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Melanin as a virulence factor of *Paracoccidioides brasiliensis* and other dimorphic pathogenic fungi: a minireview

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Abstract Melanin pigments are substances produced by a broad variety of pathogenic microorganisms, including bacteria, fungi, and helminths. Microbes predominantly produce melanin pigment via tyrosinases, laccases, catecholases, and the polyketide synthase pathway. In fungi, melanin is deposited in the cell wall and cytoplasm, and melanin particles ("ghosts") can be isolated from these fungi that have the same size and shape of the original cells. Melanin has been reported in several human pathogenic dimorphic fungi including *Paracoccidioides brasiliensis*, *Sporothrix schenckii*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Coccidioides posadasii.* Melanization appears to contribute to virulence by reducing the susceptibility of melanized fungi to host defense mechanisms and antifungal drugs.

Keywords *Paracoccidioides brasiliensis* · Melanin · Dimorphic fungi · Susceptibility · Pathogenesis

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Introduction

Melanin pigments are multifunctional polymers ubiquitous in nature as they are produced by a remarkable variety of organisms, including bacteria, fungi, plants, and animals. Melanins are negatively charged, hydrophobic, and high molecular-weight compounds, insoluble in aqueous and organic solvents and consequently are difficult to study by conventional biochemical and biophysical techniques. The knowledge about the structure of this pigment is, therefore, limited [1, 2]. They are formed by the oxidative polymerization of phenolic and/or indolic compounds and usually are dark brown or black, although melanin pigments with other colors also exist [1]. A discussion on the contribution of melanin to microbial pathogenesis has attracted considerable interest, with melanin being focused on as a putative virulence factor in fungi.

Although the synthesis of melanin in mammals is typically catalyzed by tyrosinases [3], microbes generally use the polyketide synthase pathway and/or phenoloxidases (like laccases) [4]. Recognized

human fungal pathogens that form melanin precursors by the polyketide pathway include *Exophiala (Wangiella) dermatitidis*, *Sporothrix schenckii*, *Alternaria alternata*, *Cladosporium carionii*, *Fonsecaea pedrosoi*, *Aspergillus niger*, *A. nidulans*, and *A. fumigatus* [5–9]. The production of DOPA-melanin via phenoloxidases in fungi has also raised scientific interest [10]. The synthesis of melanin in fungi by the DOPA-melanin pathway, also known as eumelanin, requires the presence of exogenous substrate in the form of certain *o*-diphenolic and *p*-diphenolic compounds, such as 3,4 dihydroxyphenylalanine (L-DOPA) [11]. The pathogenic fungi investigated in the production of DOPA-melanins via polyphenoloxidase (a laccase) include many model organisms such as *Cryptococcus neoformans* [12, 13], *Paracoccidioides brasiliensis* [14, 15], *Histoplasma capsulatum* [16], *Blastomyces dermatitidis* [17], *Candida albicans* [18], and *Coccidioides posadasii* [19].

Treatment of melanized cells by enzymatic digestion of the cell wall, proteolysis, chloroform extraction, and boiling in concentrated HCl results in the recovery of melanin ghosts [14, 20]. Atomic-force microscopy and scanning electron microscopy images reveal that the surface of melanin ghosts derived from melanized *C. neoformans* cells is composed of discrete granules with roughly uniform dimensions, which corresponds to information known for other melanins [21]. Nuclear magnetic resonance cryoporometry shows that melanin ghosts contain pores with diameters between 1 and 4 nm, in addition to a small number of pores with diameters near 30 nm [21]. Additionally, elution of graded dextrans has revealed pores in melanized and nonmelanized cells of 4.0 and 10.6 nm, respectively [22].

Some fungi like *C. neoformans* and several molds can produce melanin in the soil, and this characteristic may provide increased resistance to the environmental stressors [23, 24]. Melanized *C. neoformans* is more resistant to ingestion by environmental amoeboid [25] or nematode [26] species. Melanins also protect fungi from hydrolytic enzymes [27], UV, solar or gamma radiation [28, 29], extreme temperatures [30], and heavy metals and several other toxic compounds [reviewed in 24].

Melanins are immunologically active, however little is known about their relationship with immune systems. Some authors have shown that melanins affect macrophages and reduce proinflammatory cytokines [14, 31, 32]. For example, melanized *C. neoformans* cells can down-regulate the afferent immune response [33]. Melanization *C. neoformans* or yeast melanized in vivo elicits inflammatory changes different from nonmelanized or laccase-deficient cells and also interfere with phagocytosis of encapsulated yeast cells [34]. Melanin-binding antibody can also modify infection and melanin-binding antibodies have been isolated from mice with experimental cryptococcosis and humans infected with *F. pedrosoi* [8, 31]. Melanin-binding antibodies can influence the phagocytosis and intracellular death of diverse fungi [14].

Given the importance of melanin on fungal pathogenesis, it is timely to review recent studies that have expanded our understanding of the impact of melanin production on dimorphic fungi during the infection, the host immune defense, and the resistance to antimicrobial drugs.

Mechanism by which melanin pigments contribute to virulence in human thermally dimorphic fungi

Paracoccidioides brasiliensis

Paracoccidioides brasiliensis is the agent of the human systemic disease paracoccidioidomycosis, which affects individuals in endemic areas extending from Argentina to Central America. It is the most prevalent deep mycosis in Latin America and about 80% of diagnosed patients are from Brazil. Rural workers are the main group at risk for infection, but dwellers of urban centers located on the route of migration movements are also affected. One estimative suggests that ten million people are infected with *P. brasiliensis* and that up to 2% may develop disease [35–37]. Paracoccidioidomycosis is the 8th most common cause of death due to chronic/recurrent infections and parasitic diseases in Brazil [38]. Infection is thought to occur due to inhalation of conidia that subsequently transform into yeast forms within the lung. Although acquisition of the fungus typically results in asymptomatic infection, infection can progress to acute, subacute, and chronic clinical forms of disease [39]. The virulence of the fungi is directly related to the host immune cellular

resistance. However, there are differences in virulence between fungal isolates based on a number of attributes, such as melanin, which appear to contribute to pathogenicity.

The ability of *P. brasiliensis* to make melanin pigments was first described by Gómez et al. [15] (Fig. 1). The authors recovered pigmented particles after chemical and enzymatic treatment of conidia and yeasts grown in vitro (on water agar for conidia and in the presence of L-DOPA with yeasts). They also recovered yeast shaped melanin-like particles from infected mouse tissue after chemical and enzymatic treatment. Particles collected from cells in vitro and in vivo were reactive with antibody to melanin. A laccase-like activity was detected in protein extracts of *P. brasiliensis* that was implicated in the enzymatic synthesis of melanin in yeast cells. Additionally, in vitro conidial melanization is quite significant since conidia become pigmented when suspended in pure water indicating a capacity to synthesize melanin-like pigment in the absence of L-DOPA, which would require the fungus to either synthesize its own phenolic precursors for use via laccase or to utilize a different mechanism, such as the polyketide synthesis pathway, to produce melanin [15].

Relatively, little is known about the localization of melanin in human pathogenic fungi but it has been described in intracellular and/or extracellular (e.g., outside cell membrane) spaces. Some fungal melanin is found as part of the cell wall, often recognizable as a distinct and fairly sharply defined outer layer, and some melanin has been found in association with a fibrillar matrix projecting outward from the cell wall of many fungi. For instance, *H. capsulatum* yeast cells produce melanin in their cell wall and also have granules of melanin extending from their outer cell wall [16]. Similarly, pigmented granules have been identified on the surface of *S. schenckii* yeast [5]. In contrast, in *C. neoformans*, exogenous L-DOPA is used for the synthesis of a melanin layer next to the cell membrane and internal region of the cell wall [40]. In contrast, *C. albicans* produces small spheres of melanin and presumably secretes them as components of biofilm [18]. For *P. brasiliensis*, scanning electron microscopy reveals that melanized and nonmelanized yeast cells have a smooth surface with no major difference between them, but analysis by transmission electron micrographs shows electron-dense granules distributed throughout the cytoplasm and on the cell surface [14].

The ability of melanin to protect microbes from host defense is relevant to antimicrobial therapy because the clinical efficacies of some antimicrobial drugs are complemented by host immune defense mechanisms (reviewed by [24]). Table 1 summarizes the principal differences observed between melanized and nonmelanized yeast cells against host immune defenses and susceptibility to antifungal compounds. Melanized *P. brasiliensis* yeast cells increase resistance to phagocytosis [14]. Since melanins are charged polymers [41] their presence in the cell wall can alter the fungal cell surface charge as shown to occur with melanization in *C. neoformans* [42],

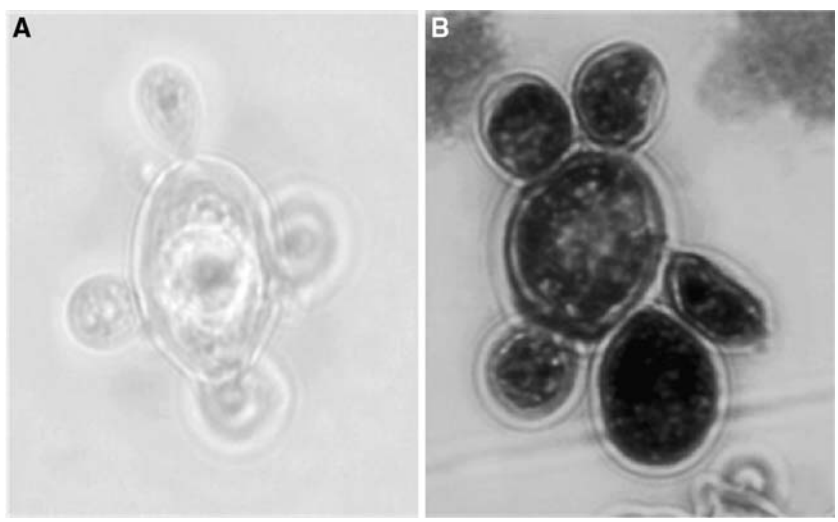

Fig. 1 Light microscopy of *P. brasiliensis* yeast cells grown in liquid medium without (**A**) and with (**B**) L-DOPA. Original magnification 100×

which may contribute to inhibition of phagocytosis. Additionally, melanin can stimulate humoral immune responses. Mice immunized with melanin ghosts from *C. neoformans* produce specific antibodies against melanin [31, 43]. Interestingly although polyclonal antibodies against *P. brasiliensis* melanin can increase phagocytosis of melanized yeast cells by macrophages-like cell lines J774.16 and MH-S (peritoneal and alveolar macrophages, respectively) in vitro [44], polyclonal antibodies against *P. brasiliensis* non-melanin, cell wall antigens do not enhance the uptake of melanized yeast cells [14]. This indicates that melanin interferes with the binding of diverse antibodies resulting in a general reduction in the internalization of melanized cells [14].

In addition to reducing ingestion, melanization protects *P. brasiliensis* against killing by macrophages [14]. Similarly, melanin production in *C. neoformans* [45], *F. pedrosoi* [46], *S. schenckii* [5] and *Exophiala* spp. [47] enhances resistance to killing by phagocytic cells. The resistance of melanized yeast cells of *P. brasiliensis* to internalization by macrophages in vitro and their increased intracellular survival if ingested can explain the higher fungal burden in the lung of Balb/c mice infected intratracheally with melanized cells, when compared with nonmelanized *P. brasiliensis* [44].

The mechanism of increased survival of pigmented *P. brasiliensis* yeast cells is in part due to the fact that melanization protects *P. brasiliensis* against injury mediated by nitrogen- or oxygen-derived free radicals [44] and also enhances resistance to H_2O_2 and hypochlorite as has similarly been shown in *C. neoformans*, *Aspergillus* spp., and *S. schenckii* (Reviewed in [24, 28]). Melanins are highly effective scavengers of free radicals [48] and have electron transfer properties [49]. Electron transfer from free radical species generated in solution to *C. neoformans* melanin has been demonstrated by electron spin resonance spectroscopy [45] and similar spectra have also been generated with melanins from *H. capsulatum*, *S. schenckii*, *P. brasiliensis*, and *Pneumocystis* spp. (Reviewed in [24, 28]).

Melanin is an antifungal resistance factor, given its ability to reduce the susceptibilities of melanized cells to antifungal drugs [50]. Notably, there is no evidence for the involvement of melanin in drug efflux pumps or in alterations in the synthesis of ergosterol or glucans in the fungal cell wall or cell membrane structures (reviewed in [24]). Melanization of *P. brasiliensis* yeast cells does not affect the cytotoxicity of amphotericin B, ketoconazole, fluconazole, itraconazole, sulfamethoxazole as measured by standard CLSI broth macrodilution procedures for assessing the susceptibility of yeast cells to antifungal drugs [14]. Similar results have previously been observed with melanized and nonmelanized *C. neoformans* and *H. capsulatum* [51]. However, an increased resistance of *P. brasiliensis* melanized cells to antifungal drugs mainly amphotericin B, and less pronounced with ketoconazole, fluconazole, itraconazole, and sulfamethoxazole, has been shown using a time-kill assay [14]. The killing assay has also shown that melanized *C. neoformans* and *H. capsulatum* are less susceptibility to amphotericin B and caspofungin, but melanization does not affect cell resistance when fluconazole or itraconazole is used [51].

Laccase is an important virulence factor in many pathogenic fungi. Two different methods have been used to demonstrate that *P. brasiliensis* produces a laccase-like enzyme [14, 15]. In spite of considerable efforts by several investigators, many aspects of the nature of laccase remain unclear. In *C. neoformans*, the ability of laccase to produce melanin pigments and prevention of iron-dependent Fenton reaction products results in enhanced dissemination of yeast forms to the brain, thus strongly correlating the enzyme with virulence [52, 53]. In *C. neoformans*, laccase appears to localize predominantly to the outer region of the cell wall where it can interact more directly with extracellullar substances and host immune products without the need for ancillary membrane or cytosolic transporters [54]. For example, oxidation of catecholamines on the outermost region of the cell wall lessens the exposure to oxidized dopamine products, which are cytotoxic in other systems [55]. Besides the oxidative effects on catecholamine, recombinant cryptococcal laccase exhibits iron oxidation activity converting Fe^{2+} to Fe^{3+}. Iron oxidase activity of laccase may protect *C. neoformans* from alveolar macrophages by oxidation of phagosomal iron to Fe^{3+} with a resulting decrease in hydroxyl radical formation [56]. In addition, laccase activity has been found to be a marker of stress, as the induction of laccase correlated with substrate starvation and presence of potentially toxic metals [57]. It is currently not known whether the *P. brasiliensis* laccase contributes to

Table 1 Melanin as virulence factor of *P. brasiliensis*—general effects and virulence

Characteristics	*P. brasiliensis*		Reference
	Melanized	Nonmelanized	
Morphology[a]			
Hyphae	Absent	o	[14, 15]
Conidia	Present	o	
yeast	Present	o	
Phagocytosis[b]			
Alveolar MØ	3.9 ± 0.5	5.8 ± 0.8	[14]
Peritoneal MØ	4.1 ± 0.4	12.3 ± 0.5	
Adherence[c]			
Alveolar MØ	11.2 ± 1	8.1 ± 0.8	[14]
Peritoneal MØ	3.2 ± 0.9	0.7 ± 0.2	
MIC[d]	No difference	No difference	[14]
Killing assay[e]	Less susceptible	Susceptible	[14]
Antifungal activity of MØ[f]			
Cfu after 6 h	4,583 ± 117	8,000 ± 408	[14]
Cfu after 12 h	3,666 ± 169	4,166 ± 236	
In vivo—cfu g/tissue[g]	30,000 ± 400	19,000 ± 800	[44]
In vitro resistance to oxidants[h]	Less susceptible	Susceptible	[44]

[a] Detection of presence or absence of melanin in vitro or in vivo

[b] Phagocytosis of melanized or nonmelanized yeast cells after 24 h of exposition to mice macrophage-like MH-S (alveolar) and macrophage-like J774.16 (peritoneal)

[c] Laterally adherent noninternalized yeast cells after 24 h of exposition to mice macrophage-like MH-S (alveolar) and macrophage-like J774.16 (peritoneal)

[d] Minimal inhibitory concentration of melanized and nonmelanized yeast cells to amphotericin b, ketoconazole, fluconazole, itraconazole, and sulfamethoxazole

[e] Antifungal killing assay of melanized and nonmelanized yeast cells to amphotericin b, ketoconazole, fluconazole, itraconazole, and sulfamethoxazole

[f] Antifungal activity of macrophages after 6 and 24 h of incubation on melanized and nonmelanized yeast cells with J774.16 previously treated with IFN-γ

[g] Cfu from lungs of Balb/c mice intratracheally infected with 3×10^5 yeast cells of melanized and nonmelanized *P. brasiliensis* after 30 days of infection

[h] In vitro exposition of melanized and nonmelanized yeast cells to chemically generated NO, oxygen-derivated oxidants, chloride-free sodium hypochlorite, and hydrogen peroxide

virulence by mechanisms other than the production of melanin.

Other fungal species

Sporothrix schenckii

Sporothrix schenckii is a thermally dimorphic fungus frequently associated with plants and soil and it is the causative agent of sporotrichosis, an important cutaneous and subcutaneous human and animal pathogen [58, 59]. The mycelial phase predominates in the environment whereas yeast-like forms develop in infected human and animal tissues [60]. Most cases of sporotrichosis are localized to the skin and subcutaneous tissues, although dissemination to osteoarticular structures and viscera may occur in both healthy and immunosuppressed individuals, particularly individuals with AIDS [61].

Existing data suggests that the conidia of *S. schenckii* produce melanin compounds via a 1,8-dihydroxynaphthalene pentaketide pathway [5]. On the selective medium Mycosel, *S. schenckii* produces visibly pigmented conidia, which are not observed when brain heart infusion and minimal broth are used; hyphae are generally not melanized [62]. However, by using techniques for production of melanin ghosts, melanin-like particles can be recovered from cells grown on Mycosel and minimal broth [63]. Monoclonal antibodies against melanin of *S. schenckii* have been generated and used to detect melanin or melanin-like compounds in conidia in vitro, and in yeast cells in vitro and in vivo [62].

Melanized *S. schenckii* yeast is less susceptible to killing by chemically generated oxygen- and nitrogen-derived radicals and UV light than melanin-deficient cells [5]. *S. schenckii* melanin also interferes with yeast cell phagocytosis and diminishes the respiratory burst mediated by human monocytes and murine macrophages [5]. Melanized conidia are more resistant to phagocytosis and killing than nonmelanized conidia and melanin has been associated with scavenging oxygen and nitrogen species [5].

Histoplasma capsulatum

Histoplasma capsulatum is a thermally dimorphic fungus that occurs most commonly in North and Central America, but the microorganism exists in many diverse areas around the world. Individuals at greatest risk for histoplasmosis are those who have occupational or recreational activities that disrupt the soil, or those who are in contact with accumulated dirt and guano in old buildings, bridges or caves where bats have roosted [reviewed in 63]. Every year, hundreds of thousands of individuals in the US and Central America are infected with *H. capsulatum*. The vast majority of infected persons are either asymptomatic or have a very mild disease that is never recognized as histoplasmosis [reviewed in 63]. Histoplasmosis may, however, progress to life-threatening systemic disease particularly in immuno-compromised individuals [64, 65].

H. capsulatum conidia, but not hyphae, produce melanin in minimal medium without the addition of phenolic substrate [16]. Yeast cells also produce melanin, but only when cultivated with phenolic compounds such as L-DOPA or (–)-epinephrine [16]. Scanning electron microscopy demonstrates that melanized conidia and yeast cells grown with L-DOPA formed tufts on the cell surface whereas cells grown in the absence of L-DOPA are smooth [16]. Transmission electron microscopy of melanized yeast cells demonstrated that the cell wall is coated with electron-dense granules [16]. Furthermore, the presence of laccase-like activity has been observed in cytoplasmatic extracts of *H. capsulatum* incubated with L-DOPA [16].

Melanization of *H. capsulatum* has been confirmed by the binding of melanin-specific antibodies to yeast cell walls in infected tissue and the recovery of melanin-like yeast-shaped particles from infected lungs subjected to treatment with acid, denaturing agents and enzymes [16]. Although the CLSI minimal inhibitory concentration (MIC) test with amphotericin B and caspofungin fail to detect differences between melanized and nonmelanized cells, time-kill assays show that melanized cells are significantly less susceptible to amphotericin B and caspofungin [51]. In contrast, no differences were found using fluconazole or itraconazole. The phenomenon of resistance to amphotericin B and caspofungin could be explained by the binding of the drugs to melanin which mitigates their antifungal actions [51].

Blastomyces dermatitidis

Blastomyces dermatitidis is a thermally dimorphic fungus endemic to the central USA that affects humans and animals. The fungus occurs in the environment as mycelia and forms yeast cells after infecting host tissue. Individuals exposed to the fungus may have an asymptomatic infection or can develop fatal pneumonia even in immunocompetent individuals [66]. In individuals with impaired immunity, the fungus behaves as an opportunistic pathogen after latent infection and can cause widely dissemination disease [67].

A melanin-like pigment from *B. dermatitidis* occurs in conidia, but not hyphae, is produced by mycelium grown on defined chemical medium or brain heart infusion, whereas yeast cells only become pigmented when grown in media supplemented with L-DOPA [17]. Melanin-binding monoclonal antibody

avidly labels particles isolated from *B. dermatitidis* conidia and yeast by serial treatment with enzymes and hot acid [17]. Additionally, tissue sections of lung from a dog with blastomycosis also reacted with the antibody, indicating that this fungus melanizes in vivo [17]. Consistent with previously described studies, there are no differences in the MIC of melanized and nonmelanized yeast cells for amphotericin B, itraconazole and voriconazole, but time-kill assays demonstrate that melanized yeast cells are significantly less susceptible to amphotericin B [17].

Coccidioides posadasii

Coccidioides posadasii is a thermally dimorphic endemic fungus in USA, Mexico, Central, and South America [68]. It grows as mycelia in desert and semiarid soils and disturbances in the soil facilitate the dispersal of arthroconidia, which are the infectious propagules. Once inhaled, the arthroconidia convert into the parasitic spherule/endospore phase in tissue [69]. Individuals exposed to this fungus may be asymptomatic, but half of immunologically competent individuals develop an atypical pneumonia characterized by cough, fever, and pleuritic pain often accompanied by rashes, sore throat, headache, arthralgia, myalgia or anorexia [69].

C. posadasii produces melanin. Utilizing the techniques described for other melanized pathogenic fungi, it was observed that the saprobic phase of *C. posadasii* produces melanized arthroconidia but not hyphae [19]. Melanin particles from arthroconidia and intact heat-killed arthroconidia were recognized by melanin-binding monoclonal antibody, indicating the presence of melanin or melanin-like deposits in the residual cell wall material [19]. The parasitic phase was also analyzed. Melanin particles, similar to intact spherule propagules, were obtained after subjecting infected tissues to denaturing enzymes and hot HCl. Additionally, lung tissue immunofluorescence revealed that only endospores within the spherules in the tissue sections reacted with melanin-binding monoclonal antibody with the cell wall of spherules being poorly labeled [19]. Also, intact spherules grown in vitro were not labeled, however spherules grown in vitro devoid of the lipid-rich, membranous spherule outer wall were readily labeled by melanin-binding antibody [19]. Hence, the lipid-rich component of *C. posadasii* effectively blocked reactivity of the melanin-binding antibody, which may affect the pigment's engagement with host effector cells.

Conclusions

Production of melanin is widespread among microorganisms. In dimorphic fungi like *P. brasiliensis*, *S. schenckii*, *H. capsulatum*, *B. dermatitidis*, and *C. posadasii*, melanin production may promote fungal survival in different environments, augment their resistance to immune effector responses in the infected host, and reduce their susceptibility to antifungal drugs.

Dimorphic fungi appear to produce DHN-melanin in conidia/arthroconidia in nature primarily via a polyketide synthetase pathway. Interestingly, there is an absence of this pigment in hyphae. Melanin production and its effects on the parasitism by yeast cells or spherules/endospores require specific substrates as shown in vitro by addition of L-DOPA or other suitable phenolic compounds in a process mediated by laccase-like enzymes. Melanin and laccase affect the virulence capacity of pathogenic microorganisms. Melanins are a challenge to the defense mechanisms of the hosts, but they are also targets for alternative antimicrobial strategies as exemplified by the fact that passive administration of monoclonal antibodies against *C. neoformans* melanin can prolong the survival of and reduce the fungal burden in *C. neoformans*-infected mice [43]. A similar result was observed by administration of glyphosate to mice infected with *C. neoformans* resulting in delayed melanization of yeast cells in vivo and prolonged mouse survival [70].

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