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Ferrochelatase is a conserved downstream target of the blue light-sensing White collar complex in fungi

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Light is a universal signal perceived by organisms, including fungi, in which light regulates common and unique biological processes depending on the species. Previous research has established that conserved proteins, originally called White collar 1 and 2 from the ascomycete *Neurospora crassa*, regulate UV/blue light sensing. Homologous proteins function in distant relatives of *N. crassa*, including the basidiomycetes and zygomycetes, which diverged as long as a billion years ago. Here we conducted microarray experiments on the basidiomycete fungus *Cryptococcus neoformans* to identify light-regulated genes. Surprisingly, only a single gene was induced by light above the commonly used twofold threshold. This gene, *HEM15*, is predicted to encode a ferrochelatase that catalyses the final step in haem biosynthesis from highly photoreactive porphyrins. The *C. neoformans* gene complements a *Saccharomyces cerevisiae hem15*Δ strain and is essential for viability, and the Hem15 protein localizes to mitochondria, three lines of evidence that the gene encodes ferrochelatase. Regulation of *HEM15* by light suggests a mechanism by which *bwc1*/*bwc2* mutants are photosensitive and exhibit reduced virulence. We show that ferrochelatase is also light-regulated in a *white collar*-dependent fashion in *N. crassa* and the zygomycete *Phycomyces blakesleeanus*, indicating that ferrochelatase is an ancient target of photoregulation in the fungal kingdom.

INTRODUCTION

Light represents both a signal that provides clues about environmental conditions and a detrimental form of radiation that can kill or mutate organisms. Many fungi are capable of sensing light to regulate growth, asexual or sexual reproduction, or pigmentation, and thereby light represents a common environmental signal that orchestrates important growth and survival strategies (Tisch & Schmoll, 2010). In the last 5 years, it has emerged that a conserved pair of proteins, homologues of the White collar 1 (WC-1) and White collar 2 (WC-2) proteins that were first characterized in the model ascomycete *Neurospora crassa*, mediate near-UV and blue-light responses in representative species across the kingdom, including other ascomycetes, basidiomycetes and the Mucormycotina that were formerly known as the zygomycetes (reviewed by Bahn *et al.*, 2007; Corrochano, 2007; Herrera-Estrella & Horwitz, 2007; Purschwitz *et al.*, 2006). Thereby, fungi are enabled to sense blue light emanating from the sky, and also sense UV light directly or via associated blue light as a proxy for concomitant exposure to potentially injurious solar irradiation. Other photoreceptors are distributed sporadically within fungi to control light responses at other wavelengths, such as the red/far-red sensor phytochrome (Blumenstein *et al.*, 2005). Curiously, in *Aspergillus nidulans*, there is evidence for the phytochrome protein forming a large complex with the blue-light sensory system controlled by the White collar proteins, as well as potentially other signalling components through interactions with the Velvet proteins (Bayram *et al.*, 2008; Purschwitz *et al.*, 2008, 2009); this further implicates the White collar complex (WCC) as a central component of light sensing. Remarkably, model fungi such as *Saccharomyces cerevisiae* and *Candida albicans*, and other hemiascomycetes have lost this light-sensing pathway, presumably as they adapted to different environmental niches such as soil and the mammalian gastrointestinal tract, in which blue-light sensing is no longer advantageous, analogous to the loss of vision in the blind fish that have evolved independently in caves around the world.

Abbreviations: LOV domain, Light-Oxygen-Voltage domain; WCC, White collar complex.

The microarray data discussed in this paper are available from the NCBI Gene Expression Omnibus (GEO) under accession number GSE21484.

WC-1 is a photoreceptor protein in which the flavin chromophore is bound to a Light-Oxygen-Voltage (LOV) domain (He *et al.*, 2002). WC-1 binds to WC-2 to form a complex that functions as a transcription factor evoking light-responsive target gene expression via GATA-type zinc finger DNA-binding domains (Froehlich *et al.*, 2002). Given the diversity of responses of fungi to light, there are likely many different downstream targets of the WC-1 and WC-2 proteins. However, there are some conserved targets that are light-induced, such as genes for β-carotene pigment production in the ascomycetes (Li & Schmidhauser, 1995; Ruiz-Roldán *et al.*, 2008) and zygomycetes (Silva *et al.*, 2006, 2008), although these are not universally conserved, since the basidiomycete *Cryptococcus neoformans* does not produce carotenoid pigments, nor is melanization induced by light exposure. The identification and functional analysis of conserved light-regulated genes should provide an understanding of the selective pressures that maintain this signalling system in some fungi but not others.

C. neoformans is a globally distributed human pathogen that is a particular threat to immunocompromised individuals (Casadevall & Perfect, 1998). It is a member of the phylum Basidiomycota and thus quite divergent from species such as *N. crassa* in the Ascomycota. Previous research has established that the *white collar* homologous genes *BWC1*/*CWC1* and *BWC2*/*CWC2* control the ability of *C. neoformans* to sense light and serve to repress mating (Idnurm & Heitman, 2005; Lu *et al.*, 2005; Yeh *et al.*, 2009). In addition, these two genes are required for tolerance to UV exposure and virulence in a mouse model of cryptococcosis (Idnurm & Heitman, 2005). Inhibition of mating could be a consequence of the transcriptional repression by light of genes within the mating-type locus, as seen by Northern blot analysis (Idnurm & Heitman, 2005). However, those studies used 24 h light or dark exposures, and the mating-type genes could be secondary targets of genes directly regulated by Bwc1–Bwc2. The genes responsible for the UV-sensitivity and reduced-virulence phenotypes are unknown.

Here we performed whole-genome microarray analysis to identify genes regulated by a short exposure to light. The two most highly regulated genes were *CFT1* and *HEM15*. *CFT1* is the homologue of the *S. cerevisiae FTR1* gene, which encodes a high-affinity iron permease. Iron metabolism is an active area of investigation in pathogens including *C. neoformans*, and *CFT1* has been characterized independently and shown to be essential for virulence in *C. neoformans* (Jung *et al.*, 2008) as well as in *Candida albicans* (Ramanan & Wang, 2000). Here we focused on the *HEM15* gene, predicted to encode ferrochelatase, an enzyme that catalyses the conversion of highly phototoxic porphyrin molecules into haem. This enzyme is conserved from bacteria to fungi and humans and plays key roles in photobiology. In fact, in humans, mutations in ferrochelatase result in a disease called porphyria, in which the accumulation of photoreactive porphyrin molecules renders afflicted individuals uniquely sensitive to light, causing photophobia (Kauppinen, 2005). We show that *C. neoformans HEM15* encodes ferrochelatase, and that ferrochelatase is light-regulated in representative species of photosensing fungi from three diverse phyla of the fungal kingdom. Ferrochelatase is thus a highly conserved transcription factor target in three major phyla of the kingdom. Our findings suggest that fungi produce ferrochelatase in response to light as a means to reduce their exposure to photosensitization via light-mediated actions on porphyrins.

METHODS

Fungal strains and culture conditions. Fungal strains used in the study are listed in Table 1. Yeast species were maintained on yeast peptone dextrose (YPD) medium and filamentous species on 5 % V8 juice pH 5 medium. The *C. neoformans* strains were revived from laboratory −80 °C glycerol stocks. The *N. crassa* wild-type and *wc-1* mutant strain, and the wild-type *Rhizopus oryzae* strain were obtained from the Fungal Genetics Stock Center [University of Missouri-Kansas City (McCluskey, 2003; McCluskey *et al.*, 2010)]. *Phycomyces blakesleeanus* wild-type and *mad* mutant strains were as reported previously (Idnurm *et al.*, 2006; Sanz *et al.*, 2009). For light-induction experiments, equal quantities of yeast cells, conidia or sporangiospores of wild-type or *white collar 1* mutant strains were inoculated onto YPD agar plates (15 cm diameter) and grown in complete darkness for 1 or 2 days depending on the growth rate of each species, after which one set was exposed to light (1600 lux, cool-white fluorescent) for 1 h. Light treatments were replicated at least once. For *R. oryzae*, a wild-type strain was grown on 10 cm diameter Petri dishes, and received white-light illumination, or wavelengths modified with Lee filters #47B blue, #58 green and #25 red (LE 4747, LE 4758 and LE 4725, Calumet). Irradience was estimated to be 22, 0.3, 4 and 5 μmol m^{-2} s^{-1}, for white, blue, green and red light, respectively. For all samples, cells or mycelia were harvested and frozen in a dry ice/ethanol bath before being lyophilized overnight.

Microarray analysis. *C. neoformans* strains JEC20 and JEC21 were cultured over a 24 h time period, and exposed to light for 0, 1, 1.25 or 2 h. These two strains are congenic, differing only in the ~20 genes found within the mating-type locus (Heitman *et al.*, 1999; Kwon-Chung *et al.*, 1992). RNA was isolated from freeze-dried samples using Trizol reagent according to the manufacturer's instructions (Invitrogen). For *C. neoformans* microarrays, 15 μg RNA was subjected to reverse transcription, and the cDNAs were labelled with Cy3 and Cy5 dyes (Amersham) and hybridized to the slides as described previously (Ko *et al.*, 2009). The microarray was constructed as part of a consortium within the *C. neoformans* community and was printed at the Duke University Microarray facility. It comprises 8000 70-mer oligonucleotides consisting of all genes predicted for the serotype D genome, primarily represented as a single spot, as well as control 70-mers from other organisms. After co-hybridization with Cy3- and Cy5-labelled cDNAs derived from dark- and light-treated samples, arrays were scanned with a GenePix 4000B scanner (Axon Instruments) and analysed using GenePix Pro v4.0 and BRB-ArrayTools (developed by Richard Simon and Amy Peng Lam at the National Cancer Institute; http://linus.nci.nih.gov/BRB-ArrayTools.html). Data were normalized using LOWESS smoothing, and differential expression was determined using a randomized variance *t*/*F* test. The threshold *P* value for determining differential expression was $P<0.05$. Six replicates were analysed, three for strain JEC21 (*MAT*α) and three for strain JEC20 (*MAT***a**), with the

Table 1. Fungal strains used in this study

Strain	Genotype	Parent or background	Reference or source
***C. neoformans* var. *grubii* (serotype A) strains**			
KN99α	Wild-type, *MAT*α		Nielsen *et al.* (2003)
AI81	*bwc1*::*NAT*	KN99α	Idnurm & Heitman (2005)
AI132	P_{H3}-*HEM15-GFP-NAT*	KN99α	This study
***C. neoformans* var. *neoformans* (serotype D) strains**			
JEC21	Wild-type, *MAT*α		Kwon-Chung *et al.* (1992)
JEC20	Wild-type, *MAT***a**		Kwon-Chung *et al.* (1992)
AI5	*bwc1*::*URA5 ura5 MAT*α	JEC43	Idnurm & Heitman (2005)
RAS009	*ADE2/ade2 ura5/ura5 LYS1/lys1 LYS2/lys2*		Sia *et al.* (2000)
AI170	*hem15*::*NAT/HEM15 ADE2/ade2 ura5/ura5 LYS1/lys1 LYS2/lys2*	RAS009	This study
AI94	*LYS2/lys2 ADE2/ade2*		Idnurm & Heitman (2005)
AI169	*hem15*::*NAT/HEM15 LYS2/lys2 ADE2/ade2*	AI94	This study
AI156	*HEM15-HYG*	JEC21	This study
AI158	*hem15*::*NAT HEM15-HYG*	AI156	This study
AI154	P_{H3}-*HEM15-GFP-NAT*	JEC20	This study
AI159	*hem15*::*NAT* P_{H3}-*HEM15-GFP-NAT*	AI154 × AI158	This study
AI165	*hem15*::*NAT*	JEC21 × *MAT***a** version of AI158	This study
***Candida albicans* strain**			
SC5314	Wild-type		Fonzi & Irwin (1993)
***S. cerevisiae* strains**			
Σ1278b	Wild-type		Grenson *et al.* (1966)
BY4742	*MAT*α *his3 leu2 lys2 ura3*		Brachmann *et al.* (1998)
AIY1	*hem15*::*KanMX MAT*α *his3 leu2 lys2 ura3*	BY4742	This study
AIY3	pTH19 *hem15*::*KanMX MAT*α *his3 leu2 lys2 ura3*	AIY1	This study
AIY4	pTH19-*CnHEM15 hem15*::*KanMX MAT*α *his3 leu2 lys2 ura3*	AIY1	This study
***N. crassa* strains**			
FGSC 4200	Wild-type		Kafer & Fraser (1979)
FGSC 4398	*wc-1*		Degli-Innocenti & Russo (1984)
***P. blakesleeanus* strains**			
NRRL1555	Wild-type		Bergman *et al.* (1973)
L51	*madA madB*		Lipson *et al.* (1980)
***R. oryzae* strain**			
RA 99-880	Wild-type		Ma *et al.* (2009)

induction experiments being conducted on independent days spanning a 9 month time frame.

Northern blot analysis. RNA was extracted from lyophilized yeast cells or mycelia using TRIzol reagent. Total RNA (15, 20 or 25 μg) was resolved on denaturing formaldehyde agarose gels, blotted to Zeta-Probe membranes (Bio-Rad), and probed with [α-^{32}P]dCTP radiolabelled DNA fragments of genes amplified with the primers listed in Table 2.

Generation of a *hem15* deletion strain of *C. neoformans*. The *HEM15* gene was targeted for gene disruption by biolistic transformation with an overlap PCR product. The 5′ and 3′ flanking regions of the *HEM15* gene were amplified from genomic DNA of strain JEC21 with primers JOHE16530/JOHE16531 and JOHE16532/JOHE16533, respectively. The nourseothricin acetyltransferase (*NAT*) gene was amplified with primers JOHE8677/JOHE11866 from plasmid pAI3. The three products were mixed in an equimolar ratio and an overlap amplification was performed with primers JOHE16530/JOHE16533. The construct was precipitated onto gold beads and wild-type strains were transformed using a PDS-1000/He particle delivery system (Bio-Rad) following reported protocols (Toffaletti *et al.*, 1993). Correct gene replacement events in strains were confirmed by PCR and Southern blot analysis. No transformants with homologous replacement events were obtained in wild-type haploid strains. The *HEM15* gene was replaced with the *NAT* marker in diploid strains RAS009 and AI94, and in a strain transformed with *HEM15* fused to a hygromycin-resistance cassette. To construct this plasmid, the wild-type *HEM15* gene was amplified by PCR and cloned into pCR2.1 TOPO (Invitrogen), and an *Xba*I–*Spe*I fragment containing the Hyg gene from pJAF15 was inserted into the *Spe*I site. To isolate *hem15*Δ haploid strains, the haploid

Table 2. Oligonucleotide primers used in this study

Primer	Sequence (5′–3′)	Purpose
JOHE16530	TCATTTGCCCTCTTGACTTC	Deletion of *C. neoformans HEM15*, 5′ flank
JOHE16531	GCTTATGTGAGTCCTCCCTGGGACGTTGGATTGCAG	
JOHE16532	CTCGTTTCTACATCTCTTCATCCTCGACATGTGAGCC	Deletion of *C. neoformans HEM15*, 3′ flank
JOHE16533	GAGCCATTCTACAAAACCAC	
JOHE8677	GAAGAGATGTAGAAACGAG	*NAT* cassette
JOHE11866	GGGAGGACTCACATAAGC	
JOHE17764	ATACCTGCTATTTGGACAAATTTAGCAGTAAAAAAAAG-AACGTTTAAAGACGTACGCTGCAGGTCGAC	Deletion of *S. cerevisiae HEM15*
JOHE17765	ATAAATAAGAGAATATACTGATATTGAGATTGTGGGATG-AATGGCCCTTAATCGATGAATTCGAGCTCG	
JOHE16534	GAGCTATGGCAAACACCG	*C. neoformans HEM1* probe
JOHE16535	AGCTGCTTGTCAGACCAG	
JOHE16536	TGGTCGAGGGCACATCAAG	*C. neoformans HEM14* probe
JOHE16537	ATGATGAATGTTACAGAC	
JOHE16538	TCGAATGCTGCCAAACCC	*C. neoformans HEM12* probe
JOHE16539	CCCATATCTTCCGGTTTG	
JOHE8713	ATGGAAGAAGAAGGTACG	*C. neoformans ACT1* probe
JOHE8714	TTAGAAACACTTTCGGTG	
JOHE16577	ATGGCCTCCATGGCACTGAG	*N. crassa hem-15* probe
JOHE16578	TGTCCAGCAAAGAACTTC	
JOHE16581	TCTACTGGTTATCATGGG	*N. crassa act-1* probe
JOHE16582	AGAAGCACTTGCGGTGCACG	
JOHE16613	GCHCCHCAYAARCMYTAYRTYGCNTT	*P. blakesleeanus hemH* degenerate primers
JOHE16622	GTYTCRATRTGRTCSWYDGTRAANGC	
JOHE16609	CACACTTTCTACAACGAG	*P. blakesleeanus act1* probe (AJ287184)
JOHE16610	ACATCTGCTGGAAGGTAG	
JOHE16799	ATGACTGCAATCCAACGTCC	Cloning *C. neoformans HEM15* cDNA
JOHE16800	CTAAGCGGCCATTTTCGTACC	
JOHE16645	CCATGGCTGCAATCCAACGTCC	Localization of *C. neoformans* Hem15
JOHE16646	CCATGGGAGCGGCCATATTCGTAC	
JOHE17187	ATGCTTTCCAGAACAATCCG	*S. cerevisiae HEM15* probe
JOHE17188	TCAAGTAGATTTCGTGATTGC	
JOHE17189	ATGGATTCTGAGGTTGCTGC	*S. cerevisiae ACT1* probe (hybridizes with *Candida albicans*)
JOHE17190	AGAAACACTTGTGGTGAACG	
JOHE17191	ACTTGCATTGCCAGAAGAGG	*Candida albicans HEM15* probe
JOHE17192	CCTTGTGCTCTTTCACTACC	
JOHE17342	CACTGGATCTCTACTTGG	*R. oryzae HEM15* probe
JOHE17343	TCGATGTGATCCTTGACG	
JOHE15199	ACGGTTCCGGTATGTGTAAG	*R. oryzae ACT1* probe
JOHE15200	TCTGTGGACAATAGAAGGAC	
JOHE16225	CCAACGGTCAGGTATGTC	*C. neoformans HMP1* probe
JOHE16226	ATCGTGCTGGACATTACC	
JOHE12182	GGATCCATGTCCCTCCTCGCCGAGTC	*C. neoformans BWC2* probe
JOHE12183	GGATCCAGATTGCTTATCTTTTTTGGG	
JOHE16221	TCTGTGACTTTCATTCAG	*C. neoformans CFT1* probe
JOHE16222	CCTTCTCAGATCCTTCTG	
JOHE16223	TGGACCTGCAGTTGGCTC	*C. neoformans HEM15* probe
JOHE16224	CTCTAAATGCAAACAGTC	

hem15::*NAT HEM15-HYG* strain was crossed to wild-type strain JEC20 (*MAT***a**) on V8 pH 7 medium or Murashige–Skoog agar (Xue *et al.*, 2007), basidiospores were micromanipulated onto YPD agar or YPD supplemented with 10 μg haematin ml^{-1}, and the phenotype of germinated progeny was determined on different medium types.

Generation of a *hem15* deletion strain of *S. cerevisiae* and its complementation with the *C. neoformans HEM15* gene. To demonstrate that the putative *C. neoformans HEM15* gene encodes a functional ferrochelatase, an *S. cerevisiae hem15* mutant was complemented with the *C. neoformans HEM15* gene. The *S. cerevisiae hem15* mutant was generated by replacing the *HEM15* ORF with the

KanMX6 construct in strain BY4742. Flanks of *HEM15* were included in primers JOHE17764 and JOHE17765, and these primers were used to amplify the *KanMX6* construct, and *S. cerevisiae* cells were transformed with the DNA fragment using a lithium acetate/PEG heat-shock method. Transformants were plated on YPD medium containing 200 μg G418 ml^{-1} and 20 μg haematin ml^{-1}. Gene replacement was confirmed by PCR and Southern blot analysis. The cDNA of *C. neoformans* was amplified from reverse-transcribed RNA of strain JEC21 and cloned into plasmid pCR2.1 TOPO, and a clone with no errors, as determined by DNA sequencing, was subcloned into the *Eco*RI site of plasmid pTH19. This plasmid comprises a 2μ circle, the *URA3* gene for selection in *S. cerevisiae*, and the *ADH1* promoter to drive expression of inserted cDNAs (Harashima & Heitman, 2005). The *S. cerevisiae hem15*Δ and wild-type BY4742 strains were transformed with the plasmid and empty vector control, and selection was on synthetic dextrose (SD) medium minus uracil and supplemented with haematin (20 μg ml^{-1}). Growth of strains was compared on media with and without haematin.

Subcellular localization of Hem15. To assess the intracellular localization of Hem15 in *C. neoformans*, the *HEM15* gene was amplified from genomic DNA of strain JEC21 with primers JOHE16645/JOHE16646 and subcloned using *Nco*I restriction enzyme sites in-frame with the GFP ORF, in a plasmid driving expression from the *C. neoformans* histone H3 promoter (Idnurm *et al.*, 2007). The construct was introduced into strains JEC20 and KN99α by *Agrobacterium*-mediated transformation (Idnurm *et al.*, 2004). A *hem15*::*NAT HEM15-GFP* strain was generated by crossing the JEC20 + *HEM15-GFP* strain with strain AI158, isolation of nourseothricin-resistant and hygromycin-sensitive progeny, and PCR analysis to determine which of these strains contained the *hem15*::*NAT* allele. Mitochondria were stained in live cells growing in YPD medium using MitoTracker Red (25 nM final concentration) according to the manufacturer's guidelines (Invitrogen). Fluorescence of cells was examined using a Zeiss Axiophot 2 Plus fluorescence microscope equipped with filters for excitation and detection of GFP and MitoTracker Red, and photographed with an AxioCam MRm digital camera.

Identification of fungal ferrochelatase homologues. The *N. crassa*, *S. cerevisiae*, *Candida albicans* and *R. oryzae* ferrochelatase homologues were identified by BLAST searches of their genome sequences. The *P. blakesleeanus* genome sequence was unavailable at the time of the experiments, so the *hemH* gene was amplified from genomic DNA of strain NRRL1555 with degenerate oligonucleotide primers (JOHE16613/JOHE16622) designed for conserved regions in fungal ferrochelatase homologues. The 672 bp PCR fragment was amplified, cloned into the pCR2.1 TOPO plasmid, and sequenced. Each homologue was amplified with the primers listed in Table 2 and used as a probe for Northern blot analyses. Amplified fragments of the actin genes of each organism were used as loading and RNA transfer controls.

RESULTS

Identification of light-regulated genes in *C. neoformans* by whole-genome transcript analysis

To identify candidate targets for regulation by light as mediated by Bwc1–Bwc2 in *C. neoformans*, transcript abundance was assessed using microarray analysis. A microarray comprising 70-mer oligonucleotides for each of the ~6000 genes in *C. neoformans* has been constructed by a consortium of *C. neoformans* research laboratories as based on the complete genome sequences of two serotype D strains (Loftus *et al.*, 2005). A series of initial microarrays used wild-type and *bwc1* mutant strains grown in the light and dark compared with a pooled reference sample of RNA isolated from all four samples. It was apparent from these preliminary studies that insufficient transcript regulation was occurring in response to light to detect Bwc1-dependent transcripts through this approach. Therefore, an induction of 1–2 h was used to identify genes regulated by light in wild-type congenic strains JEC20 and JEC21. These two strains result from back-crossing strategies, such that they are identical except for the ~20 genes that comprise the unique alleles (**a** or α) of the mating-type locus. Six replicates, three from each strain, were generated on six independent occasions, thus constituting a set of experimental replicates (rather than biological replicates, which are performed at the same time). Duplicate sets of strains were grown on YPD agar plates for 24 h, and one set was exposed to white fluorescent light (1, 1.25 or 2 h, 1600 lux white fluorescent light) shortly before harvesting the cells under safe red light conditions.

cDNAs derived from either the light or constant dark treatment were labelled with Cy5 or Cy3 dyes and competitively hybridized to the microarray slides. The signal intensity for each dye was measured and analysed. All genes that provided hybridization data for at least four replicates (this eliminated about 200 probes on the array) were considered without exclusions based on signal intensity, with the rationale that our preliminary studies had suggested that the light-regulated genes of *C. neoformans* would be expressed at low basal levels and that any noise amplified by low transcript levels would be eliminated with at least four experimental replicates. The mean induction in response to light was calculated for each spot; it should be noted that variation exists between each replicate such that using the mean potentially reduces the number of regulated genes. The full dataset is available in the NCBI GEO database for those interested in alternative analyses.

The microarray expression data revealed that no gene had a dramatic difference in transcript abundance between dark and light conditions (Table 3). Only a single gene, *HEM15*, responded to light to the extent that transcript levels were twice those observed in the dark. Many microarray studies cut off transcript changes at a twofold threshold; with such an approach this one gene alone would be considered as light-regulated in *C. neoformans*. In the *C. neoformans* analysis presented in Table 3, a stringent cut-off required at least a 1.5-fold increase or 0.5-fold decrease in transcription in the presence of light. This criterion was used, since we were uncertain whether or not we could confirm changes in abundance at levels below a 50 % increase or decrease, particularly given the complication that many genes tested showed low basal transcript abundance. In reducing the threshold, still very few genes were identified that were consistently upregulated or downregulated by light, that is 17 and 11, respectively (Table 3).

Table 3. Summary of light-regulated and other genes in *C. neoformans* strains

Replicates 1–3 are for strain JEC21 (*MAT*α) and replicates 4–6 are for strain JEC20 (*MAT***a**). $\log_2$ values are shown. For regulated genes to be included, the expression data of levels of transcript in the light versus the dark had to be present in at least four of the six experimental replicates, with a mean change in abundance set as 50 % higher or lower than in the dark. Note, however, the variation between experiments and that the mean value should be viewed accordingly. The seven genes in addition to *HEM15* that encode the enzymes for haem biosynthesis are included. Two microarray probes are present on the slides for CNA03960 (predicted to encode glyoxal oxidase). Light exposure values are in h : min.

Replicate no.	1	2	3	4	5	6	Mean	GenBank accession no.	Gene name and/or predicted protein function
Light exposure	**1 : 15**	**1 : 15**	**1 : 15**	**1 : 00**	**2 : 00**	**1 : 00**			
Mating type	**α**	**α**	**α**	**a**	**a**	**a**			
Gene									
180.m00351	1.20	1.05	1.12	1.04	0.63	1.25	1.05	CNF01700	*HEM15*, ferrochelatase
179.m00500	1.34	0.48	1.48			0.29	0.90	CNC05700	*CFT1*, high-affinity iron permease
184.m04340	0.83	0.64	2.23			−0.37	0.83	CNH00160	Conserved hypothetical protein
179.m00505	1.26	0.91	2.18	0.54	−0.43	0.24	0.78	CNC05770	292 aa, weak match to conserved proteins
179.m00102	1.12	0.52	0.24	1.08	0.53	1.14	0.77	CNC01070	*GLV2*, glycine dehydrogenase
1744.seq.067	0.71	1.16	0.80	0.81	0.84	0.23	0.76	CNH00800	*GPI1*-related, biosynthesis of GPI anchors
185.m02371	0.25	0.45	0.55	1.23	0.78	1.00	0.71	CNJ00270	Small (82 aa) protein
181.m08013	0.62	0.41	0.10	1.40	0.98	0.64	0.69	CNA02520	Small (197 aa) serine-rich protein gene family
184.m04389	0.97	0.74	0.22			0.66	0.65	CNH00690	Minor histocompatibility antigen h13
181.m08610	0.79	0.48	0.79			0.48	0.64	CNA04570	Conserved hypothetical protein, WSC domains
167.m03214	0.75	0.54	0.62	0.65	0.32	0.92	0.63	CNE00580	*GPX1*, glutathione peroxidase
176.m02473	1.05	0.76	−0.25	0.13	0.28	1.65	0.60	CNK00180	L-Serine dehydratase
181.m08425	0.60	1.22	0.18	1.03	0.16	0.39	0.60	CNA06810	Hypothetical protein
162.m02981	0.36	0.68	0.09	1.11	0.34	0.99	0.60	CNI01220	Putative ferric-chelate reductase
163.m02744	0.84	0.38	−0.24	1.21	0.22	1.15	0.59	CND02730	FK506-binding protein 4
181.m08182	1.06	0.62	0.43	0.76	0.19	0.50	0.59	CNA04240	IMP dehydrogenase
167.m03676	0.67	0.32	1.45	−0.02	0.65	0.46	0.59	CNE00390	MFS drug transporter (predicted)
167.m03276	0.08	0.03	1.22			0.81	0.53	CNE01220	*BWC2*, WC-2 homologue
179.m00031	0.22	1.65	1.81	0.10	−0.41	−1.21	0.36	CNC00310	*HMP1*, cruciform DNA-binding protein
179.m00499	0.29	0.36	0.76			0.35	0.44	CNC05690	*CFO1*, Fet3 ferroxidase
186.m03863	−0.55	−0.86	−0.08	−0.77	0.06	0.45	−0.29	CNB04260	*HEM1*, 5-aminolevulinic acid synthase
181.m08111	0.60	−0.13	0.22	0.02	0.10	−0.23	0.10	CNA03580	*HEM2*, 5-aminolevulinic acid dehydratase
179.m00209	0.14	−0.15	−0.29	−0.57	0.25	−0.13	−0.13	CNC02250	*HEM3*, porphobilinogen deaminase
176.m02384	0.13	−0.05	0.12	0.05		0.08	0.06	CNK02510	*HEM4*, uroporphyrinogen III synthase
186.m03417	0.43	−0.14	0.16			0.03	0.12	CNB00030	*HEM12*, uroporphyrinogen decarboxylase
167.m05798	0.01	−0.49	0.37	−1.32	−0.36	−0.98	−0.46	CNE00980	*HEM13*, coproporphyrinogen III oxidase
177.m03219	−0.18	−0.15	0.38	−0.32	−0.20	−0.20	−0.11	CNG03820	*HEM14*, protoporphyrinogen oxidase
181.m08767	−0.53	−0.50	0.29	−0.81	−0.65	−1.15	−0.56	CNA03960	Glyoxal oxidase
1703.seq.027	−0.36	−1.02	0.41	−1.47	−0.16	−0.80	−0.57	CNA03960	Glyoxal oxidase
179.m00221	−0.39	−0.83	−0.21	−0.91	−0.33	−0.93	−0.60	CNC02410	*ERG25*, C4 methyl-sterol oxidase
179.m00123	−0.25	−0.30	−0.17	−1.06	−0.42	−1.54	−0.62	CNC01350	Hypothetical protein
185.m02696	−1.02	−0.45	0.28	−0.56	−0.70	−1.32	−0.63	CNJ03400	Monosaccharide transporter
162.m02426	−0.32	−0.24	−1.72	0.13		−1.00	−0.63	CNI01440	Rad21/Rec8 domain protein
163.m06441	−0.50	−0.75	−0.06	−1.41		−0.44	−0.63	CND03730	Conserved hypothetical protein
184.m04377	−0.37	−0.97	0.17	−1.46	−0.25	−1.05	−0.65	CNH00540	Sugar (galactose) transporter

Table 3. cont.

Replicate no.	1	2	3	4	5	6	Mean	GenBank accession no.	Gene name and/or predicted protein function
Light exposure	1:15	1:15	1:15	1:00	2:00	1:00			
Mating type	α	α	α	a	a	a			
Gene									
1712.seq.017	−0.11	−0.62	−0.63	−2.12	−0.14	−0.47	−0.68	NA	Unannotated gene
185.m02442	−0.96	−1.11	0.32	−0.93	−0.44	−0.97	−0.68	CNJ00950	Pyruvate decarboxylase
163.m06114	−1.23	−0.32	−2.16	−0.21	−0.67	−0.12	−0.78	CND02090	Nucleoside-diphosphate-sugar epimerase
1711.seq.001	−0.38	−1.17	−0.23	−1.93	−0.18	−0.84	−0.79	AF356652	25S rRNA

Northern blot analysis was used to test the validity of the microarray expression data. A subset of downregulated genes was examined, but their transcripts were of low abundance and photoregulation could not be confirmed, so other approaches will be needed to determine whether any of these genes are repressed by light. Through Northern blot analysis, the photoregulation of the two light-induced genes with greatest induction differences, *HEM15* and *CFT1*, was demonstrated (Fig. 1). At least some genes that are light-regulated, such as *BWC2* and *HMP1*, were not within the light-regulated category by microarray, but did exhibit light induction by Northern blot analysis (Fig. 1), providing a caveat that some genes may have been excluded by using the microarray technique and analysis approach. A similar exclusion of genes has occurred in other microarray studies, including the recent report on light-regulated genes in *N. crassa* in which *ccg-1* and *fluffy*, which are known photoregulated genes, were absent (Chen *et al.*, 2009). For *C. neoformans*, *BWC2* had been previously shown to be light-regulated in a Bwc1-dependent manner (Idnurm & Heitman, 2005). *HMP1* is a homologue of a gene of *Ustilago maydis* that binds cruciform DNA structures but whose role in *U. maydis* biology is otherwise unknown (Dutta *et al.*, 1997). We were interested in this gene because of its potential role either in recombination during mating or in DNA repair. In *C. neoformans*, the homologue is regulated by multiple environmental conditions (X. Lin and others, unpublished data), as evident by the difference in transcript abundance of replicates 1 and 2 compared with replicate 3 in the Northern analysis (Fig. 1), and the homologue remains of unknown function in the fungus.

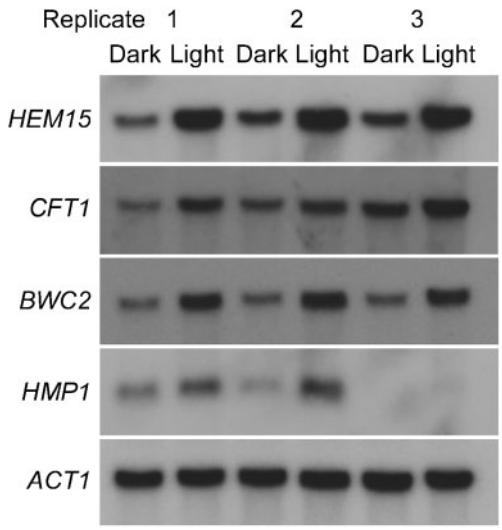

Fig. 1. Light-regulated genes of *C. neoformans*, as illustrated by Northern blot analysis. Replicates 1–3 correspond to RNA samples with the same numbers in Table 3. Photoinduction of *HMP1* in replicate 3 was evident in longer exposures of the membrane to X-ray film.

HEM15 encodes ferrochelatase, an essential, mitochondrial-targeted protein in *C. neoformans*

The most upregulated gene in response to light, based on the *C. neoformans* microarrays, was most similar to those in other fungi that encode ferrochelatase. Three experiments provided evidence that the *C. neoformans HEM15* gene indeed encodes ferrochelatase.

Ferrochelatase is essential in many organisms. We were unable to isolate *hem15* deletion mutants in *C. neoformans* haploid strains through conventional gene replacement experiments using homologous recombination, even when the medium was supplemented with exogenous haematin. This suggested that the *C. neoformans* gene is also essential. The *HEM15* gene was therefore mutated in two diploid strain backgrounds, and the strains were induced to undergo meiosis and sporulate to produce haploid basidiospores. A low germination efficiency was observed for the basidiospores (~4 %, 5/139), suggesting that there may be a dosage effect in Hem15 function (or instability of the diploid strains leading to aneuploidy). Of the five F_1 progeny, none was nourseothricin-resistant, as would be expected for a *hem15*::*NAT* deletion strain. Therefore, a second copy of the *HEM15* gene adjacent to a hygromycin-resistance cassette was introduced into a wild-type strain, producing a strain with two functional copies of the *HEM15* gene. The copy at the native locus was successfully

targeted for gene disruption, as confirmed by PCR and Southern blot analysis (data not shown). This strain (*hem15*::*NAT HEM15-HYG*) was crossed to a wild-type strain and the progeny analysed. Again, the spore germination frequency was low (~9 %, 18/208). Of the 18 progeny examined, none was Nat^R Hyg^S (*hem15*::*NAT*), further supporting the hypothesis that *HEM15* is essential in *C. neoformans*. Dissected spores from this cross were then micromanipulated onto YPD medium supplemented with 10 μg haematin ml^{-1}. Of 32 basidiospores plated, five germinated, including two 'microcolonies' that exhibited extremely slow growth. No growth was seen when the microcolony strains were grown on YPD without supplemented haematin, and they were nourseothricin-resistant and hygromycin-sensitive, confirming that they were *hem15* deletion mutants (Fig. 2).

Growth of the *hem15*Δ mutant strains in the presence of haematin resulted in barely visible colonies after 2 (Fig. 2a) to 5 days when examined by microscopy (Fig. 2b). In addition, the colonies were brown-red in colour, rather than the usual off-white of wild-type *C. neoformans*, which can be an indicator of porphyrin accumulation in the cells (Gora *et al.*, 1996). The strains were also unable to grow in the presence of light or at mammalian body temperature, even in media supplemented with haematin (Fig. 2c, d). Haematin or other haem sources such as haemin in media can rescue *hem15* deletions in other fungi (e.g. see the *S. cerevisiae* results below and in Fig. 3), and our findings indicate that *C. neoformans* can also use haem from the surrounding environment, although at very low efficiency. However, studies on iron utilization show that *C. neoformans* can efficiently utilize haem as the sole iron source (Jung *et al.*, 2008). Taken together, these observations suggest that *C. neoformans* can take up haem from its surrounding environment efficiently, since it can be used as an iron source, although it cannot use exogenous haem as a general substitute for haem generated endogenously.

The *C. neoformans HEM15* gene was used to complement an *S. cerevisiae hem15* mutant to test the function of the *C. neoformans* homologue when heterologously expressed. Hem15 has been purified from *S. cerevisiae* and shown to catalyse the incorporation of either iron or zinc into protoporphyrin IX to form haem (or zinc protoporphyrin), thus establishing its enzymic activity (Camadro & Labbé, 1988; Eldridge & Dailey, 1992; Gora *et al.*, 1996). Deletion of the *HEM15* gene results in an inviable phenotype unless the medium is supplemented with exogenous haem (Labbe-Bois, 1990). A *hem15*Δ strain was generated in the *S. cerevisiae* BY4742 strain background by replacing the ORF with the *KanMX6* cassette.

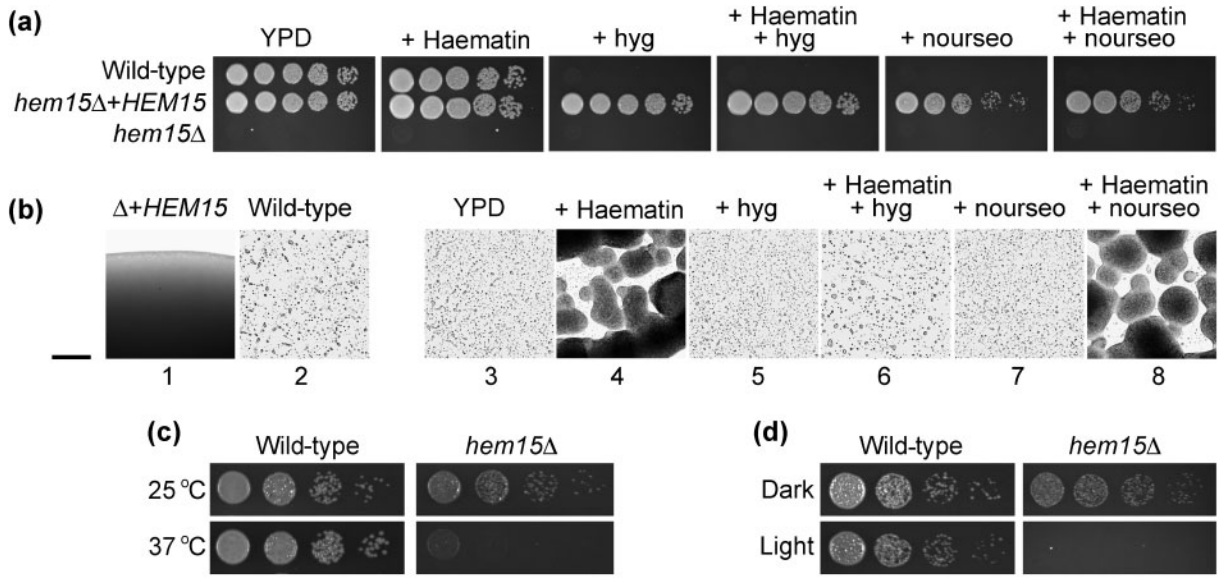

Fig. 2. *C. neoformans HEM15* is essential and *hem15* mutants exhibit an extreme growth defect even in the presence of haem. (a) Tenfold serial dilutions of *C. neoformans* cells onto YPD medium containing different drugs (hyg, hygromycin; nourseo, nourseothricin) and with or without haematin (10 μg ml^{-1}). The three strains were wild-type, *hem15*::*NAT HEM15-HYG* and *hem15*::*NAT*. Plates were incubated for 2 days and photographed. (b) Micrographs of cells from the above plates, at 5 days post-inoculation. Bar, 1 mm. Panel 1 is the edge of a colony produced by the confluent growth of the *hem15*Δ+*HEM15* complemented strain, and panel 2 is the wild-type isolate, both inoculated and grown on nourseothricin media. The remaining six panels (3–8) are the *hem15*::*NAT* isolate showing non-confluent growth, and the modest rescue of growth by exogenous haematin. (c) Tenfold serial dilutions on YPD+haematin medium, incubated at two temperatures for 2 days (wild-type) or 8 days (*hem15*Δ). (d) Tenfold serial dilutions on YPD+haematin medium, incubated in darkness or under white light for 2 days (wild-type) or 7 days (*hem15*Δ).

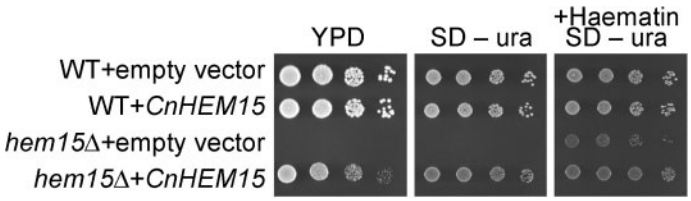

Fig. 3. The *C. neoformans HEM15* gene complements an *S. cerevisiae hem15*Δ mutant. Tenfold serial dilutions of wild-type (WT) or *hem15*Δ mutant strains of *S. cerevisiae* transformed with a control plasmid or a plasmid expressing *C. neoformans HEM15*. Cells were plated on YPD, SD medium without uracil (SD−ura), and SD−ura supplemented with haematin (20 μg ml^{-1}).

This strain was unable to grow unless haematin was added to the medium (Fig. 3), as seen previously with *hem15* mutants in other *S. cerevisiae* strain backgrounds. The *C. neoformans HEM15* cDNA was cloned into a plasmid and expressed in the *S. cerevisiae hem15*Δ mutant strain. The *C. neoformans* gene complemented the growth defect in the absence of haem (Fig. 3). Thus, by complementation of the *S. cerevisiae hem15*Δ mutant, we demonstrated that the *C. neoformans HEM15* gene encodes a functional ferrochelatase enzyme.

Ferrochelatase is localized to the mitochondrial inner membrane in eukaryotic species. As a third line of evidence for *C. neoformans* Hem15 function, we examined the subcellular localization of the *C. neoformans* gene by fusing the *HEM15* gene to the N-terminal end of GFP. The resulting *HEM15–GFP* gene was introduced into wild-type cells, or *hem15*Δ cells, where it complemented the *hem15* phenotype, demonstrating that the fusion allele is functional. The cells were stained with MitoTracker Red, which is a vital dye that results in red fluorescence of mitochondria. In unstained or untransformed wild-type cells, a low-level background fluorescence was observed, and in untransformed strains stained with MitoTracker Red, no green fluorescence was observed (data not shown). When the GFP distribution in transformed cells was compared with that of MitoTracker Red, the two co-localized either to punctate spots or to tubular structures (Fig. 4). Analysis of the predicted Hem15 protein using Psort II software suggests that there is a mitochondrial localization signal at the N terminus, which is consistent with co-localization of this protein with a mitochondrial marker. These observations support the conclusion that the ferrochelatase protein of *C. neoformans* is also localized to the mitochondria.

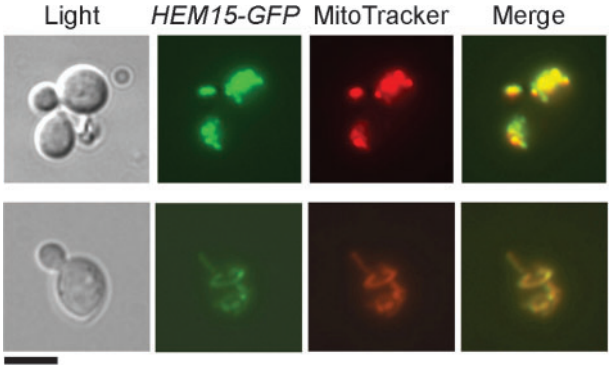

Fig. 4. Subcellular colocalization of the Hem15–GFP fusion protein with the mitochondrial marker MitoTracker Red. Globular and tubular examples of the mitochondria are illustrated. Bar, 10 μm.

bwc1 or *bwc2 C. neoformans* mutants have normal responses to oxidative and other stresses

Haem is a cofactor for many proteins, including those, such as flavohaemoglobin, required for antioxidant defences. If the Bwc1–Bwc2 complex regulates haem levels, then *bwc1* or *bwc2* mutants may show an oxidative stress-sensitive phenotype. In addition, light induces the expression of glutathione peroxidase, an enzyme known to be required for protection against oxidative stress in *C. neoformans* (Missall *et al.*, 2005). The *bwc1* and *bwc2* mutant strains have been tested previously on a number of medium types and under different conditions to reveal only a UV-sensitive phenotype (Idnurm & Heitman, 2005). The strains were tested here with growth in the light and dark on YPD medium supplemented with oxidative stress agents or DNA-damaging agents, including nitric oxide (diethylenetriamine NONOate; DETA-NONOate), methyl methanesulfonate, ethidium bromide, t-butyl hydroperoxide and H_2O_2, titrated over concentration ranges that showed growth inhibition. No altered sensitivity to these molecules was observed for the *bwc1* and *bwc2* mutant strains.

Conservation of ferrochelatase as a target for light regulation in fungal species

Haem biosynthesis is an ancient biological pathway that branches at protoporphyrin IX by the incorporation of iron into porphyrin to make haem (e.g. in eukaryotes functioning in oxidative respiration in the mitochondria) or magnesium to make chlorophyll (e.g. in eukaryotes functioning in capturing energy from light in the chloroplasts). We hypothesized that ferrochelatase could be light-regulated in other fungi because other eukaryotes such as plants and animals regulate enzymes in porphyrin biosynthesis in response to light. We conducted Northern blot analysis on ferrochelatase expression in the ascomycete *N. crassa* wild-type and a *wc-1* mutant strain, as well as the zygomycete *P. blakesleeanus* wild-type and a *madA madB*

(*wc-1 wc-2*) mutant. These species represent two phyla of the fungi that diverged from *C. neoformans* more than 460 million years ago, based on the fossil record, or earlier based on molecular clock estimates (Taylor & Berbee, 2006). To rule out ferrochelatase upregulation being a consequence of an indirect effect, e.g. degradation of haem or porphyrin intermediates by light, two non-photosensing fungi, *S. cerevisiae* and *Candida albicans*, were also included in the study. Strains were grown overnight or for 2 days (depending on growth rate) in complete darkness on YPD medium with 1 h of light exposure for one set of cultures, employing a regime similar to that used for the *C. neoformans* treatment for microarray experiments. Total RNA was purified, resolved on agarose gels, blotted to membranes, and probed with radiolabelled fragments of the ferrochelatase gene amplified from each species (Fig. 5a). The blots were stripped, and reprobed with the actin gene from each species as a control for RNA loading and transfer. For wild-type strains of *C. neoformans*, *N. crassa* and *P. blakesleeanus*, ferrochelatase transcript levels were higher after 1 h light exposure when compared with those of the strains grown in the dark. This transcript induction was not observed in the control species *S. cerevisiae* and *Candida albicans*, which have lost the *white collar* genes from their genomes during evolution. Strikingly, the upregulation of ferrochelatase was not observed in the *white collar* mutant strains of *C. neoformans*, *N. crassa* or *P. blakesleeanus*. This demonstrates that the regulation of ferrochelatase in the light-sensing fungi tested depends upon the function of this conserved photoreceptor complex.

Ferrochelatase has the potential to serve as a marker for photoinduction in fungi, especially in species in which no photobiology has yet been reported. As proof of principle, we investigated light regulation of ferrochelatase in *R. oryzae*, a Mucormycotina species closely related to *Mucor* and *Phycomyces*. In the genome of this species there are three copies of *wc-1*, as in the other two Mucormycotina genera, suggesting functional photobiology (Ma *et al.*, 2009; Sanz *et al.*, 2009). However, as far as we are aware, there is no report of light sensing in this species, and we were unable to identify a clear phenotypic change in response to light for the isolate in which the genome was sequenced. We therefore treated this strain under the dark or dark–light regime described above, as well as under blue, green and red illumination. The ferrochelatase transcript levels in *R. oryzae* were higher in the light than in the dark, and were induced by blue wavelengths and to a lesser extent by green wavelengths (Fig. 5b), demonstrating the ability of this species to sense light at wavelengths consistent with one or more of the *wc-1* homologues being functional.

DISCUSSION

Light and darkness are environmental cues commonly encountered by organisms. In the fungi, blue-light responses are controlled by the conserved WC-1 and WC-2 proteins. In previous studies of the pathogenic basidiomycete fungus *C. neoformans* it was observed that light inhibits mating (Shen *et al.*, 2002). Two genes, *BWC1*

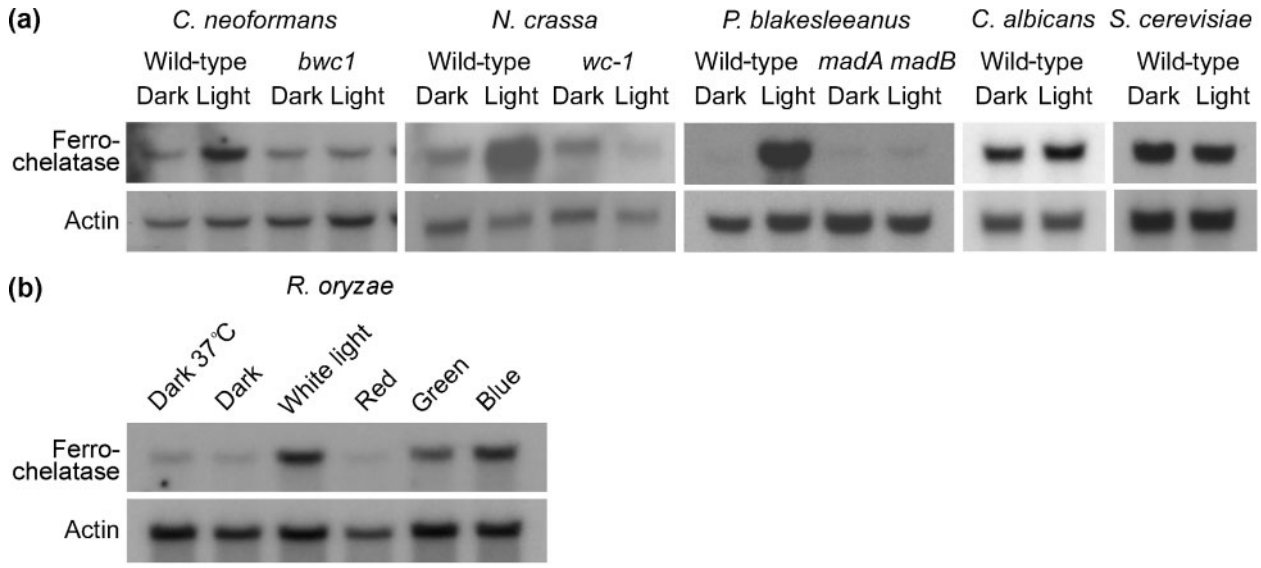

Fig. 5. Ferrochelatase *HEM15* transcript levels are light-regulated in a *white collar*-dependent fashion in fungi. Northern blot analysis of ferrochelatase gene homologues in different fungal species and strains grown in constant darkness or exposed to light for 1 h. The actin genes serve as RNA loading and transfer controls. (a) Wild-type or *white collar 1* mutant (or *wc-1 wc-2 madA madB* double mutant for *P. blakesleeanus*) strains of three species, compared with two non-photosensing ascomycete yeasts. (b) Induction of the *HEM15* homologue in wild-type *R. oryzae* exposed to 1 h light of different wavelengths.

and *BWC2*, regulate the inhibition of mating by light, and they also regulate virulence and sensitivity to UV irradiation (Idnurm & Heitman, 2005; Lu *et al.*, 2005). A homologue of *BWC1* in the basidiomycete species *Coprinopsis cinerea* is also required to mediate light sensing (Terashima *et al.*, 2005), while other basidiomycetes encode copies of *BWC1* and *BWC2* in their genomes (Sano *et al.*, 2009). Bwc1 and Bwc2 are the homologues of *N. crassa* WC-1 and WC-2, and thus are predicted to encode a transcription factor complex that binds to the promoters of light-regulated genes to induce their transcription. The downstream targets of Bwc1–Bwc2 in the light signal transduction pathway are unknown, but are of interest because of their possible roles in regulating the production of sexual spores and in virulence. One approach to the identification of these targets has been to perform forward genetic screens to identify genes mutated in strains that have altered responses to light (Yeh *et al.*, 2009). This study identified the *SSN8* gene, which encodes a general transcriptional regulatory protein. Because the WC-1 and -2 protein complex is predicted to be a transcriptional regulator, assessing transcript abundance under light and dark conditions should also be able to provide clues about downstream targets of Bwc1–Bwc2 in the light signal transduction process.

Microarray expression analysis detected few light-regulated genes in *C. neoformans*. This was a surprising result based on regulatory changes in response to light in other fungi, such as *Trichoderma* species, *N. crassa* and *P. blakesleeanus*, in which light causes large-magnitude changes in the expression of light-regulated genes (Chen *et al.*, 2009; Li & Schmidhauser, 1995; Rodríguez-Romero & Corrochano, 2004; Rosales-Saavedra *et al.*, 2006). This appears to be specific to *C. neoformans* rather than an effect of the light treatment, because a comparison with the transcript abundances in other species cultured under identical conditions showed large differences between light and dark (see Fig. 5). Genome-wide expression analyses in other fungi indicate that many genes are light-regulated, from 300–700 genes in *N. crassa* (Chen *et al.*, 2009; Dong *et al.*, 2008) to a 2.8 % estimate for *Trichoderma atroviride* (Rosales-Saavedra *et al.*, 2006). It is feasible that alternative microarray approaches would result in the detection of additional photoregulated genes, e.g. using strains over-expressing Bwc1 or Bwc2, higher light intensities, the use of blue wavelengths, growth on different media, or at different developmental stages such as during dikaryotic filament formation.

The genes identified in the microarray analysis mostly encode hypothetical or uncharacterized proteins. Genes that have been characterized include *CFT1*, which is involved in iron uptake and is required for virulence in *C. neoformans* (Jung *et al.*, 2008), and *GPX1*, which encodes a glutathione peroxidase that is required for protection against certain oxidative stresses, but not for virulence (Missall *et al.*, 2005). The most interesting gene to emerge from this analysis was *HEM15*, which by BLAST comparisons is homologous to genes encoding ferrochelatase (EC 4.99.1.1). This was the primary light-induced gene (Table 3), and warranted further study because ferrochelatase mutations cause photosensitivity in all organisms in which mutants have been examined. These organisms include the bacterium *Escherichia coli*, in which ferrochelatase mutants also become light-sensitive and negatively phototactic (Miyamoto *et al.*, 1991; Yang *et al.*, 1995), and the non-photosensing fungus *S. cerevisiae* (Abbas & Labbe-Bois, 1993; Zoladek *et al.*, 1996). Mutation of ferrochelatase is one basis of porphyria, a class of genetic disorders in humans, in which ferrochelatase impairment leads to severe sensitivity to sunlight and subsequent skin damage if exposed (Cox, 1997; Kauppinen, 2005). The basis behind the increase in photosensitity in all organisms is the accumulation of porphyrin intermediates that are highly phototoxic. Of further interest, haem is a cofactor for many proteins, including some implicated in antioxidant defence during infection, such as flavohaemoglobin of *C. neoformans* (de Jesús-Berríos *et al.*, 2003). Native biosynthesis or utilization of host haem is required for many bacterial pathogens to cause disease (reviewed by Wilks & Burkhard, 2007).

The production of haem requires at least seven dedicated enzymes in addition to ferrochelatase. One question is whether or not these other enzymes are coordinately regulated. The genes encoding the other seven enzymes in the haem biosynthesis pathway are not light-regulated at the transcript level in *C. neoformans*, based on the microarray data (Table 3). To confirm this observation by an alternative technique, transcripts of three genes (*HEM1*, *HEM12* and *HEM14*) were tested for light regulation by Northern blot analysis: none of these three genes was light-regulated (data not shown). This finding suggests that upregulation of ferrochelatase activity should be sufficient to reduce porphyrin levels in the cell, rather than a co-regulatory induction by light of every component in the pathway. Upregulation of the *CFT1* transcript levels by light may function to provide more of the iron permease to import exogenous iron to be incorporated into protoporphyrin IX to produce haem. The *FET3* iron oxidase homologue (*CFO1*) is adjacent to *CFT1* in the *C. neoformans* genome with shared promoter regions (Jung *et al.*, 2008). *CFO1* transcript abundance may be also slightly upregulated by light, as measured by microarray analysis (Table 3). The co-regulation of *HEM15* and *CFT1* but not other genes for haem biosynthesis supports a hypothesis in which haem levels increase in response to light, while the porphyrin precursor levels decrease. Thus, we hypothesize that light induction of these genes controlled by Bwc1 and Bwc2 would be consistent with the increased UV sensitivity and reduced virulence observed in strains bearing a mutation in either the *BWC1* or the *BWC2* gene. Nevertheless, the observation that the *hem15* deletion strain shows such poor growth in culture requires further analysis to support this hypothesis. In other organisms, mutation of ferrochelatase leads to haem auxotrophy, which can be

rescued by addition of haem to the medium (e.g. see *S. cerevisiae*, Fig. 3). For *C. neoformans*, additional haem only partially rescues the defect of deletion of *HEM15*. It may be that this fungus, which produces a polysaccharide capsule, has difficulties in taking up haem or in relocalization of haem within the cell after uptake.

Despite the goal of revealing the underlying links between light sensing and virulence and discovering that haem biosynthesis is light-regulated, it is still not clear how Hem15 functions to control the phenotypes observed after mutation of the *bwc1* or *bwc2* genes. A hypothesis is that haem biosynthesis is impaired in the *bwc1* or *bwc2* mutants, and this influences virulence. It may be that either normal levels of haem are required for pathogenesis or the strains are less fit due to changes in regulation, since *HEM15* is essential. Haem biosynthesis is required for virulence in both plant and animal pathogenic fungi (Kirsch & Whitney, 1991; Solomon *et al.*, 2006). The *hemH* gene encoding ferrochelatase of the α-proteobacterium *Brucella abortus* is required for virulence (Almirón *et al.*, 2001). Curiously, a LOV domain histidine kinase in the same organism is also required for light sensing and virulence (Swartz *et al.*, 2007), and it is worth speculating that these two observations may be linked in *B. abortus*, which would expand the overlapping roles of light sensing and haem biosynthesis into pathogenic bacteria.

Fig. 6 is a working model of how light and ferrochelatase function may regulate sensitivity to light in *C. neoformans*, and presumably haem levels in cells prior to inoculation into mice, which could influence the virulence outcome, although this model needs to be addressed further experimentally. A challenge in demonstrating function through the manipulation of *HEM15* is that the gene is essential, so may require careful regulation to reveal a phenotype. Future studies could focus on haem biology in *C. neoformans* and the proteins that use haem as a cofactor.

HEM15 is light-regulated across the fungal kingdom (Fig. 5). It will be of interest to see how many of the diverse and as yet unaccounted for photobiological effects in fungi (Tisch & Schmoll, 2010) are mediated by haem availability. Haem biosynthesis is regulated by the circadian clock in plants and mammals (Kaasik & Lee, 2004; Papenbrock *et al.*, 1999), and we predict that in fungi haem levels will also be regulated by the circadian clock. *N. crassa* represents the best fungal system to study the circadian clock (see recent review by Heintzen & Liu, 2007). Ferrochelatase has not been reported to be regulated by light or the clock, although microarrays have been performed on this species to examine light- and/or clock-regulated transcripts. It is possible that in the early microarrays the gene was absent, since the microarray did not cover the entire gene set (Lewis *et al.*, 2002; Nowrousian *et al.*, 2003). In two recent whole-genome studies, ferrochelatase is not discussed in the main text as a light- or clock-regulated gene. Nevertheless, the *N. crassa* ferrochelatase homologue NCU08291.2 is included amongst the original data as a gene induced by light and under the control of the circadian clock (Chen *et al.*, 2009; Dong *et al.*, 2008), providing confirmation of our Northern blot data and supporting our hypothesis. Two other genes that are light-regulated in both *C. neoformans* and *N. crassa* based on microarray studies encode glutathione peroxidase and the Hmp1 cruciform DNA-binding protein. An example of one research area to re-explore is the function of nitrate reductase in *N. crassa* circadian rhythms (Christensen *et al.*, 2004). Nitrate reductase is a haem-containing enzyme, and one can propose a system of haem availability controlled by WCC regulating the activity of the enzyme, providing inter-connected clocks.

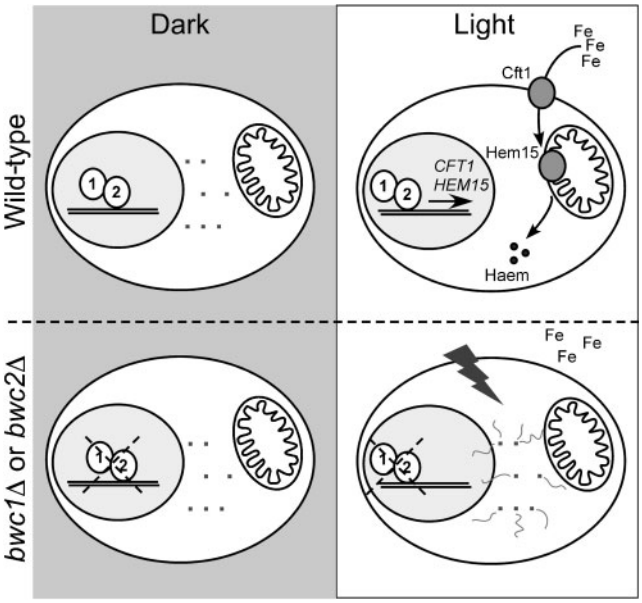

Fig. 6. Model of light-regulated changes in *C. neoformans* affecting the physiology of the fungus. In the dark, porphyrin levels are high (represented by dots) and transcription of *HEM15* and *CFT1* is at a basal level. Exposure to light triggers a conformational change in Bwc1–Bwc2 (1 and 2), leading to induction of *CFT1* and *HEM15* expression to produce proteins located on the plasma membrane (Cft1) or mitochondrial membrane (Hem15). Porphyrins are converted to haem by ferrochelatase. In the absence of Bwc1 or Bwc2, no increase in Cft1 or Hem15 occurs, and exposure of the porphyrins to light results in photooxidative damage.

The major phyla of the fungal kingdom are estimated to have diverged at least 460 million years ago, based on fossil evidence, and perhaps as long as a billion years ago (Taylor & Berbee, 2006). Our finding that ferrochelatase is regulated by the White collar system of transcription factors is a unique example of conserved regulatory function across the kingdom, and is in contrast to what is emerging from functional genomic studies on signal transduction, where the trend is for conserved transduction

pathways but divergent input and output proteins or targets (Bahn *et al.*, 2007). Why has ferrochelatase been light-regulated by the same transcription factor complex for so long? As outlined in a working model (Fig. 6), light induction of ferrochelatase should reduce the levels of highly photoactive porphyin molecules in a cell. We have previously advanced the hypothesis that the White collar proteins are under selective pressure for protection against UV irradiation (Idnurm & Heitman, 2005). We hypothesize, based on the transcript profile here, as well as the other phenotypes commonly associated with photosensing in fungi, which include upregulation of pigmentation and the DNA repair enzyme photolyase, that the original purpose of light sensing in fungi was in protection against damage caused by light and that this is responsible for its continued presence. In addition to induction of other protective properties such as pigmentation or photolyase, which are not present in all fungi, the photoregulation of ferrochelatase by the Wc1–Wc2 complex provides another, and ancient, means to reduce light-mediated damage.

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