Inventory of Supplementary Materials

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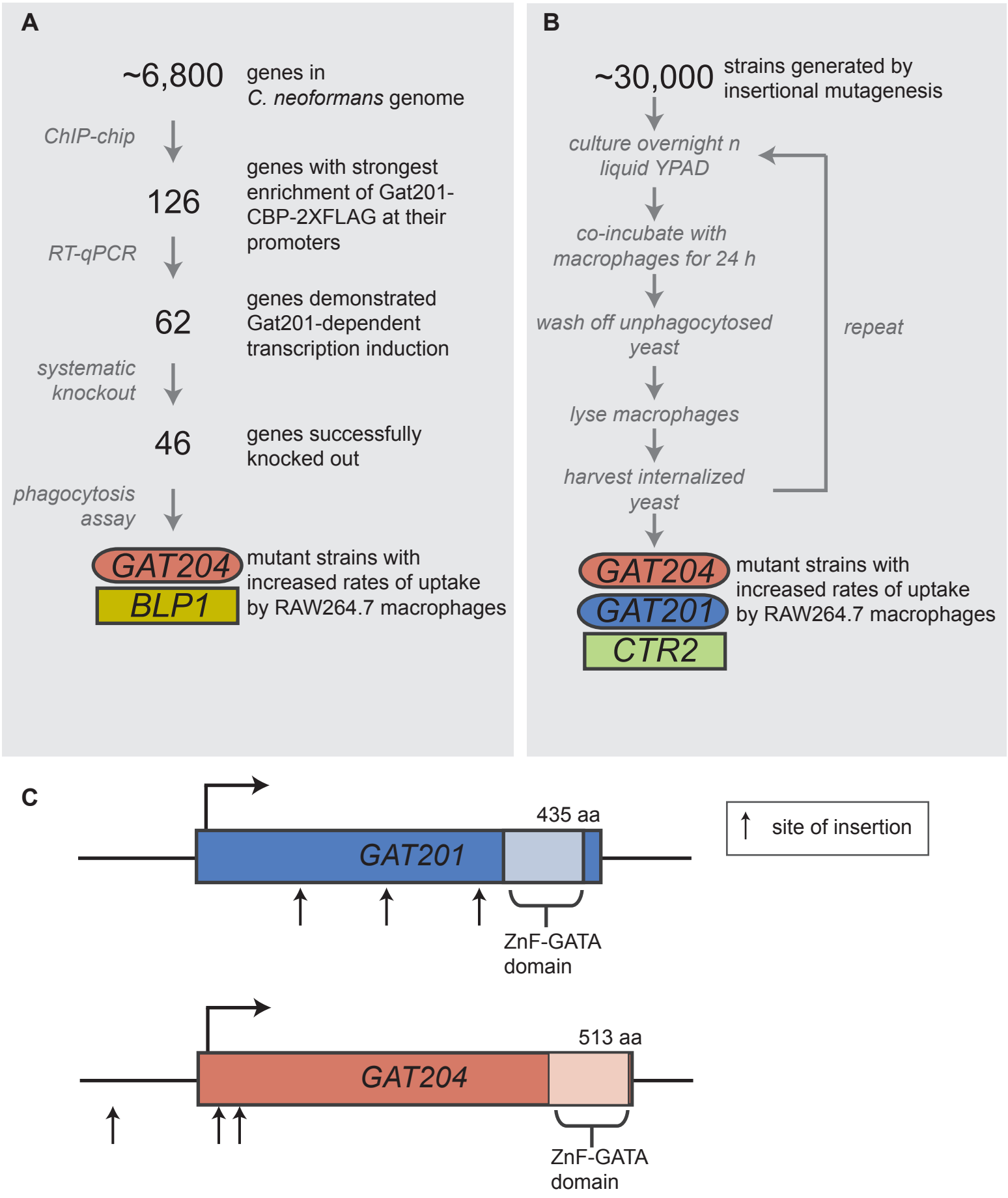
Supplementary Experimental Procedures

Supplemental References

Supplemental Figure 1



Supplemental Figure 2

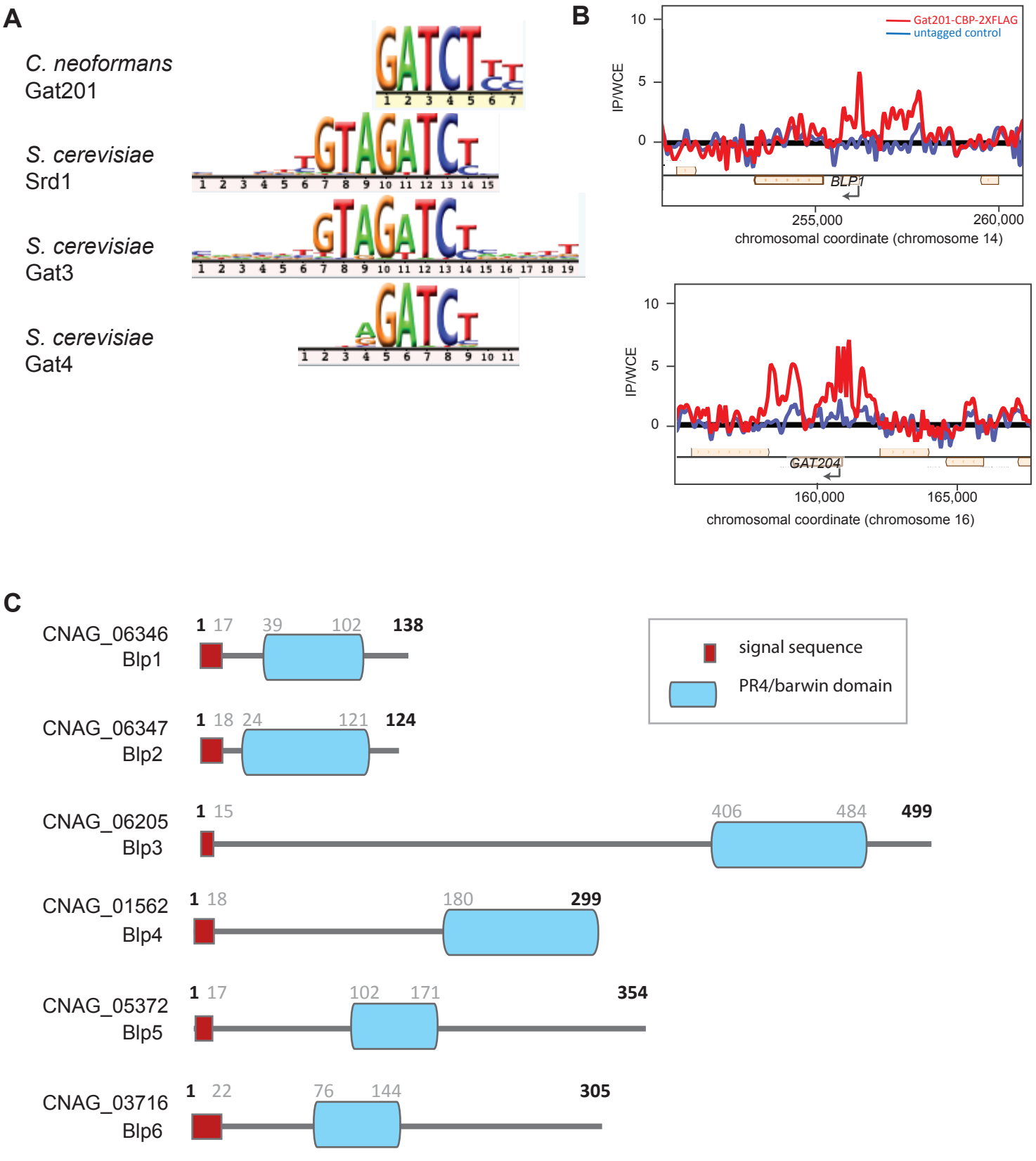


Figure S3

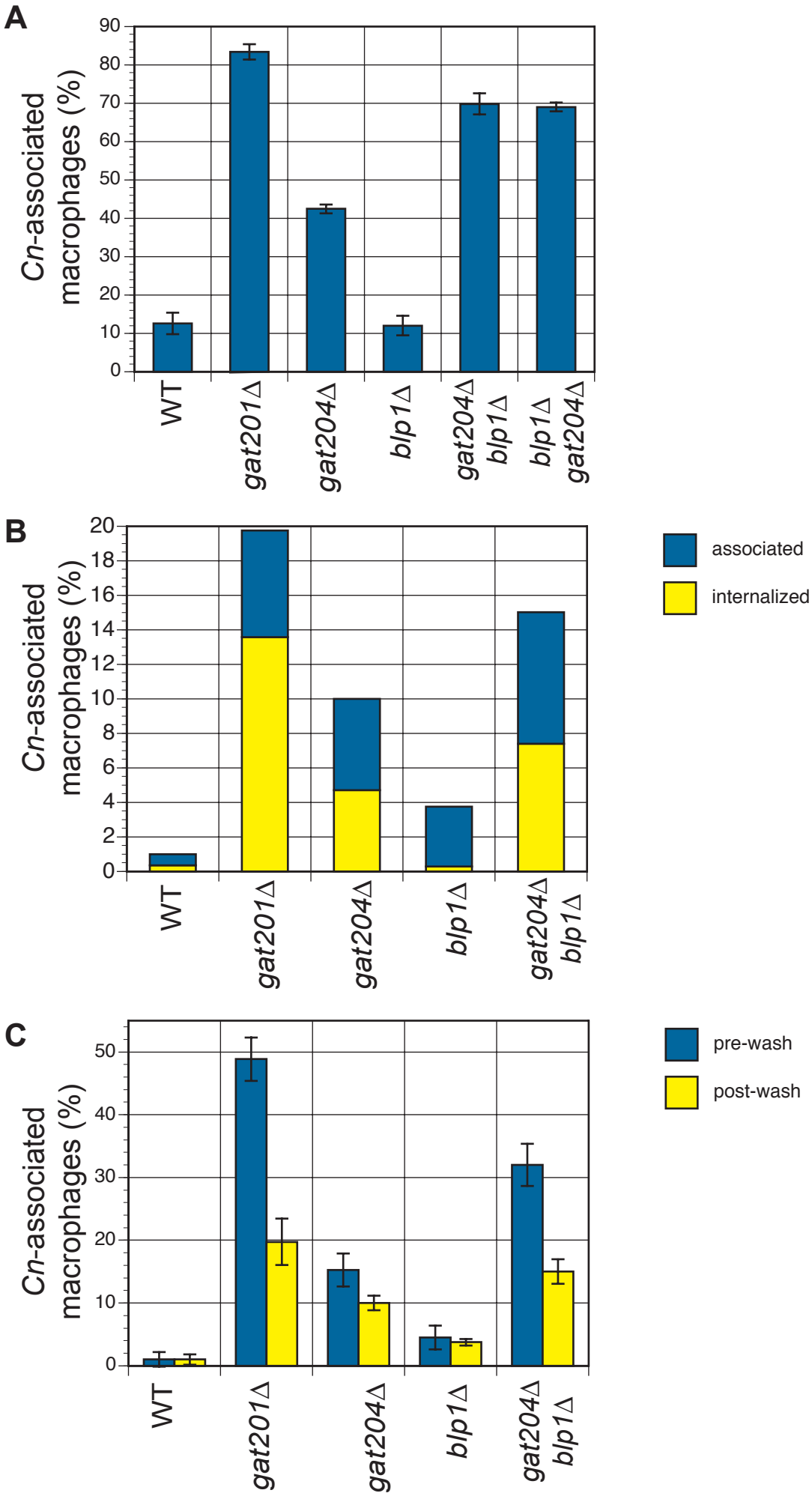

A
Cn-associated macrophages (%)
0
10
20
30
40
50
60
70
80
90
WT
gat201Δ
gat204Δ
blp1Δ
gat204Δ blp1Δ
blp1Δ gat204Δ
B
Cn-associated macrophages (%)
0
2
4
6
8
10
12
14
16
18
20
WT
gat201Δ
gat204Δ
blp1Δ
gat204Δ blp1Δ
associated
internalized
C
Cn-associated macrophages (%)
0
10
20
30
40
50
WT
gat201Δ
gat204Δ
blp1Δ
gat204Δ blp1Δ
pre-wash
post-wash

Figure S1: Two redundant approaches to identify genes required for anti-phagocytosis

(A) Approach 1. 126 genes with the strongest enrichment of Gat201-CBP-2XFLAG in their promoter regions were assayed by RT-qPCR in WT and *gat201*Δ strains. 62 genes showed Gat201-dependent transcription induction. Of these, 46 genes were knocked out and screened in a phagocytosis assay. Two strains, mutated in *GAT204* and *BLP1*, showed increased uptake by macrophages.

(B) Approach 2. Approximately 30,000 mutant *C. neoformans* strains were generated by insertional mutagenesis. These strains were co-incubated with macrophages or 24 hours prior to removal of unphagocytosed yeast by PBS washes. The internalized yeast strains were harvested from lysed macrophages and re-assayed by phagocytosis assay. Mutations in three genes were identified: *GAT204*, *GAT201* and *CTR2*.

(C) Sites of insertion of NAT^R cassette into the genes *GAT201* and *GAT204* in mutant strains identified in the screen described in (B).

Figure S2: Features of Gat201 targets

(A) A distinct motif is enriched in the promoters of Gat201-bound genes. A motif-finding algorithm in the MochiView package identified a motif enriched in the promoters of Gat201-bound genes when compared to the overall sequence of the genome. The motif shows striking similarity to already characterized binding sequences of *S. cerevisiae* GATA-family transcription factors Srd1, Gat3 and Gat4 (Badis et al., 2008).

(B) Gat201-CBP-2xFLAG binds in the promoter regions of *BLP1* and *GAT204*. Ratio of immunoprecipitated DNA (IP) to whole cell extract (WCE) plotted for each probe of the two tiling arrays at the chromosomal coordinates indicated.

(C) Schematic of Blp1 and its five family members. Blp1 is a 138 amino acid protein predicted by SMART protein analysis to have a Barwin-like domain (residues 39-102).

SignalP analysis predicts a signal sequence in the first 17 residues with a probability of 1.000. Blp1 is a member of a six-protein family, all of which contain a Barwin-like domain and a signal sequence (as predicted by SignalP). We have named these genes *BLP2-6*. *BLP1* is the only gene in this family whose mRNA is induced by Gat201, as determined by RT-qPCR (C.D.C and H.D.M., unpublished observations).

Figure S3: Additional phagocytosis experiments

(A) Quantification of phagocytosis defects. Bone marrow-derived macrophages from C57/Bl6 mice (a gift from P. Manzanillo and J. Cox) were co-incubated with the designated *C. neoformans* strains for 24 hours, then washed three times with PBS to remove unphagocytosed yeast. The macrophages were then counted to assay the number of macrophages with yeast associated. At least 100 macrophages were counted per well and each strain was assayed in triplicate.

(B) Differential staining of *C. neoformans* to distinguish yeast internalized by macrophages from those only associated externally. Phagocytosis assays were performed as described above using RAW264.7 cells. Unassociated yeast cells were removed by washing three times in PBS. Samples were then fixed in 4% paraformaldehyde, washed, then yeast cells labeled with anti-GXM and anti-β-glucan antibodies, followed by a FITC-conjugated secondary antibody, to label accessible (externally-associated) yeast cells. Samples were washing again, the macrophages permeabilized in PBS containing 0.5% SDS, and then treated again with anti-GXM antibody and TRITC-conjugated secondary antibody (to label yeast internalized by macrophages). The overage percentage of macrophages with associated yeast were determined, then the percentage of those macrophages that had either only externally associated yeast (FITC-stained; yellow bars) or contained at least one yeast cell that was completely internalized (TRITC-staining only, blue bars; note: most macrophages

with associated yeast cells are associated with multiple yeast cells). At least 100 macrophages were counted per well and each strain assayed in triplicate when determining the overall percentage of macrophages with associated yeast. A minimum of 30 (*gat201*Δ, *gat204*Δ, and *gat204*Δ*blp1*Δ strains) or 10 (wild type and *blp1*Δ strains) macrophages with associated yeast were analyzed per well to determine percent macrophages with internalized yeast versus percent with only externally-associated yeast. *gat201*Δ, *gat204*Δ, and *gat204*Δ*blp1*Δ cells all show an increase in the percentage of macrophages with internalized yeast cells, demonstrating that Gat204 and Gat204 and Blp1 together block phagocytosis of *C. neoformans* by macrophages and not just external association with macrophages.

(C) Percent of macrophages with associated yeast was determined after fixation in paraformaldehyde (pre-wash, blue bars) and after the staining and washing procedure described in part A (post-wash, yellow bars). Both macrophages and yeast cells are lost during the numerous washes of the staining procedure, but the trends remain the same. Specifically, *gat201*Δ cells show the greatest percentage of macrophages with associated yeast both pre- and post-washing and *gat204*Δ*blp1*Δ shows a significant increase in yeast-associated macrophages both pre- and post-washing compared to *gat204*Δ (Student's t-test, $p = 3.9 \times 10^{-3}$).

SUPPLEMENTAL PROCEDURES

Strains and media

C. neoformans was routinely grown in yeast culture conditions (on YPAD medium [1% yeast extract, 2% Bacto-peptone, 2% glucose, 0.015% L-tryptophan, 0.004% adenine] at 30ºC) or in tissue culture conditions (in Dulbelco's Modified Eagle Medium [DMEM] with 4.5 g/L glucose at 37ºC with 5% CO_2). All strains were constructed

by biolistic transformation into the serotype A strain H99. The construction of strains *gat201*Δ-1 and *gat201*Δ-2 was previously described (Liu et al., 2008). Gat201-CBP-2X FLAG was generated by the insertion of the epitope CBP-2XFLAG at the 3' end of the endogenous *GAT201* gene, marked with a NAT resistance cassette at the 3' end of the transformation construct. Phagocytosis measurements demonstrated that this tag did not block the function of Gat201 (C.D.C. and H.D.M., unpublished observations). *gat204*Δ was constructed by disrupting the GATA-domain of *CNAG_06762* with a NAT resistance cassette. *blp1*Δ was constructed by disrupting the entire coding sequence of *CNAG_06346* with a NAT resistance cassette. *blp1*Δ*gat204*Δ was constructed by disrupting the entire coding sequence of *CNAG_06346* with a NEO resistance cassette in the *gat204*Δ strain. *gat204*Δ *blp1*Δ was constructed by disrupting the entire coding sequence of *CNAG_06762* with a NEO resistance cassette in the *blp1*Δ strain. Strains used in this study are listed in Table 1.

Phagocytois assays

RAW264.7 macrophages ($2x10^4$ cells/well) were seeded into 96-well tissue-culture treated plates in DMEM medium and allowed to adhere overnight. *C. neoformans* cells grown in YPAD medium were washed three times with PBS then resuspended to a density of $5x10^6$ cells/ml in PBS, and 10 μl ($5x10^4$ cells) were co-incubated with the RAW264.7 macrophages in 200 μl fresh DMEM. Following 24 hours co-incubation, the macrophages were washed three times with PBS to remove unphagocytosed yeast, then fixed with 1% formaldehyde/PBS prior to visualization on an inverted light microscope. Percentage of yeast cell-associated macrophages was determined by counting the number of macrophages with yeast internalized or associated with their cell

surface, divided by the number of macrophages counted. At least 200 macrophages were assayed per well, and each strain was tested in triplicate.

For measuring the internalization of yeast cells by macrophages, phagocytosis assays were performed as described above, except $3x10^4$ cells were plated per well and the number of *C. neoformans* cells added per well were adjusted accordingly (to $7.5x10^4$ cells). Following co-incubation and fixation, wells were washing once in PBS and percentage of yeast cell-associated macrophages determined as described above ("pre-wash" data in Figure S3). Wells were then washed two more times in PBS and incubated overnight at 4°C in PBS + mouse anti-GXM (1:1000) + mouse anti-β-glucan (1:1000) antibodies. Wells were washed another two times in PBS, incubated 1hr in PBS + FITC-conjugated anti-mouse secondary antibody (1:1000), washed three times in PBS, then incubated 30 minutes in PBS + 0.5% SDS to permeabilize macrophages. Wells were washed three times in PBS, then incubated overnight at 4°C in PBS + 0.1% Triton-X 100 + mouse anti-GXM (1:1000) antibody. Wells were washed twice in PBS, incubated 1hr in PBS + 0.1% Triton-X 100 + TRITC-conjugated anti-mouse secondary antibody (1:1000), then washed a final time in PBS. Percentage of yeast-associated macrophages was determined as described above. Whether said yeast cells were internalized (TRITC staining only) or externally associated (FITC and TRITC staining) was determined for each strain background. 10 yeast-associated macrophages were analyzed per well for wild type and *blp1*Δ strains and 30 yeast-associated macrophages were analyzed per well for *gat201*Δ, *gat204*Δ, and *gat204*Δ*blp1*Δ strains. Three wells were analyzed for each strain. Microscopy was performed on an Axiovert 200M (Zeiss) microscope running Axiovision software.

Bone marrow-derived macrophage (BMDM) phagocytosis assays were performed as described for RAW264.7 cells, excepting that BMDMs were fixed in 4%

paraformaldehyde (in PBS) instead of 1% formaldehyde. BMDM cells were a gift from Paolo Manzanillo and Jeffrey Cox and were isolated as described in Shiloh et al., 2008 (Shiloh et al., 2008).

Expression profiling

Three or eight replicate cultures each of H99 and *gat201*Δ were grown overnight to saturation in YPAD at 30°C. For microarrays grown in YPAD medium, cultures were diluted to an OD_{600} of 0.1 in YPAD, grown at 30°C to $OD6_{00}$ = 1.0, and then harvested by centrifugation and snap freezing. For samples grown in DMEM, the volume of YPAD-grown culture equivalent to OD_{600} = 50 was washed three times with PBS prior to resuspension in 20 ml DMEM. The cultures were incubated for 24 hours in 15 cm tissue culture-treated dishes (Corning) at 37ºC with 5% CO_2. The cultures were harvested by centrifugation and snap freezing. RNA isolation, array hybridizations, and data analysis using SAM analysis were performed as described previously (Chun et al., 2007). Shown are the genes that have at least a two-fold average difference in gene expression between wild type and mutant following SAM analysis of the arrays from tissue-culture conditions.

ChIP-on-chip

The ChIP-chip tiling arrays were designed on 244,000 probes of 60-bp length, averaging 80-bp between probes, across the entire H99 sequence as it was published by the Broad Institute, current to 2007. *C. neoformans* strains were grown overnight to saturation in YPAD at 30°C. The equivalent volume of OD_{600} = 200 were washed three times with PBS prior to resuspension in 80 ml DMEM. The cultures split between four 15 cm tissue culture-treated dishes (Corning) and incubated for 8 hours at 37°C with 5% CO_2. The cultures were fixed with 1% formaldehyde, followed by quenching with 125 mM

glycine, then harvested by centrifugation and snap freezing. Samples were lyophilized, and chromatin immunoprecipitation performed as previously described (Nobile et al., 2009) with the following minor modifications: zirconia/silica beads (0.5 mm diameter) were used to lyse the cells via a Bead Beater-8, used for thirty minutes with frequent rests on ice. The chromatin in the cell pellet was sonicated for 1 hr in a Diagenode Bioruptor (settings: 4x15 min, 30 s on, 1 min off) at 4ºC. Immunoprecipitation was performed with an anti-FLAG antibody. Following ChIP, strand displacement amplification and labeling were performed as previously described to generate DNA probes with incorporated aminoallyl-dUTP which were then hybridized as described to the tiling arrays (Nobile et al., 2009). Analysis was performed by calculating the median of the top five probes in the 2kb flanking the start codon of each gene. The median value for the untagged strain was subtracted from the median value of the tagged strain, yielding the difference for the pair of untagged/tagged arrays. The calculation was repeated for a second duplicate pair of untagged/tagged arrays. The calculated differences were clustered using the software Cluster, and the cluster showing increased binding in both pairs was isolated for additional analysis. In this cluster, the differences were averaged between the two pairs, and the genes with greater than 1.9-fold median enrichment in tagged versus untagged arrays were selected for screening for Gat201-dependent gene expression by RT-qPCR. Phagocytosis measurements demonstrated that the tag did not block the function of Gat201 (C.D.C. and H.D.M., unpublished observations).

Binding Motif Analysis

A putative binding motif for Gat201 was identified using MochiView software v.1.37 (http://johnsonlab.ucsf.edu/sj/mochiview-software/) (Homann and Johnson, 2010). 1 kb of sequence upstream of the start codon of each gene was isolated and used as a

reference set, with which to compare the 1 kb sequences upstream from the start codon of the 62 genes transcriptionally-induced by Gat201. The Motif Finder feature of MochiView was used for this analysis, and identified the seven bp putative binding motif.

RT-qPCR

Gene expression differences observed using microarray-based transcript profiling were confirmed utilizing RT-qPCR. cDNA was synthesized using Superscript III Reverse Transcriptase (Invitrogen) and oligo-DT and random nonamer primers. Approximately 0.2 ng of cDNA was used as template in a qPCR reaction containing SYBR Green dye (Molecular Probes). Fluorescent signal was measured on an Opticon DNA Engine PCR machine (MJ Research). For each primer set, standard curves were generated using 5-fold serial dilutions of cDNA to account for differences in priming efficiencies. For each sample, values were normalized to the levels of actin (ACT1). MEU1 (CNAG_00165) qPCR primers were C1550/C1551. CNJ1810 (CNAG_04735) qPCR primers were C1554/C1555. *GAT204* qPCR primers were C2416/C2417. *BLP1* qPCR primers were: AAG AGG ATC ACC CAC AC TGG and ATC ATT GCA ACC AGG ACA CA.

A. tumefaciens*-mediated transformation of *C. neoformans

Transformation was carried out as in McClelland et al (2005) with minor changes: prior to mixing strain C601 with H99, the OD600 of C601 was adjusted to 0.5 and H99 was adjusted to 5.85. Equal aliquots of C601 and H99 were mixed together. 400 µl of C601+H99 were plated onto 0.45 um Biodyne® A membranes (PALL Life Sciences, Cat. no. 601012) placed on induction medium agar plates containing 200 µM acetosyringone. The plates were incubated at 25°C for 3 days. The membranes were then transferred to YPAD plates containing 0.1 mg/ml norseothrycin and 200 µM

cefotaxime. These plates were then incubated at 30°C for two days until *C. neoformans* growth was seen. The membranes were then washed with PBS to remove the *C. neoformans* cells and the cells from all membranes were pooled together. The resulting library was washed three times with PBS, before being frozen down in 15% glycerol. We estimated ~30,000 transformants were generated in this library.

Phagocytosis Screen with Insertional Mutant Library

$2x10^7$ RAW264.7 macrophages were seeded into 15 cm tissue culture dishes (Corning) in 20 ml DMEM medium and allowed to adhere overnight. *C. neoformans* cells from overnight cultures grown in YPAD medium with 200 μm cefotaxime were washed three times with PBS then added to the RAW264.7 macrophages in 20 ml fresh DMEM at an MOI of 10:1. Following 24 hours co-incubation, the macrophages were washed three times with PBS to remove unphagocytosed yeast, then lysed with 0.01% SDS. Cell lysis was confirmed visually on a light microscope. The lysed cells were collected and washed three times in PBS prior to resuspension in YPAD medium with 200 μM cefotaxime. The harvested yeast were then plated on YPAD agar plates containing nourseothricin and 200μM cefotaxime. Colonies that grew up were picked and individually assayed for rates of phagocytosis.

Capsule assay

C. neoformans strains were grown in YPAD overnight to saturation. $2x10^7$ cells were washed three times in PBS prior to resuspension in 2.5 ml DMEM. Cultures were incubated in 6-well tissue culture-treated dishes (BD Biosciences) for 24 hours at 37ºC with 5% CO_2. The cells were collected, fixed with 1% formaldehyde, and washed twice with PBS. The cells were incubated for 1 hour at 37ºC with a previously described mouse monoclonal antibody (mAB339) specific for the main capsule polysaccharide

glucoronoxylomannan (Belay et al., 1997). The cells were then washed twice with PBS, prior to incubation with FITC-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch) for 1 hour at room temperature in the dark. The cells were then washed twice with PBS, and 4 μL of India ink was added to 20 μL of sample. The capsule was visualized at 63x magnification using an Axiovert 200M (Zeiss) microscope running Axiovision software. To quantify capsule size, the cell diameter and capsule diameter of at least 30 cells per strain were measured.

Intranasal co-infection experiments.

C. neoformans strains were individually grown in liquid YPD cultures overnight at 30°C. Cells were washed twice in PBS then counted using a hemacytometer and an equal number of WT cells and cells of the co-infecting strain were combined to a final concentration of 1 x 10^7 cells/ml. 6 week-old female A/J (NCI) mice were anesthetized by intraperitoneal injection of ketamine (75 mg/kg) and medetomidine (0.5-1.0 mg/kg). The mice were then suspended from a silk thread by their front incisors and 50μl of the inoculum (5 x 10^5 cells) were slowly pipetted into the nares. After 10 minutes, the mice were lowered and the anesthesia was reversed by intraperitoneal injection of atiplamezole (1.0-2.5 mg/kg). Three mice were infected per inoculum. A dilution of each inoculum was also plated on Sabouraud agar plates containing 40 mg/ml gentamicin and 50 mg/ml carbenicillin. The plates were incubated for two days at 30°C, colonies were counted to verify the concentration of cells in the inoculum, and the proportion of mutant cells in the inoculum was determined by assaying 100-200 colonies for NAT resistance on YPD agar plates containing 100mg/ml NAT. Mice were sacrificed by CO_2 inhalation followed by cervical dislocation three days post-infection and the lungs were removed and homogenized in 5 ml sterile PBS. Serial dilutions of each organ sample were plated

on Sabouraud agar plates containing 40 mg/ml gentamicin and 50 mg/ml carbenicillin. The plates were incubated for two days at 30°C, colonies were isolated, and the proportion of mutant cells within each organ was determined by assaying 100-200 colonies for NAT resistance on YPD agar plates containing 100mg/ml NAT.

Supplemental References

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