# Chapter 2

## Materials and methods

## 2.1 Materials

### 2.1.1 Strains and plasmids

All *C. glabrata* and bacterial strains and plasmids used in this study are listed in Table 2.1.

### 2.1.2 Antibodies

All antibodies, their sources, clonality and dilutions used are listed in Table 2.2.

### 2.1.3 Oligonucleotides

Oligonucleotides used in this study were designed either by freely available online tool Primer 3 plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) or Generunner software. Oligonucleotides were commercially synthesised at MWG Biotech Pvt. Ltd., Bangalore, India. Oligonucleotides used in this study are listed in Table 2.3.

### 2.1.4 Chemicals, kits and culture medium components

Agarose, phenol, dimethyl sulphoxide (DMSO), sodium acetate, sodium chloride, sodium hydroxide, sodium carbonate, sodium bicarbonate, trizma base, sodium dodecyl sulphate (SDS), formamide, calcium chloride, ethylenediaminetetraacetic acid (EDTA), glycerol, polyethylene glycol, ficoll, diphenyleneiodinium (DPI), methyl methanesulphonate (MMS), camptothecin, hydroxyurea, ammonium persulphate, TEMED, acrylamide, bis-acrylamide, coomassie brilliant blue (CBB), chloroform, formaldehyde, glycine, lithium chloride, lithium acetate, menadione, isopropanol, phorbol myrsityl acetate (PMA), nuclease free water, wortmannin, bafilomycin-A, diethylpyrocarbonate (DEPC), orthrophenylenediamine (OPD), tween-20, acid washed glass beads, trypan blue, Taq DNA Polymease, trisodium citrate dihydrate and uracil were purchased from Sigma Chemicals. β-mercaptoethanol was obtained from GE Biosciences. Protease inhibitor tablets were procured from Roche. Dextrose, sucrose, agar, ammonium sulphate, potassium chloride, caffeine, magnesium chloride and sorbitol were obtained from Himedia. Hydrogen peroxide, hydrochloric acid, sulphuric acid,

methanol, acetic acid, potassium dihydrogen orthrophosphate, dipotassium hydrogen phosphate, disodium hydrogen orthrophosphate, acetone and citric acid were purchased from Qualigen chemicals. Fluconazole was procured from Ranbaxy.

Lysotracker-Red DND 99 and FM 4-64 were obtained from Molecular Probes. Hybond-N and Hybond-P membranes for nucleic acid and protein transfer, respectively, were purchased from Amersham Biosciences. SYBR-green kit for real-time PCR was procured from Eurogentech. Superscipt SS-III RT kit and Pfu polymerase were obtained from Invitrogen. Different restriction enzymes used for cloning and knock-out generation were purchased from New England Biolabs (NEB). High fidelity DNA Pfx polymerase was purchased from Finnzymes. Plasmid DNA purification, PCR purification, gel extraction and reaction clean up kits were procured from Qiagen.

Medium components for *C. glabrata* and bacterial culture *viz.*, yeast extract, peptone, tryptone, cassamino acid hydrolysate, yeast nitrogen base, yeast nitrogen base without ammonium sulphate, yeast nitrogen base without ammonium sulphate and amino acids and yeast carbon base were purchased from BD (Becton, Dickinson and Company, USA). Animal cell culture media RPMI-1640, DMEM and α-MEM were procured from Hyclone. Fetal bovine serum, glutamine and antibiotics for cell culture medium were obtained from Gibco-Invitrogen.

**Table 2.1: List of strains and plasmids used in the study**

| Strain | Genotype | Reference |
|---|---|---|
| **Yeast** | | |
| YRK19 | *ura3Δ::Tn903 G418$^R$* (BG14) | (Cormack and Falkow, 1999) |
| YRK20 | *URA3* (BG462) | De Las Peñas *et al*., 2003 |
| YRK103 | *ura3Δ::Tn903 G418$^R$ Cgyps1-11Δ::hph* | Kaur *et al.*, 2007 |
| YRK220 | *ura3Δ::Tn903 G418$^R$/pBRK202* | This study |
| YRK402 | *ura3Δ::Tn903 G418$^R$ Cgrtt107Δ::hph* | This study |
| YRK422 | *URA3 Cgrsc3-aΔ::hph* | Lab collection |
| YRK646 | *ura3Δ::Tn903 G418$^R$ Cgrtt107Δ::hph/pBRK700* | This study |
| YRK649 | *ura3Δ::Tn903 G418$^R$ Cgyps1-11Δ::hph/pBRK202* | This study |
| YRK659 | *ura3Δ::Tn903 G418$^R$ Cgrtt109Δ::nat1* | This study |
| YRK662 | *ura3Δ::Tn903 G418$^R$Cgvps15Δ::nat1* | This study |
| YRK668 | *URA3 Cgrsc3-aΔ::hph Cgrsc3-bΔ::nat1* | Lab collection |
| YRK691 | *ura3Δ::Tn903 G418$^R$ Cgrsc3-bΔ::nat1* | Lab collection |
| YRK693 | *URA3 Cgsgs1Δ::nat1* | This study |
| YRK695 | *URA3 Cgvps15Δ::nat1* | This study |
| YRK707 | *ura3Δ::Tn903 G418$^R$ Cgvps34Δ::nat1* | This study |
| YRK709 | *URA3 Cgvps34Δ::nat1* | This study |
| YRK713 | *ura3Δ::Tn903 G418$^R$ Cgacs1Δ::nat1* | This study |
| YRK753 | *ura3Δ::Tn903 G418$^R$ Cgrtt107Δ::hph/pRK202* | This study |
| YRK840 | *URA3 Cggpa1Δ::nat1* | This study |
| YRK843 | *ura3Δ::Tn903 G418$^R$ Cgvps15Δ::nat1/pRK942* | This study |
| YRK845 | *ura3Δ::Tn903 G418$^R$ Cgvps34Δ::nat1/pRK984* | This study |
| YRK939 | *ura3Δ::Tn903 G418$^R$ Cgvps15Δ::nat1/pRK202* | This study |
| YRK940 | *ura3Δ::Tn903 G418$^R$ Cgvps34Δ::nat1/pRK202* | This study |
| **Tn*7* Transposon and insertion mutants** | | |
| Tn*7* Transposon | *Tn7 R6Kγ ori URA3 npt (Km$^R$)* | Castaño *et al*., 2003 |
| YRK24 | *Cgpan1::Tn7* | This study |
| YRK547 | *Cgrtt109::Tn7* | This study |
| YRK588 | *Cgchz1::Tn7* | This study |
| YRK589 | *Cgarp7::Tn7* | This study |
| YRK590 | *Cgcti6::Tn7* | This study |
| YRK591 | *Cggtr1::Tn7* | This study |
| YRK593 | *Cgrsc3-b::Tn7* | This study |
| YRK594 | *Cgdna2::Tn7* | This study |
| YRK596 | *Cgrsc3-a::Tn7* | This study |
| YRK597 | *Cgsgs1::Tn7* | This study |
| YRK608 | *Cghfi1::Tn7* | This study |
| YRK755 | *Cgrtt107::Tn7* | This study |
| YRK846 | *Cgpho86::Tn7* | This study |
| YRK847 | *Cgrer1::Tn7* | This study |
| YRK848 | *Cgldb17::Tn7* | This study |

| | | |
|---|---|---|
| YRK849 | *Cgsro7::Tn7* | This study |
| YRK851 | *Cggea2::Tn7* | This study |
| YRK852 | *Cgact1::Tn7* | This study |
| YRK853 | *Cgsla2::Tn7* | This study |
| YRK855 | *Cgerv29::Tn7* | This study |
| YRK980 | *Cgvps15::Tn7* | This study |
| **Bacteria** | | |
| BRK2 | *E. coli* DH5α *(Δ(argF-lac)U169 supE44 hsdR17 recA1 endA1gyrA96 thi-1 relA1(φ80lacZΔM15))* | Lab collection |
| BW23473 | Δ*lac-169 robA1creC510hsd* R514 uidA::*pir endA recA* | Metcalf *et al.*, 1996 |
| **Plasmid** | **Description** | **Reference** |
| pRK74 | A CEN-ARS plasmid (pGRB2.2) of *C. glabrata* carrying *S.cerevisiaeURA3* as a selection marker. MCS sites are flanked by *S. cerevisiaePGK1* promoter at one end and by 3' UTR of *HIS3* at the other end. | Frieman *et al.*, 2002 |
| pRK202 | GFP expressing *C. glabrata* plasmid | Cormack laboratory |
| pAP599 | Contains *URA3* marker and an *hph* expression cassette (The *hph* gene is present between *S. cerevisiae PGK1* promoter and *HIS3* 3' UTR). The *hph* cassette confers hygromycin resistance ($Hyg^R$). | Cormack laboratory |
| pRK588 | 5' UTR and 3' UTR of *CgRTT107* cloned in pAP599 | This study |
| pRK613 | 5' UTR and 3' UTR of *CgRSC3-A* cloned in pAP599 | Lab collection |
| pRK849 | *CgRSC3-A* ORF (2.5 kb) cloned in Spe*I*-BamH*I* sites in pRK74 | Lab collection |
| pRK806 | *CgRSC3-B* ORF (2.4 kb) cloned in Xma*I*-Xho*I* sites in pRK74 | Lab collection |
| pRK700 | *CgRTT107* ORF (3.3 kb) cloned in Sma*I*-Sal*I* sites in pRK74 | This study |
| pRK941 | *CgRTT109* ORF cloned (1.3 kb) in BamH*I*-Sal*I* sites in pRK74 | This study |
| pRK942 | *CgVPS15* ORF (3.4 kb) cloned in Xma*I*-Xho*I* sites in pRK74 | This study |
| pRK984 | *CgVPS34* ORF (2.4 kb) cloned in Sal*I*-Xma*I* sites in pRK74 | This study |

**Table 2.2: List of the antibodies used in the study**

| Name | Dilution used | Clonality | Company | Catalog number |
|---|---|---|---|---|
| **Primary antibodies** | | | | |
| Anti-Gapdh | 1:10,000 | Polyclonal | Abcam | ab22555 |
| Anti-histone H1 | 1:1,000 | Monoclonal | Abcam | ab62884 |
| Anti-histone H2A | 1:2,000 | Polyclonal | Abcam | ab18255 |
| Anti-histone H2B | 1:5,000 | Polyclonal | Abcam | ab1790 |
| Anti-histone H3 | 1:10,000 | Polyclonal | Millipore | 06-755 |
| Anti-histone H4 | 1:500 | Polyclonal | Abcam | ab10158 |
| Anti-H3K9Ac | 1:1,000 | Polyclonal | Cell Signaling Technology | 9649 |
| Anti-H3K14Ac | 1:5,000 | Polyclonal | Millipore | 06-911 |
| Anti-H3K56Ac | 1:1,000 | Monoclonal | Abcam | ab76307 |
| Anti-H3K9$Me_3$ | 1:5,000 | Polyclonal | Cell Signaling Technology | 9754 |
| Anti-H3K27$Me_2$ | 1:5,000 | Monoclonal | Cell Signaling Technology | 9728 |
| Anti-H4K16Ac | 1:500 | Polyclonal | Abcam | ab61240 |
| Anti-H4K20$Me_3$ | 1:10,000 | Polyclonal | Abcam | ab9053 |
| Anti-H3S10P | 1:1,000 | Polyclonal | Cell Signaling Technology | 9701S |
| Anti-gamma H2AX | 1:5,000 | Monoclonal | Abcam | ab11174 |
| Anti-acetylated lysine | 1:500 | Polyclonal | Abcam | ab23364 |
| Anti-CpY | 1:8,000 | Polyclonal | Thermo Scientific | PA1-27244 |
| Anti-Epa1 | 1:10000 | Polyclonal | Cormack laboratory | |
| **Secondary antibodies** | | | | |
| Anti-mouse IgG | 1:5,000 | | Cell Signaling Technology | 7076S |
| Anti-rabbit IgG | 1:5,000 | | Cell Signaling Technology | 7074S |

**Table 2.3: List of the oligonucleotides used in the study**

| Primer name | Sequence (5'-3') | Target gene |
|---|---|---|
| **For creating knockouts and confirmation by PCR** | | |
| OgRK744 | AATGGAGATGAGGGCTCCTT | *CgACS1* |
| OgRK745 | TTCGTTTATATACTTCTGTGCCTGT | *CgACS1* |
| OgRK746 | GCGTCGACCTGCAGCGTACGCAATTTGGAAGATGA GATCTGAAA | *CgACS1* |
| OgRK747 | CGACGGTGTCGGTCTCGTAGCAATCGAGAAGGGAT ATTCTGC | *CgACS1* |
| OgRK748 | TCTTTCTTCCTATTGCATATCTCAAC | *CgACS1* |
| OgRK749 | GGCACATGCTTGTATGCACT | *CgACS1* |
| OgRK 658 | TGTTCAACAGATTGGTCTCCA | *CgGPA1* |
| OgRK 659 | GTCAAACCTTCAACGCTGCT | *CgGPA1* |
| OgRK 660 | GCGTCGACCTGCAGCGTACGTTGATTTCGTAAAATG CTGCT | *CgGPA1* |
| OgRK 661 | CGACGGTGTCGGTCTCGTAGCCATTATAGCTTCACA TTTTCCA | *CgGPA1* |
| OgRK 662 | AACTCCGACACATATTGCATGT | *CgGPA1* |
| OgRK 663 | TCCCCAGATTCATTAACAAAG | *CgGPA1* |
| OgRK 758 | TTTCTGCTAAGGCACTCAATTATG | *CgGPA1* |
| OgRK 759 | ATTAGCACAGGCGAAGCATT | *CgGPA1* |
| OgRK 299 | CGCTCGAGGTGCAGTAGTGAACTTATCGCAAGCAA | *CgRTT107* |
| OgRK 300 | CGCAAGCTTTTCATTTACTACCAGTTTGATAATTCA A | *CgRTT107* |
| OgRK 301 | GCCGCCGGCGGCCGCGGCATGCAAGGCATGTTA | *CgRTT107* |
| OgRK 302 | CGGAGCTCGTGCAGCCTCGATGCTCTATTTACAAG | *CgRTT107* |
| OgRK 303 | GTATCGAAAGCCACATTATTGA | *CgRTT107* |
| OgRK 304 | GTTCAACATGGGATTGCATTG | *CgRTT107* |
| OgRK 305 | ACAATCTAGGGCCCATGACC | *CgRTT107* |
| OgRK 306 | AGCTTTCGCTTTCCCTGATA | *CgRTT107* |
| OgRK 579 | GCAATTGAGGCCGATAAAAC | *CgRTT109* |
| OgRK 580 | GGTAAACCAACTAAACATCATACTGG | *CgRTT109* |
| OgRK 581 | GCGTCGACCTGCAGCGTACGCGTATTTTGATTGGAC TTTCTGA | *CgRTT109* |
| OgRK 582 | CGACGGTGTCGGTCTCGTAGTTCTGAGAACTAAAG AAAAACTTCAA | *CgRTT109* |
| OgRK 583 | AGGGCTCTCAACTTTTTCTGA | *CgRTT109* |

| | | |
|---|---|---|
| OgRK 584 | TCGTTCAAGGATGTTGTGGA | *CgRTT109* |
| OgRK 585 | AGCGGATACCAATGGATACTG | *CgRTT109* |
| OgRK 586 | GGAAGCCCACCATTTCAATA | *CgRTT109* |
| OgRK 573 | TTCAAAATCAGCCAGTATGAGC | *CgSGS1* |
| OgRK 574 | AGGGCGAAGTTGACACATCT | *CgSGS1* |
| OgRK 575 | GCGTCGACCTGCAGCGTACGTCTAAACAAGTGATTAGGCC | *CgSGS1* |
| OgRK 576 | CGACGGTGTCGGTCTCGTAGTTTTTATCCATTATTTAGTTTGGACA | *CgSGS1* |
| OgRK 577 | GTGCTTCCATGGAGGTTGAT | *CgSGS1* |
| OgRK 578 | GGTGGTGACGGTAGTGTGC | *CgSGS1* |
| OgRK 607 | GGAATCCTTCGAAGTGTATCTT | *CgSGS1* |
| OgRK 608 | TCTCTTTAATAGGATCAGGGAGTT | *CgSGS1* |
| OgRK 528 | AAGTCACTTCCAGGTGCTTATTTC | *CgVPS15* |
| OgRK 529 | GCGTCGACCTGCAGCGTACGCACTTCCAATTTTTGGCCTACACACT | *CgVPS15* |
| OgRK 530 | CGACGGTGTCGGTCTCGTAGCATATATTATAGGCGATGCTT | *CgVPS15* |
| OgRK 532 | GATCAAGTCATATTTAAACGAACC | *CgVPS15* |
| OgRK 619 | TTGCTAATCTTTTCTACACAGTTTTTG | *CgVPS15* |
| OgRK 620 | CATTTAAGTAATAATATTATCGA | *CgVPS15* |
| OgRK 259 | GCAGTCATCAACAATTCCAA | *CgVPS15* |
| OgRK 260 | GCTATTTGGGTGTAATATC | *CgVPS15* |
| OgRK 652 | TGATTATCACTCATAAACTGCATCAA | *CgVPS34* |
| OgRK 653 | TGGTCGACATTGACCTGTGT | *CgVPS34* |
| OgRK 654 | GCGTCGACCTGCAGCGTACGGCAGGGAACCCTTAATCCTG | *CgVPS34* |
| OgRK 655 | CGACGGTGTCGGTCTCGTAGTCTAAAAATTCGGACCCGTAG | *CgVPS34* |
| OgRK 656 | CGTAAATAGCACGTACATCCACA | *CgVPS34* |
| OgRK 657 | ACTTTTGTTTGACGCATGAG | *CgVPS34* |
| OgRK 756 | GCTGAAAATACCAATGGGAAATAA | *CgVPS34* |
| OgRK 757 | TGCTGTTCAAACGGCTTAGA | *CgVPS34* |
| OgRK 340 | CGTACGCTGCAGGTCGACGCCTTCCGCTGCTAGGCGCGCCGTG | *nat1* |
| OgRK 341 | CTACGAGACCGACACCGTCGGGCCGCTGACGAAGT | *nat1* |
| OgRK 342 | GTCTACTACTTTGGATGATAC | *nat1* |
| OgRK 343 | TCTGTTCCAACCAGAATAAG | *nat1* |

| **For qRT-PCR** | | |
|---|---|---|
| OgRK 420 | GTCGTCTTCGCAGGTTTCTC | *CgACS1* |
| OgRK 421 | TTGTGGGGTTTCTCTCAAGG | *CgACS1* |
| OgRK 839 | AGACCGCTGTGGTTGGTATC | *CgACS2* |
| OgRK 840 | AGCGAATGGACCAATTTCAC | *CgACS2* |
| OgRK 1075 | AGACAAAAATGCGGAATTGG | *CgAQY1* |
| OgRK 1076 | AAACATGAATGTGCCAACCA | *CgAQY1* |
| OgRK 686 | CTACTAAAGCGTGTTGGTGACG | *CgARN1* |
| OgRK 687 | GGCAACAATTGTCTCCAGTCTT | *CgARN1* |
| OgRK 1101 | TCCTGGAGATGTTGGCTTCT | *CgATH1* |
| OgRK 1102 | CTTTGTCCCCACAGGATGAT | *CgATH1* |
| OgRK 944 | TTGGGAAGGTGACAACAACA | *CgCMK1* |
| OgRK 945 | AGGTGAACACTTTCCGGATG | *CgCMK1* |
| OgRK 680 | TTAGGTTTGATGATGCACGAAG | *CgFTR1* |
| OgRK 681 | TGTTGTCCATACGGTTTAGCAG | *CgFTR1* |
| OgRK 459 | GAATCTGTTATCAGAGACGCTGT | *CgHHF2* |
| OgRK 460 | TTAACCACCGAAACCGTACA | *CgHHF2* |
| OgRK 408 | TCAAGGTATTACCAAGCCAGCTA | *CgHHF3* |
| OgRK 409 | TTCTCTTGGCGTGTTCAGTG | *CgHHF3* |
| OgRK 402 | GATTCCAATCCTCTGCCATC | *CgHHT1* |
| OgRK 403 | CACCTCTCAATCTTCTAGCCAAC | *CgHHT1* |
| OgRK 404 | AGCCAGGTACTGTCGCTTTG | *CgHHT3* |
| OgRK 405 | ATGGCAGCCAAGTTGGTATC | *CgHHT3* |
| OgRK 392 | AGCTGGTTCAGCTGCTAAGG | *CgHTA1* |
| OgRK 393 | TTGCAAATGTCTTGGGATGA | *CgHTA1* |
| OgRK 394 | GGTTCTGCTGCTAAGGCTTC | *CgHTA2* |
| OgRK 395 | GCCAATTCCAAGATTTCAGC | *CgHTA2* |
| OgRK 455 | GAAGAGAACCAAGGCCAGAA | *CgHTB1* |
| OgRK 456 | GCGGTTTGGATTTCTCTAGC | *CgHTB1* |
| OgRK 396 | TGCTAAAGCCGAAAAGAAGC | *CgHTB2* |
| OgRK 397 | GGTGTCTGGGTGAGTTTGCT | *CgHTB2* |
| OgRK 416 | CTTCTTCGACTGGGACTTGC | *CgICL1* |
| OgRK 417 | GTCTGGGTACTTGGCCTTGA | *CgICL1* |

| | | |
|---|---|---|
| OgRK 1071 | CACGGGGTTGCAAAACTTAT | *CgIME1* |
| OgRK 1072 | TCCGTGTCATCCCAATTTGT | *CgIME1* |
| OgRK 1067 | GCTGAGCAAGCGTTATTGTTCGC | *CgIME2* |
| OgRK 1068 | TTGCATTGATGGAGTTGACCAGA | *CgIME2* |
| OgRK 940 | CACCTAAGGTCGCTGTGTCA | *CgISA1* |
| OgRK 941 | TTCTCCACCCAGTCCATCTC | *CgISA1* |
| OgRK 418 | CATCACTTGGAGGCCAAACT | *CgMLS1* |
| OgRK 419 | AGTCCCAACGACCACAGTTC | *CgMLS1* |
| OgRK 942 | TTGGGAAGGTGACAACAACA | *CgPOX1* |
| OgRK 943 | AGGTGAACACTTTCCGGATG | *CgPOX1* |
| OgRK 1069 | CCCAGTTCACCAAGGAAGAA | *CgPRY1* |
| OgRK 1070 | CGAATTCTCCAGCCCAATTA | *CgPRY1* |
| OgRK 1073 | TAACGCAATTGCCAATCAGA | *CgRED1* |
| OgRK 1074 | TCAACCATTCGCTCAACATC | *CgRED1* |
| OgRK 930 | GCAAAAATGGATCCCTGAGA | *CgREX2* |
| OgRK 931 | GATTGTGTCTCCTGGCCACT | *CgREX2* |
| OgRK 1099 | TGCCGCTGTATTAAATGACG | *CgYAP5* |
| OgRK 1100 | TTGATAGGCCCGACTTTGAC | *CgYAP5* |
| OgRK 65 | CGACGACCCATCCCCAGGCTC | *CgYPS3* |
| OgRK 66 | ACTTAGCTCTTCATGGTAACG | *CgYPS3* |
| OgRK 1103 | GGCTCAACTTCGAGGTAAGG | *CAGL0F00341g* |
| OgRK 1104 | ACGCGTTAAGTTCCTGCTTG | *CAGL0F00341g* |
| OgRK 1077 | TTGGCGGATAAACACAATGA | *CAGL0F02057g* |
| OgRK 1078 | ATGGCCCATCCAATTTGTTA | *CAGL0F02057g* |
| OgRK 1093 | CTGCTGCTCCATCTTCTTCC | *CAGL0H09614g* |
| OgRK 1094 | CAGAGGTGACAACGGTGGTA | *CAGL0H09614g* |
| OgRK 1097 | AGCAACATACCGATGCATGA | *CAGL0H09966g* |
| OgRK 1098 | CAAGCTCATCAGGGTCGTTT | *CAGL0H09966g* |
| **For cloning** | | |
| OgRK 360 | AGTAAACCCGGGATGTCTATTGATACATCGAT | *CgRTT107* |
| OgRK 361 | ACTCCGTCGACTGTACTAAATCCTTTTATTGA | *CgRTT107* |
| OgRK 633 | CAGGATCCATGCTCCAAGCAATTCTCAAAG | *CgRTT109* |
| OgRK 634 | GGGTCGACCTATTTCTTTCTGCGAACTTGGA | *CgRTT109* |

| | | |
|---|---|---|
| OgRK 382 | TTCCCGGGATGGGTGGACAGTTGTCTTT | *CgVPS15* |
| OgRK 383 | CCTCGAGTCGCCTATAATATATGCTAGGT | *CgVPS15* |
| OgRK 768 | GTTGTCGACATGAGTGGGAAGAGTGTATCTT | *CgVPS34* |
| OgRK 769 | CTTCCCGGGCTAGGCTCTCCAGTATTGGGCT | *CgVPS34* |
| **For radiolabeling of input and output probes by PCR** | | |
| OgRK 25 | ATCCTACAACCTCTCTAG | Forward |
| OgRK 26 | TACCCATTCTAACCTCTA | Reverse |
| **For Tn*7* insertion mapping** | | |
| OgRK 183 | GTCGACCAACCAGATAAGTGA | Tn*7* end-1 |
| OgRK 184 | GCACTTCAGAAAATGAAGAGT | Tn*7* end-2 |

**Table 2.4: List of the oligonucleotides used to confirm deletion of *C. glabrata* ORFs**

| | **5' Homologous recombination** | | **3' Homologous recombination** | | **Gene internal region** | |
|---|---|---|---|---|---|---|
| **Strain** | **Forward** | **Reverse** | **Forward** | **Reverse** | **Forward** | **Reverse** |
| *Cgrtt107*Δ | OgRK 303 | OgRK 94 | OgRK 93 | OgRK 304 | OgRK 305 | OgRK 306 |
| *Cgrtt109*Δ | Og RK 579 | OgRK 344 | OgRK 345 | OgRK 584 | OgRK 585 | OgRK 586 |
| *Cgsgs1*Δ | Og RK 573 | OgRK 344 | OgRK 345 | OgRK 578 | OgRK 607 | OgRK 608 |
| *Cgvps15*Δ | OGRK 532 | OgRK 344 | OgRK 345 | OgRK 620 | OgRK 259 | OgRK 260 |
| *Cgvps34*Δ | OgRK 652 | OgRK 344 | OgRK 345 | OgRK 657 | OgRK 756 | OgRK 757 |
| *Cggpa1*Δ | OgRK 658 | OgRK 344 | OgRK 345 | OgRK 663 | OgRK 758 | OgRK 759 |
| *Cgacs1*Δ | OgRK744 | OgRK 344 | OgRK 345 | OgRK749 | OgRK 760 | OgRK 761 |

### 2.1.5 Media

#### 2.1.5.1 Yeast media

**Yeast extract Peptone Dextrose (YPD)**

1% Yeast extract

2% Peptone

2% Dextrose

**Yeast Nitrogen Base (YNB)**

0.67% Yeast Nitrogen Base

2% Dextrose

For alternate carbon source utilization experiments, dextrose was replaced with other carbon sources *viz*., sodium acetate, ethanol, oleic acid, glycerol and citric acid.

**Yeast Nitrogen Base (YNB) without ammonium sulphate and amino acids**

0.17% Yeast Nitrogen Base

2% Dextrose

**Casamino Acid (CAA)**

0.67% Yeast Nitrogen Base

2% Dextrose

0.6% Casamino acids

For preparing plates, 2% agar was added to the medium before autoclaving.

#### 2.1.5.2 Bacterial media

**Luria Bertani (LB)**

0.5% Yeast Extract

1% Tryptone

1% NaCl

**Super Optimal Broth (SOB)**

0.5% Yeast Extract

2% Peptone

10 mM NaCl

2.5 mM KCl

10 mM $MgCl_2$

10 mM $MgSO_4$

**LB-ampicillin and LB-kanamycin plates**

LB medium

50 µg/ml ampicillin

30 µg/ml kanamycin

Media and solutions were sterilized either by routine autoclaving at 121°C and 15 psi for 20 min or by filtration through membrane of 0.22 µm porosity.

### 2.1.6 Buffers and solutions

#### 2.1.6.1 Common buffers

**Phosphate-Buffered Saline (PBS)**

137 mM NaCl

2.7 mM KCl

10 mM $Na_2HPO_4$

2 mM $KH_2PO_4$

pH was adjusted to 7.3 before autoclaving.

PBS was prepared as a 10X stock solution and diluted to 1X concentration before autoclaving.

**Tris-HCl buffer**

0.5 M Trizma Base

pH was adjusted to 7.6 using concentrated HCl.

Tris-Cl buffer was prepared as a 10X stock solution and used at a 1X concentration.

**Tris-EDTA (TE) buffer**

10 mM Tris-HCl (pH 8.0)

1 mM EDTA

**Tris-Acetic acid EDTA (TAE) buffer**

40 mM Tris base

0.5 M EDTA

pH was adjusted to 8.5 with glacial acetic acid.

TAE buffer was prepared as a 50X stock solution and used at 0.5X concentration.

**Alkaline denaturing solution for DNA for membrane preparation**

0.5 M NaCl

0.25 M NaOH

Volume was adjusted with sterile water.

**Denhardt's solution (50X)**

1% Ficoll-400

1% Polyvinyl pyrollidone

1% Bovine serum albumin

Volume was adjusted with water and solution was stored at -20°C.

**Saline Sodium Citrate (SSC) buffer (20X)**

3.0 M Sodium chloride

0.3 M Sodium citrate

Volume was adjusted with water and solution was sterilized by autoclaving.

**Prehybridization Buffer**

5X SSC

5X Denhardt's solution

50% Filtered formamide

1% SDS

Volume was adjusted with sterile water.

**Post hybridization wash buffers**

**Wash buffer 1**

2X SSC

0.1% SDS

**Wash buffer 2**

1X SSC

0.1% SDS

**Stripping solution for DNA**

1% SDS

0.1% SSC

Desired volume was adjusted with sterile water. Alternatively, 0.4 M NaOH was also used to strip the bound probes from nylon membranes.

**HEPES [4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid] buffer**

1 M HEPES

pH was adjusted to 7.5 with NaOH.

HEPES was used as a buffering agent for preparing plates of YNB medium of different pH. Buffer was filter-sterilized and stored in an amber-coloured bottle.

**INOUE transformation buffer**

10 mM PIPES

15 mM $CaCl_2.2H_2O$

250 mM KCl

55 mM $MnCl_2.4H_2O$

pH was adjusted to 6.7 with 1 N KOH.

**Yeast transformation reagents**

1 M Lithium acetate

50% Polyethylene glycol

2 mg/ml carrier DNA

Dimethyl sulfoxide (DMSO)

**Zymolyase cocktail buffer for yeast colony PCR**

2.5 mg/ml Zymolyase

1.2 M Sorbitol

Zymolyase buffer was prepared in 1X PBS.

### 2.1.6.2 Buffers for extraction and analysis of genomic DNA and RNA

**Buffer A**

50 mM Tris-HCl (pH 8)

10 mM EDTA

150 mM NaCl

1% Triton-X

1% SDS

**Buffer B**

50 mM Tris-HCl (pH 7.5)

10 mM EDTA

1.1 M Sorbitol

50 mM β-mercaptoethanol (To be added just before use)

**Buffer C**

100 mM Tris-HCl (pH 7.5)

10 mM EDTA

10% SDS

**AE buffer**

3 M Sodium acetate (pH 5.3)

0.5 M EDTA (pH 8.0)

**Phenol:Chloroform:Isoamyl alcohol (25:24:1) solution**

25 ml Tris-equilibrated Phenol

24 ml Chloroform

1 ml Isoamyl alchol

**DNA sample loading buffer**

0.25% Bromophenol blue

0.25% Xylene cyanol

15% Ficoll

DNA sample loading buffer was prepared in water.

### 2.1.6.3 Buffers for protein extraction and analysis by SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis)

**Whole cell lysis buffer (Homogenizing buffer)**

50 mM Tris-HCl (pH 7.5)

2 mM EDTA

10 mM sodium fluoride

1 mM sodium orthovanadate

1 X protease inhibitor cocktail

**SDS-PAGE**

**30% Acrylamide solution**

29 g Acrylamide

1 g Bis-acrylamide

Dissolved in 100 ml $H_2O$.

**10% Sodium Dodecyl Sulfate (SDS)**

10 g SDS in 100 ml $H_2O$

**Resolving gel mix (12%) (20 ml)**

6.6 ml $H_2O$

8 ml 30% acrylamide:bisacrylamide (29:1) mix

5 ml 1.5 M Tris-HCl (pH 8.8)

200 µl 10% SDS

200 µl 10% Ammonium persulfate (APS)

8 µl N,N,N′,N′-Tetramethylethylenediamine (TEMED)

**Stacking gel mix (5%, 6 ml)**

4.1 ml $H_2O$

1 ml 30% acrylamide:bisacrylamide (29:1) mix

750 µl 1 M Tris-HCl (pH 6.8)

60 µl 10% SDS

60 µl 10% APS

6 µl TEMED

**SDS loading buffer**

130 mM Tris-HCl (pH 8.0)

20% (v/v) Glycerol

4.6% (w/v) SDS

0.02% Bromophenol Blue

2% DTT

SDS-loading buffer was prepared as a 4X stock solution in $H_2O$ and used at a 1X concentration.

**SDS-PAGE running buffer**

0.25 M Tris-HCl (pH 8.0)

1.92 M Glycine

1% SDS

Running buffer was prepared as a 10X stock solution and diluted to 1X concentration before use.

**Buffers for Western blot analysis**

**Transfer buffer (10 X stock solution)**

0.25 M Tris-HCl (pH 8.0)

1.92 M Glycine

1% SDS

Transfer buffer was prepared as a 10X stock solution and diluted to 1X concentration.

**1X Transfer buffer (1 litre)**

200 ml of methanol

100 ml of 10X transfer buffer

700 ml of water

**Tris-Buffer Saline (TBS)**

25 mM Tris

150 mM NaCl

pH was adjusted to 7.4 with HCl.

TBS buffer was prepared as a 10X stock solution and diluted to 1X concentration.

**Blocking and wash buffers (PBS-T and TBS-T)**

5% Fat-free milk

0.1% Tween-20

Volume was made to 100 ml with 1X TBS.

### 2.1.6.4 Reagents for PI3-kinase assay

**Spheroplast resuspension buffer**

0.1M KCl

15 mM HEPES (pH 7.5)

3 mM Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA)

10% Glycerol

**Phosphatidylinositol sonication buffer**

10 mM HEPES (pH 7.5)

1 mM EGTA

**PI3-kinase reaction buffer**

40 mM HEPES (pH 7.5)

20 mM $MgCl_2$

80 µM ATP

5 µCi $\gamma$-$P^{32}$ ATP

**Developing solution for thin layer chromatography (120.2 ml)**

Chloroform – 60 ml

Methanol – 47 ml

Ammonia – 4.4 ml

Water – 8.8 ml

## 2.2 Methods

### 2.2.1 Animal cell culture methods

#### 2.2.1.1 Cell lines and culture conditions

THP-1 and Lec-2 cell lines were obtained from ATCC (American Type Culture Collection). THP-1 and Lec-2 cells were cultured and maintained in RPMI-1640 and α-MEM media, respectively, supplemented with 10% heat inactivated fetal bovine serum, 2 mM glutamine and antibiotics (100 units/ml of penicillin and 100 µg/ml of streptomycin). Both cell lines were maintained at 37°C and 5% $CO_2$ in Thermo-Scientific cell culture incubator. After every 2-3 days, spent medium was replaced with fresh, pre-warmed medium. For splitting the culture, cells were harvested at 1,000 rpm for 3 min. Spent medium was discarded and cells were resuspended in 4-6 ml fresh prewarmed medium. Finally, 3-4 million cells were resuspended in 12 ml medium in 100 mm culture dishes. Cells were cultured and maintained in tissue culture incubator at 37°C and 5% $CO_2$.

#### 2.2.1.2 Isolation of primary (peritoneal) macrophages from BALB/c mice

To isolate primary peritoneal macrophages, 6-8 week old BALB/c mice were injected with 3% (w/v) thioglycollate broth (0.55% dextrose, 0.05% sodium thioglycollate, 0.5% sodium chloride, 0.05% agar) intraperitonealy (I.P. 50 µl/g body weight). After five days of injection, mice were euthanized by $CO_2$ inhalation and peritoneal macrophages were harvested by flushing the peritoneal cavity (lavage) with 10 ml DMEM medium (Zhang *et al*., 2008).

#### 2.2.1.3 Cryopreservation and revival of cell lines

Freezer stocks of THP-1 and Lec-2 cells were prepared either in commercial cell preservation medium (Gibco) or complete medium supplemented with 10% heat inactivated serum and 10% DMSO. For cryopreservation, 5-6 million cells were resuspended in 0.5 ml freezing medium in 2 ml cryopreservation vials, stored in an isopropanol bath and were transferred to -70°C freezer. After two days, freezer stocks were transferred to liquid nitrogen container till further use. To revive the cells, freezer stocks were taken out from liquid nitrogen container and transferred immediately to 37°C water bath. After 2-3 min, when freezing medium had thawed completely, cells

were transferred to a sterile 100 mm cell culture dish containing 11 ml fresh and prewarmed complete medium and cultured in tissue culture incubator at 37°C and 5% $CO_2$. After 12 h incubation, medium was replaced with fresh prewarmed medium and cells were allowed to proliferate till they acquire 80% confluence.

#### 2.2.1.4 Treatment of THP-1 monocytic cells with phorbol myrsityl acetate

THP-1 monocytes were treated with phorbol myrsityl acetate (PMA) to differentiate them to macrophages (Tsuchiya *et al*., 1982). For PMA treatment, THP-1 cells grown upto 70-80% confluence were harvested from the culture dishes at 1,000 rpm for 3 min. Harvested THP-1 cells were resuspended in 5-10 ml fresh and prewarmed complete RPMI medium. 100 µl of this cell suspension was appropriately diluted in PBS and number of viable cells was determined by trypan blue staining using hemocytometer. Cell suspension was diluted with prewarmed RPMI medium to a final density of $10^6$ cells/ml. PMA was added to this THP-1 cell suspension to a final concentration of 16 nM and mixed well. PMA-treated THP-1 cells were seeded either in 24-well cell culture plate or culture dishes and transferred to the incubator set at 37°C and 5% $CO_2$. After 12 h incubation, medium was replaced with fresh prewarmed medium and cells were allowed to recover for 12 h.

#### 2.2.1.5 Single infection assay

For infection of THP-1 cells with single *C. glabrata* strain, PMA-treated THP-1 monocytes were seeded in 24 well cell culture plate to a seeding density of 1 million cells per well. To prepare *C. glabrata* cells for macrophage infection, single colony of the desired strain was inoculated in YPD medium and allowed to grow for 14-16 h at 30°C. *C. glabrata* cells from 1 ml overnight culture were harvested, washed with PBS and cell density was adjusted to $2X10^7$ cells/ml. 50 µl of this *C. glabrata* cell suspension was infected to macrophages to a MOI of 10:1. Two hours post infection, infected THP-1 macrophages were washed thrice with PBS to remove non-phagocytosed yeast cells and medium was replaced with fresh prewarmed medium. At different time points post infection, infected THP-1 macrophages were washed with PBS three times and lysed in 1 ml sterile water. Lysates were collected by scrapping the wells with a micropipette tip, diluted in PBS and appropriate lysate dilutions were plated on YPD agar medium. Plates were incubated at 30°C and colony forming units (CFU) were counted after 1-2 days. Final CFUs per ml were determined by

multiplying CFUs with dilution factor and fold-replication was determined by dividing the CFUs obtained at 24 h time-point by 2 h CFUs.

#### 2.2.1.6 Cytokines measurement

THP-1 cells were seeded in a 24-well tissue culture plate to a cell density of 1 million cells per well, treated with PMA and were infected with yeast cells to a MOI of 10:1. Two hours post infection, cells were washed thrice with PBS and medium was replaced with fresh prewarmed RPMI medium. Plates were incubated at 37ºC for 24 h. Supernatants were collected, centrifuged at 3,000 rpm for 5 min to get rid of particulate matter, if any, and were stored at -20°C until use. Estimation of different cytokines was performed using BD OptEA ELISA kits as per the supplier's instructions.

#### 2.2.1.7 Fixing of PMA-treated THP-1 macrophages

For confocal microscopy analysis, $5X10^5$ THP-1 cells were seeded and treated with PMA in 4-chambered slides. Differentiated THP-1 macrophages were infected either with FITC-labeled or GFP-expressing *C. glabrata* strains to a MOI of 1:1. At different time intervals, medium was aspirated out from each chamber of 4-chambered slides and chamberes were washed twice with PBS. To fix the infected macrophages, 500 µl formaldehyde (3.7%) was added gently to each chamber and incubated for 15 min at room temperature. Each chamber of the slide was washed twice with PBS to remove formaldehyde solution completely. To permeabilize the fixed cells, 500 µl Triton-X (0.7%) was dispensed to each chamber and slide was incubated at room temperature for 5 min. Chambers of the slide were washed twice with PBS, separated from the slide using a chamber removal device and were air dried. Coverslips were placed on slides using Vectashield mounting medium and borders were sealed with nail paint. Slides were stored at 4°C until used for fluorescence imaging.

#### 2.2.1.8 Lysotracker staining

PMA-treated THP-1 macrophages were infected with *C. glabrata* cells to a MOI of 1:1 in four-chambered slides and incubated at 37°C and 5% $CO_2$. After 1 h coincubation, each chamber was washed thrice with PBS to eliminate extracellular yeast cells and medium was replaced with fresh prewarmed RPMI medium containing 100 nM Lysotracker Red DND-99. Infected THP-1 macrophages were incubated

under tissue culture conditions for 45-60 min and fixed in 3.7% formaldehyde as described earlier. For DAPI staining, Vectashield mounting medium containing DAPI was used and slides were visualized under confocal microscope. For heat killing, yeast cells were harvested from 1 ml culture, washed, resuspended in PBS and were incubated at 95°C for 5 min.

#### 2.2.1.9 Harvesting of macrophage-internalized *C. glabrata* cells for RNA and protein extraction

To collect macrophage-internalized yeast cells for RNA and protein extraction, $10^7$ THP-1 monocytes were seeded in 100 mm cell culture dishes and treated with PMA. PMA-differentiated THP-1 macrophages were infected with appropriate *C. glabrata* strains to a MOI of 1:1. Equal number of *C. glabrata* cells was inoculated in RPMI medium as control. Two hours post infection, non-phagocytosed yeast cells were removed by washing THP-1 macrophages thrice with PBS. At different time points, culture dishes were washed twice with chilled PBS and 2 ml chilled sterile water was added to each dish to lyse the macrophages. Corresponding cultures grown in RPMI medium were transferred to 50 ml polypropylene tubes and transferred on ice. Lysates were collected by scrapping the macrophage monolayer and transferred to 50 ml polypropylene tubes. RPMI-grown and macrophage-internalized *C. glabrata* cells were harvested by centrifugation at 2,500g for 8 min. Macrophage cell debris were removed from macrophage-internalized cells by repeated washing with chilled sterile water. Harvested *C. glabrata* cells were stored at -20ºC till further use.

### 2.2.2. Microbiological methods

#### 2.2.2.1 Strains and culture conditions

*C. glabrata* strains were routinely grown either in rich YPD medium or synthetically-defined YNB medium at 30°C with continuous shaking at 200 rpm unless otherwise stated. In general, *C. glabrata* frozen glycerol stocks were revived on YPD medium by streaking and allowed to grow for 1-2 days. *C. glabrata* strains harboring the plasmid with *URA3* as selectable marker were revived on CAA medium. To prepare liquid cell culture, single colony of each *C. glabrata* strain was inoculated either in YPD or YNB broth medium and grown for 14-16 h. *C. glabrata* strains streaked on plates were stored at 4°C for a maximum period of 2 weeks.

Bacterial strain *Escherichia coli* DH5α used for cloning purpose was revived on LB medium and grown at 37°C with continuous shaking at 200 rpm. LB medium was supplemented with appropriate antibiotics to grow bacterial strains carrying plasmids. Another *E. coli* strain, BW23473, was used to rescue the Tn*7* transposon cassette from *C. glabrata* Tn*7* insertion mutants. For plasmid DNA purification, bacterial strains were grown overnight in LB broth medium containing suitable antibiotics.

### 2.2.2.2 Growth analysis and determination of generation time

For growth analysis of a *C. glabrata* strain, single colony was inoculated in appropriate broth medium and grown for 14-16 h. Overnight grown culture was used to inoculate the test medium to an initial $OD_{600}$ of 0.1-0.3. Cultures were transferred to a shaker incubator set at 30°C and 200 rpm. Absorbance of cultures was measured using Ultraspec 2100 pro UV/visible spectrophotometer (Amersham Biosciences) at 600 nm at regular time-intervals till 48 h. Absorbance values were plotted with respect to time and generation time was determined from the logarithmic (log) phase of cell growth using the following formula.

$$\text{Generation time (G)} = (T_2\text{-}T_1)\ X\ \frac{\log 2}{\log N_f/N_i}$$

G = Generation time (h)

$T_1$ = Initial time point taken for analysis

$T_2$ = Final time point taken for analysis

$N_f$ = Number of cells at time $T_2$ (1 $OD_{600}$ of *C. glabrata* corresponds to 2 X $10^7$ cells.)

$N_i$ = Number of cells at time $T_1$ (calculated from $OD_{600}$ value as mentioned above)

### 2.2.2.3 Serial dilution spotting assay

Yeast strains were grown in YPD medium for 14-16 h at 30°C under continuous shaking at 200 rpm. Cells were harvested from 1 ml culture, washed with PBS and were diluted to an $OD_{600}$ of 1. Five ten-fold serial dilutions were prepared from an initial culture of 1 $OD_{600}$. 4 µl culture of each dilution was spotted on YNB-agar plates containing different carbon sources. For spotting on YPD plates containing different compounds, 3 µl culture of each dilution was spotted. Plates were incubated

at 30°C and images were captured after 2-8 days of incubation depending upon the medium used.

#### 2.2.2.4 Phenotypic profiling

Identified mutants were phenotypically characterized in 96-well plate format. Mutant cultures were grown in YPD medium for overnight, diluted 150-fold in PBS and 5 µl of cell suspension was spotted on different plates with a 96-pin replicator. Growth was recorded after 1-2 days of incubation at 30°C.

#### 2.2.2.5 Yeast transformation

Single colony of *C. glabrata* strains was inoculated in 10 ml YPD-liquid medium and grown at 30°C with constant shaking at 200 rpm for 14-16 h. Overnight culture was used to inoculate 10 ml YPD broth to an initial $OD_{600}$ of 0.1 and culture was grown for 4-5 h to obtain log-phase culture. Log-phase *C. glabrata* cells were harvested in 15 ml sterile polypropylene tubes by centrifugation at 4,000 rpm for 5 min. Harvested cells were washed with 10 ml sterile water, resuspended in 1 ml sterile water and transferred to a 1.5 ml microcentrifuge tube. Cells were harvested at 4,000 rpm for 5 min and resuspended in 100 µl of 100 mM lithium acetate solution. Yeast transformation cocktail was prepared in a 1.5 ml microcentrifuge tube by mixing 240 µl polyethylene glycol (50%), 36 µl lithium acetate (1 M) and 25 µl heat-denatured single stranded carrier DNA (2 mg/ml). 50 µl *C. glabrata* cell suspension and 50 µl transforming DNA was added to the transformation cocktail, mixed well and incubated at 30˚C for 45 min. 43 µl DMSO was added and cells were subjected to heat shock at 42˚C for 15 min. After the heat shock, cells were transferred to ice for 10-15 seconds, centrifuged at 4,000 rpm for 5 min and supernatant was removed. Cells were resuspended in 200 µl sterile water and spread plated on appropriate selection medium. Plates were incubated at 30˚C for 2-3 days.

#### 2.2.2.6 Screening of *C. glabrata* Tn*7* insertion mutant library

YPD-grown cultures (0.05 $OD_{600}$) of each mutant pool (96 mutants, each carrying a unique signature tag) were either inoculated in YPD medium for overnight (input) or used to infect differentiated THP-1 cells ($1X10^6$). After 2 h incubation, non-cell-associated yeast cells were removed by washing THP-1 cells thrice with PBS. At

24 h post infection, THP-1 macrophages were washed thrice with PBS, lysed in water and recovered yeast cells were used to infect THP-1 cells at a MOI of 1:10. Three rounds of macrophage infection for each mutant pool were carried out to enrich for the desired mutants in the final population. The lysate of 3$^{rd}$ round infection was inoculated in YPD medium for overnight (output). Cells were harvested, genomic DNA isolated from each input and output cell pellet and unique signature tags were PCR-amplified with $P^{32}$-labeled α-dCTP using primers complementary to the invariant region flanking each unique tag sequence. Labeled PCR products were denatured at 95°C for 10 min, chilled on ice and were hybridized to nylon membranes carrying immobilized plasmid DNA containing 96 unique tags for 14-16 h at 42°C. Membranes were washed twice with 0.1X SSC buffer and exposed to phosphorimager screen for 2-4 h. Radioactive counts for each spot were quantified using Image Quant and Fuji Multi Gauge V3.0 software. Relative percentage intensity for individual spot was calculated with respect to all spots present on each hybridized membrane.

#### 2.2.2.7 Fluorescein isothiocyanate (FITC) staining of *C. glabrata* cells

*C. glabrata* strains were grown overnight in YPD medium. Cells were harvested from 1 ml culture and washed with PBS. Cells were next washed with 50 mM $NaH_2PO_4$ and resuspended in 100 µl FITC-dextran (50 mg/ml). After incubation at 37°C for 45 min, cells were washed thrice with PBS for complete removal of FITC-dextran. Yeast cells were resuspended in 1 ml PBS and used to infect PMA-treated THP-1 cells in 4-chambered glass slide.

#### 2.2.2.8 Opsonization of *C. glabrata* cells

For opsonization, *C. glabrata* cells were incubated with 1 µg/µl human IgG for 30 min at 37°C and washed thrice with PBS. Alternatively, yeast cells were incubated with 25% human serum at 37°C for 30 min followed by three PBS washes.

#### 2.2.2.9 Harvesting of and treatment to logarithmic phase *C. glabrata* cells

For several experiments, log-phase *C. glabrata* cells were harvested and treated with different compounds. For this, single colony of a *C. glabrata* strain was inoculated in YPD-liquid medium and grown for 14-16 h at 30ºC with continuous shaking at 200 rpm. Overnight cultures were reinoculated in YPD medium to an initial $OD_{600}$ of 0.1 and grown for another 4 h. These log-phase cells were harvested by

centrifugation at 5,000 rpm for 4 min at room temperature. Harvested cells were washed with PBS and treated with different compounds *e.g.* $H_2O_2$. After treatment, cells were harvested and further processed according to the type of experiments performed.

### 2.2.2.10 Staining of *C. glabrata* vacuoles with FM4-64

A lipophilic styryl dye, FM 4-64, is a vital stain which is taken up by cells *via* endocytosis through plasma membrane (Vida and Emr, 1995). Therefore, it fluoresces only in live cells. Importantly, neither fixed cells can be stained with FM 4-64 nor cells can be fixed after FM 4-64 staining. For vacuole staining, single colony of the test strain grown on YPD plate was inoculated in 10 ml YPD medium for overnight. 100 µl overnight culture was inoculated in fresh YPD medium and incubated at 30ºC for 3 h to obtain log-phase cells. *C. glabrata* cells from 1 ml log-phase culture were harvested at 4,000 rpm for 5 min in a table top centrifuge. Supernatant was aspirated out, cells were resuspended in 50 µl YPD medium and 1 µl FM 4-64 (16 µM final concentration) was added. *C. glabrata* cells were incubated in a 30ºC water bath for 30 min. 1 ml YPD medium was added and cells were harvested at 4,000 rpm for 5 min in a table-top centrifuge. After discarding supernatant, *C. glabrata* cells were washed with fresh YPD medium and resuspended in 1 ml YPD medium. *C. glabrata* cells were incubated at 30ºC for 90 min, washed with 1 ml sterile water and were resuspended in 50 µl YNB medium. Labeled *C. glabrata* cells were observed under fluorescence microscope in red filter (730 nm).

### 2.2.2.11 Yeast colony PCR

A microtipful of cells for each yeast strain from appropriate medium was suspended in 10 µl zymolyase cocktail and incubated at 37ºC for 90 min. 2 µl of zymolyase-treated cell suspension was used as template in 25 µl PCR reaction.

### 2.2.2.12 Preparation of *E. coli* DH5α ultracompetent cells

A single colony of *E. coli* DH5α strain was inoculated in 10 ml LB medium and incubated at 37˚C for overnight. 4 ml overnight culture was inoculated in 2 lt SOB medium and incubated at 18˚C till the $OD_{600}$ reached to 0.5. Cultures were centrifuged at 2,500 g for 10 min at 4˚C and harvested cells were washed gently with 80 ml ice-cold Inoue transformation buffer. Cells were collected by centrifugation at 2,500 g for

10 min at 4˚C and gently resuspended in 20 ml ice-cold Inoue transformation buffer. To this cell suspension, 1.5 ml sterile DMSO was added and swirled gently. Cell suspension was kept on ice for 10 min and 50 µl volume was aliquoted to chilled sterile microcentrifuge tubes. Cells were immediately snap-frozen in liquid nitrogen and stored at -80˚C.

### 2.2.2.13 Bacterial transformation

*E. coli* DH5α strain was transformed with plasmids carrying appropriate inserts to clone and generate deletion strains of *C. glabrata* ORFs (Sambrook, 2001). Ultracompetent cells stored at -70°C were thawed on ice for 5-10 min. 5 µl ligated plasmid was added to 100 µl ultracompetent cells and cells were incubated on ice. After 30 min, competent cells were subjected to heat shock at 42°C for 90 seconds. Cells were immediately transferred to ice for 2-3 min. Next, 800 µl SOC (or LB) medium was added and cells were allowed to recover for 45 min on a shaker incubator set at 37°C. After the recovery, cells were centrifuged at 2,500g for 4 min. Medium supernatant was discarded and cells were resuspended in 200 µl fresh sterile LB medium. Cells were plated on LB agar medium containing appropriate antibiotics. Plates were incubated at 37°C for 12-16 h.

### 2.2.2.14 Preparation of *E. coli* BW23473 electrocompetent cells

To prepare electrocompetent cells, a single colony of *E. coli* BW23473 strain from a freshly-streaked LB agar plate was inoculated in 50 ml LB liquid medium. Culture was incubated at 37°C for 14 h with continuous shaking at 200 rpm. 25 ml overnight-grown *E. coli* BW23473 culture was transferred to 500 ml LB liquid medium and incubated at 37°C till the $OD_{600}$ reached to 0.4. Post incubation, cultures were transferred to ice and centrifuged at 1,000g for 15 min at 4°C. Cells were washed twice with 500 ml ice-cold sterile water, three times with 250 ml ice-cold 10% glycerol solution and resuspended in 1 ml 10% glycerol solution. After absorbance measurement, cell suspension was normalized to final cell density of $3X10^{10}$ cells/ml and dispensed in 50 µl aliquots to sterile ice-cold 1.5 ml microcentrifuge tubes. Aliquots were snap frozen in liquid nitrogen and stored at -70°C.

### 2.2.2.15 Transformation of *E. coli* BW23473 cells by electroporation

*E. coli* BW23473 electro-competent cell aliquots were taken out from -70ºC freezer, thawed on ice and were mixed with 1-2 µl plasmid DNA. Mixture was pulsed with the Gene Pulser® electroporation apparatus (Bio-Rad), set at 1800 Volts, 25 µF and 200 Ω, in a chilled 0.1 cm electroporation cuvette. After electric pulse, 1 ml LB medium was immediately added to the cuvette and suspension was transferred to a 1.5 ml sterile microcentrifuge tube. Cells were incubated at 37°C and 200 rpm for 1 h, centrifuged and were plated on LB-agar plates containing kanamycin (30 µg/ml). Transformants were colony purified on LB-kanamycin plates. Positive clones were verified by colony PCR and inoculated in LB-liquid medium containing kanamycin (30 µg/ml) for plasmid isolation.

### 2.2.2.16 Mutant rescue

Genomic mapping of disrupted locus in Tn*7* insertion mutants was carried out as described previously (Kaur *et al*., 2004). *C. glabrata* mutants carrying Tn*7* insertions were grown in YPD-liquid medium and genomic DNA was isolated from overnight cultures. 10 µg genomic DNA was digested either with restriction enzyme Mfe*I* or Spe*I*. Restriction enzyme-digested DNA was precipitated with 1 ml ethanol and $1/10^{th}$ volume of sodium acetate (3 M, pH 5.2). DNA pellet was washed twice with ice-cold 70% ethanol, air dried and was resuspended in sterile water. DNA was recircularized with T4 DNA ligase. Resultant circular DNA carried Tn*7* cassette flanked on both sides by the disrupted locus of the *C. glabrata* genome. Circular DNA was transformed in *E. coli* BW23473 strain which contains protein Π (the product of the pir gene) required by R6Kγ *ori* for replication. Two verified transformants were grown overnight in LB-kanamycin medium and plasmids were extracted. Purified plasmids were sequenced with primers reading outwards (OgRK 183 and OgRK 184) from both ends of Tn*7* cassette. Sequences obtained were compared, using BLAST, against *C. glabrata* genome sequence database and regions of Tn*7* insertions in *C. glabrata* were mapped.

### 2.2.3. Molecular biology methods

#### 2.2.3.1 Plasmid DNA purification

*E. coli* strains carrying plasmids were inoculated and grown overnight at 37ºC and 200 rpm in LB-liquid medium supplemented with either 50 µg/ml ampicillin or 30 µg/ml kanamycin. Cells were harvested by centrifugation at 2,500g for 5 min. Plasmids were extracted using Qiagen plasmid miniprep kit following the manufacturer's instructions. Concentration of the extracted plasmid DNAs was measured using spectrophotometer at 280 nm and stored at -20ºC.

#### 2.2.3.2 Membrane preparation

*E. coli* strains containing plasmids with unique oligonucleotide signature sequences were inoculated in LB medium containing ampicillin and grown overnight at 37°C and 200 rpm. Plasmids were extracted, quantitated and denatured in alkaline denaturing solution. Approximately, 200 ng of each plasmid DNA was transferred to the Hybond-N membrane using 96-well Dot Blot apparatus. Membranes were neutralized in 2X SSC and denatured plasmids were cross-linked to Hybond-N membranes using UV cross linker.

#### 2.2.3.3 Southern hybridization

Two Hybond-N membranes, each carrying 96 immobilized DNA with unique signature sequences, were transferred to 15 cm long hybridization bottles and labeled as input and output. 10 ml prehybridization buffer was added to each bottle and bottles were transferred to a hybridization oven set at 42ºC and 6 rpm. To prepare input and output probes, genomic DNAs from input and output *C. glabrata* cell pellets were extracted using glass bead lysis method and unique signature tags were PCR amplified with nucleotide mix containing $\alpha P^{32}$-dCTP using primers complementary to the invariant region flanking each unique oligonucleotide sequence. Following is the composition of 50 µl PCR cocktail used to prepare radiolabeled input and output probes.

10X PCR buffer – 5 µl
dNTP mix (without dCTP, 2mM) – 0.5 µl
dCTP (0.05 mM) – 1 µl

Primers (OgRK 25 and OgRK 26, 200 pM ) – 1 µl, each
Taq DNA polymerase – 0.5 µl
$\alpha P^{32}$-dCTP ( specific activity-3,000 Ci/mMol) – 2.5 µl
Water – 38.5 µl

$\alpha P^{32}$-dCTP labeled PCR products were denatured by incubating tubes at 100°C for 5 min followed by immediate chilling on ice. After 2 h prehybridization, radiolabeled denatured input and output probes were added to respective bottles and bottles were transferred to a hybridization oven set at 42ºC and 6 rpm.

**2.2.3.4 Post-hybridization washes**

After 14-16 h incubation, hybridization buffer was decanted to a radioactive liquid waste container. Membranes were washed twice with 2X SSC (saline-sodium citrate) containing 0.1% SDS for 15 min at 55°C followed by two washes with 1X SSC containing 0.1% SDS for 15 min at room temperature. Post washes, membranes were rinsed with 1X SSC buffer at room temperature and exposed to phosphorimager screen for 2-3 h.

**2.2.3.5 Data analysis**

After 2-3 h exposure, phosphorimager screen was scanned on Fuji FLA-9000 to acquire hybridization images. Next, signal intensity for each spot on the membrane for both input and output samples was quantified using Fuji Multi Gauge V3.0 software and percentage intensity for each spot relative to the whole signal intensity of the membrane was determined. To identify mutants with altered survival profiles, ratio of output (Op) to input (Ip) signal for each spot (oligonucleotide tag) present on the membrane was calculated. Mutants displaying at least 6-fold higher and 10-fold lower survival were selected as ‘‘up’ (Op/Ip = 6.0) and ‘down’ (Op/Ip = 0.1) mutants, respectively.

**2.2.3.6 Stripping of probes from hybridized membranes**

Radiolabeled-bound probes were stripped from the membrane by boiling in 1% SDS containing 0.1X SSC for 15 min. Alternatively, membranes were incubated twice in stripping solution (0.4 M NaOH) at 45°C for 30 min to remove the bound probes.

Stripping of membranes in buffer containing 0.4 M NaCl yielded slightly better results. Hybond membranes were reused for 5-10 times after stripping.

### 2.2.3.7 Genomic DNA isolation by quick genomic DNA extraction method

*C. glabrata* cells were grown overnight in 10 ml YPD liquid medium. Cells were harvested at 2,500g for 5 min, resuspended in 400 µl Buffer A (50 mM Tris-HCl, 10 mM EDTA, 150 mM NaCl, 1% Triton X-100 and 1% SDS) and were transferred to a 2 ml microcentrifuge tube. Equal volume of phenol-chloroform-isoamyl alcohol (PCI) solution was added to cell suspension and tubes were vortexed for 2-3 min. After incubation at 42˚C for 30 min on a thermomixer set at 800 rpm (Eppendorf), cell debris was removed by centrifugation at 7,500g for 10 min and aqueous phase (300-350 µl) was carefully transferred to a new 2 ml microcentrifuge tube. Genomic DNA was precipitated with 800 µl chilled absolute ethanol and 35 µl sodium acetate (3 M, pH 5.2). DNA pellet was washed with chilled 70% ethanol and dried at room temperature for 5-10 min. Genomic DNA pellet was dissolved either in 50 µl 0.1X TE or molecular biology grade water containing 0.3 µl Ambion RNase cocktail and incubated at 37˚C for 30 min for digestion of RNA. After RNA degradation, 100 µl of 0.1X TE or nuclease-free water was added to the tube and stored at -20ºC. Quality of extracted genomic DNA was checked on 0.6% agarose gel by electrophoresis.

### 2.2.3.8 Genomic DNA isolation by glass bead lysis method

Desired *C. glabrata* strain was grown overnight in YPD liquid medium and yeast cells were harvested by centrifugation at 2,500g in 15 ml polypropylene tube. Yeast cells were washed with PBS, resuspended in 500 µl lysis buffer (Buffer A) and were transferred to a 2 ml microcentrifuge tube. Yeast cells were incubated for 15 min on a thermomixer set at 65˚C and 750 rpm. After incubation, 0.5 gm glass beads (0.5 mm) and 500 µl PCI solution were added to the tube. Yeast cells were lysed three times for 45 seconds each on a bead beating apparatus with intermittent cooling on ice to prevent overheating. Cell lysates were centrifuged at 7,500g for 5 min and upper aqueous phase (300-350 µl) was transferred carefully to a new 1.5 ml microcentrifuge tube. 1 ml absolute ethanol was added and mixed well by inverting the tube 3-4 times. To precipitate genomic DNA, suspension was centrifuged at 7,500g for 10 min. Precipitated genomic DNA was washed with 70% ethanol and dried at room

temperature. Genomic DNA pellet was dissolved either in 50 µl 0.1X TE or molecular biology grade water containing 0.3 µl Ambion RNAse cocktail and incubated at 37ºC for 30 min. After RNA digestion, 100 µl of 0.1X TE or nuclease-free water was added to the tube and stored at -20ºC. Quality of extracted genomic DNA was checked on 0.6% agarose gel by electrophoresis.

**2.2.3.9 Micrococcal nuclease digestion assay**

Nucleosomal-associated DNA was extracted from RPMI-grown and macrophage-internalized *C. glabrata* cells using EZ Nucleosomal DNA prep kit (ZYMO Research), treated with micrococcal nuclease digestion for 2.5, 5, 7.5 and 10 min at 25ºC and was resolved on 2% agarose gel.

**2.2.3.10 RNA extraction**

For RNA experiments, all solutions were prepared in RNase free diethylpyrocarbonate (DEPC) water. Microcentrifuge tubes and tips used for RNA work were autoclaved twice and dried at 70˚C for overnight before use. Non-autoclavable plastic items were wiped with Ambion RNAseZap to remove RNAse contamination, if any. RNA was extracted from *C. glabrata* cells using acid phenol extraction method. *C. glabrata* cells were harvested at 2,500g for 5 min at 4˚C, resuspended in 1 ml ice-cold DEPC water and were transferred to a 2 ml microcentrifuge tube. Cells were spun down at 6,000g for 3 min at 4˚C and resuspended in 350 µl AE solution. Next, 50 µl SDS and 400 µl acid phenol (pH 4.5) solutions were added to the tube and mixed well by vortexing. The tube was incubated at 65˚C for 15 min with continuous mixing. After incubation, tube was kept on ice for 5 min and centrifuged at 7,500g for 5 min at 4˚C. Aqueous phase was transferred to a new 1.5 ml microcentrifuge tube and RNA was extracted with an equal volume of chloroform. Total RNA was precipitated at room temperature with $1/10^{th}$ volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of chilled absolute ethanol for 20 min. Precipitated RNA was collected by centrifugation at 7,500g for 5 min at 4˚C. RNA pellet was washed with chilled 70% ethanol and resuspended in 50 µl nuclease-free water. RNA concentration was determined by measuring absorbance at 260 nm. Quality of extracted RNA was examined by gel electrophoresis on 0.8% agarose gel prepared in DEPC-treated TAE buffer.

### 2.2.3.11 Synthesis of complementary DNA (cDNA)

Complementary-DNA synthesis was done using reverse transcriptase enzyme and oligo-dT primers. For this, 1 µg good quality RNA was treated with 1µl (1 unit) DNase I for 15 min to remove DNA contamination. Next, SuperScript III First-Strand Synthesis System kit (Invitrogen) was used to synthesize cDNA according to the manufacturer's instructions. cDNA synthesized was stored at -20˚C till further use.

### 2.2.3.12 Quantitative real-time PCR

Primers for real-time PCR analysis were designed using Primer3 plus software and are listed in Table 4. To extract RNA from macrophage-ingested *C. glabrata* cells, infected THP-1 cells were washed twice with PBS and lysed in 1 ml ice-cold water. Lysate was centrifuged followed by two quick washes with DEPC-treated water and washed yeast cell pellets were frozen on dry ice. For RNA extraction, yeast cells were disrupted with glass beads in trizol and total RNA was isolated using acid phenol extraction method described above. Optimal primer and cDNA concentrations were standardized and qRT-PCR was performed using ABI 7500 Fast Real-Time PCR System (Applied Biosystems). In brief, 0.5 µl cDNA, 0.1 to 0.2 picomoles of gene specific primers and 10 µl 2X MESA GREEN qPCR™ Mastermix Plus containing SYBR green dye (Eurogentec) were mixed in the wells of a 96-well PCR plate (Axygen). Final reaction volume was adjusted to 20 µl with DEPC-treated water. Transcript levels were quantified with an end-point value known as $C_t$ (cycle threshold). Expression of *TDH3*, which encodes CgGapdh, was used as an internal control. The $C_t$ defines the number of PCR cycles required for the fluorescent signal of SYBR green dye to cross beyond the background level. Fold-change in transcript expression was determined using following formula.

Fold change in expression = $2^{-\Delta\Delta Ct}$

$\Delta\Delta C_t = \Delta C_t$ treated - $\Delta C_t$ untreated

$\Delta C_t$ treated = $C_t$ value for the gene of interest under treated condition - $C_t$ value for the internal control gene (TDH3) under treated condition

$\Delta C_t$ untreated = $C_t$ value for the gene of interest under untreated condition - $C_t$ value for the internal control (TDH3) gene under untreated condition

To preclude the possibility of human RNA contamination, cDNA prepared from internalized yeast was examined for the presence of human transcripts encoding Ccl5 and histone H3. However, no amplification for human genes was observed, thus, eliminating any possible contamination of THP-1 RNA with yeast RNA.

**2.2.3.13 Microarray Analysis**

*C. glabrata* cells grown either in RPMI medium or harvested from THP-1 macrophages were collected, washed with DEPC treated water and were disrupted with glass beads in trizol. Total RNA was isolated using acid phenol extraction method and frozen at -80$^{o}$C. Quality of RNA was examined by determining the RNA integrity number (RIN) before microarray analysis. Microarray experiments were performed at Ocimum Biosolutions Ltd., Hyderabad (http://www.ocimumbio.com). Briefly, a 4x44K GE Agilent array comprised of 10,408 probes representing 5,205 ORFs of *C. glabrata* was used wherein average number of replicates for each probe was four to five. Feature Extraction software version 10.7.3.1. (Agilent) and Quantile normalization was used for data analysis. Hierarchical clustering was performed using Complete Linkage method with Euclidean Distance as distance measure. Data are the average of two hybridizations from biological replicates of each sample and raw data sets for this study are available at the Gene Expression Omnibus database (Accession number - GSE38953).

**2.2.3.14 Gel extraction of DNA**

QIAGEN QIAquick Gel extraction kit containing required buffers, spin columns and collection tubes was used to extract and purify DNA from agarose gels. Digested DNA sample was resolved on 1-1.2% agarose gel and gel piece containing desired fragment was cut on a UV-transilluminator. DNA fragment was purified as per the kit manufacturer's instructions.

**2.2.3.15 Purification of restriction enzyme-digested and PCR amplified products**

QIAGEN QIAquick PCR purification kit containing buffers, spin columns and collection tubes was used to purify DNA fragments from PCR and enzymatic digestion reactions as per the kit manufacturer's instructions.

### 2.2.3.16 Ligation

After restriction enzyme digestion, digested products were resolved on agarose gels and desired DNA fragments were extracted from the gel. Concentration of gel-extracted DNA fragments was determined using spectrophotometer and ligation reactions were set up using a molar ratio of vector to insert of 1:3 and 1:1 for sticky and blunt end ligations, respectively. Ligation mix was incubated either at 22°C for 4 h or at 16°C for 14-16 h. After incubation, $T_4$ DNA ligase was inactivated at 65°C for 20 min.

### 2.2.3.17 Cloning of *C. glabrata* ORFs

*CgRTT107* (3.3 kb), *CgRTT109* (1.3 kb), *CgVPS15* (3.4 kb) and *CgVPS34* (2.4 kb) ORFs were PCR amplified from genomic DNA of the wild-type strain using high fidelity Platinum Pfx DNA polymerase with primers carrying restriction sites for Sma*I*-Sal*I*, BamH*I*-Sal*I*, Xma*I*-Xho*I* and Sal*I*-Xma*I*, respectively. Amplified fragments were cloned downstream of the *PGK1* promoter in the pGRB2.2 plasmid. Clones were verified by bacterial colony PCR, sequencing and complementation analysis.

## 2.2.4 Other methods

### 2.2.4.1 Ethics statement

Experiments involving mice were conducted at VIMTA Labs Limited, Hyderabad in strict accordance with guidelines of The Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. The protocol was approved by the Institutional Animal Ethics Committee (IAEC) of the Vimta Labs Ltd. (IAEC protocol approval number: PCD/OS/05). Procedures used in this protocol were designed to minimize animal suffering.

### 2.2.4.2 Mouse infection assay

Experiments involving mice were conducted at VIMTA Labs, Hyderabad. 100 µl YPD-grown *C. glabrata* cell suspension (4 X $10^7$ cells) was injected into female BALB/c mice (6-8 weeks old) through tail vein. Seven days post infection, mice were sacrificed and kidneys, liver, spleen and brain were harvested. Organs were

homogenized in 1 ml PBS and fungal burden was assessed by plating appropriate dilutions of tissue homogenate on YPD plates containing penicillin and streptomycin antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin). All mice experiments were repeated twice with a set of 7-8 mice per strain in each experiment.

**2.2.4.3 Protein extraction and immunoblotting**

For protein extraction, yeast cells were suspended in 50-100 µl protein extraction buffer containing 320 mM $(NH_4)_2SO_4$, 200 mM Tris-Cl (pH 8), 20 mM EDTA (pH 8), 10 mM EGTA (pH 8), 5 mM $MgCl_2$, 1 mM DTT, 10% glycerol and protease inhibitors and disrupted using glass beads. Cell lysate was centrifuged at 7,500g and 4$^oC$ for 15 min. 30 µg of total protein was resolved on a 15% SDS-PAGE gel at 32 mA till the dye front reached the bottom. Resolved proteins were transferred to Hybond-P membrane at 350 mA for 1.5 h in the cold room. Transfer of the proteins was visually confirmed by examining marker's lane and membranes were incubated in a small box for 2 h in 5% fat free milk prepared in 1X TBST for blocking. Blocking solutions were discarded and primary antibody, appropriately diluted in 5% fat free milk prepared in 1X TBST, was added to the box containing membrane. After overnight incubation in primary antibody, membranes were washed thrice with 1X TBST for 10 min. Membranes were incubated for 2 h in appropriate secondary antibody diluted in 5% fat free milk prepared in 1X TBST. Blots were either developed by chemiluminescence based ECL-Plus western detection system or Chemidoc$^{TM}$ gel imaging system. CgGapdh was used as a loading control. To exclude the possibility of any contribution of THP-1 proteins to cell extracts prepared from macrophage-internalized yeast, two control experiments were performed. First, we probed the blots with antibodies specific for mammalian tubulin and actin. As expected, we neither detected any signal for mammalian actin nor for mammalian tubulin. In the second control experiment, we treated macrophage lysates with proteinase-K prior to the yeast pellet disruption and probed yeast lysates for different histone modifications. This proteinase-K treatment did not alter the epigenetic signature of *C. glabrata* cells. Together, these data indicate that yeast protein samples were devoid of any mammalian protein contamination.

### 2.2.4.4 Lysine deacetylase (KDAC) activity measurement

20 mg protein samples, isolated from RPMI-grown and macrophage-internalized yeast, were used to measure KDAC activity using HDAC Fluorimetric Assay/Drug Discovery Kit (Enzo Life Science) as per manufacturer's instructions.

### 2.2.4.5 Adherence assay

Adherence of *C. glabrata* cells to Lec-2 epithelial cells was measured as described previously (Cormack *et al*., 1999). Lec2 cells were seeded in a 24-well tissue culture plate at a seeding density of $5X10^5$ cells per well and allowed to adhere for 12 h. After 12 h, medium supernatant was discarded by inverting the plate in a reservoir and cells were washed thrice with PBS. Lec2 cells were fixed in 3.7% para-formaldehyde for 15 min followed by 2 PBS washes. PBS containing antibiotics, penicillin and streptomycin, was added to each well of the 24-well plate and Lec-2 cells were stored at 4°C.

For adherence measurement, strains were taken out either on YPD or CAA medium and grown at 30°C for 2 days. Single colony of a *C. glabrata* strain was inoculated in 10 ml CAA medium in a 100 ml culture flask and allowed to grow at 30°C for 16-20 h. 100 µl yeast culture was reinoculated in fresh 5 ml CAA liquid medium in a 15 ml polypropylene tube. 200 µCi of $S^{35}$(Met:Cys-65:25) INVIVO PROTWIN label mix (JONAKI, India) was added to the tube and cultures were grown at 30°C for 16-20 h for radiolabeling of *C. glabrata* cells. *C. glabrata* cells from 1 ml culture were harvested and washed three times with PBS to remove residual $S^{35}$(Met:Cys-65:25) labeling mix from medium supernatant. Next, cells were resuspended in 1 ml PBS. $OD_{600}$ was measured and cell suspensions of 0.5 $OD_{600}$ were prepared. PBS was aspirated out of the wells of 24-well plate containing fixed Lec-2 cells. 200 µl of $S^{35}$(Met:Cys-65:25)-labeled *C. glabrata* cell suspensions were added to each well. To determine the total amount of radioactivity present in labeled *C. glabrata* cell suspension, 200 µl of $S^{35}$(Met:Cys-65:25)-labeled *C. glabrata* cell suspensions were transferred to a scintillation vial containing scintillation fluid. Radioactive counts present in this fraction were considered as 'input values'. For measurement of yeast adherence to Lec-2 cells, plates were centrifuged at 1,000g for 5 min and incubated for 30 min at room temperature. Following incubation, each well

was washed three times with PBS to remove non-adherant *C. glabrata* cells and Lec-2 cells were lysed in 5% SDS. Lysates were transferred to tubes containing scintillation fluid and radioactive counts obtained were considered as 'output values'. Percentage adherence was determined using following formula.

$$\% \text{ Adherence} = \frac{\text{Output radioactive counts}}{\text{Input radioactive counts}} \text{ X } 100$$

**2.2.4.6 Colony blot assay**

Colony blot assay was performed to analyse secretion of carboxypeptidase Y (CPY) as described previously (Roberts *et al*., 1991). Single colony of a *C. glabrata* strain was inoculated in YPD medium and culture was grown till stationary phase. 0.1 $OD_{600}$ equivalent cells from this culture were spotted on CAA medium, overlaid with a nitrocellulose membrane and plate was incubated at 30˚C for 18-20 h. After incubation, nitrocellulose membrane was washed with water to remove cells and membrane-bound CPY was detected by immunoblotting with polyclonal anti-CPY antibody at a dilution of 1:10,000.

**2.2.4.7 Phosphatidyl inositol-3 kinase (PI-3 kinase assay)**

*In vitro* PI-3 kinase reactions were set up to measure PI-3P synthesized as described earlier (Whitman *et al*., 1988).

**2.2.4.7.1 Preparation of cell lysate**

A single colony of desired *C. glabrata* strain was inoculated in YPD-liquid medium and grown for 14-16 h. 50 µl overnight culture was inoculated in YPD-liquid medium for 4 h. Log-phase-grown yeast cells were harvested, washed with PBS and were inoculated at initial $OD_{600}$ of 2 and 4, into YNB-dextrose and YNB-sodium acetate liquid medium, respectively. After 4 h incubation, yeast cells were harvested by centrifugation at 2,500g for 5 min and treated with 1.2 M zymolyase for 1 h to obtain spheroplasts. Post zymolyase treatment, spheroplasts were resuspended in 100 µl resuspension buffer and an equal amount of 0.25 mm glass beads was added to lyse the spheroplasts. Using bead beater apparatus, spheroplasts were lysed and protein concentration in spheroplast lysates was determined using bicinchoninic acid assay (BCA) method and samples were stored at -20ºC till further use.

#### 2.2.4.7.2 Preparation and sonication of phosphatidylinositol-sodium salt solution

10 mg phosphatidylinositol-sodium salt (from *Glycine max*) was dissolved in 2 ml chloroform to prepare a 5 mg/ml stock solution. This solution was prepared in a small glass vial as chloroform is known to react with polypropylene. Small aliquots of stock solution were made and stored at -20ºC till further use. To avoid spillage due to vapor pressure, vials containing phosphatidylinositol-sodium salt solution were opened very carefully. To prepare sonicated phosphatidylinositol for one PI-3 kinase reaction, 2 µl of the stock phosphatidylinositol solution (10 µg) was transferred to a new 1.5 ml microcentrifuge tube. Using vacuum evaporator apparatus, chloroform was evaporated from the solution and phosphatidylinositol-sodium salt was resuspended in 5 µl sonication buffer. For sonication, a total of 20 pulses, each of 30 sec with 30 sec resting time were given on ice.

#### 2.2.4.7.3 PI-3 kinase reaction set up and phopsholipid extraction

PI-3 kinase reaction was set up in a total volume of 50 µl in a 1.5 ml microcentrifuge tube as described below.

PI-3 kinase reaction buffer = 25 µl
Spheroplast lysate = 20 µl (equivalent to 10 µg protein)
Sonicated phosphatidyl inositol = 5 µl

Reaction mix was incubated at 25ºC for 20 min and enzyme reaction was stopped by adding 80 µl HCL (1N) solution. To extract phospholipids, 160 µl chloroform:methanol (1:1) was added to the reaction mix with continuous mixing. Organic phase containing phopholipids was separated from aqueous phase by centrifugation at 7,500g for 4 min at 4ºC and transferred to a new vial. Using vacuum evaporator apparatus, solvent was evaporated and phospholipids were dissolved in 10 µl chloroform.

#### 2.2.4.7.4 Seperation of phospholipids by thin layer chromatography (TLC)

To resolve phospholipids, a TLC chamber was prepared by pouring 50 ml developing solution and sealing the chamber with aluminium foil so that developing solution can generate vapor. TLC silicagel-60 plate (Merck) was incubated at 80ºC for 4 h for activation. After 30 min of TLC chamber preparation, phospholipids extracted from *C. glabrata* cells were spotted at the bottom (1.5 cm from the lower end) of the

activated TLC silicagel-60 plate and transferred to the TLC chamber. After the solvent had migrated upwards (1.5 cm from the top), TLC plate was removed, air dried behind perspex shield, wrapped with cling plastic wrap and was exposed to phophorimager screen for 2 h. Phosphorimager screen was scanned using a Fugi-FLA 9000 scanner.