Heme sensing and trafficking in fungi

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Abstract

Fungal pathogens cause life-threatening diseases in humans, and the increasing prevalence of these diseases emphasizes the need for new targets for therapeutic intervention. Nutrient acquisition during infection is a promising target, and recent studies highlight the contributions of endomembrane trafficking, mitochondria, and vacuoles in the sensing and acquisition of heme by fungi. These studies have been facilitated by genetically encoded biosensors and other tools to quantitate heme in subcellular compartments and to investigate the dynamics of trafficking in living cells. In particular, the applications of biosensors in fungi have been extended beyond the detection of metabolites, cofactors, pH, and redox status to include the detection of heme. Here, we focus on studies that make use of biosensors to examine mechanisms of heme uptake and degradation, with guidance from the model fungus *Saccharomyces cerevisiae* and an emphasis on the pathogenic fungi *Candida albicans* and *Cryptococcus neoformans* that threaten human health. These studies emphasize a role for endocytosis in heme uptake, and highlight membrane contact sites involving mitochondria, the endoplasmic reticulum and vacuoles as mediators of intracellular iron and heme trafficking.

Keywords

Iron acquisition; Genetically-encoded sensor; Endocytosis; CFEM proteins; Nutritional immunity

1. Introduction

Metal ions and heme are essential cofactors for numerous proteins and play key roles in multiple cellular processes (Galaris et al., 2019). In the context of microbial pathogenesis, the acquisition of metal ions, especially iron and copper, is critical for the ability

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of microbes to cause disease in vertebrate hosts (Monteith and Skaar, 2021). This is true for fungal pathogens of humans including *Aspergillus fumigatus, Candida albicans,* and *Cryptococcus neoformans,* and recent reviews provide excellent overviews of the mechanisms of iron homeostasis in these fungi (Bairwa et al., 2017; Gerwien et al., 2018; Horianopoulos and Kronstad, 2019; Roy and Kornitzer, 2019; Kornitzer and Roy, 2020; Labbé et al., 2020; Stanford and Voigt, 2020; Jung et al., 2021; Misslinger et al., 2021). During disease, fungal pathogens acquire iron from iron-containing proteins and, importantly, from heme that accounts for the bulk of the iron quota in vertebrate hosts (Donegan et al., 2019). Importantly, an understanding of mechanisms of iron and heme acquisition may lead to new therapeutic approaches to combat fungal diseases. Novel approaches and new targets are critically needed because of the devastating impact of fungal pathogens in immunocompromised individuals, the emergence of new pathogens, and the limited arsenal of antifungal drugs (Fisher et al., 2020).

The analysis of heme use by fungi has benefited recently by the application of genetically encoded sensors, which are powerful tools to monitor and quantify molecules of biological interest including metabolites or environmental signals in real time and in living cells. A number of excellent reviews provide detailed descriptions of genetically encoded sensors that generally consist of a protein that senses a specific signal (e.g., a small molecule, a protein-protein interaction, or an enzyme activity) coupled to a protein that provides a fluorescent or bioluminescent readout (Greenwald et al., 2018; Lin et al., 2019; Qiu et al., 2019; Terai et al., 2019; Kostyuk et al., 2020; Marsafari et al., 2020; Zhou et al., 2020; Kim et al., 2021; Nasu et al., 2021). There are numerous examples of applications of genetically encoded biosensors in fungal research. For example, Van Genechten et al., (2021) recently reviewed the applications of fluorescent proteins and imaging tools for the detection of amino acids, glucose, glutathione, lipids, oxygen, pH changes, redox homeostasis, and signaling activities (e.g., kinase activation). Approaches that are particularly relevant for fungal pathogens include monitoring intracellular pH, e.g., in response to antifungal drugs (Liu and Köhler, 2016; Tournu et al., 2017), monitoring oxygen levels during infection (Eichhof and Ernst, 2016), detecting signaling activities (Demuyser et al., 2018), and assessing redox status during colonization of host tissue (Mentges et al., 2015; Huang et al., 2017).

In this review, we focus on recent studies of heme acquisition and trafficking in fungi, with an emphasis on the use of the genetically encoded sensors and genetic approaches to provide insights into intracellular trafficking (Hanna et al., 2016, 2017, 2018; Sweeny et al., 2018; Bairwa et al., 2020; Martinez-Guzman et al., 2020; Weissman et al., 2021). We also highlight recent studies that reinforce an appreciation of mitochondria and vacuoles as hubs of iron and heme sensing, and that showcase the role of the endomembrane system in trafficking. We conclude with perspectives on areas for future emphasis with a focus on mitochondrial functions.

2. Heme acquisition and trafficking in fungal pathogens

Heme is an iron-containing prosthetic group that serves as a cofactor and a signaling molecule for crucial cellular activities such as iron homeostasis, gas sensing, electron

transfer, cell cycle progression and proliferation, mitophagy, apoptosis and the response to oxidative stress (Donegan et al., 2019; Chambers et al., 2021; Gallio et al., 2021). Studies in the last 15 years are starting to reveal how fungi obtain iron from heme, the most abundant iron source in mammalian hosts (Ganz and Nemeth, 2015; Donegan et al., 2019). Much of our understanding of heme acquisition in fungal pathogens comes from studies with *C. albicans*, a commensal yeast normally found in the oral, gastrointestinal, and genital tracts but with the potential to cause life threatening systemic infections in immunocompromised patients (Kuznets et al., 2014; Kumamoto et al., 2020; Lopes and Lionkis, 2022). Like other pathogens, *C. albicans* must acquire essential nutrients and micronutrients from the host, but free iron is extremely scarce due to nutritional immunity. Therefore, heme, which is mostly bound to hemoglobin, represents an alternative iron source in the host. Indeed, many gram-positive bacteria such as *Bacillus anthracis* and *Staphylococcus aureus*, and gram-negative bacteria such as *Serratia marcescens* have independently acquired hemophores to hijack the host's iron from heme (Caza and Kronstad, 2013; Contreras et al., 2014). Similar strategies have been identified in *C. albicans* including a heme-iron uptake system in addition to a high-affinity, reductive iron uptake system, and transporters for siderophores (Kornitzer and Roy, 2020).

The initial analyses of *C. albicans* uptake mechanisms for heme and hemoglobin identified several subunits of the vacuolar ATPase (*VMA* genes) and components of the ESCRT (endosomal sorting complex required for transport) and HOPS (homotypic fusion and vacuole protein) sorting complexes involved in endocytosis and targeting vesicles to the vacuole (Weissman et al., 2008) (Fig. 1A). The approach to identify these endomembrane trafficking functions involved expressing the heme-binding protein Rbt5 (described below) in *Saccharomyces cerevisiae* and using Synthetic Genetic Analysis to identify yeast mutants with defective growth on hemoglobin (Weissman et al., 2008). Subsequent testing of mutants with defects in the homologous genes in *C. albicans* confirmed roles for the vacuolar ATPase, the HOPS protein Vps41, proteins of ESCRT complexes, a type I myosin (Myo5), the Sla2 actin-binding protein involved in endocytosis, and a number of transporters. The emerging model is that endocytosis internalizes extracellular components, such as heme, into cytoplasmic vesicles. The cargo-loaded vesicles then fuse with early endosomes where they are sorted by ESCRT machinery into late endosomes or multivesicular bodies.

The mechanisms of acquisition of heme from extracellular hemoglobin have been revealed by elegant studies from the Kornitzer group (Weissman and Kornitzer, 2004; Kuznets et al., 2014; Nasser et al., 2016; Roy and Kornitzer, 2019; Kornitzer and Roy, 2020; Pinksy et al., 2020). Specifically, a relay network of the CFEM (Common in Fungal Extracellular Membrane) proteins Csa2, Rbt5 and Pga7 mediates the process of heme binding for delivery to the endocytic pathway (Weissman and Kornitzer, 2004; Kuznets et al., 2014; Nasser et al., 2016). The CFEM proteins have different locations: Csa2 is exported into the medium, Rbt5 is mainly localized to the cell wall via a GPI anchor, and Pga7 is GPI-anchored to the plasma membrane. These proteins act as hemophores to bind heme (e.g., from hemoglobin), and extracellular Csa2 and Rbt5 are thought to act in a relay to shuttle heme across the cell wall to Pga7 for eventual endocytosis (Fig. 1A). It is not yet clear, however, how the hemophores are connected to the endocytic machinery and whether a plasma membrane

receptor is involved in directing heme to the endocytic/ESCRT pathway (Kornitzer and Roy, 2020). In addition to hemoglobin, other host proteins may also serve as sources of heme including human serum albumin (HSA) (Pinsky et al., 2020). Incubation with HSA stimulated the use of heme and hemoglobin by *C. albicans*, and the influence of HSA was dependent on the CFEM hemophore system. Interestingly, the drugs naproxen and salicylic acid that bind HSA interfere with heme use by *C. albicans*.

The importance of the hemophore relay system for the virulence of *C. albicans* was assessed in a mouse model of systemic candidiasis (Kuznets et al., 2014). In particular, a mutant lacking Pga7 is attenuated in virulence compared to the wild type strain or the mutant containing a reintroduced copy of the wild type *PGA7* gene. Earlier work did not reveal a virulence contribution of Rbt5 in the mouse model, and this may reflect a smaller contribution of Rbt5 to heme and hemoglobin use compared to Pga7 (Kuznets et al., 2014; Braun et al., 2000). An ortholog of Rbt5 also functions in the uptake of hemoglobin in strains of *Paracoccidioides*, a dimorphic fungal pathogen that causes paracoccidioidomycosis in Latin America (Bailão et al., 2014; de Souza et al., 2020, 2021; Seki Kioshima et al., 2021). Reduced Rbt5 expression in *Paracoccidioides* by an antisense RNA approach led to lower survival of the fungus in macrophages and in the spleens of infected mice (Bailão et al., 2014).

Similar to the system in *C. albicans*, the saprophytic yeast *Schizosaccharomyces pombe* uses a GPI-anchored cell surface protein Shu1 and the vacuolar transporter Abc3 to acquire heme (Fig. 1B). Shu1 has 4 Cys residues that are reminiscent of the canonical 8-Cys containing CFEM motif and was found to interact with Nbr1, a receptor for ESCRT-dependent endosomal microautophagy, in the presence of heme (Mourer et al., 2017). These results suggest that the Shu1-Nbr1 heteroprotein complex could be recognized by the ESCRT machinery leading to endocytosis. Additionally, *S. pombe* has another lower affinity heme uptake system dependent on the Str3 protein, and this system appears to function independently from the higher affinity Shu1-Abc3 uptake system. Str3 is a cell surface transmembrane protein belonging to the major facilitator superfamily of transporters (MFS transporters) (Normant et al., 2018; Labbé et al., 2020).

Heme use has also been studied in the fungal pathogen *C. neoformans* that causes life threatening meningoencephalitis in immunocompromised individuals (Cadieux et al., 2013; Bairwa et al., 2019, 2020). This pathogen has a global health impact in that cryptococcal disease accounts for ~15% of deaths in the HIV/AIDS population (Shroufi et al., 2021). The fungus has multiple acquisition mechanisms to obtain iron including a high-affinity, reductive uptake system, transporters for siderophores, and the ability to use heme (Kronstad et al., 2013, Bairwa et al., 2019; Horianopoulos and Kronstad, 2019). As in *C. albicans*, heme/iron uptake by *C. neoformans* involves extracellular binding by a putative hemophore, followed by endocytosis and trafficking via vesicles and ESCRT and HOPS machinery to the vacuole (Fig. 1C). Four candidate CFEM proteins have been identified in *C. neoformans* but these appear to be dispensable for heme acquisition (G. Hu, unpublished results; Bairwa et al., 2019). Instead, a secreted mannoprotein Cig1 may be a hemophore because it possesses weak heme-binding activity and is required for optimal growth of the fungal cells at physiological pH (Cadieux et al., 2013). Notably, Cig1 does not share sequence similarity to

the CFEM hemophores of *C. albicans*. Genetic screens revealed that *C. neoformans* takes up heme by clathrin-mediated endocytosis (CME) and targets it to the vacuole via endosomes and ESCRT and HOPS complexes. Components of CME, such as the Chc1, Las17, Rvs161, and Rvs167 proteins, are critical in uptake of heme by influencing the endocytosis and intracellular trafficking of heme and hemoglobin, as revealed by both heme uptake assays and heme sensor measurements described below (Bairwa et al., 2019, 2020). The ESCRT machinery generally targets monoubiquitinated membrane proteins for internalization and trafficking to the vacuole (Hurley and Emr, 2006), which are the major sites of iron storage and use in cells. It is not yet known whether the heme-Cig1 binding complex, and/or other heme-binding hemophores are ubiquinated, or if any specific receptors are involved, and targeted to ESCRT machinery in *C. neoformans*. The ESCRT proteins Vps23 (ESCRT-I), Vps22 (ESCRT-II), and Snf7 (ESCRT-III) are involved in the use of heme iron, likely by influencing the intracellular trafficking of heme to the vacuole (Hu et al., 2013; Hu et al., 2015). Furthermore, the contribution of the ESCRT machinery for heme occurs at least, in part, via activation of the Rim101 pH signaling pathway leading to expression of Cig1 (Hu et al., 2015). Loss of Cig1 alone does not cause a virulence defect in a mouse model of cryptococcosis, in comparison with the attenuation that occurs upon loss of ferroxidase Cfo1 of the high affinity uptake system (Jung et al., 2008, 2009). However, deletion of the *CIG1* gene in the background of a strain lacking Cfo1 further attenuated virulence thus revealing a contribution of the protein (Cadieux et al., 2013).

Additional studies in *C. neoformans* also revealed contributions to heme trafficking for endosomal trafficking proteins including the noncatalytic subunit of flippase (P4-ATPase) Cdc50, the Sec1/Munc18 (SM) protein Vps45, and two Vam6/Vps39/TRAP1-domain proteins, Vps3 and Vam6/Vps39 (Caza et al., 2018; Hu et al., 2017, 2021). Cdc50 functions as a chaperone to facilitate the exit of flippases and other cargo from the ER. In *C. neoformans*, Cdc50 contributes to heme uptake and internal trafficking of iron-containing molecules (e.g., to the vacuole, mitochondria, and ER), and iron processing, possibly by interaction with flippases (Hu et al., 2017). SM proteins such as Vps45 regulate vesicle trafficking and fusion by interacting with SNARE (soluble N-ethylmaleimide-sensitive attachment protein receptor) proteins that are components of the fusion machinery. In *C. neoformans*, Vps45 co-localizes with mitochondria, and is required for mitochondrial functions, calcium homeostasis, and resistance to reactive oxygen species (Caza et al., 2018). Vps45 also plays a significant role in the uptake of exogenous iron/heme and intracellular sorting of the iron permease Cft1 (Caza et al., 2018). Vam6/Vps39/TRAP1-domain proteins, Vps3 and Vam6, are essential components, respectively, of CORVET and HOPS complexes of the endosomal pathway, mediating the fusion events between endosomes and the vacuole (Bröcker et al., 2012). In *C. neoformans*, the Vam6/Vps39 protein in particular supports fungal growth on heme and influences the trafficking and expression of iron uptake proteins (Hu et al., 2021). Moreover, Vam6 is a key component of the vacuole and mitochondrial patch (vCLAMP) contact site that may participate in vesicle transport between the endomembrane system and mitochondria in fungi (Elbaz-Alon et al., 2014; Hönscher et al., 2014). A role for Vam6 supports the possibility that mitochondria contribute to the iron acquisition from heme via a connection with the vacuole. Notably, functions involved in uptake and cellular trafficking of heme iron in *C. neoformans* are all

required for virulence in a mouse model of cryptococcosis, including Cig1, Las17, ESCRT complex components, Vps45, Cdc50, Vam6, and Vps3, highlighting the importance of heme uptake and intracellular trafficking in fungal pathogenesis (Cadieux et al., 2013, Bairwa et al., 2017, 2019; Caza et al., 2018; Hu et al., 2013, 2015, 2017, 2021).

The importance of heme as an iron source has also been explored in *Aspergillus fumigatus*. This filamentous fungus is acquired via inhalation of airborne spores (conidia) and can cause invasive aspergillosis, particularly in immunocompromised individuals (Cadena et al., 2021). This disease is often fatal due to challenges in diagnosis and the limited options for antifungal therapy. There is a wealth of information on mechanisms of iron sensing, acquisition and homeostasis in *A. fumigatus* (Blatzer and Latgé, 2017; Misslinger et al., 2021). In particular, the contributions of siderophores to iron acquisition and storage, and the role of reductive iron assimilation have been studied in detail. In the context of virulence, the analysis of a mutant lacking SidA, the L-ornithine-N^5-monooxygenase, revealed that this enzyme is essential for virulence in a neutropenic mouse model of aspergillosis (Schrettl et al., 2004). In contrast, a mutant lacking the iron permease, FtrA, for high affinity, reductive iron uptake, was still able to cause disease. The availability of a *sidA/ftrA* double deletion mutant provided an opportunity to assess the ability of *A. fumigatus* to use host-related iron sources. Specifically, the mutant was unable to grow on blood agar or hemoglobin, hemin, holotransferrin or ferritin (Schrettl et al., 2004). These results led to the conclusion that *A. fumigatus* lacks mechanisms to acquire iron from these sources. Consistent with this interpretation, single, double or triple deletion mutants lacking the three genes encoding candidate CFEM hemophores in *A. fumigatus* did not reveal contributions to heme uptake or virulence (Vaknin et al., 2014).

More recently, Michels et al. (2022) revisited the possibility that *A. fumigatus* exploits heme as an iron source during infection. Lung hemorrhage, a feature of invasive aspergillosis, was examined and found to correlate with increased lung hemoglobin during the first three days of infection; a correlation with increased iron in heme was also detected. Heme uptake was assessed in fungal cultures by examining fluorescence in cells exposed to tin (IV)-protoporphyrin, a non-iron heme analog. Increased fluorescence was consistent with uptake, as was the detection of increased iron associated with heme in the cytoplasm of cells grown on heme as the sole iron source. Importantly, administration of heme during infection increased the severity of disease and increased fungal burden in the lungs (Michels et al., 2022). Pre-culture of germinated conidia with heme but not tin (IV)-protoporphyrin also resulted in increased lung damage and fungal burden upon infection. Taken together, these results suggest that *A. fumigatus* can use heme during infection, although questions remain about the mechanisms of acquisition, including the relationship with iron acquisition via siderophore production and potential influences of heme on the host during aspergillosis.

3. Development and application of a genetically encoded heme sensor in *S. cerevisiae*

Genetically encoded biosensors have been deployed in several organisms to investigate mechanisms involved in heme signaling, dynamics, trafficking, and allocation to different

subcellular compartments (Hanna et al., 2016; Gallio et al., 2021; Leung et al., 2021). These biosensors are supporting efforts to understand heme availability in cell compartments given that heme can be stably bound to proteins or present in a labile or exchangeable form to enable transfer to heme-binding proteins. The responsiveness of the heme sensors must therefore be considered in the context of the relative affinities and concentrations of the sensor versus other heme-binding proteins. A detailed discussion of these considerations has been presented by Gallio et al. (2021). Of importance to fungal cells, a ratiometric biosensor (HS1) was engineered by the Reddi laboratory for the analysis of labile or exchangeable heme in *S. cerevisiae* (Hanna et al., 2016). The yeast HS1 heme biosensor functions similar to the mammalian heme probe (CISDY) in which the fluorescence resonance energy transfer (FRET) effect of fluorescent molecules is modulated by the heme binding activities of sensory modules (Song et al., 2015; Hanna et al., 2016). Specifically, the HS1 sensor consists of a bacterial heme binding moiety cytochrome b_{562} (cyt b_{562}) inserted within the coding sequence of an EGFP protein that is N-terminally linked to the red fluorescent protein Katushka 2 (mKATE2) (Arnesano et al., 2000; Takeda et al., 2003; Hanna et al., 2016) (Fig. 2A). Heme binding to cyt b_{562} enables resonance energy transfer from eGFP to cyt b_{562} quenching almost all of the fluorescent signal while not influencing the fluorescence of mKATE2. Thus, the sensor allows the ratiometric analysis of the intracellular labile heme pools independent of sensor concentration (Arpino et al., 2012; Hanna et al., 2016; Bairwa et al., 2020) (Fig. 2B). The read out of heme binding to the sensor is therefore measured as a decrease in the GFP signal relative to the mKATE2 fluorescence.

Different versions of the biosensor have been tuned to different binding affinities and expression levels to detect a range of concentrations of heme in specific subcellular compartments without interfering with cellular physiology or heme homeostasis. For example, an HS1 variant (HS1-M7A) detected a labile heme level in the cytosol of 20 to 40 nM, whereas the levels detected in the nucleus or mitochondria remained ~4-fold lower (2.5 nM) than the cytosol (Hanna et al., 2016) (Fig. 2C). Utilizing the same probe, it was found that heme levels could vary from different subcellular compartments in response to signaling molecules such as nitric oxide (NO). In particular, it was observed that the nitric oxide donor NOC-7 induced rapid changes of labile heme in the cytosol and nucleus, with increases of two and 100-fold, respectively, possibly through the labilization of hemoproteins by weakening the affinity of bound heme to promote dissociation. Notably, a mutant screen revealed that deletion of the gene encoding the heme binding protein glyceraldehyde phosphate dehydrogenase (GAPDH) (or inhibition of heme binding to this protein) altered cytosolic labile heme levels and the influence of heme on the activity of the heme-dependent transcription factor Hap1 (Hanna et al., 2016; Sweeny et al., 2018).

To further explore the potential role(s) of labile heme, experiments with the heme sensor revealed that *S. cerevisiae* cells treated with succinyl acetone (SA), an inhibitor of heme synthesis, exhibited greater reduction of labile heme levels than total heme suggesting that yeast cells preferentially mobilize labile heme when experiencing cellular heme deficiency (Hanna et al., 2018). The same study revealed that stress induced by lead (Pb^{2+}) negatively affects total cellular heme by disrupting heme biosynthesis. Targeting of heme biosensors to specific subcellular compartments in *S. cerevisiae* also helped elucidate not only the levels

of heme but also mechanisms of heme mobilization after synthesis at the mitochondrial inner membrane (Hanna et al., 2016; Martinez-Guzman et al., 2020). For example, the use of *in vivo* pulse-chase assays using the sensors in combination with succinyl acetone (SA) revealed higher heme trafficking rates towards the nucleus than to the cytosol or the mitochondrial matrix suggesting the participation of heme as an interorganellar signal. The analysis of nuclear-mitochondrial heme mobility in different yeast mutants also revealed the contribution of various mitochondria-associated proteins to heme trafficking. For instance, the GTPases Gem1 and Mgm1, regulators of the endoplasmic reticulum-mitochondria encounter structure (ERMES) and mitochondrial fusion, respectively, both positively regulate heme transport towards the nucleus. This was in contrast to the negative regulation observed for Dnm1, a mitochondrial fission-related GTPase. Overall, the versatility and scope of heme biosensors to understand the impact of stress on heme homeostasis and the mechanisms involved in heme uptake and trafficking make of these molecular probes valuable tools for investigating heme trafficking in medically important fungal pathogens, as described in the next section.

4. Application of a genetically encoded heme sensor in the fungal pathogens *Cryptococcus neoformans* and *Candida albicans*

As outlined earlier, the mechanisms for the uptake and homeostasis of heme-iron have been examined in the fungal pathogen *C. neoformans* (Jung et al., 2008, 2009; Kronstad et al., 2013; Hu et al., 2013; Caza and Kronstad, 2013; Cadieux et al., 2013; Saikia et al., 2014; Hu et al., 2015; Bairwa et al., 2017, 2019). The generation and application of a codon optimized version (CnHS) of the yeast heme sensor (HS1-M7A) developed by Hanna et al. (2016) enabled further analysis of the intracellular heme dynamics in *C. neoformans* including the impact of oxidative stress, mitochondrial and vacuolar functions on heme homeostasis (Bairwa et al., 2020). The use of the CnHS heme probe also allowed further examination and validation of the role of clathrin-mediated endocytosis (CME) in the heme uptake process (Bairwa et al., 2019). For example, disruption of the CME machinery through chemical inhibition using chlorpromazine or deletion of the endocytic components Chc1 or Las7 induced a reduced response of the heme sensor to exogenous heme, thus supporting the role of the CME in heme internalization. The CnHS heme sensor was also used to characterize other mechanisms involved in the uptake and intracellular trafficking of heme-iron, such as the hemophore Cig1 and the vesicular trafficking regulator Vps45 (Bairwa et al., 2020; Cadieux et al., 2013; Caza et al., 2018). Importantly, the heme sensor also detected alterations in the labile heme pool when vacuolar or mitochondrial functions were compromised. For example, inhibition of vacuolar acidification or the activity of electron transfer chain complexes resulted in drastic differences of probe ratiometric values compared to the read outs obtained for the untreated fungal cells. In addition, expression of CnHS in a wild-type strain revealed a gradual increase for the ratiometric values of the probe after cells were phagocytosed by murine alveolar macrophages. The increase in the GFP signal relative to mKATE2 fluorescence suggests that *C. neoformans* may experience a restriction of its intracellular labile heme pool. This result suggests limited availability of heme within the host and/or cellular adaptation to heme homeostasis during the intracellular proliferation in phagocytic cells (Bairwa et al., 2020). Overall, the utility of the heme

biosensor validated previous observations on heme-iron homeostasis in *C. neoformans* and, importantly, expanded the molecular toolkit to more thoroughly dissect heme uptake and processing mechanisms.

Genetically encoded heme sensors have also been employed with *C. albicans* to further understand mechanisms of heme and hemoglobin use. A recent study demonstrated the ability of the fungus to use heme both as an iron source and directly, and examined the role of the heme oxygenase Hmx1 in iron acquisition from heme and hemoglobin (Weissman et al., 2021). Hmx1 appears to play a minor role in iron acquisition from these sources, but likely has a more important role in preventing heme toxicity. The use of cells encoding the heme sensor revealed that loss of Hmx1 function results in a higher labile heme concentration in the cytosol in response to heme or hemoglobin in the medium, compared to the wild type strain. As discussed above, the uptake of heme from hemoglobin and from human serum albumin carrying heme is dependent on the CFEM hemophore relay to deliver heme to the vacuole (Pinsky et al., 2020; Weissman et al., 2021). The appearance of heme in the cytoplasm therefore suggests the presence of a vacuolar transporter (e.g., similar to Abc3 in *S. pombe*). The study also revealed that heme may be taken up by another mechanism that is energy dependent and separate from the CFEM hemophore cascade (Weissman et al., 2021). Overall, the studies with *C. albicans* further demonstrate the utility of the genetically encoded heme sensor in understanding internalization dynamics and identifying uptake and processing functions.

5. Connections between iron and heme trafficking, endomembrane systems and organelles.

The connections between endocytosis and iron/heme acquisition identified for fungal pathogens (*C. neoformans* and *C. albicans*) are consistent with earlier studies in *S. cerevisiae*. In this model yeast, endocytic processes influence the activities of two iron uptake systems in the plasma membrane, a reductive, a high-affinity system involving the multicopper oxidase Fet3 and the iron permease Ftr1, and a siderophore transport system involving the Arn1-4 transporters (Li and Kaplan, 2004; Philpott, 2006; Li et al., 2010; Rutherford et al., 2001). The role of endocytosis can be illustrated with the siderophore uptake system in which the Arn1-4 proteins internalize iron bound to siderophores, and fluctuations in the level of the siderophore ferrichrome influence recycling of the Arn1 transporter from endosomes to the plasma membrane (Kim et al., 2002). Sorting of Arn1 in the Golgi to endosomal compartment was found in cells in the absence of ferrichrome, and addition of the siderophore at a low concentration (0.1 μM) resulted in relocalization of Arn1 to the plasma membrane. Interestingly, Arn1 at the plasma membrane undergoes endosome-to-plasma membrane recycling at an intermediate ferrichrome concentration (10 μM) (Kim et al., 2002). Together, these studies establish a paradigm for the connections between iron/heme trafficking and the endomembrane system including the functions of the ESCRT complexes and the vacuole in fungi (Xu et al., 2014; Gerwien et al., 2018; Rizzollo et al., 2021).

Mitochondria represent a key nexus in efforts to understand heme homeostasis because many mitochondrial proteins such as those involved in respiration bind iron ions, heme, or iron–sulfur clusters, and the organelle contains part of the machinery for heme biosynthesis (Paul et al., 2017). Additionally, mitochondrial activities such as the generation of iron-sulfur clusters and heme biosynthesis regulate iron homeostasis via iron or heme-responsive transcription factors. For example, activation of *S. cerevisiae* master iron regulators Aft1- or Aft2-dependent iron regulon requires mitochondrial iron-sulfur protein biogenesis (Babcock et al., 1997; Rutherford et al., 2005). Additionally, and as mentioned above, the Hap1 transcription factor is activated by heme (Zhang and Guarente, 1995). Similarly, the transcription factors Cir1 and HapX in *C. neoformans* are iron-binding proteins and regulate genes involved in mitochondrial iron metabolism including iron-sulfur cluster biosynthesis (Jung et al., 2006; Jung et al., 2010; Do et al., 2020). Additional connections between iron/heme sensing and the endomembrane system are also emerging. For example, Xue and colleagues (2017) reported that the protein complex endoplasmic reticulum (ER) mitochondria encounter structure (ERMES) mediated iron regulation via the transcription factor Aft1. The expression of the iron regulon controlled by the Aft1 was activated in the absence of ERMES components, resulting in accumulation of iron in the yeast cells, perhaps due to an influence on mitochondrial proteins important for regulation of the iron–sulfur cluster biosynthesis (Xue et al., 2017). ERMES functions in the exchange of materials between the ER and mitochondria, and is required for iron homeostasis in *S. cerevisiae* (Xue et al., 2017). It is possible that heme is delivered to the vacuole, mitochondria and ER via ERMES complexes in other fungi. A similar mechanism may be in place in *C. neoformans* because inhibition of mitochondrial respiration causes a decrease in the cytosolic labile heme pool (Bairwa et al., 2020). Overall, these findings support emerging insights into the roles of endomembrane trafficking systems and mitochondria in mediating iron and heme uptake.

6. Conclusions and perspectives

Genetically-encoded sensors are providing new insights into the mechanisms of heme uptake and homeostasis in pathogenic and non-pathogenic fungi. These mechanisms include hemophore dependent and independent heme acquisition systems, and a role for endosomal trafficking in heme acquisition and processing. In fact, endosomal trafficking mediated by ESCRT machinery and the involvement of vacuole and mitochondrial functions are common features in heme uptake in *C. albicans*, *C. neoformans*, and *S. pombe*. These studies suggest that the heme sensor and additional genetic approaches could further clarify aspects of heme uptake and homeostasis in other pathogenic fungi such as *A. fumigatus* and *Paracoccioides sp*. In general, the mechanisms by which fungal (and other) pathogens extract heme from host sources need to be more thoroughly examined (Tolosano et al., 2010; Kornitzer and Roy, 2020; Pinsky et al., 2020). This information will be useful in further understanding fungal pathogenesis and, potentially, for therapeutic intervention by exploiting heme uptake systems, e.g., with non-iron metalloprotophyrins (Bairwa et al., 2019).

Despite the accumulating information on heme acquisition in fungi, there are several areas where further investigation is needed. These include more detailed characterization of the different contributions of the CFEM proteins and hemophores in heme acquisition in

C. albicans and *C. neoformans*. In both species, there is evidence for additional heme uptake mechanisms, and there may be other hemophores for *C. neoformans*. Additionally, functions that act upstream (e.g., receptors and transporters) of the endocytic machinery remain to be identified in the pathogenic fungi. More mechanistic insights are also needed to understand the roles of downstream components such as endomembrane proteins and proteins at membrane contact sites that shuttle heme inside the cell. In this regard, the role of GAPDH found in *S. cerevisiae* needs to also be examined in the pathogens (Sweeny et al., 2018). Finally, the recent work on the Hmx1 heme oxygenase in *C. albicans* provides valuable insights into heme degradation or recycling, and more work is needed to build a clear picture of intracellular heme processing (Weissman et al., 2021).

The role of mitochondria in heme homeostasis in fungi deserves particular emphasis for future work. That is, additional connections between endosomal trafficking and mitochondrial functions are likely to be relevant to iron and heme transport and homeostasis. Mitochondria are vital for fungi to adapt to environmental change or to cause disease, and the majority of mitochondrial proteins are encoded by nuclear genes, translated in cytosol or at the outer mitochondrial membrane, and subsequently imported (Becker and Wagner, 2018; Béthune et al., 2019; Pfanner et al., 2019; Vardi-Oknin and Arava, 2019; Müntjes et al., 2021). In this regard, we raise the speculative possibility that iron and heme homeostasis influences mitochondrial functions via regulation of the endosomal transport of mRNA to mitochondria (Schatton and Rugarli, 2018; Tsuboi et al., 2020). mRNA localization and subsequent local translation enables spatiotemporal gene expression, which allows fungi to finely adjust cellular physiology in response to intracellular and extracellular signals (Das et al., 2021; Niessing et al., 2018). There are detailed studies on mRNA localization in fungi including *S. cerevisiae* and the plant pathogen *Ustilago maydis* that provide a foundation for studying the impact of iron/heme homeostasis on mRNA localization and mitochondrial function (Vollmeister et al., 2012; Haag et al., 2015, 2017; Salogiannis and Reck-Peterson, 2017; Béthune et al., 2019; Müller et al., 2019; Das et al., 2021). In this context, powerful methods are available to study RNA localization including localized RNA recording, APEX-seq, APEX-RIP, proximity-specific ribosome profiling, fluorescence in situ hybridization (FISH) and live cell imaging (Williams et al., 2014; Chen et al., 2015; Kaewsapsak et al., 2017; Tutucci et al., 2018; Padrón et al., 2019; Medina-Munoz et al., 2020). Finally, we also note that iron homeostasis is known to involve regulation of mRNA stability and this area should be investigated in the context of iron and heme pools in different cellular compartments of fungal pathogens (Perea-García et al., 2022; Romero et al., 2022).

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Highlights

- Heme is an abundant iron source for fungi that infect vertebrate hosts

- Proteins at the cell surface play important roles in iron acquisition from heme

- Internalization of heme depends on endocytosis in model and pathogenic fungi

- Genetically-encoded sensors support the analysis of heme uptake and trafficking

- More work is needed to understand heme processing and movement between organelles
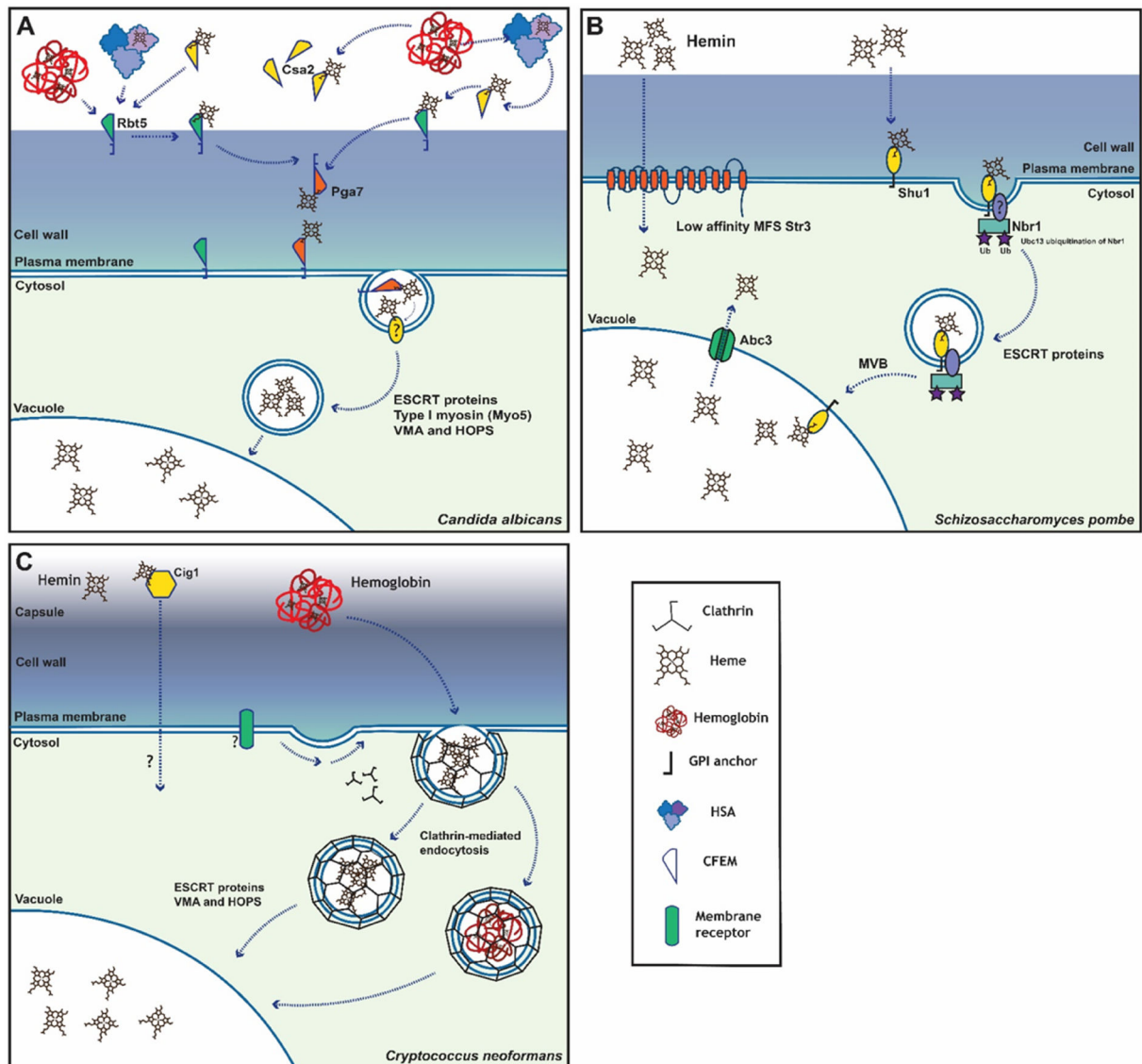
Fig. 1 - Overview of heme acquisition systems in pathogenic and model fungi. (A) *Candida albicans*, **(B)** *Schizosaccharomyes pombe* **and (C)** *Cryptococcus neoformans*.
As described in the main text, the system in *C. albicans* consists of CFEM proteins (i.e., Rbt5, Csa2 and Pga7) that form a relay network to access heme (e.g., from hemoglobin). Subsequent internalization involves endocytic trafficking and participation of the ESCRT system for delivery to the vacuole. For *S. pombe*, the Shu1 protein binds heme and translocates from the plasma membrane to the vacuolar membrane. The ESCRT system and the Nbr1 protein also participate in internalization, while the Abc3 transporter is thought to mobilize heme from the vacuole to the cytoplasm. A lower affinity heme uptake system also exists in *S. pombe* and requires the Str3 transporter of the major facilitator superfamily (MFS). The system for heme use in *C. neoformans* involves the mannoprotein Cig1 and the participation of the machinery for endocytosis as well as the ESCRT system.
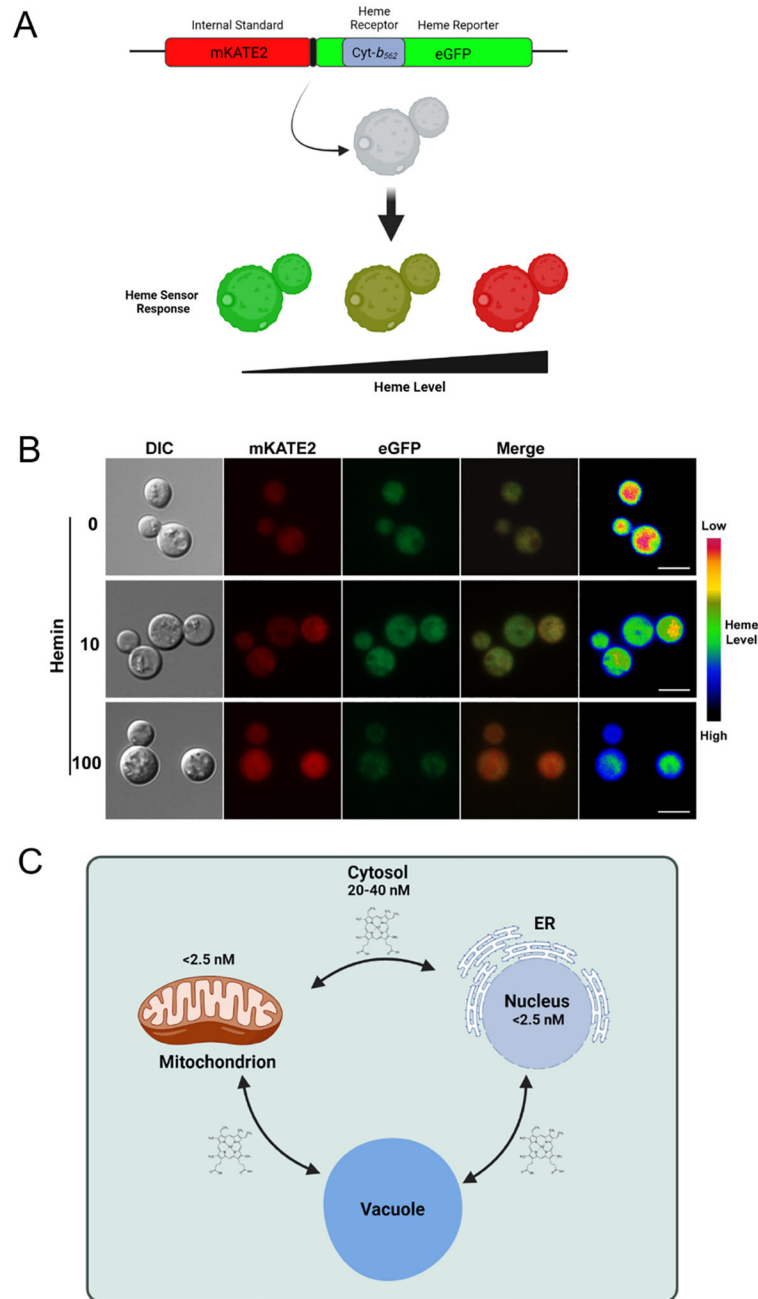
Fig. 2 - Deployment of a genetically encoded heme biosensor in *Cryptococcus neoformans*.
(A) Diagram of sensor composition including the heme binding domain of Cyt-b562 within eGFP and the mKATE2 protein to allow ratiometric measurements (Hanna et al., 2016). Heme binding quenches the GFP signal but does not influence mKATE2 thus resulting in a shift in the ratio of fluorescence from the two proteins. (B) Example of the evaluation of the labile heme pool in *C. neoformans* by microscopy. The images are from a replicate of an experiment presented by Bairwa et al. (2020). Scale bar = 5 μm. (C) Diagram of

cellular compartments and labile heme concentrations estimated by Hanna et al. (2016) for *S. cerevisiae.*