Identification of Components of the SUMOylation Machinery in Candida glabrata

ROLE OF THE DESUMOYLATION PEPTIDASE CgUlp2 IN VIRULENCE

Rahul Gujjula1,2, Sangeetha Veeraiah3, Kundan Kumar1, Suman S. Thakur4, Krishnaveni Mishra1, and Rupinder Kaur1,2

From the 1Centre for DNA Fingerprinting and Diagnostics, Building 7, Gruhalakpa, 5-4-399/B, Nampally, Hyderabad 500001, the 2Department of Biochemistry, School of Life Science, University of Hyderabad, Prof. C. R. Rao Road, Gachibowli, Hyderabad 500046, the 3Graduate Studies Program, Manipal University, Manipal, Karnataka 576104, and the 4Centre for Cellular and Molecular Biology, Habsiguda, Uppal Road, Hyderabad 500007, India

Regulation of protein function by reversible post-translational modification, SUMOylation, is widely conserved in the eukaryotic kingdom. SUMOylation is essential for cell growth, division, and adaptation to stress in most organisms, including fungi. As these are key factors in determination of fungal virulence, in this study, we have investigated the importance of SUMOylation in the human pathogen, Candida glabrata. We identified the enzymes involved in small ubiquitin-like modifier conjugation and show that there is strong conservation between Saccharomyces cerevisiae and C. glabrata. We demonstrate that SUMOylation is an essential process and that adaptation to stress involves changes in global SUMOylation in C. glabrata. Importantly, loss of the deSUMOylating enzyme CgUlp2 leads to highly reduced small ubiquitin-like modifier protein levels, and impaired growth, sensitivity to multiple stress conditions, reduced adherence to epithelial cells, and poor colonization of specific tissues in mice. Our study thus demonstrates a key role for protein SUMOylation in the life cycle and pathobiology of C. glabrata.

SUMOylation, the covalent reversible conjugation of small ubiquitin-like modifier (SUMO)5 polypeptide to lysine residues, often within the canonical consensus motif ΨKXE (Ψ and X represent a hydrophobic amino acid and any amino acid, respectively) in target proteins, is a post-translational modification that plays a key regulatory role in several cellular processes, including transcription, protein homeostasis, stress response, and development (1, 2). The process of SUMO attachment consists of the following four steps: (i) processing of the ~10-kDa precursor SUMO peptide by SUMO-specific proteases to reveal a C-terminal diglycine motif in the mature SUMO; (ii) ATP-dependent activation of the processed SUMO through the thioester bond formation between the C-terminal glycine of SUMO and the catalytic cysteine of the E1-activating enzyme; (iii) transfer of the SUMO polypeptide from the E1 enzyme to a conserved cysteine in the E2-conjugating enzyme via a thioester linkage; and (iv) E3 ligase-mediated formation of an isopeptide bond between the C-terminal glycine of the SUMO and the ε-amino group of the lysine residue within the conserved sequence on the target protein (2, 3). Besides the precursor SUMO maturation, the SUMO-specific peptidases are also able to hydrolyze the isopeptide bond between SUMO and SUMO-modified proteins thereby rendering the SUMOylation process reversible.

The SUMO polypeptide is ubiquitously present in all eukaryotes and highly conserved from yeast to mammals (1–3). SUMO modification of protein substrates has diverse functional consequences and range from increased protein stability to altered subcellular localization (1–3). Furthermore, deregulated expression of SUMOylation components has been implicated in several human diseases, including neurodegeneration, heart failure, cancer, diabetes, and infections by bacterial and viral pathogens (4, 5).

In the yeast Saccharomyces cerevisiae, SUMOylation machinery is composed of a single SUMO protein Smt3, SUMO-specific proteases Ulp1 and Ulp2, E1-conjugating heterodimeric complex Aos1-Uba2, E2-conjugating enzyme Ubc9, and four E3 ligases Siz1, Siz2, Cst9, and Mms21 (5–12). Of these proteins, Smt3, Ulp1, Aos1, Uba2, Ubc9, and Mms21 are essential for cell viability, and the substrate specificity is conferred by SUMO ligases (13, 14). Ulp2 is also known to be involved in the cleavage of poly-SUMO chains (15). SUMOylation affects about 5–10% of all yeast proteins (16–19), and SUMO-modified proteins have been implicated in ribosome function, septin ring dynamics, mRNA processing, and response to osmotic stress in S. cerevisiae (2, 12, 20, 21). Recently, SUMOylation has been shown to be required for thermal stress resistance in the pathogenic yeast Candida albicans (22).

Candida bloodstream infections, also known as candidemia, are a common occurrence in patients with immune dysfunctions and undergoing transplantation and radiation therapy.
Role of SUMOylation in the Pathobiology of Candida glabrata

TABLE 1
List of CAGLORFs identified whose orthologues in S. cerevisiae are involved in SUMOylation

| CAGLORF       | S. cerevisiae orthologue | % similarity | Known function in S. cerevisiae                              |
|---------------|--------------------------|--------------|--------------------------------------------------------------|
| SUMO activating enzymes |                        |              |                                                             |
| CAGL0G08080g   | AOS1                     | 76.44        | Subunit of a heterodimeric nuclear SUMO-activating enzyme, E1|
| CAGL0M16060g   | UBA2                     | 76.83        | Subunit of a heterodimeric nuclear SUMO-activating enzyme, E1|
| SUMO-conjugating enzymes |                     |              |                                                             |
| CAGL0M03267g   | MM221                    | 59.12        | SUMO ligase and component of the SMCS-SMC6 complex           |
| CAGL0L04290g   | SIZ2                     | 49.62        | SUMO E3 ligase                                              |
| CAGL0R02783g   | SIZ1                     | 41.96        | SUMO/Smt3 ligase                                            |
| CAGL0D08814g   | UBC9                     | 96.18        | SUMO-conjugating enzyme involved in the Smt3p conjugation pathway |
| SUMO protein   |                          |              |                                                             |
| CAGL0K05731g   | SMT3                     | 85.32        | Ubiquitin-like protein of the SUMO family                   |
| DeSUMOylation peptidases |                     |              |                                                             |
| CAGL0L08640g   | ULP1                     | 62.56        | Protease that specifically cleaves Smt3p protein conjugates |
| CAGL0K02464g   | ULP2                     | 45.88        | Peptidase that deconjugates Smt3/SUMO-1 peptides from proteins |

(23, 24). Candida often results in prolonged hospitalization in intensive care unit, high healthcare costs, and considerable morbidity and mortality (23). During last 2 decades, the incidence rate of candidemia has increased significantly with C. albicans being the most prevalent species followed by Candida glabrata (23, 25–27). C. glabrata accounts for up to 29% of total Candida bloodstream infections with a crude mortality rate of 40–45% (26–29).

Among the known virulence factors of C. glabrata, glycosylphosphatidylinositol-linked adhesins, cell surface-associated proteases, robust oxidative stress response, and the ability to form biofilms and evade immune response occupy central position (30). Although SUMOylation is known to be involved in the regulation of fungal development, differentiation, and virulence (22, 31), nothing is known about its role in the pathogenesis of C. glabrata. Here, using the reverse genetics approach, we have identified components of the SUMOylation machinery in C. glabrata and show that the deSUMOylation peptidase CgULP2 is required for biofilm formation, adhesion, and virulence of C. glabrata. We also report essentiality of C. glabrata Smt3 for cell growth and viability. Furthermore, we demonstrate for the first time a functional conservation of key SUMOylation components between S. cerevisiae and C. glabrata.

Results

Identification of Components of the SUMOylation Pathway in C. glabrata—To determine components of the SUMOylation pathway in C. glabrata, we performed whole proteome sequence and BLAST analyses, and identified C. glabrata orthologues of the proteins that are involved in SUMOylation in S. cerevisiae (Table 1). Orthologues of all the components could be identified, and their percent similarity across the complete sequence is shown in Fig. 1 and Table 1. SUMO and the E2 ligase Ubc9 protein are the most conserved between C. glabrata and S. cerevisiae with both showing over 85% identity (Table 1 and data not shown). Other SUMOylation components in both yeasts also showed significant sequence similarities as well as conserved architecture of various characterized domains (see Fig. 1 and data not shown). One striking difference was the absence of the SAP domain in the C. glabrata Siz1 ligase (see Fig. 1). The SAP domain, found in SIZ/PIAS family (Sap and Miz/protein inhibitors of activated STAT) of SUMO ligases, is implicated in DNA binding and nuclear retention (32).

In S. cerevisiae, deletion of the SAP domain results in reduced nuclear localization of the Siz1 ligase, whereas the full-length protein has a cell cycle-dependent localization. Although Siz1 is nuclear through most of the cell cycle stages, it relocates to the bud neck during cytokinesis, as SUMOylation of septins and other substrates at this stage is essential for completion of cell division (11, 14, 33, 34). Siz1 relocation is dependent on the C-terminal domain, whereas its nuclear retention is contingent on the SAP domain (32). Therefore, loss of this well conserved domain in CgSiz1 ligase raises the possibility of additional C. glabrata-specific SUMO substrates outside the nucleus.

Functional Conservation between C. glabrata and S. cerevisiae SUMO Components—Given the strong conservation of sequence and architecture of all components of the SUMOylation pathway in C. glabrata, we next examined whether some of the key components would functionally complement the S. cerevisiae mutants. For the two essential genes tested, SMT3 and ULP1, we performed plasmid shuffling experiments in S. cerevisiae knock-outs. First, we transformed the S. cerevisiae smt3Δ strain carrying the ScSMT3 gene on a URA3-based vector either with a LEU2-based plasmid expressing the CgSMT3 gene from the ADH1 promoter (CKM 379) or with the LEU2-based vector alone. The double transformants were streaked on plates containing 5-fluoroorotic acid (5-FOA) to select for ura-cil auxotrophs. We found that the S. cerevisiae smt3Δ strain expressing CgSMT3 survived, but the strain carrying the empty vector did not (Fig. 2A). This establishes that the Scsmt3Δ mutant could be complemented by CgSMT3, and therefore it could lose the ScSMT3-expressing plasmid. Through similar plasmid shuffle experiments, we next showed that CgULP1 could also complement the Sculp1Δ mutant (Fig. 2B). The ULP2 gene is not essential for cell viability in S. cerevisiae, but its deletion results in growth retardation (35). As shown in Fig. 2C, slow growth of the S. cerevisiae ulp2Δ mutant was rescued by ectopic expression of the CgULP2 gene indicative of a functional conservation between S. cerevisiae and C. glabrata Ulp2 deSUMOylase. However, CgULP2 could not complement the telomeric silencing defect of the Sculp2Δ mutant as CgULP2-expressing Sculp2Δ cells did not grow on medium containing 5-fluoroorotic acid (see Fig. 2C). These cells carry URA3 at the subtelomeric region of chromosome VII and would exhibit...
### Saccharomyces cerevisiae

| Gene   | Accession  |
|--------|------------|
| AOS1 (347) | CAGL0G09889g (336) |
| ThiF : 16 - 345 | ThiF : 12 - 335 |
| UBA2 (636) | CAGL0M01606g (632) |
| ThiF : 2 - 437 | ThiF : 4 - 442 |
| UBA e1 thiolCys : 246 - 363 | UBA e1 thiolCys : 248 - 368 |
| MMS21 (267) | CAGL0M03267g (266) |
| zf-Nse : 171 - 227 | zf-Nse : 174 - 230 |

### Candida glabrata

| Gene   | Accession  |
|--------|------------|
| NFII (726) & SIZ1 (904) | CAGL0L04290g (754) & CAGL0F02783g (839) |
| PINIT : 152 - 289 | PINIT : 157 - 294 |
| zf-MIZ : 334 - 383 | zf-MIZ : 338 - 387 |
| SAP : 43 - 77 | SAP : 18 - 52 |
| SIZ1 | CAGL0F02783g |
| PINIT : 174 - 312 | PINIT : 148 - 294 |
| zf-MIZ : 357 - 406 | zf-MIZ : 339 - 388 |
| SAP : 34 - 68 | |
| UBC9 (157) | CAGL0D00814g (157) |
| UQ_con : 8 - 152 | UQ_con : 8 - 151 |
| SMT3 (101) | CAGL0K05731g (108) |
| Rad60-SLD : 22 - 92 | Rad60-SLD : 30 - 100 |
| ULP1 (621) | CAGL0L08646g (588) |
| Peptidase_C48 : 447 - 620 | Peptidase_C48 : 414 - 583 |
| ULP2 (1034) | CAGL0J02464g (916) |
| Peptidase_C48 : 456 - 675 | Peptidase_C48 : 450 - 675 |

**FIGURE 1.** *C. glabrata* orthologues of genes encoding components of the SUMOylation pathway. *S. cerevisiae* proteins were retrieved from Saccharomyces Genome Database, and their orthologues in *C. glabrata* were identified using Blastp. The protein sequences were scanned for annotated domains using Pfam and HMMER. Maps of proteins along with their domains were generated using DOG.
growth on FOA medium only if URA3 expression was suppressed due to the telomere position effect. As expected, *S. cerevisiae* wild-type strain harboring the URA3 gene at the subtelomeric locus exhibited robust growth on plates containing 5-FOA (see Fig. 2C). These data suggest that CgULP2 cannot complement all functions of ScULP2.

Of note, we could not perform complementation studies with *C. glabrata* UBA2, AOS1, UBC9, and MMS21 genes because the corresponding heterozygous *S. cerevisiae* mutants sporulated extremely poorly (data not shown).

**Subcellular Localization of *C. glabrata* SUMO Enzymes**—In *S. cerevisiae*, SUMOylated proteins are mostly nuclear with very few proteins SUMOylated in the cytosol or other subcellular compartments. This is because most of the SUMO ligases are found in the nucleus except Siz1, which localizes to the septin ring during cell division (13, 36). To examine whether the location of SUMO enzymes was conserved between *C. glabrata* and *S. cerevisiae*, we introduced the dual His-FLAG tag sequence into *C. glabrata SMT3*, SIZ1, SIZ2, ULP1, and ULP2 gene sequences, and we transformed these plasmids into either wild-type strain (for essential genes) or the respective *C. glabrata* deletion strains. Immunofluorescence analysis with anti-FLAG antibody revealed that CgSmt3 was localized uniformly across the cell suggesting that SUMO and/or SUMOylated proteins are found both in the cytosol and the nucleus (Fig. 3). The two SUMO ligases, CgSiz1 and CgSiz2, and the desumoylating enzymes, CgUlp1 and CgUlp2, were all predominantly nuclear with only CgSiz1 and CgUlp1 showing few cytosolic spots (see Fig. 3). Interestingly, the characteristic nuclear pore complex localization of ScUlp1 was not observed in CgUlp1. Thus, despite uniform distribution of SUMOylated proteins across the cell, the enzymes involved in SUMOylation and deSUMOylation are predominantly nuclear in *C. glabrata*. These findings suggest that localization of most components of the SUMOylation pathway in *C. glabrata* is similar to that in *S. cerevisiae*.

**Disruption of *C. glabrata* ORFs That Are Potentially Involved in SUMOylation in *C. glabrata***—To examine the effect of perturbation of SUMOylation machinery on physiology and pathogenesis of *C. glabrata*, we sought to generate strains lacking one or more SUMO components. Of the set of nine genes shown in Table 1, we were able to create deletion strains for CgSIZ1, CgSIZ2, and CgULP2 genes via a homologous recombination-based strategy. We also generated a double deletion strain for CgSIZ1 and CgSIZ2 genes to investigate the role of SUMO ligases in *C. glabrata* pathobiology. Despite several attempts, we could not delete CgAOS1, CgUBA2, CgUBA9, CgMMS21, CgULP1, and CgSMT3 ORFs. Notably, *S. cerevisiae* was unable to grow on plates containing 5-FOA unless the URA3 gene was suppressed. Therefore, the disruption of *C. glabrata* URA3 expression was required for growth on plates containing 5-FOA.
orthologues of these six genes are essential for cell viability (5–10). Furthermore, using the one-step disruption and plasmid loss methodologies, we could show that CgSMT3 is required for growth in vitro in C. glabrata (data not shown).

To investigate the role of SUMOylation in cell physiology, we conducted a comprehensive phenotypic characterization of the C. glabrata mutants generated. Growth analysis of Cgsiz1Δ, Cgsiz2Δ, Cgsiz1Δsiz2Δ, and Cgulp2Δ mutants revealed that the Cgulp2Δ mutant grew about 18% slower than the wild-type (WT) strain in rich medium (Fig. 4A). The Cgulp2Δ mutant exhibited highly attenuated growth at 42 °C and in medium containing caffeine and non-fermentable carbon sources (glycerol, oleic acid, and ethanol) (Fig. 4, B and C). Compared with WT cells, the Cgulp2Δ mutant was more susceptible to the DNA-alkylating agent methyl methanesulfonate (MMS), replication fork staller hydroxyurea, thymine dimer-inducing ultraviolet (UV) radiation, and oxidative stress-inducing agent hydrogen peroxide (see Fig. 4B). Elevated sensitivity of the Cgulp2Δ mutant to aforementioned stresses was complemented by ectopic expression of the CgULP2 gene, indicating that these effects were specifically induced by the absence of CgULP2 (see Fig. 4B). Contrary to the Cgulp2Δ mutant, growth of the Cgsiz1Δ mutant remained unaffected under diverse stressful conditions, viz. DNA damage (MMS, camptothecin, and hydroxyurea), oxidative (hydrogen peroxide and menadione), thermal (37 and 42 °C), varied pH values (low (2.0) and neutral (7.0) pH), cell wall (caffeine), cell membrane SDS, non-fermentable carbon sources, and antifungal drug (fluconazole and caspofungin) stresses (see Fig. 4, B and C and data not shown). Interestingly, the Cgsiz2Δ and Cgsiz1Δsiz2Δ mutants displayed sensitivity to both UV and MMS, which could be rescued by ectopic expression of the CgSIZ2 gene (see Fig. 4B). Expression of CgSIZ1 partially restored slow growth of the Cgsiz1Δsiz2Δ mutant in MMS-supplemented medium and upon UV treatment (see Fig. 4B). Altogether, these data implicate CgSiz2 and CgUlp2 in survival of DNA damage and thermal, oxidative, and DNA damage stresses, respectively.

Disruption of CgULP2 Rendered C. glabrata Cells Hypoadherent to Lec2 Ovary Epithelial Cells—During our phenotype profiling analysis, we noticed that about 5% of the Cgulp2Δ mutant population displayed elongated pseudohypha-like structures after 48 h of growth in the YPD medium (Fig. 5A). To investigate whether this change in morphology is due to altered cell wall composition, we examined sensitivity of the Cgulp2Δ mutant to digestion with zymolyase, which hydrolyses β-glucan in the cell wall. The fungal cell wall is a complex and dynamic structure and consists of an inner layer of β-glucan-chitin complex and an outer layer of heavily O- and N-glycosylated mannoproteins (37). Compared with WT cells, the Cgulp2Δ mutant displayed resistance to zymolyase digestion, which was reversed upon ectopic expression of the CgULP2 gene in the mutant (Fig. 5B). Furthermore, cell wall chitin analysis revealed 1.5-fold elevated chitin content in the Cgulp2Δ mutant compared with WT cells (Fig. 5C). Consistently, staining with calcofluor white, which binds to chitin in the cell wall, showed a diffused signal along the cell wall in the Cgulp2Δ mutant compared with bud scar-limited staining of WT cells.
Overall, these findings are indicative of an altered cell wall architecture in the \textit{Cgulp2Δ}/H9004 mutant.

Cell wall integrity in \textit{C. glabrata} is maintained by protein kinase C (PKC)-mediated signal transduction pathway, and mutants with cell wall defects have previously been reported to exhibit constitutively activated PKC signaling cascade (38). To examine the status of the PKC-dependent signaling pathway in the \textit{Cgulp2Δ} mutant, we checked phosphorylation levels of \textit{CgSlt2}, which is the terminal mitogen-activated protein kinase (MAPK) of the PKC pathway, in the mutant. As shown in Fig. 5D. Overall, these findings are indicative of an altered cell wall architecture in the \textit{Cgulp2Δ} mutant.

FIGURE 4. \textit{CgUlp2} is required for survival of thermal and DNA damage stress. \textit{A}, growth curve analysis. Overnight-grown cultures of indicated \textit{C. glabrata} strains were inoculated into the YPD medium to an initial \textit{A}_600 of 0.1. Absorbance at 600 nm was recorded over a 48-h time course at indicated time intervals. Data represent mean ± S.E. of three to five independent experiments. Doubling times were calculated during the exponential phase of growth and are presented on the bottom right side of the graph. Statistical analysis was performed using an unpaired, two-tailed, Student’s \textit{t}-test (*, \textit{p} < 0.05). \textit{B}, serial dilution-spotting assay to assess the growth under indicated stress conditions. 3 µl of 10-fold serial dilutions of overnight-grown and 1.0 \textit{A}_600-normalized cultures of indicated \textit{C. glabrata} strains were spotted on different media. Plate images were captured after 2 days of incubation at 30 °C. Methylmethanesulfonate (MMS), hydroxyurea (HU) and hydrogen peroxide (H$_2$O$_2$) were used at the concentrations of 0.03%, 50 mM, and 50 mM, respectively. To check sensitivity to ultraviolet radiation, 3 µl of 10-fold serial culture dilutions was spotted on the YPD medium, and the plate was exposed to 40 J/m$^2$ UV radiation at 256 nm before incubation at 30 °C. \textit{C}, heat map illustrating cell growth in the presence of diverse stress-causing agents. 3 µl of 10-fold serial dilutions of overnight YPD medium-grown and 1.0 \textit{A}_600-normalized cultures of indicated \textit{C. glabrata} strains were spotted on different media. Growth profiles, recorded after 1–8 days of incubation, are color-coded and indicated at the bottom. Columns correspond to various growth condition mutants and rows to mutants. Stressful conditions used were as follows: DNA damage stress (camptothecin; 10 mM); cell wall stress (caffeine; 10 mM); antifungal stress (fluconazole (FLC; 16 µg/ml) and caspofungin (75 ng/ml)); membrane stress (SDS; 0.005%); varied pH stress (pH 2.0 and 7.0); and utilization of alternative carbon sources (glycerol (3%), ethanol (2%), and sodium acetate (2%)).

(Fig. 5D). Overall, these findings are indicative of an altered cell wall architecture in the \textit{Cgulp2Δ} mutant.
FIGURE 5. Cell wall architecture is altered in the Cgulp2Δ mutant. A, Cgulp2Δ mutant displayed pseudohyphal structures. Representative DIC of WT and Cgulp2Δ mutant grown in the YPD medium for 72 h. B, zymolyase digestion assay. Log phase cultures of indicated C. glabrata strains were treated with 50 μg/ml zymolyase, and absorbance at 600 nm was recorded at regular time intervals. Initial A<sub>600</sub> of the cultures was considered as 100%, and data (means of three (Cgsiz1ΔΔ/Cgsiz2 and Cgulp2Δ/V strains), four (Cgsiz1ΔΔ/V and Cgsiz1ΔΔ/Cgsiz1 strains), and five (WT/V and Cgulp2Δ/CgULP2 strains) independent experiments) are plotted as percentage of the starting A<sub>600</sub>. Statistical analysis was performed using an unpaired, two-tailed, Student's t test (*, p < 0.05). Statistically significant differences between WT and the Cgulp2Δ mutant are marked. C, cell wall chitin measurement. Log phase cultures of indicated C. glabrata strains were stained with 25 μg/ml calcofluor white (CFW), and fluorescence intensity was measured by flow cytometry. Signal intensity mean values ± S.E. represent data from three independent experiments. Unpaired, two-tailed, Student's t test is shown (***, p < 0.001). Statistically significant differences between WT and the Cgulp2Δ mutant are marked. D, representative confocal images illustrating calcofluor white (CFW)-stained cell walls of log phase cultures of WT and Cgulp2Δ mutant. Scale bar, 10 μm; DIC, differential interference contrast. E, Western blotting of CgSlt2 phosphorylation in indicated C. glabrata strains grown in YPD medium, YPD medium containing 16 μg/ml fluconazole (FLC), and 10 mM caffeine for 4 h at 30 °C. Whole cell protein extracts were prepared by glass bead lysis and quantified using the Thermo Scientific Pierce BCA protein assay kit. 30 μg of protein was separated on SDS-PAGE, and Western blots were developed with an anti-phospho-ERK1/2 MAPK (Thr<sup>202</sup>/Tyr<sup>204</sup>) antibody. Individual band intensity was quantified using the ImageJ software. CgSlt2 phosphorylation signal was normalized to the corresponding CgGapdh signal in each lane, and results, representative of at least three independent experiments, are presented as fold change (± S.E.) in phosphorylation levels compared with CgSlt2 phosphorylation in YPD-grown wild-type cells (considered as 1).
Role of SUMOylation in the Pathobiology of Candida glabrata

5E, the Cgulp2Δ mutant exhibited high levels of phosphorylated CgSlt2 compared with wild type and complemented strains indicating a constitutively active PKC-mediated cell wall integrity pathway. Furthermore, consistent with earlier studies, C. glabrata WT cells responded to the antifungal fluconazole and the cell wall stressor caffeine by activating the PKC cascade (see Fig. 5E). However, caffeine treatment, instead of activating the PKC pathway, resulted in down-regulation of CgSlt2 phosphorylation in the Cgulp2Δ mutant (see Fig. 5E). Of note, the Cgulp2Δ mutant was fully proficient in activation of the PKC pathway upon fluconazole exposure (see Fig. 5E). These data indicate that lack of CgUlp2 adversely affects the ability of C. glabrata cells to respond to the cell wall stressor caffeine. Importantly, these findings are in accordance with regular and elevated sensitivity of the Cgulp2Δ mutant to fluconazole and caffeine, respectively (see Fig. 4C).

Next, to investigate the effect of altered cell wall structure on the adhesion capacity of the Cgulp2Δ mutant, we examined the ability of Cgulp2Δ to adhere to Lec2 ovary epithelial cells. As a control, adherence assays were also carried out with Cgsiz1Δ, Cgsiz2Δ, and Cgsiz1Δsiz2Δ mutants, all of which displayed no detectable cell wall abnormalities (see Fig. 5, B and C). As shown in Fig. 6A, the Cgulp2Δ mutant displayed 2-fold less adherence to epithelial cells compared with that of the WT cells, which was again restored back to WT levels in the Cgulp2Δ-complemented strain. The hypo-adherence of the Cgulp2Δ mutant was unexpected and found to be, in part, due to a 3–4-fold reduced expression of two epithelial adhesin-encoding genes EPA1 and EPA6 in the mutant (Fig. 6B). Notably, Epa1 and Epa6 belong to a family of at least 23 cell wall adhesins that mediate adherence of C. glabrata cells to host epithelial cells (39–41). Epa6 has also been shown to be pivotal to biofilm formation in vitro (42). To examine the effect of EPA6 transcript levels on biofilm formation, we measured the ability of WT, Cgulp2Δ, and Cgulp2Δ-complemented strains to make biofilm on polystyrene-coated plates. We observed that the CgUlp2 disruption led to a 50% reduction in the biofilm formation capacity of C. glabrata cells (Fig. 6C). Collectively, these data indicate that CgUlp2 plays a role in regulated expression of adhesin-encoding genes, biofilm formation, and maintenance of cell wall architecture.

Perturbation of SUMOylation Affects Growth of C. glabrata—SUMOylation of protein targets is regulated in part by recruitment of SUMO ligases and isopeptidases to specific subcellular sites at specific instances (13, 43). Therefore, we tested the effect of an additional 1–2 copies of E3 SUMO ligases and isopeptidases on the physiology of C. glabrata by transforming WT cells with the pRK74 plasmid expressing CgSIZ1, CgSIZ2, CgMMS21, CgUlp1, or CgUlp2 genes from the PGK1 promoter. We found that although additional copies of CgSIZ1, CgMMS21, CgUlp1, or CgUlp2 genes had no measurable effect on growth (data not shown), an additional copy of the CgSIZ2 gene resulted in perturbed growth (Fig. 7). This effect was specific to the minimal medium as C. glabrata WT cells carrying CgSIZ2-expressing plasmid exhibited normal growth on the YPD medium (see Fig. 7). Intriguingly, additional copy of CgSIZ2 also rendered cells sensitive to MMS, again in the minimal medium (see Fig. 7). Because the CgUlp2 deletion also caused similar phenotypes, albeit in all media, including YPD,

FIGURE 6. Cgulp2Δ mutant displayed reduced adherence to the Lec2 epithelial cells. A, adherence of CAA medium-grown, 35S-labeled (Met/Cys, 65:25) C. glabrata strains to formaldehyde-fixed Lec-2 ovary epithelial cells. Data represent means ± S.E. of three to five independent experiments. Unpaired, two-tailed, Student’s t test (***, p ≤ 0.001). B, quantitative PCR analysis of EPA1 and EPA6 gene expression in wild-type and Cgulp2Δ mutant. Data (mean of three independent experiments ± S.E.) were normalized to an internal CgGAPDH mRNA control and represent fold change in expression upon CgUlp2 disruption. Paired two-tailed, Student’s t test (**, p = 0.01) is shown. C, biofilm formation of indicated C. glabrata strains. Cells were grown in RPMI 1640 medium containing 10% FBS for 48 h in a polystyrene 24-well plate. Cells were stained with crystal violet (0.4% in 20% (v/v) ethanol solution) for 45 min followed by complete destaining with 95% ethanol. Absorbance at 595 nm was recorded to measure the amount of the crystal violet stain in ethanol. Data represent means ± S.E. of three independent experiments. ***, p ≤ 0.01; two-tailed paired Student’s t test.
(see Figs. 4B and 7), we speculate that this may either be due to the increased SUMOylation of a critical substrate by CgSiz2 or absence of deSUMOylation of that substrate in the C gulp2Δ mutant.

**Global SUMOylation Pattern Is Altered in C. glabrata Mutants Lacking SUMO Ligases and deSUMOylase**—To detect the SUMO proteome of C. glabrata, we tagged the CgSmt3 protein with His6 and FLAG epitopes at the N terminus (hereafter referred to as “dual tagged Smt3”) and performed Western blots on whole cell extracts of the Cgsmt3/H9004 mutant complemented either with CgSmt3 or dual tagged CgSmt3 using anti-FLAG antibody. The dual tagged CgSmt3 was able to complement the viability defect of the Cgsmt3Δ mutant. As seen in Fig. 8A, we could detect multiple proteins only in the presence of FLAG-labeled CgSmt3 confirming that the tagged CgSmt3 was conjugated to cellular proteins. Although global protein SUMOylation was reduced in Cgsiz1Δ and Cgsiz2Δ mutants, the Cgsiz1Δsiz2Δ double mutant had hardly any detectable SUMOylated proteins (see Fig. 8A). Furthermore, the presence of differentially SUMOylated proteins in Cgsiz1Δ and Cgsiz2Δ mutants is indicative of substrate specificity of CgSiz1 and CgSiz2 ligases (see Fig. 8A). Unexpectedly, in the C gulp2Δ mutant, we could detect neither SUMOylated proteins nor free SUMO. Next, we examined the SUMOylation pattern in C. glabrata WT cells expressing additional copies of CgSiz1, CgSiz2, CgUlp1, and CgUlp2 enzymes. CgSiz1 or CgSiz2 hyper-expression led to increased protein SUMOylation, with CgSiz2 being more effective than CgSiz1 with several high molecular weight SUMOylated proteins (Fig. 8B). Interestingly, elevating the dosage of the SUMO peptidases, CgUlp1 and CgUlp2, did not reduce the SUMOylated proteins (see Fig. 8B) suggesting that deSUMOylation is very well regulated.

The result that the C gulp2Δ mutant contains neither any SUMOylated proteins nor free SUMO is intriguing. We expected to see accumulation of SUMO-conjugated proteins in the absence of a deSUMOylase. There are two possible explanations for this observation as follows: first, as in Aspergillus nidulans (31), CgUlp2 is the SUMO-processing enzyme, and therefore, in its absence, no SUMOylation can be detected. Alternatively, increased accumulation of polySUMOylated proteins in the C gulp2Δ mutant leads to their degradation by the SUMO-dependent ubiquitination pathway. To test whether CgUlp2 is required for SUMO-processing, we made a CgSMT3 construct that encodes SMT3 without the last four amino acids and terminating with the diglycine motif that could be directly used for conjugation to substrates. This construct was expressed in C gulp2Δ mutant cells. We found that expressing mature SUMO improved the growth...
Role of SUMOylation in the Pathobiology of *Candida glabrata*

FIGURE 9. Severity of the *Cgulp2Δ* mutant phenotype is partly reduced by mature SUMO. A, copy of either empty vector or full-length *CgSMT3* (SMT3) or *CgSMT3* encoding mature SUMO (mSMT3) was introduced in the *Cgulp2Δ* mutant and WT cells. Cells were tested for growth at non-permissive temperatures and plates containing MMS as indicated by spotting 5 μl of 10-fold serially diluted cultures. Improved survival of the *Cgulp2Δ* mutant could be observed in both conditions. B, *Cgst3Δ* (YRK1022) was transformed with either dual-tagged SMT3 (1 and 2; pCKM405) or dual-tagged m-SMT3 (3 and 4; pCKM469) and tested for complementation of SMT3 function by loss of WT-*CgSMT3*. YPD-Nat growth indicates presence of the pCN-PDC1 plasmid, and growth on 5-FOA plates indicates ability to lose the WT-*CgSMT3* carried on pGRB2.2 plasmid in the *Cgst3Δ* strain. C, total protein extracts were made from indicated *C. glabrata* strains, and the SUMOylation pattern was analyzed as described above. The mature SUMO but not the full-length SUMO could be detected clearly in the *Cgulp2Δ* mutant. Porceau S-stained membrane is displayed as a loading control. D, indicated *C. glabrata* strains were grown overnight in the YNB medium containing 0.1% proline as a sole nitrogen source at 30 °C and 200 rpm. Cells were harvested; *A*₆₀₀ was adjusted to 0.5 with fresh media containing 0.003% SDS and incubated for 4 h. Cultures were transferred into a 96-well plate containing 100 μl of YNB-proline media and 300 μM MG132 and incubated at 30 °C at 175 rpm. After a 2 h incubation, MMS (0.045%) was added to the media and grown for 3 h. Cultures were 10-fold serially diluted, and a 3 μl volume was spotted on YPD medium. Plate images were captured after 48 h of incubation at 30 °C.

of *Cgulp2Δ* measurably but not up to wild-type levels (Fig. 9A). Similarly, it also conferred improved resistance to MMS; in both cases, merely adding additional copies of full-length *CgSMT3* did not improve growth (see Fig. 9A). We confirmed that the mature SUMO complemented the *Cgst3Δ* phenotype using the plasmid shuffle assay described earlier (Fig. 9B). Western blot analyses revealed that now we could detect free SUMO and a very slight increase in the SUMO proteome (Fig. 9C). These observations together suggest that either providing mature SUMO improves the stability of critical SUMOylated proteins or having mature free SUMO improves survival by non-covalent associations.
Role of SUMOylation in the Pathobiology of Candida glabrata

C. glabrata cells encounter several stressful conditions in the internal milieu of macrophages, including the nutrient-limiting reactive oxygen species-generating environment (30). To gain insights into whether intracellular survival/proliferation of C. glabrata cells in macrophages involves SUMO proteome modifications, we performed co-incubation assays. Macrophages derived from the human monocytic cell line THP-1 were co-incubated for 8 h with dual tagged SMT3-expressing C. glabrata cells, and the internalized C. glabrata were harvested. Total protein from these yeast cells was compared with RPMI 1640 medium-grown C. glabrata cells for any changes in SUMO proteome. As seen in Fig. 10B, in comparison with RPMI 1640 medium-grown C. glabrata cells, macrophage-internalized C. glabrata cells showed several prominently SUMOylated proteins. This establishes that to survive and multiply within macrophages, several C. glabrata proteins are SUMOylated.

CgUlp2 Is Required for Virulence in the Murine Model of Systemic Candidiasis—Next, to examine the interaction of C. glabrata strains with altered levels of SUMOylated proteins with host immune cells, we infected human THP-1 macrophages with wild type, Cgsiz1Δ, Cgsiz2Δ, Cgsiz1Δsiz2Δ, and Cgulp2Δ mutants, and we studied their intracellular growth behavior via colony-forming unit (CFU) assay. Of note, C. glabrata wild-type cells are known to multiply in macrophages (30). All strains were phagocytosed by macrophages at the same rate of ~70–85% (wild type (73%), Cgsiz1Δ (84%), Cgsiz2Δ (74%), Cgsiz1Δsiz2Δ (80%), and Cgulp2Δ (71%)). However, compared with 4–6-fold intracellular replication of WT and Cgsiz mutants, the Cgulp2Δ mutant exhibited no increase in CFUs after 24 h of co-incubation with THP-1 macrophages (Fig. 11A). A 24-h time course analysis in the RPMI 1640 medium revealed 6-fold lower CFUs for the Cgulp2Δ mutant (Fig. 11B) suggesting that the impaired intracellular proliferation of the mutant could be due to both the diminished capacity to grow under tissue culture conditions and the elevated susceptibility to oxidative stress. Notably, C. glabrata strains expressing additional copies of Cgsiz1Δ, Cgsiz2Δ, CgUlp1, and CgUlp2 genes were able to undergo 5–6-fold multiplication in THP-1 macrophages (WT/Cgsiz1Δ (6.1-fold), WT/Cgsiz2Δ (4.5-fold), WT/CgUlp1 (5.7-fold), and WT/CgUlp2 (5.6-fold)), indicating that increased dosage of SUMO ligases and deSUMOylases had no effect on intracellular proliferation.

Next, to investigate whether components of the SUMOylation machinery are required for virulence of C. glabrata, we examined fungal burden in BALB/c mice infected intravenously either with the wild-type or the Cgsiz1Δsiz2Δ and Cgulp2Δ mutant strains. Approximately, 10- and 8-fold lower yeast CFUs were recovered from the kidneys and liver, respectively, of the mice infected with the Cgulp2Δ mutant compared with CFUs retrieved from corresponding organs of the WT-infected mice (Fig. 11, C and D). Ectopic expression of the CgUlp2 gene restored the organ fungal burden in the Cgulp2Δ-infected mice (see Fig. 11, C and D). Of note, no statistically significant differences in the fungal burden were seen between the spleen of WT- and Cgulp2Δ-infected mice (Fig. 11E).
Importantly, statistically similar yeast CFUs were obtained from all three target organs of WT- and Cgsiz1Δsiz2Δ-infected mice (see Fig. 11, C–E). Together, these data indicate an organspecific role for the CgUlp2 deSUMOylase and dispensability of CgSiz1 and CgSiz2 SUMO ligases in survival of C. glabrata in the murine model of disseminated candidiasis.

**Discussion**

In this work, we have initiated studies to understand the importance of protein SUMOylation in the pathobiology of C. glabrata. First, we identified components of the SUMOylation machinery in C. glabrata based on homology with S. cerevisiae. Second, we performed complementation studies in S. cerevisiae to confirm the predicted activities. Third, we generated C. glabrata deletion strains for non-essential SUMOylation genes and examined the effects of perturbed SUMOylation on stress response and survival in vivo. Finally, we demonstrated the essentiality of CgSmt3 for cell growth in C. glabrata. Our studies firmly establish that SUMOylation, like in S. cerevisiae, is essential in C. glabrata. This is different
from other yeast/fungi like Schizosaccharomyces pombe, A. nidulans, and C. albicans, where SMT3 is not essential for survival (22, 31, 44). We also speculate that the essential SUMO conjugation in C. glabrata may be carried out by the CgMms21 because the Cgsiz1siz2Δ mutant is viable (see Fig. 4A) and the CgMms21Δ mutant could not be generated. In addition, we could not create knock-outs for genes encoding SUMO-processing enzyme CgUlp1, SUMO-activating enzymes CgAOS1 and CgUBA2, and SUMO-conjugating enzyme CgUBC9, suggesting that these, like in S. cerevisiae, could also be essential in C. glabrata.

An initial examination of the SUMO proteome in C. glabrata revealed several proteins to be SUMOylated. Lack of SUMO ligases, CgSiz1 and CgSiz2, led to loss of the majority of SUMOylation (see Fig. 8A), although their increased dosage resulted in accumulation of additional SUMO-conjugated proteins (see Fig. 8B). Interestingly, we observed several high molecular weight SUMOylated proteins with additional copies of CgSiz2 (see Fig. 8B) suggesting that, as in S. cerevisiae (45), CgSiz2 may also be involved in polySUMOylation in C. glabrata. SUMOylation pattern studies in the Cgulp2Δ mutant revealed intriguing results as, surprisingly, we could neither detect SUMOylated proteins nor the SUMO protein in the mutant (see Fig. 8A). We reasoned that there maybe two possible explanations for this observation as follows. First, CgUlp2 is the SUMO-processing enzyme and therefore, in the absence of mature SUMO, no sumoylation takes place. Second, in the Cgulp2Δ mutant, SUMOylated proteins accumulate and are targeted for degradation by the SUMO-dependent ubiquitination pathway. Our experiments to test these possibilities suggest that CgUlp2 is unlikely to be the processing enzyme because introduction of the processed SUMO does not completely alleviate Cgulp2Δ phenotypes. Similarly, inhibition of proteasome also does not completely rescue the phenotype. However, both treatments improve Cgulp2Δ growth perceptibly suggesting that tilting the balance toward retaining SUMOylated proteins can improve Cgulp2Δ phenotypes. It further suggests that lack of a SUMOylated protein rather than deSUMOylating a critical protein(s) as a cause for Cgulp2Δ mutant phenotypes cannot be ruled out.

Three lines of evidence indicate an important role for SUMOylation in cellular stress response in C. glabrata. First, perturbing SUMOylation by deleting SUMO ligases CgSiz1 and CgSiz2 or deSUMOylating enzyme CgUlp2 resulted in diminished survival under different stress conditions (see Fig. 4, B and C). Second, C. glabrata cells respond to a changing environment, including ethanol stress and macrophage internal milieu by SUMOylating multiple cellular proteins (see Fig. 10). Third, the C. glabrata mutant disrupted for CgUlp2 exhibited altered cell morphology and cell wall architecture and the constitutively active terminal MAPK (CgSlt2) of the cell wall integrity signaling pathway (see Fig. 5). These findings along with strong survival defects of the Cgulp2Δ mutant in specific tissues in the disseminated candidiasis model (see Fig. 11, C–E) indicate that SUMOylation is pivotal to stress response in C. glabrata.

The fungal cell wall is a dynamic organelle, and any alterations to its core constituents (β-glucan, chitin, and mannoproteins) result in the activation of compensatory mechanism(s) (37). The Cgulp2Δ mutant contained elevated levels of chitin in the cell wall (Fig. 5C). Importantly, an increase in the cell wall chitin content has previously been associated with reduced susceptibility to the β-1,3-glucan synthesis-targeting echinocandin antifungals and diminished virulence in C. albicans (46, 47). However, despite elevated chitin levels, the Cgulp2Δ mutant was found to not be resistant to caspofungin (see Fig. 4C). Of note, C. glabrata mutants with perturbed SUMOylation (Cgsiz1Δ, Cgsiz2Δ, Cgsiz1ΔCgsiz2Δ, and Cgulp2Δ) did not exhibit altered sensitivity to the ergosterol biosynthesis inhibitor, fluconazole, either (see Fig. 4C). Consistently, cellular SUMOylation status remained unaffected upon exposure of C. glabrata cells to fluconazole (see Fig. 10A) suggesting that SUMOylation is dispensable for response of C. glabrata cells to azole and echinocandin antifungal drugs.

An important feature of this work is the strong conservation of the SUMOylation pathway between S. cerevisiae and C. glabrata. Similar to S. cerevisiae (48, 49), SUMOylation appears to be involved in the regulation of the telomere position effect in C. glabrata. Adherence to the host tissue, an important virulence attribute of C. glabrata, is mediated by a large family of cell wall proteins, including Epa adhesins (30, 40). The majority of the adhesin-encoding EPA genes in C. glabrata are transcriptionally silenced due to their close proximity to telomeres (40–42). SUMOylation is a reversible process, and the tight regulation of SUMO conjugation and SUMO removal is probably necessary to fine-tune substrate functions (1, 2). Our finding that a lack of the CgUlp2 deSUMOylase resulted in further repression of EPA1 and EPA6 expression and hypoadherence (see Fig. 6, A and B) indicates that removal of the SUMO modification from component(s) of the subtelomeric silencing machinery is probably pivotal to the expression of subtelomeric genes. This is particularly relevant for the virulence of C. glabrata due to the subtelomere-based localization of EPA genes that are likely to be relieved of the telomere position effect in response to host environmental cues. Notably, transcriptional activation of EPA6, which is required for biofilm formation, has been shown to be regulated by niacin levels in the mouse model of urinary tract infection (47, 50).

Taken together, our findings demonstrate for the first time a pivotal role for SUMOylation in the life cycle of C. glabrata.

**Experimental Procedures**

**Strains and Culture Conditions**—Bacterial and C. glabrata strains were routinely maintained in the LB medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) at 37 °C and YPD (1% yeast extract, 2% peptone, and 2% dextrose) medium at 30 °C, respectively. For alternative carbon source utilization, the synthetically defined YNB (0.67% YNB and 2% dextrose) medium was used. C. glabrata cultures were grown in the CAA medium (0.67% yeast nitrogen base (YNB) without amino acids, 0.6% casamino acids, and 2% dextrose) for adherence analysis. Logarithmic (log) phase C. glabrata cells were obtained after incubation of overnight cultures for 4 h in the fresh medium at 30 °C with shaking at 200 rpm. S. cerevisiae strains were routinely grown either in YPD medium or minimal medium lacking the indicated nutrient. Bacterial, S. cerevisiae, and C. glabrata strains and plasmids used in this study are listed in Table 2.
### Table 2
List of strains and plasmids used in the study

| Strain   | Genotype                                      | Reference |
|----------|-----------------------------------------------|-----------|
| YRK19    | ura3::Tn903 G418Δ                           | (57)      |
| YRK20    | URA3 or Cg462                                | (58)      |
| YRK949   | URA3 Cgsiz1Δ::nat1                           | This study|
| YRK950   | URA3 Cgsiz2Δ::nat1                           | This study|
| YRK1042  | URA3 Cgsiz1Δ::nat1 Cgsiz2Δ::hph              | This study|
| YRK971   | URA3 Cgup2Δ::nat1                            | This study|
| YRK999   | ura3::Tn903 G418Δ Cgsiz1Δ::nat1              | This study|
| YRK1000  | ura3::Tn903 G418Δ Cgsiz2Δ::nat1              | This study|
| YRK1064  | ura3::Tn903 G418Δ Cgsiz1Δ::nat1 Cgsiz2Δ::hph | This study|
| YRK990   | ura3::Tn903 G418Δ Cgup2Δ::nat1               | This study|
| YRK1057  | ura3::Tn903 G418Δ/pRK74                      | This study|
| YRK1058  | ura3::Tn903 G418Δ Cgsiz2Δ::nat1/pRK74        | This study|
| YRK1059  | ura3::Tn903 G418Δ Cgup2Δ::nat1/pRK74         | This study|
| YRK1060  | ura3::Tn903 G418Δ Cgsiz2Δ::nat1/pCKM381      | This study|
| YRK1061  | ura3::Tn903 G418Δ Cgup2Δ::nat1/pCKM382       | This study|
| YRK1074  | ura3::Tn903 G418Δ Cgsiz1Δ::nat1/pRK74        | This study|
| YRK1075  | ura3::Tn903 G418Δ Cgsiz1Δ::nat1/pRK1071      | This study|
| YRK1106  | ura3::Tn903 G418Δ Cgsiz2Δ::hph/pRK74         | This study|
| YRK1108  | ura3::Tn903 G418Δ Cgsiz2Δ::hph/pCKM377       | This study|
| YRK1110  | ura3::Tn903 G418Δ Cgsiz2Δ::hph/pCKM381       | This study|
| YRK1020  | ura3::Tn903 G418Δ/pCKM379                    | This study|
| YRK1022  | ura3::Tn903 G418Δ Cgsm3::hph/pCKM379         | This study|
| CRC6     | smt3::hsp6/pC440                            | This study|
| KRY1502  | Scup2::His3 TEL URA                         | This study|
| KRY968   | BY 4743; Scsm3::kanMX4/SMT3                  | Euroscarf |

| Plasmid | Description                                                                 | Reference |
|---------|-----------------------------------------------------------------------------|-----------|
| pRK74   | pGRB2.2, CEN-ARS plasmid with *S. cerevisiae* URA3 as selection marker. MCS | (59)      |
|         | sites are located between *S. cerevisiae* PGK1 promoter and 3' UTR of HIS3  |           |
| pRK77   | pAP599, Plasmid with *S. cerevisiae* URA3 and hph expression cassette      | (60)      |
|         | which confers hygromycin resistance. Hph gene is flanked with PGK1          |           |
|         | promoter at 5' end and 3' UTR of HIS3 at other end and entire cassette      |           |
|         | is flanked by MCS sites.                                                    |           |
| pRK625  | pCR2.1 plasmid containing the *nat1* gene                                   | Cormack laboratory |
| pRK983  | CgSMT3 5' UTR cloned in pAP599                                               | This study|
| pRK985  | CgSMT3 5' UTR and 3' UTR cloned in pAP599                                    | This study|
| pRK986  | CgSIZ2 5' UTR cloned in pAP599                                               | This study|
| pRK990  | CgSIZ2 5' UTR and 3' UTR cloned in pAP599                                    | This study|
| pRK1001 | pCN-PDC1-a high expression promoter with *nat1* gene                        | Addgene plasmid # 45325 |
| pCKM377 | CgSIZ1 cloned in pRK74                                                      | This study|
| pCKM379 | CgSMT3 cloned in pRK74                                                      | This study|
| pCKM381 | CgSIZ2 cloned in pRK74                                                      | This study|
| pCKM382 | CgULP2 cloned in pRK74                                                      | This study|
| pCKM387 | CgULP1 cloned in pBEVY-T                                                    | This study|
| pCKM394 | CgSMT3 cloned in pBEVY-L                                                    | This study|
| pCKM405 | 6XHIS3XFLAG tagged CgSMT3 in pRK1001                                         | This study|
| pCKM428 | 6XHIS3XFLAG tagged CgSIZ2 in pRK74                                           | This study|
| pCKM430 | 6XHIS3XFLAG tagged CgULP1 in pRK74                                           | This study|
| pCKM431 | 6XHIS3XFLAG tagged CgULP2 in pRK74                                           | This study|
| pCKM432 | 6XHIS3XFLAG tagged CgSIZ1 in pRK74                                           | This study|
| pCKM469 | 6XHIS3XFLAG modified CgSMT3 in pRK1001                                       | This study|
Role of SUMOylation in the Pathobiology of Candida glabrata

Construction of C. glabrata Deletion Strains and Plasmids—

C. glabrata siz1Δ, siz2Δ, and alp2A strains were created using the homologous recombination-based strategy as described previously (51). For generation of the Cg siz1Δ siz2Δ strain, 433 and 406 bp of 5′UTR and 3′UTR regions, respectively, of the Cg SIZ2 ORF were cloned in the pAP599 plasmid in such a way that Cg SIZ2 UTRs flank each end of the hph1 gene. The 2.96-kb fragment containing 5′UTR-CgSIZ2, hph1 gene, and 3′UTR-CgSIZ2 was obtained by digestion of the plasmid pRK990 with KpnI and SacI restriction enzymes and transformed into the Cg siz1Δ natt1 strain via the lithium acetate method. Transformants were selected on the YPD medium containing hygromycin and confirmed for disruption of the Cg SIZ2 ORF with the hph1 gene via PCR. To generate the Cgsmt3Δ/CgSMT3 strain, C. glabrata wild-type strain (YRK19) was first transformed with the plasmid pRK1069 containing full-length CgSMT3 gene under the PGK1 promoter. Transformants were selected for uracil prototrophy and colony purified followed by replacement of the genomic CgSMT3 locus with the hph1 gene. Disruption of the genomic CgSMT3 locus in the YRK1020 strain was achieved by transforming with a linear DNA fragment (3.91 kb), carrying 5′UTR-CgSMT3, hph1 gene, and 3′UTR-CgSMT3, obtained from the Xhol and SacI-digested pRK985 plasmid. Hygromycin-resistant colonies were checked for disruption of the CgSMT3 ORF with the hph1 gene via PCR.

All plasmids encoding C. glabrata genes were constructed by amplifying sequence-encoding full-length protein from genomic DNA and placed downstream of the PGK1 promoter in pRK74 plasmid. The His6-3×FLAG tags were constructed by first ligating an oligonucleotide encoding three copies of the FLAG sequence in the NheI/BamHI site of pRSETa plasmid. Then the cassette containing His-FLAG was isolated as an XbaI/BamHI fragment and placed downstream of the PGK1 promoter in the pRK74 plasmid. For tagging of C. glabrata proteins at the N terminus, C. glabrata genes were placed in-frame with the His-FLAG sequence in the pRK74 plasmid. Construct encoding mature SUMO was made by amplifying the coding region of SMT3 until the diglycine motif and including a stop codon in the reverse primer. Sequence of primers used and further details are available upon request.

Microscopy Analysis—For differential interference contrast (DIC) microscopy, YPD medium-grown overnight cultures of C. glabrata were subcultured to 0.5–0.6 A600 and spun down at 3000 × g for 5 min. After washing with sterile water, cells were suspended in 200 μl of 10 mM DTT and 0.1 M EDTA-KOH and incubated at 30 °C for 10 min. Cells were collected by centrifugation and resuspended in 500 μl of YPD containing 1.2 M sorbitol. 50 μl of 2.5 mg/ml zymolyase and a pinch of lyticase was added to the cell suspension and incubated at 30 °C for 45 min. Spheroplasting was monitored under a light microscope, and when complete, the cells were harvested by spinning at 2000 × g for 10 min and washed three times with YPD medium containing 1.2 M sorbitol. Finally, the spheroplasts were resuspended in 100 μl of YPD supplemented with 1.2 M sorbitol. Immunofluorescence was performed as described earlier (48). Imaging was done in Zeiss Axio Scope A1 microscope equipped with an AxioCam camera and processed using the Zen software.

Quantitative Real Time PCR—C. glabrata strains were grown in the YPD medium for 48 h followed by incubation in fresh YPD medium for 1 h. Cells were collected, and RNA was extracted using the acid phenol extraction method. First-strand cDNA synthesis was done using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen), and quantitative PCR was performed using the SYBR Green Master Mix (Eurogentec). CgGAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA was used to normalize RT-quantitative PCR data.

Western Blot—Whole cell extracts were made from overnight C. glabrata cultures using trichloroacetic acid (TCA) precipitation (49). An equal number of cells as measured by absorbance were used for protein extractions, and protein levels in samples were estimated by SDS-PAGE/Ponceau-stained blots and, in some cases, additionally by Pierce BCA kit. Protein samples were separated on 10% SDS-PAGE and transferred onto PVDF membrane. After blocking with 3% skimmed milk powder for 1 h, membrane was incubated with FLAG primary antibody (Sigma, 1:10,000) for 1 h, washed three times with TBST, and incubated with the mouse HRP-conjugated secondary antibody (Jackson ImmunoResearch, 1:15,000) for 1 h. Blots were washed three times with TBST and developed with chemiluminescent developing solutions from GE Biosciences.

Chitin Estimation—Overnight cultures were inoculated in the YPD medium to an A600 of 0.1 and incubated at 30 °C for 6 h. Cells were washed twice with PBS, normalized to an A600 of 2.0, and incubated with 2.5 μl of calcofluor white solution (10 mg/ml) for 15 min at room temperature in the dark. After washing PBS, 12.5 μl of cell suspension (~50,000 yeast cells) was diluted 24-fold in PBS and used to measure mean fluorescence intensity via flow cytometry (BD FACS ARIA III). Mean fluorescence intensity ratio was calculated by dividing the fluorescence intensity value of the mutant sample with that of the WT sample.

Biofilm Assay—The ability of C. glabrata cells to produce biofilms on polystyrene-coated plates was assessed as described previously (52). One ml of the YPD-grown logarithmic culture (1 × 10^5 cells in PBS) was added to a well of the polystyrene-coated 24-well plate and incubated at 37 °C for 90 min. After two PBS washes, 1 ml of the RPMI 1640 medium containing 10% FBS was added to each well, and the plate was incubated at 37 °C with shaking (75 rpm). After 24 h, 500 μl of the spent medium was replaced with the fresh RPMI 1640 medium, and incubation was continued for another 24 h. Unbound C. glabrata cells were removed with three PBS washes, and the plate was air-dried for 45 min and incubated with 250 μl of
Role of SUMOylation in the Pathobiology of Candida glabrata

crystal violet solution (0.4% in 20% ethanol) for 45 min. Well attached C. glabrata cells were washed four times with PBS to eliminate surplus crystal violet stain and incubated with 95% ethanol for 45 min. Absorbance of the 100-µl destaining solution was recorded at 595 nm, which is reflective of the number of biofilm-forming C. glabrata cells. Absorbance values of wells without C. glabrata cells were subtracted from those of yeast-containing wells, and the biofilm ratio was calculated by dividing the mutant absorbance units by those of WT cells.

THP-1 Macrophage Infection—THP-1 monocytes seeded at a density of 1 × 10⁵ per well of a 24-well tissue culture plate were treated with 16 nm phorbol 12-myristate 13-acetate for 12 h followed by infection with C. glabrata strains at a multiplicity of infection of 0.1. After 2 h, the wells were washed three times with PBS to remove extracellular yeast cells. THP-1 cells were lysed in water, and the number of intracellular C. glabrata cells was determined by plating appropriate dilutions of lysates on the YPD medium.

Mouse Infection Assay—Experiments involving mice were performed at the Centre for DNA Fingerprinting and Diagnostics animal facility, VITMA Labs Ltd., Hyderabad, India (www.vimta.com), in strict accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India. The protocol was approved by the Institutional Animal Ethics Committee of the VITMA Labs Ltd. (Institutional Animal Ethics Committee protocol approval number PCD/CDFD/05). YPD medium-grown C. glabrata cells (4 × 10⁵, 100 µl of PBS cell suspension) were injected into 6–8-week-old female BALB/c mice through the tail vein. At day 7 post-infection, mice were sacrificed, and the three target organs, kidneys, liver, and spleen, were collected. Organs were homogenized in 1 ml of sterile PBS, and organ fungal load was determined by plating appropriate homogenate dilutions on the YPD medium containing penicillin and streptomycin.

Other Procedures—S. cerevisiae proteins were retrieved from the Saccharomyces Genome Database, and their orthologues in C. glabrata were searched for using Blastp. Percent similarity and identity were calculated using EMBOSS Stretcher (pairwise sequence alignment) tool (53). The protein sequences were scanned for annotated domains using Pfam and HMMER. Maps of proteins along with their domains were generated using DOG (Domain Graph) (54). Zymolyase digestion, CgSlt2 phosphorylation, and adherence analysis were performed as described previously (38, 51).

Author Contributions—R. G., S. V., K. K., S. S. T., K. M., and R. K. conceived the experiments. R. G., S. V., and K. K. performed the experiments. R. G., S. V., K. M., and R. K. analyzed the data and wrote the manuscript.

Acknowledgments—We are grateful to Sridhar and Jayant Pandalikrao Hole for their help with BALB/c mice experiments. We also thank Hita Garapati for sequence comparisons and V. Joy Prashant and A. Lakshmi Annapurna for help with confocal microscopy.

References

1. Geiss-Friedlander, R., and Melchior, F. (2007) Concepts in sumoylation: a decade on. Nat. Rev. Mol. Cell Biol. 8, 947–956
2. Wilkinson, K. A., and Henley, J. M. (2010) Mechanisms, regulation and consequences of protein SUMOylation. Biochem. J. 428, 133–145
3. Wang, Y., and Dasso, M. (2009) SUMOylation and deSUMOylation at a glance. J. Cell Sci. 122, 4249–4252
4. Flotho, A., and Melchior, F. (2013) Sumoylation: a regulatory protein modification in health and disease. Annu. Rev. Biochem. 82, 357–385
5. Dohmen, R. I., Stappen, R., McGrath, J. P., Forrové, H., Kolarov, J., Goffeau, A., and Varshavsky, A. (1995) An essential yeast gene encoding a homolog of ubiquitin-activating enzyme. J. Biol. Chem. 270, 18099–18109
6. Johnson, E. S., Schwienhorst, I., Dohmen, R. J., and Blobel, G. (1997) The ubiquitin-like protein Smt3p is activated for conjugation to other proteins by an Aos1p/Uba2p heterodimer. EMBO J. 16, 5509–5519
7. Johnson, E. S., and Blobel, G. (1997) Ubc9p is the conjugating enzyme for the ubiquitin-like protein Smt3p. J. Biol. Chem. 272, 26799–26802
8. Li, S. J., and Hochstrasser, M. (1999) A new protease required for cell-cycle progression in yeast. Nature 398, 246–251
9. Ouspenksi, I. I., Elledge, S. J., and Brinkley, B. R. (1999) New yeast genes important for chromosome integrity and segregation identified by dosage effects on genome stability. Nucleic Acids Res. 27, 3001–3008
10. Li, S. J., and Hochstrasser, M. (2000) The yeast ULP2 (SMT4) gene encodes a novel protease specific for the ubiquitin-like Smt3 protein. Mol. Cell. Biol. 20, 2367–2377
11. Takahashi, Y., Kahyo, T., Toh-E, A., Yasuda, H., and Kikuchi, Y. (2001) Yeast Uvl1/Siz1 is a novel SUMO1/Smt3 ligase for septin components and functions as an adaptor between conjugating enzyme and substrates. J. Biol. Chem. 276, 48973–48977
12. Yeh, E. T. (2009) SUMOylation and De-SUMOylation: wrestling with life’s processes. J. Biol. Chem. 284, 8223–8227
13. Li, S. J., and Hochstrasser, M. (2003) The Ulp1 SUMO isopeptidase: distinct domains required for viability, nuclear envelope localization, and substrate specificity. J. Cell Biol. 160, 1069–1081
14. Reindle, A., Belichenko, I., Bylchyl, G. R., Chen, X. L., Gandhi, N., and Johnson, E. S. (2006) Multiple domains in Siz SUMO ligases contribute to substrate selectivity. J. Cell Sci. 119, 4749–4757
15. Bylchyl, G. R., Belichenko, I., and Johnson, E. S. (2003) The SUMO isopeptidase Ulp2 prevents accumulation of SUMO chains in yeast. J. Biol. Chem. 278, 44113–44120
16. Wohlschlegel, J. A., Johnson, E. S., Reed, S. I., and Yates, J. R., 3rd (2004) Global analysis of protein sumoylation in Saccharomyces cerevisiae. J. Biol. Chem. 279, 45662–45668
17. Wykoff, D. D., and O’Shea, E. K. (2005) Identification of sumoylated proteins by systematic immunoprecipitation of the budding yeast proteome. Mol. Cell. Proteomics 4, 73–83
18. Hannich, J. T., Lewis, A., Kroetz, M. B., Li, S. J., Heide, H., Emili, A., and Hochstrasser, M. (2005) Defining the SUMO-modified proteome by multiple approaches in Saccharomyces cerevisiae. J. Biol. Chem. 280, 4102–4110
19. Denison, C., Rudner, A. D., Gerber, S. A., Bakalarски, C. E., Moazed, D., and Gyggy, S. P. (2005) A proteomic strategy for gaining insights into protein sumoylation in yeast. Mol. Cell. Proteomics 4, 246–254
20. Zhou, W., Ryan, J. J., and Zhou, H. (2004) Global analyses of sumoylated proteins in Saccharomyces cerevisiae. Induction of protein sumoylation by cellular stresses. J. Biol. Chem. 279, 32262–32268
21. Abu Iqeba, A., Li, Y., Panahi, M., Zhu, M., and Wang, Y. (2014) Regulating global sumoylation by a MAP kinase Hog1 and its potential role in osmo-tolerance in yeast. PLoS One 9, e87306
22. Leach, M. D., Stead, D. A., Argo, E., and Brown, A. J. (2011) Identification of sumoylation targets, combined with inactivation of SMT3, reveals the impact of sumoylation upon growth, morphology, and stress resistance in the pathogen Candida albicans. Mol. Biol. Cell 22, 687–702
23. Pfanner, M. A., and Diekema, D. J. (2007) Epidemiology of invasive candidiasis: a persistent public health problem. Clin. Microbiol. Rev. 20, 133–163
24. Pappas, P. G., Alexander, B. D., Andes, D. R., Hadley, S., Kaufman, C. A.,
Role of SUMOylation in the Pathobiology of Candida glabrata

Freifeld, A., Anaisiss, E. J., Brumle, L. M., Herwaldt, L., Ito, J., Kontoyiannis, D. P., Lyon, G. M., Marr, K. A., Morrison, V. A., Park, B. J., et al. (2010) Invasive fungal infections among organ transplant recipients: results of the Transplant-Associated Infection Surveillance Network (TRANSNET). Clin. Infect. Dis. 50, 1101–1111

25. Pfaller, M., Neofytos, D., Diekema, D., Azie, N., Meier-Kriesche, H. U., Quan, S. P., and Horn, D. (2012) Epidemiology and outcomes of candidemia in 3648 patients: data from the Prospective Antifungal Therapy (PATH Alliance(R)) registry, 2004–2008. Diagn. Microbiol. Infect. Dis. 74, 323–331

26. Pfaller, M. A., Moet, G. J., Messer, S. A., Jones, R. N., and Castanheira, M. (2011) Candida bloodstream infections: comparison of species distributions and antifungal resistance patterns in community-onset and nosocomial isolates in the SENTRY Antimicrobial Surveillance Program, 2008–2009. Antimicrob. Agents Chemother. 55, 561–566

27. Montagna, M. T., Llovero, G., Borghi, E., Amato, G., Andreoni, S., Campon, I., Lo Cascio, G., Lombardi, G., Luzzaro, F., Manso, E., Mussap, M., Peci, P., Perin, S., Toghoria, E., Tronci, M., Iatta, R., and Morace, G. (2014) Candidemia in intensive care unit: a nationwide prospective observational study (GISIA-3 study) and review of the European literature from 2000 through 2013. Eur. Rev. Med. Pharmacol. Sci. 18, 661–674

28. Klevay, M. J., Ernst, E. J., Hollanbaugh, J. L., Miller, J. G., Pfaller, M. A., and Diekema, D. J. (2008) Therapy and outcome of Candida glabrata versus Candida albicans bloodstream infection. Diagn. Microbiol. Infect. Dis. 60, 273–277

29. Moran, C., Grussheimer, C. A., Spalding, J. R., Benjamin, D. K., Jr., and Reed, S. D. (2010) Comparison of costs, length of stay, and mortality associated with Candida glabrata and Candida albicans bloodstream infections. Am. J. Infect. Control 38, 78–80

30. Rodrigues, C. F., Silva, S., and Henriquez, M. (2014) Candida glabrata: a review of its features and resistance. Eur. J. Clin. Microbiol. Infect. Dis. 33, 673–688

31. Harting, R., Kayram, O., Laubinger, K., Valerius, O., and Braus, G. H. (2013) Interplay of the fungal sumoylation network for control of multicellular development. Mol. Microbiol. 90, 1125–1145

32. Takahashi, Y., and Ikizuchi, Y. (2005) Yeast PIAS-type Ull1/Siz1 is composed of SUMO ligase and regulatory domains. J. Biol. Chem. 280, 35822–35828

33. Johnson, E. S., and Gupta, A. A. (2001) An E3-like factor that promotes SUMO conjugation to the yeast septins. Cell 106, 735–744

34. Takahashi, Y., Iwase, M., Strunnikov, A. V., and Kikuchi, Y. (2008) SUMO conjugation to the yeast septins. Trends Genet. 24, 34311–34324

35. Coromack, B. P., Ghorri, N., and Fankow, S. (1999) An adhesin of the yeast pathogen Candida glabrata mediating adherence to human epithelial cells. Science 285, 578–582

36. de Groot, P. W., Bader, O., de Boer, A. D., Weig, M., and Chauhan, N. (2013) Adhesins in human fungal pathogens: glue with plenty of stick. Eukaryot. Cell 12, 470–481

37. Castaño, I., Pan, S. J., Zupanic, M., Hennequin, C., Dujon, B., and Cor-mack, B. P. (2005) Telomere length control and transcriptional regulation of subtelomeric adhesins in Candida glabrata. Mol. Microbiol. 55, 1246–1258

38. Iraqui, I., Garcia-Sanchez, S., Aubert, S., Dromer, F., Ghigo, J. M., d’Enfert, C., and Janbon, G. (2005) The Yak1p kinase controls expression of adhesins and biofilm formation in Candida glabrata in a Sir4p-dependent pathway. Mol. Microbiol. 55, 1259–1271

39. Sydorsky, Y., Srikkumar, T., Jeram, S. M., Wheaton, S., Vizeacoumar, F. J., Makhnevytch, T., Chong, Y. T., Gingras, A. C., and Raught, B. (2010) A novel mechanism for SUMO system control: regulated Ulp1 nuclear sequestration. Mol. Cell. Biol. 30, 4452–4462

40. Tanaka, K., Nishide, J., Okazaki, K., Kato, H., Niwa, O., Nakagawa, T., Matsuda, H., Kavamukai, M., and Murakami, Y. (1999) Characterization of a fusion yeast SUMO-1 homologue, pmt3p, required for multiple nuclear events, including the control of telomere length and chromosome segregation. Mol. Cell. Biol. 19, 8660–8672

41. D’Ambrosio, L. M., and Lavoie, B. D. (2014) Pds5 prevents the PolySUMO-dependent separation of sister chromatids. Curr. Biol. 24, 361–371

42. Walker, L. A., Munro, C. A., de Bruijn, I., Lenardson, M. D., McKinnon, A., and Gow, N. A. (2008) Stimulation of chitin synthesis rescues Candida albicans from echinocandin resistance. J. Infect. Dis. 204, 626–635

43. Pasupula, N., Easwaran, S., Hannan, A., Shore, D., and Mishra, K. (2012) The SUMO E3 ligase Siz2 exerts a locus-dependent effect on gene silencing in Saccharomyces cerevisiae. Eukaryot. Cell 11, 452–462

44. Hannan, A., Abraham, N. M., Goyal, S., Jamir, I., Priyakumar, U. D., and Mishra, K. (2015) Sumoylation of Sir2 differentially regulates transcriptional silencing in yeast. Nucleic Acids Res. 43, 10213–10226

45. Domergue, R., Castaño, I., De Las Peñas, A., Zupanic, M., Locktall, V., Hebel, J. R., Johnson, D., and Cormack, B. P. (2005) Nicotinic acid limitation regulates silencing of Candida adhesins during UTI. Science 308, 866–870

46. Borah, S., Shivarathri, R., Srivastava, V. K., Ferrari, S., Sanglard, D., and Kaur, R. (2014) Pivotal role for a tail subunit of the RNA polymerase II mediator complex CgMed2 in azole tolerance and adherence in Candida glabrata. Antimicrob. Agents Chemother. 58, 5976–5986

47. Djordjevic, D., Wiedmann, M., and McLandsborough, L. A. (2002) Microtiter plate assay for assessment of Listeria monocytogenes biofilm formation. Appl. Environ. Microbiol. 68, 2950–2958

48. Rice, P., Longden, I., and Beaays, A. (2000) EMBOS: the European molecular biology open software suite. Trends Genet. 16, 276–277

49. Ren, J., Wen, L., Gao, X., Jin, C., Xue, Y., and Yao, X. (2009) DOG 1.0: illustrator of protein domain structures. Cell Res. 19, 271–273

50. Cormack, B. P., and Falkow, S. (1999) Efficient homologous and illegitimate recombination in the opportunistic yeast pathogen Candida glabrata. Genetics 151, 979–987

51. De Las Peñas, A., Pan, S. J., Castaño, I., Alder, J., Cregg, R., and Cormack, B. P. (2003) Virulence-related surface glycoproteins in the yeast pathogen Candida glabrata are encoded in subtelomeric clusters and subject to RAP1- and Sir-dependent transcriptional silencing. Genes Dev. 17, 2245–2258

52. Frieman, M. B., McCaffery, J. M., and Cormack, B. P. (2005) Telomerelengthcontrolandtranscriptionalregulation ofsubtelomericadhesinsinCandida glabrata. Mol. Microbiol. 55, 1246–1258