungal compounds in extracellular space and thus prevents the drugs' cytotoxic effects. In *C. neoformans* and *H. capsulatum,* melanin is able to absorb amphotericin B, an antifungal commonly used in clinic, and caspofungin, the original drug from the commonly used echinocandin class of antifungals (van Duin et al. 2002). The presence of melanin and L-DOPA in the media allows the fungal cells to survive better following amphotericin treatment, and it was found that isolated melanin from *C. neoformans* was able to successfully bind amphotericin B in media. Following conditioning with melanin, this amphotericin-containing media showed an increased minimum inhibitory concentration (MIC) when used to grow *C. neoformans* cultures compared to the non-conditioned control media. This indicates the antifungal potency of the media was lost, likely due to absorption and binding of melanin to amphotericin B. Similar observations that melanin protects fungi from antifungals have been reported in *Paracoccidiodes brasiliensis*, where melanin did not effect the MIC of the drugs tested, but a smaller percentage of melanized cells were killed compared to the percentage of non-melanized cells killed by amphotericin B, ketoconazole, fluconazole, itraconazole, and sulfamethoxazole (da Silva et al. 2006). Media containing the antifungal azoles itraconazole and ketoconazole were conditioned with isolated melanin from *Madurella mycetomatis*. The melanin conditioning was found to increase the MIC (van de Sande et al. 2007). Using tricyclazole, a DHN melanin inhibitor, makes the fungi *S. brasiliensis* and *S. schenckii* more susceptible to killing by the antifungal drug terbinafine, whereas inhibition of eumelanin and supplementation with L-DOPA do not seem to affect susceptibility or MIC of terbinafine (Almeida-Paes et al. 2016). The DHN melanin-producing fungi *Alternaria infectoria* increased the production of melanin in response to certain antifungal drugs, notably, nikkomycin Z, caspofungin, and itraconazole treatment all increased melanin production (Fernandes et al. 2016).

Importantly, melanin does not bind all drugs, and not all fungal melanin contributes to drug resistance. It has been reported in *C. neoformans*, *H. capsulatum,* and *M. mycetomatis* and several other black fungi that their melanin does not confer protection from or bind to fluconazole (van Duin et al. 2002; van de Sande et al. 2007), which is a popular member of the azole class of antifungals. However, melanized *P. brasiliensis* is less susceptible to fluconazole as well as other azoles such as itraconazole, ketoconazole, and sulfamethoxazole (da Silva et al. 2006). Melanin-conditioned media using isolated melanin from *M. mycetomatis* did not show altered MIC for amphotericin B, suggesting that the *M. mycetomatis* melanin does not bind this drug as other fungal melanins do (van de Sande et al. 2007). Additionally, melanin has not been shown to play a role in the high amphotericin B resistance of *L. prolificans* (Al-Laaeiby et al. 2016; Ruiz-Díez and Martínez-Suárez 2003). In the more recent study, polyketide synthase (*PKS*) and scytalone

dehydratase (*SCD*), two genes necessary for the DHN melanin production pathway were knocked out, and the mutants were analyzed for susceptibility to antifungals. It was found that the albino mutants were not susceptible to amphotericin compared to the melanized wild type.

Knowledge of which drugs get absorbed by melanin is medically important, as it represents a way clinicians could treat melanized fungi without having to deal with the pharmacoevasive effect of pigment.

As of this writing, melanin's protective effect in live fungi and its in vitro mechanism of antifungal drug sequestration is unknown. It is possible that the melanin is directly binding these antifungal drugs and thereby sequestering them, which is supported by the apparent absorption of the drugs by isolated melanin ghosts. An additional possibility is that the melanin is interfering with the oxidative burst that these antifungals, namely amphotericin B, may induce in order to kill the fungi (Mesa-Arango et al. 2014).

3.3 Protein Binding

Melanins have been shown to bind peptides strongly. One indication of this is that isolated melanin ghosts from *C. neoformans* still have proteins associated with them via NMR analysis, and "ghosts" of melanin granules isolated from *C. neoformans* still have peptide fragments that are identifiable through mass spectrometry (Camacho et al. 2019). These peptides fragments are so tightly bound and protected by melanin that they remain in the melanin ghosts after extended acid hydrolysis, enzymatic treatment, and lipid extractions involved in the production of the melanin ghosts (Wang et al. 1996). This allows for interesting insight in understanding which proteins that peptides interact with melanin during fungal melanogenesis. Other studies indicate that melanin non-specifically binds proteins from both mammalian serum and fungal lysates (Doering et al. 1999). The protein binding was dependent upon pH—the more acidic conditions resulted in less protein binding to the melanin. This finding indicates that melanin–protein binding might be due to binds to electrostatic interactions. Melanin is a negatively charged pigment, so the protons present in acidic environments might neutralize the melanin and prevent the attraction and binding to positively charged proteins.

In the context of infection and pathogenesis, the peptide-binding role of melanin could be important. Melanins derived from *C. neoformans* were found to inactivate or absorb antimicrobial peptides produced by the host immune system, such as defensins, protegrins, and magainins (Doering et al. 1999). The media containing the antimicrobial peptides was less effective at preventing fungal growth when treated with melanin versus untreated. This represents a way in which melanin blocks the microbicidal actions of the host immune system.

Melanin was also reported as providing a line of defense against enzyme-dependent degradation and killing. There have been associations between melanization and resistance to chitinases and glucanases for decades, primarily in

Aspergillus nidulans (Bull 1970a; Kuo and Alexander 1967). Inhibition of these enzymes protects the fungi from lysis. This protection from enzymatic degradation has subsequently been reported in other fungi such as *C. neoformans* (Rosas and Casadevall 2001) and *E. dermatitidis* (Paolo et al. 2006). *C. neoformans* was shown to have increases resistance to a commercially available mixture of hydrolytic enzymes that decompose the cell wall, which included cellulase, proteinase, and chitinase. Protection from such enzymes prevents the destruction of the cell wall and the subsequent death of the cell.

Synthetic melanin was found to inhibit *Thermus icelandicus* and *Taq* DNA polymerase the context of a PCR experiment (Eckhart et al. 2000). The addition of melanin to the polymerase resulted in a higher molecular weight band when run on a non-denaturing polyacrylamide gel electrophoresis, indicating there is a binding occurring between melanin and the enzymes. The inhibition of the polymerases was dose-dependent and reversible when diluted with more PCR reaction buffer and when treated with bovine serum albumin (BSA) in a 1000-fold excess of polymerase.

3.4 Immune Detection

Melanin had historically been thought of being a molecule that is immunologically inert as many organisms produce it, and an immune reaction against melanin could result in auto-reactivity. However, the interaction between microbial melanins and the host's immune system is becoming better characterized.

3.4.1 Lectin Binding

A C-type Lectin Receptor in humans and mice, known as MelLec, binds DHN melanin produced from *Aspergillus fumigatus* conidia and other DHN-melanized fungal species (Stappers et al. 2018). C-type Lectin Receptors are common innate pattern recognition receptors (PRRs) that mediate antifungal immunity and bind to the carbohydrates present on the cell wall of fungal pathogens (Hardison and Brown 2012). MelLec also specifically binds DHN melanin and is unable to bind to DOPA-derived melanins or non-melanized fungi such as *Saccharomyces cerevisiae*. MelLec is expressed in murine and human CD31+ endothelium cells and human myeloid cell lineages, cell lines that are likely to interact with melanized fungi, for example, in the lung and digestive tract endothelium.

Another C-type Lectin, known as Pulmonary Surfactant Protein-D (SP-D), binds melanin in *Aspergillus fumigatus* (Wong et al. 2018). SP-D is a lectin that contains long stretches of collagen domains and is found in the lung. SP-D plays an important role in clearing bacterial and fungal infections (Kishore et al. 2006). The collagen domain directly binds melanin, whereas the lectin-binding domain binds the carbohydrate-rich fungal cell wall. The binding of SP-D opsonizes the fungi and

leads to its clearance by the immune system by phagocytosis, pro-inflammatory cytokine release, and cell-mediated immunity (Wong et al. 2018). *A. fumigatus* is able to produce DHN melanin (Pal et al. 2014), DOPA melanin (Youngchim et al. 2004), and pyomelanin (Schmaler-Ripcke et al. 2009). In this study, the subtype of melanin that bound SP-D was not clarified.

It would be of interest to investigate whether SP-D binds to DOPA melanins, as SP-D is an important molecule in the pathogenesis of *C. neoformans.* Opsonization of the yeast with SP-D enhances its likelihood of phagocytosis (Geunes-Boyer et al. 2012). Subsequently, the yeast survives and replicates within the phagosome by using melanin, urease, and other virulence factors to evade the antimicrobial defenses of oxidative stress and acidification (DeLeon-Rodriguez and Casadevall 2016). SP-D is protective for *C. neoformans* both in vivo and in vitro (Geunes-Boyer et al. 2012). SP-D mutant mice were less susceptible to *C. neoformans* infection, and the addition of exogenous SP-D to these knockout mice brought their susceptibly back to that of a normal mouse, resulting in accelerated infection of the lungs and dissemination to the central nervous system. SP-D also protects *C. neoformans* by acting as a direct shield to extracellular macrophage oxidative stressors (Geunes-Boyer et al. 2012). It would be interesting to examine if cryptococcal eumelanin enhances virulence through binding with the collagen domain of SP-D in addition to the binding of fungal carbohydrates, thus contributing to our multi-faceted understanding of melanin as a microbial virulence factor.

3.4.2 Cell Death and Inflammation

Melanin from *A. fumigatus* conidia inhibits the intrinsic apoptosis pathway upon phagocytosis by macrophages (Volling et al. 2011). The prevention of cell death programs allows for continued protection of the conidia within a living macrophage without further immune activation, which promotes longer survival and potential dissemination throughout the host. The anti-apoptotic effect appears due to PI3K/Akt activation; this signaling pathway is upstream of cell survival networks, regulates mitochondrial homeostasis, and also regulates transcription factors that may impact programmed cell death (Franke et al. 2003). Melanins from other *Aspergillus* species, *A. nidulans,* and *A. niger* also inhibit apoptosis. Compared to the degree that is seen in wild-type fungi, the apoptosis inhibition was limited in *Aspergillus* mutant strains that lacked polyketide synthase genes (Volling et al. 2011). It appears that the anti-apoptotic properties of melanin are not specific for the type of melanin present in the microbe. Both synthetic DOPA melanin and *Sepia officinalis*-derived DOPA melanin similarly inhibit intrinsic apoptosis pathways.

Additionally, melanin derived from *A. fumigatus* plays an anti-apoptotic role in lung epithelium, where it prevents activation of the extrinsic apoptosis pathway (Amin et al. 2014). Similarly, *A. fumigatus* conidial melanin ghosts, synthetic DOPA melanin, and *S. officinalis*-DOPA melanin inhibited TNF-activated extrinsic apoptosis, and such inhibition was not seen in mutants lacking polyketide synthase

genes. The extrinsic apoptotic pathway is typically activated by "death ligands" such as TNF, interacting with the "death receptor" such as TNF-receptor in response to microbial infection and tissue damage. Interestingly, high-melanizing isolates of *C. neoformans* inhibit TNF production in pulmonary tissue, reduce pulmonary inflammation, and consequently result in higher (but delayed) infection burdens and increased death (Huffnagle et al. 1995). Similarly, exposure of melanized *A. fumigatus* conidia to human peripheral blood mononuclear cells (PBMCs) resulted in dramatic decreases in pro-inflammatory cytokine secretion, including TNF and IL-6, compared to the non-melanized albino conidia (Chai et al. 2010).

3.4.3 Complement System Activation

Melanin could also help fungal pathogens evade immune destruction by preventing or masking complement deposition on the surface of the cell. Non-pigmented mutant strains of *A. fumigatus* that lack the *pksP, alb1,* or *arp1* genes required for DHN melanin production showed increased deposition of C3 on the surface of conidia compared to the melanized wild-type counterparts (Tsai et al. 1997). C3 is a critical part of the classical, alternative, and lectin complement pathways. Reduction in deposition of this molecule would stifle an important way in which the host recognizes, binds, and clears infections. While in these instances melanin decreases the ability of C3 deposition on a pathogen's surface, isolated melanin from *C. neoformans* and *A. niger* are able to directly bind to C3 and activate the complement system (Rosas et al. 2002). C3 binds to the surface of isolated melanin ghosts from *C. neoformans* after injection of the ghosts into the respiratory tract of BALB/c mice. While the molecule is able to bind isolated melanins, there was no difference reported for the deposition of C3 on melanized versus non-melanized *C. neoformans.* Similar results were seen in melanin ghosts isolated from *F. pedrosoi,* where the melanin elicited activation of the alternate complement pathway and deposition of C3 and C4 (Pinto et al. 2018).

3.4.4 Antibody Response

In addition to interactions between melanin and the innate immune system, melanin activates the adaptive immune response. Antibodies produced in response to melanin have been reported in *C. neoformans, F. pedrosoi, H. capsulatum, P. brasiliensis, S. schenckii, A. fumigatus,* and *T. marneffei* (Alviano et al. 2004; Morris-Jones et al. 2003; Nosanchuk et al. 1998, 2002; Urán et al. 2011; Youngchim et al. 2004, 2005). The antibodies are produced upon the injection of mice with cryptococcal melanin ghosts are mainly IgG and IgM antibody isotypes and are able to agglutinate the melanin ghosts in vitro (Nosanchuk et al. 1998). IgM specific to melanin is produced during *C. neoformans* infections after a week of infection (Nosanchuk et al. 1999). Evidence that these antibodies can play a role in host defense is provided by the finding that passive administration of

melanin-binding monoclonal antibodies prolonged survival in mice with lethal *C. neoformans* infection (Rosas et al. 2001). Consistent with this notion, antibodies from serum of chromoblastomycosis patients infected with the melanized fungi *F. pedrosoi* promoted phagocytosis, fungal killing, and fungal clearance (Alviano et al. 2004).

3.4.5 Phagocytosis

The role of melanin in phagocytosis is complex—in the case of *F. pedrosoi,* melanin elicited phagocytosis through antibody opsonization (Alviano et al. 2004), however, void of opsonins, the melanin of *F. pedrosoi* inhibits phagocytosis (Farbiarz et al. 1992). Melanin plays an anti-phagocytic role in several fungal infections. In *C. neoformans,* melanization reduced the amount of fungal cells phagocytosed by alveolar macrophages (Mednick et al. 2005). Melanized *C. neoformans* acapsular mutants showed increased phagocytosis versus non-melanized acapsular mutant yeast. The increased phagocytosis in melanized acapsular mutants suggests the capsule normally hides melanin from immune surveillance and recognition by phagocytes, such as by blocking opsonins like C3, antibodies, or C-type lectins. Similarly, melanization reduces the phagocytosis of *P. brasiliensis* yeast, *S. schenckii,* and *T. marneffei* (Liu et al. 2014; Romero-Martinez et al. 2000; da Silva et al. 2006).

Paradoxically, there are reports of correlation between clinical *Cryptococcus* isolates with high laccase activity and high rates of uptake by phagocytes (Sabiiti et al. 2014). In the study finding the correlation, there was no significant association between melanization and phagocytosis. It was suggested that the association of high laccase activity with more efficient phagocytosis could be unrelated to laccase's role in melanization and due to another function, possibly "iron scavenging or inflammation regulation" (Sabiiti et al. 2014).

4 Conclusions

Melanin is a powerful tool for all forms of life, including human pathogenic fungi. Melanized fungi are able to survive harsh environmental conditions including UV radiation, temperature, and radioactive waste. In a similar vein, melanin acts as an external shield and makes fungi better equipped to survive within humans and other host organisms (Fig. 3). Melanin helps microbes evade our immune system by countering microbiocidal peptides and neutralizing the oxidative stresses induced by reactive oxygen species. In some instances, melanin appears to block complement deposition, resulting in improved fungal survival, while in other cases, melanin binds and activates complement. The pigment also appears to commonly elicit an antibody response, producing IgM and IgG that are able to bind to melanin. Melanin also binds lectins, proteins with carbohydrate-binding domains, such as

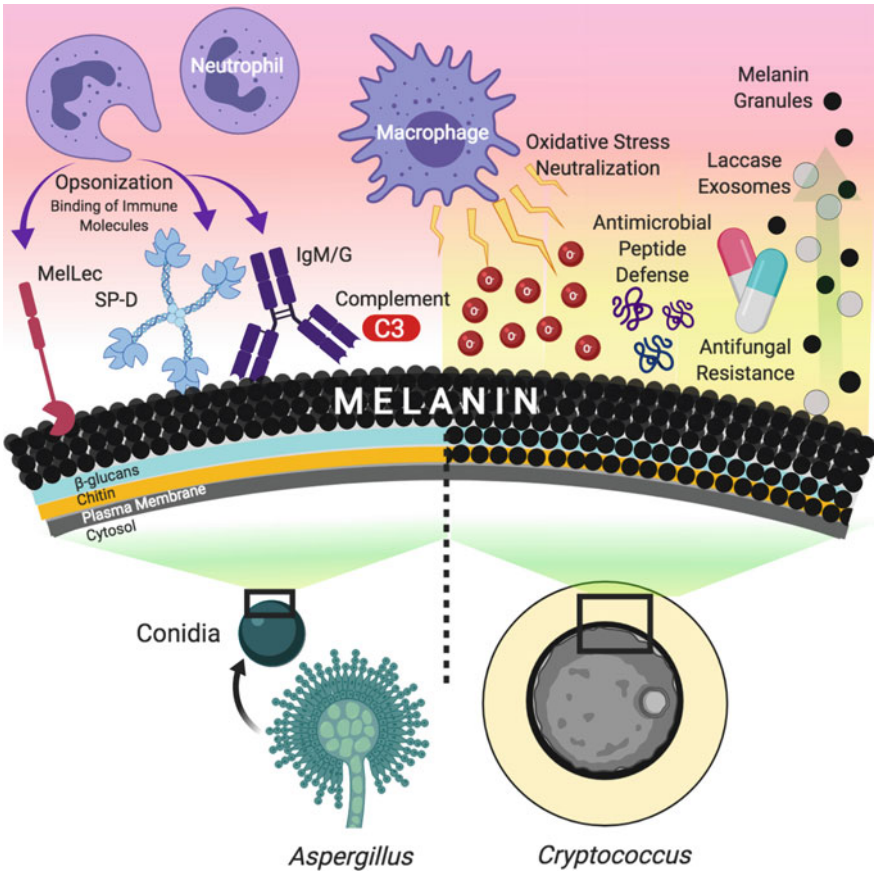


Fig. 3 Multifaceted roles of melanin in fungal pathogenesis. Melanin plays diverse roles in mediating infection during filamentous and yeast fungal infections, typified here by *Aspergillus* and *Cryptococcus,* respectively. In *Cryptococcus* spp., melanin begins depositing on the chitin/chitosan layer of the cell wall, and the entire cell wall melanizes overtime. Cryptococcal melanin is thought to be deposited in concentric layers of melanin granules, which are small vesicle-sized particles. Melanin granules may be secreted into the extracellular space, along with exosomes containing the melanin-producing enzyme laccase. The exosomes secreted by *C. neoformans* contain additional virulence factors and are thought to facilitate virulence during infection. In *Aspergillus* spp., the cell walls of conidia are melanized. The conidial melanin is deposited on the external layers of the cell wall above the glucan layers. Across melanized fungi, the pigment acts a shield for many antifungal agents within the host and extracellular environment. Melanin neutralizes reactive oxygen species released by macrophages and neutrophils. Melanin also sequesters some antifungal pharmaceuticals, antimicrobial peptides, and antifungal enzymes. In some cases, melanin also prevents the deposition and activation of the complement system, which helps prevent the fungi's clearance from the host, whereas, some melanins can promote complement deposition and activation, helping to clear the fungal infection. Melanin can elicit a melanin-specific antibody response during infection with *C. neoformans* and *F. pedrosoi,* which can be protective during infection. Similarly, C-type lectins can also bind melanin, opsonize the fungi, promote phagocytosis, and lead to clearance from the host. The collagen-like domain of the Surfactant-D lectin binds melanin. Additionally, the MelLec C-type lectin specifically binds DHN melanin, and helps elimination of DHN-producing fungi. Illustration created with BioRender software

MelLec and SP-D. The binding of lectins and antibodies result in opsonization and phagocytosis of the fungi. While these phagocytic immune defenses may spell disaster for some fungi, melanin can counter some of the reactive oxygen species present within the phagolysosome, and even further, it has been hypothesized that some fungi such as *Cryptococcus* have adapted to use phagocytosis to their advantage to disseminate infection throughout the body (Charlier et al. 2009; Santiago-Tirado et al. 2017; Sorrell et al. 2016).

Due to the importance of this pigment in effective pathogenesis, and evidence that interference with melanogenesis reduces microbial virulence, the fungal melanin system is a natural target for drug discovery. Although there are no therapeutics currently available that target melanin, this system is attractive for the development of future antifungal therapeutics. Such therapies could include drugs that inhibit tyrosinase and/or laccase, drugs that interfere with melanin deposition on the cell wall, drugs that impact melanogenesis pathway machinery, and possible vaccines and antibodies that recognize melanin and enable clearance via the immune system.

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The Overlooked Glycan Components of the *Cryptococcus* Capsule

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Abstract Pathogenic species of *Cryptococcus* kill approximately 200,000 people each year. The most important virulence mechanism of *C. neoformans* and *C. gattii*, the causative agents of human and animal cryptococcosis, is the ability to form a

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polysaccharide capsule. Acapsular mutants of *C. neoformans* are avirulent in mice models of infection, and extracellularly released capsular polysaccharides are deleterious to the immune system. The principal capsular component in the *Cryptococcus* genus is a complex mannan substituted with xylosyl and glucuronyl units, namely glucuronoxylomannan (GXM). The second most abundant component of the cryptococcal capsule is a galactan with multiple glucuronyl, xylosyl, and mannosyl substitutions, namely glucuronoxylomannogalactan (GXMGal). The literature about the structure and functions of these two polysaccharides is rich, and a number of comprehensive reviews on this topic are available. Here, we focus our discussion on the less explored glycan components associated with the cryptococcal capsule, including mannoproteins and chitin-derived molecules. These glycans were selected for discussion on the basis that i) they have been consistently detected not only in the cell wall but also within the cryptococcal capsular network and ii) they have functions that impact immunological and/or pathogenic mechanisms in the *Cryptococcus* genus. The reported functions of these molecules strongly indicate that the biological roles of the cryptococcal capsule go far beyond the well-known properties of GXM and GXMGal.

1 Introduction: The Cryptococcal Capsule

Capsular structures are common surface components of bacterial pathogens including *Streptococcus pneumoniae*, *Haemophilus influenzae,* and *Neisseria meningitidis* (Roberts 1996). *C. neoformans* and *C. gattii* are the only known eukaryotic pathogens that harbor capsules. The capsular network of *Cryptococcus* is the outermost layer surrounding the cell, concealing the cell wall and the plasma membrane (Fig. 1). The capsular barrier imposes additional difficulties for the design of new antifungal chemotherapies against cryptococcosis.

The capsule of *Cryptococcus* has been extensively studied, which is justified by its major role as a virulence factor (Bose et al. 2003; Zaragoza et al. 2009; Doering 2010; Wang et al. 2018). Structurally, the cryptococcal capsule is mainly composed by two polysaccharides. Glucuronoxylomannan (GXM) is the most abundant polysaccharide and consists of a chain of α1,3-linked mannose units with xylosyl and glucuronyl substitutions (Cherniak et al. 1998). GXM, which comprises around 90% of the total mass of the capsule, is also abundantly found in the extracellular milieu in the form of a heterodisperse, high molecular mass polysaccharide (1700–7000 kDa) (McFadden et al. 2006). The remainder 10% of the total mass of the capsule is mostly composed of glucuronoxylomannogalactan (GXMGal). GXMGal is formed by α1,6-linked galactose units with mannosyl, xylosyl, and glucuronyl substitutions (Heiss et al. 2009). The molecular mass of GXMGal corresponds to approximately 100 kDa (Zaragoza et al. 2009).

GXM and GXMGal are key players in physiological and immunopathogenic events in the *Cryptococcus* genus. Their biological roles, however, have been discussed in detail in several reviews (Doering 2010; Agustinho et al. 2018).

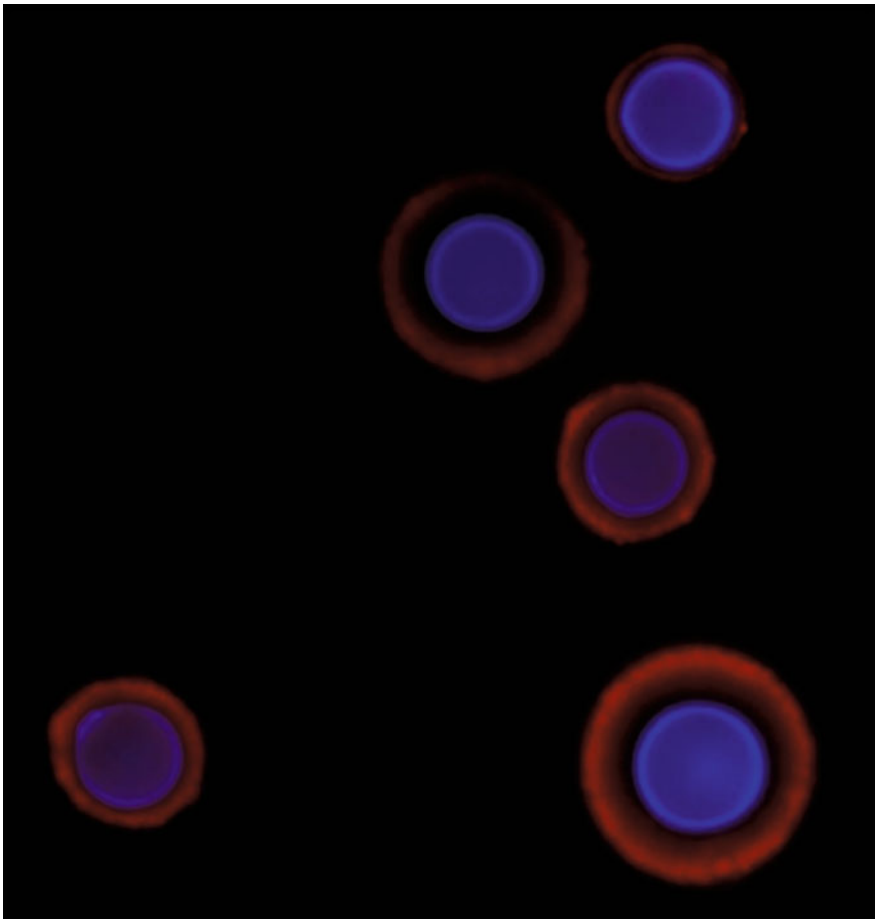

Fig. 1 Cell surface of *Cryptococcus*. Major surface components of *Cryptococcus* can be evidenced by fluorescence microscopy. Capsular structures are stained in red, while the cell wall is stained in blue. Capsule size is usually a variable within cryptococcal populations, which likely impacts pathogenic mechanisms (Fonseca et al. 2010; Albuquerque et al. 2014). Experimental details about cell surface staining of the cryptococcal surface are available in Rodrigues et al. (2008), Fonseca et al. (2009)

Other components associated with the capsule have been overlooked. Mannoproteins (MPs) are considered by a number of authors as the third glycan component of the capsule (Zaragoza et al. 2009), but the connections of these glycoproteins with the major capsular structures have not been established. Additional glycans have also been observed within the capsular network of *Cryptococcus*, including chitin-derived structures. Although these glycans are not covalently linked to the major capsule components (Ramos et al. 2012), they have been consistently detected in different layers of the cryptococcal capsule (Rodrigues et al. 2008, 2015; Fonseca et al. 2009,

2013). Both MPs and chitin-derived structures participate in the interaction of *Cryptococcus* with the host (Voelz and May 2010; Fonseca et al. 2013; Teixeira et al. 2014; Rodrigues et al. 2015). Here, we will discuss the roles of these two classes of glycans as components of the cryptococcal capsule.

2 Chitin and Capsular Architecture of *Cryptococcus*

Chitin metabolism in fungi involves a number of key structures, including its de-acetylated form (chitosan), the products of chitin hydrolysis (chitooligomers), and the enzymes involved in the balance between chitin synthesis (chitin synthases) and hydrolysis (chitinases). Each of them participates in the capsular architecture of *Cryptococcus*, as detailed in the following topics.

2.1 Chitin and Chitin Synthases

Chitin is a structural component of the fungal cell wall, accounting for approximately 2% of the wall mass (Camacho et al. 2017; Agustinho et al. 2018). This ancestral polysaccharide is a linear homopolymer composed of ß-1,4-linked units of *N*-acetylglucosamine (GlcNAc). Microfibrils of stacked chitin chains are stabilized by hydrogen bonds, assuring insolubility and mechanical strength (Doering 2010; Gonzalez et al. 2010). However, the view that chitin is an exclusive structural component providing protection from the internal hydrostatic pressure exerted on the wall by the cytoplasm and/or by environmental stresses (Gow et al. 2017) is now considered minimalist. In *Cryptococcus*, chitin is in fact a scaffold structure of the cell wall (Agustinho et al. 2018), but the polysaccharide also participates in capsular architecture (Rodrigues et al. 2008, 2018; Fonseca et al. 2009; Ramos et al. 2012) and assembly of melanin into the cell wall (Banks et al. 2005; Baker et al. 2007; Camacho et al. 2017). In addition, chitin has been demonstrated to modulate the host's immune response (Da Silva et al. 2008, 2009; Wagener et al. 2014; Wiesner et al. 2015; Ost et al. 2017). Therefore, this molecule is crucial for both cryptococcal physiology and pathogenesis.

Chitin is polymerized by membrane-associated chitin synthases, which use cytosolic UDP-*N*-acetyl-D-glucosamine as a sugar donor (Doering 2010). Eight putative cryptococcal chitin synthase genes (*CHS*1–*CHS*8) and three regulator proteins (Csr1–Csr3) have been identified in *C. neoformans* (Banks et al. 2005). It is therefore assumed that chitin synthesis is highly redundant in this fungus, which is likely related to its low susceptibility to nikkomycin, an inhibitor of chitin synthesis (Rinaldi 1999; Banks et al. 2005). Importantly, none of the chitin synthase or chitin synthase regulator genes are essential for cryptococcal viability (Banks et al. 2005; Rodrigues et al. 2018). Recent evidence, however, indicated that chitin synthesis is required for the correct capsular architecture of *C. neoformans*.

Deletion of seven out of the eight *CHS* genes resulted in aberrant capsular morphologies and altered polysaccharide dimensions (Rodrigues et al. 2018). Five of the eight *CHS* genes were required for the serological reactivity of capsular GXM, while the functionality of all eight genes was required for full polysaccharide secretion, in comparison with parental cells regularly expressing the *CHS* genes. (Rodrigues et al. 2018). These results clearly demonstrate that chitin synthesis and capsular formation are connected in *C. neoformans*. Remarkably, deletion of *CHS* genes also affected extracellular vesicle formation and chitinase activity (Rodrigues et al. 2018), which are events that also participate in capsular architecture (Rodrigues et al. 2007; Fonseca et al. 2009).

2.2 Chitooligomers

Chitooligomers or chitooligosaccharides are chitin-derived structures composed of 3–20 residues of β1,4-linked *N*-acetylglucosamine, produced enzymatically by chitinase-mediated chitin hydrolysis (Fonseca et al. 2013). These structures efficiently react with the wheat germ lectin (WGA) (Foster et al. 2004).

The demonstration that chitin-related structures are part of the capsular network in *C. neoformans* was generated by a combination of microscopic, biochemical, and pharmacological approaches. The first suggestion that chitin-derived structures and capsular components were associated was originated from the microscopic detection of chitin oligomers within the capsular network of both *C. neoformans* and *C. gattii* (Rodrigues et al. 2008). In *Cryptococcus*, WGA recognized chitooligomers with high affinity (Rodrigues et al. 2008; Fonseca et al. 2009, 2013). The chitooligosaccharides were detected at the cell wall but, unexpectedly, they were also found as projections emerging from the cell wall into the cryptococcal capsule, apparently connecting these two layers of the fungal surface. Chitooligomers formed round and hook-like structures detected within the capsule, as well as ringlike structures around the bud neck (Rodrigues et al. 2008).

The apparent association between capsular components and chitooligosaccharides was confirmed through different approaches. Chromatographic analysis revealed that hybrid glycans containing GXM and chitooligomers were found in their soluble form in culture supernatants, suggesting that formation of the complexes is a common event in the cryptococcal physiology (Fonseca et al. 2009). In the presence of the oligomers, capsular fibers increased in size, reinforcing the suggestion of intermolecular interactions between GXM and chitin-derived structures (Fonseca et al. 2009). Pharmacological inhibition of the synthesis of glucosamine 6-phosphate, a precursor of UDP-GlcNAc synthesis, resulted in decreased chitin detection and faulty capsules with clearly reduced dimensions (Fonseca et al. 2009). Structural analysis of the complexes formed by chitin and GXM revealed the requirement of chitin's *N*-acetyl groups for an efficient, non-covalent interglycan interaction (Ramos et al. 2012). The association of chitin-related structures and capsular components had a dramatic impact on the physiology of *C. neoformans*. Blocking of chitooligomers

with WGA resulted in reduced concentrations of extracellular GXM and decreased capsular dimensions (Fonseca et al. 2013). In addition, the transcription levels of genes involved in the synthesis, cellular traffic, and signaling pathways controlling capsule formation were also reduced (Fonseca et al. 2013).

The functional impact of the GXM–chitin association was not limited to the cryptococcal physiology. The interaction between chitooligomers and GXM resulted in stable, hybrid glycans with immunological functions that differed from each molecule alone (Ramos et al. 2012). Hybrid molecules were efficient inducers of lung tumor necrosis factor alpha (TNF-α), interleukin 10 (IL-10) and IL-17 in mice, suggesting that the association of GXM with chitooligomers produced molecules with unique immunological functions (Ramos et al. 2012). This observation is also in agreement with the notion that molecular interactions within the capsule can generate a number of complex structures with still unknown immunological functions.

Chitin-derived molecules were detected in outer layers of the capsule of *C. neoformans* and *C. gattii* (Rodrigues et al. 2008), suggesting that they could participate in the interaction of each pathogen with host cells. In fact, chitin-derived surface components affected the interaction of *C. neoformans* with the host at multiple levels (Fonseca et al. 2013). Toll-like receptor (TLR) 2, Dectin-1, and mannose receptor have been associated with immune responses to chitin resulting in the production of TNF-α and IL-10 (Da Silva et al. 2008, 2009; Bueter et al. 2013). Both murine and human macrophages produced IL-10 in response to cryptococcal chitin, in processes that likely required activation of NOD2 and TLR-9 (Heung 2017). WGA-treated *C. neoformans* were attenuated in virulence and had a poor capacity of dissemination to the central nervous system (Fonseca et al. 2013). Treatment of *C. neoformans* with WGA also resulted in reduced levels of interaction with host macrophages through mechanisms that required TLR-2 (Fonseca et al. 2013), suggesting that chitooligomer recognition is part of a Trojan horse mechanism of dissemination to the brain (Casadevall 2010). Accordingly, brain infection with *C. gattii* was further associated with increased chitooligomer distribution at the surface of fungal cells in mice (Rodrigues et al. 2015). Moreover, chitin stimulated Th2 responses during *C. neoformans* infection of mice through mechanisms that required polysaccharide cleavage by chitotriosidase, a mammalian chitinase (Wiesner et al. 2015). Increased chitooligomer distribution also correlated with peaks of chitinase activity in the lungs of infected mice (Rodrigues et al. 2015), suggesting that chitin hydrolysis, chitooligomer formation, capsule assembly, and pathogenesis are linked in *Cryptococcus*, as discussed below.

2.3 Chitinases

Chitooligomers are produced through chitin hydrolysis by both fungal and host chitinases (Goldman and Vicencio 2012; Ramos et al. 2012). Fungal chitinases have an important role in cell wall remodeling during growth, morphogenesis, and cell division (Adams 2004). In experimental models of cryptococcosis, expression

of chitinases varied according to the infected anatomic site (Fonseca et al. 2009). For instance, chitinase activity was induced in mice lungs infected with *C. neoformans*, but not in the brain (Overdijk et al. 1999; Vicencio et al. 2008). Intratracheal infection with *C. neoformans* also resulted in increased detection of chitinase activity in bronchoalveolar lavage fluids and lung homogenates of rats (Vicencio et al. 2008). Importantly, surface detection of chitooligomers in both *C. neoformans* and *C. gattii* was increased in lung tissues manifesting higher activity of chitinase (Fonseca et al. 2009, 2013; Rodrigues et al. 2015). Chitinase activity was also responsible for producing soluble, extracellular oligomeric structures of chitin that formed hybrid glycans with GXM during regular growth and macrophage infection, as concluded from the reduced formation of the hybrid structures in the presence of a chitinase inhibitor (Ramos et al. 2012; Rodrigues and Nimrichter 2012). Chitinase activity in *C. neoformans* under stress conditions (Rodrigues et al. 2018), which are known to induce capsule formation (Zaragoza and Casadevall 2004), was enhanced in both intracellular and extracellular fractions from *C. neoformans* (Rodrigues et al. 2018), suggesting that enzyme activity and chitooligomer formation are stimulated during capsule enlargement.

2.4 Chitosan

Differently from other fungi, chitin in *C. neoformans* is mostly de-acetylated by chitin deacetylases to form chitosan (Banks et al. 2005). The *CDA1*, *CDA2,* and *CDA3* genes are required for chitin deacetylation, but the presence of only one of these three chitin deacetylases is sufficient for chitosan production, suggesting metabolic redundancy (Baker et al. 2007). Cryptococcal chitosan levels may exceed the cellular amount of chitin by up to tenfold (Banks et al. 2005). The de-acetylated polysaccharide confers flexibility to the cell wall (Wang et al. 2018), which is essential for the molecular traffic across this cellular layer (Rodrigues and Casadevall 2018). Chitosan also contributes to the maintenance of cell wall integrity and bud separation (Banks et al. 2005; Wang et al. 2018). Importantly, chitosan is essential for melanin deposition on the cell wall of *C. neoformans* (Banks et al. 2005; Baker et al. 2007, 2011). Melanin is a cell wall pigment implicated in fungal pathogenicity (Nosanchuk and Casadevall 2006), resistance to environmental stress (Wang and Casadevall 1994; Nosanchuk and Casadevall 2006), and decreased susceptibility to antifungal drugs (Wang and Casadevall 1994; Martinez and Casadevall 2006).

Lack of chitosan critically impacts *C. neoformans* virulence (Baker et al. 2011). Fungal cells lacking chitosan synthesis manifest unstable cell walls, slow growth at 37 °C, and increased vulnerability to host defense mechanisms, with consequent inability to kill mice (Baker et al. 2011). Furthermore, chitosan-deficient *cda1cda2cda3*Δ mutants were not pathogenic and induced robust inflammatory, protective responses (Baker et al. 2011; Upadhya et al. 2016). In this context, heat-killed *cda1cda2cda3*Δ cells have been suggested as prototypes for vaccine development in the *Cryptococcus* model (Upadhya et al. 2016).

3 Mannoproteins

The cryptococcal surface contains highly mannosylated glycoproteins, namely mannoproteins (MPs). The main structural features of MPs include a signal sequence for post-Golgi secretion, a site for attachment of glycosylphosphatidylinositol (GPI) anchors, and a serine-/threonine-rich region, which bears extensive *O*-mannosylation (Levitz and Specht 2006). MPs are in close association with the cryptococcal capsule, and early estimates suggest they account for less than 1% of the capsular mass (Bose et al. 2003; Zaragoza et al. 2009). MPs are predominantly located in the inner region of the capsule, close to the cell wall (Jesus et al. 2010), but the fact that they contain secretory tags might suggest that they are transitory capsular components being transported to the extracellular space (Biondo et al. 2006; Eigenheer et al. 2007; Jesus et al. 2010; Agustinho et al. 2018).

Cryptococcal MPs are highly immunogenic and immunostimulatory (Chaka et al. 1997; Levitz and Specht 2006). MPs stimulate a massive production of IL-12 by human monocytes (Pitzurra et al. 2000). Binding of MPs to the mannose receptor of dendritic cells led to activation of T cells and protective immunity against *C. neoformans* (Specht et al. 2007; Dan et al. 2008a, b). Inhibition of mannose receptors or MP deglycosylation strongly prevented activation of T cell responses, indicating the essential contribution of mannosylation to immunogenicity (Levitz and Specht 2006). On the basis of these observations, MPs have been proposed as potential vaccine candidates against cryptococcosis (reviewed by Van Dyke and Wormley (2018)).

Bioinformatic analysis using the proteomes of *C. neoformans* and *C. gattii* revealed the putative occurrence of 43 and 36 predicted MPs, respectively (Reuwsaat et al. 2018). However, most of them remain to be characterized at the functional and structural levels. Levitz and colleagues explored the functions of *C. neoformans* MP98 in stimulating T cell responses using murine hybridomas (Levitz et al. 2001). MP98 is encoded by chitin deacetylase 2 gene (*CDA2*), which is responsible for converting chitin to chitosan. This protein, which is GPI-anchored to the plasma membrane, is associated with the cell wall. However, MP98 association with the cell wall is independent of both the GPI anchor and β-1,6-glucan (Gilbert et al. 2012). Similarly, MP88 is involved in T cell activation, sharing several structural features with MP98, which include a serine-/threonine-rich C-terminal region and a GPI anchor motif (Huang et al. 2002). Nevertheless, a supposed function for MP88 based on sequence similarity analysis has not been assigned (Levitz and Specht 2006). Other cryptococcal MPs that have been studied at the functional level include MP84 and MP115, which are recognized by serum antibodies from AIDS patients with cryptococcosis (Biondo et al. 2005). MP84 and MP115 have homology to polysaccharide deacetylase and carboxylesterase proteins, respectively. Furthermore, MP84 mediates the adhesion of *C. neoformans* to lung epithelial cells (Teixeira et al. 2014). Lastly, the *C. neoformans CIG1* gene encodes a secreted mannoprotein (Cig1) involved in a heme uptake system. Cig1 influences *C. neoformans* virulence in a mouse model of cryptococcosis but only in a strain that also lacked the high-affinity iron uptake system (Cadieux et al. 2013).

The roles of cryptococcal MPs in capsule structure and assembly have not been fully addressed. Recently, Reuwsaat et al. characterized a *C. gattii* putative MP, namely Kpr1, and suggested that it participates in capsular structure and GXM release (Reuwsaat et al. 2018). The *kpr1* null mutant cells were more sensitive to congo red, a classical cell wall stressor that disrupts beta-glucan synthesis. Gene knockout affected cell-associated cryptococcal polysaccharide thickness and phagocytosis by J774.A1 macrophages. Furthermore, recombinant Krp1 was selectively recognized by serum from patients with cryptococcosis (Reuwsaat et al. 2018). The in vivo pathogenic potential of *C. gattii*, however, was not affected by *KPR1* deletion. These data suggest a role of Kpr1 in capsule assembly in *C. gattii*.

The studies showing key biological functions of MPs contrast with the fact that only a minor fraction of cryptococcal MPs has been functionally characterized. In addition, the glycan moieties of cryptococcal glycoproteins have never been structurally determined. This scenario stimulates studies on the roles of MPs in the pathogenesis of *C. neoformans* and *C. gattii*.

4 Conclusions

It is now clear that the capsule of *Cryptococcus* is much more complex in composition and functions than initially thought. GXM and GXMGal are unquestionably major capsular components with fundamental biological functions, but the capsular network assuredly includes less abundant and even transitory components that might impact biological functions and pathogenic potential. The roles of these overlooked capsular components remain largely unexplored. Finally, intermolecular interactions within the capsular network are expected, as well as the formation of multimolecular structures with completely unknown roles in physiopathogenesis. Although the progress in the understanding on how the capsular components of *Cryptococcus* impact physiology and pathogenesis is incontestable, it seems clear that there is still much to learn about the functional multiplicity of the cryptococcal capsule.

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Extracellular Vesicles in Fungi: Composition and Functions

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Abstract The comprehension of fungal biology is important for several reasons. Besides being used in biotechnological processes and in the food industry, fungi are also important animal and vegetal pathogens. Fungal diseases in humans have a great importance worldwide, and understanding fungal biology is crucial for treatment and prevention of these diseases, especially because of emerging antifungal resistance that poses great epidemiological risks. Communication through extracellular vesicles is a ubiquitous mechanism of molecule transfer between cells and is used to transport proteins, nucleic acids, lipids, and other biologically active molecules. Several pathogens can produce and transfer extracellular vesicles, and the importance of this pathway in fungal communication with hosts and between fungal cells has been described for several species in the last years, as shown for *Saccharomyces cereviseae, Cryptococcus neoformans, Candida albicans, Paracoccidioides braziliensis, Sporothrix schenckii, Candida parapsilosis, Malassezia sympodialis, Histoplasma capsulatum*, among others. In this chapter,

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we review the role of extracellular vesicles in fungal communication, interaction with hosts and with the environment, and also highlighting important molecules found in fungal EVs.

1 Introduction

The rising importance of fungal diseases can be linked to their widespread distribution and emergence in the world (Binder and Lass-Flörl 2011; Bongomin et al. 2017), highlighting the need to understand fungal biology in order to promote adequate prophylaxis and treatment. In parallel, an alarming offset of antifungal-resistant infections raises concerns about our preparedness to respond to widespread epidemiological risks (Farmakiotis and Kontoyiannis 2017). In addition to these concerns, there is also an area of study bringing to light the influence of our own fungal microbiome on health and disease (Ghannoum et al. 2010; Huffnagle and Noverr 2013). Of particular interest are organisms such as *Candida*, *Malassezia,* and *Cryptococcus,* from which commensalism can evolve into pathogenicity depending on immunological barriers.

Although they were first described in 1984 (Harding et al. 1984), extracellular vesicles have only recently been recognized as a ubiquitous cellular communication mechanism (Raposo and Stoorvogel 2013). Their molecular composition varies depending on the cell of origin and environmental conditions (Raposo and Stoorvogel 2013). They can carry lipids, carbohydrates, proteins, or nucleic acids and associate with the bilayer surface or inside the vesicles, shielding these molecules from external interference. What were once thought of as "garbage" carriers and shed debris are now recognized as an important pathway for communication between cells, both in single-cell and multicellular organisms, that have enormous potential as biomarkers (Raposo and Stoorvogel 2013). In the case of fungi, release of EVs serves as a tool by which fungal cells communicate and interact with other organisms (Rizzo et al. 2017b), playing a role in pathogenesis and commensalism. Vesicle packaging has some advantages when compared to soluble secretion of components directly to the extracellular milieu. Vesicles promote protection of molecules sensitive to extracellular enzymatic degradation, such as circulating RNAs, which are usually found in human plasma associated with extracellular vesicles or Argonaute proteins (Arroyo et al. 2011). Compartmentalization also avoids leakage, especially when the cargo needs to be used far from the original source. A vesicle can theoretically maintain the same original concentration of a molecule that can be delivered to the target cells/tissues without significant leakage. Additionally, the synergistic effect of two or more molecules can be exerted over great distances, while for soluble secretion, there is no guarantee that the molecules will reach the target. Vesicles allow for co transportation of molecules in proximity with each other, thus allowing their synergic potential (Raposo and Stoorvogel 2013).

Extracellular vesicles are very important in host–pathogen interactions. Extracellular pathogens can communicate through EVs, sharing information about the environment and facilitating the persistence of infection, pathogen motility, and tissue tropism (Cipriano and Hajduk 2018). On the other hand, EVs released by intracellular pathogens can alter the homeostasis of host cells, inducing the differential expression of RNAs and proteins as well as affecting the production of different types of EVs during infection. EVs originating from infected cells can modulate the immune response and affect host membrane properties (Cipriano and Hajduk 2018). Current interest in EV biology is growing exponentially, and there are significant efforts to uncover the potential use of these vesicles for the biodelivery of small molecules (Kutralam-Muniasamy et al. 2015) for therapeutic purposes. Applications have already been developed for EVs of bacterial origin (Wang et al. 2018), such as adjuvants in a meningococcal vaccine (van der Pol et al. 2015). Similar studies can be potentially explored for fungal EVs.

This review will focus on what is currently known about fungal EVs, with a major emphasis on their role in pathogenesis. We will explore the molecular content of these vesicles, highlighting how a direct relation exists between EV cargo and biological effects. Additionally, studies pertaining to the nucleic acid composition of fungal EVs will be explored in depth, comparing recent findings to what is known for other eukaryotes.

2 Origin and Biogenesis

Mammalian extracellular vesicles are usually characterized by their size and origin, although distinct populations of extracellular vesicles are known to coexist (Raposo and Stoorvogel 2013). A smaller subpopulation, initially called exosomes, seems to have functional properties in several organisms. These are derived from multivesicular bodies, have a peculiar lipid composition and range in size from 20 to 100 nm in diameter. These smaller vesicles usually have a conserved cup-shaped morphology when observed by electron microscopy, a known artifact of the fixation procedure when the vesicles are observed without sectioning. When these vesicles are sectioned using techniques like cryo-EM, they appear round and not cup-shaped (Raposo and Stoorvogel 2013). Additionally, cells can shed microvesicles that bud directly from the plasma membrane are generally between 100 and 1000 nm in diameter and have a composition closer to the overall distribution of cytoplasmic proteins (Raposo and Stoorvogel 2013). Apoptotic bodies (produced when cells go through apoptosis) are between 500 and 2000 nm in size and can regulate innate immune responses when they are phagocytized by antigen-presenting cells (Kerr et al. 1972).

A variety of regulators have been associated with EV formation in fungi, although no overall key molecular component(s) have been conclusively identified (Oliveira et al. 2013). Some authors show that they may have originated from

cytoplasmic fractions, since some cytoplasmic proteins without signals for secretion are found in these vesicles (Rodrigues et al. 2013). Other authors show the role of membrane budding and multivesicular bodies in this process (Joffe et al. 2016). A membrane reshaping mechanism can also participate in EV biogenesis (Rodrigues et al. 2013). Some individual molecules are shown to affect EV release in fungi. In *C. neoformans*, SEC6 suppression (through an RNAi-expressing plasmid) caused a decrease in laccase, urease, and soluble GXM secretion to the extracellular space through the EVs. Additionally, an accumulation of intracellular vesicles was observed alongside a lack of detected EVs in the extracellular environment. SEC6 is part of the exocyst complex, which is responsible for the fusion of vesicles (post-Golgi origin) to the plasma membrane. Thus, its participation in EV formation is very likely (Panepinto et al. 2009). Other pathways have been investigated, mainly through the use of mutant strains. The Golgi reassembly and stacking protein (GRASP) and Sec4p are proteins involved in Golgi-derived secretion, while Snf7p participates in multivesicular body (MVB) genesis (Oliveira et al. 2010b; Kmetzsch et al. 2011). Mutants of *S. cerevisiae* containing defective expression of one of these proteins showed altered vesicle cargo but no effects on vesicle release. Although these studies show components that participate in the overall machinery for vesicle production, no study has yet described a key component that would be common to all fungi regarding EV release (Oliveira et al. 2013; Rodrigues et al. 2014).

The fungal cell wall is a dynamic and essential device for maintaining fungi structure and providing protection against biological and chemical hazards. It is comprised mainly of glucans, chitin, and polysaccharides, as well as pigments and glycoproteins (Nimrichter et al. 2016). EVs have been shown to pass over the fungal cell wall and actively participate in its maintenance and modeling (Brown et al. 2015). The observation of atypical molecules on the cell wall, usually associated with internal membranes, sheds light on fungal EV secretion since they had to pass through the cell wall to reach the extracellular environment. This process could also contribute to its dynamic composition, bringing transient components to the cell wall (Nimrichter et al. 2016). The first concrete evidence of EV transport across the cell wall of fungi came from *C. neoformans*. The maintenance and rebuilding of the cryptococcal capsule require delivery of GXM that is transported through vesicles across the cell wall (Rodrigues et al. 2007). Its robust structure, although necessary for adequate function, hinders the microorganism in terms of secretion, and in order to be released, fungal EVs need to interact with cell wall components (Wolf and Casadevall 2014). The presence of cell wall-degrading enzymes on EVs has already been shown for *S. cerevisiae, C. albicans* (Rodrigues et al. 2008b), *C. neoformans* (Wolf et al. 2014), *H. capsulatum* (Albuquerque et al. 2008), and *P. braziliensis* (Vallejo et al. 2012b).

Environmental and metabolic conditions can alter the intensity of EV secretion, as well as their size and content, which leads to the assumption that there are environmental influences on what a fungus secretes through EV (Rodrigues et al. 2013). One example is what happens with starved yeast cells, where they increase

their release of EVs until the glucose levels are back to normal and the cells start to internalize the EVs from the extracellular environment (Winters and Chiang 2016). The complete mechanisms involved in fungal EV genesis and release are not yet completely understood.

3 Composition

3.1 Proteins

In mammalian cells, several protein types are involved in EVs and are conserved between EVs from diverse cell types and organisms, such as RAB GTPases, cytoskeleton proteins, and tetraspanins such as Alix, TSG101, CD9, CD63, and CD81 (Raposo and Stoorvogel 2013). For fungal EVs, there is a different set of common proteins that can be found in EVs from fungal cells of different species, similar to those involved with the metabolism of proteins/carbohydrates, stress response, translation, oxidation/reduction, transport, and signaling (Vallejo et al. 2012b), while others are specific for certain fungal species. Additionally, fungal EVs have several protein components associated with virulence (Albuquerque et al. 2008; Rodrigues et al. 2008a; Vallejo and Puccia 2011; Vargas et al. 2015).

Enzymes that are recognized as virulence factors in fungal infections are present in fungal EVs. *C neoformans* laccase, part of the melanin synthesis pathway (Eisenman et al. 2007); urease; and acid phosphatase are characterized as virulence factors and are found in *C. neoformans*-derived EVs (Rodrigues et al. 2008a). Analysis of *H. capsulatum* vesicles revealed the presence of REDOX-related proteins such as catalase B, superoxide dismutase and thiol-specific antioxidant (Albuquerque et al. 2008) as well as endochitinase and glucanase, enzymes that hydrolyze components of the cell wall facilitating EV release (Albuquerque et al. 2008). Phosphatase activity was detected in the vesicles of *P. brasiliensis* following a dose-dependent ratio (Vallejo et al. 2012b). The protein content of *P. brasiliensis*-derived EVs was compared to that of *H. capsulatum, C. neoformans,* and *S. cerevisiae*. A total of 26 proteins were found to be common in all four fungi, and most were also present in the vesicle-free fraction of *P. brasiliensis*. It is interesting to note that some of the proteins present in vesicles from two or more fungi can be related to infectivity and virulence, such as superoxide dismutase (a known REDOX enzyme) or heat shock proteins (related to stress response) (Vallejo et al. 2012b). Heat shock proteins are also important immunogens of *C. neoformans* (Rodrigues et al. 2014). Several proteic allergens were also found in *Malassezia sympodialis* derived EVs (Johansson et al. 2018).

EVs can also be used to transmit prions, infectious protein particles that can self-aggregate and cause diseases such as spongiform encephalopathy. The prion SUP35 from *S. cerevisiae* can be transferred vertically and horizontally between yeast cells and is also found in *S. cerevisiae*-derived extracellular vesicles, as shown

in SUP35p in its soluble and aggregated infectious form (Kabani and Melki 2015) and for SUP35 NM (Liu et al. 2016). When these vesicles are taken up by recipient cells, they can also induce self-sustained aggregation of SUP35 NM proteins (Liu et al. 2016).

3.2 RNAs

Nucleic acid export in EVs allows for genetic flow due to the information contained in both DNA and RNA molecules. Nucleic acids present in EVs have potent abilities of posttranscriptional regulation, influencing regulatory processes in recipient cells. Several types of functional RNAs can be transported by EVs, such as small noncoding RNAs (i.e., micro RNAs and tRNA fragments), long noncoding RNAs, structural RNAs (such as vault RNA and SRP-RNA) and protein-coding mRNAs (van der Grein and Noltet Hoen 2014) that can also modulate the gene expression of recipient cells (Valadi et al. 2007).

The first comprehensive study on the RNA content in fungal vesicles (evRNAs) was published in 2015. Using next-generation sequencing (NGS), the evRNA from *S. cerevisiae*, *C. albicans*, *C. neoformans,* and *P. brasiliensis* was identified (Peres da Silva et al. 2015). Interestingly, RNAse treatment did not degrade the sRNA fraction, which is protected by the membrane of EVs. The majority of small RNAs (sRNA) from all samples were 250 nt long on average. A total of 1246 conserved miRNA-like sequences were detected, of which 20 were common to all four species. Differential expression analysis showed specific sRNA content in each species. *P. brasiliensis* contained a component of the miR-125 family, which is involved in cell proliferation, immune differentiation, growth factors, apoptosis, and nonsense-mediated mRNA decay. Some sequences found in *P. brasiliensis, C. neoformans* and *S. cerevisiae* were related to plant cells. Additionally, other types of ncRNAs (non-miRNA) were detected in the evRNA samples. A total of 114 ncRNA sequences were observed, with variations between species. Small-nucleolar RNAs (snoRNAs) and tRNAs were the most represented RNAs in all species (Peres da Silva et al. 2015).

More recently, a study on the evRNA originating from the fungus *Malassezia sympodialis* uncovered reads with a length range of 16–22 nt, similar to small RNAs. The genus *Malassezia* peculiarly does not carry homologs for the components of RNAi machinery (Dicer, Argonaute, etc.) but nevertheless showed a spike in sRNA-sized reads. A suggested explanation for this EV content is the Dicer-independent pathway present in *Neurospora crassa* may also be present in *M. sympodialis*. This is just a hypothesis, however, given the limitations of the study (Rayner et al. 2017).

EV RNAs are also important in plant–fungi interactions. Fungal small RNAs can interfere with RNAi pathways in plant hosts, impairing plant immunity (Weiberg et al. 2013). It was also shown that this cross-kingdom RNAi activity can be bidirectional (Wang et al. 2016). Another interesting mechanism involving

EV RNA in host–pathogen interactions of fungi and plants is *host-induced gene silencing* (HIGS), which implies a transfer of small RNAs from the plants to parasitic fungal cells (Tinoco et al. 2010). These small RNAs sent by plants to fungal cells can silence virulence genes (Cai et al. 2018; Wang et al. 2016). Silencing RNAs derived from transgenic plants can downregulate some RNA targets in fungal pathogens that have similarities with plant RNAs. This biotechnological tool is being used for studies and pest field control and it is possible that this also occurs in nature, although its natural occurrence has not yet been described. This phenomenon was already shown for *Blumeria graminis* (Nowara et al. 2010), *Puccinia striiformis f.sp.tritici* (Yin et al. 2011), *Puccina triticina* (Panwar et al. 2013), a species related to powdery mildew disease (Pliego et al. 2013), *Sclerotinia sclerotiorum* (Andrade et al. 2016) and *Fusarium* species (Ghag et al. 2014; Cheng et al. 2015) (reviewed by Chaloner et al. 2016).

Secreted RNA species are being increasingly recognized as important players in cellular communication and interspecies dynamics. Their role as a communication tool (Iguchi et al. 2010) results from their ability to influence the transcriptome of the recipient cells. By sending specific RNAs to a neighboring cell (e.g., a collection of miRNAs), the secretory cell can modulate its activity from a remote location. The RNA content in EVs can act as "packages of RNA data" efficiently delivering this information, and can be used both in a collaborative (e.g., mutualistic interactions between organisms) or competitive manner (e.g., pathogenesis and parasitism) (Chaloner et al. 2016).

3.3 Lipids

Lipids are important regulators of pathogenicity in fungi. In addition to functioning as virulence factors, they are also important in biofilm formation, drug resistance, formation of microdomains, and the formation of extracellular vesicles. Changes in lipid composition of fungal membranes and EVs can alter fungal pathogenicity (Rella et al. 2016).

C. neoformans-derived EVs contain the lipids sterol and glucosylceramide (Rodrigues et al. 2008b). In *H. capsulatum,* various lipids were found with particular abundance, especially phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine. These lipids are multifunctional and are key elements in the form and function of the lipid bilayer in both fungi and mammals (Albuquerque et al. 2008). In *C. albicans,* the main lipids found in EVs are ergosterol and GlcCer, a composition similar to that of *C. neoformans* (Vargas et al. 2015). GlcCer is also an important immunogenic molecule of *C. neoformans* (Nimrichter and Rodrigues 2011), and it has already been shown that antibodies against GlcCer could inhibit fungal growth (Rodrigues et al. 2000). A comprehensive study on lipid composition of fungal EVs was also published by Vallejo et al. 2012b.

3.4 Carbohydrates and Pigments

The first characterization of *C. neoformans* EVs was a result of an investigation on the transport glucuronoxylomannan (GXM), a capsular polysaccharide (Rodrigues et al. 2011). Its vesicles are able to transport GXM through the cell wall and onto the external surface of the fungi, promoting capsule assembly and maintenance. In addition to lipids and carbohydrates, pigments such as melanin can also be found in EVs, as shown in *C. neoformans* (Eisenman et al. 2007).

Concerning common pathogenic fungi, Table 1 summarizes the first studies that identified EV release in each species. The deeply studied model *S. cerevisiae* is also shown. Additionally, landmark studies on the proteomic, lipidomic, and glycomic contents of fungal EVs are referred here.

Table 1 Production of EVs was described for several fungal species and for some of the biological activity was also shown

Fungi species	Confirmed EV release	Proteins	Lipids	Glycans	Immunoactive
Paracoccidioides brasiliensis	Vallejo and Puccia (2011)	Vallejo et al. (2012b)	Vallejo et al. (2012a)	Da Silva et al. (2015)	Vallejo and Puccia (2011), Da Silva et al. (2016)
Sporothrix schenckii	Albuquerque et al. (2008)				
Candida albicans	Anderson et al. (1990), Albuquerque et al. (2008)	Vargas et al. (2015)	Vargas et al. (2015)		Vargas et al. (2015)
Candida parapsilosis	Albuquerque et al. (2008)				
Malassezia sympodialis		Gehrmann et al. (2011), Johansson et al.2018)			Gehrmann et al. (2011)
Histoplasma capsulatum	Albuquerque et al. (2008)	Albuquerque et al. (2008)	Albuquerque et al. (2008)		Albuquerque et al. (2008)
Cryptococcus neoformans	Rodrigues et al. (2007)	Rodrigues et al. (2008a)			
Saccharomyces cerevisiae	Albuquerque et al. (2008)	Oliveira et al. (2010b)			

4 Fungi Use EVS to Interact With Hosts, Other Fungi and the Environment

EVs are potent routes of intercellular communication and can also be used for interorganism interactions. Immune stimulation has been observed both in vitro and in vivo, when model cells or animals are exposed to fungal EVs (Oliveira et al. 2010a; Gehrmann et al. 2011; Vargas et al. 2015). *C. albicans* EVs can elicit a significant biological response in immune cells and can be internalized by macrophages (MOs) and dendritic cells (DCs), inducing dose-dependent NO production and secretion of IL-12, TNF-α, IL-10, and TGF-β. In addition, EV treatment increases CD86 in MOs and DCs and induces MHCII expression in DCs (Vargas et al. 2015). Exposure of NOs to *C. neoformans* EVs results in internalization and promotes dose-dependent NO production (Oliveira et al. 2010a). GXM, an important component of the fungal capsule, is also found in EVs. When used to stimulate macrophages, EVs from capsular *C. neoformans* and acapsular mutants increased the secretion of TNF-α, IL-10, and TGF-β. EVs from the acapsular strain had a larger effect on NO production, while the capsular strain EVs had a higher impact on IL-10 levels. EVs secreted by *M. sympodialis,* a fungus involved in atopic eczema, can elicit IL-4 and TNF-α response on PBMCs (Gehrmann et al. 2011). Additionally, EVs secreted by *P. brasiliensis* can also induce cytokine secretion in macrophages. EVs can also exhibit Pathogen Associated Molecular Patterns (PAMPs) and lectins on their surface. In *P. brasiliensis* EVs, exposed surface carbohydrates can be recognized by DC-SIGN, an important surface receptor present in human dendritic cells.

Another interesting infection mechanism mediated by EVs is the "division of labor" that occurs in *Cryptococcus gattii*, a pathogen that causes a life-threatening infection (Bielska et al. 2018). This happens when some cells remain dormant to allow the growth of other cells, which can be triggered from long distances when EVs are involved. EVs from a virulent strain of *C. gattii* can be taken up by macrophages and induce the proliferation of fungal cells that are naturally less resistant inside its phagosomes, promoting pathogen survival when normally these fungi would be eliminated by the macrophages (Bielska et al. 2018). In this work, the authors showed that EV proteins and RNA are necessary in the process of virulence transfer in *C. gattii*, since treating the EVs with proteases and RNases prevented the increased survival of cryptococci inside macrophages (Bielska et al. 2018).

The effects of pretreatment with EVs in infection vary for different models. Preincubation of *C. albicans* EVs with *G. mellonella* larvae prior to challenge with yeast cells results in a decrease of fungal CFUs and enhances the survivability of *G. mellonella* (Vargas et al. 2015). Additionally, pretreatment of MOs with *C. neoformans* EVs enhances their phagocytosis and microbicidal activity (Oliveira et al. 2010a), suggesting a protective effect. In the *C. albicans* model, EVs can also confer protection against infection (Vargas et al. 2015). However, some evidence that fungal EVs can enhance infection also exists. For *C. neoformans*, it has been

shown that EVs can render mice more susceptible to infection and that EVs can accumulate in sites of brain infection, increasing adhesion and transcytosis of *C. neoformans* in microvascular endothelial cells found in the human brain (Huang et al. 2012). However, it was also shown that *C. neoformans* EVS can be destabilized by serum proteins, suggesting that the host could counteract this mechanism in vivo (Wolf et al. 2012).

The interaction with the host defense mechanisms can also alter the content of fungal extracellular vesicles. For *H. capsulatum*, it was shown that binding of protective (Mab6b7) or nonprotective (Mab7b6) monoclonal antibodies that target Hsp60 to fungal cells was able to modulate the protein cargo of vesicles secreted by these fungi (Matos Baltazar et al. 2016). Antibody binding also altered vesicle size and the activity of enzymes present in fungal EVs such as laccase, phosphatase, and catalase (Matos Baltazar et al. 2016), which could potentially alter the susceptibility to host defenses. It was also shown that *H. capsulatum* vesicles are capable of reacting with sera from patients with histoplasmosis (Shi et al. 2008).

EVs can be used in communication between yeast cells outside of hosts, similar to in biofilms, and play a role in the transition of dimorphic fungi from a yeast-like morphology to a pseudo-hyphal mode in response to environmental cues, as shown in *Pichia fermentans* (Leone et al. 2018). Filamentous fungi can also secrete EVs, as shown in *Alternaria infectoria*, an environmental fungus that can also cause opportunistic infections (Silva et al. 2014). Additionally, fungi can use EVs to interact with environmental predators. *Acanthamoeba castellanii*, a known environmental predator of *C. neoformans*, can internalize *C. neoformans*-derived-free GXM and extracellular vesicles (Rizzo et al. 2017a). This internalization does not affect the viability of *A. castellanii*, but the internalization of EVs is able to modulate the response of *A. castellanii* in a way that increases the survival of the fungus; the internalization of free GXM does not have the same effect (Rizzo et al. 2017a).

5 Concluding Remarks

This review chapter aspired to bring to light the most current knowledge we have on extracellular vesicles of fungal origin, especially highlighting the nucleic acid content within these vesicles. EV release serves as a gateway for complex extracellular communication, both between the fungal cells as well as between the fungi and organisms in their proximity (e.g., host) (Fig. 1). A key aspect, which highlights the importance of studying EV biology, is the capacity for modulation of host activity at a posttranscriptional level. Further studies can better disclose the mechanisms by which this regulation occurs, and also explore the huge potential of fungal EVs in biomedical and biotechnological applications.

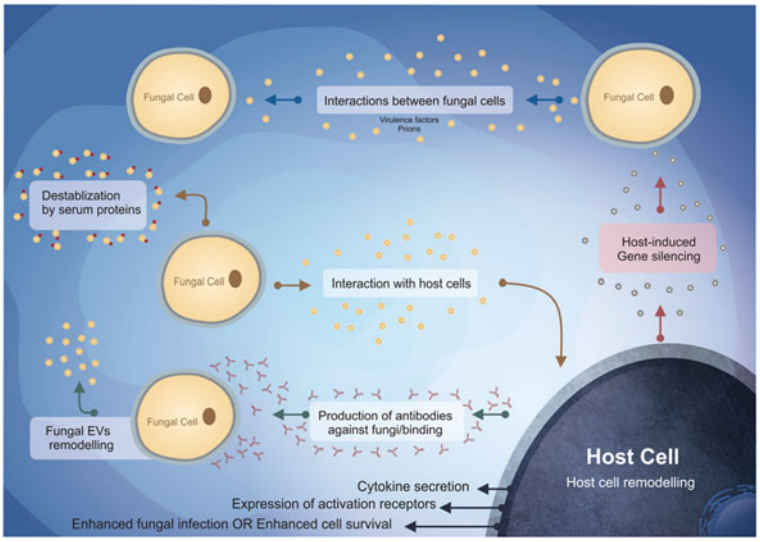

Fig. 1 Fungi use extracellular vesicles to interact with other fungi, hosts, and the environment. EVs can mediate the interaction between fungal cells, transferring virulence factors and prions. Fungal EVs can also mediate the interaction between fungi and host cells, and some of them can be destabilized by serum proteins. Fungal EVs that reach host cells can induce antibody production, and when these antibodies bind to other fungal cells, they can reprogram the content of fungal EVs. Signaling mediated by fungal EVs can also induce changes in host cells, inducing cytokine secretion and expression of surface receptors. The activity of fungal EVs can increase fungal infection or increase host cell survival, depending on the infection model. EVs released by host cells can promote gene silencing in host cells, as described for plants

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From Genes to Networks: The Regulatory Circuitry Controlling *Candida albicans* Morphogenesis

Virginia Basso, Christophe d'Enfert, Sadri Znaidi and Sophie Bachellier-Bassi

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Abstract *Candida albicans* is a commensal yeast of most healthy individuals, but also one of the most prevalent human fungal pathogens. During adaptation to the mammalian host, *C. albicans* encounters different niches where it is exposed to several types of stress, including oxidative, nitrosative (e.g., immune system),

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osmotic (e.g., kidney and oral cavity) stresses and pH variation (e.g., gastrointestinal (GI) tract and vagina). *C. albicans* has developed the capacity to respond to the environmental changes by modifying its morphology, which comprises the yeast-to-hypha transition, white-opaque switching, and chlamydospore formation. The yeast-to-hypha transition has been very well characterized and was shown to be modulated by several external stimuli that mimic the host environment. For instance, temperature above 37 °C, serum, alkaline pH, and CO_2 concentration are all reported to enhance filamentation. The transition is characterized by the activation of an intricate regulatory network of signaling pathways, involving many transcription factors. The regulatory pathways that control either the stress response or morphogenesis are required for full virulence and promote survival of *C. albicans* in the host. Many of these transcriptional circuitries have been characterized, highlighting the complexity and the interconnections between the different pathways. Here, we present the major signaling pathways and the main transcription factors involved in the yeast-to-hypha transition. Furthermore, we describe the role of heat shock transcription factors in the morphogenetic transition, providing an edifying example of the complex cross talk between pathways involved in morphogenesis and stress response.

1 Introduction

Fungal pathogens have become responsible for increasing mortality in humans over the past four decades. In developed countries, fungal infections appear as one of the primary causes of hospital-acquired infections, most of them caused by *Candida* spp.—particularly *Candida albicans*. *Candida* infections range from mild superficial infections to life-threatening invasive infections. Despite the use of antifungals and other therapeutic approaches, *Candida* infections remain associated with a high mortality rate (Kullberg and Arendrup 2016; Pfaller and Diekema 2007).

Numerous studies have shown that morphogenesis is tightly linked to the ability of *C. albicans* to cause disease. Although *C. albicans* displays an extended array of morphological transitions (e.g., chlamydospore formation, GUT phenotype, white-opaque switching, gray phenotype), the yeast-to-hypha transition appears to be an important virulence trait (Brown and Gow 1999; Gow et al. 2002; Mukaremera et al. 2017; Nemecek et al. 2006; Peters et al. 2014; Rooney and Klein 2002; Trevijano-Contador et al. 2016). Therefore, particular interest was given to the mechanisms of transition between the unicellular yeast form and either pseudohyphal or hyphal forms, often referred to as filamentous forms (Odds 1987; Sudbery et al. 2004). For instance, the yeast form is required for dissemination into the bloodstream (Bendel et al. 2003; Saville et al. 2003) and adhesion to endothelial cells (Grubb et al. 2009; Yang et al. 2014). In contrast, the filamentous form is needed for tissue invasion and for escaping from immune cells, yielding resistance toward phagocytosis (Erwig and Gow 2016; Fradin et al. 2005; Lorenz et al. 2004; Rooney and Klein 2002).

In this review, we summarize the major signaling pathways that regulate the morphogenetic transition between unicellular yeast form and filamentous forms. We report the differences between *C. albicans* and the model organism *Saccharomyces cerevisiae*, highlighting specific features of *C. albicans* related to morphogenesis and the associated cellular stress response.

2 Stimuli Inducing the Yeast-to-Hypha Transition

The yeast-to-hypha transition is triggered by different environmental cues that reflect the host environment, such as nutrient starvation, N-acetyl glucosamine (GlcNAc), serum, CO_2 levels, neutral pH, temperature, and surface contact. In laboratory conditions, several media are used to mimic these environmental cues, such as GlcNAc-containing medium or Spider medium (Liu et al. 1994; Mattia et al. 1982). However, the most widely used inducer of morphogenesis is serum (Ernst 2000; Gow 1997), whose complex composition makes difficult the identification of the factor(s) responsible for filamentation. Early studies have shown that CO_2 is an inducer of *C. albicans* morphogenesis (Persi et al. 1985) and that physiological CO_2 levels are essential for *C. albicans* pathogenicity (Klengel et al. 2005). Filamentation is favored by pH values close to neutral, but is considerably reduced at pH lower than 6, while the yeast form is predominant at pH 4 (Buffo et al. 1984). High temperatures also promote filamentation via the molecular chaperone Hsp90 (O'Meara et al. 2017; Shapiro et al. 2009) and the transcription factor (TF) Sfl2 (Song et al. 2011). Finally, surface growth triggers several behaviors, such as invasion, thigmotropism, and biofilm formation (Kumamoto 2005; Kumamoto 2008; Kumamoto and Vinces 2005).

The yeast-to-hypha transition is also influenced by quorum sensing molecules, such as homoserine lactone (HSL), the 12-carbon backbone molecule dodecanol, farnesol, and tyrosol (Chen et al. 2004; Hogan et al. 2004). These compounds block the hyphal transition by the Ras-cAMP-PKA pathway and impact on cAMP-controlled genes involved in morphogenesis and stress response (Davis-Hanna et al. 2008). In the host, HSL, dodecanol, and farnesol are secreted by bacteria, such as *Pseudomonas aeruginosa*, and regulate *C. albicans* morphogenesis through independent mechanisms (Hall et al. 2011). Additionally, farnesol is produced by *C. albicans* as a by-product of ergosterol biosynthesis (Hornby et al. 2003) and affects many physiological functions, including cell wall maintenance, iron transport and the regulation of genes encoding heat shock proteins (Hsps) (Cao et al. 2005). Recently, it has been reported that farnesol's effect on hyphal morphogenesis is linked to *EED1* gene (see below), since an *eed1* null mutant is hypersensitive to this molecule (Polke et al. 2017). Farnesol and HSL inhibit the activity of the adenylate cyclase (AC) Cyr1, and several evidences suggest that farnesol acts downstream of Ras1 (Feng et al. 1999). On the other hand, dodecanol modulates filamentation and cAMP signaling without affecting AC activity, but through a mechanism involving the transcription factor Sfl1 (Hall et al. 2011), while tyrosol is

continuously released into the culture medium by *C. albicans* itself in order to promote germ tube formation (Chen et al. 2004). Finally, several alcohols, such as amyl alcohol, have been shown to inhibit the yeast-to-hypha morphological transition in a dose-dependent manner (Chauhan et al. 2013).

3 Hypha-Associated Genes (HAGs) and Chromatin Remodeling

The genes induced during hyphal formation have been referred to as hypha-specific genes (HSGs), in contrast to genes involved during yeast growth—yeast-specific genes (YSGs). However, it has been shown that HSGs can also be expressed by yeast cells under certain conditions (Fradin et al. 2005) and that hyphal growth does not require the induction of all HSGs (Naseem et al. 2015); thus, they can be referred to as hypha-associated genes (HAGs). During the morphogenetic transition, HAGs are under the control of several TFs, such as Efg1, Tec1, Nrg1, Tup1, Rim101, Bcr1, and Rfg1 (Eckert et al. 2007; Whiteway and Bachewich 2007). During the yeast-to-hypha morphogenetic transition, Efg1 interacts with the histone acetyltransferase (HAT) NuA4 leading to an increase in histone H4 acetylation at the promoter of HAGs (Lu et al. 2008). The Hos2/Set3 histone deacetylase complex (HDAC) acts as a regulator of hyphal development through the cAMP-PKA pathway (Hnisz et al. 2010) and modulates the expression levels of the transcription regulators *BRG1, TEC1, NRG1,* and *EFG1,* acting as both repressor and activator (Hnisz et al. 2012). The GATA family TF Brg1 recruits Hda1 to the promoter of HAGs leading to chromatin modifications that prevent the binding of the repressor Nrg1 (Lu et al. 2012; see below).

The Swi/Snf chromatin remodeler complex has been reported to bind to the promoters of HAGs only in hyphae, and this binding is mediated by Efg1 (Lu et al. 2008). Thus, Efg1 recruits NuA4 to the HAGs' promoters, allowing in turn the recruitment of the Swi/Snf complex in order to activate transcription during the morphogenetic transition (Lu et al. 2008). Moreover, deletion of *SWI/SNF* or other subunits of the complex, such as Snf6, prevents hyphal growth; a *swi1* deletion strain is defective in HAGs expression and is avirulent in a mouse model of systemic infection (Mao et al. 2006; Tebbji et al. 2017).

HAGs form a large group of virulence factors that contribute to pathogenesis. These factors are molecules recognized by the host, such as cell wall proteins (adhesins) and hydrolytic enzymes that contribute to penetration and tissue damage within the host (Hiller et al. 2011). In a reconstituted human epithelium (RHE) model, the expression levels of HAGs increased within 30 min after inoculation, leading to cellular damage (Hiller et al. 2011; Spiering et al. 2010).

In order to penetrate the host tissues, *C. albicans* produces hydrolytic enzymes, such as phospholipases, lipases, and secreted aspartyl proteinases (SAPs). Genes encoding Sap4, Sap5, and Sap6 were shown to be among the most upregulated

HAGs induced by serum and elevated temperature, together with genes encoding a cell wall mannoprotein (*HWP1*), required for hyphal development and yeast adhesion to epithelium, and the adhesin-like protein 3 (*ALS3*) (Nantel et al. 2002). The agglutinin-like sequence (ALS) family is composed of glycosylphosphatidylinositol (GPI)-anchored proteins encoded by eight genes (*ALS1-7* and *ALS9*). Among them, *ALS3* is the best studied and behaves both as an adhesin and as an invasin. As an adhesin, it mediates the attachment to epithelial and endothelial cells, and to extracellular matrix; as an invasin, it binds to the E-cadherin and N-cadherin host receptors, inducing endocytosis (Phan et al. 2007). Further, *C. albicans* can use ferritin as a source of iron and Als3 is involved in this process. It has been proposed that *C. albicans*—in its hyphal form—can bind ferritin by Als3, iron release is then facilitated by acidification of the environment, and uptake is promoted by the reductive pathway (Almeida et al. 2008). Among the HAGs, extent of cell elongation 1 (*ECE1*) was identified in a screen of yeast and hyphal cells (Birse et al. 1993). Its expression was not detected in yeast, but within 30 min after hyphal induction. However, despite the association between *ECE1* expression levels and morphogenesis, *ece1* null mutants are not defective for filamentation, showing that *ECE1* is not essential for this process (Birse et al. 1993). Recently, it was shown that Ece1 is critical for epithelial damage and *C. albicans* recognition by the immune system and that *ECE1* encodes a cytolytic peptide toxin, called "Candidalysin," secreted during infection. *C. albicans* strains lacking this toxin are avirulent in an oropharyngeal candidiasis (OPC) mouse model (Moyes et al. 2016). Hyphal G cyclin 1 (*HGC1*) is another HAG that encodes a cyclin involved in the cell-cycle regulation that plays a role in polarized growth and represses cell separation of hyphae. Mutants lacking *HGC1* are defective for filamentation, but the constitutive expression of *HGC1* is not sufficient to activate morphogenesis (Zheng et al. 2004). Furthermore, the TF Ume6 has a role in *HGC1* regulation, since the *HGC1* expression levels are significantly reduced in *ume6* deletion strains, although there is no evidence of a direct regulation mechanism (Fan et al. 2013).

Morphogenesis is important for the ability of *C. albicans* to cause disease, even if it could be uncoupled to some extent from virulence based on the observation from large-scale studies (Noble et al. 2010; Perez et al. 2013) that some genes that have a role in infectivity do not have an obvious role in morphology and vice versa. Nevertheless, in these large-scale studies, filamentation was tested in only one condition (solid Spider medium), and virulence was only tested in a mouse model of systemic infection (Noble et al. 2010). Therefore, the exact correlation between morphology and virulence remains to be fully appreciated in order to define those factors that contribute independently or simultaneously to these two processes. Yet, the regulation of morphogenesis is critical for *C. albicans* virulence and a detailed understanding of the molecular pathways that regulate the yeast-to-hypha transition will provide important insights into new therapeutic strategies.

4 Morphogenetic Transition: Major Signaling Pathways

Morphogenesis in *C. albicans* is promoted mainly by the cAMP-PKA and the mitogen-activated protein kinase (MAPK) pathways described below. However, other signaling pathways such as the target of rapamycin (TOR) and regulation of Ace2 morphogenesis (RAM) pathways are known to regulate morphogenesis in certain environmental conditions, such as matrix embedding and pH responses (Fig. 1).

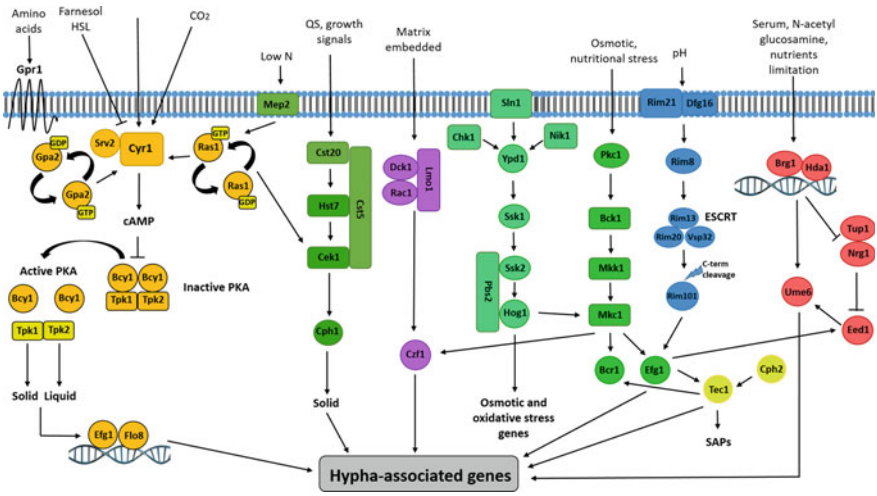


Fig. 1 Major signaling pathways of *C. albicans* morphogenesis. Several environmental factors are known to activate the yeast-to-hypha transition by affecting different components of the represented pathways. The signaling cascades lead to the activation of transcription factors, such as Efg1, Flo8, Cph1, Czf1, Bcr1, and Ume6 that directly influence the expression of hypha-associated genes. Some of these transcription factors are activated in very specific conditions. For instance, Rim101 cleavage and subsequent activation depend on the environmental pH (blue); Czf1 and Cph1 enhance filamentation in matrix-embedded conditions (purple) and on solid media (dark green), respectively. The cAMP-PKA pathway is represented in orange; the adenylate cyclase Cyr1 is directly activated by serum and CO_2. Ras1 activated state also stimulates AC activity, as well as proline and methionine through Gpr1 and Gpa2. Cyr1 associates with Srv2 to increase the intracellular levels of cAMP. cAMP binds to the regulatory subunit of the PKA Bcy1 with the subsequent release of the catalytic subunits Tpk1 and Tpk2, which regulate filamentation in solid and liquid media, respectively. The MAPK pathways are represented in green. The Cek1 pathway leads to the activation of Cph1, the Hog1 pathway whose role in filamentation has not been clarified yet, is involved in the osmotic and oxidative stress response, and the Mkc1 pathway activates several transcription factors, such as Czf1, Bcr1, and Efg1. Activation of the transcription factor Tec1 depends on Cph2 (in yellow) and takes to the SAPs expression, important for virulence. Finally, several stimuli induce the expression of Brg1 (in red), which is recruited at the promoter of HAGs and, together with Hda1, occlude the binding of Nrg1, which represses transcription in a Tup-1-dependent manner. Among the genes subsequently expressed, there is *UME6*, whose expression depends also on the transcription factor Eed1

The cAMP-PKA pathway. The cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) pathway is well conserved across eukaryotes and regulates many cellular processes in fungi. PKA is a heterotetramer consisting of two regulatory subunits that bind and inactivate two catalytic subunits. cAMP activates PKA by a conformational change that occurs upon binding. The cAMP intracellular levels are controlled by the AC Cyr1, which synthesizes cAMP from ATP, and by phosphodiesterases, which in contrast hydrolyze cAMP. AC activity is controlled by G protein-coupled receptors. There are two classes of G proteins: the monomeric small GTPases and the heterotrimeric G protein complexes, consisting of three subunits, namely α, β, and γ. Both G protein types are activated upon GTP binding and inactivated when GDP is bound. The exchange between GDP and GTP is catalyzed by GTPase-activating proteins (GAPs) or guanine exchange factors (GEFs).

In *S. cerevisiae,* the cAMP-PKA pathway is required as the major intermediate by which glucose or other nutrients regulate the cell cycle (Colombo et al. 2017; Fuller and Rhodes 2012). Furthermore, it is involved in pseudohyphal differentiation in response to nitrogen starvation and requires Gpr1, a seven-transmembrane-spanning receptor able to bind glucose (Lorenz et al. 2000), which activates the homolog of the mammalian G-α subunit, Gpa2 (Kubler et al. 1997; Lorenz and Heitman 1997). This signaling culminates in the activation of the TFs Flo8 and Sfl1 (Pan and Heitman 2002). In nitrogen starvation conditions, Gpr1 binds to Gpa2 (Harashima and Heitman 2005; Xue et al. 1998) leading to the activation of cAMP production through the AC Cyr1 (also called Cdc35) upon glucose induction (Colombo et al. 1998; Kraakman et al. 1999; Lorenz et al. 2000). α-Gpa2 does not form a heterotrimeric complex with the known yeast Gβγ subunits Ste4/18, but with Gpb1/2 and Gpg1 proteins (Harashima and Heitman 2005; Kraakman et al. 1999).

Furthermore, the G protein Ras2 can activate cAMP generation by Cyr1 (Minato et al. 1994). In fungi, Ras signaling has been associated to cell shape (Cullen and Sprague 2012), stress response (de la Torre-Ruiz et al. 2010), mating (Alspaugh et al. 2000), and nutrient sensing (Broach 2012). In *S. cerevisiae*, the role of Ras1 and Ras2 is not totally clear: current model suggests that low-level sugar phosphorylation activates the Ras-mediated localization of Cyr1 to the plasma membrane, where Gpr1-Gpa2 can trigger the signaling (Colombo et al. 2004; Thevelein and de Winde 1999). In contrast, Pde1 and Pde2, respectively low- and high-affinity cAMP phosphodiesterases, negatively regulate cAMP levels (Hu et al. 2010; Ma et al. 1999). PKA comprises a single regulatory subunit, Bcy1 (or Sra1) (Toda et al. 1987), and three catalytic subunits Tpk1, Tpk2, and Tpk3 (Toda et al. 1987). The Tpk2 subunit activates pseudohyphal differentiation, whereas Tpk1 and Tpk3 play a negative role, inhibiting filamentous growth (Pan and Heitman 1999; Robertson and Fink 1998). Tpk2 mediates the binding of the TF Flo8 and inhibits the binding of the negative regulator Sfl1 to the promoter of the *FLO11* gene, which encodes a GPI-anchored cell surface adhesin (Kobayashi et al. 1996; Rupp et al. 1999), through Flo8 and Sfl1 phosphorylation (Pan and Heitman 2002).

In *C. albicans,* the cAMP-PKA pathway mediates different cellular processes, such as cell-cycle regulation and stress response (Cao et al. 2017; Giacometti et al. 2009), but it is one of the major pathways that positively regulate filamentation, involving the TFs Efg1 and Flo8 (Bockmuhl and Ernst 2001; Cao et al. 2006) (Fig. 1). Indeed, reduced activity of PKA results in defects in filamentation (Bockmuhl et al. 2001; Sonneborn et al. 2000), and a *flo8* deletion mutant is blocked in filamentation and in the expression of HAGs, leading to avirulence in a mouse model of systemic infection (Cao et al. 2006). Many factors have been described that activate filamentation through this pathway. For instance, amino acids such as proline or methionine stimulate hyphal growth via Gpr1 and Gpa2 (Maidan et al. 2005; Miwa et al. 2004). CO_2 also activates AC by directly stimulating Cyr1 catalytic domain (Klengel et al. 2005; Parrino et al. 2017). Furthermore, serum strongly induces filamentation through the muramyl peptide, which binds to Cyr1 and stimulates AC activity (Xu et al. 2008). The cAMP levels are negatively regulated by the phosphodiesterases Pde1 and Pde2, as reported in *S. cerevisiae* (Hoyer et al. 1994; Jung and Stateva 2003). Ras1 is activated by the GAP Csc25 (also known as Cdc25). The Ras1 active state stimulates the AC activity of Cyr1, which interacts with the AC-associated protein Srv2 (also known as cyclase-associated protein Cap1) in order to increase the intracellular cAMP levels (Fig. 1). Cyr1 physically interacts with Ras1 through the Ras association (RA) domain in the presence of serum (Fang and Wang 2006). The increased cAMP binds to Bcy1 leading to the release of the two PKA catalytic subunits encoded by *TPK1* and *TPK2* (there is no Tpk3 in *C. albicans*). Either Tpk1 or Tpk2 can activate TFs such as Efg1, by phosphorylation (Bockmuhl and Ernst 2001) (Fig. 1). Both subunits have been linked to different cues: Tpk1 is reported to be critical for hyphal development on solid-inducing medium, while Tpk2 has a role in filamentation only in liquid medium (Bockmuhl et al. 2001). Although the mechanisms that activate Ras1-mediated hyphal growth are not fully understood, it is known that incubation at 37 °C induces filamentous growth through the molecular chaperone Hsp90, which has an important role in the regulation of Ras1 and AC activity in response to temperature. However, Hsp90 works via effectors downstream of PKA and distinct from Efg1 (Shapiro et al. 2009).

The Ras-cAMP-PKA pathway has been involved in virulence (Bahn et al. 2003; Hogan and Sundstrom 2009; Leberer et al. 2001). For instance, Tpk2 is required for germination and engulfment in epithelial cells and for virulence in a mouse model of OPC (Sanchez AA 2005). This pathway regulates Efg1, which is considered as the major regulator of the morphogenetic transition in *C. albicans* (Braun and Johnson 2000). *EFG1* encodes a basic helix-loop-helix (bHLH) TF that belongs to the APSES family of fungus-specific regulators (Doedt et al. 2004). The role of Efg1 in filamentation is complex: Loss of *EFG1* results in a defect in filamentation under hypha-inducing conditions (Lo et al. 1997); however, under microaerophilic or embedded conditions, hyphal development is normal in an *efg1* deletion strain (Giusani et al. 2002). Therefore, under hypoxic conditions, Efg1 functions as a repressor of hyphal growth on agar at temperatures below 35 °C (Setiadi et al. 2006), while under normoxia, Efg1 is a strong inducer of hyphal growth (Lo et al.

1997; Stoldt et al. 1997). The hypoxic repressor function is mediated by modifications at the Efg1 N-terminus (Desai et al. 2015). Genome-wide chromatin immunoprecipitation (ChIP) analysis showed that the promoter regions bound by Efg1 in yeast-form cells are different from those bound in hyphal cells (Lassak et al. 2011). These evidences suggest a double role for Efg1, either as a repressor or as an activator of filamentation, depending on the environmental cues. Indeed, while in a model of systemic bloodstream infection (increased oxygen levels) the *efg1* mutant was much less virulent than the WT (Bendel et al. 2003; Lo et al. 1997), in hypoxic niches, as in the mouse gut, the *efg1* mutant was hyperproliferative (Pande et al. 2013; White et al. 2007). Additionally, it was reported that the changes in *EFG1* expression, depending on histone modifying enzymes, are important to allow *C. albicans* to better adapt in the host, establishing optimal survival strategies (Tyc et al. 2016). A transcriptomic analysis showed an overlap between the targets of Flo8 and Efg1, and a physical interaction between these two proteins was demonstrated (Cao et al. 2006).

The MAPK pathways. The mitogen-activated protein kinase (MAPK) signal transduction pathway comprises a conserved module of three kinases: the MAP kinase kinase kinase (MAPKKK), the MAP kinase kinase (MAPKK), and the MAP kinase (MAPK). The signaling depends on three phosphotransfer steps, where the MAPKKK becomes phosphorylated and phosphorylates the MAPKK, which in turn phosphorylates the MAPK.

In *S. cerevisiae,* the MAPK cascade is used to mediate various cellular functions, such as osmolarity adaptation (Hohmann 2009), mating (Schrick et al. 1997), cell wall integrity (Levin 2005), and filamentation. Indeed, the genome of *S. cerevisiae* encodes multiple MAPKs. In order to activate morphogenesis, the G protein Ras2 stimulates the activity of the G protein Cdc42, upon nitrogen starvation signals. Cdc42 binds to GTP, becomes active, and interacts with the p21-activated kinase (PAK) Ste20, which needs to be dissociated from its negative regulator Hsl17 (Fujita et al. 1999). The Cdc42-Ste20 dimer activates the MAPK cascade: Ste11 (MAPKKK), Ste7 (MAPKK), and Kss1 (MAPK) (Cook et al. 1996; Madhani et al. 1997). Once activated, Kss1 removes repressor activity of Dig1/Dig2 dimer (also known as Rst1/Rst2) on the TF Ste12 through Dig1/Dig2 phosphorylation (Bardwell et al. 1998a, b; Cook et al. 1996). Ste12 cooperates with the TF Tec1 to bind the enhancers called filamentation and invasion response elements (FREs) and to start the transcriptional program required for filamentous growth by the activation of genes, such as *FLO11* (Chou et al. 2006; Lo and Dranginis 1998; Madhani and Fink 1997). In *C. albicans*, three different MAPK pathways have been described: the Hog1-MAPK, the Mkc1-MAPK, and Cek1/Cek2 (see below). All the MAPK signaling pathways serve as a pattern of cascades essential for *C. albicans* morphogenesis, but they are also required under different conditions and they are activated by different stimuli. Hence, in contrast to *S. cerevisiae* the MAPK pathways of *C. albicans* show a broader function, and all of them play a role in morphogenesis, suggesting the importance of phenotypic plasticity for *C. albicans* in order to adapt and survive in different host niches.

The Hog1-MAPK pathway. Activation of the Hog1-MAPK pathway culminates in the phosphorylation, activation, and nuclear translocation of Hog1. In *S. cerevisiae*, this pathway is involved in osmolarity adaptation. Two separate branches have been described, where independent transmembrane osmosensors converge at the MAPKK Pbs2, which activates the Hog1 MAPK (Hohmann 2009). The Sln1 branch, the so-called two component system, is controlled by the plasma membrane-localized sensor Sln1, a histidine kinase active under low osmolarity conditions. Sln1 inactivates Ssk1 via the phosphorelay protein Ypd1. When osmolarity increases, Sln1 is inactivated, the phosphorylation steps are interrupted, and the unphosphorylated form of Ssk1 accumulates. Ssk1 binds to the regulatory domain of Ssk2 and Ssk22 MAPKKKs, triggering their autophosphorylation and activation (Posas and Saito 1998). Then, Ssk2 and Ssk22 phosphorylate and activate Pbs2 MAPKK, which in turn phosphorylate the MAPK Hog1 (de Nadal et al. 2002; Hohmann 2002; Maeda et al. 1995; Posas et al. 1996). The Sho1 branch is controlled by two mucin-like transmembrane sensors, namely Msb2 and Hkr1 (de Nadal et al. 2007; Tatebayashi et al. 2007). These sensors might monitor the movements between the cell wall and the plasma membrane, since mucins connect the cell interior with the cell wall. Sho1 is a membrane-localized scaffold protein, recruiting components to the cell surface where remodeling is necessary (Reiser et al. 2000). Sho1 is activated under high osmolarity conditions, and the signaling to Pbs2 requires the Ste11 MAPKKK, which interacts with Ste50, Ste20, and the small GTPase Cdc42 (de Nadal et al. 2002; Posas et al. 2000).

S. cerevisiae harbors only one transmembrane histidine kinase (Sln1), but three are found in *C. albicans*, namely Sln1, Nik1, and Chk1 (Calera et al. 1999; Nagahashi et al. 1998) (Fig. 1). Deletion of Ca*SLN1* only slightly affects the osmotic stress tolerance (Nagahashi et al. 1998). The second putative kinase, Nik1, is the homolog of *Neurospora crassa Nik1* and is required for osmotolerance and filamentation (Alex et al. 1996; Alex et al. 1998; Srikantha et al. 1998). Finally, CaChk1 modulates the expression of cell surface components (Kruppa et al. 2004), showing a role in virulence and morphogenesis (Calera et al. 1999; Calera and Calderone 1999).

Homologs of *S. cerevisiae* components of the osmostress pathway (*PBS2, SSK1* and *SHO1*) have also been identified in *C. albicans*, but they exhibit different functions as compared to their counterparts in *S. cerevisiae*. Deletion of *PBS2* prevents the relay of both osmotic and oxidative stress signals to Hog1 (Arana et al. 2005). In contrast, deletion of *SSK1* prevents activation of Hog1 in response to oxidative stress. This contrasts with the observations in *S. cerevisiae* where inactivation of both the Sho1- and the Sln1 osmosensing branches prevents relay of osmotic stress signals to Hog1 (Maeda et al. 1995). Furthermore, *sho1* mutants are sensitive to oxidative stress, but Sho1 has a minor role in the transmission of the phosphorylation signal to Hog1 in response to oxidative stress, which probably occurs through the Sln1-Ssk1 branch (Roman et al. 2005). However, which histidine kinase regulates Ssk1 in response to oxidative stress is unclear, since *sln1* mutant are still able to activate the pathway in response to H_2O_2 (Li et al. 2004; Roman et al. 2005). Furthermore, a *sho1* null mutant is sensitive to cell wall

interfering compounds, showing modifications in the cell wall architecture (Roman et al. 2005). Therefore, in *C. albicans* the Hog1 pathway is involved not only in both osmotic (Arana et al. 2005; San Jose et al. 1996) and oxidative (Alonso-Monge et al. 2003; Correia et al. 2017; Du et al. 2005) stresses, but also in cell wall biosynthesis (Alonso-Monge et al. 1999, 2001; Monge et al. 2006; O'Rourke and Herskowitz 1998). Additionally, it plays a role in morphogenesis: *hog1* and *pbs2* mutants show enhanced filamentation in non-inducing conditions (Alonso-Monge et al. 1999; Arana et al. 2005). However, a direct role of Hog1 in the regulation of morphogenetic gene targets has not been established yet.

The Mkc1-MAPK pathway. In *S. cerevisiae,* protein kinase C (Pkc1) is involved in response to a large number of stresses, including osmotic (Garcia-Rodriguez et al. 2005) and nutritional (Vilella et al. 2005) stresses. Pkc1 activation leads to a MAPK module consisting of the bypass of C protein kinase (Bck1) MAPKKK that activates the Mkk1 MAPKK, which in turn phosphorylates the Mpk1 MAPK. In *C. albicans, pkc1* mutants are sensitive to osmotic stress, but do not display any filamentation defect (Paravicini et al. 1996), whereas Pkc1 activates the Mkc1 pathway that in contrast has a role in morphogenesis (Navarro-Garcia et al. 1995). The MAPK Mkc1, the homolog of ScMpk1, plays a role in cellular integrity and cell wall formation (Navarro-Garcia et al. 1995) and is essential for invasive growth under embedded conditions and for biofilm formation (Kumamoto 2005). This suggests that Mkc1 signaling is relevant for processes dependent on physical interaction with external surfaces (Eisman et al. 2006). Further, *mkc1* mutants are sensitive to high temperatures, lytic enzyme preparations, and cell wall antifungals, indicating an additional role of Mkc1 in the construction of the cell wall (Navarro-Garcia et al. 1995; Navarro-Garcia et al. 1998). Mkc1 is also phosphorylated upon oxidative stress (as described for Hog1), osmotic stress, antifungal treatment, in the presence of calcium and at low temperatures. The targeted TFs or those indirectly induced by Mkc1 remain to be identified, but possible candidates are Efg1, Czf1, and Bcr1 (Biswas et al. 2007) (Fig. 1). Bcr1 is involved in *C. albicans* hyphal development and virulence; its expression depends on the hyphal regulator Tec1 (Nobile and Mitchell 2005), a member of the TEA/ATTS family of TFs. Tec1 regulates virulence because it is required for the expression of the SAPs and for filamentation in order to escape macrophages (Schweizer et al. 2000). The SAP gene family encodes at least nine members (Sap1 to Sap9), considered putative virulence factors. Sap activity has been demonstrated to be important for attachment and penetration in the host during infection (Borg and Ruchel 1988; Ollert et al. 1993). The Sap4, Sap5, and Sap6 proteins are activated during hyphal formation (Hube et al. 1994), while the *sap4 sap5 sap6* triple mutant is avirulent in vivo (Sanglard et al. 1997). *TEC1* expression has been shown to be regulated by Efg1, providing an edifying example of how *C. albicans* can converge different signals to regulate a common set of differentially expressed genes (Lane et al. 2001) (Fig. 1).

In *C. albicans, TEC1* transcription might depend on a TF named Cph2 (Lane et al. 2001). Cph2 belongs to the myc subfamily of bHLH proteins and regulates hyphal growth independently of either the MAPK or the cAMP-PKA pathways.

A *cph2* deletion strain shows medium-specific impairment in hyphal growth (Lane et al. 2001). Further, ectopic expression of *TEC1* suppressed the filamentation defects in *cph2* null mutant, suggesting that Cph2 function in hyphal growth is mediated, in part, through Tec1. Furthermore, Cph2 may also play a direct role in the transcriptional activation of some HAGs (Lane et al. 2001).

The Cek1-MAPK pathway. The *Candida* ERK-like kinase (Cek1) MAPK pathway was first discovered in *C. albicans* because of its ability to confer a cell-cycle-specific arrest when overexpressed in *S. cerevisiae* (Whiteway et al. 1992). *S. cerevisiae* homolog of Cek1, Fus3, is part of a pathway participating in mating (Elion 2000), invasive growth (Palecek et al. 2002), and vegetative growth (Cullen et al. 2000; Lee and Elion 1999). In *C. albicans*, the Cek1-MAPK pathway ends up with the activation of the TF Cph1, a Ste12 homolog (Liu et al. 1994) (Fig. 1). Interestingly, Cph1 is required for filamentation on solid media, but not in liquid (Leberer et al. 1996; Liu et al. 1994) and is dispensable for virulence in a murine model of systemic candidiasis (Liu et al. 1994). The *efg1 cph1* double mutant fails to form filaments in hypha-inducing conditions and is avirulent in a systemic mouse model of candidiasis (Lo et al. 1997). However, under certain conditions, such as matrix-embedded, the *efg1 cph1* double mutant is still able to filament, suggesting the presence of other pathways for hyphal development that involve other TFs (e.g., Czf1). Several studies have highlighted the involvement of the Cek1-MAPK pathway in morphogenesis and filamentation. For instance, deletion of *CEK1* results in filamentation defects in media where nitrogen source is limiting (Csank et al. 1998). As mentioned before, nitrogen starvation is one of the signals that trigger the morphogenetic transition (Csank et al. 1998; Tripathi et al. 2002). *C. albicans* has two genes encoding ammonium permeases (*MEP1* and *MEP2*) that, in addition to their role as transporters, enable growth when limiting concentrations of ammonium are the only available nitrogen source. Mep2 has also a central role in the induction of filamentation on solid surface under limiting nitrogen conditions (Biswas and Morschhauser 2005). It has been proposed that Mep2 triggers the Cph1/Cek1-mediated pathway in a Ras1-dependent manner (Biswas and Morschhauser 2005), and mutations of components of this pathway lead to reduced virulence (Marcil et al. 2002). Finally, Cek1-MAPK is also activated by growth signals (Roman et al. 2005), by quorum sensing (Sato et al. 2004), and is involved in cell wall biogenesis (Roman et al. 2005) and in mating (Magee et al. 2002).

Negative regulators of morphogenesis and chromatin remodeling. The hyphal induction program is also under negative regulation by the TFs Nrg1, Rfg1 (Kadosh and Johnson 2005), and Sfl1 (see below). Another negative regulator is Tup1, but this protein does not directly bind DNA. In *S. cerevisiae*, Tup1 regulates genes involved in glucose homeostasis, DNA damage, and oxygen stress response (DeRisi et al. 1997), forming a transcriptional corepressor complex with Ssn6 (Smith and Johnson 2000). In *C. albicans*, the *tup1* mutant constitutively grows in a filamentous form, in the absence of hypha-inducing conditions (Braun and Johnson 1997). However, Tup1 is not only a repressor of filamentation, since the *tup1*

deletion strain has misshapen cell wall and is not able to grow at 42 °C (Braun and Johnson 2000).

SSN6 encodes a global transcriptional repressor, whose overexpression increases filamentation and decreases virulence (Hwang et al. 2003). As stated above, the Ssn6–Tup1 corepressor complex does not interact directly with DNA, but is targeted to specific promoters through interaction with sequence-specific DNA-binding proteins (i.e., TFs) (Keleher et al. 1992; Smith and Johnson 2000). An independent role of Ssn6 has been reported, namely through its interaction with the HDAC Rpd31. The *ssn6 rpd31* double mutant is able to form elongated cells, but is unable to extend filaments, suggesting that Ssn6 has a dual role in filamentation depending on the interaction with Rpd31 (Lee et al. 2015).

The Nrg1 TF contains a highly conserved zinc finger domain. In *C. albicans*, Nrg1 represses transcription in a Tup1-dependent manner (Braun and Johnson 2000; Murad et al. 2001). Ectopic overexpression of *NRG1* blocks hyphal growth in all conditions (Sanchez AA 2005) and attenuates virulence in a systemic infection mouse model (Saville et al. 2006). Another TF interacting with the Tup1-Ssn6 complex is repressor of filamentous growth 1 (Rfg1), homolog of ScRox1 (Khalaf and Zitomer 2001). However, *RFG1* overexpression does not inhibit hyphal development in vitro or in vivo and drives pseudohyphal formation under yeast-growth conditions (Cleary et al. 2010).

Molecular mechanisms involved in the initiation and extension of hyphal development. The two phases of hyphal development, namely initiation and maintenance, are sustained in *C. albicans* by two parallel pathways active either in air or in hypoxia plus CO_2 (Fig. 2) (Lu et al. 2013). Serum, GlcNAc, or nutrient limitation induce the expression of the activator Brg1, which is recruited to the promoter of HAGs through changes in chromatin that occlude the binding of the repressor Nrg1 (Fig. 2). Both hyphal elongation pathways require the initiation step with a transient downregulation of Nrg1 via the cAMP-dependent PKA pathway; at the same time, Brg1 and the hypha-specific TF Ume6 are upregulated (Fig. 2). Recently, Hir1, a subunit of the HIR histone chaperone complex, has been shown to be involved in hyphal initiation, providing evidence of the tight control of gene expression during yeast-to-hypha transition (Jenull et al. 2017). Afterward, a window of opportunity permits to choose the maintenance phase: either involving Brg1 and Hda1 (air), or initiating a positive feedback loop by stabilizing Ume6 (hypoxia plus high CO_2 levels) (Fig. 2) (Lu et al. 2013). In the first case, maintenance needs the HDAC Hda1 recruitment to promoters, allowing a chromatin remodeling that blocks Nrg1 access at the promoters of HAGs (Lu et al. 2011, 2014). Brg1 recruits Hda1 to promoters of HAGs under reduced Tor1 signaling for hyphal maintenance (Lu et al. 2012). In the feed-forward regulation of hyphal development, Brg1 regulates the expression of Ume6 (Lu et al. 2012). Hyphal extension and maintenance depend on the TF Ume6 and the Eed1/Def1 protein.

In *C. albicans, UME6* encodes a filament-specific transcription regulator sufficient to direct hyphal growth in the absence of hypha-inducing conditions (Zeidler et al. 2009). This differs greatly from *S. cerevisiae* Ume6, whose function is to regulate arginine catabolism (Messenguy et al. 2000), DNA repair (Sweet et al.

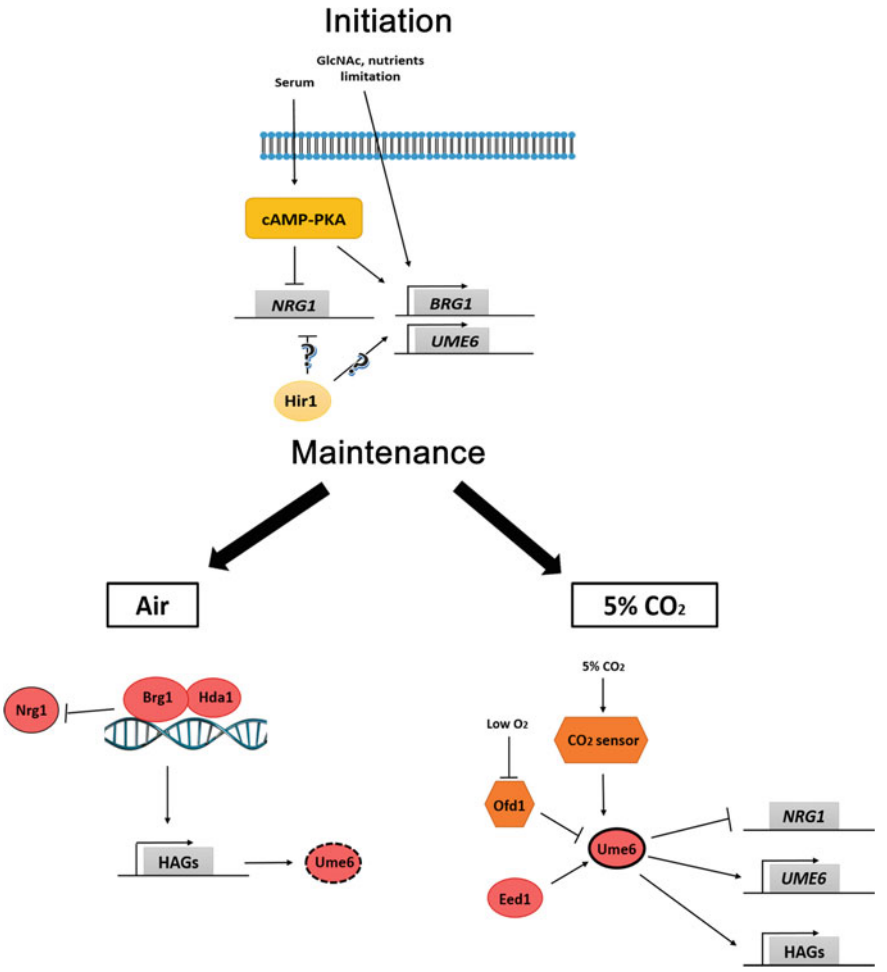


Fig. 2 Hyphal initiation and maintenance. Both hyphal elongation pathways require the initiation step characterized by a transient downregulation of Nrg1 via the cAMP-dependent PKA pathway, and upregulation of Brg1 and Ume6. Serum, GlcNAc, or nutrient limitation induce the expression of Brg1. Hir1, a subunit of the HIR histone chaperone complex, has been shown to be involved in hyphal initiation, but its exact function remains to be elucidated. The maintenance phase consists of two parallel pathways active either in normoxia or hypoxia + CO_2. In normoxia, Brg1 recruits the HDAC Hda1 to the promoters of HAGs, allowing a chromatin remodeling that prevents Nrg1 binding to these promoters. Brg1 regulates the expression of Ume6, which is unstable under normoxia and is degraded (dashed lines). Under hypoxia and in the presence of 5% CO_2, the oxygen sensor Ofd1 and an uncharacterized CO_2-sensing pathway stabilize Ume6 (thick full line), leading to hyphal development through a positive feedback loop where Ume6 binds to its own promoter, regulates HAGs expression, and inhibits *NRG1* expression. Furthermore, Ume6 is regulated by Eed1

1997), and meiosis (Rubin-Bejerano et al. 1996). *C. albicans ume6* deletion mutants are defective in hyphal growth and are attenuated in virulence (Banerjee et al. 2008), while constitutive overexpression promotes hyperfilamentation in the absence of filament-inducing conditions, tissue invasion, and virulence (Banerjee et al. 2008; Carlisle et al. 2009; Zeidler et al. 2009). Ectopic overexpression of *UME6* is able to rescue the hyphal growth defects observed in *brg1* and *hda1* mutants (Lu et al. 2012).

As described before, hyphal maintenance can occur through two different pathways (air or hypoxia plus CO_2). In response to hypoxia and 5% CO_2, the oxygen sensor Ofd1 and an uncharacterized CO_2-sensing pathway stabilize Ume6, leading to hyphal development through a positive feedback loop where Ume6 binds to its own promoter (Fig. 2). Furthermore, Ume6 is degraded under normoxia, while very stable in hypoxia or hypercapnia: it was reported that deleting Ofd1 resulted in stabilizing Ume6 in 5% CO_2, but not in air (Lu et al. 2013). Additionally, *UME6* has one of the longest 5' untranslated regions (UTRs) identified in fungi, which is predicted to form a complex and stable secondary structure. The 5' UTR region functions to inhibit Ume6 protein expression under several filamentous-inducing conditions. Overall, the translational efficiency mechanism has evolved to fine-tune morphogenesis, and in this case, the 5' UTR region of *UME6* is found to regulate the protein translational efficiency (Childers et al. 2014). Furthermore, Ume6 is regulated posttranslationally by the cell-cycle kinase Cdc28/Cdk1, which reduces Ume6 activity, and the cyclin Cln3, an essential promoter of yeast proliferation, suppresses hyphal induction through Ume6 (Mendelsohn et al. 2017).

In addition to the TFs already described, *UME6* expression depends on epithelial escape and dissemination (*EED1*, also known as *DEF1*) (Martin et al. 2011) (Fig. 1). *EED1* was identified in a genome-wide analysis in a RHE model. It was shown that an *eed1* mutant strain was still able to invade superficial oral epithelial cells, but in these cells the mutant grew as yeasts, without dissemination in tissues (Zakikhany et al. 2007). *EED1* is unique to *C. albicans,* and no homolog has been detected in the genomes of the species that belong to the CUG clade, including species closely related to *C. albicans* (Butler et al. 2009), even if orthologs of its flanking genes exist in other fungi (Martin et al. 2011). Furthermore, it seems that *EED1* does not have any DNA-binding domain; however, it is one of the key outputs of the signal transduction pathways that lead to activation of HAGs (Sudbery 2011). The reason why *EED1* has evolved specifically in *C. albicans* could be linked to its importance in the biology of filamentation and possibly to pathogenesis. In fact, Eed1 is important for hyphal development on solid media and for the interaction with host cells, but ectopic overexpression of *UME6* restored hyphal defects of *eed1* loss of function (Martin et al. 2011). The suggested model proposes that Eed1 expression is regulated by Efg1 (Martin et al. 2011) and by Nrg1 (Doedt et al. 2004; Martin et al. 2011), and this is the primary element of a cycle controlling hyphal extension. The second step involves *UME6* activation by Eed1, and this may be required to keep *NRG1* expression at low levels to allow hyphal maintenance (Martin et al. 2011). The mechanism by which Eed1 regulates Ume6 remains unclear, and it might require additional players.

5 Morphogenetic Transition: Other Signaling Pathways

The matrix-embedded response. The ability to respond to the matrix-embedding environment is significant for *C. albicans* growing within host tissues. As already mentioned, the TF Czf1 is involved in the regulation of hyphal growth under these conditions (Brown et al. 1999), but the master regulator Efg1 and the TF Cph1 are also associated with the response to the matrix (Fig. 1). Ectopic expression of *CZF1* in embedded cells promotes hyphal formation. However, *czf1* mutants exhibit a defect in filamentation only in some embedding conditions (low temperature, in the absence of strong inducers of hyphal growth). Mutants lacking both *EFG1* and *CPH1*, although defective in germ tube formation under many laboratory conditions (Lo et al. 1997), were still able to filament in the tongues of immunosuppressed gnotobiotic piglets and in embedded conditions (Riggle et al. 1999). In contrast, strains lacking both *CPH1* and *CZF1* displayed the most defective phenotype in a matrix-embedding environment, suggesting that these genes play an important role in hyphal growth under these conditions (Brown et al. 1999). Efg1 is a positive regulator of *CZF1* expression, as it binds to its promoter, and deletion of *EFG1* abolishes *CZF1* expression. On the other hand, *CZF1* expression is negatively autoregulated, through direct binding to own promoter (Vinces et al. 2006). This suggests that a different genetic program is involved in filamentous growth in embedded conditions (Vinces et al. 2006).

Another signaling pathway regulating filamentation in embedded conditions involves the G protein Rac1 (Hope et al. 2008) (Fig. 1). Rac1 is required for filamentous growth in an agar matrix (Bassilana and Arkowitz 2006) and is activated upon interaction with the GEF Dck1 (Hope et al. 2008). Rac1 and Dck1 function together with Lmo1, a protein similar to the human engulfment and cell motility (ELMO1) protein, upstream the Cek1 and Mkc1 MAP kinase pathways for invasive filamentous growth and cell wall integrity (Hope et al. 2010) (Fig. 1).

The pH-dependent response. *C. albicans* is highly tolerant to a wide range of pH conditions encountered in several niches within the human body. As already mentioned, pH fluctuations drive the morphological transition; moreover, *C. albicans* actively neutralizes the environment from either acidic or alkaline pHs. Under acidic conditions, the fungus is able to raise the pH from 4 to >7 in less than 12 h, resulting also in yeast-to-hypha transition (Vylkova et al. 2011).

In the pH-dependent filamentation, the Rim101 TF (also called Prr2 for pH response regulator) has a central role, by inducing the expression of alkaline-specific genes and repressing the expression of acid-specific genes (Fonzi 1999; Ramon et al. 1999). The extracellular pH is sensed by the transmembrane proteins Rim21 and Dfg16 (Barwell et al. 2005; Penalva et al. 2002) (Fig. 1). The protein Rim8 (or Prr1) has a crucial role, since its expression is activated in low pH conditions and mutants lacking *RIM8* are defective in pH-dependent regulation of gene expression (Porta et al. 1999). Rim20 and Rim13 are also part of the pathway: They form the endosomal sorting complexes required for transport (ESCRT), required for endocytic trafficking, and together with Vps32 (also called Snf7)

activate the transcription factor Rim101 by proteolytic cleavage of its C-terminal domain (Cornet et al. 2005; Kullas et al. 2004) (Fig. 1). Under alkaline conditions, Rim101 is active in its shorter form, while in acidic conditions, it is processed in a longer form whose function is not known (Li et al. 2004). Once activated, Rim101 binds to the promoter regions of different genes, activating *PRA1* and *PHR1* and repressing *PHR2* (Ramon et al. 1999; Ramon and Fonzi 2003; Baek et al. 2006). *PHR1* and *PHR2* are required for normal morphology at pH above or below 5.5, respectively. These genes encode GPI-anchored glycosidases that localize at the plasma membrane and are involved in cross-linking cell wall glucans (Fonzi 1999; Muhlschlegel and Fonzi 1997). *PRA1* encodes a cell wall protein whose deletion is associated to morphological defects in alkaline-induced filamentation (Sentandreu et al. 1998) and host zinc acquisition (Citiulo et al. 2012). The pH response represents another example of the dynamic interaction and adaptation of *C. albicans* to its host environment.

The Target of Rapamycin (TOR) pathway. Rapamycin, produced by the bacterium *Streptomyces hygroscopicus*, was discovered in a screen for antimicrobial activity against *C. albicans* and later found to have a potent immunosuppressive activity (Baker et al. 1978; Vezina et al. 1975). Rapamycin binds to its intracellular receptor FKBP (FK506-binding protein), and this complex inhibits the activity of a phosphatidylinositol 3-kinase-related kinase, the target of rapamycin (TOR), which is important for cell growth (Schmelzle and Hall 2000). There are two highly homologous TOR proteins in *S. cerevisiae*, Tor1 and Tor2 (Kunz et al. 1993). In addition to rapamycin-dependent function, Tor2 also regulates cytoskeletal dynamics during polarized cell growth (Helliwell et al. 1998). Furthermore, two different TOR complexes have been found in *S. cerevisiae* (Loewith et al. 2002; Reinke et al. 2004; Wullschleger et al. 2005): target of rapamycin complex 1 (TORC1), which contains either Tor1 or Tor2 and mediates the TOR-shared, rapamycin-sensitive pathway, and target of rapamycin complex 2 (TORC2), which contains Tor2 and mediates a TOR2-unique, rapamycin-insensitive, pathway. Within the TORC1 complex, Tor1 is conserved from yeast to humans. In *C. albicans*, Tor1 participates in a signaling pathway involved in cellular growth in response to nutrient availability (Rohde et al. 2008; Rohde and Cardenas 2004) and it mediates repression in cell–cell adhesion (Bastidas et al. 2009). In fact, Tor1 controls the expression of adhesin genes by interacting with the transcriptional activators Bcr1 and Efg1. In addition, it promotes the expression of the transcriptional repressors Nrg1 and Tup1 (Fig. 3) (Bastidas et al. 2009). Tor1 signaling during hyphal initiation activates the expression of the transcription factor Brg1, which recruits the HDAC Hda1 to the promoters of hypha-specific genes (Fig. 3) (Su et al. 2013). One of the TORC1 substrates is the kinase Sch9, which in *C. albicans* is involved in cellular growth, stress response, and filamentation under certain conditions. In particular, CaSch9 regulates filamentation differentially in liquid and solid media (Liu et al. 2010). This observation appears consistent with the fact that CaTor1 is required for filamentation on agar surface, but not in liquid medium (Bastidas et al. 2009). Further, Sch9 controls hypha formation in response to oxygen and CO_2 levels: *sch9* mutants showed a striking hyperfilamentation

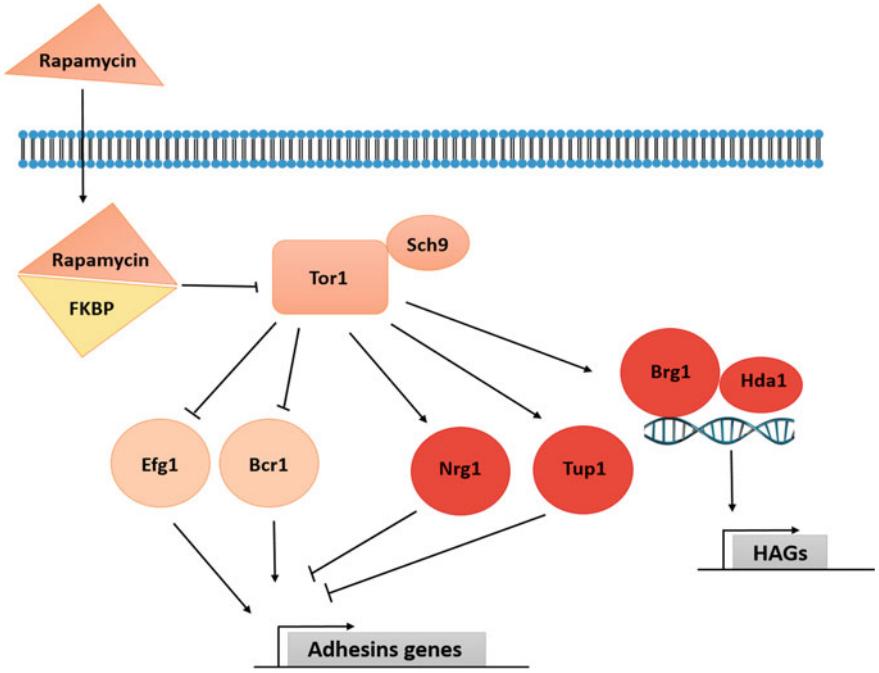


Fig. 3 TOR pathway during morphogenesis. Rapamycin binds to its intracellular receptor FKBP forming a complex that inhibits the activity of Tor1. Tor1 regulates adhesins genes expression by interacting with Bcr1 and Efg1 and promotes the expression of Nrg1 and Tup1. During hyphal initiation, Tor1 signaling activates the expression of Brg1, which recruits the HDAC Hda1 to the promoters of HAGs

under hypoxia, but only in the presence of elevated CO_2 levels (>1%) and at a high temperature (>37 °C). Notably, to generate this hyperfilamentous phenotype, the transcription factors Czf1 and Ace2, as well as both protein kinase A isoforms, were required (Stichternoth et al. 2011).

The Regulation of Ace2 and Morphogenesis (RAM) pathway. The RAM signaling pathway is involved in the regulation of polarized morphogenesis, cellular separation, and bud development in *S. cerevisiae* (Nelson et al. 2003). This pathway converges in the activation of Ace2, a TF whose activity is regulated by a set of six proteins that include the Cbk1 kinase, its activator Mob2, Tao3 (Pag1), Hym1, Sog2, and the Ste20-like protein kinase Kic1 (Fig. 4) (Bidlingmaier et al. 2001; Colman-Lerner et al. 2001; Du and Novick 2002; Nelson et al. 2003; Weiss et al. 2002). During cellular division, the Cbk1–Mob2 complex blocks Ace2 in the nucleus of the daughter cell where Ace2 accumulates and drives the expression of genes that are not expressed in the mother cell (Colman-Lerner et al. 2001; Mazanka et al. 2008; Weiss et al. 2002). In *C. albicans*, the RAM pathway is also involved in morphogenesis and cell polarity (McNemar and Fonzi 2002; Song et al. 2008). It was shown that mutations of the RAM genes lead to hypersensitivity to

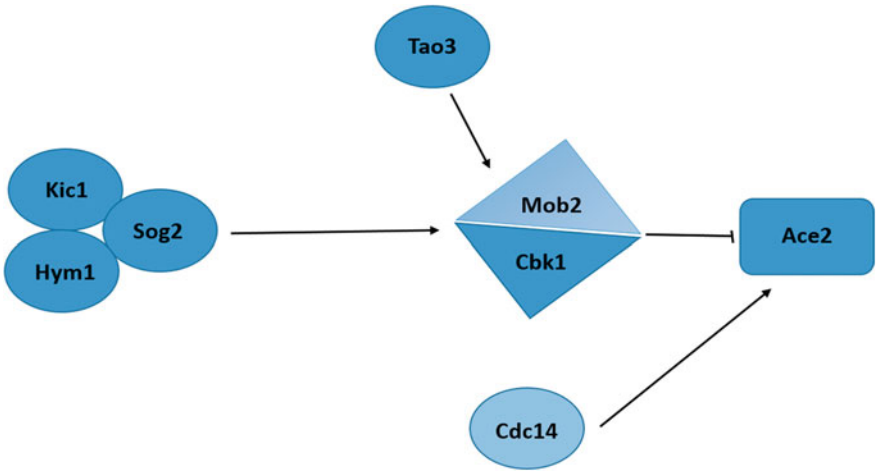
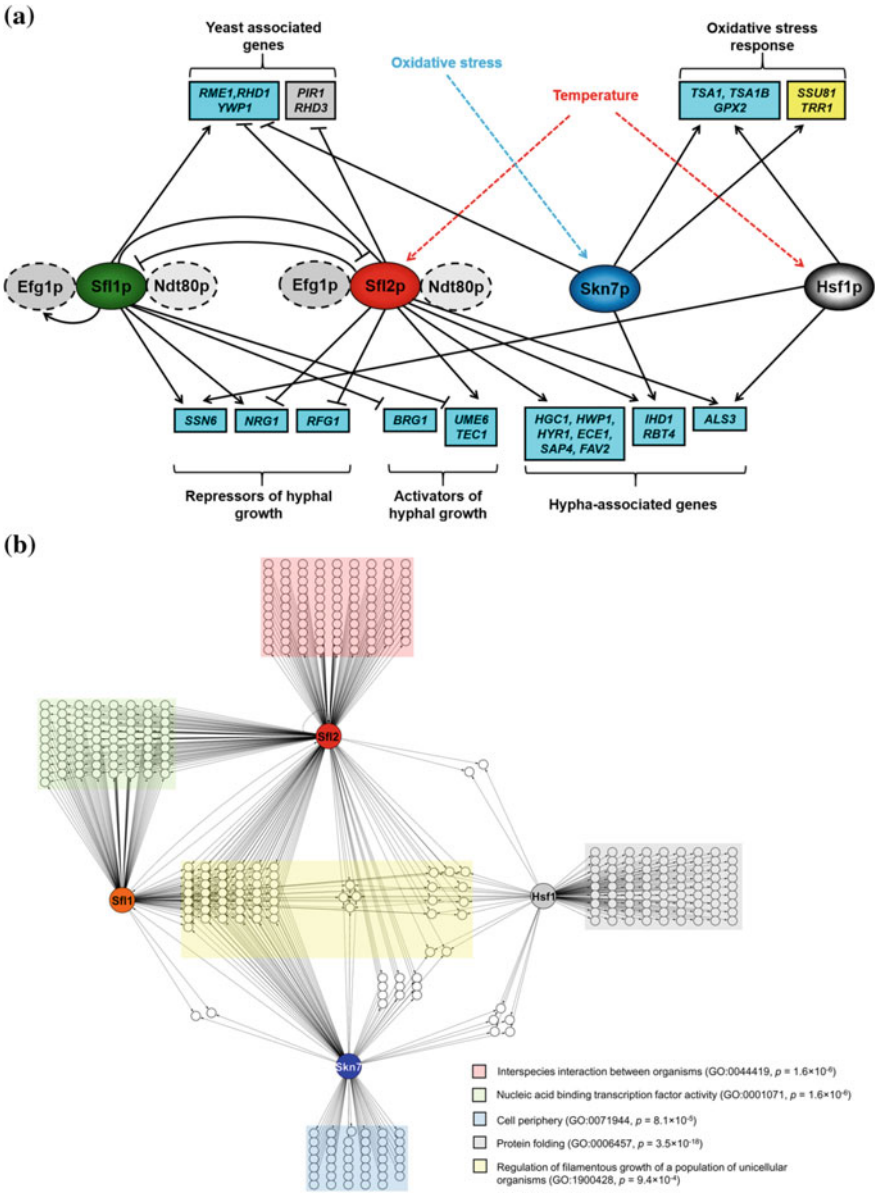


Fig. 4 Activation of RAM pathway during morphogenesis. During morphogenesis, the TF Ace2 is regulated by the kinase Cbk1 associated to its activator Mob2. This complex, whose formation is a key event for hyphal growth, is regulated by Hym1, Sog2 and the Ste20-like protein kinase Kic1 complex, together with Tao3. In addition, Ace2 localization depends on the protein phosphatase Cdc14

cell wall or membrane stresses, defects in cell separation, and loss of cell polarity. In particular, the Cbk1–Mob2 interaction is a key event for hyphal growth (Song et al. 2008). In addition, Ace2 localization depends on the protein phosphatase Cdc14 in both yeasts (Fig. 4) (Clemente-Blanco et al. 2006; Weiss et al. 2002). Furthermore, deletion of Ca*ACE2* caused an increased invasion of solid agar medium and led to abnormal pseudohyphal growth, even in the absence of external inducers. The mutant cells were also impaired for adherence to plastic surface, biofilm formation, and virulence (Kelly et al. 2004). Finally, Ace2 is found in two isoforms: a full-length Ace2L, involved in the septin ring formation during hyphal development that avoids cell separation, and a shorter form Ace2S, that regulates the expression of cell separation genes (Calderon-Norena et al. 2015).

6 Role of the Heat Shock Factors in *C. albicans* Morphogenetic Transition

In response to heat stress, cells activate an ancient signaling pathway leading to the transient expression of heat shock or heat stress proteins (Hsps). Hsps display very complex protection mechanisms, but the most conserved Hsps are molecular chaperones that prevent protein misfolding and the formation of non-specific protein aggregates, and assist proteins in the acquisition of their native structures (Richter et al. 2010).



Specific heat shock TFs are required to upregulate *HSP*s. Heat shock TFs share a common structure characterized by an N-terminal DNA-binding domain (DBD) followed by a cluster of hydrophobic amino acids organized in heptad repeats that form leucine zippers, and C-terminal heptad repeats (Su et al. 2013). In eukaryotes, *HSP* expression depends on the heat shock TF Hsf1, conserved from

◂**Fig. 5 a HSF-type regulators transcriptional network**. Sfl1 and Sfl2 regulate the expression of yeast-associated genes, and genes encoding repressors and activators of hyphal growth (blue boxes indicate common targets), by direct binding or together with Efg1 and Ndt80 (gray, dashed lines indicate hypothetical interaction). Sfl1 (dark green) induces the expression of yeast-associated genes (*RME1, RHD1, YWP1*) and of genes encoding repressors of hyphal growth (*SSN6, NRG1*) and represses genes encoding activators of hyphal growth (*BRG1, UME6, TEC1*). Sfl2 (red) is activated by temperature increase and negatively regulates yeast-associated genes, that are either common targets of Sfl1 (*RME1, RHD1, YWP1)* or unique targets of Sfl2 (*PIR1, RDH3*). Conversely, Sfl2 negatively regulates the expression of genes encoding repressor of hyphal growth, also regulated by Sfl1 (*SSN6, NRG1, RFG1*), and positively the expression of activators of hyphal growth also regulated by Sfl1 (*UME6, TEC1*), as well as hypha-associated genes (*HGC1, HWP1, HYR1, ECE1, SAP4, FAV2, IHD1, RBT4, ALS3*). Sfl1 and Sfl2 negatively regulate each other. Skn7 (dark blue) is activated during morphogenesis and oxidative stress; Skn7 induces the expression of hypha-associated genes, also targets of Sfl2 (*IHD1, RBT4*), negatively regulates yeast-associated genes also regulated by Sfl2 (*RME1, RHD1, YWP1*), and regulates the expression of several oxidative stress response genes (*TSA1, TSA1B, GPX2, SSU81, TRR1*). Hsf1 (dark gray) is activated upon temperature increase as Sfl2 and shares common targets with Sfl1 (*SSN6*), Sfl2 (*ALS3*), and Skn7 (*TSA1, TSA1B, GPX2*). **b Transcriptional circuitry of HSF-type Regulators Hsf1, Sfl2, Sfl1 and Skn7**. Hsf1 (gray sphere), Sfl2 (red sphere), Sfl1 (orange sphere), and Skn7 (blue sphere) share common direct transcriptional targets (open spheres, highlighted with green shading for Sfl1 and Sfl2 common targets and yellow shading for targets involving at least 3 HSF-type regulators). Targets that are specific to each regulator are highlighted with red (Sfl2), blue (Skn7), and gray (Hsf1) shadings. The associated functional categories (GO terms) that are significantly enriched are shown at the bottom right part of the figure. GO term enrichment analyses were conducted using the GO Term Finder algorithm (http://www.candidagenome.org/cgi-bin/GO/goTermFinder) at the *Candida* Genome Database. Binding data were taken from references (Leach et al. 2016) (Hsf1) (Znaidi et al. 2013) (Sfl2, Sfl1) and (Basso et al. 2017) (Skn7). Transcriptional network was constructed using Cytoscape version 3.4.0

yeasts to humans (Sorger and Pelham 1987; Wiederrecht et al. 1987). In response to temperature upshift, Hsf1 binds to a short highly conserved DNA sequence, namely the heat shock element (HSE), consisting of short tandem repeats of the sequence AGAAN (N is any nucleotide) found in the promoters of many heat shock genes (Fernandes et al. 1995).

Heat shock transcription factors in eukaryotes contain a helix-turn-helix DBD. In *S. cerevisiae*, the DBD is important not only to bind DNA, but also for regulation of Hsf1 function. In *C. albicans*, in addition to Hsf1, there are three transcription factors containing an HSF-type DBD, namely Sfl1, Sfl2, and Skn7 (Fig. 5). These transcription factors play a role in morphogenesis, as reflected in the overrepresentation of genes involved in the regulation of filamentous growth among their common targets (Fig. 5b).

Hsf1. In *C. albicans,* Hsf1 is required for virulence and HSE-containing genes are activated upon systemic infection (Nicholls et al. 2011), demonstrating that the connection between heat shock response and pathogenicity is crucial. In response to an acute temperature upshift, *C. albicans* induces classical heat shock genes and some other stress-regulated genes (Fig. 5a and b) (Enjalbert et al. 2003). In *C. albicans* as in other yeasts, Hsf1 is also essential for viability (Nicholls et al. 2009), and the Hsf1-HSE regulon is critical for the modulation of genes involved in protein folding under basal conditions, including the chaperone-encoding *HSP70*,

HSP90, and *HSP104* genes, as well as in response to heat stress (Leach et al. 2012; Nicholls et al. 2009).

Hsf1 physically interacts with the heat shock proteins Hsp70 and Hsp90 (Leach et al. 2012). Hsp70 and Hsp90 contribute to the Hsf1 autoregulatory circuit: in the absence of stress, Hsp90 represses Hsf1, but under thermal stress, the repression is released and Hsf1 can become active, leading to an increase in Hsp90 levels (Jarosz et al. 2010; Leach et al. 2012).

Heat shock regulation also influences *C. albicans* morphogenesis, since a mild heat shock is normally used to stimulate the yeast-to-hypha transition in experimental conditions (Fig. 1) (Odds 1988). The levels of the chaperone Hsp90 are increased during the yeast-to-hypha transition (Swoboda et al. 1995), and Hsp90 was demonstrated to regulate morphogenesis, since compromising its function induces the yeast-to-hypha transition via the Ras1-PKA pathway and attenuates virulence in systemic infection mouse model (Shapiro et al. 2009). Recently, it has been shown that *hsf1* mutants display defective filamentation on various solid hyphal inducing media (Nair et al. 2017) and that tuning the expression level of *HSF1* has a significant impact on morphogenesis (Veri et al. 2018). Indeed, overexpression of *HSF1* was sufficient to drive expression of the morphogenetic regulators *UME6* and *BRG1*, while loss of either *UME6* or *BRG1* was sufficient to block filamentation in response to *HSF1* overexpression (Song and Carlson 1998).

Sfl1 and Sfl2. Suppressor gene for flocculation (*SFL1*) was first identified in *S. cerevisiae* where it encodes a repressor for invasion and pseudohyphal formation (Robertson and Fink 1998), and it binds specifically to GAA triplet motif of the HSE elements (Zhu et al. 2009). Sfl1 behaves as a repressor of flocculation-related genes, such as *FLO11, STA1,* and *SUC2* (Kim et al. 2004; Robertson and Fink 1998; Song and Carlson 1998). However, this transcription factor can also act as a positive regulator of stress-responsive genes (Conlan and Tzamarias 2001; Galeote et al. 2007). Inhibition of transcription by Sfl1 is likely due to its interaction with the Ssn6–Tup1 corepressor complex. Besides, repression of gene expression by Sfl1 and Ssn6–Tup1 might involve a negative regulation of RNA polymerase II, since some components of the RNA polymerase II mediator complex, such as Sin4 and Srb10, are needed for full repression by Sfl1 (Conlan and Tzamarias 2001; Song and Carlson 1998). Tpk2 inhibits Sfl1 binding to DNA (Conlan and Tzamarias 2001; Pan and Heitman 2002) and, in parallel, activates Flo8 in the cAMP-signaling pathway (Conlan and Tzamarias 2001; Fujita et al. 1989). In *C. albicans,* two structural homologs of ScSfl1 have been identified, namely Sfl1 and Sfl2, and their role in morphogenesis and virulence were proven (Bauer and Wendland 2007; Song et al. 2011; Spiering et al. 2010). In fact, deletion of Ca*SFL1* leads to flocculation and promotes hyphal development, through HAGs and cell adhesion genes induction, while its overexpression blocks filamentation (Bauer and Wendland 2007; Li et al. 2007). Therefore, Sfl1 and Flo8 antagonistically regulate hyphal development in a mechanism similar to the one described in *S. cerevisiae*, where they can bind the same regions depending on phosphorylation by Tpk2 (Li et al. 2007). However, in the absence of Flo8, Sfl1 acts as a repressor at high temperature, and as an activator at low temperature, proving its dual function: in

embedded agar at 24 °C, overexpression of *SFL1* enhances filamentation, whereas deletion of *SFL1* blocks filamentous growth in a *flo8* deletion mutant (Li et al. 2007). Interestingly, both deletion and overexpression of Sfl1 attenuate *C. albicans* virulence during systemic infection (Li et al. 2007).

Sfl2 is a positive regulator of filamentous growth in *C. albicans*, displaying high amino-acid sequence homology with Sfl1 (Song et al. 2011). Both TFs are probably the products of a gene duplication event from a shared unique ancestor and have diverged to exert antagonistic functions in filamentous growth. Interestingly, exchanging the HSF-DNA-binding domains of Sfl1 and Sfl2 almost completely reversed the repressor/activator functions of the hybrids generated from the domain swapping (Song et al. 2011), suggesting that their antagonistic functions are conferred by their ability to bind DNA. A comprehensive model for Sfl1 and Sfl2 transcriptional network was recently provided (Fig. 5a and b) (Znaidi et al. 2013). According to this model, Sfl1 and Sfl2 positively and negatively regulate a common set of targets that can be divided into three categories: repressors of hyphal growth (*SSN6, NRG1, RFG1*), activators of hyphal development (*UME6, BRG1, TEC1*), and yeast-specific genes (YSGs) (*RME1, RHD1, YWP1*). In particular, while the two transcription factors can act both as repressor and activator for the first two gene categories described above, only Sfl1 upregulates and Sfl2 downregulates the yeast-specific gene category (Fig. 5a). Furthermore, Sfl1 and Sfl2 directly repress each other's expression. Lastly, Sfl1 and Sfl2 behave as «switch on/off» regulators, with Sfl1 turning off the expression of hyphal positive regulators expression and turning on the YSGs and the hyphal repressors, whereas Sfl2 turns off the YSGs and negative regulators of hyphal development and turns on activators of hyphal growth, as well as a set of HAGs, not regulated by Sfl1 (Fig. 5a). Moreover, motif discovery analyses suggest an interaction between Sfl1 and Sfl2 with the transcriptional regulators Ndt80 and Efg1, proving the complexity of the circuitry that regulates morphogenesis (Znaidi et al. 2013). Finally, evidences of the reminiscent heat shock response of these two transcription factors are reported. For instance, Sfl1 and Sfl2 can bind to the promoter of *HSP104* and *HSP70* (Znaidi et al. 2013), and one potential Sfl1 binding motif is similar to that for ScHsf1 (MacIsaac et al. 2006; Morozov and Siggia 2007). Taken together, these data suggest the specific needs of *C. albicans* to survive and adapt in warm-blooded animals, converting temperature-sensing inputs into morphogenesis output by the HSF-type transcription factors.

Skn7. The third *C. albicans* transcription factor that shares the HSF-DBD homology with Hsf1, Sfl1, and Sfl2 is named Skn7. Skn7 is highly conserved among fungi, displaying a uniform architecture consisting in an N-terminal HSF-DBD and a C-terminal receiver domain (Fassler and West 2011). Within the HSF domain, two residues known to be involved in contacting DNA and critical in combination for the protein function are conserved, namely phenylalanine 76 and leucine 83 (Basso et al. 2017; Fassler and West 2011). However, Skn7 presents a very interesting peculiarity when compared with the other HSF-type transcription factors: the presence of a response-regulator domain. This domain confers an additional regulation for transcriptional activity via phosphorylation of conserved

residues within the protein and distinguishes Skn7 from the other HSF-type DBD proteins (Hsf1, Sfl1, and Sfl2). In *C. albicans,* the mechanisms that regulate Skn7 are not known. Nevertheless, it has been reported that Skn7 is involved in the oxidative stress response (Basso et al. 2017; Singh et al. 2004) and morphogenesis (Basso et al. 2017; Chauvel et al. 2012; Singh et al. 2004) (Fig. 5a and b), but how it regulates either the adaptation to oxidative stress or the morphogenetic transition has not been defined so far. *C. albicans* adaptation to oxidative stress depends on the TF Cap1 (Wang et al. 2006; Zhang et al. 2000), and Skn7 does not share common targets with Cap1 (Basso et al. 2017; Znaidi et al. 2009), suggesting that the role of these TFs in the oxidative stress response is different. However, Skn7 seems to prevent the accumulation of reactive oxygen species (ROS) occurring during filamentation on solid medium and, interestingly, the morphogenesis regulation appears uncoupled from the protection against intracellular ROS (Basso et al. 2017). Furthermore, Skn7 positively regulates the expression of other TFs, such as Brg1, Cph1, Czf1, Eed1, Sfl2, Tec1, and Ume6, and is required for Cph1, Tec1, and Ume6 function (Basso et al. 2017), highlighting its deep interconnection with other regulators of morphogenesis.

7 Conclusion

C. albicans exhibits remarkable morphological features of adaption to host niches that differ in terms of temperature, pH, oxygen/CO_2 levels, and nutrient availability. Such environmental fluctuations are a source of stress, requiring the activation of regulatory pathways and transcriptional circuits that impact on *C. albicans* morphogenesis and promote survival within body organs. Some of the transcriptional circuitries governing *C. albicans* morphogenesis have been characterized in detail. They already highlight the complexity that lies behind modulation of their activity. As stated above, turning on and off gene expression by TFs in response to a variety of stimuli is tightly orchestrated by a series of cascades and highly interconnected signal transduction pathways, including the cAMP/PKA, the Hog1/Mkc1/Cek1-MAPK pathways or those responding to matrix embedding, pH variation, and heat shock. Through deciphering the components of the cascades activated upstream of TFs, one could provide important clues on how signals that trigger filamentous growth and stress response are sensed and transduced. On the other hand, through studying TF function and modeling the transcriptional circuitries that operate during morphogenesis, one could identify important effectors and determinants of morphological switching that act downstream of TFs, as illustrated in Fig. 5b. The knowledge gathered from deciphering the regulatory circuitries involved in morphogenesis and/or stress response in *C. albicans* could open up new avenues for identifying potential molecular targets for future antifungal drug development.

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Cryptococcal Titan Cells: When Yeast Cells Are All Grown up

Rocío García-Rodas, HC de Oliveira, Nuria Trevijano-Contador and Oscar Zaragoza

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Abstract *Cryptococcus neoformans* is a human pathogenic yeast that causes hundreds of thousands of deaths worldwide among susceptible individuals, in particular, HIV+ patients. This yeast has developed several adaptation mechanisms that allow replication within the host. During decades, this yeast has been well known for a very peculiar and unique structure that contributes to virulence, a complex polysaccharide capsule that surrounds the cell wall. In contrast to other fungal pathogens, such as *Candida albicans* or *Aspergillus fumigatus*, the role of morphological transitions has not been studied in the virulence of *Cryptococcus neoformans* since this yeast does not form hyphae during infection. However, in the last years, different groups have described the ability of this fungus to change its size during infection. In particular, *Cryptococcus* can form "titan cells," which are blastoconidia of an abnormal large size. Since their discovery, there is increasing evidence that these cells contribute, not only to long-term persistence in the host, but they can also actively participate in the development of the disease. Recently, several groups have simultaneously described different media that induce the appearance of titan cells in laboratory conditions. Using these conditions, new inducing factors and signaling pathways involved in this transition have been described. In this article, we will review the main phenotypic features of these cells, factors, and transduction pathways that induce cell growth, and how titan cells contribute to the disease caused by this pathogen.

Keywords *Cryptococcus neoformans* · Titan cell · Capsule · Morphogenesis Virulence

1 Introduction

Microscopic fungi are distributed worldwide and can be isolated from most of the environmental niches. They play important roles in multiple processes, since they are commensals of multiple organisms and participate in bioremediation and ecological balance. Furthermore, they are a frequent cause of disease in plants and animals.

In the case of humans, fungi are commensals that can be isolated from the skin, mucosa, and gastrointestinal tract. Commonly, fungi can cause superficial infections. However, in immunosuppressed patients they can cause severe and disseminated diseases (Lamps et al. 2014; McNeil et al. 2001; Singh 2001). The increasing incidence of invasive fungal infections has several important problems associated, such as the high mortality, the economical costs of prolonged hospital stays and the frequent neurological sequelae (Brown et al. 2012; Erjavec et al. 2009; Rueping et al. 2009).

The management of these diseases is complicated for many reasons. Despite the development of new diagnostic tools, such as MALDI-TOF, still the lack of

availability of early detection tests, the increasing selection of resistant strains due to the massive use of antifungals and the poor knowledge on the virulence mechanisms constitute a problem (Ruhnke and Schwartz 2016). In this context, the main factor that determines the outcome of fungal infections is the immune system of the host (Casadevall and Pirofski 2003, 2009; Pirofski and Casadevall 2008, 2017). However microbial elements have also a key role in the development of the disease. In particular, the ability to survive in the host is most probably the main factor that determines the ability of a fungus to behave as commensal or symbiotic, or even as a pathogen in immunosuppressed patients.

There are many mechanisms that allow the proliferation of pathogenic fungi within the human, such as the adaptation to the nutritional, osmotic and oxidative environment, growth at corporal temperature and the capacity to evade killing by macrophages (Alvarez et al. 2009; Fu et al. 2018; Johnston and May 2013; Robert and Casadevall 2009; Zaragoza et al. 2008). In addition, most fungi can also induce morphological transitions that contribute to the evasion of the immune response, dissemination through the organism and tissue invasion (May et al. 2016; Mayer et al. 2013). Most of these morphological transitions involve a change in the shape of the cells, and in particular the formation of hyphae and pseudohyphae. The role of these morphotypes during infection has been extensively described and reviewed in the literature [reviewed in (Huang 2012; Kadosh 2013; Mayer et al. 2013; Mitchell 1998; San-Blas et al. 2000; Sudbery 2011; Trevijano-Contador et al. 2016)].

However, in some fungi, morphogenesis does not involve a change in the shape of the cell but in the size. This is the case of the pathogenic yeast *Cryptococcus neoformans*, which is widely distributed in the environment. This fungus has a high prevalence among HIV infected patients, and it is the cause of hundreds of thousands of deaths per year (Park et al. 2009; Rajasingham et al. 2017). *Cryptococcus neoformans* is an excellent model to investigate fungal pathogenesis, since it has developed some characteristics that have a determinant role during infection (Alspaugh 2015; Coelho et al. 2014; Esher et al. 2018). For example, this fungus has a polysaccharide capsule around the cell that protects it against multiple stress factors, but that also has deleterious effects in the host [reviewed in (Agustinho et al. 2018; Ding et al. 2016; Doering 2009; O'Meara and Alspaugh 2012; Vecchiarelli et al. 2013; Zaragoza et al. 2009)].

Regarding morphological changes, *C. neoformans* also possesses a complex morphogenetic program that results in the appearance of a heterogeneous population in the lung (Feldmesser et al. 2001). Although *C. neoformans* can form pseudohyphae during infection, this is a rare phenomenon, and most of the cells present a spherical shape. However, the size of the cells is very heterogeneous, in particular, in the lung (Zaragoza 2011). The regular size of cryptococcal size is around 5–8 μ. However, in vivo, the diameter of the cells can vary from 1 to 100 μ (Fig. 1). This review will focus on the transition that results in the significant cellular enlargement that leads to the formation of the so called "titan cells".

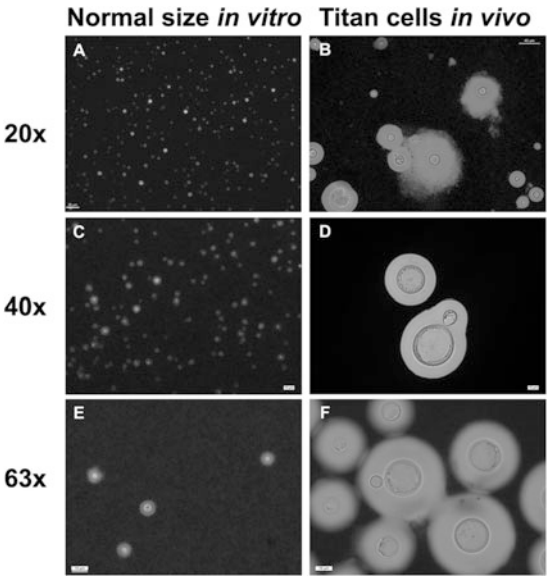

Fig. 1 Cryptococcal titan cells. Cells from *C. neoformans* obtained in Sabouraud-rich medium in vitro (**a**, **c** and **e**) and titan cells isolated from infected mice (**b**, **d,** and **f**) were suspended in India Ink and pictures were taken with different objectives: 20x (**a** and **b**), 40x (**c** and **d**), and 63x (**e** and **f**). In consequence, **a** and **b**, **c** and **d**, and **e** and **f** have the same magnification. Scale bars denote 25 μ in **a**, 40 μ in **b** and 10 μ in **c**, **d**, **e,** and **f**

2 Cryptococcal Titan Cells. A Dramatic Morphological Change

The most typical morphological transition in *C. neoformans* consists of a significant enlargement of both the capsule and cell body size (Okagaki et al. 2010; Zaragoza et al. 2010). The magnitude of the cell body increase can reach over ten-fold the regular cell size, but there are different definitions of titan cells. As recently reviewed by Zhou and Ballou, titan cells can be defined in different ways (Zhou and Ballou 2018). The initial criteria defined titan cells as those with a cell body diameter over 15 μ or total size (including the capsule) over 30 μ (Zaragoza and Nielsen 2013). However, some other authors suggest that a cell body diameter above 10 μ is enough to consider those cells as titan. Other definitions of titan cells are based on ploidy or phenotypic features, such as large vacuole or thickened cell wall. In fact, most definitions have arguments against, because there could be situations in which titan cells are found without all the classical phenotypic characteristics. Furthermore, there are some cases in which huge cells are formed due to a massive growth of the capsule, and not so much of the cell body, and these cells could also be considered as titan cells because they are also cells with a significant large size difficult to remove by the immune system.

Curiously, these cells have been almost neglected for the scientific community for decades. Although some articles reported cryptococcal cells of abnormal size in

clinical specimen (Cruickshank et al. 1973; Love et al. 1985), titan cells were not described in detail until 2010. Since then, this morphotype has been the focus of multiple studies. Besides, they have been found in further studies in clinical samples from patients with cryptococcal pneumonia (Wang et al. 2014). The main purpose of this review is to illustrate the main aspects of this type of cryptococcal cells, how they are formed, and how they contribute to adaptation to the host and to the development of disease.

2.1 Phenotypical Features of Cryptococcal Titan Cells

The first phenotypical characteristic of titan cells is their huge size compared to the cells obtained in rich media in laboratory conditions and/or isolated from the environment. The increase in diameter goes from 6 μ in vitro to around 40–70 μ in vivo (Fig. 1). Cells with even a diameter of 100 μ have been described in the lungs of infected mice (Okagaki et al. 2010; Zaragoza et al. 2010). This is a dramatic change, in particular, if the increase in volume is calculated, since it can be 1000 times larger in titan cells compared to cells of regular size. The increase in size is due to growth of both the capsule and the cell body. In addition to their large size, titan cells also present other phenotypic differences. The capsule consists of much denser polysaccharide fibbers compared to that of cells obtained in vitro, and it has reduced permeability (Zaragoza et al. 2010). Furthermore, the cell wall is also enlarged. If cells in vitro have a cell wall thickness of around 200 nm, in titan cells it can reach up to 3 μ. Titan cells cell wall contains increased amounts of chitin, significantly higher glucosamine and lower glucose than cell walls from in vitro regular size cells, which has been associated with a detrimental Th2 response in mice (Mukaremera et al. 2018; Wiesner et al. 2015).

Intracellularly, titan cells have also a large vacuole, and it has been hypothesized that this could represent a mechanism by which these cells do not need to augment their cytoplasmic content (such as mitochondria, DNA, proteins) in direct proportion to their size (Zaragoza et al. 2010). However, these cells are still polyploid (Okagaki et al. 2010; Zaragoza et al. 2010) and it has been argued that they are formed by endoreduplication. In this way, there would be several rounds of S phase without mitosis, so the cells would go several times through G1 and G2 phases, which are the phases in which there is a significant increase of the cell size, without separating the genetic content into a daughter cell. Titan cells have not lost their capacity to replicate, and in fact, when isolated from the lungs and placed in laboratory conditions, these cells can originate a large number of daughter cells (Gerstein et al. 2015; Zaragoza et al. 2010).

2.2 Which Factors Trigger Titan Cell Formation? In Vivo Studies

One of the main aspects that have been the focus in the research of titan cells is to elucidate the factors and signaling pathways that trigger their induction. Initial studies were based on in vivo experiments, in which mice were infected with different mutants and the proportion of titan cells was investigated in the lungs. Using this approach, it was first discovered that mutants lacking adenylate cyclase (Cac1) and that cannot synthesize cAMP cannot form titan cells, indicating that this pathway is required for this process (Zaragoza et al. 2010). Furthermore, the proportion of titan cells in vivo increased when mice were coinfected with strains of different mating type, and mutants defective in the pheromone receptor Ste3 were defective in this transition (Okagaki et al. 2010, 2011). This receptor is coupled to the G-protein Gpa1, which activates the activity of the adenylate cyclase (Alspaugh et al. 2002). However, mutants that do not produce cAMP are avirulent, so in vivo studies might be influenced by the decreased fitness of the yeasts. In one of these early studies, it was found that *C. neoformans* can produce a small proportion of titan cells in vitro after prolonged incubation of the yeasts in minimal media, and using this approach, it was confirmed that cAMP mutants are defective in titan cell production (Zaragoza et al. 2010). This result also suggested that titan cells are formed in response to nutritional stress. In agreement, overexpression of *PKA1* which encodes protein kinase A produces increase in cell size and ploidy of cryptococcal cells (Choi et al. 2012).

Elegant studies by Okagaki et al. demonstrated that other elements of the cAMP pathway regulate cell growth in *C. neoformans*. Among them, they identified a G-coupled receptor Gpr5 as a positive regulator of titan cell formation. Interestingly, this receptor mediates signaling through Gpa1 and Cac1. These authors also found that transcription factor Rim101 is required for titan cell development. This transcription factor is activated by cAMP and plays pleiotropic effects in the virulence of *C. neoformans.* One of the proteins that were also found to be required for titan cell formation was the cyclin Pcl1, which confirmed that this transition is regulated by cell cycle elements (Okagaki et al. 2011).

The host environment also plays an important role in the cryptococcal morphogenesis in vivo. Despite it is not fully clear which specific environmental elements trigger titan cell formation, this transition does not happen in the same way in different mouse strains that elicit different immune responses. For example, in mice that induce Th2-type immunity, the proportion of titan cells is higher than in mice that induce a Th1 response (Garcia-Barbazán et al. 2016). This result suggests that non-protective immune responses against *C. neoformans* result in a less aggressive environment that favors cellular growth of the yeasts.

3 Titan Cells Obtained In Vitro: New Factors and Signaling Pathways Elucidated

The studies that aimed a better molecular characterization of titan cells faced a great challenge since researchers did not have an effective way to obtain them in vitro. Initially, investigation of titan cells required infecting mice and then isolating the fungal cells from the lungs of the animals. This approach has been very effective, but it presents two important limitations: (1) the low amount of titan cells obtained and (2) the ethical issues and economic costs associated to the use of animals for research.

In the last years, several articles described that titan cells can occasionally appear in some media. In this way, and as stated above, Zaragoza et al. described that prolonged incubation of cryptococcal cells in minimal media resulted in the appearance of titan cells in vitro (Zaragoza et al. 2010). Then, it was found that other conditions, such as overexpression of *PKA1* or exposure to ameba or macrophage extracts also result in the appearance of titan cells in vitro (Choi et al. 2012; Chrisman et al. 2011). However, it has not been until recently that three different groups have described in vitro conditions that consistently result in the appearance of *C. neoformans* titan cells that resemble the ones found in vivo, with body size diameters close to 15 μm, enlarged capsule, increased nuclear DNA content, thicker cell wall, and capacity to generate a normal size progeny (Dambuza et al. 2018; Hommel et al. 2018; Trevijano-Contador et al. 2018). Percentage of titan cells obtained in vitro is variable depending on the protocol followed and the strains tested ranging from 14 to 70%. In two of these articles, the main induction factor was addition of serum to limited nutrient media in the presence of CO_2 (Dambuza et al. 2018; Trevijano-Contador et al. 2018). The other protocol involves prolonged incubation (3–5 days) in hypoxia in minimal media (Hommel et al. 2018), which resembles in part the conditions initially described by (Zaragoza et al. 2010). Figure 2 shows a scheme of the different methods described that induce titan cells in vitro. The possibility to obtain titan cells in vitro has opened new perspectives in this field and has also elucidated new signaling factors and signaling pathways involved in this morphological transition.

3.1 Regulation by Serum, CO_2, and Nutritional Limitation

The in vitro conditions involved nutrient limitation by transferring yeasts from a rich to a poor medium (Dambuza et al. 2018; Hommel et al. 2018; Trevijano-Contador et al. 2018). Furthermore, the addition of several inducing factors contributes to titan cell formation.

Mammalian serum, as described by Trevijano-Contador et al. and Dambuza et al., is an essential inducing factor to obtain titan cells in vitro. The addition of 5 and 10% of bovine fetal serum led to a significant increase in cell size

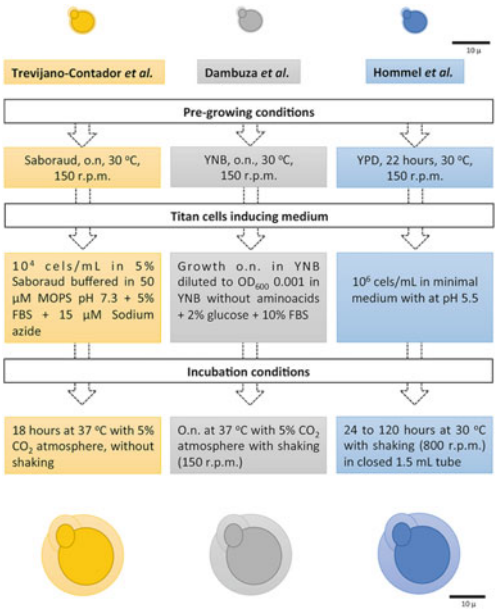

Fig. 2 Scheme of the methods to obtain titan cells in vitro. The figure shows a scheme of three methods published in 2018 by Trevijano-Contador et al. (2018) (yellow), Dambuza et al. (2018) (gray), and Hommel et al. (2018) (blue) to obtain titan cells in vitro. The methods are based on two steps: pre-growth at 30 °C with agitation (150 rpm) on Sabouraud overnight (yellow), YNB overnight (gray), or YPD for 22 h (blue), followed by an incubation on the different inducing medium, in different conditions

(Dambuza et al. 2018; Trevijano-Contador et al. 2018). The same did not occur when the serum was added to rich medium, showing the importance of the nutrient limitation in this process (Trevijano-Contador et al. 2018). These two groups also found that serum components are capable to induce titan cell formation in vitro. Polar lipids isolated from the serum were able to induce the formation of the titan cells. They also could evaluate that phosphatidylcholine (PC), induced a partial increase in cell size in vitro, but which was not of the same magnitude as of whole serum, indicating that other components are also important in this process. This is in agreement with previous reports in the literature, where is it was found that PC promotes cell growth in vitro (Chrisman et al. 2011), although these author found the effect at higher PC concentrations. However, phospholipase B seems to play a paradoxical role in titan cell formation, since mutants lacking this enzyme induce titan cell formation inside phagocytic cells (Evans et al. 2015). Pharmacological inhibition of PKC abolished the formation of titan cells (Trevijano-Contador et al. 2018). In *C. neoformans*, PKC is activated by diacylglycerol (DAG), which is produced by degradation of phospholipids by phospholipases. For this reason, these authors hypothesized that serum effect was in part due to the activation of PKC signaling in the cell (Trevijano-Contador et al. 2018).

Dambuza et al. found that two serum fractions composed of at least nine different compounds with sugar and amino acids structures, induced titan cell formation in vitro (Dambuza et al. 2018). However, Hommel et al. showed that addition of FBS to minimal medium decreased the cell size and the same was

observed with the addition of phosphatidylcholine showing that titan cells can be obtained in vitro through parallel or independent pathways (Hommel et al. 2018). It is noteworthy that the time to produce in vitro titan cells following the above-mentioned protocol is very different, and this could clearly determine the different response to the same stimuli. Besides, since quorum sensing plays a role in titan cell formation, different initial cell densities could also promote different response to the same stimuli.

Oxygen limitation also led to an increase of *C. neoformans* cell size in vitro. Addition of sodium azide, which inhibits complex IV of the respiratory chain, and statistic incubation of the cells leads to a higher proportion of cells with enlarged sizes (Trevijano-Contador et al. 2018). This result suggests that mitochondrial damage can induce a stress signal that results in cell cycle arrest and cell growth. Similarly, Hommel et al. found that induced hypoxia, either physical (lack of shaking) or chemical by adding 1 nM $COCl_2$ favors the formation of titan cells (Hommel et al. 2018; Trevijano-Contador et al. 2018). Oxygen limitation may reduce the respiration rate of the cell, which can trigger a stress signal that influences cell cycle. Furthermore, inhibition of respiration induces activation of cAMP pathway in different fungi (Fuller and Rhodes 2012), which may contribute to titan cell formation.

CO_2, which induces capsule growth in *C. neoformans* (Granger et al. 1985), also induced cell body enlargement in vitro (Dambuza et al. 2018; Trevijano-Contador et al. 2018). In *C. neoformans*, CO_2 is converted into HCO_3^- by the action of two carbonic anhydrases, Can1 and Can2, being Can2 the most expressed. Bicarbonate can activate adenylate cyclase (Klengel et al. 2005). In consequence, it was argued that CO_2 induces titan cell production through the activation of the cAMP pathway.

3.2 Regulation by Microbiome

Titan cells are mainly found in the lungs, and Dambuza et al. found that bronchial alveolar lavage (BAL) can also induce titan cell formation (Dambuza et al. 2018). BAL extracts contained lung-resident bacteria, so they also tested the impact of the host microbiome in the formation of the titan cells. Live or heat-killed *Escherichia coli* and live *Streptomyces pneumonia*, when co-incubated with *C. neoformans* in YNB, induced the formation of titan cells. The importance of the host microbiome was also evaluated in vivo. Infection of mice with *C. neoformans* previously treated with penicillin and streptomycin resulted in a reduction of titan cells in the lungs compared with non-treated animals, suggesting that the interaction between cryptococcal cells and bacteria induces cell growth of this fungal pathogen (Dambuza et al. 2018).

Bacterial components can also induce titan cell formation. Muramyl tetrapeptides (MTP) are peptidoglycan subunits that are found in the cell wall of Gram-positive,

Gram-negative, and mycobacteria and that induce the yeast-to-hypha transition in *C. albicans* (Xu et al. 2008). Dambuza et al. found that FBS contained muramyl dipeptide (MDP) and N-Acetylmuramyl-L-alanyl-D-isoglutamine (NMAiGn), which are similar to MTP, induce titan cell formation (Dambuza et al. 2018). The effect of these peptidoglycans was absent in a mutant defective in cAMP signaling, suggesting that these compounds induce titan cell formation through activation of the cAMP pathway (Dambuza et al. 2018). All together, these data highlight that titan cells in *C. neoformans* could be formed in response to the interaction with bacteria. In addition, these findings also indicate that the interaction with the microorganisms in the host can be a determinant factor influencing microbial virulence.

3.3 Regulation by Quorum Sensing

Another factor found to be important in titan cell formation in vitro is the cell density of the cultures. When cultures are inoculated at low cellular densities, the proportion of titan cells in the cultures was much higher compared to cultures inoculated with higher cryptococcal cell concentrations (Trevijano-Contador et al. 2018). Similarly, Dambuza et al. also found that larger cells are found at higher proportion when cultures were inoculated at lower optical densities (Dambuza et al. 2018).

These findings suggest that this process is regulated by *quorum sensing* (QS), which is a mechanism of cell-to-cell communication regulated by molecules that are released to the medium. Trevijano-Contador et al. found that cell-free medium from *C. neoformans* cultures inhibits the formation of titan cells in the presence of other inducing factors, such as CO_2 and serum. These data suggest that molecules released by the cells of regular size negatively regulate the process of cellular enlargement.

Some molecules where previously described as QS molecules in *C. neoformans*. The peptide Qsp1 was described as an important QS molecule required for virulence (Homer et al. 2016; Lee et al. 2007), and more recently, as an important regulator for sexual reproduction of *C. neoformans* (Tian et al. 2018). Trevijano-Contador et al. and Hommel et al. found that addition of Qsp1 to the titan cell inducing media inhibits cell enlargement in a dose-dependent manner. However, a *qsp1* mutant strain formed titan cells in vitro (Dambuza et al. 2018; Trevijano-Contador et al. 2018). This result suggests that although Qsp1 negatively regulates titan cell formation, other QS molecules could be involved. Another molecule involved in the QS phenomenon described in *C. neoformans* is pantothenic acid (PA) (Albuquerque et al. 2013), and Hommel et al. also found that PA increased the proportion of titan cells in vitro (Hommel et al. 2018). Together, these data suggest that cell-to-cell communication is an important factor that regulates titan cells formation.

3.4 New Factors Discovered Through Genomic Analysis and Gene Expression Experiments

Recently, genomic approaches have been also carried out to find genes and pathways involved in titan cell formation. Trevijano-Contador et al. compared the gene expression of regular and titan cells in vitro after 7 and 18 h of incubation in titan cell inducing conditions and found that during the development of titan cells, there is a repression of genes that encode proteins involved in cell cycle regulation (DNA repair and chromosomal condensation) which is in agreement with the idea that titan cell induction is the result of cell cycle alterations. In this analysis, they also found that in titan cells, there is an increase in the expression of genes that encode proteins from the tricarboxylic cycle, glycolysis and involved in stress response, suggesting that there is a metabolic (Trevijano-Contador et al. 2018). Interestingly, there was also an increase in genes involved in protein trafficking (vesicle secretion, COPI vesicle coating and proteins from Golgi apparatus and Golgi membrane).

One of the genes that had increased expression at both times was the one encoding calnexin (Cne1), which is a chaperone from the ER required for proper folding of glycoproteins (Ellgaard and Helenius 2003). Paradoxically, calnexin mutants had an abnormal large size even in rich medium. This result revealed an unexpected role for regulatory elements of intracellular trafficking pathways in cell size. In agreement, *atg7* mutants that are defective in recycling proteins at the proteasome have also increased cell size in vitro (Oliveira et al. 2016).

Another gene that was found upregulated in titan cells was *CIG1,* which encodes a glycoprotein involved in heme and iron uptake. In *C. neoformans*, iron limitation induces capsule growth (Lian et al. 2005). Depletion of iron from the media induces an increase in titan cell formation (Trevijano-Contador et al. 2018). Iron limitation is sensed by the cell through the G-protein Gpa1, which also activates cAMP production (Alspaugh et al. 1997). These data confirm that iron limitation is another factor that triggers this morphological transition in *C. neoformans.* Iron is required for many processes in yeasts and since its concentration is low in the host environment, microbes and the host have to compete to uptake this element.

In parallel, genome sequencing of strains with different capacity to form titan cells has been carried out. Using this approach, it was found that *PKR1* gene, a cAMP-dependent protein kinase regulatory subunit and whose disruption results in constitutive activation of this pathway, was mutated in an isolate able to overproduce titan cells (Hommel et al. 2018). This result confirms that constitutive activation of the cAMP pathways and PKA induce titan cell development.

Hommel et al. also found that a clinical isolate that had a truncation of *USV101* produced more titan cells than the wild-type strain (Dambuza et al. 2018; Hommel et al. 2018). In agreement, an *usv101Δ* mutant strain had a significantly increase in the cell size in vitro. Usv101 is a C_2H_2 transcription factor required for virulence that regulates melanin production and capsule formation (Gish et al. 2016). These findings revealed a new role for Usv101 as a negative regulator of titan cells formation.

Tetraspanin 2 (*TSP2*) plays an important role in the integrity of plasma membrane (Li et al. 2012) and appears to negatively regulate titan cells development in *C. neoformans*. Clinical isolates with a truncated *TSP2* gene have cells with larger cell size, and a similar phenotype is found in *tsp2* mutants generated in the laboratory (Hommel et al. 2018). Tsp2 is a plasma membrane that in *C. neoformans* is involved in the repression of laccase, and this phenotype is reverted by the addition of cAMP (Li et al. 2012). For this reason, Hommel et al. argued that the inhibitory effect of Tsp2 on titan cell formation is mediated through inhibition of the cAMP signaling pathway (Hommel et al. 2018).

The genome sequence of different isolates of the wild-type H99 strain that has undergone microevolution in different laboratories (Janbon et al. 2014) with different abilities to form titan cells has been also analyzed (Hommel et al. 2018). In this way, the authors associated SNPs/indels with two genes, *SGF29* (which encodes a transcription factor (Bian et al. 2011) and *LMP1* (which is required for cryptococcal virulence) with cellular enlargement (Hommel et al. 2018).

In summary, the development of new tools and media that allow the production of titan cells in vitro has allowed the identification of new genes and signaling pathways required for this morphological transition. Figure 3 represents a suggestive scheme on how all these signals and pathways could be integrated at the moment. However, we acknowledge that future work is required to fully elucidate the mechanisms of titan cell formation. For this reason, the availability of different methods that reproduce the phenomenon in vitro will open new research lines that will contribute to characterize this process.

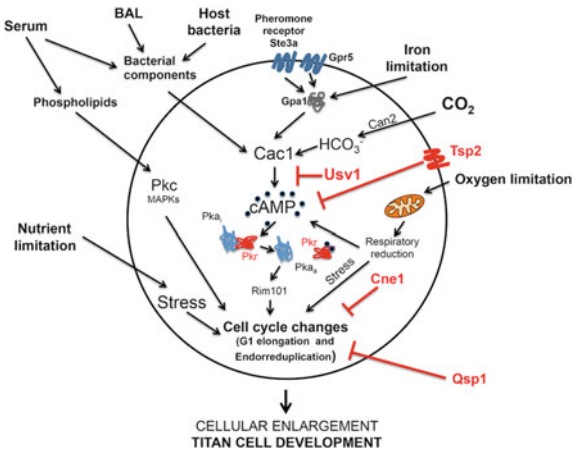

Fig. 3 Summary of different signals and pathways involved in titan cell formation. In red, we denote negative regulators of titan cell formation. See text for all the details. BAL, bronchoalveolar lavage. Cac1, adenylate cyclase. Pka_i, Protein kinase A inactive bound to the regulatory subunit Pkr. After binding of cAMP (small black circles) to Pkr, Pka becomes free and active (Pka_a). Can2, carbonic anhydrase 2. Tsp2, tetraspanin 2. Qsp1, quorum sensing peptide. Ste3a, G-coupled pheromone receptor. Gpr5, G-coupled receptor 5. Gpa1, G-protein. Cne1, calnexin

4 Importance for Cryptococcal Virulence

One of the main questions that have been the focus of different investigations is how titan cells contribute to the virulence of *C. neoformans*. One of the main processes that result in the cryptococcal disease is the dissemination from the lung to the brain. Titan cells are too big, and it is believed that they cannot cross-biological barriers nor disseminate. However, different evidences indicate that these cells are important, not only for the survival of the fungus in the host, but also because they actively participate in different pathogenic processes.

4.1 Resistance to Stress Factors

Titan cells are thought to be produced by endoreduplication, and therefore, they are polyploid while their progenies have shown different aneuploidies (Gerstein et al. 2015; Okagaki et al. 2010; Zaragoza et al. 2010). Analysis of titan cells showed that they are more resistant to stress factors and antifungals, such as H_2O_2, nitrosative oxidants, and fluconazole (Gerstein et al. 2015; Zaragoza et al. 2010). Interestingly, the progeny of titan cells is also more resistance to those stresses (Gerstein et al. 2015), which suggests that titan cells can actively participate during the pathogenesis of the disease, by producing a population of daughter cells more adapted to the host environment and to antifungal treatments.

4.2 Titan Cells Are Not Phagocytosed

Macrophages are the first line of attack of the immune system once microorganisms reach the alveoli. *Cryptococcus neoformans* has developed several mechanisms that avoid killing by phagocytic cells. This fungus is a facultative intracellular pathogen, and therefore, it is able to survive and replicate inside phagocytic cells [reviewed in (DeLeon-Rodriguez and Casadevall 2016; Feldmesser et al. 2000; Garcia-Rodas and Zaragoza 2012)]. In addition, it has also acquired some traits that difficult the internalization by macrophages. One of them is the presence of the capsule, which "hides" the main cell wall epitopes that are recognized by phagocytic cells (Kozel and Gotschlich 1982). And of course, another mechanism that inhibits phagocytosis is the production of titan cells, because they cannot be phagocytosed due to their large size. In consequence, it is reasonable to suggest that production of titan cells could be important for cryptococcal virulence.

Interaction of titan cells and macrophages has been studied by microscopy time lapse in vitro (Zaragoza et al. 2010). In this sense, different studies have shown that titan cell production results in overall lowered phagocytosis. Okagaki and Nielsen proved that there was a nonlinear relationship between titan cell formation and

phagocytosis using several mutants with different capacities of producing titan cells during murine infections. Furthermore, titan cells also protect cells of regular size from phagocytosis (Okagaki and Nielsen 2012). Overall, these data suggest that production of titan cells confers cross-protection to regular size cryptococcal cells.

In agreement to this, in vitro titan-like cells are not phagocytosed (Zaragoza et al. 2010). However, titan-like cells did not impair nor modulate phagocytosis of regular size cryptococcal cells, which means that phagocytosis and activation of macrophages in the lungs may be regulated by different factors not present in vitro (Trevijano-Contador et al. 2018).

4.3 Proliferation and Dissemination Mediated by Titan Cells

Titan cells constitute an active source of infection since they are able to produce a progeny of regular size cells (Gerstein et al. 2015; Zaragoza et al. 2010), and therefore, it may provide an advantage during establishment of infection.

Titan cells isolated from lungs of infected mice can kill the invertebrate *Galleria mellonella* similarly to cryptococcal cells of regular size (Garcia-Rodas et al. 2011), and in this model, it was described the appearance of a progeny of cells of normal size in the larvae. In mice, however, the *gpr4*Δ*gpr5*Δ strain, with limited titan cell production, showed attenuated virulence when compared to infection produced by the wild type strain resulting in lower fungal burdens (Crabtree et al. 2012). This observation suggests that production of titan cells promotes survival and proliferation of cryptococcal cells in the lungs. In agreement to this, the *otc1*Δ strain, which overproduces titan cells, is able to persist in the lungs of mice for more than two months despite that this strain has impaired in vivo replication. Therefore, titan cell production promotes cryptococcal cell survival in the host (Crabtree et al. 2012).

Furthermore, mice infected with a *gpr4*Δ*gpr5*Δ mutant showed lower fungal burden in the brain compared to those infected with the wt strain. However, no differences in fungal burden between the strains were observed during intracerebral infections. These results indicate that limited titan cell production correlates with reduced dissemination (Crabtree et al. 2012).

4.4 Polarization Toward a Th2 Response

Titan cell production increases the number of eosinophils in the lungs (García-Barbazán et al. 2016). Increased recruitment of eosinophils is characteristic of a Th2 response, typically observed during extracellular pathogens infection. However, in the case of fungal pathogens, Th2 immunity is associated with non-effective responses and lack of protection. In the particular case of *C. neoformans*, as infection progresses, induction of Th2 immune response may result in a more permissive environment for the yeast that favors fungal survival

and replication. Furthermore, the immune polarization would also facilitate the survival of the yeasts inside phagocytic cells and their replication.

In vivo studies have shown that titan cell formation depends on the dose of infection and the cryptococcal strain (Okagaki et al. 2010, 2011; Zaragoza et al. 2010). Furthermore, it is known that the percentage of cryptococcal titan cells in the population of mouse infected lungs varies during the course of infection and that the proportion of titan cells is higher when the mice are infected with low doses (Zaragoza et al. 2010). Cryptococcal cell growth also depends on host factors. It has been shown that the size of *C. neoformans* is larger in the lungs of mice deficient of B cells (Szymczak et al. 2013). In agreement, mice that develop Th2-type responses are more susceptible to the infection and present higher percentages of titan cells in the lungs (Garcia-Barbazán et al. 2016). This Th2-polarized response consists of low levels of IFN-γ and TNF-α and a poor recruitment of T and B cells in the lungs (Garcia-Barbazán et al. 2016). Besides, consistent with previous data, these mice showed more infiltrates of eosinophils instead of neutrophils. These data indicate that titan cells might induce a polarization to non-protective responses, which highlight another mechanism by which these cells contribute to the disease.

5 Future Perspectives

Cryptococcus neoformans is one of the most interesting models to investigate fungal virulence due to its ability to adapt to the lung environment. Multiple factors contribute to this adaptation, such as the polysaccharide capsule, production of melanin and growth at 37 °C. The formation of titan cells is a dramatic morphological change that can also facilitate the survival of the yeasts in the hosts. At the moment, this transition has been mainly described in the lungs (Okagaki et al. 2010; Wang et al. 2014; Zaragoza et al. 2010), but it is not known whether these cells also appear in other target organs, such as the brain or spleen, which are usually inflamed during the course of infection. Due to their significant capacity to evade the immune response, future studies are required to investigate whether titan cells also contribute to fungal colonization of different organs and niches in the human.

The recent description of conditions that promote titan cell formation in vitro can become a key contribution to better understand the biology of these cells. So far, three different groups have described conditions to induce this process, and interestingly, some of the inducing factors (serum, cell density effect, and quorum sensing) have been described simultaneously in these laboratories. One of the main conclusions of these works is that cellular enlargement is a response to multiple signals, so it is still possible that new factors will be found in the future. These approaches are limited by the fact that titan cell formation is highly dependent on the genetic background of the strain and microevolution in different laboratories. However, we anticipate that the involvement of multiple research groups in this topic and standardization of the different protocols will allow the full identification of the pathways involved in titan cell development.

Finally, the role of the immune response on cryptococcal morphology is another factor of great importance on this field. Apart from some in vivo factors, such as serum or bacterial components, there is increasing evidence that different polarization of the immune response has a determinant influence on titan cell development. This is an important aspect when extrapolated to human patients, because different persons might have different predisposition to develop titan cells. The identification of the factors of the immune response that influence cryptococcal morphology might in consequence help to predict the susceptibility of different individuals to this infection. Furthermore, future research on this topic will also contribute to anticipate the efficacy of different antifungal treatments on different patients.

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The Clock Keeps on Ticking: Emerging Roles for Circadian Regulation in the Control of Fungal Physiology and Pathogenesis

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Abstract Tic-tac, tic-tac, the sound of time is familiar to us, yet, it also silently shapes daily biological processes conferring 24-hour rhythms in, among others, cellular and systemic signaling, gene expression, and metabolism. Indeed, circadian clocks are molecular machines that permit temporal control of a variety of processes in individuals, with a close to 24-hour period, optimizing cellular dynamics in synchrony with daily environmental cycles. For over three decades, the molecular bases of these clocks have been extensively described in the filamentous fungus *Neurospora crassa*, yet, there have been few molecular studies in fungi other than *Neurospora*, despite evidence of rhythmic phenomena in many fungal species, including pathogenic ones. This chapter will revise the mechanisms underlying clock regulation in the model fungus *N. crassa*, as well as recent findings obtained in several fungi. In particular, this chapter will review the effect of circadian regulation of virulence and organismal interactions, focusing on the phytopathogen *Botrytis cinerea,* as well as several entomopathogenic fungi, including the behavior-manipulating species *Ophiocordyceps kimflemingiae* and *Entomophthora muscae*. Finally, this review will comment current efforts in the study of mammalian pathogenic fungi, while highlighting recent circadian lessons from parasites such as Trypanosoma and Plasmodium. The clock keeps on ticking, whether we can hear it or not.

1 Introduction

Consecutive sunsets and sunrises clearly illustrate the fact that we live in a highly cyclic environment, exposed to daily oscillations in several factors: light (and darkness), temperature, solar radiation, environmental humidity, as well as variations in organismal interactions. These daily fluctuations can bring upon considerable challenges, i.e., thermal and osmotic stress, DNA damage, yet, the repetitive nature of these changes makes them predictable, which has contributed to the appearance of circadian clocks. These biological clocks are molecular machines that have emerged throughout evolution allowing organisms to keep track of time, even in the absence of celestial cues, permitting, therefore, to anticipate some of these daily challenges (Dunlap et al. 2004). Relevantly, as we will discuss in this chapter, in recent years, it has become evident that circadian clocks can also help anticipating daily fluctuations in biotic variables associated with organismal interactions, as exemplified in several studies.

It is essential to stop for a minute to analyze the etiology of the adjective that defines these clocks: circadian, from the Latin *circa diem,* which implies that these clocks measure time with a period of approximately, but not exactly, 24 h. Thus, in the absence of environmental cues (as in constant darkness), circadian clocks exhibit their free-running period, which is close to (but not precisely) 24 h (i.e., 22.5 or 25 h), while in the presence of environmental cycles, they are entrained to precisely 24 h. Thus, circadian clocks are endogenous subcellular devices that

confer daily rhythms to a broad range of biological processes such as gene expression, physiology, and behavior (Dunlap et al. 2004).

Despite their relevance, circadian clock components do not share sequence similarity across phyla, and the evidence suggests that they have emerged at least three independent times throughout evolution (Rosbash 2009). Thus, although the sequence characteristics of clock elements vary across distant organisms, such as cyanobacteria, insects, fungi, or mammals, in all cases they appear to share the same basic design: a transcription-translation negative feedback loop (TTFL). In such circuitry, positive elements drive the expression of negative elements that feedback to shut down their synthesis by inhibiting the positive elements, a process that repeats itself every ~24 h (Bell-Pedersen et al. 2005; Montenegro-Montero et al. 2015; Dunlap 1999). In the case of plants, the circuitry of the central oscillator appears more complex, involving the presence of additional loops (Romanowski and Yanovsky 2015). On the other hand, in prokaryotes, most studies have focused on the cyanobacteria *Synechococcus elongatus* (Mackey et al. 2011), while no clock components have been reported in Archaea. However, rhythms in peroxiredoxins oxidation have also been described in several organisms, including Archaea, which has been postulated as evidence of a common evolutionary ancestor (of unknown molecular composition and circuitry topology) in the origin of circadian rhythms (Edgar et al. 2012). Moreover, rhythms in magnesium levels have been shown to exist in fungi, unicellular alga, and mammalian cells, pointing to the presence of additional conserved underlying clock mechanisms across taxa (Feeney et al. 2016).

As mentioned, the free-running period of a circadian clock can be entrained to external rhythmic cues, being light and temperature the most relevant ones, such that its period matches the environment, which in planet Earth implies 24 h days. Thus, clocks can be synchronized or "entrained," allowing temporal control of several processes, optimizing them to certain times of the day (Dunlap 1999; Bell-Pedersen et al. 2005; Montenegro-Montero et al. 2015). Three basic requirements are generally considered in order to classify an oscillation as truly circadian: (1) It should persists in the absence of environmental cues [i.e., in constant light or constant darkness (free-running conditions)] with a period of *circa* 24 h; (2) it must be entrained by cyclical temperature or light signals; and finally, (3) it should exhibit temperature and nutritional compensation (Montenegro-Montero and Larrondo 2013; Dunlap 1999). Such characteristics allow distinguishing circadian-based rhythms from cell-cycle-regulated or environment-driven ones, and from other metabolic or developmental rhythms (Dunlap et al. 2004).

Accumulative evidence has helped to highlight the importance of clock regulation in organismal physiology and fitness. Their transversal relevance has been recently recognized with the 2017 Nobel Prize in Medicine awarded to work conducted in Drosophila, due to its implications to biology and human health (Dibner and Schibler 2017). Examples compiled in cyanobacteria, plants, and mammals, utilizing mutants with altered clocks, have revealed that when the internal circadian period matches the one in the oscillating environment improved fitness can be observed (Ouyang et al. 1998; Dodd et al. 2005; Yerushalmi and

Green 2009; Lowrey and Takahashi 2011). Several studies have shown the importance of circadian regulation in mammals, and how alterations or misalignments can lead to metabolic disorders and disease (reviewed in (Perelis et al. 2015; West and Bechtold 2015; Paschos and FitzGerald 2017; Scheiermann et al. 2018)). On the other hand, the evidence supports the effect of clock regulation of immune and defense responses not only in mammals, but also in flies and plants (Bhardwaj et al. 2011; Wang et al. 2011; Shin et al. 2012; Zhang et al. 2013; Goodspeed et al. 2012, 2013; Korneli et al. 2014; Ingle et al. 2015; Labrecque and Cermakian 2015; Lee and Edery 2008; Scheiermann et al. 2018). Therefore, the data supports the idea that circadian clocks confer an adaptive advantage, allowing anticipation of environmental changes, including interaction with other organisms and maximizing the efficiency of some processes at certain times of the day (McClung 2006; Roden and Ingle 2009).

Neurospora crassa is a filamentous fungus considered to be a premier model for circadian studies. In this ascomycete, daily spore production (conidiation) occurs just before dawn (a time when humidity is high, and the temperature is low) which would confer an adaptive advantage allowing enhanced spore dispersion and survival (Bell-Pedersen et al. 1996). Despite the extensive characterization of clocks mechanisms in Neurospora, there is scarce molecular information regarding circadian clocks in other fungal models including pathogenic ones. Therefore, how these clocks could modulate microbial virulence, and converse with the clock of the host, are questions that remain largely unexplored.

In the next pages, we will provide a basic view of clock mechanisms in Neurospora, to then review recent molecular and phenotypic description of clocks in other fungi, including pathogenic ones. Importantly, we will also cover emergent information of clock regulation on other pathogenic organisms.

2 Circadian Clocks: Neurospora and Beyond

The fungal kingdom comprises an enormous diversity of organisms, most of which remain uncharacterized. Conservative estimations indicate that it harbors at least 5.1 million species (Blackwell 2011), distributed in five main phyla: Chytridiomycota, Zygomycota, Glomeromycota, Basidiomycota, and the Ascomycota (James et al. 2006). The latter hosts the unicellular Saccharomycotina and Taphrinomycotina subphyla as well as the Pezizomycotina subphylum of filamentous fungi (Hibbett et al. 2007). Within the Pezizomycotina, one can distinguish groups such as the Dothideomycetes, Eurotiomycetes, Leotiomycetes, Sordariomycetes, Pezizomycetes, and the Orbiliomycetes, being the last two the most basal filamentous fungi (Traeger et al. 2013; James et al. 2006). Nevertheless, despite their great diversity and abundance, molecular work describing the mechanistic details of circadian clocks has concentrated exclusively in one species, the ascomycete *N. crassa*.

2.1 Molecular and Phenotypical Rhythms in Neurospora

Along with Drosophila, work in Neurospora paved the road to the molecular dissection of circadian clocks, thanks to its straightforward genetics and the ease to visualize a circadian phenotype: daily conidiation (Dunlap 2008). After almost 30 years of the cloning of the first clock components in both organisms, this is what we know so far. In Neurospora, the circadian TTFL (also called the FRQ-WCC oscillator) is based on a heterodimer formed by the transcription factors (TFs), White Collar 1 (WC-1), and White Collar 2 (WC-2). These GATA Zn-finger proteins interact through PAS domains to form the White Collar Complex (WCC), which controls the expression of the *frequency* (*frq*) gene. When FRQ (the negative element) is produced, it dimerizes to then associate with another core-clock component: FRH (FRQ-RNA-Helicase). This FRQ-FRH complex interacts with kinases, causing progressive phosphorylation of FRQ and promoting inactivation of WCC (by phosphorylation) with the concomitant drop in *frq* mRNA levels. The existing FRQ protein continues to be progressively phosphorylated in over 100 Ser/Thr sites by several kinases including CK1, CK2, and PKA, (Guo and Liu 2010; Montenegro-Montero et al. 2015). As this happens, the interaction between FRQ/FRH and the WCC decreases and therefore WCC recovers activity, as the hyperphosphorylated FRQ is degraded by the proteasome (He and Liu 2005). As FRQ is degraded new FRQ is produced and clear oscillations in *frq* and FRQ levels can be observed daily, every 22.5 h under free-running conditions (constant darkness). Recent results have highlighted posttranslational modifications of FRQ as a major mechanism regulating the speed of the clock, revealing also that degradation of hyperphosphorylated FRQ is not a critical event for the TTFL to reinitiate a new clock cycle (Larrondo et al. 2015; Dunlap and Loros 2018).

WC-1 is not only a TF, but also a photoreceptor, as it contains a light oxygen and voltage (LOV)-sensing domain. Under free-running conditions, such as constant darkness (DD), the WCC binds to a specific sequence in the *frq* promoter known as the clock-box (*c-box*), which is both necessary and sufficient to sustain *frq* rhythmic expression (Froehlich et al. 2003). In the presence of light, WCC suffers a conformational change and, driven by LOV-LOV interactions, forms a multimer with other light-activated WCCs. This leads to changes in DNA binding specificity, and therefore light-activated WCC recognizes a different sequence in the *frq* promoter, the proximal light regulatory element (*pLRE*), allowing a massive increase in *frq* expression upon exposure to light. These changes in *frq* levels are critical for the entrainment of the clock by environmental light (Froehlich et al. 2002; Montenegro-Montero et al. 2015).

In addition to the action of WCC on both the *pLRE* and *c-box*, several chromatin-remodeling events have been shown to control proper *frq* expression at both sites (Proietto et al. 2015; Wang et al. 2014). Nevertheless, although some co-repressors such as RCO-1 have been reported as core-clock component necessary for *frq* rhythmic levels by controlling *frq* chromatin status (Zhou et al. 2013),

recent data has challenged the clock-essential role originally assigned to RCO-1 (Olivares-Yanez et al. 2016).

In general, these rhythmic core mechanisms occurring at the circadian oscillator can be visualized as daily cycles in *frq* and FRQ levels. Importantly, through the input and output signaling pathways, respectively, this oscillator can be synchronized to the environment while it temporally controls a series of processes, such as conidiation (Montenegro-Montero et al. 2015).

Light and temperature are environmental cues that allow synchronizing and entraining the circadian oscillator, through the input pathways. In the case of light, *frq* expression is acutely induced through the *pLRE*, which leads to phase advances or delays, or to a complete resetting of the clock, depending on the intensity of the light stimuli (Crosthwaite and Heintzen 2010). While temperature pulses can also lead to changes in phase, probably the most remarkable—but poorly understood—characteristic of clocks is their emergent property of temperature compensation (period remains constant over a range of physiological temperatures). In the case of Neurospora, casein kinase 2 (CK2) has been shown to mediate such property by phosphorylation reactions that balance stabilization and destabilization of FRQ (Mehra et al. 2009).

The central oscillator in return controls a series of the cellular process through the output pathways. One of the key mechanisms by which the core oscillator provides rhythmic expression of *clock-controlled genes* (*ccg*s) relays on oscillatory transcription. This is in part mediated by a hierarchical arrangement of rhythmically expressed TFs [reviewed in (Montenegro-Montero et al. 2015)]. RNA-seq data indicates that up to 40% of the Neurospora genome may be rhythmically expressed, through mechanisms that also involve posttranscriptional control (Hurley et al. 2014). Nevertheless, the contribution of transcriptional versus posttranscriptional mechanisms in *ccg*s expression is a matter of debate in Neurospora, as well as in other models (Montenegro-Montero and Larrondo 2016). For simplicity, this section will just focus on the former. Under circadian conditions, the WCC directly recognizes promoters of hundreds of genes among which several encode for TFs, such as CSP-1, ADV-1, and SUB-1 (which are referred as *first tier* TFs). Not surprisingly, mRNAs for these TFs exhibit rhythmic expression, as well as their target genes (Smith et al. 2010; Sancar et al. 2015). Thus, WCC not only rhythmically controls *frq* in the TTFL, but also is a pivotal link between the core oscillator and the output pathways, acting as the executor of a hierarchical cascade of rhythmic transcription.

In Neurospora, conidiation provides a strong phenotype for clock output which can be easily visualized through the race-tube assay: hollow glass tubes, filled with agar, in which Neurospora grows from one end to the other producing asexual spores (conidia) once a day. This assay permits calculating clock properties such as period and phase, the latter defined as the time of appearance of a clock event (i.e., band of conidia) in relation with a reference point (i.e., transition between light and dark) (Montenegro-Montero et al. 2015).

Although luciferase (*luc*) has been a popular real-time reporter in the study of all circadian systems, such as Arabidopsis, cyanobacteria, and mammals (Welsh et al.

2005), its use in Neurospora started rather late (Gooch et al. 2008). Thus, codon optimized firefly luciferase controlled by the *frq* promoter helped visualizing *frq* expression dynamics under several physiological conditions, allowing to interrogate clock mechanisms in strains with altered output (Shi et al. 2007), or exhibiting growth defects (Olivares-Yanez et al. 2016). The design of modular circadian transcriptional reporters by putting luciferase under the control of a *c-box + minimal promoter* also allows to easily examine, simultaneously, multiple strains and genotypes in 96-well plates, revealing unexpected details of the circadian oscillator (Olivares-Yanez et al. 2016; Larrondo et al. 2015; Gooch et al. 2014; Hurley et al. 2013). Likewise, fusing *luc* with *ccg* promoters allows for the study of circadian output (Sancar et al. 2011; Hurley et al. 2014). In addition, another useful strategy has been to directly fuse, by homologous recombination (Larrondo et al. 2009), the *luc* sequence to the *frq* gene at its endogenous locus, generating a FRQ-LUC translational fusion, allowing real-time monitoring of FRQ levels (Larrondo et al. 2012).

Readers interested in other aspects of Neurospora core-clock mechanisms, including the role of kinases, period determination, and a detailed analysis of input and output pathways, can consult other specialized reviews elsewhere (Montenegro-Montero et al. 2015; Guo and Liu 2010; Hurley et al. 2015).

3 Rhythms in Non-pathogenic Fungi

Compared to the previous section that contained abundant data on circadian mechanisms in Neurospora, the next paragraphs may seem disappointing, as there is scarce information on clock mechanisms in other fungi.

There have been several reports describing rhythmic fungal phenomena, some dating over 60 years ago (Uebelmesser 1954; Schmidle 1951), yet most of those circadian systems have not been molecularly dissected (reviewed in (Bell-Pedersen et al. 1996). In the case of *Sordaria fimicola*, a Sordariomycete like Neurospora, the existence of rhythms in spore discharge, had also been reported (Austin 1968). Later on, it was shown that its *frq* gene was able to complement a *N. crassa frq* mutant strain, rescuing rhythmic conidial banding (Merrow and Dunlap 1994). One of the important things that this paper came to confirm was that *frq* was actually a gene involved in clock regulation and not just a regulator of asexual reproduction (conidiation).

Other genus belonging to the class of the Sordariomycetes is Trichoderma, where genomic analyses have confirmed the presence of *frq*, *wc*-1, and *wc*-2 homologs in *T. atroviride*, *T. reesei, T. virens*, and *T. pleuroticola* (Steyaert et al. 2010). In the latter, circumstantial data suggests the production of conidial rings under DD with a period near 24 h, although no rhythmic conidial phenotype has been described for any of the other mentioned Trichoderma species (Steyaert et al. 2010). Importantly, the absence of evidence is not evidence of absence: In this case, the lack of a visible overt rhythm is, by no means, evidence of the absence of

circadian regulation. Nevertheless, the *wc*-1 and *wc*-2 homologs (*blr*-1 and *blr*-2) are known to mediate blue light-induced conidiation in *T. atroviride*, as one would expect based on the available *N. crassa* model (Casas-Flores et al. 2004).

The fungus *Pyronema confluens* belongs to the early diverging Pezizomycetes group of filamentous ascomycetes. The sequencing of its genome revealed the presence of *frq, wc*-1, *wc*-2, and *frh* homologs (Traeger et al. 2013), whereas experiments confirmed that *P. confluens frq* expression is induced by light. Moreover, its mRNA oscillates under free-running conditions (DD), peaking in the subjective morning with a circadian period that is temperature compensated. Nevertheless, no overt circadian output phenotype was observed, although from a handful of analyzed genes only two behaved as *ccg*s displaying an oscillatory expression pattern (as seen by RT-qPCR) with profiles of morning-specific genes (Traeger et al. 2013; Traeger and Nowrousian 2015). Although this work shows that this *frq* homolog oscillates, strictly speaking it does not prove that the oscillations mechanism depends on *frq*, as the latter could be just a *ccg* in *P. confluens*. Therefore, additional work is needed to confirm a central role for this gene in clock regulation in this fungus.

The presence of a functional *frq* homolog, exhibiting rhythmic expression in *P. confluens* suggests that *frq* was present in the common ancestor of the filamentous ascomycete fungi (Pezizomycotina) (Traeger and Nowrousian 2015). In silico analysis appear to suggest that *frq* has been lost several times during evolution. For example, *frq* or *frq*-like sequences appear to be absent in most members of the Eurotiomycetes, which contains genus such as Aspergillus or Penicillium (Montenegro-Montero et al. 2015; Traeger and Nowrousian 2015). In addition, a FRQ-like sequence is present in the genome of *Saitoella complicata,* a fungus in the early diverging Taphrinomycotina subphylum of the Ascomycota. Yet, there is no vestige of *frq-like* sequences in *Saccharomyces cerevisiae* or other members of the Saccharomycotina subphylum (Montenegro-Montero et al. 2015). Moreover, our additional in silico studies identified a FRQ-like protein in *Rhizophagus irregularis*, whereas recently its mRNA expression has been confirmed (Lee et al. 2018). *R. irregularis* is an early diverging mycorrhizal fungus that belongs to the Glomeromycota phylum, which is located before the divergence of Basidiomycota and Ascomycota (Montenegro-Montero et al. 2015). This argues that the idea of FRQ being the result of a recent innovation restricted to a certain group of fungi followed by subsequent losses is unlikely. Indeed, distant FRQ homologs were also identified in some basidiomycetes (Montenegro-Montero et al. 2015) mainly restricted to the Pucciniomycotina subdivision. As an example, the *Sporobolomyces roseus* genome encodes for a FRQ-like sequence that exhibits a 28% identity with *N. crassa* FRQ. While these in silico analyses (Montenegro-Montero et al. 2015; Hevia et al. 2016) further expand the extent and depth of previous studies of *frq* distribution (Salichos and Rokas 2010), additional robust phylogeny studies are required to identify the evolutionary origin of FRQ and other core-clock components, as well as the co-evolution of ancillary co-opted components.

And what happens underground, under conditions in which light information may be restricted? That is a typical scenario in which one can encounter arbuscular

mycorrhizal fungi (AMF) interacting with plant roots, yet there is evidence of WCC homologs in such organisms and confirmation that in some, these homologs are actively expressed as seen in *Rhizoglomus irregularis*, an AMF belonging to the Glomeromycota (see above). Thus, a recent report not only describes that both the *wc*-1 and *wc*-2 homologs are expressed, but that there is also a *frq* homolog which expression is increased after a light pulse (Lee et al. 2018). Nevertheless, while discussing their results, the authors erroneously state that "so far, all the fungal species with conserved *frq* and *WC* proteins have a circadian clock" (Lee et al. 2018). Such statement is inaccurate, as the mere presence of *frq* and *wcc* encoding genes is not enough to assure that a clock will be functional, as this needs to be confirmed following some of the criteria stated above and found elsewhere (Montenegro-Montero et al. 2015). However, the fact that a *frq* homolog is present and expressed in a Glomeromycota member is quite remarkable. Our own analysis of the *R. irregularis* genome indicates that the encoded FRQ sequence exhibits a 45.5% of identity to the *N. crassa* one, while two other Rhizophagus genomes (*R. cerebriforme* and *R. diaphanous*) have sequences with 45.6 and 47.6% of identity to Neurospora FRQ. It is also worth mentioning the description, based on a sophisticated ecological observatory setup, of rhythms in Arbuscular mycorrhizal hyphal growth during 24 h, which appeared to parallel circadian oscillations occurring in the plants (Hernandez and Allen 2013). At the depths at which these fungi are normally interacting with the roots, light levels are rather negligible while temperature tends to display little daily variation (Hernandez and Allen 2013). Therefore, both light and temperature may not be acting as entraining or synchronizing cues for those fungal rhythms, which raises intriguing questions regarding their circadian nature as well as their dependence and/or interrelationship with the plant clock.

In the case of the ascomycete *Aureobasidium pullulans*, belonging to the Dothideomycetes class, daily formation of concentric rings, under particular media and light conditions, was known to exist, yet it was not clear if they were a manifestation of a *bona fide* clock (Slepecky and Starmer 2009). Analysis of an *A. pullulans* strain, isolated from the Argentinian Patagonia, confirmed that the formation of such daily rings was under circadian control, as they persisted (with a period of ~24.5 h) under constant conditions (DD) and could also entrain to different light:dark (LD) cycles (Franco et al. 2017). Moreover, these rhythms were also shown to be temperature compensated, as determined by growing *A. pullulans* at temperatures ranging from 10° to 20 °C. Remarkably, this appears to be the first experimental evidence of a circadian clock running at such low temperatures, something that may not be so surprising if one considers the natural cold ecological niche of this Patagonian *A. pullulans* isolate. At the molecular level, *A. pullulans* bears homologs of *wc*-1, *wc*-2, and *frq*, and while *frq* levels did not exhibit any clear oscillations under the tested conditions, it did show a strong response to light (Franco et al. 2017).

In *S. cerevisiae,* despite the absence of a *frq* homologue, or WCC encoding sequences, interesting phenomena have been described: rhythms in amino acid uptake, cell division, and metabolism (Edmunds et al. 1979). Interestingly, careful

examination of these metabolic oscillations has revealed that they share several mechanistic similarities to circadian rhythms, including the importance of post-translational modifications mediated by CK1 (Causton et al. 2015). In addition, these yeast metabolic rhythms have been described to exhibit circadian entrainment, responding to cycle length as well as the strength of environmental signals. Nevertheless, such oscillations display a weak free-running rhythm, consistent with a weak damped oscillator (Eelderink-Chen et al. 2010). Another member of saccharomycotina, *Schizosaccharomyces pombe*, does not appear to have *bona fide* circadian rhythms, although infradian temperature compensated oscillations in heat tolerance and ultradian rhythms in cell division have been observed [reviewed in (Montenegro-Montero et al. 2015)].

Previous genomic studies had concluded that ascomycetes genomes in the Eurotiomycetes class did not have *frq* homologs (Salichos and Rokas 2010). Nevertheless, subsequent analysis by our group identified FRQ-like sequences encoded in the genomes of *Talaromyces aculeatus* and *Talaromyces stipitatus.* Interestingly, such sequences appear shorter than the one in Neurospora, presenting identities of 19.5 and 20.6%, respectively (Montenegro-Montero et al. 2015). The Eurotiomycetes class also hosts the genus Aspergilli, with iconic fungi such as *Aspergillus nidulans*. As for most Eurotiomycetes, bioinformatic analyses of the *A. nidulans* genome failed to identify a *frq* homolog, although there are genes encoding for *wc*-1 and *wc*-2 homologs, which are involved in photobiology (Fuller et al. 2015). Aspergillus lacks any distinguishable circadian rhythm in conidiation, although rhythms in sclerotia formation have been reported in *Aspergillus flavus* (Greene et al. 2003), a plant and human pathogen (see below). On the other hand, when growing *A. nidulans* under DD conditions, infradian rhythms (28–32 h) in *glyceraldehyde*-3-*phosphate dehydrogenase* (*gpdA*) mRNA levels have been described. Importantly, when analyzing *gpdA* oscillations under temperature cycles, it presents entrainment with a period of 24 h (Greene et al. 2003).

As hinted earlier, the absence of an overt circadian phenotype, such as conidiation, does not necessarily imply the absence of an underling functional circadian clock. A clear example is Neurospora. Indeed, observing rhythmic conidiation in Neurospora isolates, under laboratory conditions, may not always be trivial, and actually, the *Neurospora* strain utilized since the 60's as the "WT strain" for circadian studies is a mutant isolate, called *band* (Sargent et al. 1966). This strain exhibited a robust circadian banding phenotype (spores observed as a defined daily band of conidia), and therefore, the *band* (*bd*) mutation has been systematically crossed into strains or isolates that are subjected to circadian studies. Subsequent studies revealed that *bd* corresponds to a point mutation in the *ras*-1 gene ($ras\text{-}1^{bd}$), which enhances circadian conidiation, allowing clear visualization of this phenotype in the race-tube assay, under laboratory conditions (Belden et al. 2007).

An exciting observation, with interesting implications in organismal dynamics, was recently described for the mushroom *Neonothopanus gardneri*, a fungus present in the Brazilian forest (Oliveira et al. 2015). This basidiomycete was shown to exhibit rhythms in bioluminescence that would persist even under DD conditions, with a period of $\sim$22 h, and a peak in the subjective night. One of the hallmarks of

this study is that it provided a nice example of adaptive advantage conferred by a biological clock to a fungus: Bioluminescence emitted at night helps attracting insects, which would then facilitate spore dispersion. Nevertheless, the molecular basis of the clock that regulates this rhythmic bioluminescence in *N. gardneri* remains unknown. Still, in silico analyses have revealed that weak FRQ homologs are present in a few members of the Basidiomycota phylum, further supporting the idea that FRQ was gained and lost several times during fungal evolution (Montenegro-Montero et al. 2015; Salichos and Rokas 2010).

4 Rhythms in Plant-Pathogenic Fungi

As indicated earlier, the presence and influence of clocks in pathogenic organisms have been a poorly explored area in general, with only a handful of publications reporting on this topic for fungal pathogens. Nevertheless, in the context of plant–pathogen interactions, the insect *Trichoplusia ni* stands as a nice example of circadian modulation of a pathogen's dynamics (Goodspeed et al. 2012). Rhythmic feeding behavior has been observed for *T. ni*, with a peak at midday under a light: dark cycle (LD), that persist even under DD conditions.

Overall, there is scarce, but interesting data regarding the presence and effect of circadian rhythms in pathogenic fungi, as we narrate now.

The soybean pathogen *Cercospora kikuchii*, which produces the phytotoxin cercosporin, is an ascomycete that belongs to the Dothideomycetes class (Daub and Ehrenshaft 2000). This fungal plant pathogen displays circadian rhythm of hyphal melanization under LD, a behavior that persists for many days under DD conditions, with a period close to 24 h. Importantly, this rhythmic melanization also persists at different temperatures between 20 and 30 °C (Bluhm et al. 2010). This same publication indicated the absence of a *frq* homolog in *C. kikuchii*, or from the closely related fungus *Cercospora zeae-maydis*, as tested by PCR or EST examination, respectively. Nevertheless, in silico genomic analysis has subsequently confirmed the presence of a *frq* homolog not only in *C. zeae-maydis,* but also in other members of the Capnodiales order, to which Cercospora belongs, including the closest related fungus *Mycosphaerella graminicola* (Montenegro-Montero et al. 2015). In addition, although the hemibiotrophic fungal maize pathogen *C. zeae-maydis* does not exhibit rhythmic hyphal melanization (Bluhm et al. 2010), it presents CRP1, a homolog of the *N. crassa* WC-1 (Kim et al. 2011a). A Δ*crp*1 *C. zeae-maydis* is impaired in virulence, exhibiting loss of stomatal tropism, defects in appressoria formation, conidiation as well as in the production of cercosporin. Importantly, it has been indicated that under field conditions *C. zeae-maydis* displays an alternating lesion pattern resulting from bands of conidiophores interspersed by areas of vegetative growth, which allows for the untested hypothesis that a circadian clock is governing such phenotype (Kim et al. 2011a). If such a clock actually actively impacts its pathogenic potential, depending on the time at which inoculation occurs, remains to be assessed.

As mentioned earlier, although no *frq* homologs are present in Aspergilli (Salichos and Rokas 2010; Montenegro-Montero et al. 2015), rhythms in sclerotia formation have been documented in the human and plant pathogen *Aspergillus flavus*. These developmental rhythms are dependent on the media composition, can be entrained by temperature and light cycles, and persist even under DD, with a period that is temperature compensated between 30 and 40 °C. This period of sclerotia formation nevertheless is of 33 h, closer to an infradian rhythm, compared to the ~22.5 h seen in Neurospora (Greene et al. 2003). Recently, a study that focused on the transcriptional control of genes needed for sclerotia development, including ones participating in cellular fusion, found some parallels with events occurring in *N. crassa*, raising interesting hypothesis of potential *ccg*s controlling this rhythmic phenotype in *A. flavus* (Zhao et al. 2017).

Magnaporthe oryzae has been ranked as probably the most important phytopathogen worldwide based both in its agronomic and scientific relevance (Dean et al. 2012). This ascomycete, a great model to study plant–pathogen interactions, infects important crops including rice, causing the well-known rice blast disease, a major problem as approximately one half of the world relays on rice as a primary food source (Dean et al. 2012). The genome of this Sordariomycetes exhibits homologs of *frq*, *wc*-1, and *wc*-2 (Salichos and Rokas 2010). And while there has not been yet a molecular characterization of a functional circadian oscillator in *M. oryzae*, diverse observations have led to propose, although not yet prove, that conidiation and virulence in this fungus are under circadian control (Deng et al. 2015). Some of the data that has led to this idea is that under LD cycles *M. oryzae* presents a conidial banding pattern or that the release of conidia occurs at night time (Lee et al. 2006). It is also interesting the fact that *M. oryzae frq* mRNA oscillates under a 12:12 LD cycle, although its behavior has not been reported under constant dark (Deng et al. 2015). Probably, the most compelling data regarding the presence of *bona fide* rhythms in this fungus relates to the expression of the *twilight* (*twl*) gene, which is induced by light, and displays rhythmic oscillations under LD and DD conditions, peaking at dawn (Deng et al. 2015). In LD *frq* mRNA continues to oscillate in the absence of TWL, which indicates that TWL is not a core-clock component, although it is necessary for full virulence (Deng et al. 2015). On the other hand, MGWC-1, the homolog of *N. crassa* WC-1, although not necessary for spore production, is needed for spore release (Lee et al. 2006) and it is also required to suppress disease development in LL (constant light) (Kim et al. 2011b).

There are also reports describing mycelial banding in the plant pathogen *Sclerotinia fructigena,* from the Leotiomycetes class (Jensen and Lysek 1983). This ascomycete, also known as *Monilinia fructigena*, shows mycelial banding that is temperature compensated and that persists even under free-running conditions (Jensen and Lysek 1983). Interestingly, it was reported that out of 181 examined isolates of this fungus that exhibited rhythmic growth pattern under LD cycles, only 30 of them would still present such rhythm in DD (Jensen and Lysek 1983). Our in silico analyses confirmed the presence of *frq, wc*-1, and *wc*-2 homologs in its genome, as it has also been described for *Sclerotinia sclerotiorum*, a close relative of *M. fructigena* (Salichos and Rokas 2010).

Further general information of clock phenomena in other fungi can be found elsewhere (Ramsdale 2008; Montenegro-Montero et al. 2015).

4.1 Molecular Characterization of the Circadian Clock of the Plant-Pathogenic Fungus Botrytis cinerea

B. cinerea is an aggressive necrotrophic fungal plant pathogen, causing the killing and rotting of plant material, also known as gray mold disease (Tudzynski and Siewers 2004). The genus Botrytis includes over 20 species, of which *B. cinerea* is the most infamous one, ranking second after *M. oryzae* as the most important phytopathogen, in terms of its relevance (Dean et al. 2012). *B. cinerea*, along with the necrotrophs *S. sclerotiorum* and *M. fructigena*, belongs to the Leotiomycetes class.

B. cinerea has been reported to infect over 1000 different plant species, including several relevant commercial crops, positioning this fungus as a major agronomical problem (Veloso and van Kan 2018). This versatile necrotroph is a professional assassin, capable of killing the host cells through the controlled production of reactive oxygen species, toxins, plant degrading enzymes, and an oxidative burst produced by the plant in response to *B. cinerea*. As a result, plant cells die and serve as a substrate for fungal growth and spreading of the infection (Veloso and van Kan 2018).

Among the many primary hosts of *B. cinerea* are dicotyledonous plants such as grape vine, strawberry, tomato, ornamental flowers, as well as monocots, although the latter are considered poor hosts. Botrytis infection is not limited to only one type of tissue, and, indeed, it can infect leaves, stems, flowers, and fruits, whether they are ripe or unripe (Schumacher and Tudzynski 2012). In addition, *B. cinerea* can remain quiescent in infected flowers for long periods, until the fruit ripens. Moreover, this fungus can be active at low temperatures (i.e., as low as 0 °C), with the obvious complications as post-harvest disease can occur as products are stored or in transit to international markets (Elad et al. 2007). Although the infection process can equally occur from 0 to 30 °C (Elad et al. 2007), optimal temperature for germination and infection is 20 °C, while adequate levels of humidity are also important for infection under field conditions (Latorre and Rioja 2002).

Conidiation in Botrytis, at least for the B05.10 wild-type strain, is dependent on the presence of light (Canessa et al. 2013). In the absence of light, microconidia are first produced, while after some weeks (3–4), sclerotia, dark melanin covered structures, are formed (Schumacher and Tudzynski 2012). While *B. cinerea* macroconidia are key in the infection process, microconidia fail to successfully infect as they generate a short germinal tube not compatible with the process (Jarvis 1977).

B. cinerea exhibits high genetic variability, which impacts aggressiveness, production of secondary metabolites, virulence factors, as well as the mode of

reproduction displayed by the different isolates (Schumacher and Tudzynski 2012). For example, regarding the latter point, while in the dark the B05.10 strain forms sexual structures (microconidia and sclerotia), the T4 strain (also a popular laboratory isolate) presents an "always conidia phenotype," which means that even in the dark it produces macroconidia, failing to form sexual structures (Canessa et al. 2013). Therefore, these different behaviors to a defined stimulus serve as cautionary tale: how critical is to choose a defined and well-characterized strain to conduct phenotypic and molecular assays. In the case of *B. cinerea*, due to its genetic stability (and easy genetic manipulation), the aggressive isolate B05.10 has become the standard recipient strain for genetic modifications and virulence studies (Buttner et al. 1994; Tuzdynski and Kokkelink 2009).

Although for a long time evidence indicated that *B. cinerea* responded to light at the phenotypic level, there was no molecular data on how this was occurring, yet a likely candidate to be mediating some of the light responses was the homolog of Neurospora WC-1 (Schumacher and Tudzynski 2012). As mentioned earlier for *N. crassa*, the TF and blue light receptor WC-1 along with WC-2 form the WCC, commanding responses to light and also being a core-clock component (Chen et al. 2010). In silico analysis of the Botrytis genome not only confirmed the presence of both WC-1 and WC-2, but also of a *frq* homolog, allowing their further molecular characterization. Thus, it was possible to confirm how light, through the action of *B. cinerea* WC-1 (BcWCL1), mediates some phenotypical responses and also activates changes in gene expression in this fungus (Canessa et al. 2013). As expected, several of the tested genes that respond to light in *B. cinerea* stop responding in a BcWCL1 knockout strain. Nevertheless, and in opposition to *N. crassa* where all light responses depend on WC-1, some light-responsive genes kept on being activated by this stimulus in a Δ*bcwcl*1 genetic background (Canessa et al. 2013), exemplifying the complex and fascinating photobiology of this organism (Schumacher 2017). Importantly, among the BcWCL1 light-activated genes, was *B. cinerea frq* homolog (*bcfrq*1), suggesting that at least in the context of light-induction its regulation was comparable to the Neurospora paradigm.

Despite the existence of core-clock components in Botrytis, and their regulation by light, this fungus fails to display rhythmic spore development in DD. Nevertheless, the *B. cinerea* B05.10 strain exhibits bands under LD cycles; although they are not made in a daily fashion and surprisingly, they are enhanced in the absence of BcWCL1 (Canessa et al. 2013). This lack of an overt circadian phenotype was not necessarily evidence of the absence of a molecular clock, and, indeed, molecular exploration of Botrytis *bcfrq*1 showed that its mRNA oscillates under LD (Hevia et al. 2015), being high during the lights-on period, to then decrease during the lights-off phase. Most importantly, it was possible to verify that *bcfrq*1 expression was anticipating the dark-to-light transition, as its levels would increase before lights on, a key feature of a circadian behavior (Dunlap 1999). Another classic circadian feature exhibited by *bcfrq*1 expression was that *bcfrq*1 mRNA levels displayed a robust oscillatory pattern under DD (as seen in *N. crassa* (Aronson et al. 1994)). This rhythm is lost in LL, since expression of *bcfrq*1 remains high and insensitive to the negative feedback mechanism, consistent with

the fact that in *N. crassa*, the circadian clock is dysfunctional under constant light (Crosthwaite et al. 1995). And, just as *bcfrq*1 requires BcWCL1 to respond to light (Canessa et al. 2013), it also depends on this TF to oscillate in DD, as seen in Neurospora (Lee et al. 2003). Additional experiments demonstrated that BcFRQ1 was a core-clock component, acting as the negative element, and not just an oscillatory gene. Thus, when an additional copy of *bcfrq*1 is expressed under the control of a strong actin promoter (overexpressing strain, OE::*bcfrq*1), the levels of the endogenous *bcfrq*1 mRNA remain low, as the transcripts coming from de $actin_{prom}$-*bcfrq*1 locus are high. (Hevia et al. 2015). Therefore, the BcFRQ1 generated from the $actin_{prom}$-*bcfrq*1 locus constantly closes the negative feedback loop on the native *bcfrq*1 (controlled by its endogenous promoter) by persistently inhibiting the WCC, recapitulating the original demonstration of the Neurospora circadian negative feedback loop (Aronson et al. 1994). Clock oscillations in *bcfrq*1 mRNA levels are expected to produce daily rhythms in BcFRQ1 protein levels. A method that allows easy tracking of FRQ levels in vivo, confirming the latter, is to generate a FRQ-LUCIFERASE translational fusion, obtained by fusing the *luc* sequence to *frq* (at its endogenous locus), as already tested in Neurospora (Larrondo et al. 2012). Thus, by quantifying bioluminescence, it was possible to confirm that BcFRQ1-LUC levels oscillate under DD and also under temperature cycles. In addition, using this BcFRQ1-LUC reporter, it was possible to confirm that Botrytis entrains to temperature cycles of different period length (T), which would not occur in the absence of a functional circadian oscillator (Pregueiro et al. 2005). The relevance of all these different results is that they provided, for the first time, molecular evidence of the existence of a functional circadian oscillator in a fungus other than *Neurospora* in general and in particular in any pathogenic organism (Hevia et al. 2015).

In Neurospora, FRQ plays a key role in controlling its circadian clock but, other than that it does not seem to serve any further function in additional cellular processes (Montenegro-Montero et al. 2015). Intriguingly, FRQ in Botrytis plays extra-circadian roles as inferred from the analysis of *B. cinerea* Δ*bcfrq*1, as this mutant produces (in rich undefined media) microconidia and sclerotia (sexual development) in the presence of light (Hevia et al. 2015). Nonetheless, Δ*bcfrq*1 recovers its normal developmental program in light (production of macroconidia), by simply changing media composition, which suggests an existing connection between nutrient signaling pathways and the processes controlled by BcFRQ1, different from the ones associated to the circadian clock (Hevia et al. 2015). Indeed, the phenotypes for Δ*bcfrq*1 are observed in constant light, a condition where—as mentioned earlier—the clock is not running. These BcFRQ1 non-circadian roles may imply an active repression of sexual development in the light, under particular nutritional conditions, and current efforts in our laboratory are devoted to explore such ideas.

Considering that non-extra-circadian functions have never been described for FRQ in Neurospora makes this unexpected extra-circadian function for BcFRQ1 all the more intriguing. Whether these extra functions of clock proteins are particular of pathogenic or necrotrophic fungi are, still, an open question.

5 Circadian Modulation of Plant–Pathogen Interactions

There is compelling evidence indicating that *Arabidopsis thaliana* immunity is modulated by a circadian clock, which allows anticipating and responding to the attack of certain pathogens (Sharma and Bhatt 2014; Ingle et al. 2015). Nevertheless, most of these reports have focused on the response of Arabidopsis to microbial pathogens in which there is no molecular evidence of endogenous circadian regulation, such as the oomycete *Hyaloperonospora arabidopsidis* (*Hpa*), the bacteria *Pseudomonas syringae,* or the insect *T. ni.* Nonetheless, the characterization of a clock in *B. cinerea* B05.10 allowed analyzing the contribution of each circadian system in the outcome of a bipartite plant–pathogen interaction (Hevia et al. 2015).

5.1 The *B. cinerea- A. thaliana* Interaction*:* A Question of Time

Having simultaneous access to both *A. thaliana* and *B. cinerea* clock mutants allowed, for the first time, to analyze the contribution of a pathogen's clock to the outcome of plant–fungal organismal dynamics. The data revealed that the circadian machinery of this necrotrophic fungus is necessary to achieve a maximal relative virulence at dusk and that therefore the efficiency of the interaction between the pathogen and its host differs with the time of the day (Hevia et al. 2015). Importantly, the same conclusion was obtained when confronting the wild-type fungus to arrhythmic *A. thaliana* plants, since the leaves showed bigger lesions when inoculating the plants with Botrytis at dusk (night) than at dawn (morning), further indicating that the fungal clock is controlling the temporal aspect of the process (Hevia et al. 2015). Interestingly, the arrhythmic Arabidopsis mutants, corresponding to CCA1ox (constitutive expression of the CIRCADIAN CLOCK ASSOCIATED 1) and *Δ*975 (triple mutant in PSEUDORESPONSE REGULATORS PRR9 PRR7 PRR5), appeared to be overall more susceptible to infection than WT Arabidopsis, yet displaying bigger lesions in dusk-inoculated leaves. On the other hand, when eliminating the *B. cinerea* clock by tampering with its core oscillator components (Δ*bcwcl*1, Δ*bcfrq*1, or by OE::*bcfrq*1), it became clear that the *B. cinerea* circadian oscillator was responsible for the time-of-the-day difference in lesion size, either under DD or LD conditions. Thus, the data indicates that the fungal clock exerts control on Botrytis physiology such that it displays its maximal pathogenic potential at nighttime and that this effect disappears if the plant is infected with a *B. cinerea* clock mutant. Since it has been reported that one of the principal plant defense mechanism against necrotrophic fungi, jasmonic acid (JA), displays rhythmic levels with lows at night time (Goodspeed et al. 2012), it was plausible that such difference could explain the Botrytis-night increased effectiveness. Nevertheless, experiments conducted with WT Botrytis and Arabidopsis, utilizing environmental perturbations showed otherwise. By growing both organisms in

inverted LD cycles (e.g., daytime for Arabidopsis and nighttime for the fungus, and vice versa), it was observed that bigger lesions were obtained infecting dawn plants if the utilized fungal inoculum had dusk time. On the other hand, plants that had night (dusk) time yielded smaller lesions as they had been inoculated with fungus having morning (dawn) time. These data further confirms the weight of the fungal clock in regulating this organismal interaction, while downplaying a potential role of rhythmic JA levels in the interaction between *B. cinerea* B05.10– *A. thaliana* Col-0, at least under the tested experimental conditions (Hevia et al. 2015).

While both Δ*bcfrq*1 and OE::*bcfrq*1 arrhythmic mutant lose the ability to produce bigger lesions at dusk, they exhibit interesting differences in their overall virulence. Δ*bcfrq*1 shows increased aggressiveness at all times compared to the wild-type strain, while overexpression of *bcfrq*1 leads to equally reduced virulence at dawn and dusk, marked by very small lesions (Hevia et al. 2015). Thus, it would appear as if during parts of the day the clock is serving as a break for virulence (high *bcfrq*1 expression), which could be conceptualized as investing energy, efforts, and resources to other processes at certain hours, while concentrating on virulence at some other times. In other words, it becomes too expensive to be the best at everything 24/7, and therefore, the fungus needs to compromise and organize resources and strategies around the clock.

As it was previously indicated (see above), in the presence of constant light (LL) *B. cinerea* presents a non-functional circadian clock, since *bcfrq*1 expression is flat and low (Hevia et al. 2015), whereas the *A. thaliana* clock maintains its functionality under constant light conditions (McClung 2001). Remarkably, when WT plants are challenged with WT Botrytis in LL, differences in the size of lesions between infections carried out at dawn and dusk time were still observed. Nevertheless, such differences are no longer observed if CCA1ox arrhythmic plants are challenged with the wild-type fungus. These results demonstrate that under this particular environmental condition of constant light, the only clock that is functional, the one in Arabidopsis, has a preeminent role in determining the evolution and outcome of this organismal interaction. Likewise, a recent study also highlights the importance of the *A. thaliana* clock in the defense response against a *B. cinerea* (Ingle et al. 2015). The data, obtained with a *B. cinerea* pepper isolate, different from the B05.10 strain, also indicates that the outcome between Botrytis and Arabidopsis changes depending on the time of inoculation, such that bigger lesions are seen when the plant is inoculated at subjective night time, in concordance with previous reports (Hevia et al. 2015). Interestingly, this phenotype analyzed under LL conditions disappears in arrhythmic *elf*3-1 (EARLY FLOWERING 3) and *cca*1-*lhy* (MYB TFs CCA1 and LHY double mutant) plants. Transcriptomic data indicates that hundreds of Arabidopsis genes are differentially expressed at dawn versus dusk in response to Botrytis infection; among them, some key genes related to defense responses, which could partially explain the lower susceptibility of *A. thaliana* at dawn (Ingle et al. 2015). Importantly, several of these genes of interest correspond to TFs, targets of core-clock components, that exhibit rhythmic expression patterns, coincident with the idea that the defense responses are under strong circadian control in constant light. While differences in lesion size between

dawn and dusk were also observed in experiments conducted in LD between WT plants and the WT Botrytis isolate, the arrhythmic *elf*3-1 or *cca*1-*lhy* mutants were not tested under LD or DD (Ingle et al. 2015). Indeed, both LD and DD are conditions under which it has been reported that other arrhythmic plants challenged with WT *B. cinerea* (B05.10 strain) are still more susceptible at night which supports the fundamental role of the fungal clock in determining the outcome of the interaction (Hevia et al. 2015). Ingle and cols. also reported that under their experimental conditions, temporal changes in JA signaling appear to participate in this temporal differential response to the pathogen. JAZ transcriptional repressors participate in the activation of defense genes in response to JA and, when plant single or triple *jaz* mutants were infected, under LD or LL conditions, the plant appeared overall more resistant, and there was no difference in the size of the lesions at night compared to morning. Such results would indicate that the increased susceptibility displayed by Arabidopsis to Botrytis at dusk depends on changes in JA signaling. Interestingly, as mentioned earlier, the Botrytis strain utilized by Ingle and cols. is not the standard *B. cinerea* B05.10 strain and instead is a less characterized strain: a pepper isolate, where no circadian characterization has been conducted, and more importantly, a strain that produces conidia both in the light and in the dark (Ingle et al. 2015). As previously commented, it has been observed that the genetic diversity of *B. cinerea* sometimes translates in anomalous responses to the presence and absence of light: While some strains behave like B05.10 (conidiation in response to light, sclerotia in the dark, referred as "light-responsive strains"), different isolates exhibit diverse phenotypes such as "always sclerotia," "always conidia," and some "always fluffly," meaning that instead of changing their developmental program in light vs dark, they exhibit the above-mentioned phenotypes irrespective of the light conditions (Canessa et al. 2013). These differences, that remain to be molecularly dissected, also reflect divergence in the way light is sensed (or on how light-dependent genetic programs are implemented). Yet, this diversity of light-responsive phenotypes could also imply that the circadian clocks in some of these strains may be altered. This idea once again highlights the importance of strain selection when it comes to evaluating a particular biological process.

5.2 *Other Circadian Examples of Microbial Pathogen—A. thaliana Interactions*

Different aspects of the circadian modulation of plant immunity have been provided in the context of infections caused by oomycetes, insects, and bacteria (Sharma and Bhatt 2014; Korneli et al. 2014). It has been reported that the plant circadian clock, through its central component CCA1, modulates resistance against the oomycete *Hpa*, activating the expression of defense genes near the morning (dawn), when CCA1 expression is high (Wang et al. 2011). Not surprisingly *cca*1 clock-less

mutants display reduced resistance, whereas an arrhythmic mutant based on CCA1-overexpression displays increased overall defense response. This opposing resistance phenotype, by manipulating a central plant clock component, draws an interesting parallel to what happens when the fungal *B. cinerea* clock is turned arrhythmic by altering *bcfrq*1 levels by deletion of the gene or its overexpression (see above).

When *A. thaliana* plants are infected with *Pseudomonas syringae* pv *tomato* DC3000 and maintained under LL conditions, they exhibit decreased susceptibility when inoculated, by infiltration, at dawn compared to dusk (Bhardwaj et al. 2011). In addition, Arabidopsis arrhythmic plants (CCA1ox and *elf*-3) exhibit enhanced susceptibility at all times, as they show loss of temporal regulation in the defense response against this pathogen. Interestingly, *A. thaliana* plants display increased susceptibility at dawn to *P. syringae* pv *maculicola* DG3, if the bacteria are sprayed on the leaves, instead of infiltrated (Zhang et al. 2013). This enhanced susceptibility at dawn disappears in CCA1ox arrhythmic plants, mutants that also show overall enhanced susceptibility in both LL and LD. These studies along with other reports (Korneli et al. 2014) highlight the circadian modulation of plant immunity responses and the different outcomes depending on the time and mode of inoculation and infection. Interestingly, recent data has provided new insights, although this time from the pathogen's perspective. Extensive analyses of the ability of *P. syringae* pv. tomato DC3000 (*PsPto)* to sense different light wavelengths have shown that quality of light, and the time at which is administered, can impact virulence (Santamaria-Hernando et al. 2018). Thus, experiments carried by inoculating tomato plants with Pseudomonas, by immersion, at dusk or dawn, reported higher virulence at dawn whether the bacterial cells had been kept in the dark or given a 10-min light pulse of blue or white light. Nevertheless, when Pseudomonas cells were treated with a 10-min red light pulse previous to dusk or dawn inoculation, no time-of-the-day changes in virulence was observed and moreover, virulence appeared overall reduced. Similarly, loss of dawn-preferential virulence was also registered when analyzing different Pseudomonas photoreceptor mutants subjected to particular light treatments (Santamaria-Hernando et al. 2018). Although no circadian clock has been described for Pseudomonas, these results highlight how environmental manipulations of a pathogen can modulate key aspects of the plant–pathogen interaction, apparently overriding circadian changes in the plant. Thus, one could conceive that misleading environmental cues (red light in the night) may alter the way *PsPto* senses the environment, compromising its ability to take advantage of time-of-the-day opportunities, such as stomata opening at dawn.

6 Clock Regulation of Entomopathogenic Fungi

Several fungal entomopathogens have developed sophisticated mechanisms that allow them to manipulate host behavior, in such a way that they can increase the chances of spores discharge to infect new hosts. In this context, the term "zombie"

has been utilized to describe insects infected by a fungal or non-fungal entomopathogen that alters insect behavior and morphology, benefiting the pathogen. Interestingly, studies focused on such fungal–insect interactions have reported fascinating temporal aspects in the development of stereotypical phenotypes, or in key steps of the infectious cycle as, for example, the timing of fungal conidiation from insect cadavers.

Early field studies have reported daily rhythms in air-spore counts of several fungal species, including insect parasitic zygomycetes from the genus Entomophthora (Hamilton 1959; Lacey 1962). Thus, measurements have revealed a relative increase in Entomophthora spore levels in the last hours of the night (05:00-07:00 h), which could be interpreted as a response to environmental cues, such as increased humidity, or light from sunrise. Subsequent studies have confirmed these daily rhythms in spore release, with a peak around dawn, in different Entomophthora species such as *E. aphidis* and *E. muscae* (Wilding 1970). Part of this response could be directly modulated by light, be the product of entrained environmental conditions or overt circadian output. A report by Callaghan (Callaghan 1969) in *Conidiobolus coronatus* (*Entomophthora coronate*) indicates that a diurnal rhythm of spore discharge can be induced by alternating light:dark cycles, but it fails to persist under constant darkness and, therefore, appears to be conditioned by light at sunrise. But overall, these consistent daily rhythms of Entomophthora conidia, observed in the field (Wilding 1970), pose interesting questions regarding the underlying mechanisms. Importantly, such periodicity echoes the observations of diurnal patterns of aphids deaths, when infected with Entomophthora fungi (Milner et al. 1983). Indeed, under LD cycles, infected aphids tend to die at specific times (during the light phase), while differences in timing of death appeared to be dictated by the species of the parasitic fungus. Thus, aphids infected with the zygomycetes *Entomophthora planchoniana* and *Ervnia neoaphidis* (*Pandora neoaphidis*) preferentially died in a 4-h period window, with mortality peaks at 8 or 14 h post-dawn, respectively (Milner et al. 1983). These types of observations have led to postulate that such timing relates to the fact that entomophthoran fungi are adapted to transmit preferentially at night, as it has been postulated in studies involving the fungus *Entomophthora gammae* and the soybean looper *Pseudaletia includens*, insects that when infected die between 18:00 and 22:00 h, while *E. gammae* air conidia are reported to reach peaks after midnight (Newman and Carner 1974).

Other studies have measured *Neozygites fresenii* conidia in the air over cotton fields. This zygomycete when infecting the cotton aphid *Aphis gossypii* exhibited a clear daily rhythm of spore discharge reaching a peak at night, between 01:00 and 05:00 h (Steinkraus et al. 1996). As discussed by the authors, such timing of spore discharge puts conidia in the air at a time of the day (night) at which temperatures are rather low and humidity levels high, conditions that could favor survival and germination.

Interestingly, other reports contain similar observations for different fungal-insect systems. Thus, conidia of *Entomophaga maimaiga* appear to be preferentially discharged from dead gypsy moth larvae (*Lymantria dispar*) between

02:00 and 08:00 h (Hajek and Soper 1992). Importantly, the data suggests that the fungus also manipulates the larvae to die mainly in the afternoon, such that sporulation can occur at night, although not of the same day (Nielsen and Hajek 2006). Nevertheless, the evidence also appears to indicate that light cues and not merely a circadian clock may be responsible for these specific timings in the *E. maimaiga*-gypsy moth system (Nielsen and Hajek 2006).

Some recent studies have also focused on the timing of stereotypical behavior and death postures adopted by infected insects. The soldier beetle, *Chauliognathus pensylvanicus*, adopts a peculiar final posture attached to flowers, with its mandibles in a final "grim death grip" when infected by the zygomecete *Empusa lampyridarum*. Lock in place by its mandibles, the soldier beetle body is lean upward at a 45° angle with wings raised as in a flight position, which clears space for conidiophores to emerge from the abdomen of the dead insect. Remarkably, preliminary data indicates that this "grim death grip" tends to occur in the early morning of one day, while the wing-opening and conidiation process does not occur until the dark hours of the following morning, about 15–22 h after the death grip (Steinkraus et al. 2017).

The fungus *E. muscae* infects house flies (*Musca domestica*), killing them, using the insect tissue as food source and as a launching platform for abundant spore dissemination. To maximize that, the fungus manipulates the fly behavior such that in its last hours of life, the fly climbs up, affixes itself in place, and leaves its wings in a final up position, providing plenty of space for spores to be launched from reproductive structures emerging from the fly's abdomen. Interestingly, it has been reported that the time of the day at which flies stop moving, to then die, is not random and that instead it tends to occur around sunset (Krasnoff et al. 1995; Bellini et al. 1992). Recent studies, utilizing a *E. muscae* "Berkeley" isolate, capable of infecting *Drosophila melanogaster*, have verified a time preference for the fly last locomotory movements (Elya et al. 2018). Thus, in their very last day of life (about 4 days after being inoculated with the fungus), infected flies stop moving between 0-5 h before sunset (Elya et al. 2018). Remarkably, such behavior, both in regular house flies, or in *E. muscae* "Berkeley" infected *D. melanogaster*, it is only observed under LD cycles, since if flies are maintained in constant darkness (DD) they die at random hours throughout the day, instead of in a gated fashion (Elya et al. 2018; Krasnoff et al. 1995). As flies can sustain clear circadian rhythms in DD, these results could suggest that rhythmic light cues—processed by the fungus or by the fly—may be needed to confer a time-of-the-day preference to timing of death. Nevertheless, a relevant piece of information is presented in the study of Krasnoff and cols., as they report that the absence of time-of-the-day preference exhibited for infected flies housed under DD, can be overcome by entraining them under three 12:12 LD cycles before releasing them into DD (Krasnoff et al. 1995). This could be taken as that the Drosophila clock needs strong entrainment (before DD), to see such a phenotype. Nevertheless, the fact that the timing of last locomotor activity in the LD-to-DD protocol exhibited a period closer to 21 h, instead of a 24 h rhythm, was interpreted by the authors as evidence of a free-running fungal clock controlling this phenotype (Krasnoff et al. 1995). Thus,

such attractive hypothesis suggests that after 3 days of LD entrainment, the fungal clock would free run in the absence of external cues, manifesting its endogenous period, controlling the time of last movement of the fly. On the other hand, under LD conditions, external cues would synchronize the fungal clock to 24 h, and therefore, the phenomena would manifest itself with that periodicity, around the time of sunset (Krasnoff et al. 1995). Interestingly, transcriptomic analysis of *E. muscae* "Berkeley" as it infects *D. melanogaster*, confirmed the expression of the blue light-sensing WC-1 homolog in *E. muscae* (Elya et al. 2018), and although this fungus does not seem to harbor a *frq*-like gene, the existence of a functional circadian clock is plausible. Since it is now possible to study this entomopathogenic fungus in a Drosophila model, classic circadian experiments can be conducted to test if the fungal or the fly clock (or a cross-talk between them) is responsible for this temporal phenotype. Indeed, different Drosophila clock mutants, in combination with environmental perturbations, can be utilized to evaluate the contribution of the fly clock to this phenomenon, as it has been done in the past to assess the relevance of the fly clock in the circadian modulation of the response to bacterial infections (Lee and Edery 2008).

There is no doubt that zombie ants have captivated the attention of general public due to the clear change in behavior experimented by the ants, and the dramatic death and post-mortem phenotypes caused by fungal growth. Interestingly, a timing component is distinguishable in the infection process (de Bekker et al. 2014). Thus, field studies in a Thai rain forest have looked into the kinetics of the interaction between the fungus *Ophiocordyceps unilateralis s.l.* and its host ant *Camponotus leonardi*. Ants infected with this Sordariomycetes showed locomotor activity restricted to certain part of the day, followed by a clear preference for leave biting (Hughes et al. 2011). Before they die, these ants show stereotypical behavior that finalizes in a death grip, as they bite plant leaves to then be killed by the fungus. Interestingly, in the analyzed population, the timing of this final biting is synchronized around noon. It is also noticeable the fact that the ants exhibit a marked preference for abaxial leaf veins (Hughes et al. 2011). Thus, these observations allow speculating that such specific behavior could be controlled by a fungal clock. Moreover, the preference of abaxial versus adaxial side of the leaf could also be somehow controlled by the fungus. Indeed, it is known that light-sensing components in general not only allow responding to light or darkness, but also tuning responses depending on light-spectrum composition. Thus, the red/far-red light ratio is different on top versus the bottom side of the leaf, something that could be sensed by the fungus, eliciting changes that allow further controlling the ant behavior.

A laboratory-experimental setup to study the effect of an American *O. unilateralis s.l.* species isolate, on its natural host *Camponotus castaneus*, has been recently achieved. This setup that was kept under strict light:dark 24 h cycles showed that infected ants adopted the biting position at 09:00 h (3 h after lights on), to then die around 14:00 h (de Bekker et al. 2015). Such timing appears phase advanced compared to what was observed in the wild, in Thailand (Hughes et al. 2011), which is biting around noon followed by death 6 h later. These phase

differences could be associated with species-specific determinants or with particular characteristics of the experimental setup.

The new American isolated species of the *O. unilateralis* complex (de Bekker et al. 2015), now named *Ophiocordyceps kimflemingiae*, has served as a substrate for genomic and transcriptomic studies which have opened the door to explore the circadian biology of this ant fungal parasite (de Bekker et al. 2017). Thus, analysis of the *O. kimflemingiae* genome confirmed the presence of the Neurospora clock homologs *frq*, *wc*-1, and *wc*-2, as well as associated clock component. De Bekker and cols. where also able to identify laboratory conditions in which to grow *O. kimflemingiae* such that its morphological growth would resemble the one adopted when growing it in the *C. castaneus* ant. Moreover, they were able to analyze *C. castaneus* gene expression over 48 h by RNA-seq, with 4 h interval resolution. The analysis of the times course, one under LD and the other under DD conditions, revealed a different number of oscillating transcripts. In LD, over 300 genes showed oscillatory expression, whereas in DD the number was only 154. In LD, the *frq* homolog showed a rhythmic pattern, although in DD it did not pass the threshold to be classified as such. Interestingly, among the oscillating genes in LD, there were several predicted to be involved in parasite–host interactions, and 14 genes encoding for TFs, which suggests a potential hierarchical transcriptional cascade leading to ample *ccg* expression. Also, among the rhythmic transcripts, there was overrepresentation of genes encoding for 41 small-secreted and 15 secreted proteins, including five enterotoxins and six proteases in the latter category. Moreover, among the secreted enzymes, there were a tyrosinase and tyrosine phosphatase (de Bekker et al. 2017), which had been already identified as genes of interest in a previous study (de Bekker et al. 2015). And, although the overlap between the total number of rhythmic genes under LD and DD conditions was small (only 26 candidates), the above-mentioned type of genes encoding for small-secreted and secreted proteins was overrepresented (5 and 6, respectively). The temporal pattern of these 11 genes is also interesting, as nine peaks in the dark phase in LD, or during the subjective night in DD. And, among these night-expressed genes, there were ones encoding for chloroperoxidase, exo-beta-D-glucosaminidase, and enterotoxin, amid other attractive ones. Of interest is also the day-peak expression of two rhythmic genes encoding for TFs and for a histidine phosphotransferase.

In general, the analysis of the time courses revealed that in LD 52% of the rhythmic genes were up-regulated during the light period. On the other hand, analysis of the DD time course indicated that the majority of the rhythmic genes (64%) had peaks of expression during the subjective night. Based on diverse analyses of their data, the authors conclude that the majority of the day-active genes observed in LD are probably only light-driven and not necessarily clock regulated, whereas among the night-active rhythmic genes, clock-control is more likely to be occurring. Thus, while an important number of TFs reach maximal expression in light, several rhythmic genes that encode for secreted components get expressed during the night and may play important roles in the interaction with the ant.

The fact that ant foraging behavior (which is under tight clock regulation) becomes disrupted when the fungal infection is in place suggests that somehow the fungus can alter the host circadian regulation or the temporal control of the ant behavior. Interestingly, limited transcriptomic data of infected ants revealed changes in the expression of two clock components of infected versus not infected ants, which is commented by the authors as a circumstantial, but provoking piece of information that could help explaining their altered behavior (de Bekker et al. 2014).

7 Circadian Regulation of Human Pathogenic Fungi: An Area Awaiting for Systematic Exploration

While there is scarce information regarding the regulation of virulence of fungal phytopathogens, there is even less data reporting the existence of clock phenomena, mechanisms, or daily regulation of virulence of human pathogenic fungi.

Nevertheless, there is an increasing number of reports describing rhythmic modulation of the mammalian immune system and how the ability to respond to different immune challenges varies throughout the day (Labrecque and Cermakian 2015; Scheiermann et al. 2018). And, although there are several nice examples of circadian modulation of the innate immunity in response to bacteria, as well as viruses (Curtis et al. 2014; Scheiermann et al. 2018), this has not been systematically addressed for fungal pathogens.

And while, as mentioned earlier, there is no description of clock regulation of human pathogenic fungi, this section will revise some reports that are setting the pace for future exploration in this area. Thus, there have been studies on the effect of light on the physiology of important human fungal pathogens. One of the earlier reports focused on *Cryptococcus neoformans*, a fungus that represents a serious threat for immunocompromised individuals, showing that it was capable of sensing blue light through a WC-1 ortholog. Deletion of this gene led to attenuated virulence in a mouse model (Idnurm and Heitman 2005). Similarly, a WC-1 homolog was also identified in the plant and human pathogen *Fusarium oxysporum*. While deletion of the *F. oxysporum wc*-1 does not affect virulence in tomato, it decreases virulence when tested in mice, indicating that this gene is important to achieve full virulence (Ruiz-Roldan et al. 2008).

Until today, there is not enough evidence to interpret the phenotypes of these particular *wc*-1 mutants to defects in circadian regulation. Instead, these defects may originate on altered genetic programs due to the absence of White Collar TFs and the inability to mount some responses to light. Indeed, functional WC-1 signaling appears important to cope with stress, and some of these mutants, for example, appear more sensitive to oxidative stress (Fuller et al. 2015). In the case of *Aspergillus fumigatus*, oxidative stress sensitivity only becomes evident when another light-sensing component, the phytochrome *fphA*, is simultaneously deleted

(Fuller et al. 2013). Nevertheless, the existence of a functional clock in this organism remains an open question.

In the interphase of the host–pathogen dynamics, there are plenty of examples of how time of the day may influence the ability of a mammalian host to overcome bacterial and viral insults. And although some of these examples go almost 50 years back (Feigin et al. 1969), the molecular understanding of how clock regulation modulates immunity is still work in progress. Thus, for example, it is known that in macrophages about 8% the transcriptome oscillates daily, which includes different components involved in pathogen recognition (Keller et al. 2009). Recently, this circadian paradigm was tested in the context of *A. fumigatus* infection, particularly assessing if macrophage activity against this pathogen, or its clearance from the lungs was under circadian regulation (Chen et al. 2018). Thus, the authors measured Dectin-1 expression in macrophages, as this receptor can play a key role in recognizing fungal components. Nevertheless, they did not observe circadian expression of Dectin-1, nor clock expression of other receptors such as Dectin-2 or TLR4. Likewise, they failed to observe rhythmic phagocytosis of labeled *A. fumigatus* spores by macrophages. Nevertheless, when testing if clearance of *A. fumigatus* from the lungs changed throughout the day, they observed significant differences if inoculation had occurred at ZT0 vs ZT12, a mechanism that could depend on other macrophage receptors and/or some other immune cells, such as neutrophils. Interestingly, this twofold enhanced clearance of the fungus, when animals had been inoculated at night, was only evident when smaller fungal inocula (10^5 spores vs 10^7 spores) were utilized (Chen et al. 2018).

In toto, while these observations indicate that light is an environmental variable capable of modulating virulence, little is known on how circadian variables may modulate fungal pathogenic potential or the interaction dynamics between pathogenic fungi and a mammalian host. It would be interesting to envision strategies, based on concepts such as chronotherapy, being applied in the efficient treatment of fungal pathogens, either by defining the best times and doses for antifungal administration or by perturbation of the clock of the pathogen (i.e., by distinct disruptive photoperiods).

Additionally, it will be informative to witness what emerges out of comprehensive microbiome studies not only focused on bacterial components, but also addressing the fungal composition and temporal variation of different mammalian associated microbiota. Indeed, recent studies have shown that the intestinal microbiota, in both mice and humans, exhibits diurnal compositional and functional oscillations (Thaiss et al. 2014). Such studies, focused on the bacterial aspects of the gut microbiota, have revealed unexpected effects of this microbial rhythm on the host circadian oscillations (Thaiss et al. 2016). Therefore, it would be interested not to only unveil if, for example, the natural skin or mucous fungal microbiota may exhibit daily changes, but to also understand whether such hypothetical oscillations could modulate the growth of opportunistic or pathogenic fungi.

8 Evidence of Clock Regulation in Other Pathogenic Organisms

Several reports describe the existence of daily rhythms of parasitic infections affecting mammalian hosts, although none of those examples include fungi [reviewed in (Rijo-Ferreira et al. 2017b)]. The manipulation of time variables in organismal interaction dynamics appears as a smart way to play around defense/susceptibility daily fluctuations of the host, as well as maximizing the chances of encountering new fresh victims. Thus, some of these rhythms seem to have adapted to the final host, as seen for the trematodes *Schistosoma mansoni* (Mouahid et al. 2012). Indeed, this parasite presents a specific developmental stage as it emerges from snails to then swim in fresh water, where it infects its hosts. While the emergence of this infectious stage is rhythmic and its chronotype appears to match its diurnal host (humans), a new night chronotype was described to be able to infect rats, a nocturnal animal (Mouahid et al. 2012). Such adaptation could be interpreted as the ability of the parasite to use endogenous circadian regulation to adapt to a time at which a new suitable host is present.

Herein, we will mention two different examples for which molecular data can explain, at least in part, reported rhythms in parasites.

8.1 Trypanosoma

Daily rhythms in the amount of blood parasites have been described for different organisms, including various Trypanosoma species such as *T. rotatorium* in the blood of frogs, or *T. congolense* and *T. lewisi* in the blood of rodents (Rijo-Ferreira et al. 2017b).

In the case of *T. brucei,* the causal agent of sleeping sickness in humans, no daily rhythmic levels of this parasite have been detected in bloodstream. Nevertheless, a hallmark of this disease is the disruption of the sleep/wake cycle, along with alteration of core body temperature and the timing of endocrine secretion, which strongly suggest a circadian alteration of the host caused by the parasite. Indeed, studies have shown that mice infected with *T. brucei* suffer circadian alteration that manifests as phase advance of the host clock (associated with a period shortening), and abnormal activity during the rest phase. Notably, the period shortening is not only occurring at the organismal level, but also at the cellular level, both in vivo and in vitro (Rijo-Ferreira et al. 2018).

In vitro studies have revealed that *T. brucei* has an intrinsic circadian clock that modulates its metabolism as well as gene expression. Time courses of cultivated *T. brucei,* in its bloodstream form, showed that $\sim$10% of its genes exhibit rhythmic expression, as measured by RNA-seq. Since light appeared as a weak entraining signal, temperature was assessed as an entraining cue. A time course of temperature-entrained (32/37 °C) bloodstream *T. brucei,* yielded $\sim$1490 genes

(∼15% of the genome) oscillating, while a time course collected under constant temperature (after the parasites had been temperature-entrained for 3 days), revealed 1092 rhythmic transcripts (∼11 of the genome). Interestingly, a similar experiment was conducted with *T. brucei* procyclic forms, adapted to the tsetse fly gut environment, with a protocol involving a 23/28 °C temperature entrainment, or under constant temperature after this entrainment, yielding 1123 and 854 rhythmic genes, respectively. What is remarkable is that comparing the circadian gene sets obtained from the bloodstream or the procyclic (insect) forms reveals only 127 genes in common (Rijo-Ferreira et al. 2017a). Thus, the *T. brucei* clock appears to be able to sense the environment (fly vs bloodstream) and temporally control different gene sets, to better face the evolutionary constrains and challenges that each microenvironment imposes.

The *T. brucei* clock was shown to be temperature compensated, and also to be running in vivo, as tested in a mouse model. Many of these cycling genes impact metabolic pathways, ATP levels, and even resistance of the parasite to the commonly used drug suramin. Therefore, the parasite is not always the same throughout the day, in terms of its expression pattern and also on the way it interacts with its local environment. Analysis of the *T. brucei* genome does not allow explaining the underlying clock mechanisms driving these rhythms. Yet, it has been postulated that posttranscriptional mechanisms may be playing an important role, in accordance with the observed behavior of the asynchronous expression of polycistronic units in this parasite (Rijo-Ferreira et al. 2017a).

8.2 Plasmodium

An interesting and puzzling observation is that replication of malaria parasites occur in synchrony with the circadian rhythm of the host (Mideo et al. 2013). In its blood stage, Plasmodium, the causing agent of Malaria, exhibits a synchronous asexual cycle, from the invasion of the red blood cells until their timely bursting. Such cycles, which are associated with cyclic fevers in the host, last 24 h or multiples of 24 h, depending on the species (Hawking et al. 1968). Recent studies conducted with *Plasmodium chabaudi*, in a mouse model, have shown that inflammation-induced hypoglycemia negatively affects *P. chabaudi* replication, while active proliferation of the parasite occurs during food intake, in synchrony with the host circadian cycle. Thus, circadian fluctuations of TNFα and food intake play a key role in synchronizing the parasite with the host rhythm such that if time of food availability is inverted, then the cycle of *P. chabaudi* is also inverted, whereas no rhythm is observed in diabetic mice (Hirako et al. 2018). Thus, rhythms associated with the time of feeding and metabolism, and not light-entrained rhythms at the level of the mouse suprachiasmatic nucleus, appear key in determining the phase of *P. chabaudi* oscillations (Prior et al. 2018).

9 Conclusions

Despite the overwhelming diversity of existing fungal species, almost everything that we know about circadian mechanisms in fungi has been deciphered in *N. crassa*. Nevertheless, there are a large number of observations that indicate that rhythmic phenomena are common in different fungi, pathogenic, and non-pathogenic ones. Only in recent years, molecular data has emerged depicting the clockworks in other fungi, providing the tools to directly assess the role of circadian regulation in diverse processes, such as fungal virulence. The implementation of real-time reporters such as luciferase has boosted the speed by which fundamental aspects of circadian mechanisms can be addressed. As this type of research expands to different fungi, it will be possible to understand what is transversal and what is particular to different species. Moreover, the abundance of fungal genomes can help in providing new insights into the evolution of fungal clock components, with lessons that can be also extrapolated to metazoans. As time passes, our knowledge about how hours are measured within cells keeps on advancing.

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Histoplasma Capsulatum: Mechanisms for Pathogenesis

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Abstract Histoplasmosis, caused by the dimorphic environmental fungus *Histoplasma capsulatum*, is a major mycosis on the global stage. Acquisition of the fungus by mammalian hosts can be clinically silent or it can lead to life-threatening systemic disease, which can occur in immunologically intact or deficient hosts, albeit severe disease is more likely in the setting of compromised cellular immunity. *H. capsulatum* yeast cells are highly adapted to the mammalian host as they can effectively survive within intracellular niches in select phagocytic cells. Understanding the biological response by both the host and *H. capsulatum* will facilitate improved approaches to prevent and/or modify disease. This review presents our current understanding of the major pathogenic mechanisms involved in histoplasmosis.

1 Introduction

Histoplasma capsulatum is an environmental dimorphic fungus (Wheat et al. 2007). Human infection occurs after the fungus (in the form of microconidia or hyphal fragments) is inhaled, travels through the respiratory system, and reaches the alveoli. In the alveoli, it transforms into a yeast form, a process that can occur both inside or outside of phagocytes (Deepe et al. 2008). *H. capsulatum* infections are primarily acquired, and there is no person-to-person transmission with the rare exception of organ transplantation (Lenhart et al. 2004). Once *Histoplasma* enters the host, it must evade immune-mediated and intracellular defenses, and find a favorable niche for growth and reproduction, which may include dissemination and the development of a state of latency within granulomas. Host phagocytes play a central role in the pathogenesis of histoplasmosis, as they are the vehicles for dissemination, spreading initially to the lymph nodes and later to multiple organs (Guimarães et al. 2006). The pathogen's ability to evade inflammatory responses and the intensity of the host immune response determine the severity of symptoms and clinical presentation, and whether a state of latency develops with the potential for reactivation (Huffnagle and Noverr 2008; Casadevall and Pirofski 2003). The processes involved for this pathogen to survive and cause host damage are discussed here, and they highlight the complexity of the interactions between the host and pathogen. An understanding of pathogenesis provides insight into the clinical manifestations, reasons for reactivation, and future therapeutic targets.

2 History

Histoplasma was first described in 1906 by Dr. Samuel Taylor Darling. His first report was on the autopsy findings of a 27-year-old carpenter from Martinique who was working in the Panama Canal (Darling 1906). He likened his findings to what had been observed in protozoan infections. Dr. Darling coined the name

H. capsulatum when he saw the invasion of histocyte-like cells with these encapsulated organisms. Histoplasmosis was later referred to as Darling's Disease. Subsequently, in 1912, the correct description of the organism was determined by pathologist Henrique da Rocha-Lima who recognized it as a fungus (Baum and Schwarz 1957). Since then, *Histoplasma* taxonomically has been divided into three groups based on geographic distribution and clinical manifestations: var. *capsulatum* which is the most common worldwide, var. *duboisii* found in Africa, and var. *farciminosum* known to be a horse pathogen. To refine phylogenic classifications, Kasuga et al. performed phylogenetic analyses on 137 individual isolates representing the three original classifications of *Histoplasma.* Using DNA sequence variation, eight clades were genetically identified with seven of them representing isolated groups. The clades are called: North American class 1, North American class 2, Latin American group A, Latin American group B, Australian, Netherlands, Eurasian, and the African clade. Overall, seven of these clades represent isolated groups except for the Eurasian clade which originated from the Latin American group A clade. Isolates classified in one of the three original categories were intermixed and were found to be present in multiple phylogenetic clades thus making the original nomenclature obsolete (Kasuga et al. 2003). Kasuga's clade classification is used today to identify *Histoplasma* fungus; however, new information on cryptic speciation may lead to further refinements of *H. capsulatum* classifications (Sepúlveda et al. 2017).

3 Epidemiology

As evident from the clade grouping, *H. capsulatum* is distributed throughout the world, including Asia, Africa, Australia, North, Central, and South America (Chakrabarti and Slavin 2011; Loulergue et al. 2007; McLeod et al. 2011; Colombo et al. 2011). In North America, a higher incidence has been reported in the Ohio and Mississippi River Valleys via identification of a high frequency of positive skin testing (Manos et al. 1956). In South America, there is a predominance of disease in Brazil, Ecuador, Venezuela, Paraguay, Uruguay, and Argentina (Guimarães et al. 2006). A possible cause for this difference in overall distribution may be associated with features of the soil in these areas and climate differences (Wheat et al. 2007). Histoplasmosis is the most prevalent endemic fungal infection in the US. It is estimated that 50 million people have latent infection, and 500,000 new infections estimated annually (Nosanchuk and Gacser 2008; Retallack and Woods 1999; Baddley et al. 2011). Despite wide distribution, there is still a significant underdiagnosis and hence underreporting of this disease in the US. This was evident in a multistate epidemiological surveillance analysis of histoplasmosis performed in US from 2011–2014 (Armstrong et al. 2018). It was noted that over a 3-year period,

only 3409 histoplasmosis patients were diagnosed in 12 states including: Alabama, Arkansas, Delaware, Illinois, Indiana, Kentucky, Michigan, Minnesota, Mississippi, Nebraska, Pennsylvania, and Wisconsin. Of those identified infected individuals, most patients were asymptomatic; although, patients who presented with symptoms had a significant risk of mortality as 7% of hospitalized patients with histoplasmosis died (Armstrong et al. 2018). Given its prevalence and potential for causing severe disease, further study and a deeper understanding of pathogenesis are crucial in development and implementation of diagnostic and treatment strategies, with goals of improving patient outcomes and increasing disease awareness.

4 Clinical Manifestations

There is a broad range of presentations for histoplasmosis. Disease signs and symptoms are mediated by the host immune status with immunosuppressed patients being at higher risk for more severe disease. Other factors that play a role in the severity of the disease include the virulence of the fungal strain and the amount of inhaled inoculum (Knox and Hage 2010). Most often individuals will be asymptomatic after the acquisition of the fungus, but 1% will present with symptoms associated with the infection (Kauffman 2007). Symptomatic clinical presentations include acute pulmonary disease, disseminated disease, and chronic pulmonary histoplasmosis. Additional forms of disease can occur (Wheat et al. 2016).

4.1 Acute Histoplasmosis

Symptomatic patients usually present within one to 3 weeks from exposure. They can progress to develop fever, chills, dry cough, chest pain, myalgias, and headaches, often described as a flu-like syndrome. There are no specific imaging findings, but often mediastinal lymphadenopathy and infiltrates can be seen (Nadel et al. 2005). Acute disease can manifest as pneumonia, less frequently as pericarditis or with rheumatologic syndromes including arthritis, arthralgias and erythema nodosum, and mediastinal fibrosis particularly in patients with HLA-A2 in whom fibrosis is associated with abnormal host inflammatory response (Kataria et al. 1981; Wheat et al. 1983; Rosenthal et al. 1983; Davis et al. 2001; Peebles et al. 2000). Other possible manifestations of the acute histoplasmosis are broncholithiasis and pulmonary nodules, occasionally leading to compression syndromes due to their size (Arrigoni et al. 1971; Goodwin and Snell 1969). Nodules can also represent the residual changes of acute disease, and can be seen in

radiographs as single or multiple calcified and noncalcified lesions, often described as "coin" lesions that can mimic and cause concern for malignancy (Galetta et al. 2007).

4.2 Disseminated Histoplasmosis

Disseminated disease most often occurs in individuals exposed to *H. capsulatum* who are immunosuppressed, but a large inoculum infection can result in severe disease in ~29% of individuals without immunological disorders (Larrabee et al. 1978). The risk for dissemination is particularly increased in patients with HIV/AIDs, particularly with a CD4 count less than 200 cells per μl, but chronic steroid use and immunosuppression secondary to malignancy are additional risk factors for disease (Kauffman 2007; Knox and Hage 2010; Histoplasmosis Risk & Prevention; Nacher et al. 2014). Symptoms of disseminated disease include fever, weight loss, and respiratory complaints. Clinical findings of lymphadenopathy, hepatomegaly, and splenomegaly, together with bone marrow toxicity on laboratory evaluation are associated with disseminated disease. Skin and mucosal lesions can also manifest. Disseminated disease can involve multiple other organs including endovascular, central nervous system, gastrointestinal and suprarenal glands (Wheat et al. 2016). Since symptoms are nonspecific, even disseminated disease can at times evade recognition, and if not treated, can progress to chronic or latent disease.

4.3 Chronic Pulmonary Histoplasmosis

Chronic pulmonary disease typically occurs in older individuals with previously injured lung tissues (Goodwin et al. 1976). It may follow acute pulmonary infection or develop secondary to persistent, slowly progressive disease, typically a complication of the acute disease. With acute disease, there is a progression of disease from pulmonary infiltrates to fibrosis and finally cavitation (Wheat et al. 1984). Apical bullae can also be part of chronic disease. Patients present with symptoms of fever, weight loss, cough, and dyspnea (Kauffman 2007). Bronchopleural fistulas are a serious complication of chronic disease (Goodwin et al. 1976). Due to the presence of cavitary lesions in chronic pulmonary histoplasmosis, it can be confused with tuberculosis or other mycotic infections and coinfections are possible (Wheat et al. 2016).

5 Diagnosis and Treatment

Although an in-depth discussion on diagnosis and treatment is beyond the scope of the current work, it merits a brief overview. There are currently multiple diagnostic modalities that include antigen detection, serology, pathology, and culture that

when combined can achieve high sensitivity and specificity (Azar and Hage 2017). Culture of *Histoplasma* is the gold standard for diagnosis but obtaining adequate tissue and fluid samples for culture can be difficult. Considering fungal growth patterns, it can take more than 2 weeks to observe mycelial growth, which can significantly delay diagnosis. Positive cultures are more likely to be found in disseminated disease due to the presumed higher fungal burden in the host. When histopathology is available, finding yeast cells in tissue is consistent with *Histoplasma* and supports the diagnosis. Another important diagnostic method is antigen detection. Antigen testing (i.e. detection of *Histoplasma* polysaccharide by antibodies) can be done on any body fluids and tissues depending on the suspected site of infection but is most often tested in the urine (Azar and Hage 2017; Nadel et al. 2005; Assi et al. 2011). Additionally, urine and other body fluid antigen levels can be helpful in monitoring treatment response. Serology is more useful for chronic and subacute disease. Various techniques include immunodiffusion, complement fixation enzyme immunoassay, and radioimmunoassay. Finally, molecular methods are not yet FDA-approved, but these approaches can nevertheless be helpful due to high specificity and faster processing time (Azar and Hage 2017).

The decision to treat is based on the severity of the disease as well as the host immune status. Treatment recommendations are largely based on the 2007 update by the Infectious Disease Society of America (Wheat et al. 2007) and the 2011 American Thoracic Society statement (Limper et al. 2011). When the disease is classified as mild acute pulmonary histoplasmosis there is no indication for treatment, unless symptoms are present for more than 4 weeks or if the patient is immunocompromised, in which cases itraconazole is recommended. If the disease is classified as moderate, then the treatment of choice is itraconazole, which should be initiated regardless of the duration of symptoms. Other possible therapeutic options include voriconazole and posaconazole. For patients identified as having moderately severe to severe acute pulmonary disease, the first line of treatment is liposomal amphotericin B, followed by itraconazole. The use of steroids is recommended at initiation of therapy if a patient is hypoxemic or presents with respiratory distress. Unfortunately, if host immunosuppression persists then the medication will need to be given lifelong. When treating disease with itraconazole, serum drug level monitoring should be performed 2 weeks after therapy is started and every 3–6 months while receiving treatment (Wheat et al. 2007).

Despite having a wide geographic distribution, histoplasmosis continues to be under-recognized. There is a range of disease states discussed above and clinical severity determined to a large extent by the immune status of the host. Additionally, as new immunotherapies are developed, the number of people at risk for disease is expanding. To enrich our understanding of disease epidemiology, treatment development and prevention modalities further, we will further focus on the pathogenesis of this disease.

6 Pathogenesis

6.1 Introduction

H. capsulatum is a member of the family Ascomycetes (Kwon-Chung et al. 1974). It has a mold or mycelial form and a yeast morphology (Nosanchuk and Gacser 2008). The mycelial phase is primarily present in the environment, in nitrogen enriched soil which is the case when it is contaminated with bird or bat droppings. Fungal hyphae measure from 1.25 to 2 μm in diameter, and occasionally, hyphal forms can be seen during human infection, particularly in the setting of endocarditis (Hutton et al. 1985; Svirbely et al. 1985). There are two types of conidia in the mycelial form; macroconidia ranging in size from 8 to 15 μm in diameter, and microconidia ranging in size from 2 to 5 μm in diameter; it is the latter that can effectively be inhaled and travel as far as the host alveoli (Fig. 1). The mycelial-to-yeast phase transition in *H. capsulatum* is primarily induced by changes in temperature. The yeast form is ovoid, measuring from 2 to 5 μm in diameter. The yeast cells reproduce by polar budding giving them their characteristic narrow budding base and the appearance of a bridge between mother and daughter cells (Nadel et al. 2005). The yeast phase predominates in host tissues with an optimal growth rate at 37 °C (Maresca and Kobayashi 1989).

The yeast is the pathogenic form of *H. capsulatum* causing acute disease, or it can become latent, able to potentially reemerge in the setting of immunosuppression. However, *H. capsulatum* has many obstacles to overcome to cause host injury. It must bypass mucosal barriers during acute inoculation, evade host immune cell responses, and find its niche within host macrophages. Table 1 is a summary of the host-pathogen responses to various components of the immune response. When

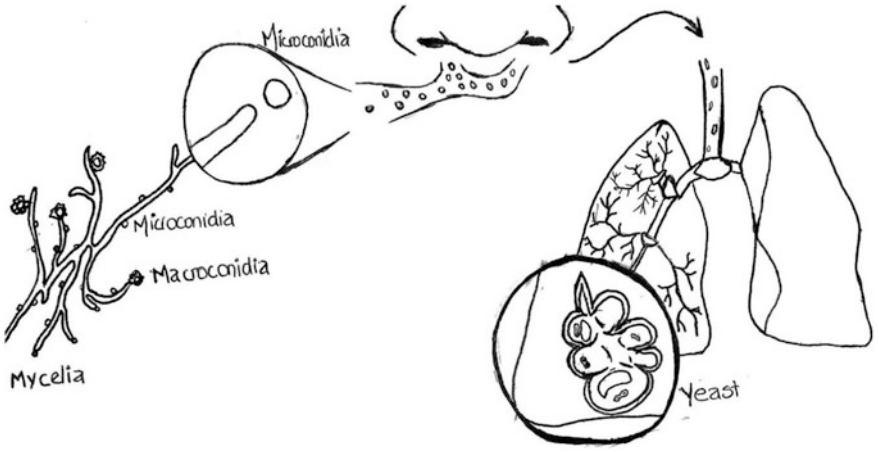

Fig. 1 A depiction of the acquisition process of mycelial *Histoplasma* from the environment to the lung with the transformation to the yeast form upon deposition in alveoli

Table 1 Summary of the immune system components, their response to an invading pathogen, and *Histoplasma's* protective mechanisms

	Host mechanisms	Pathogen response	References[a]
Host entry	None	Phase transition from mycelial to yeast form	(Sacco et al. 1983; Medoff et al. 1987; Nemecek et al. 2006)
Innate defense	Mucociliary clearance, mucus production	None needed	(Elansari et al. 2016; Rizzi et al. 2006; Symptoms of Histoplasmosis)
Surfactant	Pathogen opsonization Alters *Histoplasma* yeast permeability—Surfactant proteins A and D (SP-A and SP-D)	Rapid entry into macrophage—intracellular localization shields from the action of pulmonary surfactants	(McCormack et al. 2003; Han and Mallampalli 2015; Carreto-Binaghi et al. 2016)
Macrophages	Detects fungal cell components and initiate pro-inflammatory response Attacks phagocytized pathogen with reactive oxygen and nitrogen species Creates an acidic environment in the phagosome which allows for activation of hydrolases upon lysosomal fusion Creates nutritionally deficient environment Attract other immune cells	Avoids immune response on entry Inactivates reactive oxygen and nitrogen species Prevents acidification and lysosomal fusion Produces its own nutrients or has mechanisms for transport to obtain them from the environment Induce apoptosis to further disseminate	(Long et al. 2003; Guimarães et al. 2011b; Garfoot et al. 2017; Missall et al. 2004; Newman et al. 1994)
Dendritic cells	Cytotoxic to the pathogen Acts as an antigen presenting cell and secretes cytokines to further propagate the immune response	No active defense to this cell	(Clark and Kupper 2005; Zhou et al. 2001; Gildea et al. 2001)
Neutrophils	Traps the yeast forms intracellularly in a fungistatic response using enzymes contained within azurophilic granules to impede growth	No active defense to this cell	(Newman et al. 1993, 2000; Gray et al. 1989)

(continued)

Table 1 (continued)

	Host mechanisms	Pathogen response	References[a]
Natural killer cells	Cytotoxic to the pathogen Acts as an antigen presenting cells and secretes cytokines to further propagate the immune response	No active defense to this cell	(Cain and Deepe 1998; Cohen et al. 2011; Tewari and Von Behren 2000)
Adaptive immunity	Cytotoxic to pathogen Further propagate the immune response to eventually trap the pathogen within a granuloma	Utilizes macrophages as a sanctuary Reactivates with changes to the host's immune status	(Allen and Deepe 2006; Heninger et al. 2006; Tristão et al. 2012; Shi et al. 2008; Nosanchuk et al. 2003)

[a]Sample references provided, please see text for full list of references and details on each topic

H. capsulatum successfully enters macrophages, it can effectively avoid host effector responses and multiply. Within these cells, the fungus can disseminate throughout the host organs. These processes highlight the complexity of the interaction of the host and *Histoplasma* and the interconnected nature of the host response.

6.2 Transformation to the Pathogen

H. capsulatum pathogenesis begins immediately upon contact with the host. Upon entry into to the host and the shift to mammalian body temperature, the mycelial form ceases to be metabolically active. Thereafter, "shunt pathways" mediated by cysteine and sulfhydryl compounds are thought to induce morphogenesis to the yeast form (Sacco et al. 1983). This transition process usually takes from hours to days (Sacco et al. 1983). The transition can be inhibited by sulfhydryl blocking agents, which lock the fungus in the mycelial phase. Significantly, using this blocking system, treated *H. capsulatum* is unable to cause disease in well-established animal models (Medoff et al. 1987), which highlights the necessity of the transformation into the yeast form for virulence.

More recent studies have evaluated genes involved in the transition process. Nemecek et al. evaluated *DRK1* (dimorphism regulating kinase). Silencing of this gene reduced *H. capsulatum* virulence and suppressed additional factors important to pathogenesis such as *CBP1* and *AGS1*, which were no longer expressed (Nemecek et al. 2006). Nguyen et al. demonstrated that *RYP1* (required for yeast phase growth), which is part of the WOPR family of genes, was similarly required for effective morphogenesis and virulence (Nguyen and Sil 2008). Webster et al. identified *RYP2* and *RYP3* that are part of the Velvet family of genes, as necessary

for morphogenesis (Webster and Sil 2008). While all the functions of each of these factors have not been elucidated, Beyhan et al. attempted to clarify the interactions between Ryp1, Ryp2, and Ryp3, and identified another transcription factor Ryp4 involved in the transformation process (Beyhan et al. 2013). Using whole genome transcriptional profiling, they observed that 96% of yeast phase transcripts were dependent on the expression of these transcription factor genes, which were found to associate and interact with upstream regions and impact expression of genes that controlled not only transition but also virulence and therefore pathogenesis. Further studies are needed to identify additional factors involved in controlling transformation into the yeast form as well as characterizing possible pathways for interaction. Understanding these processes may lead to the identification of potential therapeutic targets in the future. However, the transformation from the environmental mycelia morphology to the yeast form is only the beginning of the process of successfully infecting the host.

6.3 Mucosal Barriers

At the onset of infection, aerosolized microconidia enter host airways, and the most common types of diseases described are sinus and lung infections. Regarding the former disease state, it is unclear if this is just a part of dissemination or the result of the nares being the entryway of the pathogen (Elansari et al. 2016; Rizzi et al. 2006; Symptoms of Histoplasmosis). In pulmonary and disseminated disease, *Histoplasma* microconidia bypass initial innate defenses, such as nasal and pharyngeal mucus, mucociliary clearance, and initial antibody defenses, likely due to their small size and the negative pressure present with inhalation. As *Histoplasma* spores travel down the respiratory tract, they undergo phase transition to yeast. In the alveoli, yeast encounter the first of the host defenses that impact their survival.

6.3.1 Surfactant Proteins

During acute infection or initial inoculation, microconidia and newly transitioned yeast forms that successfully pass the filtering systems of the upper airways and reach the alveoli, encounter pulmonary surfactant proteins. Surfactant is a complex fluid that is composed mostly of phospholipids and four proteins (SP-A, SP-B, SP-C, and SP-D), which have different biological functions. SP-A and SP-D are part of the collectin family as they contain a collagen-like region that is part of the structure of the C-type lectin domain (McCormack et al. 2003). These hydrophilic surfactant components play a role in lung immunity (Han and Mallampalli 2015; Carreto-Binaghi et al. 2016). SP-A and SP-D bind viruses, bacteria, fungi, and parasites through a carbohydrate recognition domain (CRD), opsonizing the pathogens to enhance phagocytosis and clearance by neutrophils and macrophages (Nayak et al. 2012; Carreto-Binaghi et al. 2016; van de Wetering et al. 2004).

However, this is not the primary way in which surfactant impacts the survival of *Histoplasma*. Surfactant proteins have inherent fungicidal properties. McCormack et al. showed that *Histoplasma* yeast cells grown in the presence of SP-A and SP-D are greatly inhibited (McCormack et al. 2003). This decrease in viability was associated with a calcium-dependent, surfactant protein-mediated increase in permeability of *Histoplasma* cells. The complete mechanism leading to increased permeability of the yeast cells is not fully understood. The authors proposed that calcium binding leads to conformational shifts in CRD which expose hydrophobic proteins that disrupt the yeast cell wall. Also, SP-A deficient mice were more susceptible to infection compared to wild type. Interestingly, there was a minimal decrease in clearance of the pathogen in these mice, and the authors proposed that this may be due to the continued presence of SP-D or rapid phagocytosis of *Histoplasma* by macrophages. The growth of the pathogen within macrophages was uninhibited by the presence of SP-A and SP-D (McCormack et al. 2003; Carreto-Binaghi et al. 2016). Hence, the primary role of surfactants is to impede further entry of the pathogen into host tissues and cells.

6.4 Developing a Niche Within Host Macrophages

Once they reach alveoli, *H. capsulatum* yeast encounter cells of innate immune system. While shown to interact with various cell types, as part of the innate immune response, yeast cells establish their niche within alveolar macrophages. *Histoplasma* enters the macrophage via phagocytosis and is mediated by complement receptors as this does not require opsonization of the pathogen. This is important as the lungs are a site poor in serum opsonins (Le Cabec et al. 2002). Utilization of this mechanism of phagocytosis is beneficial to the pathogen as it does not trigger additional fungicidal pathways, which aids in the organism's ability to persist within a cell (Long et al. 2003). Additionally, when initially entering the cell, the pathogen must further alter its cell wall to avoid recognition by other macrophage receptors (Garfoot and Rappleye 2016; Garfoot et al. 2016, 2017). Once ingested once in the cell cytoplasm, *H. capsulatum* is contained within a phagocytic vacuole (Long et al. 2003). To persist within this vacuole, it must evade further protective responses to an invading organism. The ways that it alters normal function include inactivating phagasomal reactive nitrogen and oxygen species, prevention of phagosomal acidification, and potentially lysosomal fusion. Lastly, while *Histoplasma* is contained within the macrophage, it must be able to create and transport its own nutrients. The pathogen, if successful, can continue to grow and divide in this host cell, and eventually will travel to the nucleus to induce host cell apoptosis so that it can infect other cells and disseminate (Pitangui et al. 2015).

6.4.1 Entry into the Macrophage

Complement Receptor-Mediated Phagocytosis

The first step in the interaction between the yeast form and the macrophage is ingestion via complement receptor-mediated phagocytosis. The main receptors on the surface of alveolar macrophages are LFA-1 (CD11a/CD18), CR3 (CD11b/CD18) and CR4 (CD11c/CD18) (Bullock and Wright 1987). Each of these receptors has a unique β subunit and a common α subunit. When the CD18 subunit of these receptors is blocked, 50–90% of *Histoplasma* binding is impeded (Garfoot and Rappleye 2016; Newman et al. 1990).

The receptor–ligand protein on *Histoplasma's s*urface that is primarily involved with internalization is a heat shock protein (Hsp). Hsp are regulators of protein folding and are upregulated during times of stress (Cleare et al. 2017; Long et al. 2003; Guimarães et al. 2011b). These proteins are distinguished and named for their molecular weight and each plays a different role in fungal pathogenesis. For instance, Hsp70 is upregulated during the morphogenic shift from the environmental filamentous form to the pathogenic yeast phase (Cleare et al. 2017; Leach and Cowen 2013). Hsp60 exists on the cell wall of *Histoplasma* yeast forms and it is the ligand of the CR3 receptor on host macrophages. Interestingly, it is unclear what promotes its expression specifically on the yeast form cell wall (Long et al. 2003).

In a study by Guimarães et al., other functions of Hsp60 within *Histoplasma* were evaluated (Guimarães et al. 2011b). Hsp60 interacted with 126 unique fungal proteins and the number of interactions increased with temperature increase, suggesting that this protein has a wide breadth of cellular functions (Guimarães et al. 2011b). Significantly, it is crucial in the initial steps leading to persistence in the host macrophages. This is evident in a study by Gomez et al., where vaccination with recombinant Hsp60 effectively protected mice challenged with a lethal inoculum of *Histoplasma* (Gomez et al. 1995). A later study by Guimarães et al., had a similar finding using passive immunity with monoclonal antibodies to Hsp60. In their inoculated mice, they observed that certain Hsp60-binding antibodies prolonged survival, decreased fungal burden and organ damage, and increased Th1-type cytokine levels (IL-2, IL-12, TNFα) (Guimarães et al. 2009).

The interaction between Hsp60 and CR3 may be the reason that *Histoplasma* can enter the macrophage without triggering additional inflammatory cascades (Long et al. 2003). However, the interaction of these proteins is not an isolated event in terms of successful entry into the macrophage. The organism has additional changes it will need to undergo to further evade the host immune response.

Alterations in the Cell Wall

Macrophages have additional cell wall receptors that can recognize fungal-pathogen-associated molecular patterns (PAMPs) to trigger an attack on an invading pathogen. One such receptor is Dectin-1. This receptor recognizes

β-glucan which comprises a large part of the *Histoplasma* cell wall structure. This interaction of the glucan and Dectin-1 triggers a pro-inflammatory response from the macrophage. The primary mechanism that *H. capsulatum* utilizes to evade Dectin-1 is the production of α-linked glucans that surround and conceal β-glucans (Garfoot and Rappleye 2016; Garfoot et al. 2016).

These α-glucans are synthesized by α-(1,3)-glucan synthase (Ags1). Rappleye et al. demonstrated that reduction or loss of α-glucans results in a significant reduction in virulence (Rappleye et al. 2004). Additional genes are involved in the process of α-glucan synthesis. Marion et al. identified an α-(1,4)-amylase (Amy1) and the gene responsible for producing this protein, *AMY1* (Marion et al. 2006). As a part of the α-amylase family of enzymes, Amy1 may generate α-(1,4) linked oligosaccharides that are then used by Ags1 to generate α-(1,3) linked glucans, or it may be responsible for transglycosylation of the final product. Another gene identified in this study was *UGP1*. This gene generates a UTP-glucose-1-phosphate uridylltransferase that produces UDP-glucose monomers. These are used by Ags1 to generate a-(1,4) and a-(1,6) linked glucans. With silencing of *UGP* , there was a decrease in substrate and therefore a loss of α-(1,3)-glucan synthesis (Marion et al. 2006).

Strains that have significant quantities of α-(1,3)-glucan as part of their cells walls are called chemotype 2 strains. Some strains of *Histoplasma* lack α-glucan production as an evasive mechanism yet these strains show no difference in their virulence and are called chemotype 1 strains. This is true of the Panamanian G186A and North American isolate G217B (Edwards et al. 2011). In a study by Edwards et al., genome analysis of the promoter region of the Ags1 gene in α-glucan-deficient strains showed a large interrupting sequence that demonstrated a decrease in gene product expression in vitro (Edwards et al. 2011). However, *AGS1* mRNA was detected upon lung infection in mice. The authors proceeded to generate mutant yeast forms that lacked *AGS1* and no defects were seen in their ability to infect the lung and disseminate. These strains were also used in the study described above by Rappleye et al. Those authors found a decrease in virulence with the loss of *AGS1*, and they argue that α-(1,3)-glucan is still part of the cell wall of these strains but it may be modified and undetectable by standard methods (Rappleye et al. 2004). Both studies also propose that chemotype 1 strains may utilize unique mechanisms, other than the manipulation of their cell wall structure, to evade interaction with Dectin-1 (Edwards et al. 2011; Rappleye et al. 2004).

It is possible that secreted glucanases may be the unique or predominately utilized mechanism of certain strains to evade additional interactions with macrophage receptors. The glucanase Eng1 was studied by Garfoot et al., using strains G186A and G217B (Garfoot et al. 2016). When *ENG1* expression was silenced, the growth of the organism itself was not impacted. However, in vivo Eng1 deficiency led to reduced infectivity and increased pro-inflammatory marker production. Hence, Eng1 is a secreted glucanase that reduces the amount of β-glucan exposed to the macrophages (Garfoot et al. 2016).

Exg8 is another studied glucanase whose target is also cell wall β-glucan. Exg8, like Eng1, is only produced by the pathogenic yeast form of *Histoplasma*. A second

study by Garfoot et al., using strains G186A and G217B, found that the loss of Exg8 led to a modest attenuation in contrast to Eng1 loss (Garfoot et al. 2017). Also, Exg8 did not significantly impair yeast cell interaction with Dectin-1. Exg8 in this study is characterized as an exo-glucanase and Eng1 is an endo-glucanase. It was proposed then that the structure of cellular β-glucans is not simple chains with exposed terminal ends but loops; hence, the endo-glucanase Eng1 is of greater importance in evasion of Dectin-1. These glucanases maintain the virulence of strains that lack α-glucan production, but they do not alter the receptor–ligand binding ultimately leading to internalization by the macrophage, i.e., CR3 binding to Hsp60 (Garfoot et al. 2017).

6.4.2 Challenges to Overcome Once Within the Macrophage

The processes discussed thus far have focused on the initial entry and phagocytosis by macrophages. Once inside, *Histoplasma* survives within a phagocytic vacuole in the cytoplasm. To persist and grow in this compartment, it needs to overcome additional challenges. Most of the fungus' actions are defensive in nature. It must inactivate reactive oxygen and nitrogen species released into the vacuole, prevent phagosome acidification and lysosomal fusion, and produce and transport needed nutrients to overcome nutritional immunity of the macrophage.

Inactivating Reactive Oxygen Species

Ingestion of the fungus by macrophages triggers an oxidative burst, which is a surge in the production of reactive oxygen species by the phagosome membrane associated complex, NADPH oxidase (Missall et al. 2004). This enzyme reduces oxygen to superoxide; this is secreted into the phagosome and can cause considerable damage to the microbe within it. This attack occurs extracellularly to the organism as superoxide is charged and does not cross the cell membrane of the pathogen (Garfoot and Rappleye 2016; Youseff et al. 2012).

Superoxide is not the only chemical that is potentially fungicidal within the phagosome. It can be broken down into hydrogen peroxide or it can combine with nitric oxide, and both can cause damage to microbes (Missall et al. 2004). Hydrogen peroxide can also be further broken down into hydroxyl radicals. Hydrogen peroxide, unlike superoxide, can pass through the cell membrane of an organism since it carries a neutral charge, and has the potential to cause damage both extra-and intracellularly (Youseff et al. 2012). Overall, the susceptibility of a fungus to generated reactive oxygen species is organism dependent (Missall et al. 2004). For instance, *Histoplasma* yeast cells endure concentrations of reactive oxygen species that would kill other yeast, like *Candida* (Youseff et al. 2012). *Histoplasma* as an intracellular pathogen needs to be able to combat both extracellular and intracellular free radicals while in the phagosome. These survival mechanisms are crucial to the virulence of the organism; hence, production of certain enzymes only occurs within the pathogenic yeast form (Garfoot and Rappleye 2016; Youseff et al. 2012).

Eissenberg et al. suggested that *H. capsulatum* may not induce an oxidative burst in all macrophages it invades (Garfoot and Rappleye 2016; Eissenberg and Goldman 1987). The mechanism of this early finding has never been fully elucidated. One explanation is that murine peritoneal macrophages were used, which do not behave the same as human macrophages (Eissenberg and Goldman 1987; Youseff et al. 2012). In a 2012 study by Youseff et al., using functional assays on murine intraperitoneal macrophages, further characterized the process of respiratory burst by macrophages infected with *Histoplasma* (Youseff et al. 2012). They first observed that the yeast forms infected inactivated macrophages. They then created a strain that lacked superoxide dismutase (Sod3), which is needed for clearance of free radicals by the fungus within the phagosome. Both the wild-type and mutant strains infected inactivated macrophages, but the survival of the mutant strain was decreased by a small but significant amount. This finding indicates that even in the resting state, macrophages continue to produce reactive oxygen species. The authors also found that a *Histoplasma* superoxide dismutase decreased free radical levels to baseline within 10–15 min of exposure (Youseff et al. 2012). Additionally, they observed that activation with certain cytokines, like IFN-γ and TNFα that are released by CD4 cells, resulted in a further enhancement in the production of reactive oxygen species by macrophages (Youseff et al. 2012). This highlights the connections and cooperation between the innate and adaptive immune response that are discussed in further detail below.

Hydrogen peroxide is produced extracellularly by the phagosome and as a by-product of superoxide production. To combat hydrogen peroxide, *H. capsulatum* produces 3 catalases, CatA, CatB, and CatP (Johnson et al. 2002). CatA is produced by the mycelial form of certain strains and production is inducible in the presence of hydrogen peroxide (Holbrook et al. 2013; Guimarães et al. 2008). CatB and CatP are constantly produced by the yeast form. They differ in that CatB exerts its role extracellularly while CatP works intracellularly, but both function to reduce hydrogen peroxide into water and oxygen to prevent damage to the microbe (Guimarães et al. 2008; Holbrook et al. 2013). In a study by Holbrook et al., the authors evaluated the relative importance of each catalase by studying the impact of their removal in generated mutant strains compared to a wild type (Holbrook et al. 2013). Loss of CatB had no significant impact on virulence in vivo and only minor effect on survival in culture. The proposed explanation is that superoxide may be more abundant and play a more significant role as a fungicidal agent. Mutants that lacked both Sod3 and CatB were evaluated, and there was no additive effect in decreased survival and virulence in vitro and in vivo with the additional loss of CatB. The conclusion made was that CatB and CatP alone are not sufficient to protect the yeast form from damage by generated free radicals, and superoxide is the major fungicidal agent in the phagosome. However, the loss of both catalases in a strain while maintaining Sod3 function was evaluated, and their loss resulted in a minor reduction in virulence. Loss of CatP or CatB alone did not impact virulence to the same degree as seen when both enzymes are silenced. The authors asserted here that there is a redundancy in the function of these enzymes, and it is likely that this is a result of the nature of their target molecule. Hydrogen peroxide carries no

charge, and so unlike superoxide, it can pass freely through the pathogen's membrane. Unfortunately, wherever it travels it is flanked by a catalase on either side (Holbrook et al. 2013; Garfoot and Rappleye 2016).

The pathogen, if successful in neutralizing these reactive oxygen species, is another step closer to claiming its niche where it can persist, grow within, and utilize it to disseminate throughout the host.

Inactivating Reactive Nitrogen Species

As mentioned, superoxide can also combine with nitric oxide, which generates reactive nitrogen species that the organism must also contend with (Garfoot and Rappleye 2016; Chao et al. 2008; Lane et al. 1994; Nakamura et al. 1994; Nittler et al. 2005). Activated macrophages utilize the enzyme nitrogen oxide synthase to produce nitric oxide. Activation of this enzyme is induced by the initial infection and by IFN-γ stimulation of macrophages (Garfoot and Rappleye 2016; Lane et al. 1994; Nakamura et al. 1994). Nitric oxide, once generated and transformed into a reactive nitrogen species, can cause DNA and membrane damage, inhibit cell replication, and can inactive crucial cellular enzymes (Nittler et al. 2005). Reactive nitrogen species in response to infection with *Histoplasma* leads to a fungistatic, not fungicidal, result (Nittler et al. 2005; Garfoot and Rappleye 2016).

Nittler et al. utilized functional genomics to identify a set of genes that were induced in response to reactive nitrogen species in *Histoplasma* infection. They identified 153 gene transcripts that were upregulated with infection, but their function remained unclear, and no core group of genes could be identified (Nittler et al. 2005). One gene found was *NOR1*, and this was noted to have high sequence homology with other nitric oxide reductases seen in other fungal and bacterial pathogens that facilitate the conversion of nitric oxide to nitrous oxide, which is no longer toxic to the cell (Nittler et al. 2005). Chao et al. were able to confirm with cell culture, mass spectroscopy, and nitrous oxide detection that Nor1 reduced reactive nitrogen species into less toxic substances in the infected macrophage (Chao et al. 2008). It was also found to have constitutive expression in the mycelial form and inducible expression in the yeast form of *Histoplasma* (Nittler et al. 2005; Chao et al. 2008).

It is evident that *H. capsulatum* has defense mechanisms in place to evade both reactive oxygen and nitrogen species. But these are not the only dangers to the pathogen when it exists intracellularly and within a phagocytic vacuole.

Prevention of Phagosome Acidification and Lysosomal Fusion

As a phagosome matures it creates an acidic pH internally to enhance microbicidal activities against encompassed microbes (Isaac et al. 2013). Vacuolar ATPases (V-ATPase) are pumps on the membrane that are used to bring protons to the inside of the phagosome. Subsequently, the phagosome fuses with a lysosome that contains multiple hydrolases that work best at this generated low pH. These enzymes then further attack an invading microbe. Therefore, for the microbe to persist, it must find a way to manipulate its environment within the macrophage to create a more basic pH and potentially prevent lysosomal fusion.

Strasser et al. found that inhibition of macrophage V-ATPase had no effect on *H. capsulatum* survival, and there was no change in internal pH (Strasser et al. 1999). They also found that there was diminished phagosome-lysosomal fusion. The authors go on to assert that V-ATPase was not required for the acidification of phagosomes containing *Histoplasma*. It was thought also that *Histoplasma* itself contains a pH sensing ability for it to respond to changes in its environment (Strasser et al. 1999). Isaac et al. utilized genetic screening of insertional mutants of *Histoplasma* to identify mutants that were unable to lyse host macrophages (Isaac et al. 2013). They identified HMG CoA lyase (*HCL 1*) as being required for growth within the macrophage and later lysis of the cell particularly in glucose-deficient and leucine-rich environments. Indirectly, the authors found that this enzyme was also important to maintaining phagolysosomal pH. In mutants lacking this enzyme, there was an accumulation of acidic species when leucine was utilized as the primary energy source while grown in unbuffered media. Hcl1 mutants were able to persist in macrophages, but their growth was significantly restricted compared to wild-type strains (Isaac et al. 2013). Unfortunately, in vivo studies with mice models did not show a difference in virulence of the mutant strains compared to the wild type. This would also suggest that there are additional pathways that require alteration to inhibit pathogenesis, and further studies are needed to explore that (Isaac et al. 2013; Garfoot and Rappleye 2016).

Newman et al. further investigated the role of pH in *H. capsulatum* survival in human macrophages (Newman et al. 2005). They found that intraphagosomal pH was about 6.5 when viable yeast forms were present, but there was no change in pH noted using heat-killed cells, fixed yeast cells or nonpathogenic yeast known to cause acidification when digested by macrophages. This would indicate that an acidic pH may in fact not be needed for killing. The authors asserted that additional studies are needed to further explore this relationship out; particularly, if there is a target or signal pathway needed to initiate changes in pH that aid in clearance of *Histoplasma* (Newman et al. 2006).

The prevention of lysosomal fusion may be another method utilized by *Histoplasma* as part of its pathogenesis. P338D1 mice and J774.2 cell line models have normal fusion of these intracellular compartments (Strasser et al. 1999; Taylor et al. 1989; Eissenberg et al. 1988). However, the RAW 264.7 cell line and human macrophages show a decrease in phagosome-lysosome fusion (Newman et al. 2006). It remains unclear why this is the case. Based on the above studies by Newman and Strasser, an acidic pH does not seem to limit nor is it necessary for lysosomal hydrolase function (Newman et al. 2006; Strasser et al. 1999). Additional study in this area would be of value not only to understand how the organism may impact this interaction but for the potential of therapeutic interventions that could increase clearance of *H. capsulatum*.

Nutrient, Essential Metal, and Nucleic Acid Acquisition

At this point, if the pathogen defends itself from reactive oxygen and nitrogen species, prevents lysosomal fusion and maintains a basic pH, as alluded to earlier, there are still certain nutrients it will need to acquire that impact its growth and

survival. The phagosome is generally nutrient-poor (Garfoot and Rappleye 2016). This mechanism of limiting nucleic acids, iron, and vitamins has also been termed nutritional immunity (Woods 2016; Garfoot and Rappleye 2016). It is a dynamic process and one that the macrophage possesses to defend itself. Therefore, the pathogen needs to be able to utilize what is present or produce its own nutrients to meet its metabolic needs (Garfoot and Rappleye 2016; Woods 2016).

Iron

Histoplasma must find a way to deal with iron deficiency in its environment, so it can continue to proliferate while in the phagosome. Newman et al. investigated the role of intracellular iron and its impact on yeast cell growth (Newman et al. 1994). Initial cultures of the pathogen with iron chelators suppressed growth in a concentration-dependent pattern, and the effect was reversed with the supplementation of iron. Chloroquine had a similar effect on growth, but it did so by raising endocytic pH and induced human macrophages to kill yeast cells. Its effects were reversed using iron supplementation that was soluble at a basic pH. Chloroquine's effects were both time- and dose-dependent and impacted fungal burden and dissemination. This study highlighted the importance of iron to *Histoplasma* survival and begs the question of potential therapeutic options (Newman et al. 1994).

Within the host, iron is bound and transported into a cell by surface transferrin molecules. Once in the cell, the iron is free or can be bound to ferritin. This binding to ferritin is upregulated in the setting of infection. Lane et al. found that IFN-γ released by T cells during infection caused a decrease in cell surface transferrin receptors (Lane et al. 1991). This suggests that cytokine stimulation also plays a role in limiting iron availability within the macrophage. Intracellular unbound iron is a potential source of this essential metal for *Histoplasma*. When iron is lacking in its surroundings, the yeast cells secrete hydroxamates that function as siderophores or iron chelators (Howard et al. 2000; Garfoot and Rappleye 2016). Howard et al. observed that *H. capsulatum*-derived siderophores are detected 4 days after inoculation of the media. Expression can be suppressed with increasing concentrations of iron in the environment. Also, the authors found that there is not just one siderophore but 5 different ones (Howard et al. 2000). Hwang el al. later identified the gene *SID1,* which produces the enzymes that catalyze the first step in siderophore production. Strains deficient in this gene showed significant depression in growth (Hwang et al. 2008).

In addition to the chelating activity of siderophores, Timmerman et al. observed the utilization of enzymatic reductants by *Histoplasma* to reduce iron and thus allowed for its uptake (Timmerman and Woods 1999). The expression of these enzymes was upregulated when the pathogen was grown in iron-deficient conditions. These included secreted extracellular glutathione-dependent ferric reductase, extracellular non-proteinaceous ferric reductants, and cell surface ferric reducing agents. Subsequently, they evaluated the relationship between reductases, siderophores, and discussed pH changes leading to the release of iron from transferrin. It was initially believed that an acidic pH allows an organism to gather essential metals while within the macrophage (Isaac et al. 2013; Strasser et al. 1999). Iron is

bound to the transferrin receptors, and when the pH falls from 7.0 to 6.0 there is a 50% dissociation of iron and even more when the pH falls below 6.0. Timmerman et al. noted the function of extracellular glutathione-dependent ferric reductase as well as chelation by siderophores despite a pH of 7, and they questioned the true impact of pH changes on iron acquisition. Regardless, they proposed a model for iron acquisition that included this process. The postulate that siderophores, ferric reductants, and changes in pH can function separately to pull iron off of molecules like transferrin and transport it into the cell. Additionally, iron bound to siderophores and iron released by transferrin with pH changes can act as the substrate for ferric reductants and, once reduced, can also be transported into the cell (Timmerman and Woods 2001). The process of iron acquisition is crucial to survival for the pathogen, however, the exact mechanism is complex and there is more to be learned about the genes and enzymes that are involved (Winters et al. 2008). Additionally, other metals, like zinc, are implicated in pathogenesis, but their role and interaction with the pathogen require further study (Garfoot and Rappleye 2016; Dade et al. 2016).

Nucleic Acids

Histoplasma can overcome an iron deficient environment; similarly, it must be able to adapt to the nucleic acid deficient environment of the macrophage. To understand how *Histoplasma*, build their DNA, studies have focused on developing auxotrophs that are incapable of their own nucleic acid production. Nucleic acid auxotrophs were first derived by Woods et al. (Woods et al. 1998; Woods 2016). The *URA5* gene when interrupted, in a more recent study by Rappleye et al., created uracil auxotrophs that had a decrease in growth unless supplemented with uracil in cell culture (Woods et al. 1998; Rappleye et al. 2004). Additionally, in this study, adenine auxotrophs were created using gene disruption of *ADE2*, which showed a decrease in growth in cell culture (Woods 2016; Rappleye et al. 2004). Supplementation of a pyrimidine was required for continued growth. However, if the pathway was further damaged, such that its synthetic function is compromised, even with supplementation, there is no growth or proliferation of the pathogen (Garfoot and Rappleye 2016; Rappleye et al. 2004; Woods et al. 1998). So, it would seem then that *Histoplasma* is capable of independent production and potentially transport of nucleic acids when needed.

Vitamins

Histoplasma can synthesize its own essential vitamins while within the macrophage (Garfoot and Rappleye 2016; Garfoot et al. 2014). Garfoot et al. identified vitamin synthesis pathways within the genome, and then using a medium lacking in nutrients they were able to demonstrate that *Histoplasma* can produce all essential vitamins except thiamine (Garfoot et al. 2014). They proposed that the pathogen may gather this vitamin from the host and have scavenging mechanisms to aid in this process. Additionally, they studied the impact of riboflavin, pantothenate and biotin synthesis on growth and proliferation of the pathogen. They disrupted *RIB2*, *PAN6,* and *BIO2*. *RIB2* mutants persisted in the lungs but they did not replicate

in vivo, and a similar decrease in virulence was seen when *PAN6* was disrupted. *RIB2* mutants had only partial restoration of growth with supplementation in the cell media. *BIO2* gene disruption did not impact the virulence of the organism, and it is proposed that there is availability within the host to make up for this induced dysfunction. The authors asserted that information from this study explains more about the vitamins that are available in the phagosome and thus suggests potential therapeutic targets (Garfoot et al. 2014).

6.4.3 Inducing Apoptosis and Dissemination

H. capsulatum, by utilizing the previously discussed interactions with the host macrophage, can thrive in its intracellular niche. However, for infection to be propagated in the host and disseminate to other organs in the body, the pathogen needs to induce apoptosis of or otherwise leave the macrophage and infect subsequent phagocytes. This process will also potentially activate components of the adaptive immune response to allow for further control by the host and taking residence within granulomas. Pitangui et al. described the movement of *H. capsulatum* yeast cells within the macrophage. They found that yeast aggregate 5 h after infection around the cell nucleus and this leads to DNA damage and cell death (Pitangui et al. 2015). Deepe et al. further defined how apoptosis occurs and what cytokines are involved in this process. It is particularly intricate, and they describe the utilization of extrinsic pathways that are mediated by and lead to an increased expression of TNF-α and activation of caspases 1 and 3. Additionally, there is an increase in IL-10 production, which is counterintuitive as this is a cytokine that inhibits apoptosis in neighboring cells. The authors propose that these two cytokines produce a net effect that benefits the pathogen. There is the initial induction of apoptosis, and with the stimulation of neighboring cells with IL-10, the yeast can go on to infect phagocytes that will accommodate their survival (Deepe and Buesing 2012). The question that arises from this is whether *Histoplasma* triggers cell death, or if this is entirely a passive process that occurs when the fungal burden is too high within the macrophage.

Calcium Binding Protein (Cbp1) is specifically produced by *H. capsulatum* yeast cells, and it is involved in promoting cell growth in calcium limited settings (Batanghari et al. 1998). However, it also plays a role in cell death and proliferation (Sebghati et al. 2000). Isaac et al. evaluated the mechanisms used by Cbp1 when involved with cell death (Isaac et al. 2015). The authors screened 14,000 insertional mutants to find those that grew at high levels intracellularly but would not lyse cells, and three such mutants were identified, all lacking *CBP1* expression. Even with sufficient growth without *CBP1,* there was no cell lysis, indicating that his process was actively induced, as opposed to a passive cell death by the pathogen. Using whole genome sequencing, *CBP1* was found to be required for induction of stress-responsive genes that modulate cell death as well as activate caspases 3 and 7 (Isaac et al. 2015). This is further confirmed in a recent study by English et al. which suggests that there is an integrated stress response regulated by Cbp1 that

induces the expression of other proapoptotic genes like *CHOP* and *TRIB3*; however, the entire mechanism by which Cbp1 induces cell death is not known and is an area of further research and potential drug targeting (English et al. 2017).

6.5 Host Cellular Immune Response

Although the macrophage is a principal player in the host-pathogen interaction, *H. capsulatum* can interact with a variety of cells before it reaches its niche within the macrophage (Deepe et al. 2008). Deepe et al. demonstrated that the yeast cells preferentially invade different phagocytic cell subpopulations at different times after initial infection. They demonstrated that the yeast cells were present in neutrophils, dendritic cells (DCs) and macrophages, from days 1 to 7 after inoculation; however, DCs contained proportionately more yeast cells by day 1, with this shifting towards neutrophils and then macrophages on subsequent days (Deepe et al. 2008). DCs, neutrophils, and natural killer cells can effectively kill *H. capsulatum* yeast cells and, thus, their host-pathogen interaction is distinct from that described so far for macrophages.

6.5.1 Dendritic Cells

DCs first encounter *H. capsulatum* in the alveoli, and they serve as a link to subsequent host defenses. DCs precursors originate in the bone marrow and mature into more specialized cells that are found in the skin, and most solid organs including the lungs, specifically in airway epithelium, parenchyma, submucosa, alveolar septal wall, and alveolar surfaces (Thind et al. 2015; Sertl et al. 1986; Holt and Schon-Hegrad 1987). These cells are the primary antigen presenting cells of the innate immune system, and serve as a connection to the adaptive immune response as they interact with T cells, once they leave the tissues and travel to the lymph nodes (Clark and Kupper 2005). Immature DCs engulf entire organisms via receptor or non-receptor medicated phagocytosis at the tissue level. These cells process the pathogen and mature to then become antigen presenting cells (Thind et al. 2015). Also, once activated, DCs release cytokines like IL-12 and TNFα to recruit other DC, additional phagocyte populations, stimulate the adaptive immune response, and aid in granuloma formation (Zhou et al. 2001).

DCs efficiently engage *H. capsulatum*. Gildea et al. observed that after 6 h of incubation 75% of DCs in culture had ingested at least one yeast cell (Gildea et al. 2001). DCs binding of *Histoplasma* occurs via a fibronectin receptor on the DC surface called very late antigen-5 (VLA-5) (Gildea et al. 2001). This was an unexpected finding as there are higher levels of CD18 on the cell's surface. The authors initially hypothesized that CD18 would be utilized as was the case with macrophages. They also observed that DCs inhibited the growth of and killed phagocytized yeast cells unlike what had been observed in macrophages. This is

similar to what was observed when microconidia are ingested by DCs as their transformation into yeast cells was inhibited (Newman et al. 2011). The authors proposed that the difference in the receptor interactions of these cells with *Histoplasma* may impact the intracellular survival of the pathogen; although, the mechanism for the preferential receptor binding is unknown (Gildea et al. 2001). Subsequently, Gomez et al. identified the ligand for VLA-5 on the surface of *H. capsulatum* yeast cells, which turned out to be a 20-kDa protein called cyclophilin A (Gomez et al. 2008).

Once phagocytosis occurs, DCs can be both fungistatic and fungicidal. Human DCs phagolysosomal fusion occurs unimpeded (Gildea et al. 2005). Once inside this vesicle, there is either restriction of growth or killing of the organism, which largely occurs through hydrolases. Nitric oxide and oxygen free radicals do not seem to play a role in DC fungicidal activity, as in macrophages (Thind et al. 2015; Gildea et al. 2005). Despite the effective protection provided by the DCs, pathogens that are not phagocytized will encounter additional host defenses that can still impact their survival.

6.5.2 Neutrophils

In addition to early interactions with DCs after acquisition of *H. capsulatum*, the fungus also encounters neutrophils (Deepe et al. 2008). These cells rapidly arrive at the site of infection to engage the pathogen as part of the innate immune response (Thind et al. 2015). Human neutrophils are fungistatic against *H. capsulatum*. Antimicrobial proteins in neutrophils are contained in azurophilic granules (Newman et al. 1993). Within these granules, there are two families of proteins known as defensins and serprocidins, and two additional proteins with unique structures: lysozyme and bactericidal-permeability-increasing protein (BPI). Their interactions were rigorously evaluated by Newman et al. (Newman et al. 2000). Defensins (HNP-1, HNP-2, and HNP-3) are derived from 29 to 30 amino acids, differing only in a single N-terminal amino acid. Each of these when incubated with yeast cells showed concentration-dependent inhibition of growth. Of the three, HNP-2 had the greatest inhibitory activity. Their effect was also noted to be additive (Newman et al. 2000).

Serprocidins are a group of four proteins. Cathepsin G is one of these proteins, it is a neutral protease of molecular mass 29–31 kDa, and it is the only member of this family that inhibits growth of *Histoplasma*. Alone, this protein has inhibitory activity, and its effects are additive when associated with defensins (Newman et al. 2000). BPI is a protein within the granule with significant fungistatic ability toward *H. capsulatum*. It has a molecular mass of 50–60 kDa, characterized by a lysine-rich amino-terminal and carboxy-terminal regions (Gray et al. 1989). This protein inhibits the growth of yeast cells in a concentration-dependent manner and it has an additive effect when combined with either defensins or cathepsin G (Newman et al. 2000).

The entire mechanism of fungistasis by these proteins has not been fully elucidated (Newman et al. 1993, 2000). Newman et al. have described the discrepancy noted in *Histoplasma* endemic areas where many individuals who presumably have been exposed to the fungi at some point had negative skin tests when exposed to histoplasmin. It is hypothesized that the initial response from neutrophils may be sufficient to clear the organism from the host without the need or time for activation of the adaptive immune response, more likely in the setting of a small inoculum (Newman et al. 1993).

Both neutrophils and DC directly impact survival of *Histoplasma*. DCs through cytokine release will impact later control of the infection as well. Natural killer cells are also part of the initial host defenses, and they are connected to this process as they are recruited by DCs and macrophages.

6.5.3 Natural Killer Cells

In a study of intranasally infected mice, Cain et al. examined the inflammatory reactions and cytokine responses with active disease progression (Cain and Deepe 1998). Increased levels of IL-12 were observed by day 3 of infection followed by the increased expression of IL-2 and IFN-γ starting on day 5 till day 10. All of these declined as of day 14. It was observed that myeloid cells had increases in their expression by day 5, and by day 7 they peak with the additional presence of natural killer cells. It is by day 10 that T cells and B cells start to predominate among the inflammatory cell types at sites of disease. This highlights the involvement of natural killer cells in response to infection, and a potential connection between the initial myeloid cell response via stimulating cytokines.

Cohen et al. attempted to further define the connections between myeloid cells and the natural killer cells response to infection with various fungal pathogens (Cohen et al. 2011). Natural killer cell responses were reduced in the presence of *H. capsulatum* yeast cells and associated DCs were unable to produce IL-12. Additionally, when *Histoplasma* cells were grown with DCs lacking Dectin-1 on the cell surface, there was also a reduced natural killer cell response. The authors propose a general mechanism for natural killer cells' function during a systemic fungal infection. They postulate that antigen presenting cells, like DC or macrophages, produce IL-12 in response to fungal cell wall components leading to natural killer cell activation. Also, their data showed that natural killer cells enhance the production of IL-12 from antigen presenting cells as a positive feedback loop (Cohen et al. 2011).

Natural killer cells, akin to DCs, are directly cytotoxic to the target pathogen; however, this mechanism is not fully understood regarding infection with *Histoplasma* (Tewari and Von Behren 2000). Natural killer cells, also like DCs, continue to link the innate and adaptive host response to this pathogen. Activated natural killer cells produce IFN-γ (Zhou et al. 2001), which leads to activation of CD8 and CD4 T cells for further control of the infection and potentially granuloma formation.

6.6 *Adaptive Immune Response, Granuloma Formation and Reactivation*

The pathogen at this point has now bypassed the mucosal level obstacles, cellular host defenses and is manipulating the macrophage to further disseminate. But as alluded to earlier there is a complex network of cytokine production and the adaptive immune responses that are the host's last line of defense. The adaptive immune response once effectively activated can either clear the organism or lead to granuloma formation. If the latter occurs, there is the potential for reactivation of the organism. Reactivation is a response to impaired immunity (Allen and Deepe 2006). This can occur in the setting iatrogenic immunosuppression with the use of immunomodulating therapy that impacts cytokine function, in conditions like HIV/AIDS where there is a loss of T cell function, and with additional systemic conditions that impact the immune response (Heninger et al. 2006). Ultimately, the pathogen, though potentially controlled after acquisition, seems to never truly be eliminated by host defenses.

The adaptive immune response and granuloma formation is complex and not completely understood, but murine models have been developed to examine this process further (Allen and Deepe 2006; Heninger et al. 2006). As mentioned earlier DC act as antigen presenting cells in lymphoid tissue and activate T cells; therefore, they are a link between the innate and adaptive immune response. Natural killer cells and macrophages serve to connect these responses via cytokine production leading to cell activation. Heninger et al. used a murine model to further characterize granuloma formation (Heninger et al. 2006). On day 5, after infection, macrophages are present at the tissue, and the tissue has a vasculitic appearance that may indicate extravasation of immune cells. Granuloma formation occurred in the liver by day 7. The liver granulomas formed were noted to grow and reach their maximum size by day 10. IL-10 and TGF-β were elevated early in granuloma formation, with the latter coming from infected macrophages. The liver granulomas decreased in size after day 10 as immune stimulation waned, but pulmonary granulomas did not diminish in size. Hence, the organ itself has an impact on granuloma characteristics and control responses to the pathogen (Heninger et al. 2006).

Notably, 70% of the liver granuloma was made up of macrophages and there was an abundance of IFN-γ. Subsequently, these macrophages produced TNF-α. DCs and neutrophils were present to a lesser extent as were CD4 and CD8 T Cells. Early on there were more CD4 cells, but their ratio equalized as time progressed. Additionally, there was a low level of B cells present within the granulomas, which may be a potential link to the formation of protective antibodies. Moreover, there was a diversity of T and B cells present, indicating a diversity in recruitment processes as opposed to single cell type entry, multiplication and granuloma formation (Heninger et al. 2006).

Allen et al. further elucidated the importance of T and B cells in the granuloma, and their role in the control of disease (Allen and Deepe 2006). The authors

intranasally infected mice and then depleted them of CD4 and CD8 T cells 42 days later. The mice developed persistent disease when both cell types were eliminated. Also, latently infected mice subjected to B cell and CD4 cell depletion resulted in disease reactivation. These findings demonstrate that there is cooperation between CD8 T cells and B cells, and that this potentially explains why the depletion of either CD4 or CD8 T cells alone was insufficient to permit reactivation. The role of B cells in the immune response is complicated and has yet to be fully defined. Tristão et al. found that *Histoplasma* cell-free antigens (CFAg) are present during murine infection, and antibodies were generated against these antigens. These antigens competed with actual fungal surface antigens in antibody formation and recognition. So, as with other components of the immune response, this pathogen may have escape mechanisms against antibodies as well (Tristão et al. 2012). However, disease modifying monoclonal antibodies (mAb) have been described and include antibodies to cell surface displayed histone 2B (Nosanchuk et al. 2003; Shi et al. 2008), M antigen (Guimarães et al. 2008; Nosanchuk et al. 2012), and heat shock protein 60 (Guimarães et al. 2009, 2011a, b). However, there are antibodies that do not improve disease outcomes, such as a mAb to H1C (Lopes et al. 2010), and some mAb can enhance disease, such as an IgG2b isotype to heat shock 60 (Guimarães et al. 2009). Hence, the role of antibody therapy in histoplasmosis is complex and requires further investigation.

The Heninger study found that IFN-γ within these granulomas was produced mostly by CD4 and CD8 T cells (Heninger et al. 2006). As discussed, this cytokine activates macrophages to induce killing via reactive oxygen and nitrogen species. The macrophage will also produce TNF-α. To stress the importance of these cytokines in the control of disease, there have been studies and case series that evaluate the loss of these cytokines and how that impacts disease progression and reactivation. Clemons et al. treated rodent models with IFN antibodies and developed *IFN* knockout mice, and compared these to controls. Depletion of the cytokine and gene disruption both resulted in a loss of resistance to lethal infection and early mortality in the mice (Clemons et al. 2000). In a subsequent study by Clemons et al., IFN was evaluated as an adjuvant therapy to amphotericin B in infected mice, and the combination of the cytokine with amphotericin was superior to drug therapy alone (Clemons et al. 2001). This experimental work is supported by a case report from Zerbe et al., where a patient with an inherited IFN-γ receptor deficiency developed recurrent disseminated histoplasmosis (Zerbe and Holland 2005).

TNF-α is also a key regulator of disease (Deepe 2005). It is clinically relevant as seen in the study by Lee et al. where the authors reviewed post-licensure adverse effects of TNF inhibitors and found 10 cases of reactivation of *Histoplasma* after treatment with these agents (Lee et al. 2002). In mouse models, TNF depletion or inhibition leads to higher mortality in both primary and secondary infection (Allendoerfer and Deepe 1998; Deepe 2005). Granulomas continue to form within the tissues with an increase in inflammatory changes seen specifically in the lungs, likely due to an increase in fungal burden (Allendoerfer and Deepe 1998). Additionally, in primary infection, without TNF-α, there is a decrease in nitric oxide production and this may lead to poor clearance of the organism (Deepe 2007).

Unexpectedly, no change to IFN levels has been observed in this setting, which indicates independent production, and a possible codependent relationship with TNF-α as both are required for clearance of the organism (Allendoerfer and Deepe 1998; Deepe 2005). In secondary infection, without TNF-α, there is an upregulation of IL-4 and IL-10 which diminish protective immunity. In fact, blockade of both cytokines in TNF-α depleted mice improved survival (Allendoerfer and Deepe 1998; Deepe 2007). More recent studies are evaluating the T cells populations recruited when TNF-α is missing and how this may impact potential therapeutic interventions (Deepe and Gibbons 2008; Kroetz and Deepe 2012). Ultimately, further study is required to understand the relationships between this cytokine and the various immune cell responses in mice, and eventually expansion to human models to see if these same changes exist.

It is evident that the interactions of the adaptive and innate immune response, eventual granuloma formation and potential reactivation are each complex and interconnected connected processes. However, further characterization and definitions of the elements involved are beneficial as this can lead to therapeutic targets against disease. An example of this is evaluated in a study by Lazar-Molnar et al., as they examined the receptors involved in apoptosis (Lázár-Molnár et al. 2008). Programmed cell death-1 receptor (PD-1) is an immune inhibitory receptor that is part of the CD28:B7 family. It is expressed on activated T cells, B cells, and myeloid cells. PD-1 receptor and ligand binding inhibit cytokine production in vitro. In PD-1-deficient mice, there is protection from *Histoplasma capsulatum* infection. In wild-type mice, blockade of the PD-1 pathway generated increased survival by 70%. This is a potential pathway to be manipulated as a future therapeutic target (Lázár-Molnár et al. 2008).

6.7 New Pathogenic Mechanisms

Extracellular Vesicles

Extracellular vesicles are lipid bilayered structures that contain lipids, phospholipids, polysaccharides, nucleic acid, proteins, and other compounds. Extracellular vesicles have been described in all biological kingdoms (Zamith-Miranda et al. 2018). In fungi, extracellular vesicles have been shown to transport diverse compounds that include factors associated with virulence (Joffe et al. 2016; Rodrigues et al. 2008). *H. capsulatum* yeast cells produce extracellular vesicles that carry virulence factors such as heat shock protein 60, catalases, laccases, and phosphatases (Albuquerque et al. 2008; Matos Baltazar et al. 2016). Fungal extracellular vesicles can modulate host–pathogen interactions (Zamith-Miranda et al. 2018; Vargas et al. 2015); hence, *H. capsulatum* extracellular vesicles are postulated to impact pathogenesis. This is supported by the fact that the contents of *H. capsulatum* vesicles are recognized by immune human sera (Albuquerque et al. 2008). Additionally, binding of mAb to *H. capsulatum* heat shock protein 60 to yeast cells

induces a change in extracellular cargo loading and the characteristics of released vesicles are distinct from those isolated from untreated yeast cells (Matos Baltazar et al. 2016), which suggests that this process is dynamic and there is an interplay between the fungus and the host immune system. Targeting processes associated with the loading and release of extracellular vesicles is a promising potential approach to modifying the virulence of the fungus.

7 Concluding Remarks

H. capsulatum is the most prevalent endemic fungus worldwide. Nevertheless, histoplasmosis remains under-recognized and it is thus under-reported. Disease caused by *H. capsulatum* has a wide range of presentations and varying degrees of severity of clinical manifestations that impact treatment decisions. The process of infection and damage to the host is complex, and involves a dynamic interaction between host defense mechanisms, both at the mucosal and cellular level, and the pathogen's evasive and reactive responses. If the pathogen is successful, it can cause acute, local, or disseminated disease, or develop a latent state within tissue granulomas with the potential for reactivation. Further study and understanding of the pathogenesis of *Histoplasma* are needed. New knowledge is essential as it can open the door for therapeutic interventions that can positively impact clinical outcomes.

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Transcriptome Sequencing Approaches to Elucidate Host–Microbe Interactions in Opportunistic Human Fungal Pathogens

Hrant Hovhannisyan and Toni Gabaldón

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Abstract Infections caused by opportunistic human fungal pathogens are a source of increasing medical concern, due to their growing incidence, the emergence of novel pathogenic species, and the lack of effective diagnostics tools. Fungal

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pathogens are phylogenetically diverse, and their virulence mechanisms can differ widely across species. Despite extensive efforts, the molecular bases of virulence in pathogenic fungi and their interactions with the human host remain poorly understood for most species. In this context, next-generation sequencing approaches hold the promise of helping to close this knowledge gap. In particular, high-throughput transcriptome sequencing (RNA-Seq) enables monitoring the transcriptional profile of both host and microbes to elucidate their interactions and discover molecular mechanisms of virulence and host defense. Here, we provide an overview of transcriptome sequencing techniques and approaches, and survey their application in studying the interplay between humans and fungal pathogens. Finally, we discuss novel RNA-Seq approaches in studying host–pathogen interactions and their potential role in advancing the clinical diagnostics of fungal infections.

1 Introduction

Over the last two decades, the incidence of fungal infections (also known as mycoses) has increased dramatically (Bitar et al. 2014; Pfaller and Diekema 2007; Oren and Paul 2014), particularly in hospital-associated (nosocomial) conditions (Turner and Butler 2014; Chapman et al. 2017). Fungal infections range from superficial skin lesions to life-threatening invasive infections, including fungemia. Superficial skin or mucosal infections affect around 25% of the global population (Havlickova et al. 2008), and although they are relatively easy to manage, they collectively constitute a high burden. Invasive mycoses are life-threatening and can be associated to high mortality rates of up to 38–63%, depending on several factors such as the health status of the patient and the infecting strain (Klingspor et al. 2015; Flevari et al. 2013). It has been estimated that invasive fungal infections kill around 1.5 million people worldwide every year (Brown et al. 2012).

The phylogenetic diversity of pathogenic fungal species is high—from estimated 2.2–3.8 million fungal species, nearly 300 can cause human infections, which is likely to be an underestimation (Nature Microbiology Editorial 2017; O'Brien et al. 2005; Hawksworth and Lücking 2017; Blackwell 2011). Although most common fungal pathogens belong to several major clades, such as *Candida* (Pfaller and Diekema 2007, 2010), *Aspergillus* (Dagenais and Keller 2009), and *Cryptococcus* (May et al. 2016), each of these groups can comprise numerous distinct pathogenic lineages. For example, *Candida* species, which are considered to be the most frequent and invasive opportunistic fungal pathogens (Guinea 2014), are spread across the *Saccharomycotina* phylogenetic tree (Gabaldón et al. 2016). *C. glabrata*, which is usually the second most abundant *Candida* pathogen, after *C. abicans* (Guinea 2014), is phylogenetically much closer to the biotechnology workhorse *Saccharomyces cerevisiae* than to other pathogenic *Candidas* (Gabaldón and Carreté 2016). Similarly, pathogenic members of the so-called CTG clade, such as *C. albicans*, (Kim and Sudbery 2011), *C. parapsislosis*, and others, have numerous non-pathogenic sister species and clades. This phylogenetic dispersion points out

that the ability to infect humans has emerged multiple times independently in genetically distinct backgrounds (Gabaldón et al. 2016). Due to the high diversity and the difficulties to classify these pathogens from their physiological or morphological traits, the phylogenetic relationships between these fungi have been poorly resolved. Only the recent advent of molecular and genomic sequencing technologies has enabled accurate resolution of phylogenetic relationships and, as consequence, the taxonomic nomenclature of these species is still undergoing major revisions (Brandt and Lockhart 2012).

Novel pathogenic species are identified regularly, and the increase of incidence of previously rare species has been documented multiple times (Papon et al. 2013; Rhodes et al. 2017; Short et al. 2014). It is as yet unclear what factors drive the emergence of novel pathogens. Changes in the use of chemical products in industry or clinical care, or movement of products related to international commerce, can favor the global spread of certain species. In addition, biological factors resulting from evolutionary adaptation of microbes to novel niches can trigger the emergence of novel pathogens. One of the proposed mechanisms of emergence of novel pathogenic species in fungi is hybridization (Mixão and Gabaldón 2017), which has been related to the formation of pathogenic lineages such as *Cryptococcus neoformans x Cryptococcus gatii* (D'Souza et al. 2011), *Malassezia furfur* (Wu et al. 2015), *C. metapsilosis* (Pryszcz et al. 2015) and *C. orthopsilosis* (Pryszcz et al. 2014; Schröder et al. 2016).

Another major challenge posed by fungal pathogens is the increasing rate at which drug and multidrug resistance (MDR) is reported (Pfaller et al. 2009), which is often caused by the ability of fungi to evolve resistance phenotypes (Sanglard 2016). The problem of resistance to one or several drugs is worsened by the limited number of available antimycotic agents, which is currently restricted to few chemical families (Kathiravan et al. 2012). For example, the incidence of *C. glabrata* invasive infections has increased from 18% (in 1992–2001) to 25% (in 2001–2007), with concomitant fluconazole resistance rates increasing from 9% to 14%, respectively, in the USA (Pfaller et al. 2009). *Candida auris* is another striking example of MDR pathogenic yeast, which exhibits resistance to the main classes of antifungals (Sarma and Upadhyay 2017). Being first described in 2009 in Japan (Satoh et al. 2009), *C. auris* rapidly became a notorious pathogen causing outbreaks in hospitals throughout the world (Chowdhary et al. 2017). While the concern of research and medical community toward this pathogen is high, we still lack sufficient knowledge and effective approaches for controlling *C. auris* infections, highlighting the importance for public health of the emergence of antimycotic resistance.

As a consequence of high diversity, emergence of new pathogens, and increasing drug resistance, the diagnostic arsenal for the detection of the causative agent and the determination of the best treatment is limited (reviewed in Kozel and Wickes 2014; Griffin and Hanson 2014). Classical diagnostics methods have serious limitations. Culture-based methods using blood samples can take several days and do not provide high specificity and sensitivity, missing, for example, over 50% of the cases of documented candidiasis (Berenguer et al. 1993). Moreover, some fungal

species are non-culturable in conventionally used media. To overcome these issues, recently novel molecular-based diagnostic tools have been developed mainly including those based on polymerase chain reaction (Khot and Fredricks 2009) or mass spectrometry (Chalupová et al. 2014). More recently, with the rapid development of nucleic acid sequencing technologies, next-generation sequencing (NGS) might become a promising tool for microbial diagnostics (Smeekens et al. 2016; Zoll et al. 2016). Nevertheless, all of the aforementioned methods have their limitations from both clinical and economical perspectives (described in Kozel and Wickes 2014; Griffin and Hanson 2014).

Considering all these factors, it is evident that investigation of host–fungus interactions is crucial for overcoming the threats that pathogenic fungi currently impose. Firstly, knowing the specific host-evasion and virulence mechanisms used by diverse fungal pathogens may pave the way for the discovery of novel drug targets or the design of new treatment approaches. Secondly, many fungal pathogens are also commensal species that are part of the normal human microbiota. Hence, there is a need to understand what triggers may turn a commensal behavior into an invasive and virulent one. In addition, response from host cells and tissues toward different fungal pathogens may also provide important clues toward more efficient ways to avoid and control infection. Finally, understanding host–pathogen interactions may open new avenues for diagnostic approaches that are able to differentiate between commensal or infective behavior by detecting specific biomarkers. Although several studies have advanced our understanding of host–pathogen interactions for some of the more common species, the interplay between humans and fungal pathogens is, overall, still poorly understood.

NGS techniques, which allows obtaining sequence data on unprecedented scales and low costs, have represented a revolution in biological research (Goodwin et al. 2016), and the investigation of host–pathogen interaction is no exception (Hu et al. 2011; Westermann et al. 2012, 2017). In particular, whole transcriptome analysis by means of RNA-Seq has opened a new window to understanding gene regulation and how it changes as a result of interactions between the host and the pathogen, which potentially can shed light on the mechanisms of pathogenicity, host defense, and their interplay in various conditions (Wolf et al. 2018). In this review, we focus on the application of whole transcriptome sequencing in addressing host–fungus interactions during infection. We will first discuss the methodological concepts and peculiarities of RNA-Seq in the context of host–microbe interaction studies, then survey past studies of human–fungus interactions based on transcriptome sequencing. Finally, future perspectives in the field including the potential of emerging technologies for the study or diagnosis of fungal infections will be discussed.

2 Whole Transcriptome Analysis Methods

RNA plays a key role in the majority of cellular processes. Hence, investigation of the identity, function, and abundance of transcribed RNA molecules (i.e., transcripts) is crucial for understanding cellular behavior. Advances in the field of RNA biology were mainly driven by the development of novel technologies and methods allowing researchers to study different aspects of transcripts in an increasingly efficient way. A brief chronological overview of those techniques is discussed below, and a more in-depth comparison is provided in Table 1.

Table 1 Comparison of different transcriptomics technologies

	EST/SAGE	Microarray	RNA-Seq
First description	Sutcliffe et al. (1982), Velculescu et al. (1995)	Lockhart et al. (1996), Schena et al. (1995)	Bainbridge et al. (2006), Wang et al. (2009)
Major technology	Sanger-based sequencing of short random fragments (~500 bp)	Hybridization to complementary probes	Next-generation sequencing
Period in active usage Lowe et al. (2017)	1999–2004	2000–2014	2009–present
Popularity Lowe et al. (2017)	Almost obsolete	Decreasing	Increasing
Throughput	Low/middle	High	High
Major applications	Gene expression profiling	Gene expression profiling	Gene expression profiling, discovery of novel transcripts/isoforms of any length, gene fusion, variant detection
Discovery and quantification of novel transcripts	Yes	No	Yes
Dynamic range	Up to 3×10^5 Morrissy et al. (2009)	10^3–10^4 Black et al. (2014)	$>10^5$ Black et al. (2014)
Reproducibility (R^2)	0.96 Dinel et al. (2005)	0.99 Chen et al. (2007), SEQC/MAQC-III Consortium (2014)	0.99 Marioni et al. (2008), SEQC/MAQC-III Consortium (2014)
Complexity of data analysis	Middle	Middle, includes image processing and differential expression analysis	High (mainly command line), includes multiple steps depending on specific goal
Complexity of laboratory procedures	High	Low	High

Initial studies of RNA molecules were performed using methods such as northern blotting (Alwine et al. 1977), reverse transcriptase qPCR (Rappolee et al. 1988), and expressed sequence tags (ESTs) (Adams et al. 1991), which enabled to investigate individual molecules or small sets of transcripts. The first studies of transcriptomes, i.e., the whole set of RNA transcripts in a cell (or bulk of cells) at a given time point, begun in the mid 1990s, with the development of the serial analysis of gene expression (SAGE) method (Velculescu et al. 1995), based on Sanger sequencing technology (Sanger et al. 1977). SAGE and its derivatives (e.g., LongSAGE, RL-SAGE, SuperSAGE) in the beginning of 2000s were largely replaced by fluorescent hybridization-based RNA microarray technologies (Schena et al. 1995), which proved to be more cost-effective, as compared to previous methods. Finally, in the late 2000s, the advent of NGS superseded microarrays by RNA-Seq which provided unprecedented levels of resolution in a high throughput, unbiased, and relatively cheap manner (Bainbridge et al. 2006; Wang et al. 2009). In addition to considerations of throughput and cost, RNA-Seq presented the advantage over microarrays in that it did not require the design of probes and could explore the entire transcriptome in an unbiased manner, enabling the discovery of novel transcripts, even in the absence of a reference genome. In the last decade, RNA-Seq has been further developed, incorporating longer sequencing reads as well as increasing its versatility by being coupled to other approaches such as, for instance, target-enrichment (Amorim-vaz et al. 2015) or structure-specific digestion (Wan et al. 2013; Saus et al. 2018). Today, RNA-Seq is the major method used in transcriptomics studies.

As many other NGS-based techniques, RNA-Seq comprises two major steps: The first includes the study design and sequencing of the samples, whereas the second includes all downstream bioinformatics analysis. With current technologies, the particularities of each of the two stages can vary significantly depending on the main goal of the study. Hence, there is no universal procedure for addressing all possible biological questions that can be addressed with a transcriptomics approach (Conesa et al. 2016). Nevertheless, some generalities can be drawn. In the following sections, we will focus on the main principles of each of the two steps, underscoring, when applicable, the peculiarities that are most relevant for host–fungus interaction studies. We will first discuss steps usually performed for the dominating sequencing-by-synthesis NGS technology, implemented by Illumina, while other emerging approaches, such as nanopore and PacBio sequencing, will be discussed later in our review.

2.1 Study Design

Study design refers to the initial setup of the project, which is a crucial prerequisite for any RNA-Seq study. The project has to be planned carefully according to its main goals and taking into account the peculiarities of the addressed biological problem. Formally, the study design can be divided into experimental design and sequencing design.

2.1.1 Experimental Design

Experimental design refers to the overall type of the study ("control-vs-treatment," timecourse, observational study, and different combinations of these types) and how it is planned and performed from both logical and technical perspectives. A poorly planned experimental design can possibly result in spurious and/or misleading results. For instance, in a "control-vs-treatment" in vivo study of the influence of antimycotic drug on the gene expression of the fungus, "control" and "treatment" cases need to be selected. If "control" samples were obtained only from young people whereas the "treatment" samples came from significantly older people, then the age of the donors could be a potential confounding factor, preventing from distinguishing whether the observed gene expression changes in fungus were due to the antimycotic agent or the age of the host. To avoid such kind of confounding effects, study donors for both control and treatment group have to be as similar as possible from various perspectives, controlling for factors such as age, sex, diet, or the presence of concomitant diseases. Another example illustrating poorly planned experimental design could be a time series study of a fungal pathogen interacting with host cells in vitro. Without performing time-matched controls for the fungus, it might not be possible to differentiate between the effect of the host–microbe interactions from the potential effect of time or growth of the fungus in the given medium. For instance, some nutrients in the medium may be exhausted, triggering physiological changes in the fungal cells, which may be wrongly interpreted as a consequence of interaction with the host. To overcome this limitation, ideally host cell-free controls have to be made at the corresponding time points of the experiment (Fig. 1).

Thus, as illustrated by the examples above, a well-planned experimental design is a crucial first step for a meaningful RNA-Seq-based study. Critically assessing previous studies investigating similar questions (perhaps on other pathogens or hosts) and extensive discussions between all project partners (e.g., clinical doctors treating the patients, personnel collecting the samples, personnel responsible for the statistical comparisons) can help to achieve a good planning and avoid potential design flaws.

2.1.2 Sequencing Design

The design of the sequencing approach itself refers to the main factors to consider for sample collection, storage, preparation, and sequencing per se. While sample collection and storage methods heavily depend on a particular project, a general recommendation is to perform these two steps in the same way for all samples within a project to avoid possible confounding effects. For further sequencing, the important aspects to consider are RNA extraction protocol, library preparation protocol, read type and length, number of replicates, sequencing depth, randomization of samples and sequencing runs. The combination of the aforementioned

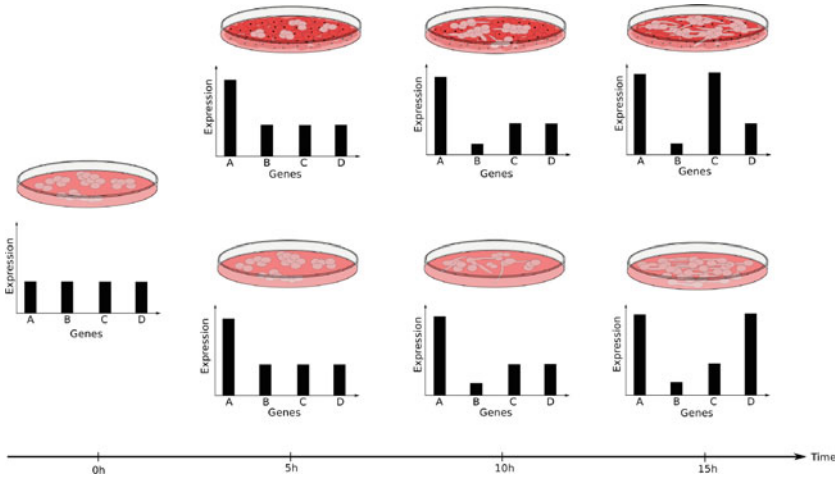

Fig. 1 A graphical representation of a time series experimental design of interaction between human cell line and a pathogenic fungus. Left-most sample represents a zero time-point control, from left to right samples—5-, 10-, and 25-h time points are the host–fungus interacting samples (above) and time-matched host cell-free fungal controls (below). Bar plots represent the expression levels of four fungal genes A, B, C, and D. The overall scheme illustrates the importance of time-matched fungal controls in the experimental design: In case of their absence, gene A at time point 5, 10, 15 h and gene B at time points 10 and 15 h could be spuriously interpreted as up- and down-regulated, respectively, as a consequence of host–fungus interaction. Controls samples allow to distinguish the effect of media and time from the effect of host–pathogen interaction, showing that only gene C is specifically up-regulated at 15 h as a consequence of interaction

parameters entirely depends on the specific goal of the project, and some general recommendations are given in Table 2 and are discussed in more details in the text.

For fungi, several commercial kits are available for high-quality and high-yield RNA extraction. An important factor to consider on this step is whether rRNA depletion or poly-(A) selection is required, since usually transcriptome studies are focused on mRNAs, while ribosomal RNA can constitute the vast majority of RNA in a cell (i.e., up to 60% in an exponentially growing *Saccharomyces cerevisiae* cell (Warner 1999), and its removal will provide higher resolution for the mRNAs (Zhao et al. 2014; O'Neil et al. 2013). Moreover, different strategies to enrich specific mRNA molecules have been recently developed, which will be discussed in more detail in the following section of our review.

The specific sequencing library preparation protocol is yet another factor to consider, especially with regards to its ability to generate strand-specific data. Early library preparation protocols were not capable of preserving information about the strand of DNA from which a transcript originated, thus biasing, for example, gene expression analysis by anti-sense transcription (Zhao et al. 2015). Today several so-called strand-specific (or stranded) protocols are available, such as dUTP (Borodina et al. 2011; Parkhomchuk et al. 2009), RNA-ligation (Lister et al. 2008),

Table 2 General recommendations of sequencing design based on addressed biological question

Type of analysis	Minimal recommended sequencing depth (in mln of aligned reads)[a]	Read length	Read layout	Replicates	Popular software	Additional information
Differential gene expression (DGE) analysis	>10 mln Liu et al. (2014b)	50 bp can be sufficient	Single-end can be sufficient	>3	Mapping—STAR, HISAT2, TopHat2; Pseudomapping—kallisto, Salmon; differential expression analysis—DESeq2, edgeR, limma-voom	Provided in the text
Differential isoform usage and alternative splicing (AS) analysis	>100 mln reads Liu et al. (2013)	>50 bp, longer reads are recommended	Paired-end is recommended	>3	Mapping—STAR, HISAT2, TopHat2; Alignment-free—kallisto, Salmon; differential expression analysis—DEXseq, MISO, Cuffdiff	Read length plays critical role in finding alternative isoforms
De novo assembly and novel transcript discovery	20–60 mln[b] Francis et al. (2013)	>50 bp, longer reads are recommended	Paired-end is recommended	1[c]	Trinity, Oases, SOAPdenovo-Trans, StringTie, Cufflinks	Sequencing parameters for performing de novo assembly depend heavily on the complexity of the of the transcriptome (i.e., levels of AS)
Allele-specific expression (ASE)	>55 mln (not well established) Castel et al. (2015)	>50 bp, longer reads are recommended	Paired-end is recommended	>3	Mapping—STAR, HISAT2, TopHat2, ASE—MBASED, Phaser, ASEReadCounter (GATK)	In general, allele-specific expression analysis based on RNA-Seq data is a relatively new area, and most of the software require variant calling file to perform ASE and only perform SNP level analysis

(continued)

Table 2 (continued)

Type of analysis	Minimal recommended sequencing depth (in mln of aligned reads)[a]	Read length	Read layout	Replicates	Popular software	Additional information
Small RNA-Seq (identification of miRNA, tRNA, snoRNA, etc.)	>1–8 mln Metpally et al. (2013), Campbell et al. (2015)	50 bp can be sufficient	Single-end can be sufficient	>3	Mapping—Bowtie, BWA; Identification—miRDeep, miRDeep2, miRanalyzer,	As a rule, discovery of small RNAs requires more sequencing depth than differential expression analysis

[a]Numbers are given for human transcriptome. For differential expression analysis, given numbers of reads are applicable for highly and moderately expressed transcripts

[b]The data is for non-model organisms

[c]In general case, replications are not strictly necessary as in DGE analysis, but are recommended for reproducibility of the results

mln = million

SMART (Zhu et al. 2001) for retaining strand information of transcripts. A comprehensive benchmark of these protocols is given in Levin et al. (2010).

The length of the reads (short stretches of cDNA that are actually sequenced) is one of the major parameters of the sequencing design. The read length of Illumina-based sequencing varies from 25 to 300 bases depending on the model of sequencing machine (Kwon-Chung et al. 2011). As an additional option to increase the capabilities of obtained data, one can perform paired-end (PE) sequencing instead of single-end (SE). In the former case, the cDNA fragment is sequenced from both ends, hence doubling the amount of the information obtained from it. As a general rule, longer reads coupled with paired-end long-insert size sequencing provide higher mapping rates to reference genomes, more accurate transcript discovery, the ability to detect larger indels, among other advantages. However, PE sequencing and longer reads come with higher price, which may compromise the number of replicates if budget is limited. Moreover, some particular goals could be sensitively achieved even with short SE reads (Chhangawala et al. 2015). For example, considering that in *Candida albicans* introns are not abundant in the genome and their length is usually short (Mitrovich et al. 2007), long PE reads are not critical for most of the typical downstream analysis.

In the context of typical RNA-Seq applications, such as differential gene expression (DGE), replicates and sequencing depth are two crucial and interconnected parameters. When choosing the number of biological replicates one has to consider the intrinsic biological variability of the studied system, the technical variability of the experimental procedures, and the desired statistical power of the experiment. As a general rule, the number of biological replicates included in the study should be at least 3, while the recommended number ranges from 6 to 12 biological replicates (based on *S. cerevisiae* data) depending on the specific goals of the study (Schurch et al. 2016). Several approaches and calculators have been implemented to perform RNA-Seq power analysis to help deciding the number of replicates in an RNA-Seq design (Hart et al. 2013; Yu et al. 2017; Guo et al. 2014).

Sequencing depth (or library size) denotes the total number of reads for each sample to be sequenced. Higher sequencing depth allows more precise transcript detection and expression quantification, but also might suffer from transcriptional noise and false-positive calls of DGE (Tarazona et al. 2011). Thus, once again the optimal sequencing depth depends on the addressed question and the system under study.

As mentioned above, replication and library size are interconnected parameters and for a particular sequencing design one can wonder whether for a fixed budget it is more advisable to add more replicates to the study or to perform deeper sequencing. Liu et al. (2014b) have evaluated the impact of both factors on DGE analysis. The study revealed that, in the case of human transcriptomic data, increasing the sequencing depth over 10 million reads has diminishing incremental effects for power of detection of differentially expressed (DE) genes, whereas an increase in the number of biological replicates significantly enhances the power of detection. Authors of the study also suggest a metric of cost-effectiveness of RNA-Seq design as a trade-off between number of replicates and sequencing depth.

The last but not least step in experimental design in the case of projects considering a high number of samples is to randomize the distribution of samples on different sequencing lanes or runs. This step is meant to avoid possible confounding factors such as different instrumental biases (lane effects, PCR duplicates, etc.) as well as difficult to control human factors.

2.2 Bioinformatics Data Analysis

As any high-throughput sequencing technology, RNA-Seq generates massive amounts of data (i.e., a typical DGE RNA-Seq analysis of yeast with two conditions and three replicates yields at least 100–150 million reads) which have to be thoroughly analyzed using bioinformatics approaches. Considering that RNA-Seq has numerous applications, the complete pipeline for bioinformatics analysis varies depending on the specific goals of the project. With regards to host–pathogen interaction studies, one of the most frequent RNA-Seq applications is the analysis of differential gene/transcript expression (Westermann et al. 2017). Here, we will briefly describe the main steps of this bioinformatics approach (Fig. 2).

The general initial step for any NGS-based data analysis is quality control (QC) of the raw data produced by the sequencing machine. As a general rule, raw reads are stored in the standard *fastq* format (Cock et al. 2009), which provides associated per base quality scores. For basic QC, several software solutions can be used, such as FastQC (Andrews 2010), HTQC (Yang et al. 2013), or NGS QC (Patel and Jain 2012). The main parameters to assess include, among others, per base sequence quality, GC content, or the presence of overrepresented sequences and/or those corresponding to the library adapters. When some quality parameters are not satisfactory, one can perform several actions including read trimming to cut out low-quality reads or bases, or removing adapter sequences coming from the library preparation step. Popular software to perform read trimming are Trimmomatic (Bolger et al. 2014), Skewer (Jiang et al. 2014), among others. However, in the case of DGE analysis trimming has to be performed gently since the harsh trimming can affect the results of read mapping and differential expression calls (Williams et al. 2016). For this reason, if the quality of the data is still unsatisfactory after trimming, it is advisable to resequence the sample or the library.

After ensuring the high quality of the sequencing data, downstream analysis depends on the presence or absence of a reference genome or transcriptome of the studied organism. In the case of human fungal pathogens, most common species like those belonging to *Candida*, *Aspergillus*, or *Cryptococcus* groups have available reference genomes in both specialized and generic public databases such as *Candida* Genome Database (Binkley et al. 2014), *Aspergillus* Genome Database (Cerqueira et al. 2014), FungiDB (Stajich et al. 2012), or RefSeq (Pruitt et al. 2007). Alternatively, in the case of absence of reference, the transcriptome can be reconstructed de novo, using, for example, the Trinity package (Haas et al. 2013) or SOAPdenovo-Trans (Xie et al. 2014).

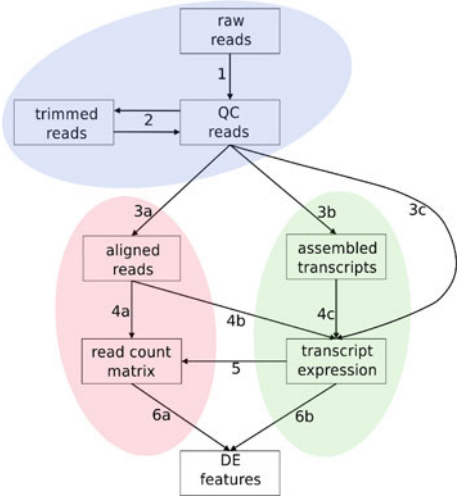

Fig. 2 General representation of RNA-Seq differential expression analysis pipeline. The numbers correspond to different steps of the analysis: 1—quality control of raw data; 2—read trimming (if necessary), 3a—read mapping to a reference genome, 3b—*de novo* assembly of transcripts 3c—pseudomapping strategy (requires reference transcriptome), 4a—read summarization, 4b—reference-guided transcriptome assembly (used for transcript identification), 4c—transcript quantification, 5—transcript-level quantifications can be converted to gene-level count, which improves gene-level inferences, 6a—differential gene/transcript/exon/feature analysis based on read counts, 6b—differential gene/transcript/exon/feature analysis based on relative expression values. Shades indicate different major strategies of the analysis: blue—general step of raw data quality control and read trimming; pink—downstream analysis when the reference genome is available, mainly includes gene-level inferences, green—when the reference genome is not available and includes transcript-level inferences

When a reference genome or transcriptome is available, downstream bioinformatics analysis implies mapping of reads to this reference. This computationally demanding task can be achieved by splice-aware read mappers (despite the fact that splicing is not as common in yeasts as in more complex eukaryotes). Numerous RNA-Seq mappers exist today, and choosing one might not be a trivial task. In fact, many researchers have addressed this question by performing benchmarks comparing different mappers, sometimes reaching contradicting conclusions (Otto et al. 2014; Kim et al. 2013; Dobin and Gingeras 2013; Baruzzo et al. 2017). Nevertheless, the most popular mappers include STAR (Dobin et al. 2013), TopHat2 (Kim et al. 2013), HISAT2 (Kim et al. 2015). After reads are mapped to the reference, quality control of the overall mapping is required. Most software tools provide basic mapping statistics, which include, among others, overall mapping rate, unique mapping rate, rate of multimappers (reads that map equally well to different locations in the reference), number of identified splices. Unique mapping rate is one of the crucial parameters and usually with high-quality raw data and a good enough reference genome the optimal values range from 85% to 95% of uniquely mapped reads. If the value is significantly lower, it might indicate low

quality of reads, poor quality of reference genome assembly or the nature of reference genome itself—for instance, genomes with large amount of repeats might result in increased numbers of multimapped reads. The next step after read mapping is read summarization, which involves the calculation of the number of reads that overlap particular genes, which is proportional to the expression levels. This procedure relies on gene annotations (gff or gtf files) and popular software to accomplish this task are htseq-count (Anders et al. 2015) and featureCounts (Liao et al. 2014), with the latter being more flexible in dealing with multimapped reads.

The two previous steps described gene-level analysis to obtain the information about expression values based on genome alignments. However, today several so-called pseudo-alignment algorithms are available that allow the assessment of expression levels of individual transcripts, rather than of genes. Examples of such algorithmic implementations include Salmon (Patro et al. 2017) and kallisto (Bray et al. 2016). For organisms with high rates of alternatively spliced transcripts, it has been recently demonstrated that transcript-level estimates could improve gene-level inferences (Soneson et al. 2015). The final step of DGE bioinformatics analysis is the assessment of DGE between groups of samples. Many studies have been performed to evaluate the most effective models and corresponding software for assessing differential expression (Schurch et al. 2016; Soneson and Delorenzi 2013; Bullard et al. 2010; Seyednasrollah et al. 2015; Rapaport et al. 2013). Readers are referred to (Conesa et al. 2016) for more details on this matter. When sufficient number of biological replicates (three or more) are available, software tools such as DESeq2 (Love et al. 2014), edgeR (Robinson et al. 2010), and *limma* (Ritchie et al. 2015) generally perform well in most of circumstances. To choose the genes that are differentially expressed, one has to set a cutoff for both fold change of gene expression between conditions and p value of statistical significance of this change, and usually, these thresholds are arbitrary and depend on the desired level of stringency.

Since overall RNA-Seq analysis consists of many wet-lab and data analysis steps, it is advisable to confirm a subset of the obtained results with an alternative approach. For instance, one can perform quantitative PCR on a subset of DE genes to ensure the reliability of the obtained results. Once the DE genes have been identified, researchers can proceed with further in-depth analysis, which, among others, usually includes gene ontology (GO, The Gene Ontology Consortium 2017) or gene set enrichment analysis (GSEA, Subramanian et al. 2005), pathway analysis (Emmert-Streib and Glazko 2011) and gene co-expression and network analysis (Schulze et al. 2016). GO and GSEA are two different approaches addressing a similar question—whether DE set or subset of genes is enriched in a specific biological function, process or cellular location. For instance, when differential expression analysis reveals hundreds of DE genes, these methods help to get an overall insight which biological functions are altered in a given condition. Similarly, pathway analysis allows to identify specific molecular pathways which are dysregulated in the studied system. As in GO and GSEA, pathway analysis is performed based on statistical test of enrichment between sets of gene lists.

Gene co-expression and network analysis on the other hand allows to quantitatively assess genes which are changing the expression levels systematically in a similar manner, revealing gene-gene interactions. Especially this kind of analysis is relevant when a time series dual RNA-Seq data is available, which enables to detect the interacting co-expressed genes of the host and the pathogen (Schulze et al. 2015).

For a more detailed discussion about the best practices of RNA-Seq-related analysis, including both study design and bioinformatics data analysis, readers are referred to the recent review by Conesa et al. (2016).

2.3 Dual RNA Sequencing

Dual RNA sequencing (dual RNA-Seq) is a relatively new methodology (Westermann et al. 2012) of simultaneous sequencing of RNA that originates from two (or more) organisms. Originally dual RNA-Seq was developed in the context of host–pathogen interaction studies, allowing to profile the gene expression of both counterparts at the same time, but in principle can be used to study the interactions between any cohabiting organisms. The main idea behind this method is to sequence the mixture of RNA that contains transcripts from two or more organisms. The mixture of RNA could be obtained by direct extraction of RNA from both species (e.g., when studying interaction between co-cultured bacterial species) or it can be extracted separately for each species and then mixed into one sample. The latter approach is more suitable for host–fungus interaction studies, since RNA extraction protocols for fungi include a cell wall disruption step, which can degrade the RNA content of the host cells. After sequencing a mixed sample, the reads from both species are separated bioinformatically by mapping the mixture of reads to both reference genomes simultaneously. When the reads are successfully separated, the data analysis is largely the same as in the case of standard RNA-Seq. Hence, the above-mentioned recommendations of experimental and sequencing design for common RNA-Seq are also applicable in dual RNA-Seq.

Despite the fact dual RNA-Seq methods are in their infancy, they have already been proven to be an efficient tool for dissecting the interplay between hosts and pathogens (Bruno et al. 2015; Aprianto et al. 2016; Dutton et al. 2016; Nuss et al. 2017; Thänert et al. 2017). Nevertheless, this approach has some technical limitations that need to be overcome. Firstly, for in vivo studies, particularly those involving fungi, the amount of microbial cells and its corresponding RNA is extremely low, as compared to the host side. The RNA-Seq of the sample, heavily shifted toward the host, yields a negligible amount of fungal reads, precluding detailed analysis of fungal transcriptome. To date, the most efficient way to overcome this problem is by using targeted enrichment of fungal transcripts (Amorim-vaz et al. 2015), which we discuss later. Second, since the dual RNA-Seq method is new, specific software and data analysis pipelines still do not exist. The major bioinformatics problem that can arise in dual RNA-Seq experiments is cross-mapping of reads to the wrong reference genome, since the mixture of reads is

mapped to both references simultaneously, biasing downstream analysis. Thus, specific data analysis pipelines should be implemented in order to remove that kind of reads. Despite these difficulties, dual RNA-Seq holds a great potential in resolving interactions between species on a transcriptome-wide manner.

3 RNA-Seq-Based Studies to Understand Human–Fungus Interactions in *Candida*, *Aspergillus* and *Cryptococcus* Clades

RNA-Seq has emerged as a versatile tool for studying host–pathogen interactions at the transcriptomic level. The majority of transcriptomic studies for elucidating pathogenic mechanisms in fungi so far has been performed in vitro by exposing the pathogen to different experimental conditions that try to mimic stress factors encountered in the host. These include, among many others, low pH, oxidative stress, or different temperatures (Cottier et al. 2015; Yang et al. 2016; Brown et al. 2016; Lin et al. 2013; Cheon et al. 2017). However, a limited number of transcriptomic studies have been performed in vivo, readily characterizing transcriptome responses of the pathogen, host, or both during their direct contact as it takes place during a real infection. Although this approach faces numerous challenges, it is still crucial for disentangling genuine human–fungal interactions. Here, we provide an overview of significant insights gained from transcriptomic studies. For simplicity, we will focus on the three major clades of fungal pathogens, namely *Candida*, *Cryptococcus*, and *Aspergillus,* as *r*esearch on other fungal pathogens generally lag behind. A schematic summary of surveyed studies is given in Fig. 3.

3.1 Candida

The most well-studied opportunistic pathogen from *Candida* species is *Candida albicans*, and its virulence mechanisms and host–fungus interactions have been extensively reviewed in Wilson and Hube (2014). Briefly, primary pathogenic mechanisms of *C. albicans* explored to date include hyphae formation (Sudbery et al. 2004) alongside with the expression of virulence factors, such as candidalysin (Moyes et al. 2016), adhesins (e.g., *HWP1*, *HGT2*) (Nobile et al. 2006; Martin et al. 2013), invasins (e.g., *ALS3*) (Liu and Filler 2011), and secreted proteases (e.g., *SAP4-6*) (Naglik et al. 2003). Hyphae formation and the expression of virulence factors promote initial adherence to the host tissue followed by invasion (either induced endocytosis or active penetrations) and damage. In turn, host defense against infecting *Candida* is mainly presented by the action of macrophages and neutrophiles (reviewed in Moyes et al. 2014; Wilson and Hube 2014). After phagocytosing the fungal cell, neutrophils expose a variety of factors to block

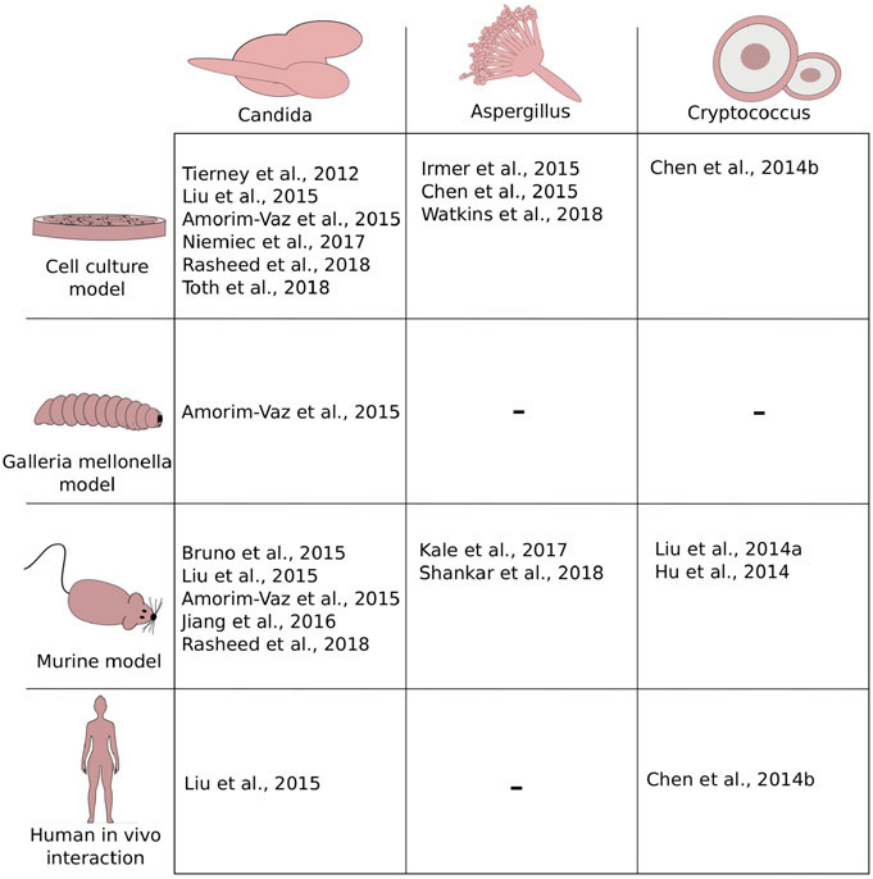

Fig. 3 Host–pathogen interactions transcriptomic studies of *Candida*, *Aspergillus,* and *Cryptococcuss* species in different experimental models

hyphae formation and eventually kill the fungus, including nutrient starvation, production of antimicrobial peptides and enzymes (e.g., defensins, lactoferrin, ellastase), oxidative burst, formation of neutrophil extracellular traps (NETs).

As mentioned above, quantities of yeast cells in an infected patient sample are generally very small, which poses many challenges for the analyses (Rosenbach et al. 2010; Bruno et al. 2015). As a consequence, many previous studies have been performed using animal or tissue culture models, where higher loads of the pathogen can be present and larger quantities of tissue are available. One of the first studies using RNA-Seq to decipher host–pathogen interactions of *C. albicans* was carried out by Tierney et al. (2012). In this study, the authors performed an in vitro timecourse model experiment of interaction between *C. albicans* and *M. musculus* bone marrow-derived dendritic cells (BMDCs) with further RNA sequencing and network analysis to identify and predict interspecific interactions. With the

aforementioned techniques, the authors predicted and subsequently experimentally verified a mechanism by which *C. albicans* escapes host immune response mediated by a reorganization of its cell wall, which in turn is triggered by the release of complement-activating and opsonin protein PTX3 from dendritic cells.

In a more recent study, Bruno et al. (2015) used a murine model of vulvovaginal candidiasis (VVC) coupled with RNA-Seq to study the transcriptome and its alterations in mice and *C. albicans*. This study demonstrated that expression of the NLRP3 inflammasome, which triggers caspases and the maturation of proinflammatory cytokine interleukin 1 beta—the hallmark of VVC immunopathogenesis, was elevated in infected mice. Moreover, *Nlrp3* −/− infected mice showed significantly lowered levels of polymorphonuclear leukocytes (PMNs), alarmins, and inflammatory cytokines. These findings suggested an important role of NLRP3 inflammasome in response to *C. albicans* in VVC. On the other hand, the authors have also attempted to analyze *C. albicans* response to host; however, the in-depth analysis of *C. albicans* transcriptome was precluded by a very low amount of fungal reads obtained from infected vaginal samples (on average ∼80 thousand reads of *C. albicans* compared to ∼103 million mouse-derived reads). Nevertheless, the analysis of highly expressed *C. albicans* genes revealed a robust expression of hypha-associated *SAP 4, 5,* and *6*, while mutants of these genes were inducing significantly lower inflammatory response.

Another comprehensive RNA-Seq-based study of *C. albicans* and host interaction was done by Liu et al. (2015). Here, the authors analyzed host–pathogen interactions in both in vitro and in vivo conditions. In the former case, two human cell cultures were used, namely human endothelial and oral epithelial cell cultures, while in vivo investigation was carried out in both murine model and using real clinical samples from patients. The time series analysis using not only infected samples but also controling on each corresponding time point (non-infected human samples and *C. albicans* cells in the growth media) allowed the researchers to reveal that, surprisingly, *C. albicans* showed a minimal transcriptional response to host cells, which was indicated by a low number of DE genes compared to the control samples. This result points out that most of the *C. albicans* genes involved in host interaction with the studied cell types are also similarly expressed in the growth medium (M199 and DMEM media). Nevertheless, this fact does not lessen the importance of these genes in host–pathogen interaction, but rather once again demonstrates the relevance of careful study design. Thereafter, focusing mainly on the host side the authors demonstrated a distinct transcriptional response of different cell lines to *C. albicans*. To identify molecular pathways governing host response, the authors used network analysis and identified numerous previously reported up-regulated pathways like MAPK1/3, TLR7, EGF, and novel pathways such as PDGF and NEDD9. Further, in-depth wet-lab analysis has shown that the last two pathways play a crucial role in endocytosis of *C. albicans* cells in a cadherin-independent manner in cell cultures. Moreover, it was demonstrated that both pathways are also implicated in pathogen interaction in disseminated mouse infection model, while in case of in vivo human infection NEDD9 was intact. Overall, this study is an excellent example where a combination of carefully

planned RNA-Seq design and thorough bioinformatical and follow-up wet-lab analysis can successfully reveal novel mechanisms of host–fungal interaction.

A recent study by Niemiec et al. (2017) has evaluated the interaction between *C. albicans* and human neutrophils by means of RNA-Seq. The authors assessed the transcriptome of neutrophils exposed to the fungus in either yeast or hyphal morphotypes, as well as the transcriptomic response of those morphotypes to intact neutrophils and NETs. The analysis revealed that the core response of neutrophils is largely similar for the two *C. albicans* morphotypes, with only 11% of DE genes being specific to the interaction with the hyphal morphotype. The core response to fungi included inflammasome induction and release of numerous cytokines, which shows that despite their short life span neutrophils are also orchestrating complex immune response to *C. albicans*. On the other hand, *C. albicans* response was also mainly morphotype-independent, while the reaction to either intact neutrophils or NETs was markedly distinct. Overall, fungal response was primarily dominated by metabolic genes, controlled by the regulators of transcription as Tup1p, Cap1p, Hap43p, with the latter being the major regulator in *C. albicans* of evasion from neutrophils.

As highlighted previously, one of the major limitations of investigating mutual host–fungus interaction (especially in vivo studies) is a very low proportion of fungal cells as compared to host cells (Rosenbach et al. 2010; Bruno et al. 2015). This problem refers not only to RNA-Seq, but to any other high-throughput NGS technique. Subsequent analysis of such kind of a "host-biased" sample generally does not yield enough fungal data for a comprehensive description of the fungal transcriptome. Previous attempts to solve this issue had serious limitations—some of them were altering true gene expression levels (Andes et al. 2005; Thewes et al. 2007), while the others did not provide transcriptome-wide resolution (Geiss et al. 2008). To overcome this issue, Amorim-Vaz et al. (2015) used, for the first time, RNA-Seq coupled with SureSelect targeted RNA enrichment technology. This technology is based on the use of biotinylated oligonucleotide baits directed to target RNA molecules of interest, which are then enriched by probe hybridization and subsequent pool down. Importantly, this enrichment procedure has been shown to not interfere or change the transcriptional profile of the sample. The authors used two animal models of *C. albicans* infection—murine model of kidney infection and a *Galleria mellonella* larvae model—and investigated host–pathogen interplay at early and late stages of infection. Consistent with previous studies, the RNA-Seq analysis of the bulk sample showed that barely 0.1–1% of the reads belonged to the pathogen. However, after applying the enrichment approach, the number of reads aligned to *C. albicans* dramatically increased up to 1670-fold, while biasing the expression levels only for 3% of genes. DGE analysis of *C. albicans* genes showed consistent results with previously published studies including up-regulation of genes involved in cell host adhesion, hypha formation, and iron acquisition. Moreover, the analyses revealed new, previously uncharacterized targets in both *C. albicans* and hosts for further exploration. Overall, this study demonstrated that targeted enrichment can be successfully applied for in vivo host–pathogen studies to describe both counterparts in a transcriptome-wide manner.

3.1.1 Non-albicans *Candida* Species

Other *Candida* species are less frequently reported than *C. albicans* in infection cases, but nevertheless they collectively account for ~50% of the cases. After *C. albicans*, the most widespread species in infections are *C. glabrata*, *C. parapsilosis,* and *C. tropicalis,* generally in this order (Guinea 2014). Despite the importance of these species in fungal infection epidemiology, their virulence mechanisms and host interactions are significantly less studied than those of *C. albicans*. So far, only a handful of studies have been performed for transcriptional profiling of these species in the context of host–pathogen interactions, and none of them was deeply focused on both counterparts.

Candida glabrata is the second most widespread *Candida* species that causes human infections. Phylogenetically, this yeast is much closer to *S. cerevisiae* than to *C. albicans,* and it does not form true hyphae and has high intrinsic resistance to azole class of antifungal drugs (Gabaldón and Carreté 2016). Rasheed et al. (2018) investigated the role of yapsins (*CgYps*)—cell surface-associated aspartile proteases of *C. glabrata*—in the interaction with human THP-1 macrophages and in systemic murine infection. First, to clarify the role of yapsins in fungal homeostasis on gene expression level, the authors performed RNA-Seq of mutant strain *Cgyps1–11*Δ, which lacks all 11 yapsins, and compared it to the wild-type (WT). Downstream analysis uncovered 35 down- and 89 up-regulated genes in the mutant, with enriched GO categories of "ion transport," "oxidation-reduction process" and "sterol import," and "carbohydrate metabolic process," "fungal-type cell wall organization" and "tricarboxylic acid cycle," respectively. Using biochemical staining assays, the authors further demonstrated the altered cell wall composition of the *Cgyps1–11*Δ mutant in β-glucan, chitin, and mannan content, largely caused by the deletion of *CgYps1* and *CgYps7* yapsins. While the application of RNA-Seq was restricted to the above-mentioned analysis in this study, the authors additionally used microarray technology to describe human THP-1 macrophages response to *C. glabrata* WT and the forementioned mutant. Broadly, the microarray profiling showed that THP-1 cell line responds differently to WT and mutant *C. glabrata* strains: In the former case, human DE genes were involved in inflammatory response, chemotaxis, and chemokine-mediated signaling pathways, while in the latter case the cells expressed genes involved in viral response. In addition, the authors elucidated the role of IL-1β in *C. glabrata* interaction, showing that its production is likely to be deleterious for fungal survival in macrophages and that yapsins play a pivotal role in suppressing the production of host's IL-1β.

To clarify the role of yapsins in vivo, BALB/c mice were infected with WT and *Cgyps1–11*Δ mutant. Overall, WT *C. glabrata* colonized and disseminated in numerous mouse organs, while the mutant strain had a significantly lower survival, demonstrating that yapsins are required for colonization and dissemination of the fungus. Finally, to uncover the roles of each of the yapsins in infection and fungal survival, the authors performed murine infection models with different combinations of single, double, and triple mutants of yapsin genes. Altogether, organ-specific survival effects of different yapsins were identified.

Another recent study (Whaley et al. 2018) focused on *C. glabrata* addressed the susceptibility mechanisms of the fungus to fluconazole, identifying the gene which negatively regulates the resistance levels. By screening a large collection of single gene mutants, the authors found that the strain with deleted JJJ1 gene (*GL0J07370g*), increased the minimum inhibitory concentration (MIC) to fluconazole 16-fold as compared to WT. This finding was further supported by deleting this gene in a *C. glabrata* clinical strain. Since the main mechanism of *C. glabrata* resistance to azoles is defined by over-expression of the transcription factor PDR1, which directly activates efflux-pump genes such as *CDR1*, *PDH1,* and *SNQ2*, the authors demonstrated that deletion of *JJJ1* increased the resistance through Pdr1-dependent up-regulation of *CDR1*. To further investigate the effect of *JJJ1* deletion on the overall transcriptome of *C. glabrata*, the authors have performed RNA-Seq using Ion Torrent technology. The analysis identified 119 and 149 up and down-regulated genes, respectively, many of which had been previously identified by microarray analysis.

Candida parapsilosis is a member of CTG clade alongside with *C. albicans* and *C. tropicalis*. It is considered to be the third most frequent opportunistic *Candida* pathogen. As for *C. glabrata*, a restricted number of studies have been performed to clarify host–pathogen mechanisms on transcription level. To our knowledge, the only RNA-Seq-based host–pathogen interaction study with *C. parapsilosis* was performed recently by Toth et al. (2018), focusing mainly on the fungal side. The authors employed a timecourse in vitro infection model of *C. parapsilosis* with human THP-1 monocytes with further RNA-Seq of fungal transcriptome to identify potential molecular targets for future antimycotic agents. RNA-Seq analysis revealed 19 highly up-regulated *C. parapsilosis* genes, which were selected for further investigation. By constructing deletion mutant strains of each of those genes and performing the screening of the mutants for different properties, the authors narrowed the search of virulence factors to three transcriptional regulator genes *CPAR2_100540*, *CPAR2_200390,* and *CPAR2_303700*. Further in-depth analysis demonstrated that these three genes play an important role in nutrient acquisition and alternative carbon source utilization, hyphae and biofilm formation, and sensitivity to low temperatures, respectively.

As for *C. tropicalis*, two studies have been performed that used RNA-Seq to understand yeast–hyphal transition. Wu et al. (2016) performed RNA sequencing of three *C. tropicalis* clinical isolates in yeast and filamentous forms. Differential gene expression analysis showed up-regulation of several genes, including *SAP2*, *SAP3*, *ALS3*, *LIP1*, which have been previously reported to be involved in hyphal transition in *C. albicans*.

Jiang et al. (2016) have studied 52 clinical isolates of *C. tropicalis* by estimating different parameters of pathogenicity, such as biofilm formation, hyphal morphology, and hemolytic activities. Based on the ability to form hyphae, two groups of strains (three highly and three lowly hyphae-producing strains) were further selected for performing in vivo murine infection model with subsequent RNA sequencing of *C. tropicalis*. RNA-Seq analysis between two groups has shown 206 DE genes in highly hyphae-producing strains, enriched in aspartic-type

endopeptidase activity, metal homeostasis, and oxidative response. On the other hand, several uncharacterized DE genes were revealed, which might also have an impact on *C. tropicalis* pathogenicity.

3.2 Aspergillus

Aspergillus is a genus within the *Ascomycota* phylum that comprises over 300 species (Samson et al. 2014). *Aspergillus* species have a high and diverse economic and social impact, since they massively spoil food products (Dijksterhuis et al. 2013), serve for various biotechnology productions (Pel et al. 2007), and some are human pathogens (Kwon-Chung and Sugui 2013). From the latter perspective, the most frequent human pathogen is the soil-associated fungus *Aspergillus fumigatus,* accounting for 90% of *Aspergillus*-caused infections, which as in the case of *Candida* affects mainly immunocompromised individuals (Perfect et al. 2001; Paterson and Lima 2017). *A. fumigatus* produces hydrophobic microscopic spores known as conidia, which are ubiquitous in the environment and are the main cause of infections (Latgé 1999). After being inhaled by immunocompromised individuals, conidia can reach pulmonary alveoli and start germinating, forming hyphae and mycelia. This causes a wide range of nosologies collectively called aspergillosis, with invasive forms reaching 50–95% mortality rates (Abad et al. 2010).

Numerous studies involving transcriptome profiling have been performed to study pathogenic mechanisms of *A. fumigatus*. Most of them have been carried out in vitro by exposing the fungus to different environments resembling the interaction with the host or to different stresses (Losada et al. 2014; O'Keeffe et al. 2014; Gibbons et al. 2012; Wang et al. 2015b). However, only few recent studies addressed host–pathogen interactions based on RNA-Seq of more realistic in vivo models focusing either on host, pathogen, or both simultaneously.

Using RNA-Seq, Irmer et al. (2015) investigated the response of *A. fumigatus* exposed to human blood in vitro, mimicking the host environment encountered by the fungus when it germinates and penetrates to blood vessels. The authors took samples at two time points of 30 and 180 min after incubating with either human blood or with minimal medium, as a control. The experiment was performed in duplicate, and all samples were compared to pre-cultured *A. fumigatus* mycelia. Differential gene expression analysis between pre-cultured fungus and blood-exposed samples revealed 410 up-regulated and 367 down-regulated genes after 30 min of exposure to blood, and 266 up-regulated and 318 down-regulated genes after 180 min. Those numbers of genes were obtained after subtracting the DE genes from the comparison between control samples and fungi grown on minimal media. After differential expression analysis, the authors performed comprehensive GO enrichment analysis. Briefly, four categories of genes were analyzed—early up-regulated genes, early up-regulated and then down-regulated genes, solely late down-regulated genes and late up-regulated genes. Functional

analysis of early up-regulated genes showed enrichment in metabolism, cell-rescue, transport, virulence- and protein synthesis-related genes. Genes that were up-regulated after 30 min but down-regulated after 180 min were largely similar to the ones only up-regulated at 30 min or only down-regulated after 180 min, and thus, their functional enrichments were also similar. After 180 min, enrichment categories remained largely the same compared to early up-regulation, but, obviously, were depressed, indicating slow-down of overall fungal metabolism. A modest number of enriched categories was found in late up-regulated genes, including iron starvation, detoxification, and stress. Overall, the gene expression patterns and functional analysis suggested that human blood is not a hostile environment for *A. fumigatus*, which first senses the environment and then shuts down several important pathways of energy-consuming metabolism after the first hours and thus can not effectively grow in blood.

Kale et al. (2017) performed a time series dual RNA-Seq analysis of two immunosuppressed mice models (one treated with chemotherapy and another one with corticosteroid) challenged with a particular *A. fumigatus* strain (Cea10) to assess how do host-pathogen interactions vary between two distinct immunocompromised states in pulmonary aspergillosis. Dual RNA-Seq yielded 16-29 mln reads, depending on the sample, from which 98% mapped to the mouse genome and the rest to the fungal counterpart. Further differential expression analysis of the host side revealed that the two immunocompromised models showed distinct patterns of gene expression response to the pathogen, showing that host response to *A. fumigatus* depends on the type of immunosuppression. Functional enrichment analysis of DE genes showed enrichment in numerous immunological processes related to cytokines, chemokines, and their receptors in the chemotherapeutic model, whereas for the corticosteroid model a limited number of cytokine-related genes were DE. More highlighted differences in functionally enriched categories between the two models were found with regard to metabolic processes—the chemotherapeutic model was enriched with urea cycle, pentose phosphate metabolism, nucleotide and vitamin metabolism, while corticosteroid-treated model in inositol phosphate metabolism, fatty acid oxidation, terpenoid biosynthesis, thiamine biosynthesis, etc. Additionally, the authors identified novel genes from pathogen-sensing gene families of *Tlrs*, *Clecs*, and *Nlrs,* which had not been previously described in pulmonary aspergillosis.

On the fungal side, the comparison of gene expression profiles of *A. fumigatus* in different mice models has shown a large proportion of similar DE genes (n = 3345), with a restricted number of model- and time-point-specific DE genes (n = 128–204). Nevertheless, the analysis of fungal secreted proteins, which are important for fungal pathogenesis, showed that *A. fumigatus* has a temporal and model-specific activation of these proteins. However, it has to be noted that the analysis of a large proportion of fungal genes (5175, $\sim$60% of total genes) was precluded due to very low expression values, which once again demonstrates the problem of low concentration of fungal genetic material for in vivo dual RNA-Seq experiments.

Previously, it has been demonstrated that virulence varies among different *A. fumigatus* strains (Fuller et al. 2016; Kowalski et al. 2016) and moreover that the

host immune response against different strains is also distinct (Rizzetto et al. 2013). Thus, opposite to Kale et al., Watkins et al. (Watkins et al. 2018) have recently made RNA-Seq gene expression profiling of two *A. fumigatus* strains *Cea10* and *Af293* interacting with human airways cell line A549 to elucidate the differences and commonalities between the virulence mechanisms of aforementioned strains. The experiment comprised two time points (6- and 16-h post-infection) of infected human cells with two fungal strains and time-matched controls for fungal samples (i.e., fungi without host cells). The study focused only on the fungal side, thus the authors did not perform controls for the host counterpart. Since the ratio of fungal and human cells in the infection models was close to 1:1, the RNA-Seq successfully recovered enough amount of fungal reads (53±29.3 million reads per sample) for robust downstream analysis. Differential expression analysis revealed 7888 genes across conditions, and PCA and hierarchical clustering with those genes showed that samples clustered largely according to time points and by strain (on 16-h time point) and that controls were positioned close to infected samples. Taken together, the patterns of sample distribution and clustering highlighted that changes of fungal transcriptional profiles are largely due to growth and metabolism dynamics, rather than to a response to the lung epithelial cells. To dissect the genes that are specifically involved in the infection process, the authors compared transcriptional profiles of time-matched infection and control samples. In this case, a modest response to human cells was found (n = 128–619 DE genes) with 70% of them being strain-specific, indicating the virtual lack of strong conservative response of *A. fumigatus* strains at least in the analyzed conditions. Nevertheless, a small proportion of genes (n = 47) that were similarly expressed in both strains was found, and the mutants of seven of these genes were shown to have attenuated virulence.

Chen et al. (2015) investigated the interactions of A549 epithelial cell line with *A. fumigatus*, but unlike in Watkins et al., this study focused on the host side. Here, the authors infected cell cultures with *A. fumigatus B5233* for 8 h and performed fungal-free control at similar conditions, with further RNA isolation and sequencing of the host cells. Differential expression analysis between infected and intact cells revealed in total 302 up- and 157 down-regulated genes. GO enrichment analysis showed that down-regulated genes were enriched in ion transport, skeletal system developments, and vascular development. On the other hand, similar to other studies, up-regulated genes were functionally enriched in numerous immune-associated processes such as chemotaxis, inflammatory response, response to bacterium, and also in cytoskeleton remodeling, which also has been reported earlier (Jia et al. 2014). To further investigate the role of specific host genes in fungal response, the authors chose two genes—*ARC* and *EGR1*—involved in cytoskeleton rearrangements, since it is known that *A. fumigatus* conidia are able to internalize in the host cells. Western blotting indicated that corresponding proteins of that genes were up-regulated during the course of infection. Moreover, inhibition of expression of *ARC* and *EGR1* genes by RNAi decreased the internalization rates of conidia by 20 and 40%, respectively.

Another in vivo murine infection model of host–aspergillus interactions was made by Shankar et al. (2018). Here, unlike in the above-mentioned study by Kale et al. (2017), the authors investigated invasive aspergillosis in immunocompetent mice and focused on kidney infection. The study comprised a timecourse infection model at five different time points up to eight days after infection. At all time points and for control animals, infected kidneys were homogenized and subjected to RNA-Seq. Initially, the authors aimed for resolving host–pathogen interaction of both counterparts; however, as usual for in vivo studies, fungal side did not yield interpretable amount of data. Thus, further analysis was focused only on the host transcriptome. Overall, differential expression analysis revealed more than 14,000 DE genes throughout the course of infection in mice. Although notable up-regulation was observable from the first day after infection, functional enrichment was only observed after five days post-infection. Enriched terms included leukocyte aggregation, acute inflammatory responses, positive regulation of chemokines, and other immune response processes. A more in-depth investigation of up-regulated genes showed the activation of several genes such as *Ccr5*, *Cxcr3*, *Ccr2*, or *Cxcr4*, which are directly involved in activation and recruitment of Th-1 and Th-17 T-helper cells. In turn, after activation of Th cells, the up-regulation of different proinflammatory cytokines such as *IFN-c*, *IL-27*, *IL-18*, *IL-24* was detected. Conversely, down-regulated genes were associated with iron and heme binding, electron carrier activity, and aromatase activity. However, in the case of iron-regulation associated genes, the pattern of down-regulation was explained by suppression of P450 related genes, since many other key components of iron homeostasis like *Nos1*, *Nos2*, *Ltf* were systematically up-regulated.

3.3 Cryptococcus

Unlike *Candida* and *Aspergillus*, the *Cryptococcus* genus belongs to the phylum *Basidiomycota* (http://www.asmscience.org/content/book/10.1128/9781555816858.ch01). There are two main pathogenic *Cryptococcus* species, *Cr. neoformans* and *Cr. gattii,* which are environmental non-host-specific pathogens infecting a wide range of hosts including insects, plants, and mammals. In the case of humans, *Cr. neoformans* is mainly an opportunistic pathogen, while *Cr. gatii* can infect immunocompetent individuals [reviewed in (Kwon-Chung et al. 2015)]. In recent decades, the incidence on cryptococcosis has increased drastically which is mainly associated with an emergence of HIV and increasing numbers of organ transplant recipients. The main types cryptococcal infections are cutaneous cryptococcosis, pulmonary cryptococcosis, and cryptococcal meningitis, with the latter being fatal if not treated on initial stages. In the developing part of the world, it has been estimated that these two species cause around one million infections with mortality rates reaching 70% and causing 650,000 deaths per year (Brown et al. 2012; Park et al. 2009). As it is the case of *Aspergillus* conidia, cryptococcal spores

or dried yeast cells enter the host organism through inhalation or through direct interaction in the case of skin-related infections.

As for *Candida* and *Aspergillus*, the pathogenicity mechanisms of *Cryptococcus* species have been more extensively studied in vitro by exposing them to different environmental conditions (O'Meara et al. 2013; Brandão et al. 2018; Zhang et al. 2014). However, in vivo or ex vivo transcriptomic studies of *Cryptococcus*-host interaction are limited to several recent studies. Moreover, some studies using RNA-Seq were performed for refining the genome and transcriptome annotations of *Cryptococcus* species (Janbon et al. 2014; Gonzalez-Hilarion et al. 2016; Ferrareze et al. 2017), which are not covered by our review.

The first study investigating transcriptome of *Cr. neoformans* cells interacting with the host environment was carried out in 2014 by Chen et al. (2014b). The authors performed RNA-Seq of two *Cr. neoformans var. grubii* strains, G0 and HC1, taken directly from the cerebrospinal fluid (CSF) of two patients with cryptococcal meningitis. Additionally, the same fungal cells were grown in two conditions—ex vivo CSF and YPD, followed by RNA sequencing and comparison with in vivo obtained fungi. Initial analysis showed that gene expression profiles of both strains in each condition were very similar; thus, the strains at the given conditions were considered as biological replicates. Differential gene expression analysis between pairs of conditions identified 129, 45, 256 DE genes when comparing ex vivo versus YPD, in vivo versus YPD, and in vivo versus ex vivo, respectively. This shows that transcriptomes from in vivo and YPD samples are more similar than in ex vivo CSF samples. Compared to ex vivo cells, in vivo samples were enriched with cellular biosynthetic GO terms, indicating that *Cr. neoformans* cells within the host are transcriptionally more active, which might be explained by interaction with the immune system. On the other hand, as expected, samples exposed to CSF (in both cases) comparing with those with YPD had multiple DE genes previously reported to be important for *Cr. neoformans* virulence, such as *CFO1* (Jung et al. 2009), *ENA1* (Idnurm et al. 2009), and *RIM101* (O'Meara et al. 2010). The authors also identified 100 strain-specific differentially expressed genes, which were enriched in transporter genes. Additionally, the high sequencing depth allowed the authors to perform variant calling of sequenced strains and compare their genotypes with the reference. Variant calling showed a substantial genomic variation between the analyzed strains and the reference genome—50,155 and 156,880 SNVs were identified in G0 and HC1, respectively, which demonstrates that diverse *Cr. neoformans* strains have largely similar transcriptomic responses to the host environment. Taking advantage of the high depth and quality of the sequencing data, the authors performed de novo assembly of the transcriptomes of strains identifying novel genes related to transport, localization, and membrane constitution. Taken together, this work was the first study addressing the question of virulence mechanisms of phylogenetically diverse strains of *Cr. neoformans* obtained directly from host using RNA-Seq and thorough methods of bioinformatics data analysis.

In another study Liu et al. (2014a) compared the transcriptomic profiles of brain tissues in mice, infected by WT *Cr. neoformans* and double knock-out mutant for

the genes of inositol transporters Itr1a and Itr3c, which were previously shown to be involved in fungal virulence through their role in uptaking inositol from the host. Gene expression profiles obtained by RNA-Seq were generated for control mice and were compared in a pairwise manner with those from mice infected by the two fungal strains. Differential expression analysis identified 1133 up- and 1600 down-regulated genes in WT-infected mice, while *itr1aΔ itr3cΔ* mutant strain showed altered expression of 552 up-regulated and 278 down-regulated genes. Three hundred and seventy-one genes were shared between mice infected by each of the two strains. GO enrichment analysis showed that many enriched functional terms are shared across the two different infections, including cellular death and survival, cell-to-cell interaction, involvement in neurological disease. However, mice infected by the mutant strain extensively activated immune-related responses, such as inflammation, humoral immune response, free radical scavenging. In stark contrast, none of the immune response pathways was significantly enriched in WT-infected mice. Moreover, terms related to cell death and necrosis were enriched only in WT-infected mice. To assess changes in the pathogen that were resulting in different host responses, the authors measured the size of fungal capsule and the secretion of glucuronoxylomannan (GXM)—two important factors for *Cr. neoformans* virulence. While the capsule size was similar in the two strains, the secretion of GXM was significantly reduced in the *itr1aΔ itr3cΔ* mutant. This result was also confirmed by immunohistologic staining of GXM in mouse brain tissue, showing that animals affected with mutant had less GXM around brain lessons. Overall, this study demonstrated the role of inositol transporters in host–pathogen interactions, linking their function with the secretion of GXM and altered composition of the fungal capsule, which in turn elicits a highlighted host immune response.

Hu et al. (2014) investigated the ability of environmental *Cryptococcus neoformans* strains to undergo microevolutionary changes promoting the increase of virulence during serial host passages. The authors used nine haploid serotype A *Cr. neoformans* strains isolated mainly from soil. Each strain was inoculated into mice sequentially four times over four months. Each following passage to a new mouse was performed using fungal colonies isolated from brains of the precedent mouse. Two strains were revealed with prominent increase of virulence, which in both cases reduced the time of mice death of the first and the last passages by 4-fold ($\sim$25 h in the first infected mouse and $\sim$ 6 h in the fourth infected mouse). To disentangle the transcriptomic changes of highly adapted strains compares to their environmental predecessors, the authors performed RNA-Seq of aforementioned two strains and one control strain that did not show virulence changes across the passages. RNA-Seq analysis revealed four genes with significantly higher expression in evolved strains compared to predecessors. One of them, *Fre3* (*CNAG_06524*), was shared between two species. Using RNA interference, the authors identified that *Fre3* functions as an iron reductase without copper reductase activity. To confirm the role of the gene in pathogenicity, they over-expressed *Fre3* in WT background, which recapitulated the increased adaptive virulence

phenotype. Overall, the study shows how RNA-Seq can be used to address the important process of environment-to-mammal transition of *Cr. neoformans*, identifying the role of iron reductase *Fre3* in the adaptation to the host.

4 Emerging Technologies in RNA-Seq

4.1 Single-Cell RNA-Seq

The term RNA-Seq is generally referred to sequencing of RNA, which was isolated from the population of cells (bulk RNA-Seq). Thus, the results obtained from bulk RNA-Seq constitute an averaged signal from the sum of individual cells, while each cell (or a sub-population of cells) might have its own transcriptomic patterns. The limitation of sequencing the bulk RNA was overcome by two major technological advances: efficient cell sorting with single-cell isolation and the availability of efficient protocols for the amplification of minute amounts of RNA from these single cells (Kolodziejczyk et al. 2015). Today, these two methods and their derivatives allow performing single-cell RNA sequencing (scRNA-Seq), disentangling transcriptional profiles of thousands (even hundred of thousands) individual cells (Fan et al. 2015; Zheng et al. 2017; Rosenberg et al. 2018). Despite challenges related to cost, technology, and data analysis (Weinreb et al. 2018; Kolodziejczyk et al. 2015; Stegle et al.2015), scRNA-Seq is now one of the most precise methods in transcriptomics studies. However, as compared to studies on mammals, it has not been used much to study microbial cells (Rosenthal et al. 2017; Kolisko et al. 2014; Wang et al. 2015a) and host-pathogen interaction studies (Avraham et al. 2015; Saliba et al. 2016). One recent advancement in this field was reported in Avital et al. 2017, where the authors developed a method for single-cell dual RNA-Seq for mouse macrophages and *Salmonella typhimurium* cells during infection, revealing three distinct stages of macrophage response to the pathogen. On the other hand, to our knowledge there are no studies addressing human–fungal interaction on the single-cell level. This technology holds a great potential to unravel specific expression patterns governing the switches between fungal morphotypes, quorum sensing, switches from commensalism to pathogenicity and switches between the stages of infection. Moreover, single-cell transcriptomics approaches can decipher how the host senses and reacts to the pathogen at different infection stages and deconvolve the expression patterns of different cell types, especially in context of in vivo studies.

4.2 Long-Read Sequencing

Today, the dominating sequencing technology "sequencing-by-synthesis" of Illumina, also known as second-generation sequencing, generates relatively short

reads (25–300 bp) with a very high throughput and high accuracy. Despite the great advantage of the last two features, short reads are often problematic in some specific tasks, such as assembly of complex and repetitive genomes or accurate reconstruction of transcript isoforms. To overcome this problem, Illumina has recently implemented so-called TruSeq synthetic long-read technology, previously known as Moleculo (McCoy et al. 2014). This experimental and data analysis approach splits the molecule into smaller pieces and uses barcodes to tag the adjacent sequences. Further sequencing and bioinformatics data analysis reassembles the initial sequence, thus allowing to obtain synthetic long reads.

Moreover, in the last decade, the advent of third-generation sequencing has opened new avenues in biomedical research, allowing to sequence much longer reads [up to several hundred kbs (Jain et al. 2018)]. Moreover, their single molecule sequencing technology is PCR free, which eliminates potential PCR amplification biases. However, today long-read sequencing comes with two major disadvantages, which are low throughput and high error rates as compared to sequencing-by-synthesis technology. The two major technologies for long-read sequencing are operated by Oxford Nanopore (ON) and Pacific Biosciences. The details of each technology are reviewed in (Lu et al. 2016) and (Rhoads and Au 2015), respectively. Although long-read sequencing was initially used in genomics field to assemble more contiguous and resolved genomes, today the technologies have also been validated in transcriptomics applications, mainly in transcript discovery (Sharon et al. 2013; Chen et al. 2017; Garalde et al. 2018) and at lesser extent in gene/transcript expression profiling (Byrne et al. 2017). To fill the gap of low throughput of long-read technologies and thus allow reliable expression evaluation, so-called hybrid sequencing can be used, which utilizes both short and long-read sequencing data (Ning et al. 2017; Wang et al. 2018). The third-generation sequencing has already advanced our knowledge about the transcriptomes of even well-studied organisms, identifying numerous previously uncharacterized transcripts and splicing events (Chen et al. 2014a; Byrne et al. 2017; Chen et al. 2017; Sharon et al. 2013; Au et al. 2013). Moreover, it already has been reported that ON can be effectively used in microbial diagnostics (Quick et al. 2015; Mitsuhashi et al. 2017; Schmidt et al. 2017), providing the potential of identifying the pathogen in 2–4 h.

Overall, long-read sequencing technology can dramatically further our knowledge of transcriptomes of poorly studied organisms, as is the case of most of human fungal pathogens. In this case, novel-species-specific transcripts can become promising biomarkers for fungal diagnostics and discovery. On the other hand, when applied to host–pathogen interaction studies, it might allow the precise reconstruction of novel pathogen-specific transcripts, like lncRNAs, in the host side, which have been already shown as immune response regulators (Heward and Lindsay 2014; Ouyang et al. 2016; Jiang et al. 2018).

Nevertheless, the third-generation sequencing is still in its infancy, and further improvements and validations of the technology are necessary in order to make it more versatile and popular in biomedical research.

4.3 Potential Applications of RNA-Seq in Fungal Diagnostics

Next-generation sequencing methods have become increasingly popular in the clinics, especially in the context of diagnosis of cancers (Luthra et al. 2015) and Mendelian diseases (Jamuar and Tan 2015). Moreover, today these techniques have already penetrated to microbiology labs, allowing to achieve high precision of microorganism detection (Turabelidze et al. 2013; Shaw et al. 2016), identify drug resistance (Stoesser et al. 2013; Wain and Mavrogiorgou 2013), control outbreaks (Reuter et al. 2013; Sherry et al. 2013), and study microorganisms that are difficult to grow using conventional culturing methods (Berenguer et al. 1993). However, despite the fact that the incidence of fungal infection is steadily increasing, so far the efforts in applying NGS in microbial diagnostics have been mainly focused on bacteria and viruses. Fungal pathogens possess features that make them difficult for management under the paradigm of traditional microbiology diagnostic methods, such as rapid emergence of antimycotic drug resistance, emergence of new pathogenic species, high biodiversity. Thus, the necessity of novel analytical tools such as NGS in fungal diagnostics becomes inevitable. On the other hand, a distinction of different NGS tools in their applicability for diagnostic purposes has to be done. While DNA sequencing plays a major role for species detection, identification, and characterization, RNA-Seq holds a great potential in identifying biomarkers (in form of novel transcripts) and gene/transcript expression-level signatures specific to different species or for different stages of infection. Nevertheless, to achieve this kind of diagnostics, additional research efforts have to be performed. Precisely, here is where the emerging technologies can immensely further the potential for RNA-Seq diagnostics. For instance, inherent problems such as the low amount of fungal RNA in patient samples can be effectively solved using probe enrichment, while the further identification of novel transcripts is addressable by long-read or hybrid sequencing. Moreover, single-cell RNA-Seq approach could be applied to decipher transcriptomic differences between cell populations, increasing the potential resolution of diagnostics. On the other hand, prices and turn-around time of these technologies are yet to achieve levels that make them suitable for the clinical settings. However, with current trends of diminishing prices, smaller and easier to handle machines, and faster turn-around times. the future of RNAseq-based diagnostics may be approaching. Taken together, RNA-Seq and related methods open promising avenues for fungal diagnostics, but nevertheless still a considerable research and technical developments have to be carried out to truly uncover this potential.

5 Concluding Remarks

In the recent decade, the advent of transcriptome sequencing technologies has opened exciting possibilities for exploring gene regulation and how it varies in different contexts at a level of detail and throughput that surpasses the most optimistic expectations of the previous decade. Many biological disciplines are taking advantage of this new era in transcriptomics, and host-pathogen interaction studies are no exception. As a result, our knowledge about the molecular mechanisms of the interplay between various microbes and their hosts has greatly advanced in this time frame. While the application of RNA-Seq for unraveling human–fungal interactions is just gaining momentum, it is already clear that the use of this technology and its derivatives will be the main trajectory in the field for the coming years. Despite its versatility, today RNA-Seq faces several natural and technical barriers, specifically in human–fungal interaction studies. While in vivo studies are complicated by extremely low amount of pathogen cells, infection models do not entirely reconstitute the whole complexity and peculiarities of human–fungal interactions. On the other hand, RNA-Seq is still relatively expensive and requires specific expertise in study planning, bioinformatics data analysis, and interpretation of results. Moreover, current mainstream technologies are limited in several technical aspects. Nevertheless, the technological advancements in the field are occurring at a fast pace, and they are already partially overcoming most of the aforementioned limitations. We anticipate that dual host–pathogen RNA-Seq analyses in both in vivo models and patients will multiply in the coming years, as current limitations are overcome, and will constitute the basis of key advancements in our understanding of host–pathogen interactions during commensalism and infection. Finally, although there are still many technical and practical impediments for the use of RNA-Seq for diagnostic purposes, we foresee a great potential that may be realized as key biomarker genes of the process of infection are discovered and technical developments enable bringing fast, accurate, and affordable RNA-Seq-based technologies to the clinics.

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Host Genetic Signatures of Susceptibility to Fungal Disease

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Abstract Our relative inability to predict the development of fungal disease and its clinical outcome raises fundamental questions about its actual pathogenesis. Several clinical risk factors are described to predispose to fungal disease, particularly in immunocompromised and severely ill patients. However, these alone do not entirely explain why, under comparable clinical conditions, only some patients develop infection. Recent clinical and epidemiological studies have reported an expanding number of monogenic defects and common polymorphisms associated

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with fungal disease. By directly implicating genetic variation in the functional regulation of immune mediators and interacting pathways, these studies have provided critical insights into the human immunobiology of fungal disease. Most of the common genetic defects reported were described or suggested to impair fungal recognition by the innate immune system. Here, we review common genetic variation in pattern recognition receptors and its impact on the immune response against the two major fungal pathogens *Candida albicans* and *Aspergillus fumigatus*. In addition, we discuss potential strategies and opportunities for the clinical translation of genetic information in the field of medical mycology. These approaches are expected to transfigure current clinical practice by unleashing an unprecedented ability to personalize prophylaxis, therapy and monitoring for fungal disease.

1 Introduction: Genetic Regulation of the Host-Fungus Interaction

An increasing number of fungal diseases has been documented over the past two decades. Although we are constantly exposed to fungi, only a few species can cause disease in healthy individuals. Opportunistic and otherwise commensal fungi can instead trigger life-threatening infections in individuals with acquired or treatment-induced immunodeficiencies, such as patients receiving hematopoietic stem-cell (HSCT) or solid organ (SOT) transplantation, or undergoing anticancer therapy (Pfaller and Diekema 2010). Many species of fungi are responsible for invasive infections, although more than 90% of all reported fungal-related deaths result from infection with species belonging to one of four genera: *Candida*, *Aspergillus*, *Cryptococcus* and *Pneumocystis*. These diseases are estimated to affect more than 2 million people annually and, in some cases, can have mortality rates that exceed 50% (Brown et al. 2012).

Despite several clinical risk factors typically associated with fungal disease have been disclosed, the actual mechanisms underlying infection in the human host remain largely undefined. Like many infectious diseases, fungal infections are characterized by significant interindividual variability in their development, progression and resolution. While a significant contribution to infection might be credited to virulence traits and the ability of fungi to adapt to the human host, recent evidence has highlighted a dominant role of heritable factors (Cunha et al. 2013; Smeekens et al. 2013b; Wojtowicz and Bochud 2014). Our current understanding of the genetic basis of fungal disease has stemmed from the study of individuals with rare monogenic defects and from cohort-based studies to identify common polymorphisms associated with disease (Lionakis and Levitz 2017). In addition, mouse studies illustrating disparities in susceptibility to experimental infection between inbred strains have also strengthened the concept of genetic control of susceptibility to fungal disease (Durrant et al. 2011; Radovanovic et al. 2011).

Clinical and basic research during the last decade has provided exciting new insights into the molecular and cellular players involved in the host-fungus interaction and antifungal host defense (Netea et al. 2015; van de Veerdonk et al. 2017). The improved understanding of the genetic mechanisms that regulate host immunity to fungi represents an opportunity for the development of new and more effective approaches to preventing and treating fungal diseases. In addition, future studies addressing the genetic architecture of both the host and the fungus and how it regulates the outcome of their interaction are expected to further support clinical translation and personalized medical interventions in fungal diseases (Oliveira-Coelho et al. 2015; Rello et al. 2018). In this review, we explore recent advances in the immunogenetics of fungal disease and how it modulates innate immunity to the two major fungal pathogens *Candida albicans* and *Aspergillus fumigatus*. Also discussed is how an improved understanding of the genetic regulation of the host-fungus interaction is expected to reform the clinical management of fungal disease by paving the way toward precision medical interventions based on individual host genetics.

2 Pattern Recognition Receptors in Fungal Immunity

Our understanding of innate immunity was revolutionized in the early 90s with the groundbreaking concept of selective recognition of conserved pathogen-associated molecular patterns (PAMPs) by germline-encoded pattern recognition receptors (PRRs) (Takeuchi and Akira 2010). Although there are remarkable differences in the way different classes of pathogens are perceived by the immune system, the first step in developing a proper innate immune response is widely acknowledged to require fungal sensing by PRRs (Fig. 1). Owing to its inherent dynamic composition and variable cellular localization of the different constitutive components during the interaction with the host, the cell wall is considered the most abundant source of fungal PAMPs, such as β-1,3-glucans and mannans (Gow and Hube 2012; Latge et al. 2017).

The main families of PRRs include Toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) (Netea et al. 2012). Following the recognition of their cognate ligands, PRRs induce mechanisms responsible for pathogen clearance, including the secretion of cytokines and chemokines, phagocytosis and production of reactive oxygen species (ROS), and orchestrate complex immunoregulatory processes resulting in the activation of adaptive immunity (Patin et al. 2018). The proficiency of fungal recognition and interaction with the membrane-associated PRRs also relies to a large extent on the opsonization by different families of soluble pattern recognition molecules, including collectins, pentraxins, ficolins and components of the complement pathway (Bidula and Schelenz 2016). Of note, studies have highlighted that these molecules may be exploited as a possible alternative or adjuvants to

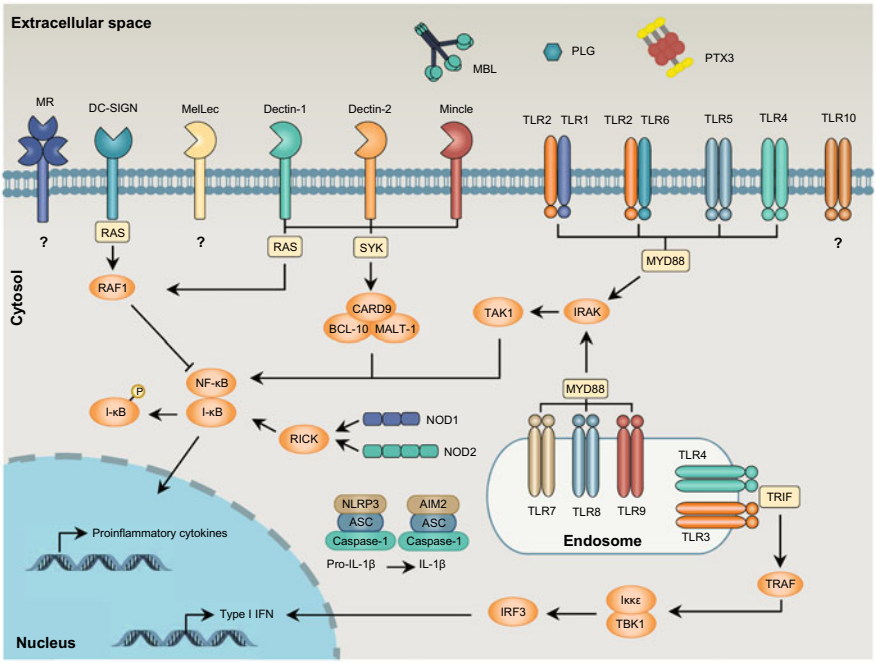

Fig. 1 Overview of PRRs and downstream signaling pathways. PRRs are expressed on the cell surface or are present in endosomes or the cytosol, where they detect PAMPs and activate downstream signaling pathways leading to the transcription of cytokines and chemokines, whereas soluble PRRs bind and opsonize fungi, facilitating their elimination. Upon stimulation, TLRs activate two disparate pathways that involve myeloid differentiation primary response protein 88 (MYD88) and/or TIR domain-containing adaptor protein inducing IFN-β (TRIF). Crosstalk between TLR signaling cascades underlies the activation of different cellular processes, including the transcription of proinflammatory cytokines and chemokines, and type I IFN. On the other hand, CLRs trigger intracellular signaling pathways typically linked to the activation of the SYK kinase and CARD9, resulting in a cascade of downstream signaling and cellular activation that regulate and fine-tune NF-κB activation and cytokine gene expression. The stimulation of NLRs also regulates NF-κB activation via receptor-interacting protein-like interacting caspase-like apoptosis regulatory protein kinase (RICK), whereas the NLRP3 and AIM2 inflammasomes process bioactive proinflammatory cytokines, such as IL-1β

currently available antifungal therapy to increase their efficacy (Lo Giudice et al. 2012; Marra et al. 2014). Besides pathogen-derived molecules, PRRs are also critically important in responding to products released from damaged host cells during infection, including nucleic acids, alarmins and metabolic products, collectively termed damage-associated molecular patterns (DAMPs) (Cunha et al. 2012). Regardless of the PRRs or soluble molecules implicated in fungal recognition or opsonization, the activation of antifungal immune responses requires a coordinated regulation of the function and cellular localization of individual or cooperating receptors.

The role of TLRs in innate immunity was initially proposed following the observation that *Drosophila* lacking the hematocyte receptor Toll were extremely susceptible to infection with fungi and Gram-negative bacteria (Lemaitre et al. 1996). This discovery prompted the identification of mammalian TLRs shortly thereafter (Rock et al. 1998). Similar to other PRRs, TLRs are primarily expressed in cells of the immune system, including monocytes, neutrophils, basophils, eosinophils and natural killer cells, but also in cells of epithelial, endothelial and stromal origin (Netea et al. 2012). TLRs are characterized by containing extracellular domains with leucine-rich repeats that interact directly with PAMPs and cytoplasmic domains highly homologous to the sequences in the interleukin (IL)-1 and IL-18 receptors. After binding to their specific ligands, TLRs activate signaling cascades that promote the translocation of transcription factors to the nucleus, where they induce transcription of genes involved in inflammatory responses and mechanisms of pathogen clearance (Takeuchi and Akira 2010).

The large family of CLRs includes members such as dectin-1, dectin-2, mannose receptor, dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), macrophage inducible C-type lectin (Mincle), macrophage C-type lectin (MCL), and the recently identified MelLec (Brown et al. 2018). These receptors have carbohydrate recognition domains and bind microbial polysaccharides and, for this reason, they have been widely implicated in the activation and regulation of antifungal immune responses (Salazar and Brown 2018). Among CLRs, dectin-1 was the first to be identified and is currently the best described receptor able to orchestrate the activation of adaptive immune responses to fungi (Dambuza and Brown 2015). Dectin-1 recognizes β-glucans (Brown and Gordon 2001) and triggers intracellular signaling pathways that, synergistically and through cross-regulatory mechanisms, culminate in the activation of nuclear factor (NF)-κB and cytokine gene expression (Geijtenbeek and Gringhuis 2009). Although most of the CLR family members have been implicated in antifungal immunity in one way or another, the fungal ligands for most of them remain elusive. One exception was recently provided by the identification of MelLec as a specific receptor for 1,8-dihydroxynaphthalene (DHN)-melanin, a process required for eliciting protective immunity to *A. fumigatus* (Stappers et al. 2018). We also refer the reader to several excellent reviews on the biology of CLRs (Brown et al. 2018; Dambuza and Brown 2015; Salazar and Brown 2018).

Besides the mainly membrane-bound TLRs and CLRs, cytoplasmic PRRs are activated by pathogens following cell invasion or release of PAMPs into the cytoplasm. Although the fungal ligands that are recognized by NLRs have not been identified, their activation during infection results in the assembly of multimeric protein complexes named inflammasomes that are primarily responsible for converting inactive pro-IL-1β and pro-IL-18 into bioactive cytokines (van de Veerdonk et al. 2015). The NLR family pyrin domain containing 3 (NLRP3) and the NLR family CARD domain containing 4 (NLRC4) inflammasomes were specifically credited with a prominent role in the immune response to *C. albicans* (Borghi et al. 2015; Cheng et al. 2011; Hise et al. 2009; Tomalka et al. 2011). The NLRP3 and AIM2 inflammasomes were instead shown to form a dual cytoplasmic surveillance

system that orchestrates protective immune responses against *A. fumigatus* (Karki et al. 2015). The contribution of NLRs to the immune response against fungi nonetheless extends beyond inflammasome formation. Fungal chitin has been demonstrated to dampen inflammatory responses to *C. albicans* by inducing the immunoregulatory cytokine IL-10 via the activation of NOD-containing receptor 2 (NOD2) and TLR9 (Wagener et al. 2014). More recently, NOD1 was found to play an inhibitory role in the host defense against *A. fumigatus* by suppressing dectin-1 expression and cytokine responses responsible for optimal fungal killing (Gresnigt et al. 2017). Taken together, these observations support the existence of highly dynamic regulatory mechanisms modulated by intracellular fungal recognition with important consequences to the overall antifungal immune response.

3 Genetic Defects in Pattern Recognition Receptors and Susceptibility to Fungal Disease

Genetic variation in the genes encoding PRRs can influence susceptibility to diseases caused by a wide range of fungal pathogens (Carvalho et al. 2010). As for most infectious diseases, genetic defects in the different families of PRRs have been widely linked with susceptibility to fungal disease (Netea et al. 2012). These associations highlight the complexity of the host genetic signatures influencing antifungal host defense. Here, we discuss the most relevant genetic variation in PRRs, its association with susceptibility to infection by *Candida* and *Aspergillus*, and their functional consequences to the activation of innate immune responses.

3.1 Toll-like Receptors

Following the early description of TLRs, genetic variation in these genes was proposed to underlie significant interindividual differences in susceptibility to infectious and inflammatory diseases (Netea et al. 2012). Although monogenic or primary immunodeficiencies affecting TLR signaling promote large effect sizes, this is typically a rare (or very rare) event on the general population that has not been implicated in the development of fungal disease thus far. However, TLRs are characterized by remarkable genetic diversity due to strong selective pressures during their evolution, particularly in the number of single nucleotide polymorphisms (SNPs) that lead to substitutions in amino acid residues (Quach et al. 2013). As such, before the advent of next-generation sequencing and genome-wide association studies, polymorphisms in TLRs were considered biologically plausible targets for involvement in susceptibility to infectious diseases, including fungal infections (Cunha et al. 2010b). Table 1 summarizes relevant genetic variants in TLRs and their association with susceptibility to infection by *Candida* and *Aspergillus*.

Table 1 Genetic variation in TLRs and susceptibility to infection by *Candida* and *Aspergillus*

Gene (s)	SNP(s)	Nucleotide change	Amino acid change	Disease(s)	References
TLR1	rs5743611	G > C	R80T	IPA	Kesh et al. (2005)
	rs4833095	C > T	N248S	IPA	Kesh et al. (2005)
	rs5743618	T > G	S602I	Candidemia	Plantinga et al. (2012)
TLR2	rs5743704	C > A	P631H	RVVC	Rosentul et al. (2014)
TLR3	rs3775296	G > T	–	IPA	Carvalho et al. (2012b)
TLR4	rs4986790	A > G	D299G	IPA and CPA	Bochud et al. (2008), Carvalho et al. (2008), de Boer et al. (2011), Koldehoff et al. (2013)
	rs4986791	C > T	T399I	IPA	Bochud et al. (2008), Koldehoff et al. (2013)
TLR5	rs5744168	C > T	R392X	IPA	Grube et al. (2013)
TLR6	rs5743810	C > T	S249P	IPA	Kesh et al. (2005)
TLR9	rs5743836	C > T	–	ABPA	Carvalho et al. (2008)

The first nucleotide (and corresponding amino acid) is the ancestral nucleotide and therefore is considered the wild-type allele. *SNP* single nucleotide polymorphism; *IPA* invasive pulmonary aspergillosis; *RVVC* recurrent vulvovaginal candidiasis; *CPA* chronic pulmonary aspergillosis; *ABPA* allergic bronchopulmonary aspergillosis

The TLR4 sequence variants rs4986790 (D299G) and rs4986791 (T399I) constitute an haplotype that has been reported to alter the leucine-rich repeat region of the receptor and decrease the efficiency of ligand recognition (Arbour et al. 2000). The presence of this haplotype in donors of allogeneic HSCT was associated with the development of invasive pulmonary aspergillosis (IPA) in the corresponding patients (Bochud et al. 2008). Although this association was validated in independent HSCT cohorts (de Boer et al. 2011; Koldehoff et al. 2013) and in immunocompetent individuals suffering from chronic aspergillosis (Carvalho et al. 2008), other studies have failed to replicate the findings (Carvalho et al. 2009; Fisher et al. 2017; Kesh et al. 2005). The TLR4 haplotype was reported to delay immune reconstitution after transplant (Koldehoff et al. 2013), and this could represent a key mechanism justifying its association with IPA in specific patient populations. The exact mechanism by which TLR4 variants may influence antifungal immune responses remains however unknown – particularly since no fungal ligands (or endogenous molecules released during infection) have been identified to date – and this may also support the lack of association with other fungal diseases, including candidemia (Plantinga et al. 2012).

Genetic variation in TLRs other than TLR4 has also been proposed as an important risk factor for fungal disease. Earlier studies have reported associations of the polymorphisms rs5743611 (R80T) and rs4833095 (N248S) in TLR1 and rs5743810 (S249P) in TLR6 with IPA (Kesh et al. 2005), but only TLR6 has been shown to be required for the human immune response to *A. fumigatus* (Rubino et al. 2012). TLR1 also appears to be an important repository of genetic variability

increasing susceptibility to candidemia (Plantinga et al. 2012). Although the precise mechanism(s) whereby TLR1 influences the risk of candidemia remains elusive, the variant rs5743618 (I602S) has been shown to impair the trafficking of TLR1 to the cell surface and to result in decreased NF-κB activation and proinflammatory cytokine responses to TLR1 agonists (Johnson et al. 2007; Wurfel et al. 2008). In addition, and despite it was not associated with systemic *Candida* infection (Plantinga et al. 2012), the rs5743704 (P631H) SNP in TLR2 was implicated in the development of idiopathic recurrent vulvovaginal candidiasis (RVVC) (Rosentul et al. 2014). Finally, the variant rs5743836 in the promoter of TLR9 was associated with the development of allergic bronchopulmonary aspergillosis (ABPA) (Carvalho et al. 2008), although this study still requires confirmation.

Another interesting example regards TLR5, the receptor for flagellin expressed by flagellated bacteria (Hayashi et al. 2001), and in which a SNP leading to an early stop codon (R392X) has been shown to disrupt flagellin recognition (Hawn et al. 2003). In HSCT recipients, the presence of this variant was associated with the development of IPA (Grube et al. 2013), thereby suggesting a likely important antifungal function of TLR5 in the non-hematopoietic compartment. However, functional data is not available, and additional studies are warranted to identify the so far unsuspected mechanisms (and the ligand) by which TLR5 might influence susceptibility to IPA. In any case, the fact that R392X presents with a relatively common frequency without imposing a primary immunodeficiency phenotype suggests a non-essential role for TLR5 in host defense (Wlasiuk et al. 2009).

Despite classically acknowledged as a prototypical receptor for double stranded RNA (Zhang et al. 2013), TLR3 has been implicated in fungal recognition and activation of adaptive immune responses. In particular, the regulatory variant rs3775296 in TLR3 was demonstrated to increase the risk of IPA after HSCT (Carvalho et al. 2012b). Cross-presenting dendritic cells harboring this variant displayed an impaired expression of TLR3 and sensing of fungal RNA, which ultimately resulted in the defective priming of memory CD8(+) T-cell responses to *A. fumigatus*. This study was recently supported by evidence demonstrating a similar association with severe asthma with fungal sensitization (Overton et al. 2017). Although there is no evidence implicating this regulatory variant in susceptibility to infections by *Candida*, the non-synonymous SNP rs3775291 (L412F) in TLR3 was however detected more frequently in patients suffering from chronic mucocutaneous candidiasis (CMC) (Nahum et al. 2011).

This study represents a critical example of how genetic defects in TLRs (and other PRRs) may influence adaptive immune responses, in addition to fungal sensing and innate immunity. Although this has yet to be addressed, there is an unequivocal need to consider the genetic profile of the patient during diagnostic approaches based on the measurement of fungal-specific adaptive immune responses (Koehler et al. 2018; Potenza et al. 2013). The same applies to immunotherapeutic strategies focused on the direct or indirect manipulation of cytokines, since there are many examples of genetic variants that influence cytokine production and function (Cunha et al. 2017; Johnson et al. 2012). In conclusion, the success of novel diagnostic and immunotherapeutic approaches for fungal diseases

will only be possible if guided by personalization based on the interindividual variability in immune function, particularly for genetic variants with well-established functional consequences.

3.2 C-Type Lectin Receptors

Given the well-established role of CLRs in the coordination of antifungal immune responses, their genetic variation has been extensively implicated in susceptibility to fungal disease (Table 2). The importance of dectin-1 in the recognition of β-glucan and activation of antifungal immunity has been demonstrated in mouse studies but also in patients with recurrent fungal infections carrying the early stop codon polymorphism rs16910526 (Y238X) (Ferwerda et al. 2009; Taylor et al. 2007). This variant results in a truncated form of dectin-1 lacking several amino acids within the carbohydrate recognition domain, which underlies its decreased expression at the surface of myeloid cells and a defective production of cytokines, particularly IL-17, after stimulation with β-glucan or *C. albicans* (Ferwerda et al. 2009). Following the initial identification of Y238X, and given its specific impact on the activation of Th17-mediated immunity, it has since been vastly implicated in mucosal and gastrointestinal fungal colonization (De Luca et al. 2013; Plantinga et al. 2009; Usluogullari et al. 2014). This functional axis is further demonstrated by primary immunodeficiencies due to rare mutations in STAT3, IL-17RA and IL-17F that impair Th17 immunity and are associated with CMC and aspergillosis (Lionakis and Levitz 2018). Because the cellular localization of the different dectin-1 isoforms regulates the signaling quality of antifungal immunity (Carvalho et al. 2012c; Fischer et al. 2017), it is also tempting to speculate that this may be one additional mechanism through which the Y238X variant may contribute to infection. Of note, another non-synonymous variant in dectin-1 (rs16910527; I223S) was instead associated with lower levels of interferon (IFN)-γ and the risk of oropharyngeal candidiasis in HIV patients (Plantinga et al. 2010). This suggests that different pathogenetic mechanisms are likely in place depending on the specific structural consequences of genetic variants to dectin-1 function.

Although the clinical phenotype of patients carrying the dectin-1 stop codon is relatively mild and less severe than that of patients with classic CMC (Puel et al. 2010), the Y238X variant was found to strongly predispose HSCT recipients to the development of IPA (Chai et al. 2011; Cunha et al. 2010a). Additional variants in dectin-1, but also dectin-2 and DC-SIGN (CD209), were likewise correlated with the development of IPA in hematological patients (Fischer et al. 2016; Sainz et al. 2012). Importantly, the genetic deficiency of dectin-1 in both the hematopoietic and non-hematopoietic compartments was disclosed to synergize towards risk of infection (Cunha et al. 2010a), a finding that was validated in the largest HSCT patient cohort at-risk of IPA collected to date (Fisher et al. 2017) and that highlights the key role of dectin-1 in antifungal immunity across multiple cell types. Among the many biological processes that are regulated by dectin-1 in response to fungi,

Table 2 Genetic variation in CLRs and susceptibility to infection by *Candida* and *Aspergillus*

Gene(s)	SNP(s)	Nucleotide change	Amino acid change	Disease(s)	References
CARD9	rs4077515	G > A	S12N	ABPA	Xu et al. (2018)
CD209	rs4804800	G > A	–	IPA	Sainz et al. (2012)
	rs11465384	C > T	–	IPA	Sainz et al. (2012)
	rs7248637	A > G	–	IPA	Sainz et al. (2012)
	rs7252229	C > G	–	IPA	Sainz et al. (2012)
CLEC1A	rs2306894	C > G	G26A	IPA	Stappers et al. (2018)
CLEC7A	rs16910526	T > G	Y238X	*Candida* colonization, RVVC and IPA	Chai et al. (2011), Cunha et al. (2010a), De Luca et al. (2013), Fisher et al. (2017), Plantinga et al. (2009), Usluogullari et al. (2014)
	rs16910527	A > C	I223S	Oropharyngeal candidiasis	Plantinga et al. (2010)
	rs7309123	G > C	–	IPA	Fischer et al. (2016), Sainz et al. (2012)
	rs3901533	G > T	–	IPA	Sainz et al. (2012)

The first nucleotide (and corresponding amino acid) is the ancestral nucleotide and therefore is considered the wild-type allele. *SNP* single nucleotide polymorphism; *ABPA* allergic bronchopulmonary aspergillosis; *IPA* invasive pulmonary aspergillosis; *RVVC* recurrent vulvovaginal candidiasis

the production of ROS through the NADPH oxidase system and the activation of downstream clearance mechanisms is critically important, as reflected by the extreme susceptibility of patients with chronic granulomatous disease (CGD) to aspergillosis (Grimm et al. 2013; Kyrmizi et al. 2013). The role of dectin-1 in immunity to infection may however extend beyond the immediate activation of antifungal effector mechanisms. For example, recognition of β-glucan has been demonstrated to confer innate immune memory to infection – a process referred to as trained immunity (Netea and van der Meer 2017) – by regulating multiple processes of cellular metabolism (Arts et al. 2016; Bekkering et al. 2018; Cheng et al. 2014). In the future, it will be critical to assess the extent to which the Y238X variant predisposes to fungal disease by impairing the induction of "natural" trained immunity as the result of our constant exposure to fungi.

Mutations in caspase recruitment domain-containing protein 9 (CARD9), the adaptor molecule that transduces signals from dectin-1 and other CLRs, have been identified in patients suffering from mucocutaneous fungal infections (Glocker et al. 2009). Importantly, neutrophils from CARD9-deficient patients were found to display impaired phagolysosomal killing of unopsonized *C. albicans*, a phenotype that was independent of dectin-1 and NADPH oxidase activity, thereby explaining the variable clinical presentation of fungal infection in patients suffering from

dectin-1 and CARD9 deficiency and CGD (Gazendam et al. 2014). Human CARD9 deficiency was also found to predispose to extrapulmonary aspergillosis with sparing of the lungs through a mechanism involving the defective accumulation of neutrophils in infected tissue (Rieber et al. 2016). Of note, the common polymorphism rs4077515 (S12N) in CARD9 was recently implicated in the risk of ABPA (Xu et al. 2018). Mechanistically, work performed in knock-in mice expressing the mutated form of human CARD9 revealed the remarkable contribution of S12N to the activation of NF-κB subunit RelB, which in turn promoted the production of IL-5 in alveolar macrophages and the recruitment of eosinophils to drive Th2 cell-mediated allergic responses. Given the key role of CARD9 in orchestrating the signals collected from the different CLRs, it will be interesting to assess whether this or other common genetic variants may impact susceptibility to other forms of aspergillosis and eventually other fungal infections.

MelLec (encoded by the *CLEC1A* gene) was recently characterized as the functional receptor for DHN-melanin from *A. fumigatus* (Stappers et al. 2018). This discovery was accompanied by the identification of a non-synonymous variant rs2306894 (G26A) in the cytoplasmic tail of MelLec, suggesting an influence on intracellular signal transduction rather than on the recognition of DHN-melanin. Accordingly, the presence of G26A in HSCT donors was found to strongly increase the risk of IPA in the corresponding recipient as the result of a broad defect in cytokine production by myeloid cells (Stappers et al. 2018). Although there is still much to be learned about the mechanisms through which MelLec orchestrates the immune response against *A. fumigatus* (Casadevall 2018), these findings demonstrate the key role of myeloid-expressed MelLec and its genetic variation to the human host defense against *A. fumigatus*.

3.3 NOD-like and RIG-I-like Receptors

Apart from the involvement of NLRs in inflammasome formation, the function of canonical receptors such as NOD1 and NOD2 in the host defense against fungi remained until recently poorly studied. Several polymorphisms in NOD1 and NOD2 have been typically associated with the development of many infectious and inflammatory diseases, namely Crohn's disease (Caruso et al. 2014), but not fungal infections. A recent genetic screening in HSCT patients and corresponding donors revealed however that the donor rs2066842 (P268S) variant was associated with protection from IPA (Gresnigt et al. 2018). Mechanistically, mononuclear cells harboring this variant displayed enhanced phagocytosis and killing capacity whereas NOD2 activation instead reduced the antifungal potential of these cells. Further supporting the suppressive effect of NOD2 activation on antifungal effector functions, NOD2 deficiency conferred resistance to experimental aspergillosis in a mouse model of infection. Altogether, the P268S variants in NOD2 represents one of the few available examples associated with genetically-determined protection

Table 3 Genetic variation in NLRs and RLRs and susceptibility to infection by *Candida* and *Aspergillus*

Gene (s)	SNP(s)	Nucleotide change	Amino acid change	Disease(s)	References
NLRP3	rs74163773	12,9,7,6 VNTR	–	RVVC	Jaeger et al. (2016), Lev-Sagie et al. (2009)
NOD2	rs2066842	C > T	P268S	IPA	Gresnigt et al. (2018)
IFIH1	rs3747517	G > A	H843R	Candidemia	Jaeger et al. (2015)
	rs1990760	C > T	A946T	Candidemia	Jaeger et al. (2015)

The first nucleotide (and corresponding amino acid) is the ancestral nucleotide and therefore is considered the wild-type allele. *SNP* single nucleotide polymorphism; *VNTR* variable number of tandem repeats; *RVVC* recurrent vulvovaginal candidiasis; *IPA* invasive pulmonary aspergillosis

from fungal infection and highlights the interesting possibility to block NOD2 signaling as a therapeutic intervention in IPA.

There is an intricate relationship between dectin-1 signaling and inflammasome formation in response to both *C. albicans* and *A. fumigatus* (Cheng et al. 2011; Said-Sadier et al. 2010). The variable number of tandem repeats (VNTR) rs74163773 in intron 4 of NLRP3 was reported to be more frequently detected in women suffering from RVVC (Lev-Sagie et al. 2009), a finding that was recently validated in a larger multicenter cohort (Jaeger et al. 2016). Importantly, both studies demonstrated that the levels of IL-1β were influenced by the number of intronic repeats carried by the patients, suggesting a regulatory role of this VNTR on NLRP3 inflammasome activity.

An important role in host defense against *C. albicans* has also been ascribed to genetic variants in other cytosolic receptors, namely RLRs. In particular, the missense variants rs1990760 (A946T) and rs3747517 (H843R) in MDA5 (*IFIH1*) were shown to be associated with systemic *Candida* infection as the result of an altered cytokine response (Jaeger et al. 2015). Like TLR3, this receptor for viral RNA also showed unsuspected effects in antifungal immunity, thereby suggesting intracellular receptors for fungal nucleic acids as novel targets amenable to therapeutic manipulation. Table 3 illustrates relevant genetic variants in NLRs and RLRs associated with fungal disease.

3.4 Soluble Pattern Recognition Receptors

In addition to the membrane-bound PRRs discussed in detail above, there are several soluble molecules that are endowed with the ability to interact with and bind to microbial polysaccharides without transducing intracellular signals and that function as opsonins to facilitate phagocytosis (Bidula and Schelenz 2016). Table 4 summarizes relevant genetic variants in soluble PRRs and their association with susceptibility to fungal disease. The mannose-binding lectin (MBL), a CLR that

Table 4 Genetic variation in soluble PRRs and susceptibility to infection by *Candida* and *Aspergillus*

Gene (s)	SNP(s)	Nucleotide change	Amino acid change	Disease(s)	References
MBL2	rs5030737	C > T	R52C	CPA	Crosdale et al. (2001), Vaid et al. (2007)
	rs1800450	G > A	G54D	RVVC	Babula et al. (2003), Donders et al. (2008), Giraldo et al. (2007), Nedovic et al. (2014), Wojitani et al. (2012)
PLG	rs4252125	A > G	D472N	IPA	Zaas et al. (2008)
PTX3	rs2305619	A > G	–	IPA	Cunha et al. (2014, 2015), Fisher et al. (2017)
	rs3816527	A > C	A48D	IPA and mold infection and colonization	Cunha et al. (2014, 2015), Wojtowicz et al. (2015)
	rs1840680	G > A	–	IPA and CPA	Cunha et al. (2014), He et al. (2018)

The first nucleotide (and corresponding amino acid) is the ancestral nucleotide and therefore is considered the wild-type allele. *SNP* single nucleotide polymorphism; *CPA* chronic pulmonary aspergillosis; *RVVC* recurrent vulvovaginal candidiasis; *IPA* invasive pulmonary aspergillosis

binds carbohydrate patterns from microorganisms and activates the lectin pathway of the complement system, stands out as one of the most well-known examples (Foo et al. 2015). Many studies have established genetic variation to be a major regulator of the levels and function of MBL in as much as 8% of individuals in the general population (Sprong and van Deuren 2008). Interestingly, these individuals do not display any obvious clinical phenotypes, suggesting that MBL deficiency may be, to a large extent, compensated by the redundancy of the humoral innate immune system, namely by the vast set of molecules that possess similar opsonic properties. Although not presenting as an outright immunodeficiency, the genetically-determined defect in the function of MBL is acknowledged as an important risk factor for infection, particularly in immunocompromised hosts. There are several known combinations of non-synonymous and promoter variants in the gene encoding MBL, either affecting the expression levels, its functional activity or both (Carvalho et al. 2010). Several studies have proposed a role for the rs1800450 (G54D) variant in MBL in the development of RVVC (Babula et al. 2003; Donders et al. 2008; Giraldo et al. 2007; Wojitani et al. 2012), and this was recently confirmed in a meta-analysis (Nedovic et al. 2014). In addition, the levels of circulating MBL were found to vary significantly during the course of invasive candidiasis (Damiens et al. 2012), although the extent to which genetic variation regulated this phenotype was not assessed. The same holds true for IPA, in which low circulating concentrations of MBL were detected in infected patients (Lambourne et al. 2009). Although there is no evidence for a contribution of genetic

variants in MBL to invasive disease, the development of chronic pulmonary aspergillosis was nonetheless linked with the presence of variable MBL alleles at codon 52 (Crosdale et al. 2001; Vaid et al. 2007). Because most of the studies that have addressed genetic variation in MBL and risk of fungal disease in the past were flawed by the limited number of patients analyzed, these associations need to be revisited in larger and well-characterized cohorts.

Another soluble PRR that has received a great deal of attention in the field of fungal diseases in the past is the long pentraxin-3 (PTX3) (Foo et al. 2015). This molecule has been shown to bind microbial moieties from a wide range of microorganisms, including bacteria, viruses, and fungi, particularly *A. fumigatus* (Garlanda et al. 2002). Although classic immunodeficiencies have not been linked to PTX3 deficiency, common polymorphisms have been disclosed as important risk factors across different infectious diseases, namely *Pseudomonas aeruginosa* colonization in cystic fibrosis patients (Chiarini et al. 2010) and urinary tract infections (Jaillon et al. 2014). Remarkably, and according to its nonredundant role in immunity to *A. fumigatus* in mouse models of infection (Garlanda et al. 2002), genetic variation in PTX3 was identified as a major risk factor for IPA after HSCT (Cunha et al. 2014). These findings were validated in a large, independent study (Fisher et al. 2017) and extended across different clinical settings, including solid organ transplant recipients (Cunha et al. 2015; Wojtowicz et al. 2015) and patients with chronic obstructive pulmonary disease (Cunha and Carvalho 2018; He et al. 2018). Collectively, these studies highlight genetic variation in PTX3 as robust host-derived markers for IPA and lay the foundations for well-designed clinical trials assessing their validity in the clinical setting.

Earlier studies have suggested binding of galactomannan to PTX3 (Garlanda et al. 2002). However, definitive evidence about the actual fungal ligand recognized by PTX3 is still lacking. This is in line with reports showing binding of PTX3 to the cell wall of *C. albicans* (Tierney et al. 2012), although no studies have been performed to date exploring the contribution of genetic variation in PTX3 to the risk of infections caused by *Candida*. Whatever the ligand(s) involved, PTX3 deficiency was found to hamper the normal alveolar expression of the protein and, at a cellular level, it impaired the antifungal effector mechanisms of neutrophils, namely phagocytosis and killing (Cunha et al. 2014). The specific impact of PTX3 deficiency on neutrophil function was corroborated by the loss of the genetic association in patients that developed IPA during severe neutropenia. However, additional mechanisms of antifungal host defense may also be influenced by PTX3 deficiency. The recent demonstration that PTX3 is a critical molecule bridging neutrophil function and B-cell function, namely class switching, plasmablast expansion and antibody production, represents one such example (Chorny et al. 2016). In addition, binding of PTX3 to myeloid differentiation protein 2, an adapter of the TLR4 signaling complex, is critically required for immune protection in experimental aspergillosis (Bozza et al. 2014). This raises the interesting possibility that the combined genetic deficiency of PTX3 and TLR4 might underlie a higher risk of IPA than the single defects alone, a hypothesis that requires confirmation.

The measurement of PTX3 in the bronchoalveolar fluids of mechanically ventilated patients was recently proposed to be endowed with the ability to identify microbiologically confirmed pneumonia (Mauri et al. 2014). The alveolar concentrations of PTX3 are known be determined at the genetic level (Cunha et al. 2014), and, as such, one could expect a further improvement to the diagnostic performance of PTX3 by knowing in advance the genotypic profile of the patient. In addition, PTX3 deficiency was also shown to influence the levels of alveolar cytokines in hematological patients suffering from IPA and to impact their ability in discriminating infection (Gonçalves et al. 2017). More important, reinstating the normal levels of PTX3 in vitro with the recombinant protein was sufficient to restore the efficacy of the antifungal effector functions of neutrophils (Cunha et al. 2014). Although data in the clinical setting is so far lacking, these observations support the potential applicability of PTX3 in novel prophylactic or therapeutic approaches for IPA in patients at-risk (Carvalho et al. 2012a). As stated above, the combined use of antifungal therapy and PTX3 treatment has been found to improve the efficacy of the drug alone in animal models of IPA (Lo Giudice et al. 2012; Marra et al. 2014).

Another example of a soluble PRR characterized by relevant genetic diversity regards plasminogen. By mining genetic data obtained from an unbiased screen of survival data in different strains of mice subjected to experimental aspergillosis, the non-synonymous variant rs4252125 (D472N) was correlated with the risk of IPA in patients undergoing HSCT (Zaas et al. 2008). These findings support the importance of additional pre-clinical studies testing different models of infection and evaluating additional immune-related readouts to assist in the discovery of human genetic variation with an important contribution to the risk of infection.

4 Clinical Translation of Host Genetics in Medical Mycology

Many studies over the past decade have implicated genetic variation in PRRs in the risk of developing fungal disease, particularly under predisposing clinical conditions such as those in the hematology setting (Cunha et al. 2011b; van der Velden et al. 2011). However, a great deal of work is still required to identify the actual causative alleles and their functional consequences, as well as to precisely pinpoint the biological mechanisms through which these influence the risk of infection. We have currently a large amount of genetic links to susceptibility to fungal disease at our disposal; however, clinical data supporting the translation of these critical insights into improved patient outcomes has been practically inexistent (Cunha et al. 2011a). This can be largely attributed to the relatively small effect size of the identified variants (since many carriers will not develop infection), which may negatively influence the discriminatory ability of the genetic profile to inform clinical decision-making. Although this limitation will hardly be countered soon

and the use of genetic information to predict the risk of fungal disease is unlikely to alter clinical practice in the near future, the predictive performance of the genetic information may benefit from advanced genetic screening strategies and mathematical models. The integration of host and pathogen genetic data into stratification models that also consider the clinical characteristics of the patients are expected to improve our current ability to predict risk and progression of disease, including the response to treatment and its duration, and adverse events. A first, modest step toward this goal was recently taken in a study that demonstrated that the concerted analysis of selected genetic and clinical factors into a predictive model could be used to guide preemptive therapy in hematological patients (White et al. 2018).

Although genetic data in the clinical setting has historically been investigated with the goal to develop risk stratification and diagnostic strategies, recent studies illustrating how genetic variation regulates processes of host immunity raises the exciting possibility that it may also be exploited to guide immunotherapy. Most of the clinical trials performed to date have failed to account for the significant impact that genetic variation may pose on subgroups of individuals, and this may partly explain the disappointing outcomes of trials involving anti-inflammatory agents for the treatment of sepsis (Cohen et al. 2015). There is therefore an urgent need to identify and characterize relevant genetic variation in the stratification of patients enrolled in clinical trials of immunomodulatory agents (Rello et al. 2018).

The application of next-generation sequencing and systems biology approaches provide a powerful tool to identify essential genes and pathways in the host-fungus interaction at a level of complexity that was not possible beforehand (Dix et al. 2016). Although in the field of fungal diseases, genetic association studies at the genome-wide level are scarce, a few examples exist which have enabled the identification of novel players controlling susceptibility to infection (Kumar et al. 2014; Smeekens et al. 2013a). For example, the integration of transcriptional data and functional genomics revealed an unanticipated role of type I IFN in the host defense against *Candida* (Smeekens et al. 2013a). Polymorphisms in type I IFN genes were found to modulate cytokine production and to drive a skew from a Th17 to a Th1 type of response, thereby predisposing to candidemia in critically ill patients. More recently, other functional genomics approaches combining whole genome information and immunological screenings have also provided invaluable evidence about the genetic regulation of cytokine production in humans in response to different stimuli, including fungi (Li et al. 2016a, b). By correlating genome-wide SNP genotypes with cytokine abundance in response to fungal stimulation, several cytokine quantitative trait loci (QTL), i.e., genetic variants that control cytokine production, were identified (Li et al. 2016a). Among them, a cytokine QTL at the NAA35/GOLM1 locus markedly influenced the levels of IL-6 produced and was associated with susceptibility to candidemia. Altogether, these studies support the need to consider the genetic contribution to immune phenotypic variation (e.g., cytokine production and immune cell function) to generate accurate maps of the human genomic architecture regulating susceptibility to fungal disease.

5 Conclusions

Our current understanding of the immunobiology of human fungal diseases has derived largely from studies addressing polygenic susceptibility. These studies have not only revealed the individual contribution of genetic variants but have also highlighted the complex interactions between adding effects of variants with small or modest effect sizes to the immune response. The current state-of-the-art is however not adequate for the needs of clinicians since it does not allow the accurate prediction of which individuals might develop fungal disease or that may benefit the most from intensive diagnostic surveillance or alternative prophylaxis and treatment strategies. Clinical translation of patient genetics will benefit from the concerted action of clinicians and researchers into the establishment of larger and well-characterized patient cohorts, as well as comprehensive functional testing to identify the biological mechanisms of association with infection. By unraveling molecules and pathways whose expression or function may be regulated at the genetic level may allow the possibility to target them with personalized immunotherapeutics aimed at restoring lacking or defective immune components (Cunha and Carvalho 2012). In conclusion, an improved understanding of how specific genetic signatures regulate susceptibility to fungal disease may revolutionize the field of medical mycology, opening new horizons and laying the foundations for personalized medical interventions based on individual genomics in patients who are at risk.

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Fungi as Part of the Microbiota and Interactions with Intestinal Bacteria

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Abstract The human microbiota consists of bacteria, archaea, viruses, and fungi that build a highly complex network of interactions between each other and the host. While there are many examples for commensal bacterial influence on host

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health and immune modulation, little is known about the role of commensal fungi inside the gut community. Up until now, fungal research was concentrating on opportunistic diseases caused by fungal species, leaving the possible role of fungi as part of the microbiota largely unclear. Interestingly, fungal and bacterial abundance in the gut appear to be negatively correlated and disruption of the bacterial microbiota is a prerequisite for fungal overgrowth. The mechanisms behind bacterial colonization resistance are likely diverse, including direct antagonism as well as bacterial stimulation of host defense mechanisms. In this work, we will review the current knowledge of the development of the intestinal bacterial and fungal community, the influence of the microbiota on human health and disease, and the role of the opportunistic yeast *C. albicans*. We will furthermore discuss the possible benefits of commensal fungal colonization. Finally, we will summarize the recent findings on bacterial–fungal interactions.

1 Development and Composition of the Intestinal Microbiota

For a long time, it was assumed that the unborn child is sterile and that the initial contact with commensal microbes occurs during birth (Stinson et al. 2017). This led to the official doctrine that any microbes found in the uterine cavity must be pathological and hazardous to the unborn child. However, in the last few years a growing body of scientific studies reported the presence of either microbial DNA or viable microbiota in the placenta, the amniotic fluid, the meconium, or the umbilical cord blood. As mother–offspring pairs share microbial signatures between placenta, amniotic fluid, and meconium, it is suggested that the early gut colonization may be initialized prenatally by a distinct trajectory of maternal microbes (Kundu et al. 2017; Collado et al. 2016).

The establishment of the human intestinal microbiota is influenced by multiple factors. One important factor is the mode of delivery, which affects diversity and colonization pattern of the infant gut microbiota. Vaginally delivered infants harbor in all body habitats bacterial communities that are in composition most similar to the vaginal communities of their mothers (Dominguez-Bello et al. 2010). The microbiota of children delivered by cesarean section is most similar to the skin communities of their mothers (Dominguez-Bello et al. 2010). While dominant bacterial taxa found in vaginally delivered infants are *Lactobacilli*, *Prevotella*, *Atopobium,* or *Sneathia* spp., typical skin taxa in samples from caesarian section delivered babies include *Staphylococcus, Corynebacterium*, and *Propionibacterium* spp. as well as *Klebsiella*, *Veillonella*, and *Clostridiaceae*. (Dominguez-Bello et al. 2010; Kundu et al. 2017; Rutayisire et al. 2016). There is more and more evidence that the early microbiota colonization may influence the development of diseases later in life (Goulet 2015). For instance, a low microbial diversity in infancy precedes the onset of allergic disease (West 2014), and sensitization to food in children

at three months and one year was correlated with a high abundance of *Enterobacteriaceae* and a low abundance of *Bacteroidaceae* in the gut microbiota (Azad et al. 2015). Furthermore, there is growing evidence that an aberrant gut microbe composition develops as result of the delivery mode, e.g., caesarian section affects the subsequent regulation of immune responses (Knight and Girling 2003; Rutayisire et al. 2016). It is reported that the risk of developing celiac disease, asthma, obesity, or type-1-diabetes (T1D) is increased in children delivered by caesarian section as compared to vaginally born infants (Black et al. 2015; Kuhle et al. 2015; Adlercreutz et al. 2015).

In addition, intake of colostrum and breast milk possibly supplies neonates with the unique microbial pattern of the mothers' breast milk (Martin et al. 2007). Using this route, the most frequent genera which are vertically transferred from mother to child are *Lactobacillus, Staphylococcus, Enterococcus,* and *Bifidobacterium* (Kundu et al. 2017; Makino et al. 2011; Fernandez et al. 2013).

Upon introduction of dietary supplements, solid food, and withdrawal from breast milk, the intestinal microbiota acquires greater complexity, individuality and increasingly resembles the microbiota of a typical omnivore (Kundu et al. 2017; Palmer et al. 2007). In parallel with increasing age during adolescence and puberty, the population of aerobes and facultative anaerobes decreases gradually and the number of obligate anaerobes increases (Hopkins and Macfarlane 2002). In addition, gender-specific diversifications of the gut microbiota arise during this period of life (Markle et al. 2013). Moreover, the adolescent microbiome was found to differ functionally from that of adults, expressing genes related to development and growth (Hollister et al. 2015). The adult microbiota harbors a core community of colonizers and is more stable compared to that of early life (Palmer et al. 2007; Rajilic-Stojanovic et al. 2012). However, it is still amenable to changes by environmental perturbations such as changes in nutrition (David et al. 2014), seasonal variations or temperature fluctuations (Chevalier et al. 2015), or even altitude (Zhang et al. 2016). Changes in the composition of the intestinal microbiota also account for changes in gut metabolites. Using comparative metabolomics, clear differences between gut metabolite productions can be observed between humans following omnivore, vegetarian, vegan, or a Mediterranean diet (De Filippis et al. 2016; Wu et al. 2016). However, to which extent these metabolome differences are due to diet-associated differences in the microbiota or nutrient composition of the diet itself remains to be determined.

2 Microbiota in Health and Disease

The defined composition of the intestinal microbiota is a unique feature of each individual. As demonstrated above, this characteristic composition within each individual is not static, but rather subject to frequent changes due to environmental, nutritional, or iatrogenic influences.

The impact of the intestinal microbiota on the development of a wide variety of human pathologies is the subject of intense ongoing research. In this context, developing strategies to precisely modulate the microbiota composition in order to restore gut homeostasis in a host-specific manner is thought to offer novel therapeutic approaches for the treatment of microbiota-influenced human diseases. To achieve this ambitious aim, several fundamental questions have to be answered: (1) How exactly does a certain microbiota composition influence the progress or outcome of a distinct pathology on a molecular level? Answering this question might also lead to the discovery of novel, so far neglected host-specific therapeutic targets. (2) Does the presence of certain commensal microbes enhance the risk of developing a particular disease? (3) Is the presence of such a specific disease-promoting commensal a general phenomenon or is it restricted to a certain individual host or of host group sharing particular genetic predispositions? And finally: (4) Is there a host-specific distinct ideal microbiota composition that completely prevents potential disease-driving events?

One of the most intriguing observations supporting the hypothesis of microbiota-triggered human diseases is the effect of transplanting feces from human patients into germ-free (GF) mice. For example, fecal transplantation from multiple sclerosis (MS) patients into GF mice results in a more prominent experimental autoimmune encephalomyelitis (EAE) outcome compared to using feces from healthy donor controls (Berer et al. 2017). Correspondingly, fecal transplantations from patients suffering from irritable bowel syndrome (IBS) (De Palma et al. 2017) or Parkinson's disease (PD) (Sampson et al. 2016) induced stronger disease phenotypes in germ-free susceptible mice compared to mice transplanted with feces from healthy human donors. However, as for many of these studies, it is not completely clear if this disease-inducing dysbiotic microbiota is the cause of the pathology or just a secondary consequence emerging from other disease-promoting events in the donor host.

There are diverse interactions between gut microbes and host cells at intestinal mucosal interfaces: (1) Commensal microbes can directly interact with host cells. (2) The host, in turn, reacts to microbes by either tolerance or inflammation, thereby potentially leading to microbiota composition shifts. (3) Members of the microbiota interact with each other, and (4) environmental factors can influence this complex interplay. It is important to note that these interactions are very dynamic and interconnected. Under homeostatic conditions, the different variables are counterbalanced leading to a highly diverse microbiota that contributes to health maintenance. However, changing one of these variables can promote dysbiosis, characterized by strongly reduced microbiota diversity, possibly leading to pathology-driving effects.

In the following, we will briefly summarize the different mechanisms by which the composition of microbiota impacts human health.

2.1 Interactions Between Bacteria

Usually, a balanced or homeostatic microbiota composition helps the host to combat infections with enteropathogenic bacteria, viruses, or fungi (Ubeda et al. 2017). Therefore, a microbiota shift toward dysbiosis might facilitate the establishment of infections caused by enteropathogens. It is important to note that, on one side, microbiota alterations due to external influences facilitate pathogen invasion and repopulation. On the other side, gastrointestinal (GI) infections affect the microbiota composition.

There are several mechanisms how certain microbiota members influence the survival or colonization properties of other intestinal microbes. These intermicrobial interactions strongly affect the overall microbiota composition leading to a highly diverse and balanced microbiota and helping to prevent overgrowth of pathobiotic or pathogenic microorganisms: Commensal microbes can impact survival or repopulation of other microbiota members either directly or indirectly via modulation of the host immune system leading to feedback effects on certain microbes (Buffie and Pamer 2013).

Some bacteria are reported to secrete molecules affecting the abundance of other intestinal bacteria. For instance, the commensal symbiont *Bacteroidetes thetaiotaomicron* secretes a factor that represses toxin production from enterohemorrhagic *Escherichia coli* strains (de Sablet et al. 2009). Comparably, certain *Bifidobacterium* spp. produce soluble factors which promote protection against pathogenic *E. coli* and *Citrobacter rodentium* strains (Gagnon et al. 2004). The probiotic strain *E. coli* Nissle 1917 secretes microcins limiting the expansion of pathobiotic or pathogenic *Enterobacteriacaeae* strains under inflammatory conditions, thereby helping to re-establish homeostasis (Sassone-Corsi et al. 2016). *Bacillus thuringiensis* was reported to inhibit *Clostridium difficile* by secretion of a strain-specific bacteriocin (Rea et al. 2010). Notably, these bactericins and microcins seem to act in a target-specific manner, not influencing other microbiota components (Sassone-Corsi et al. 2016; Rea et al. 2011).

Another important factor is the competition for adhesion sites and nutrients. For example, *Lactobacillus reuteri* expresses a mucus-binding protein facilitating adherence to the host mucosa (Kankainen et al. 2009). This effect is thought to contribute to limiting the primary adhesion of pathogens like *Clostridium difficile*. *Bacteroidetes thetaiotaomicron* metabolizes plant-derived monosaccharides, thereby limiting growth of the pathogen *C. rodentium* (Kamada et al. 2012). However, not only competition occurs at such mucosal interfaces but also synergistic effects are observed. For example, *Bacteroidetes longum* upregulates carbohydrate-metabolizing enzymes of *Bacteroidetes thetaiotaomicron*, thereby supporting this strain's growth (Sonnenburg et al. 2006).

Additionally, microbiota components indirectly impact repopulation of other consortium members via modulation of the host immune system. Such modulation includes, among others, the induction of antimicrobial peptide (AMP) secretion, the production of antimicrobial C-type lectins (Sonnenburg et al. 2006), induction of

plasma cell-derived IgA (Ivanov et al. 2009), or modulation of T cell-mediated immunity (Gaboriau-Routhiau et al. 2009).

2.2 *Intestinal Epithelial Barrier and Translocation of Microbiota Components*

The intestinal lumen and its content are separated from underlying sterile tissues by the intestinal mucosal barrier. This barrier is a dynamic structure that allows for communication between microbiota components, metabolites, and the host organism; facilitates absorption of nutrients; and aids in sensing invading pathogens (Bischoff et al. 2014). It is built up by intestinal epithelial cells (IECs), a mucus layer covering IECs at the luminal side, and antimicrobial peptides (AMPs) (Antoni et al. 2014). The integrity of this barrier is of crucial importance since it prevents from uncontrolled translocation of luminal content into other body compartments, which could result in local or systemic inflammation and disease (Valentini et al. 2014).

Barrier function is regulated by both the microbiota and host immune cells. IECs as well as immune cells express pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), mediating detection of distinct bacterial components, called microbe-associated molecular patterns (MAMPs). PRR-mediated detection of MAMPs by IECs results in the activation of intracellular signaling pathways leading, for instance, to secretion of AMPs which, in turn, contribute to intestinal homeostasis (Ayabe et al. 2000; Vaishnava et al. 2011). Therefore, defects in PRR-mediated signaling in IECs increase susceptibility to intestinal infections due to reduced secretion of AMPs but also decreased mucus formation (Frantz et al. 2012; Bhinder et al. 2014). Mucus is produced by goblet cells, and the resulting layer prevents the over-activation of host cells by separating the luminal content from the intestinal epithelium (Fig. 1). An additional factor of barrier integrity is the presence of tight junction proteins which mediate impermeability of the IECs cell layer. Commensal bacteria contribute to barrier integrity through the production of certain metabolites. For example, the generation of short-chain fatty acids (SCFAs) promotes mucus secretion from goblet cells (Burger-van Paassen et al. 2009), while indole, generated from tryptophan by tryptophanase-expressing bacteria, supports tight junction protein function by activating the pregnane X receptor on IECs (Shimada et al. 2013; Venkatesh et al. 2014).

The integrity of the epithelial barrier is influenced not only by microbial metabolites or MAMPs but also by host immune cells. Main mediators of this are cytokines, secreted by various leukocytes. In response to mucosal injury, host immune cells secrete IL-6 which promotes IEC proliferation, therefore, contributing to wound healing (Kuhn et al. 2014). A comparable effect is observed for IL-5 and IL-13, which induce cell proliferation via interposed macrophage activation (Seno et al. 2009). On the other hand, pro-inflammatory cytokines such as TNF or IFNγ

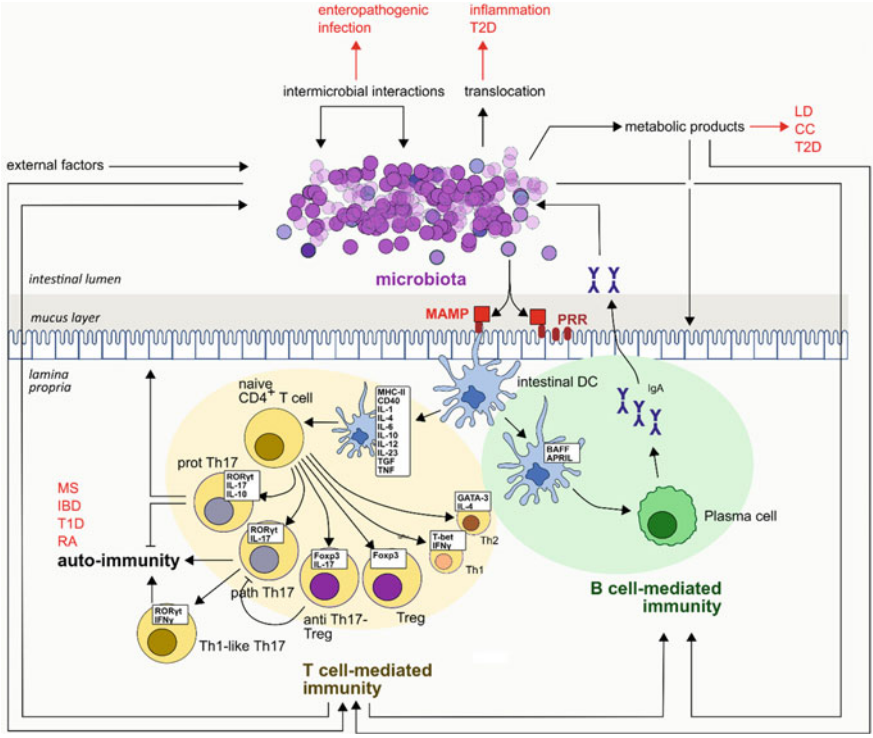

Fig. 1 Mechanisms of microbiota-mediated influences on host pathologies. The composition of the intestinal microbiota is influenced by external factors: Intermicrobial interactions and colonization resistance effects influence the microbiota's ability to fight enteropathogenic infections. Translocation of bacteria or bacterial components affects diseases such as type-2-diabetes (T2D) or can cause inflammatory reactions. Metabolites produced by the intestinal microbiota impact the progress of, e.g., liver disease (LD), colonic cancer (CC), or T2D. These metabolites also affect the integrity of the intestinal barrier and T cell-mediated immunity. Microbe-associated molecular patterns (MAMPs) derived from intestinal commensals or pathogens are sensed by host pattern recognition receptors (PRRs), subsequently influencing B cell- and T cell-mediated immunity, mainly via activation of intestinal dendritic cells (DCs) which are located within the lamina propria. These DCs influence the transepithelial secretion of IgA by plasma cells, which, in turn, impacts the intestinal microbiota. Microbiota-mediated influence of intestinal B cell immunity is mediated either T cell-dependent or T cell-independent. Surface expression of T cell-activating molecules and the cytokine secretion pattern of DCs directs polarization of naïve $CD4^+$ T cells into various different T cell phenotypes: T helper 1 (Th) 1, Th2, regulatory T cells (Treg), pathological (path) or protective (prot) Th17, anti-Th17 Tregs or Th1-like Th17 cells. Transcription factors and key cytokines characterizing each subpopulation are indicated in white boxes. Path Th17 and Th1-like Th17 cells can influence the pathology of autoimmune diseases such as multiple sclerosis (MS), inflammatory bowel disease (IBD), type-1-diabetes (T1D) or rheumatoid arthritis (RA). Prot Th17 cells help to maintain the integrity of the intestinal epithelium. These adaptive immune responses can also affect the microbiota composition. Illustration by A. Steimle

have opposite effects, weakening barrier integrity, i.e., through the suppression of β-catenin/T cell factor signaling (Nava et al. 2010). Importantly, all these host immune cell-mediated effects can be induced by microbiota components, i.e., through MAMP sensing by PRRs (Fig. 1), representing an indirect way of microbiota-mediated influences on barrier integrity.

Dysfunction of the intestinal epithelial barrier and the resulting increased permeability have been linked to various pathologies and were observed in patients with inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), various liver diseases (LD), acute pancreatitis (AP), type-1-diabetes (T1D) and type-2-diabetes (T2D), chronic kidney disease, depression, and other diseases, reviewed in (Fukui 2016). However, it is often unclear whether an increase in barrier permeability and subsequent bacterial translocation is just a secondary effect of ongoing inflammatory processes or a primary pathology-inducing effect.

However, in some cases, an association between translocated intestinal bacteria and onset of disease has been reported, i.e., for type-2-diabetes (T2D) (Sato et al. 2014) and liver disease (LD) (Gkolfakis et al. 2015). It is furthermore hypothesized that even the blood–brain barrier permeability is affected by the intestinal microbiota (Lavasani et al. 2010), therefore influencing neurodegenerative diseases and psychiatric conditions. This effect was suggested to be due to bacterial translocation under microbiota dysbiosis, finally leading to inflammatory reactions in the central nervous system (CNS) (Maes et al. 2012, 2013).

2.3 Microbial Metabolites

Among others, the host benefits from intestinal microbes by their ability to digest certain nutrients which cannot be metabolized by the host itself. Certain microbe-generated metabolites furthermore provide profound effects on the health of the host organism, such as vitamin B or K. Best studied are the effects of short-chain fatty acids (SCFAs) and long-chain fatty acids (LCFAs). SCFAs are beneficial for the host by providing nutrition for enterocytes, stimulating epithelial proliferation, and promoting epithelial barrier function. Furthermore, SCFAs promote tolerance of the intestinal immune system (de Goffau et al. 2014), while LCFAs promote the expansion of inflammation-triggering Th1 and Th17 cells (Haghikia et al. 2015). In addition, some SCFAs have a direct impact on intestinal pathobionts and thereby contribute to colonization resistance.

Microbe-derived metabolites do not only affect the intestinal tissue but also other organ systems, most prominently the liver. For example, SCFAs positively affect glucose homeostasis (Lin et al. 2012). On the other hand, not only bacterial endotoxins but also bacterial metabolites such as ethanol, ammonia, or acetaldehyde reach the liver via the gut–liver axis where they might consequently affect liver function (Gkolfakis et al. 2015). This illustrates that alterations in microbiota composition can lead to changes in metabolite production and thereby affects

pathologies such as obesity, dyslipidemia and their subsequent influence on type-2-diabetes.

2.4 Interaction of Microbiota and the Immune System

The intestinal microbiota provides a considerable influence on the host immune system. This is exemplified by experiments with GF mice which showed that maturation of the immune system requires the presence of intestinal microbiota (Hamada et al. 2002; Fukata and Arditi 2013; Shi and Mu 2017).

This effect is mediated not only by the indirect mechanisms outlined above but also by direct interactions with the intestinal immune system. MAMPs play a pivotal role in activating or silencing the host's innate as well as the adaptive immune system. Several cell types at GI mucosal interfaces express PRRs including enterocytes and phagocytes. PRRs include cytoplasmic and membrane-associated TLRs, C-type lectin receptors, NOD-like receptors, and RIG-I-like receptors. PRR activation can result in pro- or anti-inflammatory responses, depending on the MAMP being recognized. One of the most prominent MAMPs is lipopolysaccharide (LPS) of Gram-negative bacteria which is sensed by the host TLR4/MD-2 receptor complex. Interestingly, some commensal bacteria such as many *Bacteroides* spp. harbor lipopolysaccharide structures which fail to activate the TLR4/MD-2 receptor complex. This is thought to contribute to tolerate in the presence of beneficial commensal bacteria. However, PRR-mediated MAMP sensing is important for recognition and subsequent clearance of enteropathogens as well as for epithelial cell proliferation, maintenance of tight junctions, and AMP release as outlined above (Buffie and Pamer 2013; Sharma et al. 2010).

Importantly, PRRs are not evenly expressed on IECs but display a polarized distribution pattern. Extracellular TLRs, for instance, are usually expressed only at the basolateral plasma membranes of enterocytes (Yu and Gao 2015). This appears to be necessary in order to maintain immune balance and avoid over-activation of innate and adaptive immune responses since PAMPs are effectively sensed when luminal content crosses the epithelial barrier but are rarely recognized at the luminal side of enterocytes.

Besides enterocytes, PRRs are also expressed by phagocytes. Among them, intestinal dendritic cells (DCs) fulfill a crucial role by linking the innate with the adaptive immune system. Intestinal DCs can sense microbes either with participation of neighboring goblet cells (McDole et al. 2012), with the support of $CX3CR1^{+}$ phagocytic cells (Mazzini et al. 2014), or via direct sampling of luminal antigens by DC dendrites extending through the epithelium into the lumen (Farache et al. 2013). DCs play an important role in establishing a protective adaptive immunity against pathogens. Furthermore, they crucially contribute to tolerance of commensal bacteria and food antigens (Shiokawa et al. 2017). DCs impact B cell- as well as T cell-mediated immune responses via the expression of certain surface molecules or secreted proteins. This process is tightly regulated and dependent on

the sampled antigen. DCs can either display a mature phenotype which is characterized by strong expression of T cell-activating surface molecules and pro-inflammatory cytokines leading to inflammatory processes, or a tolerogenic phenotype favoring maintenance of immune homeostasis (Steimle and Frick 2016).

T cell-mediated immunity shaping is one of the main consequences resulting from activation of intestinal DCs. Naïve $CD4^+$ T cells encountering activated DCs can develop into different T cell subsets. These subsets are characterized by the expression of certain transcription factors, surface markers, or the secretion of key cytokines which are T-bet and IFNγ for Th1 cells, GATA-3 and IL-4 for Th2 cells, or Foxp3 and CD25 for Tregs. Th17 cells play an ambivalent role. While $ROR\gamma t^+$IL-17^+IL-10-Th17 cells are attributed to inflammation-driving properties, $ROR\gamma t^+$IL-17-IL-10^+Th17 cells are reported to have homeostasis-promoting functions, i.e., by supporting the integrity of the intestinal barrier. Additionally, $ROR\gamma t^+$Th17 cells can develop into inflammation-promoting so-called $ROR\gamma t^+IFN\gamma^+$Th1-like Th17 cells (Fig. 1). This dichiotomic role of Th17 cells was recently excellently reviewed by Stockinger et al. (Stockinger and Omenetti 2017).

Concerning adaptive immune responses, T cell populations are not the sole immune cells being affected by the intestinal microbiota. B cell populations, i.e., intestinal plasma cells, are also influenced by luminal microbes. Intestinal plasma cells secrete IgA which is transported transepithelially into the GI lumen. Here, they coat bacteria leading to reduced bacterial interaction with host immune components. Interestingly, pathobiotic commensals are preferably coated with plasma cell-derived IgA. This process strongly contributes to homeostasis and antagonizes dysbiosis. Intestinal plasma cell-induced IgA secretion can be either T cell-mediated or T cell-independent (Fagarasan et al. 2010). IgA-producing plasma cells are influenced indirectly, via a T cell-dependent regulation loop, or directly by intestinal dendritic cells, i.e., via microbiota-dependent regulation of the expression of B cell-influencing factors such as BAFF or APRIL (Tezuka et al. 2011) (Fig. 1).

Based on these implications, it does not seem surprising that the intestinal microbiota impacts the outcome or progression of inflammatory disorders, especially autoimmune diseases. However, little is known about the definite mechanisms by which the microbiota specifically affects disease progress. Prominent contributing factors include a disturbed microbiota-mediated regulation of cathepsin S expression and/or activity, a protease of antigen-presenting cells (APCs) influencing subsequent T cell activation, and a dysregulated systemic Th17 response which is strongly affected by the local intestinal immune system (Steimle et al. 2016). Apart from Th1 and Th2 responses, characterized mainly by IFNγ and IL-4 secreting $CD4^+$ T cells, respectively, a so-called Th17 response has gained more and more attention when it comes to microbiota-mediated immunological disorders. Th17 cells are found primarily in the gut under homeostatic conditions (Ivanov et al. 2009). However, they seem to be able to migrate to other organs or compartments under dysbiotic conditions further promoting disease progress (Krebs et al. 2016). The Th17 response plays an ambivalent role in this context since Th17 cells may express significantly different properties, depending on their particular

cytokine secretion pattern, from disease-driving to protective functions. Furthermore, the outcome of experimental autoimmune encephalomyelitis (EAE), a mouse model for MS, is highly dependent on the microbiota composition, and enhanced Th17 cell activation is associated with a more severe pathology in this model (Lee et al. 2011).

Microbiota-triggered T cell immune responses are in fact shared among all microbiota-influenced autoimmune disorders (AIDs) where they contribute strongly to the extent of the respective pathology. In fact, AIDs such as MS, IBD, rheumatoid arthritis (RA), and T1D are linked to dysregulated Th17 responses in human patients (Chen et al. 2016; van Langelaar et al. 2018; Tang et al. 2017; Stadhouders et al. 2018; Fores et al. 2018; Baharlou et al. 2016), an association that is experimentally supported in mouse models for these diseases (Lee et al. 2011; Wu et al. 2010; George et al. 2016).

T cell immunity is strongly shaped by the intestinal microbiota, as demonstrated by the complete absence of Th17, Th1, and regulatory T cells (Tregs) in GF mice (Geuking et al. 2011). Tregs aggregate in the mammalian intestine helping to maintain the host's immune homeostasis (Littman and Rudensky 2010; Ivanov et al. 2008). The importance of these cells is underlined by the observation that a depletion of Tregs results in abnormal repopulation of microbiota-directed $CD4^+$ T cells leading to pathological inflammatory reactions (Kamada et al. 2013). AID patients often exhibit intestinal irregularities such as reduced numbers of intestinal Tregs resulting in enhanced inflammatory reactions or impaired gut barrier function (Wu and Wu 2012; Rosser and Mauri 2016). This is often traced back to a general microbiota dysbiosis in these individuals, characterized by reduced α-diversity. In fact, human AID patients provide a distinct microbiota composition compared to healthy controls. This was, for example, demonstrated in patients suffering from T1D (Giongo et al. 2011; d'Arminio Monforte et al. 2014; Davis-Richardson et al. 2014; Li and Atkinson 2015), MS (Bhargava and Mowry 2014; Miyake et al. 2015), RA (Scher et al. 2013; Maeda et al. 2016; Liu et al. 2013), IBD, or systemic lupus erythematosus (SLE) (Hevia et al. 2014). Of course, most of these studies are correlation-based studies, not elucidating if microbiota dysbiosis is cause or consequence of the disease. Usually, it is assumed that a certain microbiota composition is not the exclusive cause of such pathologies since the induction of the disease usually requires a certain predisposition, which is mostly genetic, and/or specific environmental influences.

Most of the microbiota-related research, especially concerning the influence of intestinal microbes on human health, focuses on commensal and enteropathogenic bacteria, and their direct or indirect impact on the host. Surprisingly little is however known about interactions between members of the intestinal microbe consortium and how these interactions affect microbiota composition and the host. One group of understudied organisms in this context are fungi.

3 Commensal Fungi as Part of the Microbiota

Currently, studies of the human microbiota often focus on bacteria only. Historically, this can be mostly explained by technical reasons. On the one hand, the range of microbes detected in culture-based analyses depends on the media used and thereby excludes viruses and most protozoa. While many fungi do grow on media for bacterial cultivation, they are vastly outnumbered by bacteria in the healthy gut (Qin et al. 2010), and thus difficult to assess quantitatively. Sequencing-based approaches, on the other hand, are often technically biased by the DNA extraction protocols toward isolation of bacterial genomes (Underhill and Iliev 2014), and targeted sequencing of 16S rDNA limits the range of included microbes to bacteria.

While it is true that fungi are vastly outnumbered by bacteria (Qin et al. 2010), one has to keep in mind the size and volume differences between bacteria and fungi and the resulting differences in biomass. It was estimated that the average bacterium relates to a yeast cell as a human to an elephant (Chaudhuri 2016). In addition, fungi might fill a unique niche by producing metabolites specific to fungi. And in fact, recent studies dissecting the mycobiome identified 66 genera of fungi present in human stool samples of which *Saccharomyces*, *Candida,* and *Cladosporium* were the most abundant genera (Hoffmann et al. 2013). Still, how the mycobiome is shaped and how it influences the human host is largely unknown.

3.1 The Birth of a Mycobiota Community

Similar to the bacterial microbiota, the human mycobiota is inherited during and after birth directly from the mother and other individuals living in close contact (Bliss et al. 2008; Nagata et al. 2012). Studies on the mycobiota of newborns revealed that the most abundant fungal species found in the intestinal tract were *Candida parapsilosis*, *C. tropicalis*, *C. albicans*, *Saccharomyces cerevisiae*, and *C. orthopsilosis*, corresponding to the mother's vaginal microbiota. For vaginally born babies, *C. albicans* was the most dominant fungus on the skin (Juyal et al. 2013; Ward et al. 2018). After birth, the intestinal fungal population within an individual is subject to changes influenced by different internal and external factors. In contrast to the bacterial population, in which diversity reaches a maximum at adulthood and declines with old age (Yatsunenko et al. 2012), the mycobiota seems to follow an opposite trend: Diversity seems to be lowest during adulthood and higher in infants and the elderly (Strati et al. 2016) (Fig. 2). Cross-kingdom competition and colonization resistance mediated by the complex bacterial microbiota likely contribute to this, but according to our knowledge no studies have directly addressed this hypothesis. External factors shaping our entire microbiota and likely also affecting our mycobiota are, for instance, lifestyle (Evans et al. 2014; Misic et al. 2015),

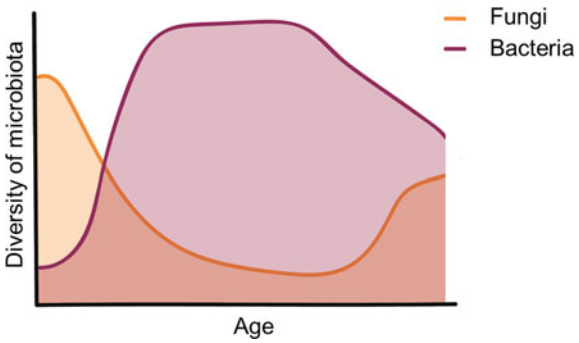

Fig. 2 Diversity of bacteria and fungi in the gut over time. The diversity of the microbiota is changing with age. Fungi are dominant after birth when bacterial diversity is low. After bacterial species become more abundant, the fungal diversity drops. This trend is reversed with high age. Illustration by M. Kapitan

nutrition (David et al. 2014), and external acquisition of new microorganisms (Strati et al. 2016).

3.2 Yeasts Commonly Associated with Humans

Some *Saccharomyces* and *Candida* spp. are commonly associated with humans. Among those, some species are thought to be strictly host-associated, while others have also environmental niches or purpose during food production. One example for the latter is the baker's yeast *S. cerevisiae*. This yeast is probably the fungus that had the largest impact on human life, culture, society, and science (Chambers and Pretorius 2010) and is one of the dominant fungal species found in the gastrointestinal (GI) tract (Hoffmann et al. 2013). Its environmental niche seems to be fruit, especially grapes, on which *S. cerevisiae* ferments sugars if the peel is damaged (Goddard 2008). This fermentation capacity has been exploited by humans for the production of bread and alcoholic beverages. Because of its natural niche on fruit and its widespread use in food production, it is not surprising that this yeast gets introduced frequently into the human GI tract. *S. cerevisiae*, like other environmental fungi, survives passaging through the GI tract (Strati et al. 2016). It is, however, not fully clear whether occurrence of *S. cerevisiae* in stool indicates transient passage or whether *S. cerevisiae* permanently resides in the gut. Because of its widespread and safe utilization, *S. cerevisiae* was even considered to be used as a probiotic (Pennacchia et al. 2008). A close relative, the yeast *S. boulardii*, is already used as a commercially available probiotic (Kelesidis and Pothoulakis 2012). As already demonstrated for the first time in the 1980s in animal and human experiments, its major benefits seem to be the reduction of *Clostridium difficile* growth (Massot et al. 1984) and positive effects on the intestinal immune system (Corthier et al. 1986).

The genus *Candida* comprises over 150 related species of which 15 are known to cause opportunistic infections in humans. Out of these, the most common causes of disease are *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis,* and *C. krusei*

(Yapar 2014). *C. parapsilosis*, *C. tropicalis,* and *C. krusei* account for approximately 17, 10, and 2% of fungal bloodstream infections worldwide, respectively (Pfaller et al. 2011). While these fungi can be found as part of the human microbiota, for instance, *C. tropicalis* in the gut (Roilides et al. 2003) and *C. parapsilosis* on the skin (Trofa et al. 2008), they all occur also in the environment (Carruba et al. 1991; Yang et al. 2012). The carriage rate of *C. krusei* in humans seems to be quite low, yet it is an important fermenter of cacao seeds (Nielsen et al. 2005) and therefore has an environmental niche intertwined with humans. *C. tropicalis* is widely present in soil (Yang et al. 2012), and *C. parapsilosis* can be found in a variety of environmental niches such as soil, water, plants, and insects (Gadanho and Sampaio 2005; Medeiros et al. 2008; Suh et al. 2008).

In contrast, *C. albicans* and *C. glabrata*, the two most commonly isolated *Candida* species associated with candidiasis, are found predominantly or even exclusively in association with warm-blooded hosts. *C. glabrata* is genetically more closely related to *S. cerevisiae* than to other *Candida* species (Kurtzman and Robnett 1998). It grows exclusively as yeast and is a colonizer of the human oral cavity and GI tract (Anderson 1917). Interestingly, colonization rates are rather low but positively correlated with age (Malani et al. 2011). Whether an environmental niche for *C. glabrata* exists is still debated (Gabaldon and Carrete 2016). It is associated with birds from where it could be transmitted directly or indirectly to humans or other animals, but it is not yet fully clear if these hosts are the natural niche or just a temporary reservoir for this fungus. One study found *C. glabrata* on spoiled oranges (Koc et al. 2007), but that might be due to contamination by bird droppings. *C. albicans* has been found only in association with warm-blooded animals including humans. It is present on most mucosal surfaces like the GI and urogenital tract, the mouth, but also the skin of at least 60% of healthy individuals (Calderone and Clancy 2012). In the absence of underlying risk factors, infections with *C. albicans* are usually superficial. Oral infections, also known as thrush, and skin infections, such as diaper's rush, are found especially in infants, in which a stable microbiota has yet to develop (Mohamadi et al. 2014). Oral candidiasis occurs predominantly in HIV-positive individuals, but also in the elderly, where it is often associated with oral prostheses (Bianchi et al. 2016), which may influence local oral microbiota composition. 75% of otherwise healthy women experience at least one vaginal infection in their life, often following the use of antibiotics and/or oral contraceptives that influence the vaginal environment (Brown et al. 2012). Severe, life-threatening disseminated *Candida* infections usually only occur in immunocompromised individuals. In addition to immunosuppression, indwelling catheters (as a source of biofilm-related infections), and traumatic or surgical breaches of the intestinal barrier are common risk factors for candidiasis (Yapar 2014). It was demonstrated that the majority of *C. albicans* bloodstream infections (BSIs) are caused by endogenous strains from the patient's own gut (Odds et al. 2006), illustrating the role of the gut as a source for opportunistic fungal infections. Furthermore, antibiotic treatment predisposes not only for mucosal but also systemic *Candida* infections (Pappas 2006). Depletion of the bacterial microbiota is frequently associated with enhanced fungal colonization which in turn correlates

with the likelihood of the development of candidiasis (Azevedo et al. 2015). On the other hand, it was demonstrated that a significant proportion of *C. albicans* infections are polymicrobial, so *Candida* is accompanied by bacteria in the respective specimen (Kim et al. 2013; Pulimood et al. 2002; Bouza et al. 2013; Klotz et al. 2007; Hermann et al. 1999). This clearly indicates that bacterial–fungal interactions could play an important role in the development of invasive candidiasis. As *C. albicans* is by far the best-studied *Candida* species, we will in the following focus on this species to discuss what is known so far about the life of *Candida* in the gut, and its interactions with the host and bacteria in this niche.

3.3 *Life of* Candida Albicans *in the Gut*

The intestinal environment varies significantly along the length of the GI tract and across the diameter of the intestine (Zheng et al. 2015; Khutoryanskiy 2015; Fischbach and Sonnenburg 2011; Flint et al. 2012). The astonishing transcriptional and metabolic versatility of *C. albicans* allows the fungus to thrive within these diverse environments and also facilitates survival during local and systemic infections (Brown et al. 2014; Ene et al. 2014). Interestingly, the expression pattern of *C. albicans* colonizing the murine gut appears to be distinct from that observed during tissue invasion (Rosenbach et al. 2010; Thewes et al. 2007; Walker et al. 2009; Zakikhany et al. 2007), suggesting a specific adaptation to a commensal lifestyle in this host niche. This is supported by studies that analyzed the role of distinct transcription factors for *C. albicans* survival in different host niches. For example, *C. albicans* Efg1 is important for virulence during systemic candidiasis, but in the gut, *EFG1*-deficient *C. albicans* strains were found to be more efficient colonizers (Pierce et al. 2013). Contrarily, the transcription factor Efh1 appears to actively promote *C. albicans* commensalism but is dispensable for systemic infections (White et al. 2007). Reciprocal roles for transcription factors have also been described in the context of iron acquisition: The transcriptional repressor Sfu1, which reduces iron uptake, is required for the persistence of *C. albicans* in the gut. In contrast, the positive regulator Sef1, mediating enhanced iron acquisition, is essential for virulence in a mouse model of systemic infection (Chen et al. 2011).

Another important feature of *C. albicans* that appears to be differentially regulated in the gut, compared to other host niches, is morphogenesis. One of *C. albicans*' most remarkable features is its morphological flexibility (Noble et al. 2017). The typical growth form at temperatures at or below 30 °C in the laboratory is the budding yeast, also known as "white cells." Upon certain environmental cues (e.g., shift to 37 °C, elevated levels of CO_2, contact to surfaces, serum components), *C. albicans* is, however, able to filament and form pseudohyphae or true hyphae (Noble et al. 2017; Sudbery 2011; Wang and Xu 2008). While yeasts proliferate faster, hyphae are capable of penetrating tissue, leading to invasion (Berman and Sudbery 2002) and also contributing to immune evasion (Torosantucci et al. 2004). Therefore, it is not surprising that filamentation is

associated with virulence in systemic, but also mucosal infections (Katagiri et al. 2018). Interestingly, hyphae are rarely observed in the gut, even though this niche provides strong filamentation cues such as body temperature, high CO_2, and contact. Consequently, this observation implies that even stronger hyphal repression signals occur in this niche. While it is tempting to speculate that members of the microbial gut community may contribute to filamentation repression, filamentation was not observed in GM mice colonized with only *C. albicans* (Böhm et al. 2017), suggesting that it is the intestinal environment, rather than the microbiota, that promotes growth in the yeast form. Furthermore, several studies found that genetically forcing *C. albicans* into the filamentous form reduces colonization levels (Böhm et al. 2017; Rosenbach et al. 2010; Vautier et al. 2015).

In addition to the "white cell" morphotype *C. albicans* can undergo transition into several other yeast phenotypes that are metabolically distinct from each other. Opaque cells, for instance, grow as elongated, spiky single cells bigger than white cells and are able to mate (Hickman et al. 2013). The white to opaque transition is regulated by a network of different transcription factors of which the two main regulators are Efg1 and Wor1, which repress each other (Morschhäuser 2010). While Wor1 promotes cells to switch to the opaque phenotype, Efg1-dominated cells are white. At 37 °C, however, opaque cells are instable and switch back to the white form (Slutsky et al. 1987), which makes mating inside the gut niche unlikely.

Recently, two other phenotypes have been discovered. The "gray cell" type is interconnected with the white–opaque transition, but is independent of the mating-type locus (Tao et al. 2014). While the role of opaque and gray cells for commensalism remains unclear, the morphotype GI-induced transition (GUT) was observed after multiple transitions of *C. albicans* through the mouse gut. Of note, similar to opaque cells, overexpression of Wor1 is necessary for the switch to GUT cells. However, GUT cells are elongated but smooth compared to opaque cells and thus morphologically distinct (Pande et al. 2013). GUT cells outcompete white cells in a mouse colonization model probably due to improved adaptation to the gut niche and a more efficient metabolism (Pande et al. 2013). Taking into consideration that Efg1 is involved in filament formation (Stoldt et al. 1997) and low Efg1 levels in *C. albicans* promotes colonization of the mouse gut (Pierce et al. 2013), it appears that these different yeast cell types have distinct roles in different host niches, regulated by Efg1 and Wor1. While Efg1 is essential for yeast-to-hypha transition and therefore virulence (Lo et al. 1997), Wor1 seems to play a major role during commensalism and therefore colonization.

Remarkably, although *C. albicans* grows predominantly as yeast in the murine intestinal tract, it was shown that the local population expressed several genes that were previously assumed to be exclusively involved in or associated with hyphal growth (Herwald and Kumamoto 2014; Rosenbach et al. 2010). Furthermore, no filamentation was observed in GF mice while earlier studies reported a low level of filamentation in the gut of antibiotic-treated, *C. albicans*-colonized mice (Böhm et al. 2017). This could indicate that a tightly regulated expression of hypha- and virulence-associated fungal attributes in the gut is supporting *C. albicans* during its interaction with the microbiota.

3.4 *Antagonistic Interactions Between Commensal Bacteria and* Candida Albicans*—Limiting Fungal Colonization*

The potential of the commensal microbiota to limit fungal proliferation in the gut appears obvious given the clinical observation that depletion of the commensal microbiota by prolonged use broad-spectrum antibiotics frequently results in fungal overgrowth (Ianiro et al. 2016; Eggimann et al. 2015; Mason et al. 2012b; Erb Downward et al. 2013; Pasqualotto et al. 2006). Colonization resistance by the commensal microbiota can be achieved in different ways, as outlined in Sect. 2.1. However, whether distinct members of the microbiota are responsible for colonization resistance against *C. albicans* and by which mechanisms this is mediated is not yet well understood.

One of the best-studied examples for antagonistic *Candida*–bacteria interactions are lactic acid bacteria (LAB). They are characterized by their ability to ferment glucose to lactic acid, but they also produce bactericins and anti-fungal peptides (De Vuyst and Leroy 2007) (Fig. 3). Lactobacilli are the dominant species of the vaginal microbiota where they prevent urogenital infections by other microbes (Vasquez et al. 2002), including *C. albicans*, by acidification of the vaginal mucosa through lactic acid (Aroutcheva et al. 2001).

Work by Mason et al. aimed to clarify how *C. albicans* and lactobacilli interact in the gut and stomach after antibiotic treatment in mice. In the presence of *C. albicans,* lactobacilli are repressed in their capacity to recolonize the stomach after antibiotic treatment is discontinued. Instead of lactobacilli, enterococci became the dominant species. This effect was reversed when fungal cells were absent (Mason et al. 2012a). Similar results were observed in the colon, where the fungus also led to faster recovery of the *Bacteroidetes* population (Mason et al. 2012b). One possible explanation is that *C. albicans* directly antagonizes lactobacilli: Yet again, fungal promotion of enterococcal growth, which then leads to repression of other Gram-positive bacteria by secretion of bactericins, could also explain the observations (Sawa et al. 2012). Lactobacilli can potentially antagonize *C. albicans* by the production of SCFAs, mainly acetate, propionate, and butyrate (Pessione 2012). SCFAs not only promote intestinal barrier function, they have also been shown to inhibit *C. albicans* filamentation and therefore invasiveness (Noverr and Huffnagle 2004) (Fig. 3). However, in the colon lactobacilli usually comprise less than 2% of the overall microbiota and the majority of SCFAs are produced by a few other bacterial taxa and species. The most important producer for propionate, for example, is the mucus-degrading bacterium *Akkermansia muciniphila* (Derrien et al. 2004), while butyrate is mainly produced from resistant starch by *Ruminococcus bromii* and a few other species (Louis et al. 2010; Ze et al. 2012). Yet, lactobacilli-derived molecules might contribute significantly to colonization resistance in distinct intestinal niches or in distinctly composed microbiota. Also, effects mediated by other colonization resistance mechanisms appear possible.

Besides lactobacilli, other anaerobic intestinal bacteria can mediate colonization resistance. In the presence of Gram-negative bacteria, IECs and Paneth cells secrete

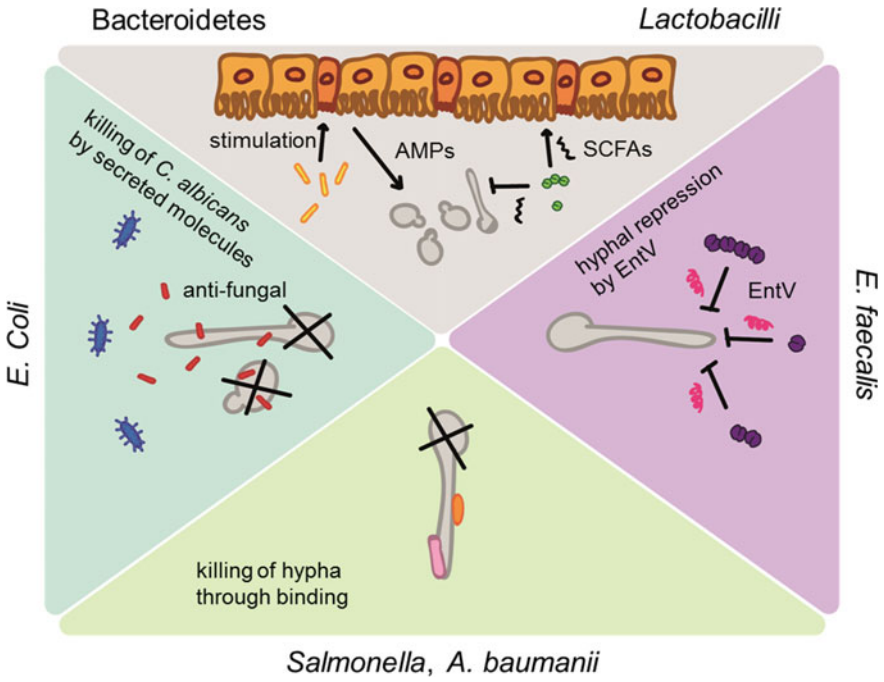

Fig. 3 Antagonistic interactions between fungi and bacteria. *Top*: Bacteroidetes species can stimulate intestinal cells to produce more HIF1α which leads to secretion of antimicrobial peptides (AMPs) that in turn promote colonization resistance against *C. albicans* in a mouse model. Lactobacilli can secrete short-chain fatty acids which are the main source of energy for enterocytes and inhibit filamentation and invasiveness of *C. albicans* in vitro. *Right*: *E. faecalis* can secrete a small peptide termed EntV that inhibits filamentation in nematodes. *Bottom*: *Salmonella* species and *Acinetobacter* (*A.*) *baumanii* directly bind to the filamentous form of *C. albicans* which leads to killing of the fungus in vitro. *Left*: *E. coli* secretes an anti-fungal peptide during magnesium depletion that inhibits fungal growth in vitro. Illustration by M. Kapitan

RegIIIγ, a C-type lectin which is highly active against Gram-positive bacteria (Cash et al. 2006). Experiments in mice showed that this immune stimulation can protect the host against vancomycin-resistant *Enterococcus faecium* colonization (Brandl et al. 2008). Similar mechanisms have been found for fungi: Fan et al. (Fan et al. 2015) demonstrated that the presence of two anaerobic bacterial species, *Bacteroides thetaiotamicron* and *Blautia producta*, was sufficient to clear *C. albicans* from the mouse gut. In the presence of fungi, these bacteria stimulated intestinal cells to express more HIF1α, a regulator of the innate immune system (Nizet and Johnson 2009) promoting expression of cathelicidins, a group of antimicrobial peptides (Peyssonnaux et al. 2005). In mice co-colonized with *C. albicans* and either of these anaerobic bacteria, HIF1α and the cathelicidin

LL-37-CRAMP were significantly upregulated. In humans, LL-37 is active against *Candida* (Lopez-Garcia et al. 2005). These findings demonstrate that commensal bacteria can potentially reduce colonization with fungi indirectly in the colon.

3.5 *Interactions Between Pathogenic Bacteria and* Candida Albicans

Dysbiosis facilitates increased colonization not only with *C. albicans*, but also with opportunistic bacterial pathogens (Bien et al. 2013). A broad spectrum of bacteria commonly found within the gut can cause disseminated infections in the host (Klastersky and Aoun 2004; Mootsikapun 2007). Given that mixed infections involving *C. albicans* and bacteria are not uncommon (Kim et al. 2013), and considering that dysbiosis favors both, growth of *C. albicans* and facultative pathogenic bacteria, in the gut simultaneously (Behnsen et al. 2014; Bien et al. 2013; Nerandzic et al. 2012), the type of interactions between these bacteria and the fungus might have clinical relevance. If fungal–bacterial interactions in the dysbiotic gut are antagonistic, the relative risk of infection might not be affected. In contrast, synergistic interactions might lead to an increased risk and warrant prophylactic treatment.

Acinetobacter baumanii is a Gram-negative bacterium recently emerging as a nosocomial opportunistic pathogen (Lee et al. 2007) difficult to treat due to development of resistances against a variety of antibiotics (Dijkshoorn et al. 2007). Interactions with *C. albicans* have been described as antagonistic. During in vitro coincubation, the bacterium can bind to *C. albicans* hyphae via the *A. baumanii* outer membrane protein A (OmpA) which results in hyphal apoptosis (Gaddy et al. 2009) (Fig. 3). Similarly, coinfection in a *Caenorhabditis elegans* nematode model resulted in reduced lethality mediated by inhibition of fungal filamentation (Peleg et al. 2008). However, it yet needs to be determined if interactions in the mammalian gut are likewise antagonistic.

The most commonly *C. albicans*-associated bacterial group are Gram-positives (Hermann et al. 1999). A variety of interactions have been found for *Streptococcus* (Bamford et al. 2015; Falsetta et al. 2014; Yu et al. 2018) and *Staphylococcus* species, especially *Staphylococcus aureus* (Harriott and Noverr 2010; Kong et al. 2016; Krause et al. 2015; Schlecht et al. 2015; Carlson 1983a, b). However, these interactions are mostly taking place outside of the gut. *Enterococcus faecalis* on the other hand shares a variety of niches with *C. albicans* (Hayashi et al. 2005; Horsley et al. 2013; Komiyama et al. 2016), and interactions in the gut are highly likely (Mason et al. 2012b). The high association between *C. albicans* and *E. faecalis* in clinical settings suggest synergistic relationships or at least neutral coexistence. Yet, the interactions described so far experimentally were antagonistic: In 2013, Cruz et al. (Cruz et al. 2013) observed that nematodes coinfected with *E. faecalis* and *C. albicans* survive longer compared to the respective single infections. This was

partly due to inhibition of hyphae by a small peptide later identified and termed EntV (Graham et al. 2017) (Fig. 3). The peptide alone was shown to be able to protect mice from *C. albicans* infection in an oropharyngeal candidiasis (OPC) model by inhibiting hyphal formation and is also able to dissolve mature fungal biofilms. It is, however, unclear if EntV is expressed in the gut and whether interactions in this niche are also antagonistic.

Escherichia coli is a highly diverse bacterial species (Becker et al. 2014; Meador et al. 2014), and thus, it is not surprising that it also appears to be heterogenic in its behavior toward *C. albicans*. Recently, the *E. coli* strain MG1655 was found to secrete a fungicidal molecule that killed *C. albicans* during in vitro co-cultivation under magnesium-depleted conditions (Cabral et al. 2018). In contrast, enterohemorrhagic *E. coli* (EHEC) led to enhanced invasion and damage of enterocytes in vitro during coinfection, likely mediated by upregulation of hypha-associated genes like *EFG1* and *HWP1* (Yang et al. 2016) (Fig. 3). Such fungal–bacterial synergism was also observed in vivo during intraperitoneal coinfection of mice. While no mice died during single infection with either species, coinfection with both microorganisms led to drastically enhanced mortality (Klaerner et al. 1997). One strain of *E. coli* was furthermore shown to enhance fungal attachment to the bladder mucosa of rats and led to higher virulence during urinary tract infection (Levison and Pitsakis 1987). Of note, it was also demonstrated that supernatants of *E. coli*, similar to *Pseudomonas aeruginosa*, could inhibit biofilm formation in vitro (Holcombe et al. 2010; Bandara et al. 2013). Another in vitro study observed promotion of growth and proliferation of *E. coli* K12 in the presence of *C. albicans*. This effect was dependent on iron acquisition mechanisms from both microbial species. Deletion of either the fungal genes *SEF1* or *SFU1*, transcription factors involved in iron uptake (Chen et al. 2011), or the bacterial ferric transporters FepD or FebG abolished growth promotion (Chenault and Earhart 1992). Interestingly, this growth promotion also occurred with *E. coli* mutants lacking their own siderophores. The authors suggested that a fungal siderophore-like molecule might support *E. coli* in taking up environmental iron. Even though the mechanism is poorly understood, it might be potentially important in the gut under iron limitation occurring in certain microniches. *C. albicans* could help stabilize the microbiota in its vicinity by yet unknown secreted factors depending on the environmental setting and interacting partner.

Metal acquisition is also important during *Salmonella* infection. *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) colonization leads to severe inflammatory diarrhea (Hohmann 2001). In response to *Salmonella* infection, the host restricts iron as well as zinc and manganese, triggered by induction of the cytokine IL-22 (Godinez et al. 2008). IL-22 leads to secretion of different antimicrobial peptides such as RegIIIβ and RegIIIγ (Stelter et al. 2011), two C-type lectins able to kill Gram-negative and Gram-positive commensal bacteria but not *S. typhimurium* (Cash et al. 2006; Stelter et al. 2011). Two other downstream targets of IL-22 are lipocalin-2, which binds to the bacterial siderophore enterochelin preventing iron acquisition (Raffatellu et al. 2009), and calprotectin, a zinc and manganese chelator (Corbin et al. 2008; Sohnle et al. 1996). This leads to

repression of commensal bacteria like *E. coli* and overgrowth of *S. typhimurium* (Behnsen et al. 2014). IL-22 has, however, no effect on *C. albicans* (Kagami et al. 2010). Direct antagonism between *C. albicans* and *S. typhimurium* has been also described in a nematode model where bacteria attached to hyphae and actively killed fungal cells over a type III secretion system (Kim and Mylonakis 2011) (Fig. 3).

In contrast to potentially harmful interplays with certain bacteria, *C. albicans* protects the host by antagonistic interactions with pathogens like *Clostridium difficile* (Markey et al. 2018). *C. difficile* is a Gram-positive obligate anaerobic spore-forming bacterium which can cause severe diarrhea and colitis, often with a lethal outcome (Burke and Lamont 2014). A major risk factor for infection is antibiotic treatment, which allows *C. difficile* to overcome colonization resistance provided by the commensal microbiota (Pepin et al. 2005). One last resort against reoccurring *C. difficile* infection is fecal microbiota transplantation (van Nood et al. 2013). It was previously shown that *C. albicans* can allow the obligate anaerobe *C. difficile* to grow under aerobic conditions during co-cultivation. This effect was independent of fungal biofilm formation which has been known to promote anaerobic growth (Fox et al. 2014); on the contrary, the bacterium secreted a molecule *p*-cresol which inhibited hyphal formation and led to conversion of hyphae back to yeast cells (van Leeuwen et al. 2016). This implies that *C. albicans* might provide a niche for the bacterium to thrive in. However, a recent in vivo study demonstrated that pre-colonization of mice with *C. albicans* protects the animals against a lethal challenge with *C. difficile* (Markey et al. 2018). This protective effect was not due to higher colonization resistance in the presence of the fungus, even though microbiota composition was significantly altered for some bacterial species in *C. albicans*-colonized mice, e.g., *Akkermansia* and *Bifidobacterium* species. *Clostridium*-derived toxin concentration was also unaltered in both groups. However, fungal colonization resulted in higher levels of pro-inflammatory IL-17A which attracts neutrophils (Nakagawa et al. 2016; Rubino et al. 2012) and promotes epithelial integrity via tight junctions (Lee et al. 2015) (Fig. 4).

While the exact mechanism by which *C. albicans* protects the host from pathogenic bacteria remains to be identified, these findings demonstrate that intestinal commensal fungi might protect the host against microbial threats under certain circumstances. Indeed Jiang et al. (Jiang et al. 2017) could show that mono-colonization by either *C. albicans* or *S. cerevisiae* after antibiotic treatment could protect mice from chemically induced colitis. Mice carrying commensal *C. albicans* did not show signs of colonic shortening or inflammation and in this regard behaved like mice with intact microbiota. Strikingly, intestinal mono-colonization with both fungi mediated protection against mortality upon pulmonary Influenza A virus infection. Responsible for this protection were mannans present in the fungal cell wall (Ruiz-Herrera et al. 2006) which led to the accumulation of protective immune cells. This work demonstrates that while antibiotic treatment favors fungal growth, this might not necessarily be detrimental,

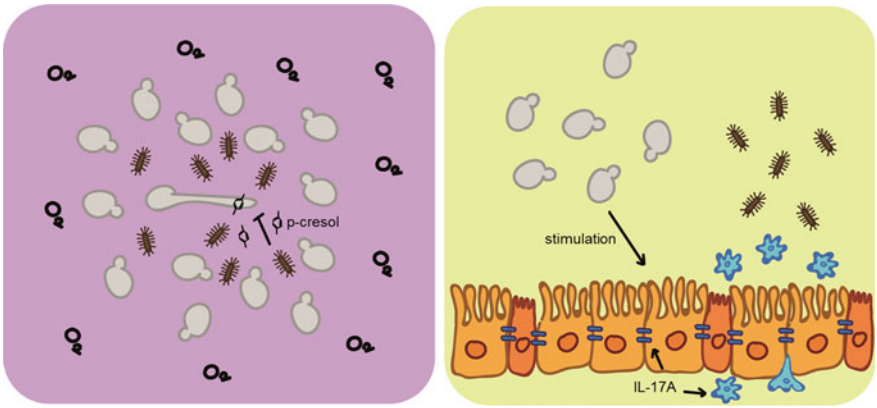

Fig. 4 Interactions between *C. albicans* and *Clostridium difficile*. *Left*: The obligate anaerobic bacterium *C. difficile* is able to grow in the presence of *C. albicans* in vitro under normoxic conditions. This effect is independent of fungal biofilm formation. In fact the bacterium secretes p-cresol, a small molecule that prevents fungal filamentation and therefore biofilm formation. *Right*: Intestinal colonization with *C. albicans* protects mice against *C. difficile*-induced killing. The fungus stimulates intestinal cells to secrete more IL-17A which boosts enterocyte regeneration, tight junctions' expression and attracts protective immune cells. Illustration by M. Kapitan

but that under distinct circumstances, fungi can also counteract infections by other microbes.

4 Conclusion

Historically, the view on *C. albicans* and other commensal fungi was dominated by their ability to cause opportunistic infections and their role during food digestion. Comparatively little is, however, known on the role of fungi as part of the microbiota in the gut. While it is obvious that bacteria mediate colonization resistance against *C. albicans*, its interactions with distinct bacterial species and strains appear to range from antagonistic to synergistic and likely depend on the environment in which these interactions take place. Under discrete circumstances, *C. albicans*, which occurs in the gut microbiota of the majority of humans and appears to be adapted to the human host, may even have beneficial effects on the host. With the growing understanding about the role that the microbiota has in different aspect of human life and well-being, it becomes increasingly clear that we also need a better understanding of fungal functions inside the bacterial community. For this, interactions between fungal and bacterial species need to be investigated in more detail. Identifying mechanisms of interactions and the environmental factors that affect these interactions will provide a basis for personalized risk assessment in

patients with dysbiosis. And even though fungi are mainly considered relevant as pathogens, they may provide benefits to the host that yet remain to be discovered.

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