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All about that fat: Lipid modification of proteins in *Cryptococcus neoformans*

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Abstract

Lipid modification of proteins is a widespread, essential process whereby fatty acids, cholesterol, isoprenoids, phospholipids, or glycosylphospholipids are attached to polypeptides. These hydrophobic groups may affect protein structure, function, localization, and/or stability; as a consequence such modifications play critical regulatory roles in cellular systems. Recent advances in chemical biology and proteomics have allowed the profiling of modified proteins, enabling dissection of the functional consequences of lipid addition. The enzymes that mediate lipid modification are specific for both the lipid and protein substrates, and are conserved from fungi to humans. In this article we review these enzymes, their substrates, and the processes involved in eukaryotic lipid modification of proteins. We further focus on its occurrence in the fungal pathogen *Cryptococcus neoformans*, highlighting unique features that are both relevant for the biology of the organism and potentially important in the search for new therapies.

Keywords

Cryptococcus; palmitoylation; myristoylation; prenylation; isoprenylation; GPI-anchored proteins; lipid modification; protein lipidation

Introduction

Although the first report of protein lipidation was published in 1951, demonstrating the existence of “proteolipides” (Folch and Lees, 1951), this paradigm-shifting concept was not widely accepted for several decades. The subsequent discoveries that fungal mating pheromones are prenylated (Kamiya *et al.*, 1978) and that certain viral coat proteins are palmitoylated (Schmidt *et al.*, 1979) were compelling, however, and soon followed by the discovery of myristoylated and glycosylphosphatidylinositol (GPI)-anchored proteins in the 1980s (Aitken *et al.*, 1982; Ferguson *et al.*, 1985). Other less frequent modifications were identified in the following years, and it is now clear that “proteolipides” are present in all of the kingdoms of life. Most broadly, these processes consist of the co- or post-translational modification of proteins with one or more lipid groups (Table 1), potentially influencing their structure, function, stability, and/or localization (Menon, 2008). In recent years, multiple examples of such events and their impact on biological systems have been

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demonstrated. In parallel, advances in proteomics have allowed whole cell profiling of modified proteins, yielding a global understanding of their prevalence and range. While most fundamental studies in this area have been performed in mammalian cell lines or the model yeast *Saccharomyces cerevisiae*, lipid modifications certainly play important regulatory roles in other systems. Their occurrence in pathogenic microbes had been relatively unexplored, until recent reports detailing lipid modifications of proteins from bacteria (Hicks and Galan, 2013; Ivanov and Roy, 2013), parasites (Goldston *et al.*, 2014), and fungi. The last group includes medically important organisms such as *Candida albicans* (Richard *et al.*, 2002; Piispanen *et al.*, 2011), *Aspergillus fumigatus* (Fontaine *et al.*, 2003; Fortwendel *et al.*, 2012; Fang *et al.*, 2015), and *Cryptococcus neoformans* (Selvig *et al.*, 2013; Nichols *et al.*, 2015; Santiago-Tirado *et al.*, 2015).

In this review we first introduce the major forms of protein lipidation, including their biological function and the enzymes that mediate these modifications. We then focus on what is known about lipid modification of cryptococcal proteins, including myristoylation, prenylation, palmitoylation, and GPI anchor addition, with particular focus on contributions to virulence and potential drug targets. Processes that have not been demonstrated in fungi (e.g. N-palmitoylation, cholesterol attachment, and several modifications specific to bacteria and archaea) are not addressed; these are reviewed elsewhere (Eichler and Adams, 2005; Miura and Treisman, 2006; Nakayama *et al.*, 2012). The rapid advances in the field of protein lipidation and the demonstrated role of these modifications in microbial virulence make it an exciting time to consider how they impact cryptococcal biology and pathogenesis. We hope this review will be a useful resource for fungal biologists and stimulate interest in this fascinating area of research.

Overview of lipid modifications of protein

The major classes of lipid modification are N-myristoylation, isoprenylation, S-acylation, and GPI-anchor addition (Table 1 and Fig. 1A). Each of these modifications is mediated by a family of enzymes that recognizes both the lipid and protein substrates. The lipids are covalently linked to target proteins, mostly through thioester or thioether links with the sulfur group of cysteine (Cys), but also via amide or oxyester bonds to glycine (Gly) or serine (Ser) residues. These chemical bonds are important for the consequences of the lipid modification as they impart certain characteristics to the process, such as the irreversibility of an amide linkage versus a thioester bond, which is reversible.

N-myristoylation

N-myristoylation is the generally co-translational addition of myristate, a 14-carbon fully saturated fatty acid, to an amino-terminal Gly that has been exposed by removal of the protein's initiating methionine (Met). Although analysis of different myristoylproteins have led to the general consensus sequence MGXXX(S/C/T), the presence of the Gly residue as the second codon is the only absolutely required motif for the modification; mutants with a Gly deletion or substitution are often used as negative controls for myristoylation. Myristate addition aids in membrane targeting, often in combination with a second membrane-binding signal, which may be a poly-basic stretch of amino acids or another lipid modification such

as palmitoylation (below). Myristoylation may also induce structural changes in response to signals such as GTP or calcium binding, thereby regulating protein function. This irreversible modification is catalyzed by myristoyl-CoA:protein N-myristoyltransferase (NMT), an enzyme present in all organisms that have been examined to date, from yeast to humans. Because the modification is required for the correct function of substrate proteins, NMTs are essential for viability in organisms including the fungi *C. albicans* (Lodge *et al.*, 1994b), *A. fumigatus* (Fang *et al.*, 2015), and *C. neoformans* (Lodge *et al.*, 1994a). Biochemical and structural analyses of NMTs suggest that they are potential therapeutic targets for diseases ranging from cancers to parasitic and fungal infections (Georgopapadakou, 2002; Panethymitaki *et al.*, 2006; Das *et al.*, 2012).

Isoprenylation

Protein prenylation (also known as isoprenylation) is the addition of farnesyl (15-carbon) or geranylgeranyl (20-carbon) isoprenoids to a Cys residue in the target protein C-terminus. This irreversible modification is often required for stable association of substrate proteins with membranes, although it frequently occurs in conjunction with other fatty acid modifications. Prenylation occurs at the Cys of a consensus sequence known as the CAAX box (Cys-aliphatic-aliphatic-X). After prenylation the -AAX is removed and the protein is capped by methylation (Nguyen *et al.*, 2010). The 'X' residue of the CAAX box generally determines whether a substrate will be modified by farnesyl transferase (FTase, when X is Met, Ser, Gln, or Ala) or geranylgeranyl transferase I (GGTaseI, when X is Leu, Ile, or Glu), although this is not absolute (*C. neoformans* is an extreme case discussed below). FTase and GGTaseI are heterodimers with one common subunit, and can occasionally modify the same sites. GGTaseII is structurally distinct and only modifies Rab proteins (>60 in mammals) at the C-terminal consensus sequence CXC, CC, or CCX (Gutkowska and Swiezewska, 2012). It works in concert with a third protein, Rab escort protein (REP), and typically transfers two geranylgeranyl groups.

Similar to N-myristoylation, prenylated substrates act in a variety of essential processes, so absence of prenylation is lethal. One well-studied group of prenylated proteins is the small GTPase superfamily. This includes fungal pheromones involved in mating, differentiation, and expression of virulence traits and key players in signal integration, cell growth, and differentiation, such as Ras proteins. Because activated forms of Ras are present in most cancers, inhibitors of farnesylation (FTIs) are of great interest. Multiple such compounds have been described, supporting the feasibility of prenylation as a therapeutic target in pathogenic fungi and parasites (Eastman *et al.*, 2006; Hast *et al.*, 2011). Few GGTase inhibitors, however, have been developed or investigated, mostly because of their high toxicity *in vivo* (Shen *et al.*, 2015b).

S-acylation

Protein S-acylation is the addition of a fatty acid, usually palmitate (a 16-carbon fully saturated fatty acid), to the side chain of Cys residues. Unlike N-myristoylation and prenylation, there is no known consensus sequence for this modification beyond the requirement for a Cys, which may occur anywhere in the peptide, sometimes close or adjacent to another lipid modification site. Protein S-acylation, commonly termed

palmitoylation for its most common form, is a diverse and versatile reaction, occurring at multiple sites within the cell and targeting cytosolic as well as transmembrane proteins. S-acylation of soluble proteins mediates membrane association, sometimes in conjunction with other lipid modifications. Acylation of transmembrane proteins typically regulates their stability or intracellular trafficking and consequent localization. For example, lack of palmitoylation of integral membrane proteins in the ER or Golgi may lead to aggregation (Fig. 2A left), while properly modified proteins are directed to the PM (Fig. 2A right). Palmitoylation of PM transmembrane proteins can promote segregation into lipid rafts (Fig. 2B right) or control protein stability by preventing the ubiquitination of lysines (Lys) in proximity to the palmitoylation site (Fig. 2B left).

Palmitoylation is catalyzed by protein S-acyltransferases (PATs), enzymes characterized by the presence of a DHHC (Asp-His-His-Cys zinc finger) domain. This family of proteins was discovered in *S. cerevisiae* (Lobo *et al.*, 2002; Roth *et al.*, 2002) and is present in all eukaryotes examined to date. Although it is not known whether the process itself is essential, dysregulation of palmitoylation is associated with several diseases and is also required in various pathogens for efficient infection (Blanc *et al.*, 2013). A critical and unique feature of PAT modification is that it is reversible, with palmitate removal mediated by the action of palmitoyl-protein thioesterases (PPTs). PAT and PPT enzymes work in concert, allowing for precisely regulated dynamic changes in the extent of protein palmitoylation and its functional consequences, such as intracellular localization. One of the best studied examples of such regulation is the evolutionarily conserved modification of Ras proteins (discussed below).

It is common for organisms to have multiple PATs: there are seven in *S. cerevisiae* and 24 in humans. Possible explanations for this widespread apparent redundancy include differing subcellular localization of the enzymes that enables spatial control of protein modification (Ohno *et al.*, 2006; Howie *et al.*, 2014), or distinct substrate specificity (Roth *et al.*, 2006; Ohno *et al.*, 2012). Although the mechanism of specificity remains elusive, recent work in several pathogens, including *C. neoformans*, has identified specific PATs that regulate critical pathogenic processes through the modification of unique protein substrates (Jones *et al.*, 2012; Frenal *et al.*, 2013; Santiago-Tirado *et al.*, 2015).

GPI-anchor addition

GPI-modification involves the transfer of a glycolipid to a newly-synthesized protein, displacing a segment of the original C-terminus and conferring membrane association. The conserved core GPI structure is composed of phosphatidylinositol (PI) linked to a linear glycan consisting of glucosamine, three mannose residues, and a phosphoethanolamine that mediates protein attachment (Fig. 1A); species-specific modification with additional ethanolamine, sugar, or lipid groups may also occur. The core GPI is primarily synthesized at the endoplasmic reticulum (ER), where it is transferred to proteins within the secretory pathway, anchoring them in the luminal membrane of that compartment (Muñiz and Zurzolo, 2014). The anchored proteins then proceed, via secretory vesicles, to the plasma membrane, where they become displayed on the outer surface of the cell (Fig. 1B).

Proteins destined to be GPI-anchored contain two signal sequences, an ER-targeting domain in the N-terminus (a signal peptide) and a GPI-attachment site in the C-terminus (known as the ω site); both are removed during protein processing (Orlean and Menon, 2007). Like myristoylation and prenylation, GPI attachment is irreversible. However, under certain circumstances the anchored protein can be released from membrane association through the action of PI-specific phospholipase C (PI-PLC) (Fig. 1B(iv)). In organisms with a cell wall, GPI-anchored proteins may be transferred, along with part of the GPI glycan, from the plasma membrane to covalent linkage with β -1,6-glucan polymers of the cell wall (Fig. 1B(iii)); this fate may be determined by additional sequences surrounding the GPI anchor site (Frieman and Cormack, 2004). Cell-wall linked proteins may be released by glucanases (Fig. 1B (v)). GPI modification is evolutionarily conserved and essential in all eukaryotes, and has been studied in fungi, protists, and mammals. In pathogenic fungi, GPI-anchored molecules have been shown to mediate adherence to tissues (Kempf *et al.*, 2009) and resistance to host immune cells (Siafakas *et al.*, 2006; Shen *et al.*, 2015a). GPI structures also anchor fungal surface polysaccharides (Costachel *et al.*, 2005), although this topic is beyond our scope in this review.

***Cryptococcus neoformans*, a model for fungal biology and pathogenesis**

Fungi share basic cellular architecture with animal cells, and their genes are evolutionarily and functionally related. This makes fungal infections particularly challenging to treat, resulting in over 1.5 million deaths each year worldwide (Brown *et al.*, 2012). Over one-third of these deaths are caused by *Cryptococcus neoformans*, an encapsulated basidiomycetous yeast (reviewed in (Srikanta *et al.*, 2014)). Cryptococcosis, which initially came to attention as an AIDS-defining illness, remains a leading cause of morbidity and mortality among AIDS patients (Park *et al.*, 2009). Recent studies also show a steady increase in the incidence of disease in immunocompetent individuals, who exhibit worse response to treatment (Panackal *et al.*, 2015). Because current therapies are inadequate and the disease burden high, there is a clear need for better and novel anticryptococcal agents. Given that protein lipidation regulates many cellular processes, including those required for virulence and pathogenesis, understanding the mechanisms and consequences of these modifications may open the way for new antifungal treatments.

Myristoylation in *C. neoformans*

Three cryptococcal proteins have been annotated as myristoylated (Table 2; Loftus *et al.*, 2005; Janbon *et al.*, 2014), although this modification has only been demonstrated experimentally for Arf1 (Lodge *et al.*, 1994b). ADP-ribosylation factors (Arfs) are small G proteins involved in the regulation of coated vesicle formation during intra-Golgi trafficking. Unlike other small G proteins, which are prenylated, Arf1 family members have an N-terminal amphipathic helix that is myristoylated. Although this modification alone is insufficient to stably anchor these proteins at membranes, the combination of myristoylation with electrostatic interactions between the amphipathic helix and membrane phospholipid headgroups is enough to secure membrane association. Studies in *S. cerevisiae* have shown that this reversible association occurs in response to GTP binding, and subsequent genetic and structural studies have revealed a fascinating mechanism (Fig. 3A; Gillingham and

Munro, 2007). Upon GDP to GTP exchange, a conformational change frees Arf1's amphipathic and myristoylated helix for membrane association. Once the protein is inactivated by GTP hydrolysis, these changes are reversed, returning the helix and myristate to an internal hydrophobic pocket and promoting protein release from membranes. Through these cycles of activation and membrane association, Arf1 regulates the formation of vesicles that shuttle cargo between secretory compartments. These events are likely conserved in *C. neoformans* because Arf1 is the most abundant myristoylprotein during vegetative growth (Lodge *et al.*, 1994b) and inhibition of Arf1 myristoylation in this organism yields morphological defects characteristic of blocks in secretory traffic (Lodge *et al.*, 1998). The other two cryptococcal proteins predicted to be myristoylated, Gpa1 and Vac8, are key regulators of cellular responses to environmental stresses. Gpa1 is one of the three GTP-binding alpha subunits of the heterotrimeric G protein coupled receptor (GPCR). Lipidation of this subunit is also conserved in animals and affects both its localization as well as interactions with its binding partners in the GPCR (Fig. 3B(i) – (iii)). Vac8 seems to be restricted to the fungal kingdom and is involved in vacuolar function (Honscher and Ungermann, 2014).

Cryptococcal protein myristoylation became a focus of intense study in the early 1990s as a possible new antifungal target, a topic of particular urgency because the pipeline for antifungals effective against *C. neoformans* had been dry since the discovery of amphotericin B (AmB) in the 1970s. At the time several observations supported this strategy, including that *C. neoformans* myristoylates proteins (Langner *et al.*, 1992), that the single cryptococcal NMT is essential for viability and virulence (Lodge *et al.*, 1994a), and that the peptide substrate specificities between model yeast and human NMTs are different (Duronio *et al.*, 1992). In this context, most studies centered on biochemical investigations of the cryptococcal Nmt protein itself, with the objective of identifying compounds that might exploit fungal-specific features of the protein for therapeutic gain. NMT demonstrates specificity for both lipid and protein substrates, which varies between organisms. For example, surveys of myristate analogs showed differential recognition by the NMTs of *C. neoformans*, other fungi, and humans. One of these, 4-oxatetradecanoic acid, exhibits fungicidal activity against *C. neoformans* (Langner *et al.*, 1992). Furthermore, human and cryptococcal NMTs have different specificity for peptides derived from known myristoylproteins (Lodge *et al.*, 1994a). Given the essentiality of protein N-myristoylation in *C. neoformans* and the unique features it exhibits, it may be feasible to target this activity. Consistent with this idea, an NMT point mutant that abrogates activity also renders the fungus avirulent in a rabbit model of cryptococcal meningitis (Lodge *et al.*, 1994a).

Protein prenylation in *C. neoformans*

As in other organisms, the cryptococcal prenylation machinery consists of three enzyme complexes, FTase and GGase I and II. The first to be identified and characterized was the FTase-specific subunit encoded by *RAM1* (Vallim *et al.*, 2004). *RAM1* was found to be essential, prompting the investigators to test several FTIs previously developed as anti-cancer therapies for fungicidal activity. Manumycin A, an inhibitor of the human FTase for Ras, was found to kill fungal cells rapidly (<4 h) with minimum inhibitory concentrations close to those for AmB (Hast *et al.*, 2011). The subunit common to cryptococcal FTase and

GGTase I is encoded by *PFT1* (Lin *et al.*, 2010), while *CDC43* encodes the unique subunit of GGTaseI (Selvig *et al.*, 2013). The latter gene is not essential, suggesting that farnesylation is the dominant form of prenylation in *C. neoformans*. Notably, while in other organisms, including humans, substrate proteins may be alternatively prenylated by GGTaseI in the absence of FTase activity, this seems to be less efficient in *C. neoformans*.

Pheromones and small G proteins are the two major classes of prenylated proteins in fungal pathogens. Both play important roles in signal transduction, as they mediate inter- and intracellular communication, respectively, and are fundamental for the ability of fungi to react and respond to environmental conditions. The lipopeptide pheromones are farnesylated, while small G proteins of the Ras, Rho, and Rab families are either farnesylated or geranylgeranylated (Table 2). Homology to other fungal sequences was used to identify the genes encoding *C. neoformans* lipopeptide pheromones, *MFa1-4* (*MFa1* and *MFa2* are identical while *MFa3* and *MFa4* are divergent alleles) and *MFa1-3* (Davidson *et al.*, 2000; McClelland *et al.*, 2002). *Mfa1* and *Mfa1* induce filamentation of opposite mating type cells in a CAAX-box dependent manner. Prenylation, natively with farnesyl groups, is required for processing and secretion of the mature pheromones and for their activity, although geranylgeranyl modification can also yield fully functional pheromones (Michaelis and Barrowman, 2012). In addition to acting as mating pheromones in a paracrine fashion, *Mfa1-3* also act in an autocrine fashion (Shen *et al.*, 2002): upon nutrient starvation, *Mfa1* stimulates MAT α cells to filament and undergo haploid fruiting, leading to sporulation (Lin *et al.*, 2005).

Of the small G proteins of *C. neoformans*, Ras1 has been studied in the greatest detail (Fig. 4). This protein has a CAAX motif that mediates farnesylation and two other nearby Cys residues that are palmitoylated. Mutagenesis studies of the three Cys residues showed that farnesylation mediates initial association of the protein with endomembranes, a prerequisite for palmitoylation, and that subsequent palmitoylation promotes trafficking of Ras1 to the plasma membrane (Nichols *et al.*, 2009). Consistent with this model, inhibition of farnesylation by manumycin A caused delocalization of Ras1 from membranes in a dose-dependent manner. Interestingly, the CAAX motif of Ras1 ends with a Leu (CAAL), which should direct its modification by GGTaseI. However, deletion of *CDC43*, which encodes the unique subunit of GGTaseI, had no effect on Ras1 localization, whereas inhibition of FTase does. Other small G proteins in *C. neoformans*, such as Cdc42 and its paralog Cdc420, Rho1 and its paralog Rho10, and Rac2, also have CAAL motifs and their homologs are GGTaseI substrates in other organisms. However, only Cdc42 was completely mislocalized in response to deletion of cryptococcal *CDC43*, while Rho10 was partially affected (Selvig *et al.*, 2013). This suggests that in *C. neoformans* the CAAL motif is insufficient to provide prenylation specificity, consistent with structural and biochemical analyses of *C. neoformans* FTase that show differences in the substrate binding pocket and high affinity for CAAL motifs *in vitro* (Hast *et al.*, 2011). Furthermore, in contrast to the nonpathogenic model yeasts, farnesylation seems to be the dominant cryptococcal isoprenyl modification, supported by the fact that *RAM1*, but not *CDC43*, is essential, and that most G proteins are affected only when Ram1 is inactivated. Different enzyme specificity and an altered role for farnesylation and geranylgeranylation in *C. neoformans* are notable differences when

compared to humans. These might allow the development of protein prenylation as a target for antifungal drugs.

Palmitoylation in *C. neoformans*

The best studied palmitoylated protein in *C. neoformans* is the signaling protein Ras1. This protein has two potential Cys sites for acylation and palmitoylation of either is sufficient for activity, presumably because one palmitate is enough to correctly localize Ras1 to the plasma membrane (Nichols *et al.*, 2009). Whether both sites are modified *in vivo* is not known. The *C. neoformans* genome encodes 7 PAT proteins, none of which are essential, probably due to functional redundancy (Nichols *et al.*, 2015). Demonstrating this redundancy, multiple PATs modify cryptococcal Ras1, in contrast to model yeasts and humans where each Ras protein is generally modified by a single PAT (Lobo *et al.*, 2002; Zhang *et al.*, 2013; Young *et al.*, 2014). The dominant modifier of *C. neoformans* Ras1, however, is encoded by *PFA4*; deletion of this gene partly phenocopies a *ras1* deletion and alters Ras1 localization. The mutant also displays additional phenotypes unrelated to Ras function, including virulence defects *in vitro* and *in vivo* (Nichols *et al.*, 2015; Santiago-Tirado *et al.*, 2015).

Pfa4 was identified in a screen for fungal regulators of *C. neoformans* internalization by macrophages, where cells lacking the corresponding gene were recognized and phagocytosed with unusual avidity. This and several other *pfa4* phenotypes are not shared with other PAT mutants and are not complemented by active site mutants of Pfa4, suggesting they are due to dysfunction of one or more Pfa4-specific substrates (Santiago-Tirado *et al.*, 2015). To globally identify Pfa4 substrates and mechanistically explain these defects, a chemical reporter for palmitate was used to label modified proteins in wild-type and *pfa4* mutant cells. Next, using bioorthogonal chemistry proteomics, the palmitoylated proteins in both strains were identified and Pfa4-specific substrates determined by comparison of the proteomic profiles (Santiago-Tirado *et al.*, 2015).

The Pfa4-palmitoylome includes proteins involved in cell wall synthesis, membrane trafficking, signal transduction, and membrane transport, which explain most of the *pfa4* mutant phenotypes (Table 2). The top Pfa4 substrate is the class IV chitin synthase Chs3, a target that is conserved with *S. cerevisiae* Pfa4. The functional consequence of the modification is also conserved, as in both organisms unmodified Chs3 is mislocalized to endomembranes rather than reaching the plasma membrane (Fig. 2A; (Lam *et al.*, 2006; Santiago-Tirado *et al.*, 2015). Interestingly, another *C. neoformans* class IV chitin synthase, Chs1, is also a Pfa4 substrate, suggesting that palmitoylation is a feature of this particular class of enzymes. Several other Pfa4 substrates are also integral membrane proteins although the specific consequences of their palmitoylation are not yet known. Another notable palmitoylation substrate is Rho11, the only one of the three cryptococcal Rho1 paralogs with a Cys adjacent to its CAAX box. Palmitoylation at this site likely works in conjunction with isoprenylation to localize this protein to the plasma membrane; Rho homologs in yeast and mammals that are not palmitoylated instead have a stretch of polybasic residues to serve this function (Moissoglu and Schwartz, 2014; Sanchez-Mir *et al.*, 2014). Finally, Ras1 (the only palmitoylated protein in *C. neoformans* known prior to the proteomic study), was found in

proteomic data sets from both wild type and the *pfa4* mutant, with the expected reduction in palmitoylation in the latter.

As with prenylation, palmitoylation in *C. neoformans* has several features that distinguish it from model yeasts. In a large-scale analysis of *S. cerevisiae* PATs and their substrates (Roth *et al.*, 2006), some general patterns were identified in the substrates of specific PATs. For example, the PAT Akr1 modifies soluble proteins with palmitoylation as the sole lipid modification; the Erf2-Shr5 complex mediates palmitoylation of Ras2 and other substrates that are also prenylated or N-myristoylated; Swf1 modifies single transmembrane domain proteins at a Cys adjacent to the transmembrane domain on the cytosolic side; and Pfa4 modifies large, multispreading membrane proteins, including the chitin synthase Chs3. Although some of these generalities hold true for fission yeast as well (Zhang *et al.*, 2013; Sanchez-Mir *et al.*, 2014), they do not seem to extend to *C. neoformans*. Instead, it appears that Pfa4 modifies most of the expected Erf2 substrates along with some substrates attributed to *S. cerevisiae* Pfa4. Notably, several fungal-specific proteins implicated in pathogenesis (e.g., chitin synthases and transporters of nickel and siderophores) are substrates of Pfa4, making inhibition of Pfa4 a viable strategy to treat fungal infections. Additionally, the closest human PAT homolog, DHHC6, is only 21% identical to the cryptococcal protein at the amino acid level, with the homology restricted mostly to the DHHC domain. Identifying and inhibiting major PATs in pathogenic fungi, such as Pfa4 in *C. neoformans*, might be a promising strategy for the development of new antifungals.

GPI-anchored proteins in *C. neoformans*

Proteomic studies have identified numerous GPI-anchored proteins in *C. neoformans*, including α -amylase, chitin deacetylases, and the β -1,3-glucanosyltransferase Gas1 (Table 2) (Eigenheer *et al.*, 2007). Among these, the best characterized is Plb1, a phospholipase B1 (Djordjevic *et al.*, 2005). As with GPI-anchored proteins in other systems, the corresponding gene encodes signals for both entry into the secretory pathway and GPI-addition. Once attached, the GPI-anchor directs Plb1 to plasma membrane lipid rafts (Siafakas *et al.*, 2006), where it may remain, be released by cryptococcal PI-specific phospholipase C (PI-PLC) (Chayakulkeeree *et al.*, 2008), or be transferred through covalent linkage to the cell wall glucans (Siafakas *et al.*, 2007). Cell wall-localized Plb1, in turn, may be released by β -glucanases, four of which are encoded in the cryptococcal genome and found in cell wall extracts (Eigenheer *et al.*, 2007). Consistent with these events (summarized in Fig. 1B), the protein is detected in all of these compartments as well as in cell supernatant fractions. Another well-studied GPI-protein of *C. neoformans* is the chitin deacetylase Cda2. Surprisingly, while the GPI-anchor of Cda2 mediates its plasma membrane association, it is not required for its cell wall association, which is non-covalent (Gilbert *et al.*, 2012). This result, which challenges the assumption that GPI-proteins in the cell wall are covalently crosslinked to β -1,6-glucan, is supported by recent similar observations in *C. albicans* (Caminero *et al.*, 2014).

Since GPI-anchored proteins are central to multiple pathogenic mechanisms, investigating differences between host and pathogen GPI biosynthetic pathways may suggest therapeutic targets. Although the GPI core structure (described above; Fig. 1A) is conserved in all

eukaryotes, it can be modified in a species-specific manner with additional sugars, lipids, or phosphoethanolamines. The inositol ring is also acylated in a subset of mammalian and most fungal GPIs. In model yeasts and mammals the added fatty acid is generally palmitate, although a variety of other acyl chains can be incorporated. Notably, *C. neoformans* incorporates a more limited repertoire of fatty acids (Franzot and Doering, 1999) at this position, due to substrate specificity of the inositol acyltransferase, Gwt1 (Umemura *et al.*, 2003). This may explain why inhibitors of *S. cerevisiae* Gwt1 are active against a broad range of yeasts and molds, but not against *C. neoformans* (McLellan *et al.*, 2012; Watanabe *et al.*, 2012). This difference suggests that it may be possible to identify inhibitors of the cryptococcal Gwt1 that do not affect the human enzyme.

Conclusions and future perspectives

Recent advances in chemical biology and proteomics have rapidly expanded our list of known lipid-modified proteins in *C. neoformans*. However, the functional significance and consequence of these modifications still are unknown for most of these proteins. Given the significant novel features of cryptococcal lipidation already discovered, there is much yet to be learned. Some of these features may serve as therapeutic targets, but this will require deeper understanding of their biology. Nonetheless, fatty acylation enzymes such as Nmt and Gwt1 show promise as drug targets, and recent studies of other enzymes, like FTase and PATs, are also encouraging.

The elucidation of protein lipidation in *C. neoformans* will also advance our understanding of fundamental biology that is relevant to all forms of life. While the core mechanisms and consequences of protein lipidation have been conserved throughout evolution, there are clear differences in molecular details, as with the findings in *C. neoformans* that traditional GGTaseI substrates are instead farnesylated and that PATs exhibit substrate ranges distinct from those of their homologs in model yeasts. In addition to the potential exploitation of these differences for therapeutic gain, they show that fungal models are valuable assets as tools in furthering our understanding of complex cellular processes.

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Fig. 1. GPI-anchored proteins in fungi

(A) Chemical structure of a GPI core, with the two acyl chains (x and y , which may differ in length and degree of saturation) of the phosphatidylinositol (PI) embedded in the membrane. The inositol phospholipids are most commonly diacyl-PI, but may also be lysoacyl-, alkylacyl-, or alkenylacyl-PI or inositolphosphoceramide. In fungi (as well as mammals), the C2 position of the inositol ring is often acylated (denoted by the asterisk), but in *C. neoformans* this substitution is mostly palmitate. (B) After anchor addition (and potential palmitoylation) in the ER lumen (pink), GPI-proteins enter the secretory pathway where they are further modified and trafficked in secretory vesicles to the PM (i); fusion of these vesicles with the PM exposes the GPI-protein cargo on the extracellular surface. Once at the cell surface, GPI-proteins can segregate into lipid rafts (purple phospholipids; ii) or, alternatively, can be translocated with part of their anchor (cleaved between glucosamine and mannose) to covalent linkage with cell wall (CW) β -1,6-glucan polymers (green tubes; iii). PM- or CW-localized GPI-proteins can be released into the extracellular space by the action of PI-specific phospholipases (PI-PLC; iv) or CW β -glucanases (v). The CW is a complex structure that surrounds the entire cell (Doering, 2009), but for clarity it is depicted only at the right of the cartoon and only the β -glucan fibers are shown.

Fig. 2. Palmitoylation acts as a cue for traffic and sorting towards specific membranes

(A) Palmitoylation of integral membrane proteins in the ER or Golgi directs them to the PM (right). If this does not occur, the proteins are retained in the ER, where they aggregate (left). (B) Palmitoylation may also direct PM transmembrane proteins into lipid rafts (purple phospholipids; right). For these proteins, the absence of palmitoylation results in mislocalization. In a subset of palmitoylated proteins, the modified Cys is near a Lys that is targeted for ubiquitin (Ub) addition; palmitoylation of the Cys prevents this from occurring. In the absence of lipidation the Ub is added, targeting the protein for lysosomal degradation (left).

Fig. 3. Consequences of myristoylation on protein function

(A) The Arf GTPase cycle illustrates myristoylation-dependent membrane recruitment. In the inactive GDP-state, the myristoyl moiety (black zigzag) is sequestered in a hydrophobic pocket of the protein while the polypeptide samples membranes via its amphipathic helix (multicolored cylinder). GEF (GDP-exchange factor) binding transiently stabilizes Arf (blue) at the membrane and mediates GTP loading; this displaces GDP, leading to closure of the pocket and exclusion of the myristate. The insertion of the excluded myristate into the lipid bilayer leads to stable membrane association of the activated Arf (green), which can then recruit effectors and regulate membrane trafficking (orange arrow). Subsequent inactivation of Arf, catalyzed by its GAP (GTPase activating protein), reverses the process, releasing Arf from the membranes to restart the cycle. (B) Lipidation is critical for G-alpha protein function. While unmodified G-alpha (blue) is cytosolic, myristoylation of this protein mediates its transient membrane retention at the ER as it moves into the Golgi (i). This makes the protein accessible to a Golgi-localized PAT, which adds a second lipid modification (ii). This combination firmly anchors the protein to membranes and provides a signal for trafficking to the PM. There the dually-modified alpha subunit interacts with its partners, the G- $\beta\gamma$ heterodimer (itself typically prenylated and sometimes additionally palmitoylated) and a 7-transmembrane domain sensory receptor, forming a signaling-competent unit: the G protein coupled receptor (GPCR; (iii)).

Fig. 4. Lipidation directs Ras localization and function

(i) FTase farnesylates cytosolic Ras proteins (lower right). This mediates their association with the ER membrane where further processing of the CAAX box occurs. Farnesylated Ras then traffics to the Golgi (lower left) where it is palmitoylated by a Golgi-associated PAT (ii) and the dually modified protein (dark blue) proceeds to the PM via the secretory pathway. At the PM Ras can be activated by guanine nucleotide exchange factors (GEFs; (iii)); the active GTP-bound form (green) functions as a signaling molecule (orange arrow) until it is inactivated by GTPase activating proteins (GAPs; (iv)). PM-localized Ras may also be depalmitoylated by a PPT (v), leading to its dissociation from the PM. It then diffuses and samples various cellular membranes, rejoining the cycle at the Golgi where its membrane localization is stabilized by palmitate addition.

Table 1

Lipid species	Lipid structure	Consensus sequence [#]	Enzyme family	Functional example
Myristate (N)		-GXXX(S/C/T)-	NMT	ADP-ribosylation factor (ARF) function ¹
Farnesyl (SC)		-CAAX	Fase	Pheromone processing and secretion ²
Geranylgeranyl (SC)		-CAAX, -CC-, -CXC-, -CCX	GGTase I, GGTase II	Membrane association ³
Palmitate (S)		-C-	PATs	Localization to PM ⁴
GPI anchor (N)	phosphatidylinositol [*] or inositol phosphoceramide	ø site	PIGs	Tethering proteins to outside leaflet of PM ⁵

(N) indicates amide bond, (SC) indicates thioether bond, and (S) indicates thioester linkage.

[#] Bold letter indicates linkage site.

^{*} The phosphatidylinositol (PI) moiety of GPI anchors is most commonly diacyl-PI, but may alternatively be lysoacyl-, alkylacyl-, or alkenyl-lacyl-PI.

¹ Gillingham and Munro (2007),

² Michaelis and Barrowman (2012),

³ Shen *et al.* (2015b),

⁴ Blanc *et al.* (2013),

⁵ Muñoz and Zurzolo (2014)

Table 2Lipid modified proteins in *C. neoformans*

Protein	Description	Ref.
N-myristoylation		
Arf1	Arf family small GTPase	1
Gpa1	α subunit G protein	1
Vac8	Vacuolar protein	
Prenylation		
Cdc42	Rho family small GTPase	2
Mfa1-4	Mating pheromone	3
Mfa1-3	Mating pheromone	4
Rac2	Rho family small GTPase	2
Ras1	Ras family small GTPase	2,5
Rho10	Rho family small GTPase	2
GPI anchor addition		
Cda1-3	Chitin deacetylases	6,7
Gas1	β -1,3-glucanosyltransferase	6
Glo1-3	Glyoxal oxidases	6
MP88	Mannoprotein	6
Plb1	Phospholipase B1	7,8
CNBE3490	α -Amylase	6
CNBH1590	Aspartic protease	6
CNBM0110	MP88-like	6
S-palmitoylation		
Cck1,2	Casein kinases	9
Chs1,3	Chitin synthases	9
Gpa1	α subunit G protein	9
Nic1	Nickel transporter	9
Ras1	Ras family small GTPase	5,9
Rho11	Rho family small GTPase	9
Sin3a	Transcriptional regulator	9
Sit1	Siderophore	9
Stt1	Sugar transporter	9
Sod1	Superoxide dismutase	9,10
Vac8	Vacuolar protein	9
CNAG_02458	GTPase activating protein	9
CNAG_03796	S/T-protein kinase	9
CNAG_03824	Phosphate transporter	9
CNAG_04484	Vesicle tether complex	9
CNAG_05229	Stomatin-family protein	9
CNAG_05615	t-SNARE	9
CNAG_05933	Vesicle tether complex	9

Protein	Description	Ref.
CNAG_06214	No known domains	9

Text in **bold** denotes experimental validation. 1, Lodge *et al.* (1994b); 2, Selvig *et al.* (2013); 3, Davidson *et al.* (2000); 4, McClelland *et al.* (2002); 5, Nichols *et al.* (2009); 6, Eigenheer *et al.* (2007); 7, Gilbert *et al.* (2012); 8, Djordjevic *et al.* (2005); 9, Santiago-Tirado *et al.* (2015); 10, Siafakas *et al.* (2006).