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The contribution of the White Collar complex to *Cryptococcus neoformans* virulence is independent of its light-sensing capabilities

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Abstract

The White Collar complex is responsible for sensing light and transmitting that signal in many fungal species. In *Cryptococcus neoformans* and *C. deneoformans* the complex is involved in protection against damage from ultraviolet (UV) light, repression of mating in response to light, and is also required for virulence. The mechanism by which the Bwc1 photoreceptor contributes to virulence is unknown. In this study, a *bwc1* deletion mutant of *C. neoformans* was transformed with three versions of the *BWC1* gene, the wild type, *BWC1*^{C605A} or *BWC1*^{C605S}, in which the latter two have the conserved cysteine residue replaced with either alanine or serine within the light-oxygen-voltage (LOV) domain that interacts with the flavin chromophore. The *bwc1*+*BWC1* strain complemented the UV sensitivity and the repression of mating in the light. The *bwc1*+*BWC1*^{C605A} and *bwc1*+*BWC1*^{C605S} strains were not fully complemented for either of the phenotypes, indicating that these *BWC1* alleles impair the light responses for strains with them. Transcript analysis showed that neither of the mutated strains (*bwc1*+*BWC1*^{C605A} and *bwc1*+*BWC1*^{C605S}) showed the light-inducible expression pattern of the *HEM15* and *UVE1* genes as occurs in the wild type strain. These results indicate that the conserved flavin-binding site in the LOV domain of Bwc1 is required for sensing and responding to light in *C. neoformans*. In contrast to defects in light responses, the wild type, *bwc1*+*BWC1*, *bwc1*+*BWC1*^{C605A} and *bwc1*+*BWC1*^{C605S} strains were equally virulent, whereas the *bwc1* knock out mutant was less virulent. Furthermore, pre-exposure of the strains to light prior to inoculation had no influence on the outcome of infection. These findings define a division in function of the White Collar complex in fungi, in that in *C. neoformans* the role of Bwc1 in virulence is independent of light sensing.

Graphical abstract

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Keywords

Cryptococcus; Galleria mellonella; Medical mycology; Photobiology

1. Introduction

The effects of light on fungi impact aspects of their biology as diverse as sporulation, circadian clock function, primary metabolism, and the biosynthesis of secondary metabolites such as toxins or pigments (Fuller et al., 2016; Tisch and Schmoll, 2010). Those responses are controlled by a small number of photosensory proteins (Fischer et al., 2016; Idnurm et al., 2010; Rodriguez-Romero et al., 2010). Among them the blue light receptor White Collar-1 (WC-1), first characterized in the model ascomycete *Neurospora crassa*, appears to have originated early and then been well-conserved during the evolution of the fungi.

In *N. crassa* the light-regulated processes are controlled by WC-1 and WC-2, which form a GATA-type zinc finger transcription factor complex. WC-1 contains a number of domains: for DNA binding, two PAS domains (named after the Per, Arnt and Sim proteins, in which they were first observed) involved in protein-protein interactions, two putative transcriptional activation domains, a nuclear localization signal, and a LOV domain which is a specialized type of PAS domain involved in environmental sensing of light, oxygen and voltage (Froehlich et al., 2002; He et al., 2002; Wang et al., 2015). The chromophore flavin adenine dinucleotide (FAD), accommodated in the LOV domain, is essential for the light sensing activity of WC-1. A motif of NCRFLQ amino acids that contributed to the α 'A helix is a highly conserved region among LOV domains, as the cysteine (C) residue is essential for the covalent attachment of the flavin chromophore to the sensor protein in the presence of light (Cheng et al., 2003). WC-1 interacts with a partner protein WC-2, another GATA-type transcription factor, to form the White Collar complex (WCC) that activates the expression of light-responsive genes upon light stimulation (Collett et al., 2002; Froehlich et al., 2002; Liu et al., 2003).

The availability of fungal genome sequences has allowed the identification of photoreceptors, especially *wc-1* and *wc-2* orthologs, in varied fungal species. Mutation of these *white collar* genes in most cases abolishes the effects of blue light on the species.

However, analysis of such mutants has also revealed phenotypes in the absence of light, such as changes in sporulation or secondary metabolite production (Fuller et al., 2016; Fuller et al., 2015), leading to questions about how this complex functions above and beyond its perception of light.

One property that may be unrelated to light status is virulence in pathogenic species. In the human pathogenic fungi *Cryptococcus neoformans* and *C. deneoformans* [their previous names are *C. neoformans* var. *grubii*/serotype A and *C. neoformans* var. *neoformans*/serotype D, respectively (Hagen et al., 2015)], light inhibits mating and induces a protective response against ultraviolet light. Mutation of the orthologs of *N. crassa wc-1* and *wc-2* impairs these effects (Idnurm and Heitman, 2005; Lu et al., 2005; Verma and Idnurm, 2013). An additional phenotype associated with mutating the *BWC1* or *BWC2* genes in *C. neoformans* and the related species *C. deuterogattii* is a reduction in virulence in animal models of disease (Idnurm and Heitman, 2005; Zhu et al., 2013). Cryptococcosis refers to a set of diseases caused by the *C. neoformans* species complex, currently made up of seven species that are most problematic when growing in the lungs and central nervous systems of people (Heitman et al., 2011). While a number of factors and genes have been identified that control virulence, mutation of the WCC does not impact on any of these that are known to date (Alspaugh, 2015; Brown et al., 2014).

The role of light or the WCC in fungal virulence is more widespread than the *Cryptococcus* species. For instance, mutation of the WC homologs also alters the pathogenicity in plant pathogens. In the maize leaf pathogen *Cercospora zeae-maydis*, the WC-1 ortholog is required for tropism toward the host stomata and lesion formation (Kim et al., 2011a). In the rice blast pathogen *Pyricularia (Magnaporthe) oryzae*, light represses asexual spore releases and a dark-phase immediately after pathogen–host contact plays a critical role in successful disease development, with spore release and the light-dependent repression accomplished by the photoreceptor MGWC-1 (Kim et al., 2011b; Lee et al., 2006). A T-DNA insertion within the WC-2 ortholog also causes a decrease in pathogenicity of *P. oryzae* (Jeon et al., 2007). Mutation of *wc1* in *Fusarium oxysporum*, which is normally a plant pathogen, causes a decrease in virulence in a mouse model of disease (Ruiz-Roldán et al., 2008). In the gray mold pathogen *Botrytis cinerea*, loss of BcWCL1 results in attenuated virulence in the host plant when light is present, and this is due to BcWCL1 being required for coping with excessive reactive oxygen species generated by the host's oxidative burst and photooxidative stress (Canessa et al., 2013). A light-responsive transcription factor, BcLTF1, downstream of BcWCL1 is responsible for anti-oxidative stress activities and is thus required for advanced host infection by *B. cinerea* (Schumacher et al., 2014). In addition, the influence of the circadian clock, which is controlled by light, on the disease outcome in the *Botrytis cinerea*-*Arabidopsis thaliana* interaction is dependent on the effects of the clock and light from the fungal pathogen, rather than changes in plant defense systems (Hevia et al., 2015). In addition to the genetic contributions of the WCC to virulence, pre-exposing fungi to light can impact subsequent virulence outcomes (Campbell and Berliner, 1973; Oliveira et al., 2018).

It is not yet clear in the pathogenic fungi how light or the light-sensing proteins contribute to virulence. This study used *C. neoformans* as the model organism to test if light sensing is responsible for the function of the conserved White Collar complex in virulence.

2. Materials and Methods

2.1. Generation of plasmids

The LITMUS 28i vector (New England Biolabs, Ipswich, MA) was double digested with the restriction enzymes KpnI and BamHI. The *BWC1* open reading frame and adjacent 5' and 3' regulatory regions were amplified from the genomic DNA of strain KN99α with the primer pair PK003/PK004, and then cloned into linearized LITMUS 28i using T4 DNA ligase, yielding the LITMUS 28i-*BWC1* vector. Primer sequences are given in Table 1. A plasmid without errors in *BWC1* was identified by sequencing the inserts. The *URA5* gene was also amplified from KN99α DNA with the primer pair PK027/PK028, and the product was cloned into the pCR®2.1-TOPO vector (Invitrogen, Grand Island, NY), resulting in plasmid pCR®2.1-TOPO-*URA5*. Subsequently, both the LITMUS 28i-*BWC1* and pCR®2.1-TOPO-*URA5* plasmids were digested by EcoRI, and the *URA5* fragment was ligated with the linearized LITMUS 28i-*BWC1*, resulting in the plasmid LITMUS 28i-*BWC1-URA5*. Specific mutations were introduced into this plasmid at the site of the conserved codon for cysteine within the LOV domain, by means of the QuikChange® Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). The cysteine residue is amino acid number 605 of GenBank accession XP_012048765.1. The primers used for the mutations are listed in Table 1. After mutagenesis, the resultant plasmids were confirmed to be otherwise error-free by DNA sequencing.

2.2. Fungal strains used in the experiments

C. neoformans strains used in this study are listed in Table 2. They were maintained on yeast peptone dextrose (YPD) medium. Crosses, between strains of *MATa* and *MATα* mating types, were conducted on V8 medium (5% Campbell's V8 juice, 4% agar, pH 5). The *bwc1* mutant strain AI81 (*MATα*) and *ura5* base pair substitution mutant strain JF99 (*MATa*) were crossed to generate progenies of the *bwc1 ura5* double mutant, via micromanipulation of the basidiospores. A *bwc1 ura5* strain of *MATα* plated on minimal medium (yeast nitrogen base; YNB) plus 1 M sorbitol was transformed with the plasmids LITMUS 28i-*BWC1-URA5*, LITMUS 28i-*BWC1*^{C605A}-*URA5*, or LITMUS 28i-*BWC1*^{C605S}-*URA5* via the biolistic system (Bio-Rad, Hercules, CA), with standard parameters (Toffaletti et al., 1993). Transformants were screened by PCR to select those in which the vectors were correctly incorporated into the genome at the *URA5* locus by homologous recombination. The *bwc1 ura5 MATα* strains transformed with either wild type or site-mutated *BWC1* alleles were crossed with the *bwc1 ura5 MATa* strain obtained earlier, to isolate progeny of the *MATa* mating type with the *BWC1* alleles integrated into the *URA5* locus.

2.3. Assessment of inhibition of mating by light in crosses between strains

Dikaryotic filaments form during the sexual cycle of *C. neoformans*. Crosses between strains were compared to each other for their filamentous growth ability on V8 medium at pH 7 as influenced with continuous white fluorescent light (1600 lux) in contrast to culturing in

darkness. To quantify the effect of light on mating, a cell fusion assay was conducted (Idnurm and Heitman, 2005). An auxotrophic *C. neoformans* strain AI202 (*MATa*, *ade2*) was crossed with the *MATa* strains of *bwc1*, *bwc1+BWC1*, *bwc1+BWC1^{C605A}*, and *bwc1+BWC1^{C605S}*. Among the progenies from each cross, the *MATa* isolates with genotypes of *ade2*, *bwc1 ade2*, *bwc1+BWC1 ade2*, *bwc1+BWC1^{C605A} ade2*, and *bwc1+BWC1^{C605S} ade2* were obtained. These strains were subsequently used to make inter-species hybrids by crosses with the *bwc1* mutant or wild type strains of *C. deneoformans* with the *MATa lys1* genotype. After 24 h incubation in dark or continuous white light at room temperature, each set of mixed yeast cells was suspended in distilled water to the concentration of 10^7 /ml. Aliquots (200 μ l) of each cell suspension were pipetted to YNB medium, and the cells were subsequently spread evenly on each plate. Colony growth on the minimum medium indicates a prototrophic dikaryotic or diploid strain resulting from successful cell fusion between two auxotrophic parents (*C. neoformans ade2* and *C. deneoformans lys1*). Colony numbers were recorded to quantify the cell fusion events for each set of crosses.

2.4. Analysis of UV sensitivity

Strains were first cultured to logarithmic phase in 5 ml of liquid YPD medium contained in light-proof 50 ml volume black tubes (Argos Technologies, Elgin, IL), and then the cell suspensions were serially diluted in a dark room with illumination by a dim red light (GBX LED safelight, Kodak, Rochester, NY). The serial dilutions were dotted onto two YPD agar plates: one was irradiated with a UV dose of 120 J/m² using a XL-1500 UV Crosslinker (Spectronics Corporation, Long Island, NY), and the other was not irradiated. The plates were then incubated at 30°C for two days.

2.5. Northern blot and qPCR analyses to measure transcript abundances of genes

Strains were first grown to logarithmic phase in liquid YPD medium, and equal quantities of yeast cells for each strain were inoculated onto YPD agar plates (15 cm diameter) and grown in complete darkness for 23 h, after which one set was exposed to light for 15 min or 1 h (1600 lux, cool-white fluorescent lights). After growth and light induction, the cells of each strain were collected from the agar surface, washed with distilled water, and pelleted in a centrifuge. This procedure was conducted in a dark room with illumination by dim red light. RNA was extracted from lyophilized yeast cells using TRIzol RNA reagent (Invitrogen). Total RNA (20 μ g) was resolved on denaturing formaldehyde agarose gels, blotted to Zeta-Probe membranes (Bio-Rad, Hercules, CA), and probed with [α -³²P] dCTP radiolabeled DNA fragments of genes amplified with the primers listed in Table 1. Levels of transcripts on northern blots were quantified in ImageJ (Schneider et al., 2012).

For qPCR, total RNA was reverse transcribed with Superscript III (Invitrogen). SYBR Green (Fermentas, Vilnius, Lithuania) was used to detect the amplification of DNA from the cDNAs by quantitative PCR in an ABI7300 machine (Bio-Rad), with the amplification primers for PCR listed in Table 2. The 2^{-C_T} method was used for relative quantification of genes (Livak and Schmittgen, 2001) whereby the actin (*ACT1*) gene was the constitutively expressed gene.

2.6. Yeast two-hybrid assays to test for protein interactions between Bwc2 and the Bwc1 isoforms

The plasmids pGBD.c1 and pGAD.c1 containing the cDNA of *BWC1* or *BWC2* had been created previously (Idnurm and Heitman, 2005; James et al., 1996). Both pGBD.c1-*BWC1* and pGAD.c1-*BWC1* were used as templates to introduce a point mutation to the flavin-binding site of the LOV domain in *BWC1* via the QuikChange Site-Directed Mutagenesis kit. The correct mutations in the resultant plasmids were confirmed by sequencing. Subsequently, the *Saccharomyces cerevisiae* reporter strain PJ69-4A (James et al., 1996) was co-transformed with the following plasmid combinations: pGBD.c1 + pGAD.c1, pGAD.c1-*BWC1* + pGBD.c1-*BWC2*, pGBD.c1-*BWC1* + pGAD.c1-*BWC2*, pGAD.c1-*BWC1*^{C605A} + pGBD.c1-*BWC2*, pGBD.c1-*BWC1*^{C605A} + pGAD.c1-*BWC2*, pGAD.c1-*BWC1*^{C605S} + pGBD.c1-*BWC2*, pGBD.c1-*BWC1*^{C605S} + pGAD.c1-*BWC2*. Double transformants were selected on yeast nitrogen base medium lacking leucine and tryptophan. The interactions between the Bwc1 isoforms and Bwc2 were measured by growth in the absence of adenine and β -galactosidase activity.

2.7. Virulence assays for *C. neoformans* strains in the insect model *Galleria mellonella*

Larvae of wax moth (*Galleria mellonella*) purchased from Vanderhorst Wholesale (Saint Marys, OH) were infected as previously described (Mylonakis et al., 2005). Aliquots of 200 μ l *C. neoformans* cells (2×10^7) were spread evenly on solid YPD medium and placed in light or dark (wrapped with foil). After overnight culture, the yeast cells were harvested by flooding with phosphate buffered saline (PBS) and scraping with cell scrapers. The cultures in dark were harvested in the dark room with the aid of red safe light. The concentration of resulted cell suspensions was adjusted to 2×10^7 per ml. For inoculation, 5 μ l of the cells were injected into the rear proleg of each larva via a 10 μ l Hamilton syringe. For each strain, 12 larvae were inoculated and subsequently incubated at 37°C in darkness, and examined daily for survival, with survival between groups compared using log-rank tests. The virulence experiments were performed on two separate occasions.

2.8. RNA-seq analysis

Five strains of *C. neoformans* were inoculated in tissue culture medium (10% horse serum, 90% RPMI) at a cell concentration of 10^5 /ml. They were cultured in complete darkness at 5% CO₂ and 37°C for 24 hours. The yeast cells were subsequently harvested under dim red light. Total RNA of these samples was extracted using the TRIzol reagent. 15 μ g of each RNA sample was treated with DNase I, and the DNase I-treated samples were then diluted to 100 μ g/ μ l. Each sample was processed and sequenced at the DNA Core Facility, University of Missouri, on an Illumina HiSeq2000 with 100-bp paired reads. All reads were mapped to the *C. neoformans* reference genome (EnsemblFungi, Genome Assembly: CNA3) by using TopHat v1.3.0. The aligned reads were then analyzed for the number of fragments per kilobase of transcript per million mapped reads using Cufflinks v1.0.3. Statistically-significant differences between strains were determined by CuffDiff. The differentially expressed genes among strains were clustered by R Heatmap2 package. RNA-sequence data are available at GenBank under accession PRJNA491293.

3. Results

3.1. The flavin-binding site in the LOV domain of Bwc1 is required for full light responses in *C. neoformans*

Plasmid vectors were created with three versions of the *BWC1* gene: a wild type copy, and those that cause a cysteine to alanine or serine substitution in the flavin-binding part of the encoded protein. These substitutes were based on previous work on the *Neurospora crassa* WC-1 and oat phototropin proteins (Cheng et al., 2003; Salomon et al., 2000), with the rationale that the new isoforms would retain their other functions apart from a loss in the ability to transmit the light signal. These constructs were transformed into a *bwc1* knock-out mutant strain and integrated into the *URA5* locus in the genome by single cross-overs through homologous recombination to ensure no genome positional variation in gene expression, with strains with integration of the plasmids into the correct site identified by PCR analysis (data not shown). A strain with the *bwc1* deletion allele and carrying the empty *URA5* plasmid integrated at the *URA5* locus was created for comparison.

The UV sensitivity was compared among these strains (Figure 1). After UV exposure, the complemented strain (*bwc1*+*BWC1*) recovered as well as the wild type strain. However, the *bwc1* strains with point mutations at the conserved cysteine site, *BWC1*^{C605A} or *BWC1*^{C605S} (this allelic information is abbreviated to *BWC1*^{C-A} or *BWC1*^{C-S}, respectively, for simplicity on all the figures), were considerably more sensitive to UV irradiation.

A second response to light in *C. neoformans* and *C. deneoformans* is the inhibition of mating. Compared to in darkness, the production of the dikaryotic mating filaments was prominently repressed by light in the wild type strain and the complemented strain (Figure 2). However, light treatment did not effectively block filamentation in the *bwc1* knock out strain or the *bwc1*+*BWC1*^{C605A} and *bwc1*+*BWC1*^{C605S} site mutation strains. The effect of light on mating of these strains was then quantified via a cell fusion assay in which the parental strains have auxotrophic mutations, that are cross-complemented when the strains fuse to form a diploid or intra-species dikaryon (Idnurm and Heitman, 2005). The ratios of cell fusion events in the light versus those in darkness were lower in both wild type and complemented strains than in the knock out and site mutation strains (Figure 3). These results confirmed that a functional LOV domain is required for sensing light by Bwc1 to inhibit mating in *Cryptococcus* species.

3.2. The mutation of the cysteine in the flavin-binding site of LOV domain in Bwc1 has no effect on the physical interaction between Bwc1 and Bwc2 proteins

In contrast to *N. crassa* WC-1 and homologs of this protein in the ascomycetes, the predicted Bwc1 protein in *C. neoformans* lacks a DNA binding domain. Thus, Bwc1 must interact with Bwc2 such that the two proteins can together transcriptionally regulate expression of downstream genes. Yeast two-hybrid analysis was conducted to test whether the mutation at the conserved cysteine site of the Bwc1 protein rendered any negative effect on the interaction between Bwc1 and Bwc2. In *S. cerevisiae* strains expressing the fusions with the Gal4 activation domain (AD) or DNA binding domain (BD), i.e. AD-Bwc1 and BD-Bwc2, AD-Bwc2 and BD-Bwc1, AD-Bwc1^{C605A} and BD-Bwc2, AD-Bwc2 and BD-Bwc1^{C605A},

AD-Bwc1^{C605S} and BD-Bwc2, AD-Bwc2 and BD-Bwc1^{C605S}, the reporter genes *ADE2* and *lacZ* were induced, as shown by cell growth in the absence of adenine and increased levels of β -galactosidase activity (Figure 4). In contrast, the *S. cerevisiae* strain expressing the AD and BD domains alone showed no growth on the adenine-deficient medium and expressed a minimal level of β -galactosidase activity. These findings demonstrate that Bwc1 and Bwc2 can physically interact, irrespective of the mutation at the conserved cysteine site in the LOV domain of the Bwc1. This indicates that changing the cysteine residue to alanine or serine does not abolish protein folding or stability of Bwc1, at least as measurable through a yeast two hybrid assay.

3.3. The flavin-binding site in the LOV domain of Bwc1 is required for the increase in transcript abundance of genes in *C. neoformans* in response to light

The ferroxidase-encoding gene *HEM15* is light-regulated by White Collar in diverse fungi (Canessa et al., 2013; Idnurm and Heitman, 2010). The transcript level of this gene, in response to light exposure, was measured in wild type, the *bwc1* knock out mutant, *BWC1* complemented, and the *BWC1*^{C605A}, *BWC1*^{C605S} site mutation strains. Northern blot analysis showed that a 15 min or 1 h exposure to light could slightly induce the expression of *HEM15* in the wild type, and *BWC1*, *BWC1*^{C605A} and *BWC1*^{C605S} site mutation strains (Supplemental Figure 1). In contrast, the expression of *HEM15* remained low in the *bwc1* knock out mutant in response to light. *HEM15* is expressed at a low level, making its detection and then quantitative comparisons by northern blots challenging. We therefore switched to a PCR-based method to detect this transcript.

Quantitative real time PCR was used to quantify the expression of *HEM15* and of *UVE1*, encoding a DNA repair endonuclease and a downstream target of the light signal via the White Collar complex in fungi (Verma and Idnurm, 2013). Like *HEM15*, the expression level of *UVE1* is low and therefore difficult to detect by northern blotting. Light treatment induced the expression of *HEM15* and *UVE1* in wild type and the *BWC1* complementation strains, while there was only a basal level of expression in the *bwc1* knock out mutant, *BWC1*^{C605A} and *BWC1*^{C605S} site mutation strains irrespective of the light/dark conditions (Figure 5). These data further indicate that the conserved cysteine site in the LOV domain is required for the ability to respond fully to light signals via the Bwc1-Bwc2 complex in *Cryptococcus* species.

3.4. The cysteine in the LOV domain in Bwc1 is dispensable for virulence of *C. neoformans*

The larvae of the wax moth (*Galleria mellonella*) were used as the animal model to determine the virulence of *C. neoformans* strains. The wild type, *bwc1* knock out, *bwc1*+*BWC1* complementation, and *bwc1*+*BWC1*^{C605A} and *bwc1*+*BWC1*^{C605S} site mutation strains were used to inoculate 12 larvae of the wax moth *G. mellonella* per strain. The effect of exposure to light on the virulence of each strain was tested by comparing larvae infected with *C. neoformans* cultured in the light or under constant darkness.

Survival rates were the same for the strains of wild type, *bwc1*+*BWC1*, *bwc1*+*BWC1*^{C605A} and *bwc1*+*BWC1*^{C605S} (Figure 6). However, the larvae infected with the *bwc1* knock-out

mutant survived longer than the other strains ($P=0.00261$ and $P=0.00208$ between wild type and *bwc1* deletion mutant inoculated with light-exposed or dark cultured strains, respectively). Pre-treatment of strains with light did not alter the virulence of the *C. neoformans* strains when compared to when the strains were cultured in darkness and all manipulations including the inoculation into the larvae was performed under safe red light ($P=0.66$ between larvae infected with the wild type strain cultured in the light vs. dark).

These results indicate that in contrast to its impact on the UV sensitivity and inhibition of mating, that the mutation of the conserved cysteine site for flavin binding in the LOV domain does not attenuate the virulence of *C. neoformans*. Hence, the Bwc1-Bwc2 transcription factor complex regulates the virulence of *C. neoformans* in a light-independent manner.

3.5. Analysis of gene expression in *C. neoformans* *bwc1* mutant strains

Towards understanding what genes may be misregulated when the *BWC1* gene is mutated, five strains were grown under conditions in constant darkness and aiming to mimic conditions within a vertebrate host. RNA was extracted from the samples, and gene expression estimated by RNA-sequencing and analysis of transcript abundance by comparing the *bwc1* deletion strain to the wild type, *bwc1+BWC1*, *bwc1+BWC1^{C605A}* and *bwc1+BWC1^{C605S}* strains (supplemental table 1). Genes were up-regulated and 192 down-regulated more than 2-fold in these four strains, as illustrated in the heat map representation of the data (supplemental figure 2). Comparison of the genes to those known to be involved in virulence, e.g. as defined in large scale studies of genes required for virulence (Liu et al., 2008), revealed no candidate that might explain the virulence differences. One exception was one of the most differentially expressed genes, *MPR1*, which has recently been shown to encode a metalloprotease required for crossing the blood brain barrier in mouse models of cryptococcosis (Na Pombejra et al., 2018; Vu et al., 2014).

4. Discussion

The LOV domains are essential for the light sensing capabilities of photosensory proteins in plants, fungi and bacteria (Briggs and Spudich, 2005), and LOV-domain containing proteins are implicated as virulence factors in pathogenic fungi and bacteria. In fungi, the most common and best-characterized LOV domain protein is White Collar 1 (Fischer et al., 2016). This gene, and a second gene *wc-2*, were first identified and cloned based on mutant phenotypes in the ascomycete *N. crassa* (Ballario et al., 1996; Linden and Macino, 1997). Both genes are required for sensing blue light. With the advent of genome sequencing projects for many fungi, it became clear that this gene pair is found throughout many fungal lineages. However, these homologs have roles beyond sensing light, such as during growth under conditions of no light, although such “dark” functions have received little attention to date. Some of the best appreciation of these functions comes from the role the WC complex plays in the circadian clock machinery of *N. crassa*. In this species the complex allows light to serve as an indicator of time-of-day, and then is part of the feedback loop during the rest of the day that is required for circadian timing (Baker et al., 2012).

The LOV domains have a conserved structure and amino acid residues for interacting with chromophores such as flavin adenine dinucleotide or flavin mononucleotide. Of these residues, an invariant cysteine in the α 'A helix is critical for the formation of a covalent adduct with the C (4a) position of the flavin in response to light exposure (Crosson and Moffat, 2001; Crosson and Moffat, 2002). Mutations to one of the nucleotides encoding the cysteine residue are predicted to block the formation of the light-induced adduct between the LOV domain and the flavin chromophore, thus impairing the capability of the protein to transmit the light signal.

We therefore tested the role of light in the virulence-related function of the Bwc1 protein of the human pathogen *C. neoformans* by making and then testing strains with amino acid substitutions in the LOV domain. The work reported here is on *C. neoformans*: we also used the same approach to create an equivalent strain set of strains in *C. deneoformans* using constructs specific to *C. deneoformans* (data not shown). Because the *C. deneoformans* background used has a low level of virulence, the results of the *C. neoformans* strains are reported here. Two specific alleles of *BWC1* were generated that substitute either an alanine or serine for the cysteine residue required for adduct formation to the flavin molecule in response to light. The equivalent residue in LOV domains inactivates light sensing in phototropin of *Arabidopsis thaliana* or WC-1 of *N. crassa* (Cheng et al., 2003; Christie et al., 2002). These *C. neoformans* *BWC1* alleles have loss-of-function phenotypes for two light-dependent effects, i.e. the inhibition of mating and protection from UV light. However, the two alleles have different effects on the protein properties, with the *BWC1*^{C605A} allele allowing some light-dependent induction of *HEM15*, which is consistent with the phenotypes of the equivalent mutations introduced into the *N. crassa wc-1* gene. That is, Cheng et al. (2003) found that the substitution of a serine for cysteine blocked the increase in transcript levels of both the *frq* and *al-3* genes in response to light, but the alanine substitution strain had nearly normal induction of *frq* yet abolished induction of *al-3*. It is worth noting that while this adduct formation is considered a requirement for transmission of the photon signal, flavin may still interact with these modified LOV domains or with other amino acid residues. The *BWC1*^{C605S} allele renders the encoded protein even less sensitive to light, and the strains carrying the two altered *BWC1* alleles are equally pathogenic as wild type. Taken together, it can be concluded that the cysteine residue within the conserved NCRFLQ motif of the LOV domain in Bwc1 is essential for the capabilities of *C. neoformans* to fully respond to light yet it is dispensable for regulating virulence of this fungal pathogen.

Since the WCC proteins possess domains for both signal input (LOV domain) and output (Zn finger DNA binding domain), it is possible for them to have both light dependent- and independent-regulatory roles. Fungi in which deletion of the genes encoding the WCC results in phenotypes when cultured in darkness include *Trichoderma atroviride* (Fiedl et al., 2008; Sánchez-Arreguín et al., 2012), *Trichoderma reesei* (Castellanos et al., 2010; Gyalai-Korpos et al., 2010; Tisch and Schmoll, 2013), *Aspergillus nidulans* (Bayram et al., 2010; Purschwitz et al., 2008), *Alternaria alternata* (Pruß et al., 2014) and *Fusarium fujikuroi* (Castrillo and Avalos, 2015). This study therefore provides the genetic evidence to divide the role of Bwc1 into two aspects, one being light sensing to impact UV sensitivity and mating, and the other being light independent and impacting virulence (Figure 7).

Photosensory proteins with LOV domain are also found in the bacteria, although they use different signal transmission domains compared to the zinc finger DNA binding domains in fungal White Collar complexes (Herrou and Crosson, 2011; Losi et al., 2015). Of those bacteria characterized to date, animal pathogens in which light sensing components impact virulence are *Brucella abortus* (Swartz et al., 2007) and plant pathogens are *Pseudomonas syringae* and *Xanthomonas citri* (Kraiselburd et al., 2012; Moriconi et al., 2013). Experiments that generate specific alleles that alter these photosensors may further elucidate how LOV domain proteins contribute to microbial pathogenesis.

One additional consideration, as suggested by the ‘O’ in the abbreviation of the domain name LOV, is that proteins containing this domain may also have the ability to sense changes levels of reactive oxygen species (ROS). The *bwc1* mutant has no change in sensitivity to ROS under in vitro conditions (Idnurm and Heitman, 2005); however, this does not preclude a possibility of sensing altered ROS levels within an animal during disease progression.

Future directions for research in *C. neoformans* would be to define which genes that are regulated by the White Collar complex contribute to virulence. A preliminary experiment used RNA-sequencing on the strains grown in constant darkness to reveal over 300 genes encoding hypothetical proteins that may be regulated by the complex, but because most of those genes have no established role in virulence in *C. neoformans* or other fungi it is currently unclear why loss of the genes causes the reduction in virulence. Identifying such genes that contribute to virulence could reveal unknown factors that affect the disease outcomes of this major human pathogenic fungus.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- The blue light sensing complex is required for virulence of *Cryptococcus neoformans*
- Mutation of a specific cysteine residue for transmission of the light signal blocks light responses
- Strains with the cysteine substituted with other amino acid residues have wild type levels of virulence
- The contribution of the White Collar 1 homolog to virulence is independent of light signaling

Fig. 1.

The UV sensitivity is higher in strains of *C. neoformans* with either the *bwc1* deletion mutation or the two amino acid substitutions. Ten-fold serial dilutions were plated onto YPD medium, and one set irradiated with UV light (120 J/m²). Photographs show colonies 2 days later.

Fig. 2.

Production of the sexual filaments is inhibited in response to light in wild type strains, but not in crosses with the *bwc1* mutant or the two strains carrying the mutant allele of *BWC1*. Strains of *C. neoformans* was crossed to either the *C. deneoformans* strain (A) JEC30 with the wild type copy of *BWC1* or (B) strain AI42 with the *bwc1* gene deleted.

Fig. 3.

Cell fusion inhibition by light is lost in the two LOV domain mutant alleles of *Bwc1*. Strains carrying the *ade2* mutation with the genotypes listed were mixed with *C. deoformans* strains JEC30 (*lys1*) or AI42 (*lys1 bwc1*) and cultured in the light or darkness for 24 h. Cultures were scraped from the plates and plated onto minimal medium. Colony forming units are a measure of cell fusion events. **(A)** Colonies indicate diploid or dikaryotic strains after cell fusion. **(B)** Quantification of cell fusion events; significant differences between data values are indicated by different letters above the histograms.

Fig 4.

The Bwc1^{C605A} and Bwc1^{C605S} isoforms physically interact with Bwc2 in yeast two hybrid assays. The cDNAs of either gene were cloned adjacent to the activation (AD) or DNA binding (BD) domains of *S. cerevisiae* Gal4. Constructs were transformed into an *S. cerevisiae* reporter strain. The β -galactosidase activity and growth on medium without adenine (ade) indicate interactions between Bwc2 and the Bwc1 alleles to reconstitute the Gal4 protein. There was no statistical difference between the β -galactosidase activities of the wild type vs. mutant isoforms.

Fig. 5.

The induction of two conserved light-regulated genes is impaired in the *bwc1* mutant and the two strains carrying the modified forms of *BWC1*. Quantitative real time PCR of the expression of two light-regulated genes in five strains of *C. neoformans*. Fold change in transcript levels in response to 15 min exposure to light relative to the constant darkness for each strain, normalized against the actin gene. The histograms marked with asterisks are significantly different from each other using Student's t test ($P < 0.05$).

Fig. 6.

C. neoformans strains with the *BWC1*^{C605A} and *BWC1*^{C605S} alleles are as virulent as wild type, and pre-exposure to light does not impact virulence. Wax moth larvae were inoculated with strains grown in complete darkness and manipulated under safe red light, or exposed to light. Survival was monitored each day. The survival difference for larvae inoculated with the *bwc1* mutant compared to the other strains is statistically significant (e.g. in light compared to wild type $P = 0.00261$, log-rank test).

Fig. 7.

A model for how the Bwc1-Bwc2 complex impacts on *C. neoformans* biology. In the presence of light, blue wavelengths are absorbed by a flavin molecule, the signal transmitted to alter transcription, and mating inhibited while resistance to UV is increased. In darkness, or when the flavin-binding capabilities of Bwc1 are impaired, transcription still occurs to regulate genes that are required for virulence. Such virulence genes currently remain undiscovered.

Table 1.

Oligonucleotide primers used in this study.

Name	Sequence (5'–3')	Function and location
PK003	GGTACCATAAGTTTGCTGCGAAGC	Forward primer to amplify <i>BWCI</i> , with KpnI cut site
PK004	GGATCCGTGATGTGGGCAGTTACG	Reverse primer to amplify <i>BWCI</i> , with BamHI cut site
PK010	CGGTATCGAAGACAATCC	Forward primer to amplify <i>URA5</i> (outside the <i>URA5</i> used in construction)
PK011	TGGGAGGAAGAGGATGAG	Reverse primer to amplify <i>URA5</i> (outside the <i>URA5</i> used in construction)
PK018	GGGACTTTGAAGAAAACGAGCATT CGACCGAGAAGTTG	Reverse primer to amplify the left part used to replace Cys site with Ala in <i>BWCI</i> gene
PK019	CAACTTCTCGGTCGTAATGCTCGTT TTCTTCAAAGTCCC	Forward primer to amplify the right part used to replace Cys site with Ala in <i>BWCI</i> gene
PK020	GGCTGACGGAATCTCTAG	Forward primer to sequence the Cys site mutation
PK021	TATAGGCACAACAGTGAC	Reverse primer to sequence the Cys site mutation
PK027	CAACTTCTCGGTCGTAATTGCAAAG AGCGAAGTTGC	Forward primer to amplify <i>URA5</i>
PK028	GGGACTTTGAAGAAAACGGATGTT GGCTGTCAATC	Reverse primer to amplify <i>URA5</i>
PK035	GGGACTTTGAAGAAAACGTCTATTA CGACCGAGAAGTTG	Reverse primer to amplify the left part used to replace Cys site with Ser in <i>BWCI</i> gene
PK036	CAACTTCTCGGTCGTAATAGACGTT TTCTTCAAAGTCCC	Forward primer to amplify the right part used to replace Cys site with Ser in <i>BWCI</i> gene
HEM15F	TGGTAGAGGCTTTTGCAC	Real time PCR of <i>HEM15</i>
HEM15R	TCACCACGGTTGACAATG	Real time PCR of <i>HEM15</i>
UVE1F	TGCATCCTGGTCAGTTTACGC	Real time PCR of <i>UVE1</i>
UVE1R	CATATGGATAATCATGACAC	Real time PCR of <i>UVE1</i>
ACT1NF	CAATGGTATTGCCGACCG	Real time PCR of <i>ACT1N</i>
ACT1NR	CTTCGCGATCCACATCTG	Real time PCR of <i>ACT1N</i>

Table 2.Strains of *Cryptococcus* used in this study.

Name	Species*	MAT	Genotype	Origin and notes
JEC30	D	a	<i>lys1</i>	(Moore and Edman, 1993)
AI42	D	a	<i>lys1 bwc1::NAT</i>	(Idnurm and Heitman, 2005)
AI81	A	alpha	<i>bwc1::NAT</i>	(Idnurm and Heitman, 2005)
KN99a	A	alpha	Wild type	(Nielsen et al., 2003)
AI202	A	a	<i>ade2</i>	(Idnurm, 2010)
JF99	A	a	<i>ura5</i>	(Nichols et al., 2004)
AIZCN1	A	alpha	<i>bwc1::NAT ura5</i>	Progeny of JF99 × AI81
AIZCN2	A	a	<i>bwc1::NAT ura5</i>	Progeny of JF99 × AI81
AIZCN4	A	alpha	<i>bwc1::NAT ura5::URA5+BWC1</i>	Created by transforming P-Litmus28i-Bwc1 (serotype A)-URA5 (serotype A) into strain AIZCN1
AIZCN12	A	a	<i>bwc1::NAT ura5::URA5+BWC1</i>	Progeny of AIZCN4 × AIZCN2
AIZCN30	A	alpha	<i>bwc1::NAT ura5::URA5+BWC1</i>	Progeny of AIZCN4 × AIZCN2
AIZCN34	A	alpha	<i>bwc1::NAT ura5::URA5+BWC1^{C605S}</i>	Created by site-directed mutation
AIZCN36	A	alpha	<i>bwc1::NAT ura5::URA5+BWC1^{C605A}</i>	Created by site-directed mutation
AIZCN66	A	alpha	<i>ade2</i>	Progeny of AI81 × AI202
AIZCN68	A	alpha	<i>bwc1::NAT ade2</i>	Progeny of AI81 × AI202
AIZCN70	A	alpha	<i>bwc1::NAT ura5::URA5+BWC1 ade2</i>	Progeny of AIZCN30 × AI202
AIZCN72	A	alpha	<i>bwc1::NAT ura5::URA5+BWC1^{C605A} ade2</i>	Progeny of AIZCN36 × AI202
AIZCN74	A	alpha	<i>bwc1::NAT ura5::URA5+BWC1^{C605S} ade2</i>	Progeny of AIZCN34 × AI202

* species: A = *C. neoformans*, D = *C. deneoformans*