Supporting Information

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

Methee Chayakulkeeree[1,2], Simon Andrew Johnston[3], Johanes Bijosono Oei[1], Sophie Lev[1], Peter Richard Williamson[4] Christabel Frewen Wilson[1], Xiaoming Zuo[1], Ana Lusia Leal[5,6], Marilene Henning Vainstein[6], Wieland Meyer[5], Tania Christine Sorrell[1,5], Robin Charles May[3], Julianne Teresa Djordjevic[1*]

[1]Fungal Pathogenesis Laboratory, Centre for Infectious Diseases and Microbiology, Sydney Medical School-Western, Westmead Millennium Institute, University of Sydney at Westmead Hospital, Westmead NSW, Australia; [2]Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand; [3]School of Biosciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT United Kingdom. [4]Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, NIH Bethesda, MD, USA; [5]Molecular Mycology Research Laboratory, Centre for Infectious Diseases and Microbiology, Sydney Medical School-Western, Westmead Millennium Institute, University of Sydney at Westmead Hospital, Westmead NSW, Australia; [6]Centro de Biotecnologia-UFRGS Laboratório de Biologia de Fungos de Importância Médica e Biotecnológica Av. Bento Gonçalves, 9.500-Prédio 43.421-Lab.220 Bairro Agronomia CEP:91501-970 Porto Alegre/RS;

*Corresponding Author: Julianne T. Djordjevic: Telephone: +61 2 9485 7046 Fax: +61 2 9891 5317 Email: julie_djordjevic@wmi.usyd.edu.au

Running Title: CnPlb1 Secretion and Pathogenicity are *SEC14*-dependent

Strategy for targeted Cn*SEC14* gene deletion and reconstitution

Targeted gene deletion The plasmids pJAF and pCH233 were used as a template for PCR amplification of the 1.8-kb Neo^r cassette (*ACT1* promoter:neomycin phosphotransferase-encoding gene:*TRP1* terminator) and 1.6-kb Nat^r cassette (*ACT1* promoter:nourseothricin acetyltransferase-encoding gene:*TRP1* terminator) respectively, using primers NEOF and NEOR. Primer names and sequences are presented in Table S1. For Cn*SEC14-1* gene disruption, a 669-bp and a 725-bp fragment comprising the 5' and 3' untranslated regions, respectively, were amplified from genomic DNA using primer pairs SEC141-1F/SEC141-2R and SEC141-5F/SEC141-6R, respectively. For Cn*SEC14-2* disruption, a 554-bp and a 627-bp fragment comprising the 5' and 3' untranslated regions, respectively, were amplified with primer pairs SEC142-1F/SEC142-2R and SEC142-5F/SEC142-6R, respectively. For Cn*SFH5* disruption, a 587-bp and a 666-bp fragment comprising the 5' and 3' untranslated regions, respectively, were amplified with primer pairs SFH5-1F/SFH5-2R and SFH5-5F/SFH5-6R, respectively. Each PCR product was gel-purified using a QIAquick Gel Extraction Kit (Qiagen Pty. Ltd., VIC, Australia). For each gene disruption, purified PCR products corresponding to a 5' untranslated region, the Neo^r or Nat^r cassette and the 3' untranslated region, all with overlapping ends, were combined and used as a template for overlap PCR using primers SEC141-1F/SEC141-6R, SEC142-1F/SEC142-6R, SFH5-1F/SFH5-6R for Cn*SEC14-1*, Cn*SEC14-2* and Cn*SFH5* disruption, respectively (Figure S1A). The hybrid DNA constructs, each approximately 3 kb, were gel-purified and used to transform strain H99 using a biolistic delivery protocol, and stable neo or nat resistant recombinants, created by homologous integration of the hybrid gene into the genome, were selected on YPD agar containing geneticin (G418) or nourseothricin, respectively. To confirm that the hybrid gene had replaced the native gene by homologous recombination, PCR was performed using flanking primers (1 and 2) which annealed upstream (5') and

downstream (3') of the integration site of the hybrid construct (Figure S1A). The size of the PCR product was used to distinguish deletion mutants from WT. The absence of ectopic integrations of each hybrid gene within the genome was confirmed by Southern hybridization (Figure S2). ***Gene reconstitution*** The Cn*SEC14-1* and Cn*SEC14-2* genes, including approximately 600-bp of the promoter (upstream) and 300-bp of the terminator (downstream), were amplified by PCR from H99 genomic DNA using primers SEC141-recF/SEC141-recR and SEC142-recF/SEC142-recR, respectively. The Neo^r cassette was amplified using primers NEOF and NEOR. The PCR-amplified Cn*SEC14-1* or Cn*SEC14-2* fragment and the Neo^r fragment were excised from an agarose gel and co-gel-purified using a QIAquick Gel Extraction Kit (Qiagen Pty. Ltd., VIC, Australia). Two-fragment overlap PCR was achieved using primers NEOF/SEC141-recR and NEOF/SEC142-recR for Cn*SEC14-1* and Cn*SEC14-2* reconstitution, respectively (Figure S1B). The two resulting hybrid fragments were transformed into Cn*Δsec14-1* separately using biolistic delivery as above and transformants were selected on geneticin (G418).

Confirmation of targeted Cn*SEC14* gene disruption and reconstitution by Southern blot

To confirm that homologous recombination had occurred at the correct site, a set of primers was designed to PCR amplify across the disrupted region to determine the difference in size between the fragment from the WT and each deletion mutant (results not shown). Targeted gene deletion at only the correct site and the ectopic integrations of Cn*SEC14-1* or Cn*SEC14-2* into the chromosome of CnΔ*sec14-1* was confirmed by Southern blotting. The reconstituted strains were also confirmed by PCR using NEOF/SEC141-recR and NEOF/SEC142-recR primer pairs, respectively (not shown).

Table S1 Oligonucleotides used for gene deletion procedures and Cn*SEC14-1* expression

Oligonucleotide	Type	Sequence	Reference or source
ScSEC-F	Forward	5’-ATGAGCGCCTCTGACCCTCT-3’	This study
ScSEC-R	Reverse	5’-TTAAACAGCCGTCGCGGTAT -3’	This study
NEOF	Forward	5’-CATGCAGGATTCGAGTGGCATG-3’	(Chayakulkeeree et al., 2008)
NEOR	Reverse	5’-GGAGCCATGAAGATCCTGAGGA-3’	(Chayakulkeeree et al., 2008)
SEC141-1F	Forward	5’-ACCCTGTGAACGGACTTGTT-3’	This study
SEC141-2R	Reverse	5’-CATGCCACTCGAATCCTGCATGGCCATTGCGGATATACCTTG-3’	This study
SEC141-5F	Forward	5’-TCCTCAGGATCTTCATGGCTCCGTAAATCCTACAAGGCCGAGTT-3’	This study
SEC141-6R	Reverse	5’-TTCCGCTTTTCTCAGAGCAT-3’	This study
SEC141-ins5F	Forward	5’-GGTGCTTGACCATAATTCTGTTG-3’	This study
SEC141-ins3R	Reverse	5’-GAGGAGAGCAGTTTGGACGA-3’	This study
SEC141-recF	Forward	5’-TCCTCAGGATCTTCATGGCTCCATAATGGGGGTGTTGCTGAG-3’	This study
SEC141-recR	Reverse	5’-CCTTTCCCAATGACAAATCC-3’	This study
SEC142-1F	Forward	5’-CGACAGTTGTACGGTCTGGA-3’	This study
SEC142-2R	Reverse	5’-CATGCCACTCGAATCCTGCATGCTCATTAAACCCTGGCTGGA-3’	This study
SEC142-5F	Forward	5’-TCCTCAGGATCTTCATGGCTCCGCCTGTGGTGGGTAAACAAT-3’	This study
SEC142-6R	Reverse	5’-TGCGGTCAGCTCCTCTTAAT-3’	This study
SEC142-ins5F	Forward	5’-GGAAAACCCCAAAACGAAGT-3’	This study
SEC142-ins3R	Reverse	5’-TTCTTCGGCTCTCCATGTCT-3’	This study
SEC142-recF	Forward	5’-TCCTCAGGATCTTCATGGCTCCAAAGCACAGACCATTTGGCTA-3’	This study
SEC142-recR	Reverse	5’-CGACAGTTGTACGGTCTGGA-3’	This study
SFH5-1F	Forward	5’-CTAGCAGCAACTGCCCAATC-3’	This study
SFH5-2R	Reverse	5’-CATGCCACTCGAATCCTGCATGAGGATGCTTCGACGACAGAC-3'	This study
SFH5-5F	Forward	5’-TCCTCAGGATCTTCATGGCTCCAGGGAAGGACCCAGTGACTT-3’	This study
SFH5-6R	Reverse	5’-CCAGGCAAAAGGAACGACTA-3’	This study
SFH5-ins5F	Forward	5’-GGCGAAATCATTGATGCCTA-3’	This study
SFH5-ins3R	Reverse	5’-CTTGGCAGGTGAGTGTCATC-3’	This study

Table S2 Oligonucleotides used for semiquantitative RT-PCR

Oligonucleotide	Type	Sequence	Reference or source
ACT1-RTF	Forward	5’-ATGGTATTGCCGACCGTATG-3’	(Chayakulkeeree *et al.*, 2008)
ACT1-RTR	Reverse	5’-CTCTTCGCGATCCACATCTG-3’	(Chayakulkeeree *et al.*, 2008)
SEC141-RTF	Forward	5’-CTGTTCCGAGGAGGTTGGAC-3’	This study
SEC141-RTR	Reverse	5’-TGATGATGAACATGTGGCCC-3’	This study
SEC142-RTF	Forward	5’-CCGAGCTCGACAGTCTAATGC-3’	This study
SEC142-RTR	Reverse	5’-TCGCGGTATCCCCCAAAT-3'	This study
SFH5-RTF	Forward	5’-GGATCTTGACCGGTTCCTTC-3’	This study
SFH5-RTR	Reverse	5’- GGCCGCCATAAGCTCAATAG-3’	This study

Table S3 Summary of expected fragment sizes detected by Southern blot

Strain	Probe	Enzyme	Expected Size (bp)
WT (H99)	5’ SEC14-1	*Tsp*RI	1326
Cn*Δsec14-1* (replaced with Nat^r)			1076
Cn*Δsec14-1* (replaced with Nat^r)/Cn*SEC14-1* (with Neo^r)			1076 and 1411
WT (H99)	5’ SEC14-2	*Hha*I	1471
Cn*Δsec14-2* (replaced with Neo^r)			1258
Cn*Δsec14-1* (replaced with Nat^r)/*CnSEC14-2* (with Neo^r)			1471 and 935
WT (H99)	5’ SFH5	*Apo*I	794
Cn*Δsfh5* (replaced with Neo^r)			2283
Cn*Δsec14-1* (replaced with Nat^r)/ Cn*Δsfh5* (replaced with Neo^r)			2283
Cn*Δsec14-2* (replaced with Neo^r)/ Cn*Δsfh5* (replaced with Nat^r)			1982

Figure S1. Constructs for Cn*SEC14* gene deletion and reconstitution

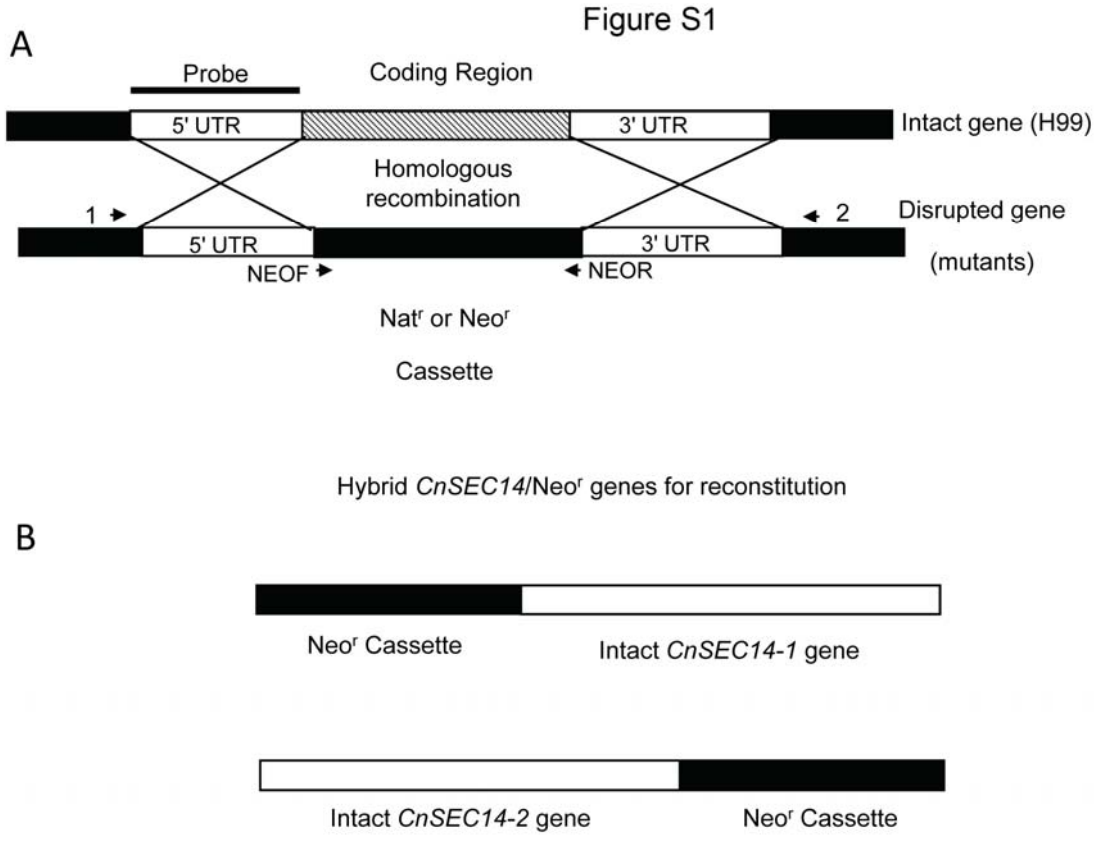
(A) *Gene deletion*- The noursiothricin (Nat^r) or neomycin (Neo^r) cassette, and approximately 600 bp of each of the 5' and 3' untranslated regions (UTR) of Cn*SEC14-1,* Cn*SEC14-2* or Cn*SFH5,* were PCR amplified in separate reactions using primer sets NEOF/NEOR (for Nat^r or Neo^r fragment), SEC141-1F/SEC141-2R (for 5' Cn*SEC14-1*) and SEC141-5F/SEC141-6R (for 3' Cn*SEC14-1*) , SEC142-1F/SEC142-2R (for 5' Cn*SEC14-2*) and SEC142-5F/SEC142-6R (for 3' Cn*SEC14-2*), SFH5-1F/SFH5-2R (for 5' Cn*SFH5*) and SFH5-5F/SFH5-6R (for 3' Cn*SFH5*) (Table S1). The downstream primer for amplification of the 5' UTRs and the upstream primer for amplification of the 3' UTRs contained overhanging sequences homologous to the adjacent Neo^r. The three PCR products, which contained overlapping ends, were "stitched" together in a final PCR and the hybrid gene created, was introduced into the H99 genome using biolistic transformation. For the single gene deletions, Nat^r was used to replace the open reading frame of Cn*SEC14-1* and Neo^r was used to replace the open reading frame of Cn*SEC14-2* and Cn*SFH5*. For the double deletion mutants, *CnSFH5* was disrupted within Cn*Δsec14-1* and Cn*Δsec14-2* using Neo^r and Nat^r, respectively. To confirm homologous recombination, genomic DNA prepared from either the WT or the mutants was used as a template for PCR amplification using primers 1 and 2. Primer 1 is designated SEC141-ins5F, SEC142-ins5F or SFH5-ins5F and primer 2 is designated SEC141-ins3R, SEC142-ins3R or SFH5-ins3R, as the sequence shown in Table S1. **(B)** *Gene reconstitution*- Overlap PCR was also used to attach Neo^r to intact Cn*SEC14-1* and Cn*SEC14-2* and each hybrid DNA was used to genetically complement the Cn*Δsec14-1* mutant using biolistic transformation.The 0.6 kb region to which the probe was directed for confirmation of targeted gene deletion/reconstitution by Southern blot (S2) is underlined.

Figure S2. Confirmation of targeted Cn*SEC14* gene disruption and reconstitution by Southern blot The 0.6 kb 5' untranslated regions of Cn*SEC14-1*, Cn*SEC14-2*, and Cn*SFH5 (See S1)* were PCR-amplified using primers SEC141-1F/ SEC141-2R, SEC142-1F/ SEC142-2R, and SFH5-1F/SFH5-2R, respectively, and used as a probe for Southern blotting. Genomic DNA extracted from H99 WT and all the deletion mutants was digested with *Tsp*RI, *Hha*I or *Apo*I and subjected to Southern blotting with the correct probe. The expected fragment sizes of DNA detected by Southern blot for each strain are listed in Table S3. WT, Wild type; S1, Cn*Δsec14-1*; S2, C*nΔsec14-2*; S5, Cn*Δsfh5*; S1S5, Cn*Δsec14-1/*Cn*Δsfh5*; S2S5, Cn*Δsec14-2/*Cn*Δsfh5*; S1R1, Cn*Δsec14-1/*Cn*SEC14-1*; S1R2, Cn*Δsec14-1/*Cn*SEC14-2*.

Figure S3. Effect of temperature SDS and calcofluor white on *C. neoformans* growth (A) Cells were grown in YPD medium overnight and cell morphology was visualized under the light microscope. Cell clumping was demonstrated in CnΔ*sec14-1* and CnΔ*sec14-1/*CnΔ*sfh5*. (B and C) Overnight YPD broth cultures of *C. neoformans* strains were pelleted, washed twice with PBS and resuspended in PBS at a concentration of 10^6 cells/ 5 µl. Serial 10-fold dilutions (10^6 to 10^1 cells /5 µl) were prepared and 5 µl of each were spotted onto YPD or YNB agar with/without 0.05% SDS or calcofluor white (as indicated). Macroscopic growth was recorded after incubation at 30º C or 37º C (as indicated) for 3-5 days. Only Cn*Δsec14-1* and Cn*Δsec14-1/*Cn*Δsfh5* exhibit retarded growth in the presence of SDS and calcofluor white. CnΔ*sec14-1* phenotypes were restored or partially restored to WT by genetic reconstitution with either Cn*SEC14-1* or Cn*SEC14-2* respectively.

Figure S4 Creation of an *iSEC14-1* RNAi strain in Cn*Δsec14-2* (*iSEC14-1*) and determination of Cn*SEC14-1* RNA suppression in *iSEC14-1*

(A) To create the *iSEC14-1* strain, a fragment of Cn*SEC14-1* cDNA (approximately 500 bp) was cloned into the KUTAP-2 expression vector twice, but in opposite orientations, and linked by the *LAC1* intron I. In brief, the sense strand was connected to the GPD promoter by an *Eco*RI site and to the *LAC1* Intron I by a *Xho*I site. The antisense strand was connected to the *LAC1* intron I and the KUTAB-2 vector by a *Xho*I and an *EcoR*I site, respectively. For the control strain (iControl), only one correctly-orientated 500-bp Cn*SEC14-1* cDNA fragment was cloned into the KUTAB-2 vector using *Eco*RI sites. Both *iSEC14-1* and iControl vectors were transformed into Cn*Δsec14-2*/FOA (uracil auxotroph) and transformants were selected on uracil deficient medium. **(B)** The Cn*SEC14-1* mRNA level in both sets of transformants was determined by qRT-PCR using primers shown in Table S2.



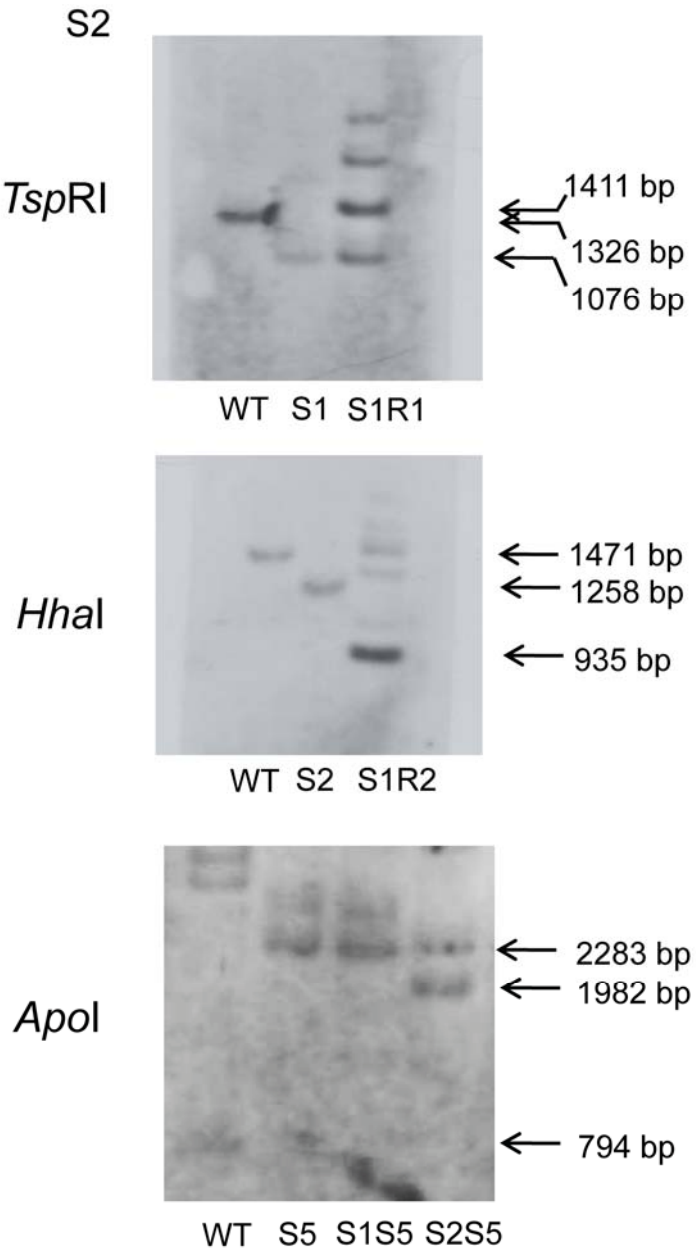
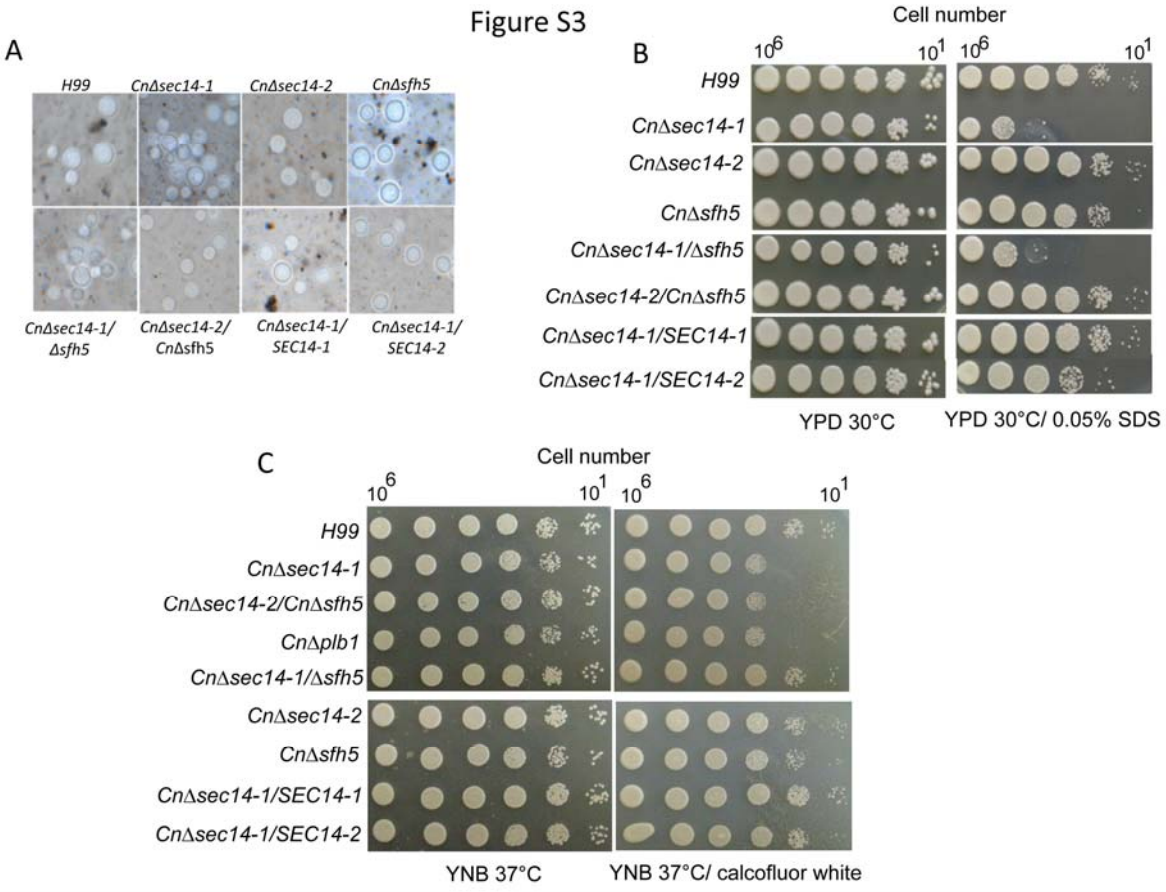
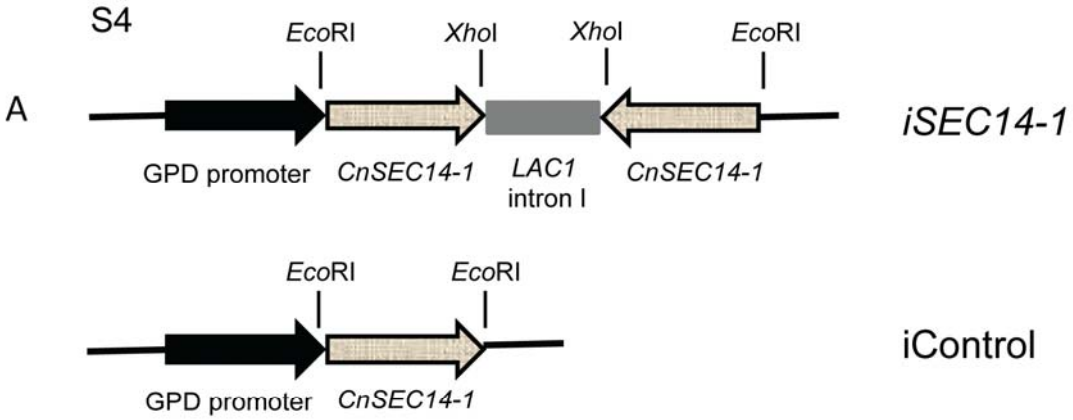
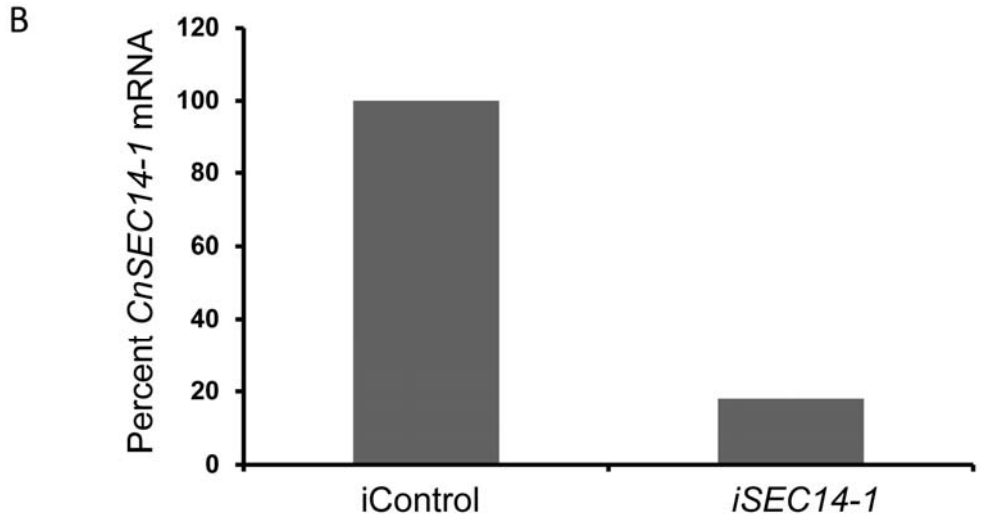

S2
TspRI
1411 bp
1326 bp
1076 bp
WT S1 S1R1
HhaI
1471 bp
1258 bp
935 bp
WT S2 S1R2
ApoI
2283 bp
1982 bp
794 bp
WT S5 S1S5 S2S5

Figure S3


A
H99
CnΔsec14-1
CnΔsec14-2
CnΔsfh5
CnΔsec14-1/ Δsfh5
CnΔsec14-2/ CnΔsfh5
CnΔsec14-1/ SEC14-1
CnΔsec14-1/ SEC14-2
B
Cell number
10^6
10^1
H99
CnΔsec14-1
CnΔsec14-2
CnΔsfh5
CnΔsec14-1/Δsfh5
CnΔsec14-2/CnΔsfh5
CnΔsec14-1/SEC14-1
CnΔsec14-1/SEC14-2
YPD 30°C
YPD 30°C/ 0.05% SDS
C
Cell number
H99
CnΔsec14-1
CnΔsec14-2/CnΔsfh5
CnΔplb1
CnΔsec14-1/Δsfh5
CnΔsec14-2
CnΔsfh5
CnΔsec14-1/SEC14-1
CnΔsec14-1/SEC14-2
YNB 37°C
YNB 37°C/ calcofluor white

S4


A
EcoRI
XhoI
XhoI
EcoRI
GPD promoter
CnSEC14-1
LAC1 intron I
CnSEC14-1
iSEC14-1
EcoRI
EcoRI
GPD promoter
CnSEC14-1
iControl


B
Percent CnSEC14-1 mRNA
120
100
80
60
40
20
0
iControl
iSEC14-1