SEC14 is a specific requirement for secretion of phospholipase B1 and pathogenicity of *Cryptococcus neoformans*

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Summary

Secreted phospholipase B1 (CnPlb1) is essential for dissemination of *Cryptococcus neoformans* to the central nervous system (CNS) yet essential components of its secretion machinery remain to be elucidated. Using gene deletion analysis we demonstrate that CnPlb1 secretion is dependent on the Cn*SEC14* product, CnSec14-1p. CnSec14-1p is a homologue of the phosphatidylinositol transfer protein (PITP) ScSec14p, which is essential for secretion and viability in *Saccharomyces cerevisiae*. In contrast to CnPlb1, neither laccase 1 (Lac1)-induced melanization within the cell wall nor capsule induction were negatively impacted in Cn*SEC14-1* deletion mutants (CnΔ*sec14-1* and CnΔ*sec14-1*CnΔ*sfh5*). Similar to the Cn*PLB1* deletion mutant (CnΔ*plb1*), CnΔ*sec14-1* was hypo-virulent in mice and did not disseminate to the CNS by day 14 post infection. Furthermore, macrophage expulsion of live CnΔ*sec14-1* and CnΔ*plb1* (vomocytosis) was reduced. Individual deletion of Cn*SEC14-2*, a closely-related Cn*SEC14-1* homologue, and Cn*SFH5*, a distantly-related SEC fourteen-like homologue, did not abrogate CnPlb1 secretion or virulence. However, reconstitution of CnΔ*sec14-1* with Cn*SEC14-1* or Cn*SEC14-2* restored both phenotypes, consistent with functional genetic redundancy. We conclude that CnPlb1 secretion is *SEC14*-dependent and that *C. neoformans* preferentially exports virulence determinants to the cell periphery via distinct pathways. We also demonstrate that CnPlb1 secretion is essential for vomocytosis.

Introduction

Cryptococcus neoformans is a facultative intracellular basidiomycete responsible for an estimated one million cases of meningitis and 675,000 deaths per year in HIV-infected

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patients alone (Park et al., 2009). Although predominantly an environmental saprophyte, *C. neoformans* causes systemic infections in humans and may have evolved as a pathogen capable of surviving macrophage defence mechanisms, via engaging in phagocytic interactions with soil amoeba (Alvarez & Casadevall, 2006). After inhalation, *C. neoformans* establishes a lung infection and can disseminate to other organs, especially the CNS, where it causes severe life-threatening meningoencephalitis.

Three major virulence mechanisms of *C. neoformans* are laccase 1 (Lac1)-mediated production of the stress-protecting pigment, melanin (Zhu et al., 2001), elaboration of the polysaccharide capsule, which prevents yeast desiccation and has immunomodulatory properties (Zaragoza et al., 2009) and secretion of phospholipase B1 (CnPlb1) (Cox *et al.*, 2001, Noverr *et al.*, 2003, Santangelo *et al.*, 2004). Secretion of CnPlb1 into host tissue has been inferred by detection of CnPlb1 breakdown products in cryptococcal lung lesions using magnetic resonance spectroscopy (Wright et al., 2002) and detection of specific antibody in serum from infected patients (Santangelo et al., 2005).

CnPlb1 contains phospholipase B (PLB) and lysophospholipase (LPL) activities that hydrolyse one (LPL) or both (PLB) ester linkages on membrane phospholipids, respectively (Chen et al., 2000), releasing fatty acids as a potential energy source (Wright et al., 2007). CnPlb1 also contains lysophospholipase transacylase (LPTA) activity which is essential for the incorporation of macrophage-derived arachidonic acid into cryptococcal lipids during cryptococcus-phagocyte interactions (Wright et al., 2007). This arachidonic acid serves as a reservoir for eicosanoid production by *C. neoformans* which suppresses macrophage activity, thus diminishing the host immune response (Noverr et al., 2003). Targeted disruption of the CnPlb1 encoding gene (Cn*PLB1*), which abolishes secretion of all three phospholipase activities, reduces virulence in animal models and prevents dissemination of cryptococci to the CNS (Cox et al., 2001, Noverr et al., 2003). *C. neoformans* also survives and replicates within the phagolysosome of lung macrophages (Feldmesser et al., 2000), the acidic pH of which is optimal for CnPlb1 activity (Chen et al., 2000). *C. neoformans* escape from macrophages occurs via a lytic process or via a non-lytic extrusion mechanism that maintains the viability of both *C. neoformans* and the macrophage (Ma *et al.*, 2006, Alvarez & Casadevall, 2006).

The externalization and/or secretion of Lac1, capsular glucuronoxylomannan (GXM) and CnPlb1 is dependent on fungal secretion pathways. Recent studies in *C. neoformans* defined preferential pathways for export of Lac1 and GXM, which utilise *SEC6*-dependent exosomes of the lysosomal trafficking pathway and *SAV1*(*SEC4*)-dependent vesicles, respectively (Panepinto *et al.*, 2009, Yoneda & Doering, 2006). Although the glycosylphosphatidylinositol (GPI) anchor regulates the cellular export and secretion of CnPlb1 (Djordjevic *et al.*, 2005, Siafakas *et al.*, 2007) and N-linked glycosylation is an essential requirement (Turner et al., 2006), components of CnPlb1-specific secretion machinery are unknown. Despite these studies in *C. neoformans*, much of our knowledge about secretion in fungi is derived from the non-pathogenic model ascomycete, *S. cerevisiae* (Harsay & Bretscher, 1995, Muniz & Riezman, 2000, Vorisek, 2000). One key component is the phosphatidylinositol transfer protein (PITP), ScSec14p, which regulates *trans*-Golgi export pathways and is essential for viability (Bankaitis *et al.*, 1989, Curwin *et al.*, 2009). ScSec14p links protein traffic and Golgi lipid homeostasis via its lipid sensory function (Bankaitis et al., 2010). However the role of *SEC14* homologues in secretory processes in pathogenic fungi remains to be elucidated.

We have identified three *SEC14* homologues in *C. neoformans:* Cn*SEC14-1* and Cn*SEC14-2* which are highly homologous to Sc*SEC14*, and a *SEC14*-like homologue (Cn*SFH5*) which is most homologous to Sc*SFH-5*. Using gene deletion analysis we

investigated the role of *SEC14* homologues in secretion of CnPlb1, melanization and capsule induction and how this impacted on virulence using a murine model of cryptococcosis. We also compared the rate of non-lytic expulsion of the Cn*SEC14-1* deletion mutant from macrophage phagolysosomes (vomocytosis), with that of CnΔ*plb1*. Our results reveal that secretion of CnPlb1, but not melanization and capsule induction, is Cn*SEC14-1*-dependent, that reduced CnPlb1 secretion in Cn*SEC14-1* deficient mutants coincides with attenuated virulence and dissemination in an animal model, and that CnPlb1 secretion is an essential requirement for vomocytosis.

Results

Identification of *SEC14* homologues in *C. neoformans*

The putative cryptococcal Sec14p homologues were identified by BLAST searching the *C. neoformans* Serotype D database with *S. cerevisiae* Sec14p and its homologues. Two putative CnSec14 phosphatidylinositol transfer proteins (PITPs), CNG04130 (designated CnSec14-1p) and CNA00270 (designated CnSec14-2p), sharing a 59% and 71% similarity with ScSec14p, respectively, were identified. In addition to ScSec14p, *S. cerevisiae* has five <u>Sec</u> <u>f</u>ourteen like <u>h</u>omologues (Sfh) which share a 42-79% similarity with ScSec14p. A third putative PITP (CNE04320) sharing a 36% similarity with ScSec14p (E-value 8.0E-05) was also identified in serotype D. This protein was most similar (47%) to ScSfh5p and was named CnSfh5p (Figure 1). All three cryptococcal proteins contain the cellular retinaldehyde-binding protein (CRALBP) and TRIO guanine exchange factor structural domain (CRAL/TRIO) typical of PITPs. Due to the greater degree of similarity between CnSec14-1p and CnSec14-2p (86%) compared to ScSec14p and ScSfh1p (79%), the cryptococcal genes were designated Cn*SEC14-1* and Cn*SEC14-2* in preference to using the *SFH* nomenclature. The serotype D amino acid sequences were used in a BLAST search of the serotype A database and sequences CNAG_03153 (chromosome 8), CNAG_00036 (chromosome 1) and CNAG_02104 (chromosome 6) were retrieved and designated CnSec14-1p, CnSec14-2p and CnSfh5p based on homology. A phylogenetic tree depicting the Sec14p similarities among *C. neoformans* serotypes and *S. cerevisiae* is shown in Figure 1.

To determine whether the Cn*SEC14* homologues identified have a similar function to their *S. cerevisiae* orthologues, we exploited a *SEC14* temperature sensitive-mutant of *S. cerevisiae*, (Sc*SEC14^{TS}*) which is permissive to growth at 25-30°C but cannot survive at 37°C. WT *S. cerevisiae* and Sc*SEC14^{TS}* were transformed with pCXJ28 (empty vector) or empty vector containing Cn*SEC14-1* or Cn*SEC14-2* cDNA. The results in Figure 2 show that all strains grew at 25°C, with Cn*SEC14-2*-transformed cells growing slightly better than Cn*SEC14-1*-transformed cells in both WT and Sc*SEC14^{TS}*. Overexpression of Cn*SEC14-1* in WT reduced growth slightly consistent with mild toxicity. As expected, Sc*SEC14^{TS}* transformed with empty vector did not grow at 37°C. However, growth at 37°C was restored in Sc*SEC14^{TS}* by overexpression of either Cn*SEC14-2* or Cn*SEC14-2*, confirming that both Cn*SEC14-1* and Cn*SEC14-2* have a similar function and are orthologues of Sc*SEC14*.

Cn*SEC14* gene deletion in *C. neoformans*

Cn*SEC14-1*, Cn*SEC14-2* and Cn*SFH5* were deleted in *C. neoformans* var. *grubii* strain H99 as described in Experimental Procedures and Supporting Information (Figure S1, Table S1), creating CnΔ*sec14-1*, CnΔ*sec14-2* and CnΔ*sfh5*, respectively. Cn*SFH5* was also deleted in combination with either Cn*SEC14-1* or Cn*SEC14-2*, creating CnΔ*sec14-1*/CnΔ*sfh5* and CnΔ*sec14-2*/CnΔ*sfh5*. However, in contrast to CnΔ*sec14-1*/CnΔ*sfh5* and CnΔ*sec14-2*/CnΔ*sfh5* disruption of *CnSEC14-2* in CnΔ*sec14-1* to produce the CnΔ*sec14-1*/CnΔ*sec14-2* double deletion mutant, did not produce any transformants despite three attempts with the

use of controls. This is presumably because the absence of both *CnSEC14-1* and *CnSEC14-2* is lethal. Cn*Δsec14-1* was also reconstituted with either Cn*SEC14-1* or its closely related Cn*SEC14-2* homologue, creating Cn*Δsec14-1*/Cn*SEC14-1* and Cn*Δsec14-1*/Cn*SEC14-2*, respectively. It is important to note that in Cn*Δsec14-1*/Cn*SEC14-2*, Cn*SEC14-2* is ectopically over expressed in addition to being expressed from its endogenous genetic locus. In contrast, Cn*SEC14-1* is only ectopically overexpressed in Cn*Δsec14-1*/Cn*SEC14-1*. Gene deletion at only the correct locus and genetic reintegration of the full-length gene into the deletion mutants, were confirmed by PCR and Southern hybridization (Supporting Information Figure S2, Table S3).

Cn*SEC14* gene deletion and phospholipase B1 secretion in *C. neoformans*

To determine whether Cn*SEC14-1* or its homologues, play a role in CnPlb1 secretion, CnPlb1 activity (measured as LPL and LPTA) and protein levels were determined in YNB culture supernatants using a radiometric enzyme assay and anti-Plb1 western blotting, respectively (Figure 3). Cn*Δsec14-1* and Cn*Δsec14-1*/Cn*Δsfh5* secreted a reduced amount of enzyme protein (Figure 3B) and LPL and LPTA activities were ~5-fold and 3.5-fold lower than in the H99 WT, respectively (Figure 3A). In contrast, secretion of active CnPlb1 by Cn*Δsec14-2* was not reduced. Genetic reconstitution of Cn*Δsec14-1* with Cn*SEC14-1* or Cn*SEC14-2* partially restored the activity of secreted CnPlb1 back to H99 WT levels.

In addition to being secreted, some CnPlb1 remains associated with cell wall β-glucans by the GPI anchor remnant (Djordjevic et al., 2005, Siafakas et al., 2007). CnPlb1 levels in β 1-3-glucanase treated cell walls were therefore assessed by western blotting (Figure 3C) to determine whether the reduction in CnPlb1 secretion coincides with the reduced presence of Plb1 in the cell wall. Relative to the H99 WT, Cn*Δsec14-2* and Cn*Δsfh5*, cell wall-associated CnPlb1 was in fact reduced in Cn*Δsec14-1* CnPlb1 was present in the Cn*Δsec14-1* cytosol confirming that CnPlb1 is being synthesized. It has been demonstrated that yeast strains with disrupted genes that encode GPI anchored proteins have cell wall defects as exhibited by their sensitivity to SDS and calcofluor white (Plaine et al., 2008). Thus we examined the sensitivity of Cn*Δsec14-1* to growth in the presence of each inhibitor. Similar to the *PLB1* deletion mutant, Cn*Δplb1* (Siafakas et al., 2007) Cn*Δsec14-1* and Cn*Δsec14-1*/Cn*Δsfh5* were sensitive to SDS and exhibited clumping (Supporting Information Figure S3 A and B). Impaired growth in SDS correlates with enhanced accessibility of the detergent to the membrane as a result of a compromised cell wall barrier. Furthermore, all three mutants were sensitive to calcofluor white (Figure S3 C). The cell wall defect is putatively due to the absence of GPI anchored CnPlb1 in the cell wall. All phenotypes were restored in Cn*SEC14-1* by genetic reconstitution with Cn*SEC14-1*, or restored/partially restored by genetic reconstitution with Cn*SEC14-2* (Figure S3). Despite the cell wall defects in Cn*Δplb1*, Cn*Δsec14-1* and Cn*Δsec14-1*/Cn*Δsfh5*, all strains exhibited comparable growth to H99 WT at 37°C (Cox et al., 2001, Noverr et al., 2003) (Figure S3).

Cn*SEC14* is not required for melanin or capsule production in *C. neoformans*

Lac1-induced melanin production is dependent on Lac1 transport to the cell periphery via the lysosomal secretory pathway (Panepinto et al., 2009). In the cell wall, Lac1 synthesizes melanin from externally-derived phenolic compounds such as L-DOPA, which is present in mammalian brain. To determine whether Cn*SEC14-1* or its homologues affect Lac1 transport to the cell wall, melanin production was measured following growth on L-DOPA agar. The extent of melanin pigmentation was similar in H99 WT and all Cn*SEC14* mutants (Figure 4A). Additionally, similar amounts of Lac1 were detected in cell walls by western blotting (Figure 4B).

Capsule formation is dependent on vesicular transport of GXM to the cell periphery, which is blocked in *SAV1(SEC4)*-deficient mutants (Yoneda & Doering, 2006). To determine whether capsule formation is also dependent on an intact Cn*SEC14-1* dependent pathway, capsule size was measured following growth under capsule inducing conditions (Figure C and D). Capsule was not reduced in any of the mutants. Rather, strains in which CnPlb1 secretion was absent (CnΔ*plb1*)(Cox et al., 2001) or attenuated (CnΔ*sec14-1*, CnΔ*sec14-1/ sfh5*) (Figure 3), had larger capsule/cell body ratios than all the other strains.

Effect of Cn*SEC14/SFH* deletion on *C. neoformans* virulence

The virulence of the Cn*SEC14/SFH5* deletion mutants was tested in an inhalation model of cryptococcosis. Only mice infected with CnΔ*sec14-1* [and CnΔ*sec14-1*/CnΔ*sfh5* (not shown)] survived longer (median survival 27 and 24 days) than H99 WT-infected mice (median survival 19 days), (Figure 5A). Prolonged survival correlated with reduced CnPlb1 secretion (Figure 3). The median survival of mice infected with CnΔ*sec14-2*, CnΔ*sfh5* and CnΔ*sec14-2*/CnΔ*sfh5* was similar to that of H99 WT (P>0.05). Reconstitution of CnΔ*sec14-1* with Cn*SEC14-1* or Cn*SEC14-2* restored virulence. The effect on virulence of CnΔ*sec14-1*, in which CnPlb1 secretion is blocked, was also compared to that of CnΔ*plb1*, which neither synthesizes nor secretes CnPlb1 (Cox et al., 2001, Siafakas et al., 2007) (Figure 5B). Although CnΔ*plb1* was previously found to be hypo-virulent in animal models, these studies were performed in different mouse strains and/or using different inoculation routes/doses (Cox et al., 2001, Noverr et al., 2003, Santangelo et al., 2004). Mice infected with CnΔ*plb1* survived a median of 7 days longer than mice infected with CnΔ*sec14-1*. We note in Fig 5B, that the median survival times for mice infected with H99 WT and CnΔ*sec14-1* (Day 16 and 22, respectively) were shorter than in Fig. 5A (Day 19 and 27, respectively) and that the difference in the median survival time between mice infected with H99 WT and CnΔ*sec14-1* was 6 and 8 days, respectively. The differences may reflect batch to batch variation in mice susceptibility to *C. neoformans* infection. The CnΔ*plb1*/Cn*PLB1* reconstituted strain has previously been shown to exhibit virulence similar to that of H99 WT and thus was not tested here (Cox et al., 2001, Noverr et al., 2003, Santangelo et al., 2004).

The hypovirulence of CnΔ*sec14-1* and CnΔ*plb1* relative to WT, correlated with reduced lung burdens at 3, 7 and 14 days post-infection and failure to disseminate to the brain within 14 days (Figure 5C and D). However by Day 14 the organ burdens for CnΔ*sec14-1* were significantly higher than those of CnΔ*plb1*. Reconstitution of CnΔ*sec14-1* with Cn*SEC14-1* restored lung organ burdens back to H99 WT levels at Day 3, 7 and 14. However, reconstitution of CnΔ*sec14-1* with Cn*SEC14-2* only restored lung organ burdens back to H99 WT levels at Day 3. By Day 14 only H99 WT, CnΔ*sec14-1*/*SEC14-1* and CnΔ*sec14-1/ SEC14-2* had disseminated to the CNS (Figure 5D). No dissemination was observed on day 3 and 7 for any strain (not shown). The brains of two animals were harvested and cultured upon succumbing to illness (day 19 for CnΔ*Sec14-1* and day 25 for CnΔ*plb1*). In agreement with previous findings, CnΔ*plb1* was absent from brain tissue. However, cryptococci (1.18 $\times 10^5$ CFUs per gram) were cultured from brains of animals infected with CnΔ*sec14-1* (not shown), indicating that dissemination did eventually occur.

CnSec14 and CnPlb1 are required for efficient vomocytosis

C. neoformans can escape macrophage killing via a phagolysosomal extrusion process known as vomocytosis. As CnPlb1 is essential for creating a macrophage-derived reservoir of arachidonic acid for eicosanoid production which can alter macrophage function (Wright et al., 2007, Noverr et al., 2003) we hypothesized that vomocytosis would be altered in CnPlb1-deficient strains of cryptococci. Vomocytosis was reduced by ~60% in CnΔ*plb1* and fully restored in the reconstituted strain (Figure 6A). In CnΔ*sec14-1*, vomocytosis was

reduced by ~40%; it was partially restored by reconstitution with Cn*SEC14-1* (Figure 6B). However, reconstitution of CnΔ*sec14-1* with Cn*SEC14-2* showed little difference with CnΔ*sec14-1* (P=0.09) and, although lower than that of H99 and CnΔ*sec14-1/CnSEC14-1*, this difference was not statistically significant (P=0.34).

Investigating potential compensatory mechanisms for the absence of Cn*SEC14*

In contrast to *S. cerevisiae* (Bankaitis et al., 1989) and *C. albicans* (Monteoliva et al., 1996), deletion of Cn*SEC14* homologues is not lethal. Thus we investigated whether expression of other *SEC14* homologues was up regulated in the absence of Cn*SEC14-1* as part of a potential compensation mechanism. qRT-PCR was used to quantify the expression of Cn*SEC14-1*, Cn*SEC14-2* and Cn*SFH5* in all mutant strains (Figure 7). As expected, the respective mRNAs corresponding to the gene deleted, were absent from CnΔ*sec14-1*, CnΔ*sec14-2* and CnΔ*sfh5* (Fig.7A) and CnΔ*sec14*-1/CnΔ*sfh5* (Fig. 7B). In CnΔ*sec14-1*, levels of Cn*SEC14-2* and Cn*SFH5* mRNA were increased 3-fold and 1.7-fold, respectively. In CnΔ*sec14-2*, CnΔ*sfh5* and CnΔ*sec14-2*/CnΔ*sfh5*, levels of Cn*SEC14-1* mRNA were reduced by 10%, 25% and 60% respectively. While the Cn*SEC14-1* mRNA level in the CnΔ*sec14-2*/CnΔ*sfh5* mutant was only 40% of the H99 WT, this was sufficient to achieve a full virulence phenotype. In fact, when Cn*SEC14-1* mRNA was inhibited by RNAi in CnΔ*sec14-2*, a Cn*SEC14-1* mRNA level as low as 18% of the H99 WT (Supporting Information, Figure S4) was sufficient to achieve a H99 WT phenotype (not shown). Reconstitution of CnΔ*sec14-1* with Cn*SEC14-1* restored Cn*SEC14-1* mRNA to 75% of the H99 WT level, and was sufficient to restore all virulence phenotypes. Reconstitution of CnΔsec*14-1* with Cn*SEC14-2*, which also restored virulence, led to a 9-fold increase in expression of Cn*SEC14-2* mRNA (Figure 7B). This is most likely due to the presence of more than one copy of Cn*SEC14-2* in CnΔ*sec14-1/SEC14-2* (the native gene and the ectopically-introduced copy).

Discussion

We have demonstrated that the cell wall association and secretion of the key cryptococcal virulence factor, CnPlb1, is Cn*SEC14-1* dependent. In contrast, neither the presence of Lac1 in the cell wall and its ability to synthesize melanin, nor the externalization of capsule, are dependent on *SEC14-1*. These results are consistent with the presence of distinct secretion pathways for virulence determinants in *C. neoformans*.

Our data demonstrates that two strains defective (CnΔ*sec14-1*) or deficient (CnΔ*plb1*) in CnPlb1 secretion have similar phenotypes in vitro (sensitivity to two cell wall perturbing agents, hyper encapsulation and reduced vomocytosis) and are hypovirulent in an mouse inhalation model of cryptococcosis. Attenuated virulence of both strains coincided with reduced lung burdens and failure to disseminate to the CNS by day 14 post infection. Our evidence suggests that the reduced presence of CnPlb1 in CnΔ*sec14-1* supernatants and the cell walls is due to reduced CnPlb1 traffic from the Golgi apparatus to the cell wall, rather than the lack of attachment of exported CnPlb1 to a defective cell wall, as the latter would have resulted in hyper secretion. Furthermore, similar to CnΔ*plb1* the reduced presence of GPI-anchored CnPlb1 in CnΔ*sec14-1* cell walls is most likely to be a factor contributing to the cell wall defect. Although CnΔ*sec14-1* and CnΔ*plb1* shared similar phenotypes, some phenotypes were more severe in CnΔ*plb1*. In particular CnΔ*plb1* was less virulent than CnΔ*sec14-1*, failed to disseminate to the CNS over the entire infection time course and was expelled from macrophages less efficiently than CnΔ*sec14-1*. These differences may be attributed to the fact that secretion of CnPlb1, and potentially other proteins, is not blocked completely in CnΔ*sec14-1*, potentially due to functional redundancy of *SEC14* homologues. Alternatively, the possibility exists for CnPlb1 acting co-ordinately with CnSec14-1 to regulate Golgi phosphatidylcholine levels which are important for vesicle formation and

secretion. Sec14p in *S. cerevisiae* has been reported to down regulate ScPlb1-mediated turnover of phosphatidylcholine (Schnabl et al., 2003). Thus the lack of CnPlb1 in CnΔ*plb1* could alter Golgi lipid levels and thereby influence the traffic of other proteins, contributing to the increased severity of the phenotype compared to CnΔ*sec14-1*.

Sec4/Sav1 and Sec6-dependent intracellular vesicular trafficking pathways have been described for transport of capsular polysaccharide (GXM) and Lac1, respectively (Yoneda & Doering, 2006, Panepinto et al., 2009) and there is evidence that the Sec6-dependent intracellular vesicles are the precursors for extracellular exosomes containing Lac1 and urease (Panepinto et al., 2009). PI3 kinase (VPS34), which has been implicated in protein transport to the multivesicular body (the source of exosomes), is also essential for Lac1 activity (Hu et al., 2008). We therefore conclude that, as Lac1 traffic to the cell wall is not disrupted in CnΔ*sec14-1*, Cn*SEC14-1* does not regulate protein traffic from the lysosome to the plasma membrane in *C. neoformans*. Clearly, cryptococcal virulence factors are transported to the cell periphery via more than one pathway.

The apparent exclusivity of the Cn*SEC14*—dependent pathway for export of CnPlb1 may be a consequence of the protein nature of the cargo. In *S. cerevisiae* GPI anchored proteins are sequestered into ER-derived ergosterol and sphingolipid-enriched membrane vesicles (Muniz & Riezman, 2000, Sutterlin *et al.*, 1997) which are the precursors of lipid rafts in the plasma membrane (Bagnat *et al.*, 2000, Klemm *et al.*, 2009) and we demonstrated the association of CnPlb1 with cryptococcal membranes possessing raft like properties (Siafakas et al., 2006). However, we also found the non-GPI anchored protein, superoxide dismutase (SOD), sequestered within rafts (Siafakas et al., 2006) arguing against raft-like vesicles being the primary transporters of GPI anchored proteins in *C. neoformans*. In *S. cerevisiae* there is support for the presence of three parallel pathways of protein exit from the ER; one specific for GPI anchored proteins and controlled by the p24 family member Emp24 and possibly by the inositol deacylase Bst1, the GPI anchor-remodelling phospholipase Per1 and the COPII components Sec12 and Sec16; and the other two for non-anchored and transmembrane proteins (Castillon et al., 2009). Whether CnSec14 homologues in *C. neoformans* preferentially regulate cellular export of all GPI-anchored proteins or all proteins not exported via the lysosomal pathway remains to be determined. Finally, our data that Cn*SEC14-1* regulates distinct protein export pathways in *C. neoformans* is supported by a recent finding in *S. cerevisiae* where Sc*SEC14* was found to regulate export of Bgl2, but not Hsp150, Cts1, Scw4, Scw10, Exg1, Cis3 and Ygp1 (Curwin et al., 2009). Interestingly, none of these proteins are predicted to be GPI anchored. Furthermore, ScSEC14 also regulates retrograde protein traffic from the endosome to the *trans*-Golgi (Curwin et al., 2009).

In contrast to *S. cerevisiae* (Bankaitis et al., 1989) and *C. albicans* (Monteoliva et al., 1996), deletion of Cn*SEC14* homologues is not lethal. The most likely explanation for a viable *C. neoformans* phenotype following deletion of Cn*SEC14-1* is that other *SEC14* homologues, and in particular Cn*SEC14-2*, compensate in part for the absence of Cn*SEC14-1*. The Cn*SEC14-2* compensatory effect is further supported by our inability to create a viable CnΔ*sec14-1*/CnΔ*sec14-2* mutant. We observed that both Cn*SEC14-1* and Cn*SFH5* have an effect on Cn*SEC14-2* mRNA levels. Notably Cn*SEC14-2* mRNA increased 3-fold in CnΔ*sec14-1*, but did not coincide with virulence restoration, and increased 9-fold in CnΔ*sec14-1*/Cn*SEC14-2* but did coincide with virulence restoration. The 9-fold increase is most likely due to the presence of two copies of Cn*SEC14-2* contributing to the final mRNA level. However, Cn*SEC14-2* levels dropped to 24% in CnΔ*sec14-1*/CnΔ*sfh5*, coinciding with this strain being hypovirulent, and 35% in the virulent CnΔ*sfh5* strain, consistent with the presence of *CnSFH5* having a positive influence on Cn*SEC14-2* gene expression. The results also indicate that Cn*SEC14-2* mRNA levels as low as 24% are sufficient for *C.*

neoformans viability in the absence of the two *SEC14* homologues. Interestingly, attempts to suppress Cn*SEC14-1* mRNA in Cn*Δsec14-2* using RNAi, failed to further compromise the Cn*Δsec14-1* phenotype despite this methodology reducing the Cn*SEC14-1* mRNA level to as low as 18% of the H99 WT. This finding raises the question as to why Cn*SEC14-1* mRNA levels are so high in the first place, an observation that has also been made for *SEC14* mRNA in *S. cerevisiae* (Bankaitis et al., 2010). We also observed that over expression of Cn*SEC14-1*, but not Cn*SEC14-2*, was growth inhibitory to *S. cerevisiae*.

CnPlb1 is essential for efficient replication of *C. neoformans* at its primary site of infection (the lungs), and for entry into the blood stream and dissemination to the brain (Cox et al., 2001, Santangelo et al., 2004). We now demonstrate that Cn*Δsec14-1* is hypovirulent in mice. This may be due in part to low levels of CnPlb1 secretion. CnPlb1 can facilitate cryptococcal entry into the lungs by hydrolysis of dipalmitoyl phosphatidylcholine (PC) in lung surfactant (Santangelo et al., 1999) and/or promote cryptococcal adherence to pulmonary epithelium (Ganendren et al., 2006). *C. neoformams* also survives and replicates within the acidic phagolysosome of alveolar and interstitial macrophages following phagocytosis (Feldmesser et al., 2000), an environment conducive to optimal CnPlb1 enzyme activity (Chen et al., 2000).

In experimental mice, inhaled cryptoccoci are rapidly ingested by alveolar and pulmonary macrophages (Santangelo et al., 2004) and a role for mononuclear phagocytes in conveying cryptococci through the blood stream and across the blood brain barrier has recently been demonstrated (Charlier et al., 2009). Macrophages expel phagolysosomes containing viable cryptococci via a non-lytic extrusion process called vomocytosis which does not trigger host cell death (Ma *et al.*, 2007, Ma et al., 2006, Alvarez & Casadevall, 2006, Alvarez & Casadevall, 2007, Johnston & May, 2010). Vomocytosis from lung or mononuclear macrophages may serve as a mechanism for escape of *C. neoformans* into the blood stream allowing dissemination to the CNS. In this study, we found that vomocytosis was markedly impaired when CnPlb1 secretion was absent or attenuated in Cn*Δplb1* and Cn*Δsec14-1*, respectively, an effect that may be related to the role of CnPlb1 in eicosanoid production and suppression of macrophage function (Wright et al., 2007, Noverr et al., 2003) or intracellular proliferation of *C. neoformans* at early time points post-phagocytosis (Cox et al., 2001). This reduced rate of vomocytosis coincided with reduced or absent rates of dissemination to the CNS. Other factors reported to be important in vomocytosis are depolymerization of the actin cytoskeleton (Jones, 2007, Alvarez & Casadevall, 2006, Ma et al., 2006, Johnston & May, 2010).

In summary, we have defined a Cn*SEC14-1*-dependent CnPlb1 secretion pathway in *C. neoformans* which is not utilised for export of two other major virulence determinants, Lac1 and capsular material. We have shown that the two CnPlb1 secretion defective strains, Cn*Δsec14-1* and Cn*Δplb1*, in which CnPlb1 activity is attenuated either by deleting Cn*PLB1* or by blocking CnPlb1 secretion via deletion of Cn*SEC14-1*, are hypovirulent in mice as a consequence of reduced or absent disseminationto the brain. We also present evidence for the first time, that cryptococcal expulsion from macrophages is CnPlb1-dependent. Reduced vomocytosis in the CnPlb1 deficient mutants may contribute to the failure of *C. neoformans* to disseminate to the CNS.

Experimental Procedures

Identification of *SEC14* homologues in *C. neoformans*

The putative amino acid sequences of Sec14-1p and its homologues in *C. neoformans var neoformans* (Serotype D) were identified by performing a BLAST search of the *C. neoformans* Serotype D database (http://genome.slu.edu/blast.html) using the *S. cerevisiae*

*Sc*Sec14, *Sc*Sfh1 and *Sc*Sfh5 sequences as a query. The serotype A homologues were then identified by performing a BLAST search of the *C. neoformans* var. *grubii* strain H99 protein databases (http://www.broadinstitute.org/annotation/genome/cryptococcus_neoformans/ MultiHome.html) using the serotype D sequences as a query. GenBank accession numbers assigned to the three serotype A sequences are as follows: Cn*SEC14-1*: HM567405; Cn*SEC14-2*: HM567406; Cn*SFH5*: HM567407.

Strains, Media and Plasmids

Wild type (WT) *C. neoformans* var. *grubii* strain H99 (serotype A, *MAT*α, ATCC 208821), and the CnΔ*plb1* and CnΔ*plb1/PLB1* deletion mutants, also derived from H99 (Cox et al., 2001), were used in this study. The strains CnΔ*sec14-1*, CnΔ*sec14-2*, CnΔ*sfh5*, CnΔ*sec14-1*/CnΔ*sfh5*, CnΔ*sec14-2*/CnΔ*sfh5*, CnΔ*sec14-1*/Cn*SEC14-1*, CnΔ*sec14-1*/ Cn*SEC14-2* were also created from H99. All strains were cultured in yeast extract-peptone-dextrose (YPD) medium with the addition of 2% Bacto agar when solid medium was required. H99 transformants containing genes disrupted with a neomycin resistance cassette (Neo[r]) or nourseothricin resistance cassette (Nat[r]) were initially grown on YPD agar plates containing 1 M sorbitol for 3 h and subsequently transferred onto YPD agar supplemented with 200 μg/mL geneticin (G418; Invitrogen, CA, USA) or 100 μg/mL nourseothricin (clonNAT; Werner BioAgents, Jena, Germany), respectively. Stable transformants were maintained in YPD medium. Sabouraud dextrose (SAB) agar, SAB Brain Heart Infusion Agar Plus Drugs [SABD-5 g neopeptone (Difco), 20 g glucose, 26 g Brain Heart Infusion Agar (Difco), 7.5 g agar, 0.025 g gentamicin and 0.25 g chloramphenicol/L] and synthetic Yeast Nitrogen Base (YNB) medium were used in some experiments as indicated. DOPA agar, consisting of glucose-free asparagine medium (1-g/L L-asparagine, 0.5-g/L $MgSO_4$, 3-g/L KH_2PO_4, 1-mg/L thiamine) plus 1 mM L-DOPA, was used to demonstrate melanin pigment production. Capsules were induced on RPMI agar (RPMI, 2% glucose, 0.165M MOPS pH7, 1.5% bacto agar, 0.03% glutamine) by incubation at 37°C in a 5% CO_2 atmosphere for 3 days. Plasmids pJAF and pCH233, were a gift from John Perfect. *Saccharomyces cerevisiae* wild type strain, M2915-6A (MATa, *leu2, ade2, ura3*), and the temperature sensitive *SEC14* (TS) mutant purchased from ATCC® 208248™ [MATa *sec14-3 ade2-1*(ochre mutation) *trp1-delta ura3-52 mal SUC2 CUP1 gal2*], were grown in YPD or YNB without uracil following transformation with the pCXJ28 constructs.

Cn*SEC14* complementation in *S. cerevisiae*

Cn*SEC14* cDNA cloning—The cell pellet from an overnight YPD culture of H99 was centrifuged, frozen at -80°C, lyophilized and disrupted by vortexing with glass beads (2 mm). RNA was extracted using TRI reagent according to the manufacturer (Sigma-Aldrich, MO, USA) and 5 μg was treated with DNaseI (Invitrogen, CA, USA). cDNA was generated using the Superscript III First-Strand Synthesis kit (Invitrogen, CA, USA). Cn*SEC14-1* and Cn*SEC14-2* (CNAG_03153 and CNAG_00036.2, respectively, Broad Institute *Cryptococcus neoformans var. grubii* strain H99 Database www.broadinstitute.org/annotation/genome/cryptococcus_neoformans) were amplified from cDNA, using sense/antisense primers 5′-ATGGCCACCACAGACTTCCT-3′ / 5′-CTAAACACCTCCGGCAGCCT-3′ and 5′-ATGAGCGCCTCTGACCCTCT-3′/ 5′-TTAAACAGCCGTCGCGGTAT-3′, respectively. The PCR products were cloned into pCR®2.1-TOPO® (Invitrogen, CA, USA). DNA sequencing of the clones revealed at least five Cn*SEC14-2* splice variants with the expected size (0.8 kb). Using the flanking *Eco*RI sites in pCR®2.1-TOPO®, the Cn*SEC14-2* cDNA splice variant encoding a protein with the highest amino acid similarity to the predicted CnSec14-2 and ScSec14 sequences (96% and 57% respectively), and the Cn*SEC14-1* cDNA were subcloned into the *S. cerevisiae*

galactose-inducible expression vector, pCXJ28, creating pCXJ28_Cn*SEC14-2* and pCXJ28_Cn*SEC14-1*, respectively.

***CnSEC14* complementation of an *S. cerevisiae* *SEC14*[TS] mutant—**Wild type *S. cerevisiae* strain M2915-6A and the *ScSEC14*[TS] mutant were transformed with pCXJ28 (empty vector) or pCXJ28_Cn*SEC14-1*/Cn*SEC14-2* using the lithium actate/polyethylene glycol method (Burke et al., 2000) and transformants were selected on uracil dropout medium containing 2% glucose. Complementation was deemed to be successful by the growth of the Cn*SEC14*-transformed *Scsec14*[TS] mutant at the restrictive temperature (37°C) after 4 days on the selection plates supplemented with 2% galactose instead of glucose to induce expression of the heterologous gene.

Targeted *SEC14* gene deletion in *C. neoformans*

Gene deletion strategy—Native Cn*SEC14-1*, Cn*SEC14-2* and Cn*SFH5*, were selectively deleted in *C. neoformans* strain H99, individually and in combination, by homologous recombination with exogenously-introduced hybrid gene constructs. The constructs contained an antibiotic resistance marker flanked by the 5′ and 3′ UTR of the gene to be deleted, and were created by overlap PCR (Davidson et al., 2002) as described previously (Chayakulkeeree et al., 2008). The hybrid gene construction strategy and the biololistic homologous recombination method are described, in detail, in Supporting Information (Figure S1), with the oligonucleotide primers listed in Table S1. ***Gene Reconstitution*** As only Cn*Δsec14-1* displayed attenuated pathogenicity relative to H99 WT, it was genetically-reconstituted with either Cn*SEC14-1* or Cn*SEC14-2* to satisfy the molecular Koch's postulates of virulence (Falkow, 1988) and to investigate whether Cn*SEC14-2* can complement Cn*SEC14-1* function as described in Supporting Information (Figure S1). Targeted gene deletion and gene reconstitution were confirmed by Southern blotting as described previously (Chayakulkeeree et al., 2008). Genomic DNA was prepared from H99 WT, Cn*Δsec14-1*, Cn*Δsec14-2*, Cn*Δsfh5*, Cn*Δsec14-1*/Cn*Δsfh5*, Cn*Δsec14-2*/Cn*Δsfh5*, Cn*Δsec14-1*/Cn*SEC14-1* and Cn*Δsec14-1*/Cn*SEC14-2*, digested and probed as described (Figure S2, Table S3 Supporting Information).

Quantitative real time-PCR (qRT-PCR)—RNA from *C. neoformans* strains (H99 and deletion mutants) was prepared, DNaseI treated and used as a template for oligo-dT primed cDNA synthesis as described above. cDNA (5 μl) diluted 1:25, was used as a template for qRT-PCR using SYBR Green Supermix (Invitrogen, CA, USA) and various primer sets; Cn*SEC14-1*: (SEC141-RTF and SEC141-RTR); *CnSEC14-2*: (SEC142-RTF and SEC142-RTR); Cn*SFH5*: (SFH5-RTF and SFH5-RTR) and *ACT1*: (ACT1-RTF and ACT1-RTR), each at a final concentration of 0.1 μM. Primer sequences for qRT-PCR are shown in Table S2, Supporting Information. Using the standard curve method, each specific mRNA was normalized to the housekeeping gene, *ACT1*, using the MxPro 3005p Real-time PCR System (Stratagene, CA, USA). PCR conditions were: 50°C for 2 min followed by 95°C denaturation for 2 min, and 45 cycles consisting of denaturation at 95°C for 15 sec and annealing/extension at 60° C for 45 sec. A melt curve was also performed from 60°C to 95°C with fluorescence monitored at 0.5°C intervals to establish presence of a single PCR product. Data were evaluated with the MxPro software (Stratagene, CA, USA).

Melanin production—*C. neoformans* strains (H99 and deletion mutants) were grown overnight at 30°C in YPD broth and the cells were pelleted by centrifugation, washed twice with sterile PBS and resuspended in PBS at 10^6 cells/5 ul. Aliquots (5 ul) were spotted onto DOPA agar (Missall et al., 2005) and plates were incubated at 30° C for 4 days.

Analysis of CnPlb1 and CnLac1 in cell walls by Western blotting—YNB broth-grown overnight cultures of *C. neoformans* (H99 and deletion mutants) prepared from equal cell numbers (1.5×10^9), were pelleted by centrifugation and the supernatants (representing the secreted CnPlb1 fraction) were dialysed and lyophilized. Cell wall fractions containing CnPlb1 and Laccase were prepared by disruption of the cell pellet using a MiniBeadbeater-8 cell disrupter and zirconia/silica beads, followed by differential centrifugation, as previously described (Siafakas et al., 2006). Briefly, cell lysates were centrifuged for 10 min ($3,500 \times g$) and the supernatants were retained. Cell pellets were further disrupted by probe sonication, and recentrifuged as above. CnPlb1 and CnLac1 were released from the cell wall-enriched pellets by treatment with β-1,3-glucanase. The supernatants prepared from the cell lysates were combined and centrifuged [$135,000 \times g$ (45,000 rpm)] for 1 h at 4°C to obtain the cytosolic fraction (supernatant). CnPlb1 in the cell wall and cytosolic fractions were assessed by western blotting as previously described (Siafakas et al., 2006) using an anti-Plb1 peptide antibody (1 μg/ml). Laccase in the cell wall was detected using an anti-Lac1 antibody (2 μg/ml). Signals were detected by ECL.

Radiometric phospholipase activity assay—CnPlb1 specific activities (lysophospholipase [LPL] and lysophospholipase/transacylase [LPTA]) in lyophilized secretions prepared as described above, were assayed radiometrically as described previously (Chen et al., 2000). A unit of activity was defined as a micromole of the product, DPPC, formed in the LPTA assay or as a micromole of the substrate, LysoPC, degraded in the LPL assay at 37°C and pH 4.0.

Murine inhalation model of cryptococcosis

The procedures described using animals are approved and governed by the Sydney West Area Health Service Animal Ethics Committee, Department of Animal Care. Pathogenicity studies and organ burden analyses were conducted in 6- to 8-week-old BALB/c mice obtained from the Animal Resource Centre, Floreat Park, Western Australia. Animals were anaesthetized by inhalation of methoxyflurane. **Survival study**- Groups of 10 mice (H99 and deletion mutant strains) were inoculated intranasally with each *C. neoformans* strain (0.5×10^6 yeast cells in 50 μl saline) and observed daily for signs of ill-health. As $Cn\Delta sec14$-1 and $Cn\Delta sec14$-1/$Cn\Delta sfh5$ exhibited clumping, one clump was counted as one cell. Mice which had loss 20% of their pre-infection weight, or which showed debilitating clinical signs prior to losing 20% of their pre-infection weight, were euthanized by CO_2 inhalation followed by cervical dislocation. Otherwise they were sacrificed after 34 days. **Organ burden**- Groups of nine mice (H99 and deletion mutant strains) were inoculated intranasally with each *C. neoformans* strain (0.5×10^6 yeast in 50 μl saline). As $Cn\Delta sec14$-1 exhibited clumping, one clump was counted as one cell. Three mice from each group were sacrificed 4, 7, and 14 days after inoculation (Day 0). Lungs and brains were harvested, weighed, homogenised and yeast colony forming units were quantified using SABD spread plates after 2-3 days incubation at 30°C.

Cryptococcus expulsion assay—J774A.1 macrophages were cultured in Dulbeccos's modified Eagles medium (DMEM) with 10% fetal calf serum (Gibco, Invitrogen, Paisley, UK), 50 U/ml penicillin and 50 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. J774 (1×10^5) were plated in 24-well plates 18 h before phagocytosis of cryptococci. J774 cells were activated with PMA for 1 h before cryptococci (opsonised with mouse monoclonal anticapsular antibody, 18B7, provided by Arturo Casadevall) were added in DMEM for 2 h at 37°C. Time lapse images were captured on a Nikon TE2000 with Digital Sight DS-Qi1MC camera, 20× objective (Ph1 PLAN APO), using NIS elements AR software (Nikon, Richmond, UK). Images were captured every 90 sec for 18 h. The microscope stage was enclosed in a temperature controlled and humidified environmental

chamber (Okolabs, NA, Italy) with 5% CO_2 at 37°C. Expulsion of cryptococcal cells was scored as described previously (Ma et al., 2006).

Statistics—Student's *t*-test and one-way ANOVA were used to compare means between 2 groups and more than 2 groups of sample, respectively, where applicable, using SPSS version 16 statistical software. For the murine model of cryptococcosis, an estimation of differences in survival (log-rank test), using the Kaplan-Meier method, was performed and the survival curves plotted using the SPSS version 16 statistical software. In all cases, a P-value <0.05 was considered statistically significant. For the cryptococcal expulsion assay, statistical significance was calculated using Fisher's exact test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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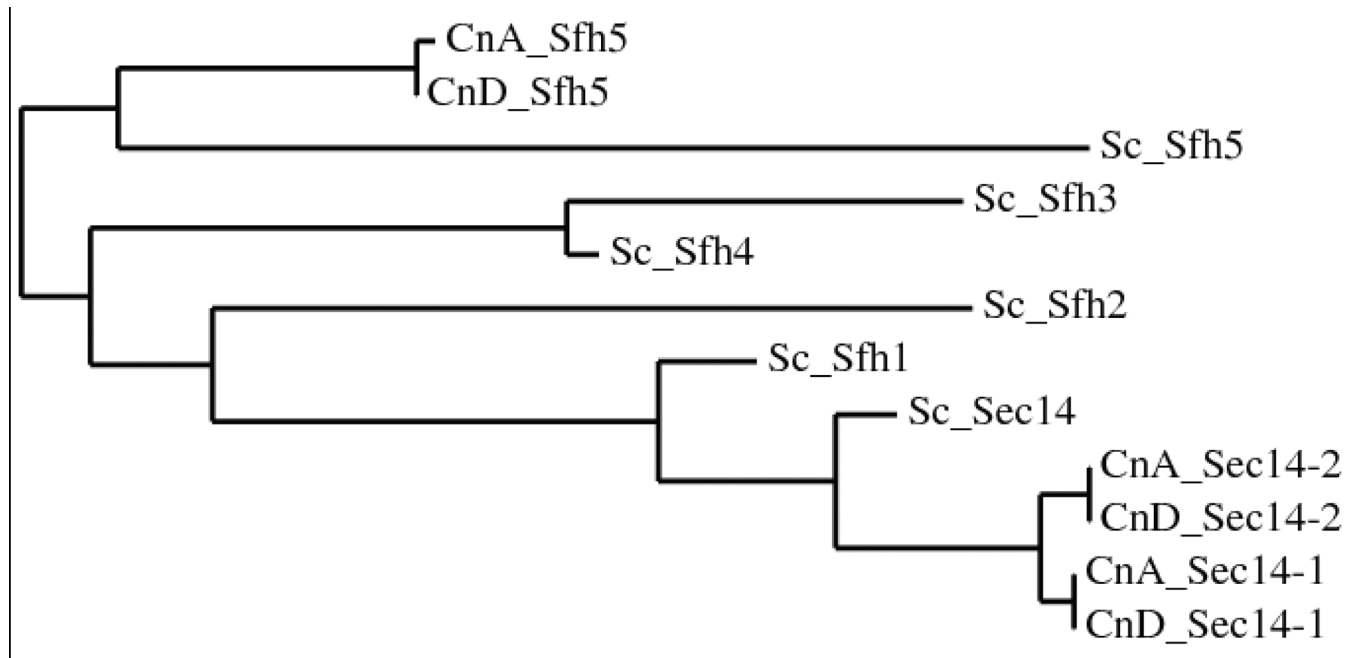
Figure 1. Phylogenetic tree summarizing Sec14p similarities
The tree was created using a Web-based service at
http://www.phylogeny.fr/version2_cgi/advanced.cgi (ClustalW alignment, default curation,
phylogeny and tree rendering). The similarities of *Cryptococcus neoformans* serotype A and
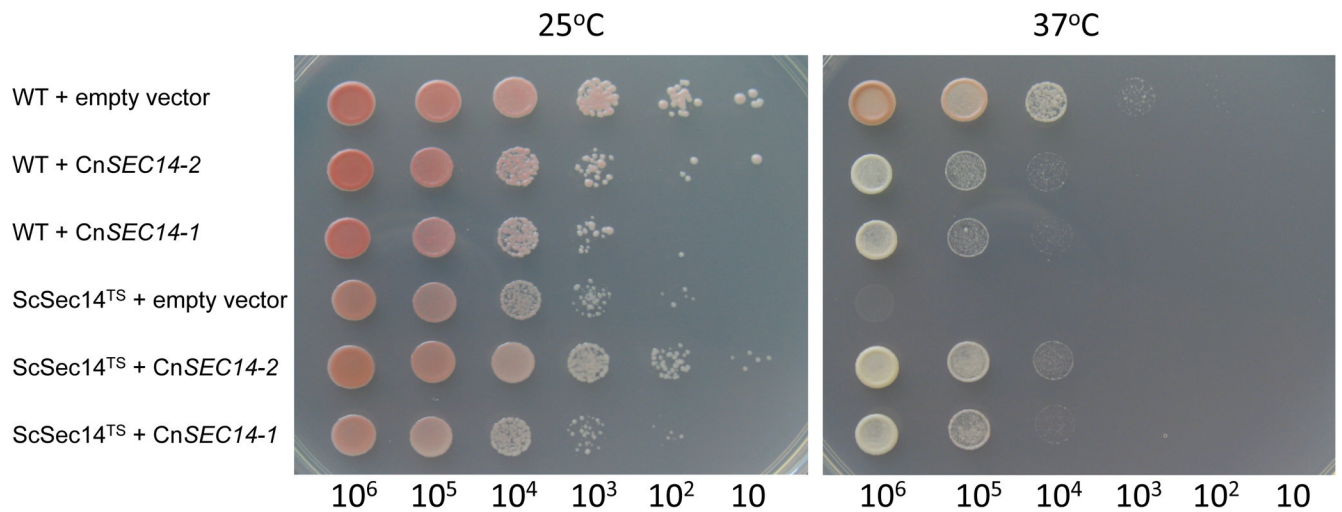D sequences are compared to those of *Saccharomyces cerevisiae*.

Figure 2. Overexpression of Cn*SEC14-1* and Cn*SEC14-2* in an Sc*SEC14^{TS}* mutant restores growth at the restrictive temperature (37 °C)

WT *S. cerevisiae* (M2915-6A) and Sc*SEC14^{TS}* were transformed with pCXJ28 (empty vector) or with pCXJ28 containing Cn*SEC14-1* or Cn*SEC14-2* cDNA. Cn*SEC14* expression was induced by incorporating galactose as the sole carbon source. The growth of serial 10 fold dilutions of each strain was compared at 25°C and 37°C.
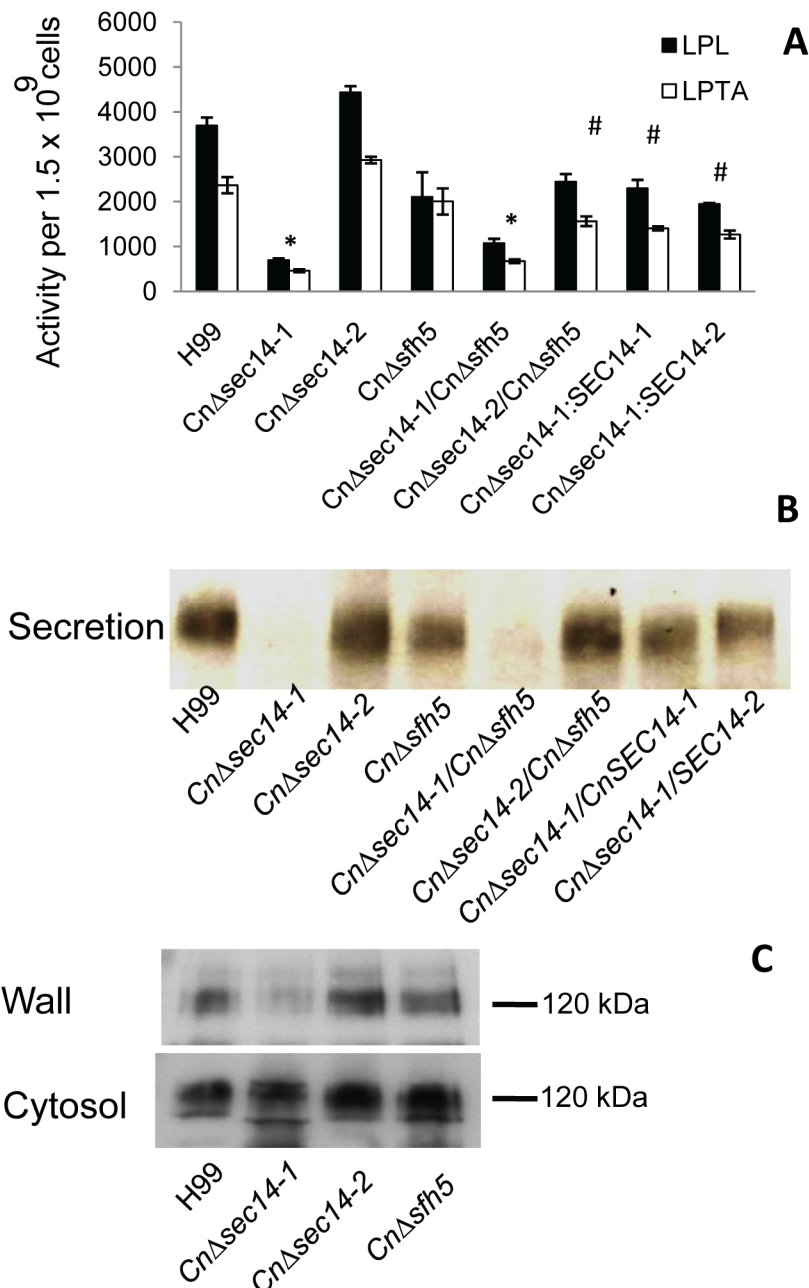
Figure 3. CnPlb1 export to the cell wall, and secretion, in *C. neoformans*, is Cn*SEC14-1* dependent

The supernatants (secretions) from 24 h *C. neoformans* YNB broth cultures were assessed for CnPlb1 activity (LPL and LPTA) by radiometric enzyme assay (A) and CnPlb1 protein by anti-Plb1 western blotting (B). In (A), total LPL/LPTA activity is expressed as Units (nmoles/per min) of substrate hydrolysed/reformed per 1.5×10^9 cells. * indicates statistically significant differences (p<0.05 unpaired T test) in secreted LPL and LPTA activity associated with Cn*Δsec14-1*, relative to WT and the other strains. # indicates statistically significant differences (p<0.05 unpaired T test) in secreted LPL and LPTA activity associated with Cn*Δsec14-2*/Cn*Δsfh5* and the Cn*Δsec14-1* reconstituted strains, relative to WT and Cn*Δsec14-2*. In (B) secretions prepared from 2.5×10^8 cells were

analysed by SDS PAGE and anti-Plb1 western blotting. Total protein secreted by equal cell numbers from each strain are: WT(344ug); CnΔ*sec14-1*(150ug); CnΔ*sec14-1*/CnΔ*sfh5*(160ug); CnΔ*sec14-2*(403ug); CnΔ*sfh5*(314ug); CnΔ*sec14-2*/CnΔ*sfh5* (308ug); CnΔ*sec14-1*/Cn*SEC14-1*(308 ug), CnΔ*sec14-1*/Cn*SEC14-2*(189 ug). (C) For CnΔ*sec14-1*, CnΔ*sec14-2* and CnΔ*sfh5*, CnPlb1 in cell wall and cytosolic fractions prepared from the secreting cells in (A) and (B) was detected by anti-Plb1 western blotting as in (B). The MW of Plb1 125 kDa) was in agreement with the expected MW of glycosylated protein.
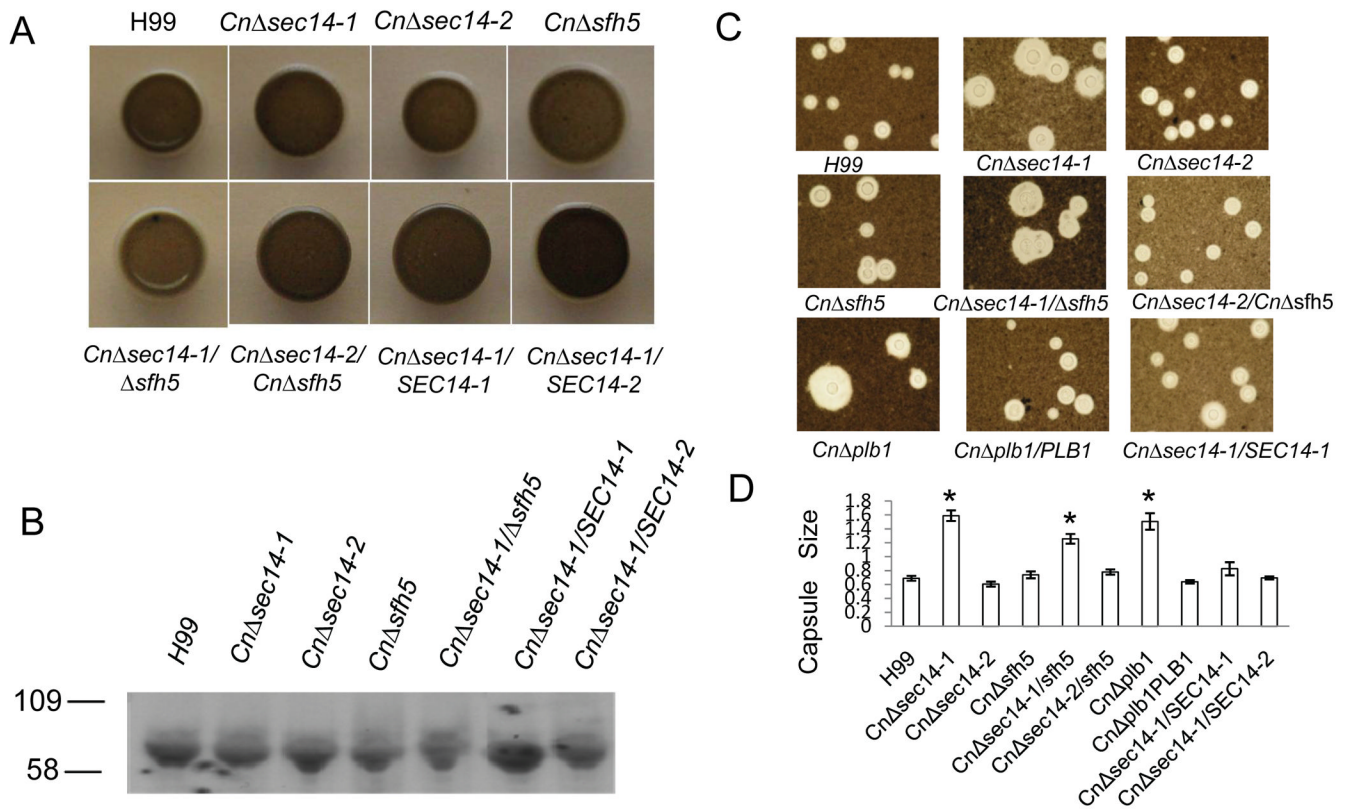
Figure 4. Cn*SEC14-1* is not required for Lac1-induced melanin production or capsule formation in *C. neoformans*

(**A**) H99 WT and mutant strains were grown overnight in YPD broth and 10^6 cells were spotted onto DOPA agar and incubated at 30°C for 3 days to allow melanin pigment production (**B**) Cell wall fractions prepared from equal numbers of cells grown as described in Figure 2 were subjected to western blotting using anti-Lac1 antibodies. Lac1 bands (~75 kDa) were detected by ECL following exposure to hyper film (**C**) Following induction of capsule, cells were stained with India ink and visualized by light microscopy (1000× mag). In (**D**), the mean capsule/cell body size ratio (n=13-34) and standard error are shown. *indicates that strains in which CnPlb1 secretion was absent (Cn*Δplb1*) or attenuated (Cn*Δsec14-1*, Cn*Δsec14-1/sfh5*) had larger capsule/cell body ratios than other mutants and H99 [P<0.05, One way ANOVA]. Due to space restrictions Cn*Δsec14-1/SEC14-2* is not shown in C but, as indicated in D, has a similar ratio to H99.
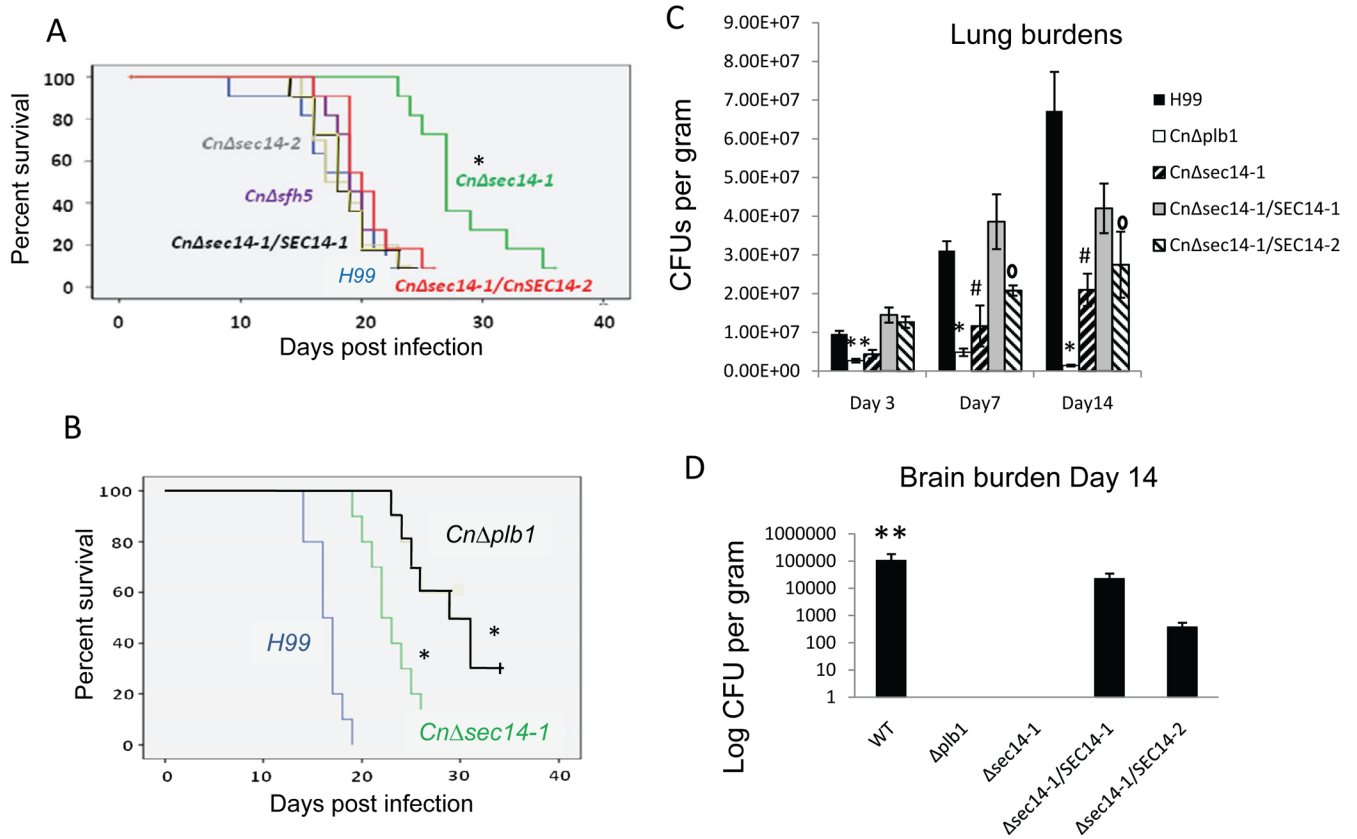
Figure 5. Cn*SEC14-1* is required for virulence

Survival study (A and B) Mice were inoculated at T=0 and euthanized after losing 20% of their starting weight. Mice asymptomatic at 34 days post infection were also euthanized. An estimation of survival differences (log-rank test) using the Kaplan-Meier method was performed where a P-value <0.05 was considered statistically significant. *indicates that the different survival time for that group, relative to all other infection groups, is statistically significant. **Organ burden (C and D)** Groups of 9 mice (n=3 for each group) were inoculated with the indicated strains, as above. Three mice from each group were euthanazed 4, 7, and 14 days post inoculation. Lungs (C) and brains (D) were homogenised and CFUs per gram of tissue determined. No dissemination to the brain was observed on day 3 and 7 for any strain (not shown). By 14 days post-infection, only H99, Cn*Δsec14-1*/Cn*SEC14-1* and Cn*Δsec14-1*/Cn*SEC14-2* had disseminated. * indicates that organ burdens are statistically different to H99 and the reconstituted strain (where relevant), [#] indicates that organ burdens are statistically different to H99, the reconstituted strain and Cn*Δplb1*, ° indicates statistically significant difference to WT and ** indicates a statistically significant difference to both deletion mutants but not to Cn*Δsec14-1*/Cn*SEC14-1* or Cn*Δsec14-1*/Cn*SEC14-2* (statistical significance is indicated by a P value <0.05 using an unpaired T-test and $\log_e$ values).
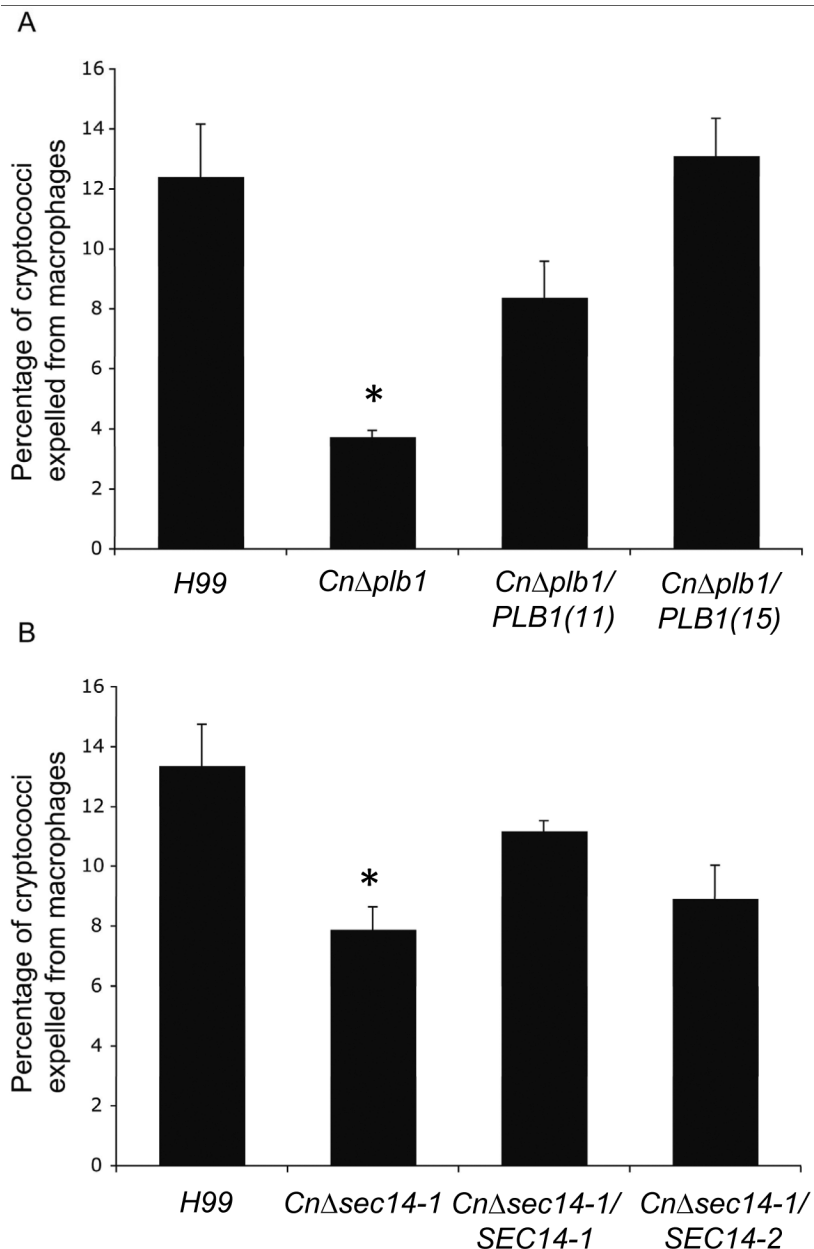
Figure 6. Absent or attenuated CnPlb1 secretion coincides with reduced expulsion of *C. neoformans* from macrophages

The percentage of cryptococci expelled was calculated from counts of expulsion events over 18 h time lapse movies. With both CnΔ*plb1* (A) and CnΔ*sec14-1* (B) the incidence of expulsion was significantly reduced* when compared to the parental H99 strain (P=6×10⁻⁵ and P= 4.6×10⁻³ respectively). Reconstitution of CnΔ*plb1* with Cn*PLB1* [CnΔ*plb1/ PLB1(11)*, CnΔ*plb1/PLB1(15)*] and of CnΔ*sec14-1* with Cn*SEC14-1*, rescued the expulsion phenotype [H99 vs CnΔ*plb1/PLB1(11)* (P= 0.181); H99 vs CnΔ*plb1/PLB1(15)* (P=0.806); CnΔ*plb1* vs CnΔ*plb1/PLB1(11)* (P=0.00851); CnΔ*plb1* vs CnΔ*plb1/PLB1(15)* (P=0.00002), H99 vs CnΔ*sec14-1*/Cn*SEC14-1* (P=0.563); CnΔ*sec14-1* vs CnΔ*sec14-1*/ Cn*SEC14-1* (P=0.167). Each strain was tested at least three times independently. Error bars are SD.
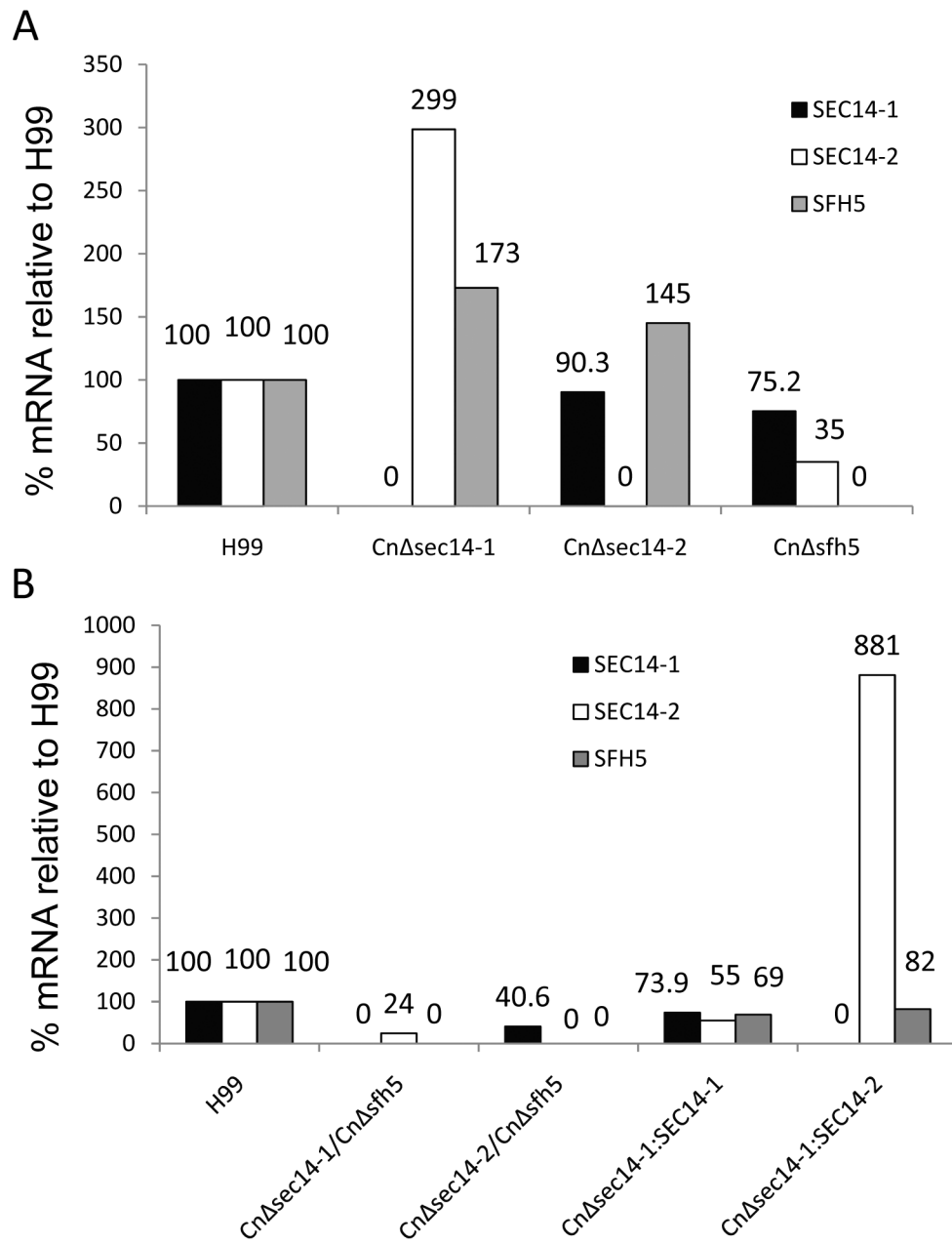
Figure 7. Comparison of Cn*SEC14/SFH* mRNA in H99 and the various deletion mutants by qRTPCR

(A) H99 WT and the single deletion mutants and (B) H99 and the double deletion mutants and reconstituted strains. For each cDNA, a Cn*SEC14/SFH* and actin (*ACT1*) PCR were performed in separate reactions and the amount of each product was determined from a standard curve. The Cn*SEC14/SFH:ACT1* ratio for each strain was expressed as a percentage relative to the Cn*SEC14/SFH:ACT1* ratio in H99WT which was set at 100%. The Y-axis designates the % of mRNA in each mutant, relative to WT. All PCR reactions were performed at least in duplicate. Some strains (CnΔ*sec14-1* and CnΔ*sec14-2*) were analysed 3 times as they were used as controls for the different batches of assays performed and consistently showed no *SEC14-1* and *SEC14-2* mRNA, respectively.