

SUMO-mediated protein homeostasis in *Candida glabrata*

Thesis submitted for the degree of

Doctor of Philosophy

work carried out by

Dipika Gupta

Reg.no.18LBPH01

under the supervision of

Prof. Krishnaveni Mishra



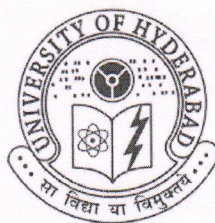
DEPARTMENT OF BIOCHEMISTRY

SCHOOL OF LIFE SCIENCES

UNIVERSITY OF HYDERABAD

HYDERABAD – 500 046

INDIA



UNIVERSITY OF HYDERABAD
SCHOOL OF LIFE SCIENCES
DEPARTMENT OF BIOCHEMISTRY

DECLARATION

I, **Dipika Gupta**, hereby declare that this thesis entitled “**SUMO-mediated protein homeostasis in *Candida glabrata***” submitted by me under the guidance and supervision of **Prof. Krishnaveni Mishra**, is an original and independent piece of research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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Reg. No. 18LBPH01

Dipika Gupta
Signature of the student



CERTIFICATE

This is to certify that the thesis entitled “**SUMO-mediated protein homeostasis in *Candida glabrata***” submitted by **Dipika Gupta** bearing registration number **18LBPH01** in partial fulfilment of the requirements for award of Doctor of Philosophy in the School of Life Sciences is a bonafide work carried out by her under my supervision and guidance.

The thesis is free from plagiarism and has not been submitted previously in part or in full to this or any other University or Institution for the award of any degree or diploma.

The student has following publications prior to submission:

- **Gupta, D.;** Garapati, H. S.; Kakumanu, A. V. S.; Shukla, R.; Mishra, K. SUMOylation in Fungi: A Potential Target for Intervention. *Comput Struct Biotechnol J* 2020, 18, 3484–3493. <https://doi.org/10.1016/j.csbj.2020.10.037>.

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S801	Research Methodology/ Analytical Techniques	4	Pass
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Supervisor

Prof. KRISHNAVENI MISHRA
Department of Biochemistry
School of Life Sciences
University of Hyderabad
Hyderabad-500046. (TS), INDIA


Head of the Department

जीवरसायन विभाग / Department of Biochemistry
जीव विज्ञान संकाय / School of Life Sciences
हैदराबाद विश्वविद्यालय / University of Hyderabad
हैदराबाद / Hyderabad-500046. INDIA


Dean of the School

संकाय अध्यक्ष / Dean
जीव विज्ञान संकाय / School of Life Sciences
हैदराबाद विश्वविद्यालय / University of Hyderabad
हैदराबाद / Hyderabad-500 046.



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REG. NO. 18LBPH01
NAME OF THE STUDENT DIPIKA GUPTA
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LIST OF ABBREVIATIONS USED

AOS	Activation of Smt3p
AIDS	Acquired immunodeficiency syndrome
ABPA	Allergic bronchopulmonary aspergillosis
AIR	Aminoimidazole ribonucleotide
ANOVA	Analysis of variance
ASP	Arabidopsis SUMO protease
ABC	ATP binding cassette
BLAST	Basic local alignment search tool
βME	Beta mercaptoethanol
CO ₂	Carbon dioxide
CAA	Casamino acid
CD	Catalytic domain
CWI	Cell wall integrity
CPA	Chronic pulmonary aspergillosis
CD	Circular dichroism
CFU	Colony forming unit
CP	Core particle
CTD	C-terminal domain
DAVID	Database for annotation, visualization, and integrated discovery
DeSI	DeSUMOylating isopeptidase
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EM	Electron microscopy

ETC	Electron transport chain
ERAD	Endoplasmic reticulum-associated protein degradation
ECL	Enhanced chemiluminescence
EPA	Epithelial adhesion
ERG	Ergosterol gene
EDTA	Ethylene-diamine-tetra acetic acid
EMBOSS	European molecular biology open software suite
FDR	False discovery rate
FBS	Fetal bovine serum
FLP	Flippase
FRP	Flippase recognition target
FDA	Food and drug administration
FGAR	Formylglycinamide ribonucleotide
FPPL	Fungal priority pathogens list
GO	Gene ontology
GAA	Glacial Acetic Acid
GST	Glutathione S-transferase
GAR	Glycineamideribonucleotide
HPLC	High-performance liquid chromatography
HIS	Histidine
HRP	Histone deacetylase
HDAC	Horse-radish peroxidase
h	Hour(s)
HIV	Human immunodeficiency virus
H ₂ O ₂	Hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HU	Hydroxyurea

IBS	Illustrator for biological sequences
IMP	Inosine 5'-monophosphate
ICU	Intensive care unit
ISBN	International standard book number
IPA	Invasive pulmonary aspergillosis
IPTG	Isopropyl β -D-1-thiogalactopyranoside
LiAc	Lithium Acetate
L	Litre
LCMS	Liquid- chromatography mass spectrometry
LB	Luria-Bertani broth
MS	Mass spectrometry
MMS	Methylmethanesulfonate sensitivity
μ	Micro
m	Mili
MitoSOX	Mitochondrial superoxide
M	Molar
MD	Molecular dynamics
MM/GBSA	Molecular mechanics with generalized born and surface area
MeV	Multiple experiment viewer
NAC	N-acetylcysteine
n	Nano
NCBI	National center for biotechnology information
NEM	N-ethylmaleimide
NEB	New England Biolabs
Ni-NTA	Nickel-nitrilotriacetic acid
NAT	Nourseothricin acetyltransferase
NTD	N-terminal domain

NPC	Nuclear pore complex
NUP	Nucleoporin
ORFs	Open reading frames
OD	Optical density
OPLS	Optimized potentials for liquid simulations
OTS	Overly Tolerant to Salt
PMSF	Phenylmethanesulfonyl fluoride
PMA	phorbol 12-myristate 13-acetate
PBS	Phosphate buffered saline
PGK	Phosphoglycerate kinase
PRA	phosphoribosylamine
PRPP	5-phosphoribosyl-1-pyrophosphate
PIPES	Piperazine-1,4-bis(2-Ethanesulphonic Acid)
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethylene glycol
PCR	Polymerase chain reaction
PVDF	Polyvinylidene fluoride
pH	Potential hydrogen
PCA	Principal component analysis
PDB	Protein data bank
PHYRE	Protein homology/analogy recognition engine
PIAS	protein inhibitor of activated STAT
RAD	Radiation sensitive
RCP	Ramachandran plot
RANBP	Ran-binding protein
ROS	Reactive oxygen species
RING	Really interesting new gene

RP	Regulatory particle(s)
RNA	Ribonucleic acid
RNF	Ring finger protein
RT	Room temperature
RMSD	Root mean square deviation
RPM	Rotations per minute
SGD	Saccharomyces Genome Database
SRplot	Science and research online plot
sec	Second(s)
SENP	Sentrin/SUMO-specific protease
STAT	Signal transducer and activator of transcription
SIR2	Silent information regulator 2
SPA	Simple pulmonary aspergilloma
ssDNA	Single-stranded DNA
SEC-MALS	Size exclusion chromatography-multiangle light scattering
SUMO	Small ubiquitin-like modifier
SDS	Sodium dodecyl sulphate
SD	Standard deviation
SG	Stress granules
SIM	SUMO interaction motif
STUbLs	SUMO-targeted ubiquitin ligases
SOB	Super optimal broth
SMT	Supressor of mif two
SLX	Synthetic Lethal of unknown [X] function
TMT	Tandem mass tag
3D	Three-dimensional
TCA	Trichloro acetic acid

TEABC	Triethylammonium bicarbonate
TFA	Trifluoroacetic acid
TBST	Tris-buffered saline with 0.1% Tween20 detergent
2D	Two-dimensional
UBA	Ubiquitin activating
UBC	Ubiquitin-conjugating
ULS	Ubiquitin ligase for SUMO conjugates
ULP	Ubiquitin-like proteases
UPS	Ubiquitin-proteasome system
UV	Ultraviolet
WT	Wild type
WHO	World health organization
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
YPD	Yeast extract peptone dextrose
YNB	Yeast nitrogen base
Y2H	yeast two-hybrid

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CHAPTER 1 INTRODUCTION

1.1 THE BURDEN OF FUNGAL INFECTIONS

Infections, caused by fungi pose a significant risk and threat to public health due to their widespread effect on diverse populations. Fungal infections or mycosis are harmful not only to humans but also to plants and animals. Fungal infections range from systemic infections like aspergillosis and candidiasis that can be serious, to common superficial infections like ringworm and athlete's foot ¹⁻³.

Pathogenic fungi include *Aspergillus* species, *Magnaporthe oryzae* (primarily infects plants), *Candida* species, and *Cryptococcus neoformans*. Diseases like pulmonary aspergilloma, aspergillosis, candidiasis, and cryptococcal meningoencephalitis are commonly caused by these pathogenic fungi ^{4,5}. A significant surge in fungal infection, including approximately 1.5 million deaths, has been recorded in the recent past. Individuals with poor immune systems, including those with HIV/AIDS, organ transplant recipients, individuals undergoing chemotherapy, or patients with prolonged stay in the Intensive Care Unit (ICU) are at higher risk of severe fungal infections ⁶⁻⁹. In fact, the fungal burden on humans was traditionally underestimated and therefore research was underfunded. However recently, WHO released a first-ever Fungal priority pathogens list (FPPL), bringing into focus the importance of fungal infections and antifungal resistance (ISBN 978-92-4-006024-1). Agriculture faces challenges from fungal attacks on staple crops like rice, wheat, corn, and soybeans, resulting in potential crop losses of up to 70% and impacting food security ^{10,11}. Plant pathogenic fungi like *Magnaporthe oryzae* cause rice blast disease, posing a major threat to agriculture ¹². Rust fungi species, including *Puccinia graminis* and *Puccinia striiformis* cause rust diseases in wheat, leading to reduced crop yields. Emerging fungal pathogens present new challenges to agriculture and forestry ¹³⁻¹⁵. Moreover, aspergillosis affects various animal species, including birds and mammals, causing respiratory distress and systemic illness. Among animals, especially in livestock, fungal infections result in economic losses in the agricultural sector, while wildlife populations are affected, impacting biodiversity and overall ecosystem health ^{3,16-18}.

1.2 ANTIFUNGAL DRUGS

Antifungal drugs are commonly used to treat fungal infections in both plants and animals. These drugs exhibit a considerable level of specificity, offering protection against disease and aiding in the recovery process for the affected individuals¹⁹. The three main classes of antifungals that are commonly used are azoles, echinocandins, and polyenes. The targets of these drugs and common means of resistance are described below.

Azoles: A class of antifungals known as azoles (Ketoconazole, Fluconazole, Itraconazole, Miconazole, and Voriconazole) prevents the synthesis of ergosterol, which is an essential component of the fungal cell membranes. Azole drugs are highly potent and often used to treat fungal infections as ergosterol is not produced in plants or animals and therefore is fungus specific. Resistance to azoles has now been seen in many fungi. There are several ways in which azole resistance can arise: a) Overexpression of efflux pump: the expression of efflux pumps, like ABC transporters, can be upregulated by fungi. These pumps actively remove azole drugs from fungal cells, preventing their accumulation to harmful levels. b) Target site alterations: Alterations in lanosterol 14 α -demethylase, the target enzyme (encoded by the *ERG11* gene), can decrease the enzyme's affinity for azole medications, consequently decreasing their efficacy (Figure 1)^{8,20,21}.

Echinocandins: Echinocandins prevent the production of β -(1,3)-D-glucan, an essential component of the fungal cell wall by targeting the glucan synthases. The following mechanisms can lead to resistance to echinocandins: a) Mutations in the FKS genes: The β -(1,3)-D-glucan synthase subunits are encoded by the FKS genes (*FKS1*, *FKS2*, and *FKS3*), which are the target of echinocandins. These genes can be mutated to change the structure of the enzyme, which lowers the echinocandins' affinity for binding. b) Activation of compensatory pathways: By triggering compensatory pathways like the cell wall integrity (CWI) pathway, certain fungi can preserve cell wall integrity even with the compromised glucan synthesis and counteract the effects of echinocandins (Figure 1)^{8,22,23}.

Polyene: In the fungal cell membrane, ergosterol binds to polyenes like amphotericin B, leading to the creation of pores that cause leakage of cellular contents (Figure 1). There are several

mechanisms by which resistance to polyenes can arise: a) Decreased ergosterol content: in order to decrease the number of target sites available for polyene binding, fungi may lower the amount of ergosterol in their cell membranes b) Alterations in membrane composition: the composition of lipids in membranes can be altered by fungi, leading to the reduction of polyene binding affinity through the addition of substitute sterols ^{20,21,24}.

Apart from these specific drugs, nucleoside analogues like Flu Cytosine which interferes with fungal DNA synthesis, and Griseofulvin that damages the mitotic spindle are also used (Figure 1) ^{19,21,24}.

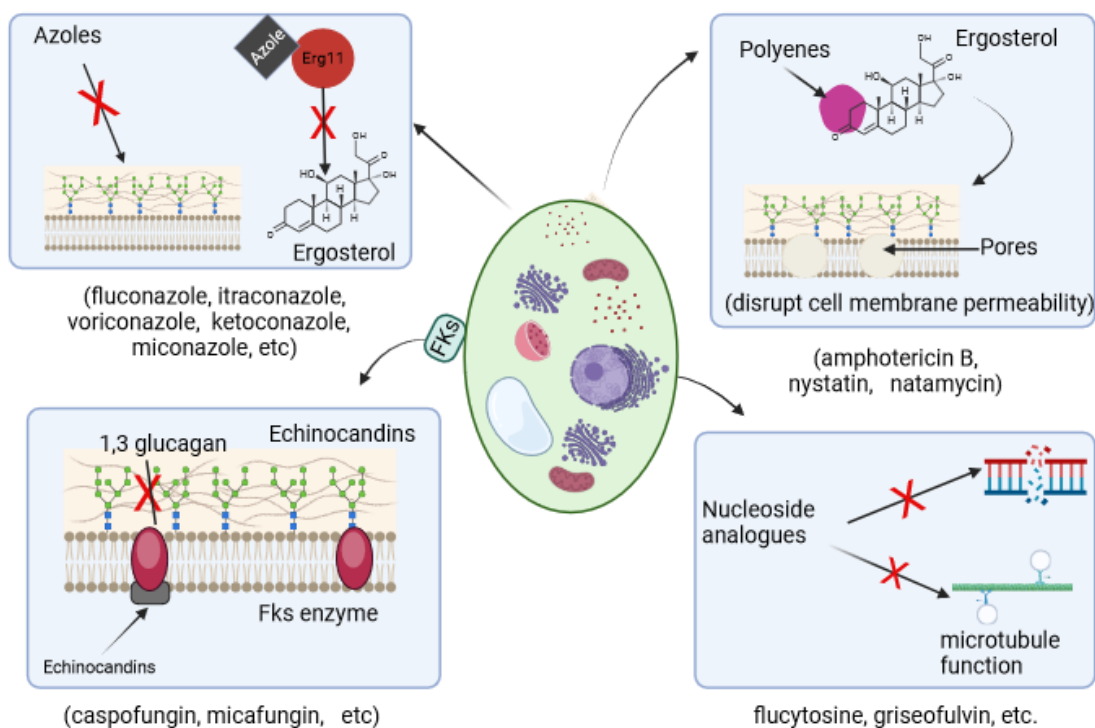


Figure 1. Classification of antifungals along with their mode of actions

However, the overuse of these antifungals, particularly azoles, has resulted in multidrug resistance.

Drug resistance is one of the most common causes of high mortality in fungal infections. Fungi are underappreciated as pathogens, and research on them receives far less funding than other illnesses with similar death tolls, such as malaria ^{2,25}. As a result, new antifungals haven't been introduced in a long time. It is essential to identify novel targets for the development of

antifungals as currently only two targets, that is, biosynthesis of the cell wall and cell membrane have been targeted. Since fungi are eukaryotes and also are closely related to mammals (as they both belong to the same group Opisthokonts), there aren't as many sufficiently different targets available. A successful target should be a) existing in the pathogen; b) essential for pathogenesis; c) druggable; and d) either completely absent from the host or sufficiently different from the pathogen target.

This thesis is an attempt to establish the SUMO-mediated protein homeostasis pathway as a potential target for antifungal drug development in the pathogenic yeast *Candida glabrata*.

1.3 *Candida glabrata*

Candida glabrata, a haploid, pathogenic yeast belonging to the ascomycetous genus *Candida* is commonly found in the human microbiota, including the oral cavity, vaginal tract, and intestinal tract. In a healthy individual, *C. glabrata* coexists with other human microbiota bacteria without posing any threat; however, it can infect patients with compromised immune systems, including those with AIDS, those who have received organ transplants, and those who stay in the intensive care unit for an extended period. Hence it is an opportunistic pathogen^{9,26}. Compared to *Candida albicans*, a human pathogenic yeast in the same genus, it is more closely related to *Saccharomyces cerevisiae*²⁷. The pathogenicity of *Candida glabrata* is governed by various virulence factors, some of which are outlined below:

1. Adhesion and Biofilm Formation: Adhesins located on the cell surface of *Candida glabrata* play a vital role in its ability to adhere to host tissues, a critical initial step in the onset of infections. Proteins like Epa1 and Awp2 facilitate this adherence process. Additionally, the capability to form biofilms enhances its persistence on both biotic and abiotic surfaces^{27–30}. This feature increases resistance to host defenses and antifungal treatments, underscoring its significance in the pathogenicity of *Candida glabrata*.
2. Immuno-evasion and Immune Modulation: *Candida glabrata* has the capacity to control the host immune response, which allows for immune system evasion. The yeast can use mechanisms that interfere with phagocytosis and intracellular killing processes to reduce the effectiveness of phagocytes, such as neutrophils and macrophages. It is also possible

for the organism to control the production of cytokines, which will impact the inflammatory response of the host^{29,31}.

3. Iron Acquisition: Iron, an essential micronutrient, is indispensable for both the survival and pathogenicity of *C. glabrata*. It can obtain iron from the host environment through various mechanisms such as secreting siderophores (e.g., triacetylfusarinine C) and using host iron-binding protein (e.g., transferrin)^{26,27}.
4. Cell Wall Composition: The structure of the fungal cell wall plays a pivotal role in interactions with the host. In the case of *C. glabrata*, its unique cell wall composition, featuring mannoproteins and beta-glucans, is essential for its pathogenicity. These components actively participate in immune recognition, adhesion processes, and the evasion of host defences²⁶.

There are other pathogenic factors in *C. glabrata* that play an essential role in its ability to cause infections, influencing the establishment, persistence, and severity of infections. These factors include: a) morphological plasticity: In contrast to *C. albicans*, *C. glabrata* typically does not form true hyphae. Nevertheless, it has the potential to display a pseudohyphal growth form, and this morphological flexibility may play a role in its capacity to invade host tissues^{32,33}, b) hemolytic activity- *C. glabrata* exhibits hemolytic activity, inducing the breakdown of red blood cells. This activity may contribute to tissue damage and the spread of infection, c) osmotic stress response- *C. glabrata* has a robust osmotic stress response, enabling it to survive in various osmotic conditions encountered within the host. This adaptability is critical for colonizing different host niches, d) *Candida* drug efflux pumps- *C. glabrata* is known for its high expression of drug efflux pumps, enhancing its resistance to antifungal agents. These pumps actively remove drugs from the fungal cell, reducing their effectiveness and posing challenges to treatment, e) sterol biosynthesis pathway- alterations in the sterol biosynthesis pathway, including changes in ergosterol production, can impact the susceptibility of *C. glabrata* to antifungal drugs. Disruptions in this pathway may contribute to antifungal resistance, f) protease activity- *C. glabrata* produces various proteases capable of degrading host proteins. This proteolytic activity may contribute to tissue invasion and immune evasion^{26,27,34}.

The progression of the *Candida glabrata* infection involves a series of sequential steps, encompassing adherence, immune evasion, tissue invasion, and systemic dissemination. The

initiation of infection by this organism involves adhering to the host surfaces, facilitated by adhesins, particularly EPA (epithelial adhesin) proteins, which promote the attachment of yeast to mucosal surfaces for effective colonization. Evading the host immune system is a strategic aspect of *C. glabrata* infection process, demonstrated by its resistance to phagocytosis by neutrophils and macrophages, ability to survive within phagocytes, and inhibition of immune signaling pathways. Subsequent to adherence and colonization, *C. glabrata* can invade host tissues, causing localized damage. In individuals with compromised immune systems, the yeast may disseminate systemically, resulting in more severe infections ^{26,27}.

One such prevalent and critical fungal infection in humans is Candidemia which plays an essential role in invasive candidiasis. This infection poses a severe threat due to its rapid progression and significant impact on major organs, resulting in a high fatality rate. Various *Candida* species contribute to invasive candidiasis including *Candida albicans*, *Candida glabrata*, and *Candida tropicalis*. Notably, *Candida glabrata* has emerged as the second leading cause of mortality associated with candidiasis ²⁶. Its multidrug-resistant nature, particularly its resistance to antifungal drugs such as azoles and echinocandins, intensifies the challenge in fungal treatment ³⁵. The high mortality rate, ranging from 40-60%, underscores the urgent need to discover new antifungals to treat fungal infections. To address this challenge, we are exploring the protein SUMOylation pathway in *Candida glabrata* as a potential target for identifying novel antifungals.

1.4 SUMOYLATION: A POTENTIAL ANTIFUNGAL TARGET

During SUMOylation, a post-translational modification process, a small ubiquitin-like protein (SUMO) is covalently attached to the target protein, enabling it to interact with other proteins. In yeast, the SUMO protein is coded by the *SMT3* gene. SUMOylation of target proteins can lead to multiple consequences (Figure 2). SUMOylation could lead to changes in protein subcellular localization, the interaction of one protein with another, and alteration in catalytic functions. In multiple cellular physiological pathways like DNA repair, intracellular transport, cell cycle progression, transcription, and immune response, SUMOylation is known to play an important regulatory role ³⁶. Also, SUMOylation has been found to play a crucial role in adaptation to

stress conditions, cell division, and growth, making it a suitable target for the development of antifungals.

A cascade of enzymatic actions leads to SUMOylation of targets. While basic machinery appears conserved in all organisms studied so far, the SUMOylation system has expanded in some organisms. For instance, there are potentially 8 isoforms of the SUMO gene in *Arabidopsis thaliana*; humans possess 4 SUMO isoforms, viz, SUMO-1, -2, -3, and -4, while *S. cerevisiae* expresses a single SUMO paralogue, called Smt3p³⁷.

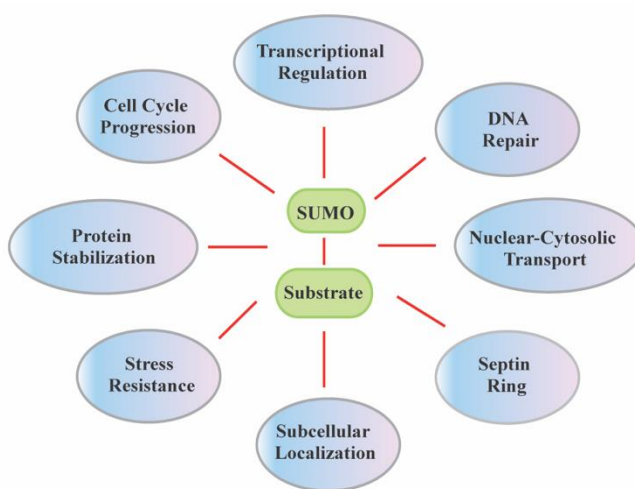


Figure 2. Schematic representation of various physiological processes that are regulated by SUMOylation.

1.4.1 Mechanism of SUMOylation

The process of SUMOylation involves multiple steps. SUMO first undergoes proteolytic processing that involves the removal of a few C-terminal amino acids, revealing a diglycine motif at the C-terminus to become mature SUMO. The ubiquitin-like proteases (Ulp) family of proteases in yeast catalyzes SUMO maturation. An E1 activating enzyme, an E2 conjugating enzyme, and one of several SUMO E3 ligases are required for SUMO conjugation. In step one of SUMO conjugation, ATP-dependent E1 activating enzyme (Aos1/Uba2 in yeast), forms a high-energy thioester bond with the SUMO C-terminus^{38,39}. This step requires ATP hydrolysis. Then the transfer of SUMO from the E1 activating enzyme to the SUMO conjugating enzyme involves the formation of a thioester linkage between the C-terminal glycine of SUMO and the catalytic

active site of a cysteine residue of the E2 conjugating enzyme. In yeast, the single E2 conjugating enzyme is encoded by Ubc9 and is responsible for catalyzing conjugation reactions. Subsequently, the SUMO moiety attaches to the target protein by forming an isopeptide bond between the C-terminal glycine of SUMO and ϵ - amino group of lysine residue of the target protein ^{40,41} (Figure 3). While *in vivo* E3 ligases aid in the process of transferring SUMO to the target, *in vitro* the E2 enzyme is adequate for conjugating SUMO to a lysine residue in the substrate ⁴².

In contrast to many E2 conjugation enzymes involved in ubiquitination, Ubc9 is the sole SUMO-conjugating E2 enzyme found in eukaryotes. *UBC9* is essential in *S. cerevisiae*, just like many other SUMOylation components, including Smt3, Aos1, and Uba2 ^{43,44}. *In vivo*, E3 ligases are involved in the transfer of SUMO from Ubc9 to the target proteins. Various E3 ligases contribute to the substrate range and specificity in the SUMOylation process. So far, identified SUMO E3 proteins which mainly comprise members of PIAS (protein inhibitor of activated STAT) proteins possessing the SP-RING domain are Siz1, Siz2, Mms21, and Zip3 in *S. cerevisiae*. On the other hand, RANBP2 (Ran-binding protein 2), a component of the nuclear pore complex, functions as a SUMO ligase despite lacking the canonical RING domain for catalytic activity ^{45–48}. Moreover, histone deacetylase 4 (HDAC4), KRAB-associated protein 1 (KPA1), Pc2, and Topors, have also been identified as potential SUMO ligases ³⁷.

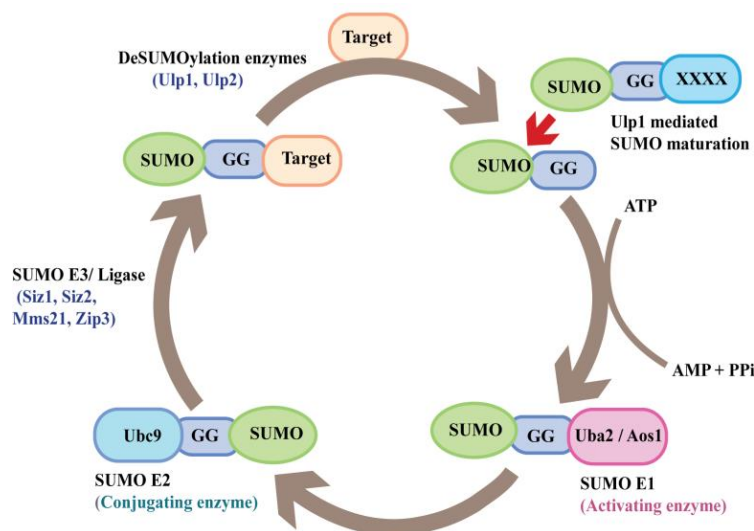


Figure 3. Schematic depiction of the SUMOylation pathway.

1.4.2 Mono- and polySUMOylation

MonoSUMOylation occurs when a single SUMO protein becomes covalently bound to a target protein. It can regulate the activity, stability, and localization of the target protein. This modification exerts significant influence over diverse cellular functions, such as transcription, DNA repair, and cell cycle progression and also modulating protein-protein interactions ^{40,49}. For example, monoSUMOylation of Rad52 (a key player in homologous recombination) is critical in its role in DNA repair and may influence its interactions with other repair factors ⁵⁰. Similarly, Sir2 monoSUMOylation regulates its translocation from the telomere to the nucleolus ⁵¹.

In contrast, polySUMOylation involves the sequential addition of multiple SUMO proteins to a substrate, resulting in the formation of SUMO chains. The SUMO-conjugating enzyme (E2) and the SUMO ligase (E3) carry out this modification. Specific SUMO E3 ligases are involved in prompting polySUMOylation, ensuring the efficient transfer of SUMO from the E2 enzyme to the target protein. The specific lysine residue on the target protein undergoing polySUMOylation can vary, as SUMO can be attached to multiple lysines within the target protein. For example, in yeast, Smt3 can be conjugated to lysines via positions 11,15, and 19 while in humans, SUMO2/3 are known to form polymeric chains on lysine 11 ^{52–56}.

The consequences of polySUMOylation are diverse, impacting various cellular processes. Notably, polySUMOylation of nucleoporins is crucial for the proper functioning of the nuclear pore complex, regulating nucleocytoplasmic transport ^{48,57}. Additionally, it is involved in DNA repair mechanisms, influencing the recruitment of repair factors to damaged sites and contributing to genome stability. It also contributes to regulating the cell cycle and mitosis by affecting the stability and activity of key cell cycle regulators. This modification also regulates the turnover of the target protein ^{58–62}. PolySUMOylation dynamics is regulated by the deSUMOylases, such as Ulp in fungi and SENP in higher organisms that can cleave SUMO from target proteins thus protecting the protein from ubiquitination and consequent degradation.

Moreover, polySUMOylation serves as a signal for recognition by the ubiquitinating machinery. These polySUMOylated proteins are polyubiquitinated and degraded in the proteasome. This

intricate interplay between SUMOylation and ubiquitination contributes to the dynamic regulation of cellular processes and protein homeostasis.

1.4.3 SUMOylation motif

Short amino acid sequences known as "SUMOylation motifs" are recognized by the enzymes that facilitate the covalent attachment of Small Ubiquitin-like Modifier (SUMO) proteins to target proteins. The SUMOylation machinery is guided by these motifs to particular lysine residues in the target proteins. The consensus SUMOylation motif is commonly identified by a core of $\Psi KxE/D$, where Ψ represents a hydrophobic amino acid, K is the lysine that is SUMOylated, x can be any amino acid, and E/D can be either glutamic or aspartic acid^{36,49,63}. Notably, the most highly conserved position is glutamate^{64,65}. ΨKxE serves as the E2 Ubc9 recognition site, and the conjugation process depends on the interaction between the motif and the enzyme^{66–68}. Even though this motif is present in one-third of the proteins, to achieve precise SUMOylation, other factors, such as the steric environment in SUMO attachment signals are also important⁶⁹.

Apart from the core sequence, additional variations around this motif also influence the site of SUMOylation. These mainly include negatively charged amino acid-dependent SUMOylation motifs (NDSMs), as well as phosphorylation-dependent SUMOylation motifs (PDSMs). The clusters of residues downstream of the ΨKxE core exhibit strong interactions with both the substrate and Ubc9, hence enhancing SUMO conjugation^{70–72}. Moreover, the inverted motif $[(E/D)xK\Psi]$ has been reported to be SUMOylated⁷³. In sum, the precise combination of factors that determine SUMOylation is still obscure.

1.4.4 SUMO interacting motif

SUMO interaction motif (SIM) refers to the short amino acid sequences that are involved in the non-covalent binding of SUMO proteins to target proteins. The first conserved SUMO interacting motifs were defined in the year 2000⁷⁴. SIMs are typically characterized by a hydrophobic core, often composed with valine (V), leucine (L), or isoleucine (I) residues, flanked by acidic or polar amino acids. The consensus sequence for SIMs is usually $V/L/I-x-V/L/I-V/L/I$, where "x" represents any amino acid^{74–76}. SIMs are frequently found in areas of unstructured regions. The presence of negatively charged residues can play a crucial role in

determining the SUMO/SIM interaction affinity, polarity, and paralogue specificity, probably through salt bridges and hydrogen bonding with conserved basic residues on the surface of SUMO ^{75,77}. There are currently three different types of SIMs that have been identified: SIMa ($\Psi\Psi x\Psi\text{Acn}$), SIMb ($\Psi\Psi\text{DLT}$), and SIMr ($\text{Acn}\Psi x\Psi\Psi$) ^{78,79}. In these SIMs, Ψ represents a hydrophobic residue (V, I, or L), n is a number between two and five, and Ac stands for Asp, Glu, or Ser. Ser, while not being an acidic amino acid, is frequently present in SIMs, most likely because it is a phosphorylation target ⁷⁹. In comparison to ubiquitin-binding domains, there is a limited number of SIMs are known ⁸⁰. This could imply that more forms of SIMs have yet to be discovered.

1.4.5 SUMO deconjugating enzymes

The process of SUMOylation is dynamic and reversible. De-conjugases or deSUMOylating enzymes or SUMO-specific proteases cleave the SUMO protein from the target protein. Ulp1 and Ulp2 (ubiquitin-like proteases) are two SUMO proteases found in yeast. These are isopeptidase-related enzymes that sever the isopeptide bond between the target protein and SUMO. Ulp1 is present at the nuclear pore complex (NPC) and is required for cleaving both the SUMO precursor and also SUMO conjugates from target proteins. Ulp1 also plays a critical role in the regulation of cell division by deSUMOylating several components of the septin ring. Ulp2, which is found in the nucleoplasm ⁸¹, is important for dismantling polySUMO chains apart from also deSUMOylating monoSUMO from nucleolar and nuclear proteins ⁸². The two proteins also appear to deSUMOylate a distinct set of SUMO conjugates ⁸¹. Ulps (SENPs in higher eukaryotes) share a conserved ~200-amino-acid catalytic domain that is typically found near their C-terminus.

In plants and animals, there is an expansion in the SUMOylation machinery. SUMO isoforms are abundant in higher animals and plants. SUMO isopeptidases and ligases have also expanded. In these organisms, the SUMO isopeptidase and SUMO ligase both seem to be at least somewhat selective for the SUMO isoform. Mammals have at least six SENPs, including SENPs 1, 2, 3, 5, 6, and 7. While SENP6 and SENP7 are more Ulp2-like, SENP1-3 and SENP5 are more Ulp1-like ⁸³. SENP proteins can be grouped into three major families. The first family, which consists of SENP-1 and SENP-2, is effective at eliminating all the mammalian SUMO isoforms (SUMO-1–3). The second family contains SENP-3 and SENP-5, which selectively remove SUMO-2/3

from substrates. Additionally, SUMO-2/3 is selectively removed from substrates by SENP-6 and SENP-7⁸⁴. In contrast to SENPs, which are primarily found in the nucleus, two new types of SUMO proteases have also recently been discovered. Dimer DeSI1 (DeSUMOylating isopeptidase 1), which is found in both the cytosol and the nucleus, and DeSI2, which is primarily found in the cytoplasm^{85,86}. DeSI1 may deconjugate SUMO-1 and SUMO-2/3 and exhibit isopeptidase activity⁸⁵. Seven SUMO-specific proteases have been found in the *Arabidopsis* genome. These are categorized as ULP1-like SUMO proteases, including ULP1a/ELS1 (Ub-like protease 1A/ESD4 like SUMO protease 1), ULP1b, ULP1c/OTS2 (Overly Tolerant to Salt2), ULP1d/OTS1 (Overly Tolerant to Salt1), and ESD4 (Early in Short Days 4). SUMO proteases that are similar to ULP2 are known as ULP2a and ULP2b/ASP1 (*Arabidopsis* SUMO protease1). Additionally, phylogenetic analysis of ULP1d/OTS1 and ULP1c/OTS2 shows that they are more similar to yeast ScUlp2⁸⁷⁻⁸⁹. These findings highlight how higher eukaryotes have a more complex SUMOylation pathway than yeast.

1.4.6 SUMOylation in pathogenic fungi

While research on *S. cerevisiae* has uncovered the mechanisms underlying SUMOylation as well as its functional consequences, several labs have recently looked at SUMOylation in pathogenic fungi. These studies have revealed that SUMOylation has a substantial impact on physiology and fungal pathogenicity. I briefly outline some of the findings on SUMOylation in pathogenic fungi below⁴¹.

In both *C. albicans* and *C. glabrata*, protein SUMOylation has been investigated. The SUMO gene, *SMT3*, is required for survival of *C. glabrata*. Changes in SUMOylation have an impact on the potential for growth, stress resistance, and DNA repair in *C. glabrata*. Ulp2 was discovered to be necessary for virulence, adhesion, and biofilm formation and its deletion in *C. glabrata* increased sensitivity to stress (Figure 4)⁹⁰. *SMT3* is not required for survival in *C. albicans*, however, *smt3* mutants have slow growth that is exacerbated by stressful environments, making them sensitive to a wide variety of perturbations, such as temperature, oxidative, and cell wall stressors⁹¹. *SMT3* inactivation in *C. albicans* results in a heterogeneous population of slowly expanding, elongating cells that resemble pseudohyphae (Figure 4)⁹¹. Loss of SUMO ligases also resulted in an increase in hyphal production in *C. albicans*, potentially increasing its

virulence⁹². These mutants were also vulnerable to genotoxic, thermal, and cell wall stressors, which may indicate that SUMO conjugation is crucial for differentiation and combating stress.

The species of *Aspergillus* and *Cryptococcus* are toxic to both plants and animals. Invasive pulmonary aspergillosis (IPA), chronic pulmonary aspergillosis (CPA), simple pulmonary aspergilloma (SPA), and allergic bronchopulmonary aspergillosis (ABPA) are all caused by *Aspergillus* infections^{4,93}. Both pulmonary cryptococcosis and cryptococcal meningoencephalitis have been linked to the *Cryptococcus*. An opportunistic human pathogenic fungus called *Cryptococcus neoformans* primarily targets immunocompromised individuals^{94,95}. Infections caused by *Cryptococcus* have a significant mortality rate and exhibit resistance to many widely used antifungal drugs. In *Cryptococcus*, SUMOylation has not been specifically studied⁹⁶. However, a large-scale investigation that looked for a potential therapeutic molecule for *C. neoformans* discovered that SUMO activating enzyme (*ΔaosI*) ortholog knockouts reduced the growth of *C. neoformans* capsules and biofilms on medical equipment (Figure 4)⁹⁷. The virulence of *C. neoformans* is decreased by the loss of capsule formation. In this investigation, numerous other proteasomal elements as well as NEDD (an additional ubiquitin-like molecule) were discovered to be crucial for capsule formation. This implies that in the absence of these pathways, the pathogen's virulence traits may be reduced.

The three *Aspergillus* species that produce aflatoxin most frequently are *A. fumigatus*, *A. flavus*, and *A. carcinogen*. A prominent source of food contamination is aflatoxin, which is present in foods like almonds. Studies on *A. flavus* revealed that SUMO-conjugated protein accumulated at higher temperatures, but the deletion of the SUMO gene negatively impacted the pathogenicity and capacity for colony formation⁹⁴. The entire SUMOylation machinery in *A. nidulans* has been found and described. *A. nidulans* has a single gene *SumO*, that encodes SUMO and also contains the E3 ligases *SizA*, *SizB*, and *MmsU*^{98,99}. *SumO* protein is necessary for cellular differentiation in this fungus even though it is not required for fungal vegetative development. *A. nidulans ΔsumO* cells exhibit enhanced susceptibility to the DNA-damaging chemical methyl methanesulfonate (MMS) and the DNA synthesis inhibitor hydroxyurea (HU) in addition to impaired conidiation¹⁰⁰. Additionally, *ΔsumO* cells exhibit self-sterility, indicating that SUMOylation of important targets is necessary for the production of viable meiotic progeny in *A. nidulans*. Proteins were tagged using a method called "SUMOlock" to identify 149

SUMOylated proteins (SUMOylome)⁹⁹. This study demonstrated that a significant portion of the SUMOylated proteins was involved in DNA repair, RNA processing, and transcription regulation. It's interesting to note that the deletion of UlpB or all three SUMO ligases together had very severe effects on conidiation and growth (Figure 4). Another intriguing finding from this research was that although *sumO* deletion was generally well tolerated, deletion of *ulpB* was not, indicating that deSUMOylation of key substrates is required for growth.

The plant pathogen *Magnaporthe oryzae* causes rice blast disease. It is a filamentous ascomycete. Deletion of the SUMO (*smt3*), E1 (*aos1*, *uba2*), and E2 enzymes in *M. oryzae* caused a variety of abnormalities, including defects in septum formation, conidiation, sensitivity to stress, mycelial development, and pathogenicity^{12,101}. It has been shown that at least four septins are SUMOylated, and any alterations to the consensus SUMOylation sites in each septin cause the septins to separate in the appressoria and impair virulence⁶⁰. These mutants developed obtrusively and with delayed host penetration. Additionally, compared to the wild type, SUMO pathway mutants (*smt3*, *aos1*, *uba2*, and *ubc9*) showed increased sensitivity to DNA damaging stress. (Figure 4)¹².

These results collectively show that although SUMOylation is not necessary for survival in pathogenic fungi (except in *C. glabrata*), it is required for stress response, differentiation, and pathogenicity in all fungi so far investigated⁴¹.

putative SIMs^{79,110,111}. On the other hand, Uls2 forms a heterodimer composed of Slx5 and Slx8. Slx5, also known as Hex3 possesses two SIMs and a RING finger domain^{105,111,113}. In humans, RNF4 (Ring Finger Protein 4) is the only studied STUbL. It has been implicated in the regulation of diverse cellular processes, including DNA repair, transcriptional regulation, and stress response. Anomalies in RNF4 levels have been linked to a variety of illnesses, including cancer^{114,115}. This emphasizes the relevance of STUbLs in cellular homeostasis. By connecting the ubiquitin and SUMO pathways, STUbLs provide an additional regulatory mechanism for controlling the levels of SUMOylated substrate forms.

1.5 SPECIFIC OBJECTIVES OF THE CURRENT STUDY

As discussed above, SUMOylation plays a significant role in regulating fungal development, differentiation, and virulence, and appears to be critical for the pathogenesis of various fungi. Our laboratory had identified the SUMOylation machinery in *C. glabrata* and demonstrated that the SUMOylation pathway was required to combat stresses like high temperature and DNA damage. We also showed that CgUlp2 was required for virulence of *C. glabrata*⁹⁰. Based on these studies we wondered if SUMOylation could serve as a good target of intervention for fungal infection. Since SUMOylation appeared to be critical for survival or combating stress or for virulence in many fungi^{41,90,91,116}, it could serve as a pathway for targeting antifungals. We, therefore, followed up our previous observations. As we had seen that loss of CgUlp2 led to reduced protein SUMOylation, we had hypothesized that this reduced protein SUMOylation was due to increased degradation of polySUMOylated proteins. If that were true, then the STUbL pathway was operational in *C. glabrata*. Therefore, in this thesis, we set out to

- 1) Identify and characterize the STUbLs and STUbL-mediated protein homeostasis in *C. glabrata*.
- 2) Since the loss of CgUlp2 leads to reduced capacity for survival in macrophages and systemic candidiasis, we attempted to decipher the structure of the CgUlp2-SUMO complex to design specific drugs that can disrupt this interaction.

CHAPTER 2 MATERIALS AND METHODS

2.1 MOLECULAR BIOLOGY METHODS

2.1.1. Polymerase Chain Reaction

The polymerase chain reaction (PCR) was performed using Vent DNA Polymerase to ensure the error-free amplification for cloning, generation of mutants, and protein expression studies whereas Taq DNA polymerase was used for screening purposes. Both the polymerases were supplied by New England Biolabs (NEB). The following was the setup for the PCR reaction used for amplification. For Taq Polymerase, standard Taq Buffer was utilized, and for Vent Polymerase, ThermoPol Buffer was employed.

Table 1. Setting up the PCR reaction

Component	Volume
10X buffer	2.5 µl
10mM forward primer	0.5 µl
10mM reverse primer	0.5 µl
10mM dNTPs	0.5 µl
Template DNA 100ng	1 µl
Polymerase	0.125 µl
MiliQ	19.875 µl

The PCR reaction was conducted in the manner described below:

Initial denaturation		95°C
Denaturation	30 cycles	95°C
Annealing		55-65°C
Extension		72°C
Final extension		72°C
Hold		4°C

The product was amplified using an Eppendorf thermal cycler. The PCR products were resolved and verified via agarose gel electrophoresis. If necessary, a Qiagen gel extraction kit was used to purify the separated products.

2.1.2. Molecular cloning

2.1.2.1. Ultracompetent cells preparation

The Inoue method, as outlined in Sambrook and Russell (2006), was employed to prepare ultracompetent cells of the DH5 α strain of *Escherichia coli*¹¹⁷. Initially, a single colony of bacteria was inoculated in SOB broth and allowed to grow for 16 hours at 37°C to make the primary culture. The next day, a secondary culture was initiated by transferring 1/100th volume of primary culture in 100mL of SOB broth, followed by incubation at 18°C with shaking at 180 rpm until the OD600 reached 0.4-0.5. After cooling the flask with ice, the cells were quickly extracted using centrifugation at 12,000 rpm for 10 minutes at 4°C. The supernatant was carefully discarded, and the cell pellet was washed with cold, distilled water before being resuspended in 80 mL of Inoue transformation mix (containing 50mM MnCl₂·4H₂O, 15mM CaCl₂·2H₂O, 250mM KCl, 500mM PIPES pH6.7). The suspension was centrifuged again for ten minutes at 4°C at 12,000 rpm, and the supernatant was discarded with caution to avoid disturbing the pellet. The cells were then resuspended in 20 mL of ice-cold Inoue buffer, supplemented with 1.5 mL of DMSO, and the mixture was gently rotated. After allowing the cell suspension to rest on ice for ten minutes, it was divided into 100 μ L aliquots and then snap-frozen in liquid nitrogen. Further, the tubes containing aliquots were maintained at -80°C until use.

2.1.2.2. DNA transformation into bacterial cells

100 μ L of ultracompetent DH5 α cells of *E. coli* were retrieved from from -80°C storage and thawed on ice. Following this, 2 μ L of plasmid DNA was added to the cells, and the mixture was allowed to stand on ice for 20 minutes. Subsequently, the cells underwent a heat shock treatment at 42°C for 90 seconds, after which the tube was promptly returned to ice for a further five minutes. Upon completion of the heat shock, 900 μ L of LB broth was added to the cells, and the mixture was incubated in a thermomixer set at 37°C for 40 minutes with gentle agitation. Following this incubation period, 100 μ L of the cell suspension was spread onto a LB plate with appropriate antibiotics, which was then incubated in a 37°C for 14-16 hours for further analysis.

Instead of using LB media, 900µl of SOB was utilized to transform the ligated DNA construct. Additionally, the cells were given a 60-minute recovery period at 37°C. The cell suspension was spun for one minute at 13000 rpm. After removing 900µl of the supernatant, the cells were resuspended in the residual media and distributed on LB plates containing the relevant antibiotics.

2.1.2.3. Plasmid isolation

The alkaline lysis method was used to isolate plasmids from bacteria. In 3 mL of LB broth with the proper antibiotic for selection, a single colony of bacteria was inoculated. The culture was grown overnight at 37°C and 180 rpm of continuous shaking. Next, by centrifuging at 13,000 rpm for one minute, the culture was harvested and pelleted. The excess liquid was then removed by decanting the media and inverting the tube onto a piece of tissue. The pellet was mixed with 200µl of ice-cold Solution-I (25 mM Tris-Cl pH 8.0, 10 mM EDTA pH 8.0), and dissolved by pipetting or vigorously vortexing the tube for 10 seconds. Solution-II (0.2 % NaOH, 0.2 % SDS) was added (200µl), and the tube was gently inverted to mix. After that, 200µl of Solution-III (3 M potassium acetate, 11.5 % glacial acetic acid) was added, and the mixture was gently mixed by repeatedly inverting the tube four to five times. The tube was then instantly kept on ice for five minutes. Subsequently, the tubes were spun for 10 minutes at 4°C at 13,000 rpm. After collecting the supernatant carefully in a new Eppendorf tube (without disturbing the pellet) 420µl of isopropanol (0.7 volumes) was added to it. In order to mix, the tube was inverted four or five times and then centrifuged it to precipitated the DNA at 13,000 rpm for ten minutes at room temperature. The resulting pellet was washed with 70% ethanol and allowed to air dry in order to get rid of any remaining alcohol. Finally, the plasmid DNA was resuspended in 40µl of TE-RNase (10 mM Tris-Cl pH 7.4, 1 mM EDTA pH 8.0, 0.1 mg/mL of DNase free RNase) and allowed to incubate for 30 minutes at 37°C. Routinely, the plasmid concentration and purity were assessed using agarose gel electrophoresis.

2.1.2.4. Screening of the clones by restriction digestion

The above-described procedure was used to isolate the plasmids, and restriction digestion was used to verify that the insert was present. Via agarose gel electrophoresis, the recombinants were distinguished by size differences between the insert-carrying and empty vectors. In order to

verify the clones, subclone the insert into a different vector, and digest the PCR product flanking restriction sites, restriction digestions were carried out. The reaction mixture was made in the following manner for a 10 µl reaction in order to facilitate DNA digestion using high-fidelity restriction enzymes from NEB. At 37°C, those reaction mixtures were incubated for a minimum of one hour. Subsequently, the digested samples were subjected to agarose gel to determine the presence of DNA and isolated for further use if required.

Table 2. Setting up restriction digestion

Component	Volume
10X CutSmart Buffer	1 µl
DNA (1-2µg)	2-4 µl (depending on concentration)
Enzyme (4 units)	0.2 µl
MiliQ water	4.8-6.8 µl

2.1.3. Site-directed mutagenesis

Point mutations in the *CgULP2* gene were created by site-directed mutagenesis. For the PCR reaction, the high-fidelity KOD polymerase was employed. Following the reaction, DpnI was used to digest the product. Since DpnI only cleaves at methylated sites, the PCR product remains intact while the template plasmid is consumed. This step is crucial as it prevents the proliferation of colonies derived from the parental template, considering the significantly higher transformation efficiency of circular template plasmids compared to linear PCR products. Following digestion, 10 µl was transformed with ultracompetent cells and incubated for 16 hours at 37°C to form colonies. The primers used to produce the *CgULP2* mutations are listed in Appendix 8.2.

2.1.4. cDNA synthesis and Quantitative Real-Time PCR

C. glabrata cells were grown in YPD at 30°C for 16 hours, it was sub-cultured till it reached an OD₆₀₀ of 1. Cells were treated with DNA damaging agent (0.04% MMS), and hydroxyurea (100mM) and allowed to grow for additional 2 hours. For thermal stress, *C. glabrata* cells were

shifted to 42°C and incubated for 2 hours. The cultures were then pelleted down and stored at -80°C for RNA extraction.

For RNA extraction, the Ribo Pure kit from Invitrogen was used, followed by cDNA conversion using the SuperScript III First-strand Synthesis System for RT-PCR (Invitrogen) with 1 µg RNA as a template. SYBR green master mix (Invitrogen) was employed for quantitative reverse transcriptase PCR. *Cg-Act-1* was used to normalize RNA levels and also served as endogenous control.

Table 3. Reaction mixture for cDNA synthesis

Component	Volume
5X cDNA synthesis Buffer	4µl
5mM dNTP Mix	2µl
RNA Primer	1µl
RT enhancer	1µl
Verso Enzyme Mix	1µl
Template (RNA)	1-5µl
Nuclease free water	to 20µl
Total Volume	20µl

Before cDNA preparation, the RNA preparations were routinely tested for DNA contamination by performing Act1 PCR. Only DNA-free RNA preparations were used for expression studies. qRT-PCR was used to measure the transcript levels. The fold change was computed using the $\Delta\Delta C_t$ method.

2.2 YEAST GROWTH AND MUTANT STRAIN GENERATION

2.2.1. Genomic DNA isolation

In 5 mL of YPD broth, a single colony of *C. glabrata* was grown for 16 hours. The cells were extracted using a 5-minutes, 3000 rpm centrifugation. Subsequently, 500 µl of breaking buffer (10 mM Tris-Cl pH8.0, 1 mM EDTA pH8.0, 100 mM NaCl, 2% TritonX-100, 1% SDS) was used to resuspend the cell pellet. A 2.0 mL microfuge tube that had been roughly filled with

glass beads to the 200µl mark was then filled with the cell suspension. 200µl of a Phenol: Chloroform: Isoamyl alcohol (25:24:1) solution was mixed to this. After wrapping the tubes with parafilm tape, they were vortexed for two minutes at a high speed. Then, the same tube was filled with 200µl of TE-pH8.0, and it was quickly vortexed for 30 seconds. After five minutes of centrifuging at 13,000 rpm, the top aqueous layer containing DNA was carefully transferred to a sterile microfuge tube. Subsequently, 1 mL of 100% ethanol was added to the tube, and the contents were thoroughly mixed by inversion. The mixture was centrifuged again at 13,000 rpm for five minutes, resulting in the formation of a DNA pellet. The pellet of DNA was left to air dry while the supernatant was disposed off. 40 µl of TE with RNase was used to resuspend the pellet. The DNA quality and concentration were evaluated by agarose gel electrophoresis.

2.2.2. Yeast transformation

The yeast colony was grown overnight in 5 mL of YPD after being inoculated. Cells were harvested by centrifuging them for 5 minutes at 3000 rpm. During the next step, cells were resuspended in sterile double distilled water and pelleted after discarding the supernatant. Further, cells were resuspended in 100mM LiAc and pelleted down again. This pellet was then given 350µl of the transformation mixture.

Table 4. Components of the transformation mixture

Components	Volumes
PEG 3500 50% w/v	240 µl
LiAc 1 M	36 µl
Single stranded DNA (2mg/ml)	50 µl
Plasmid DNA 1µg + Double distilled water	24 µl
The total volume of the transformation mixture	350 µl

After vortexing the cells to resuspend them, the mixture was put in a thermomixer and incubated it for 40 minutes at 42°C. After centrifuging the cells for 5 minutes at 3000 rpm, the supernatants were removed by pipetting. After being redissolved in 100 µl of distilled water, the pellet was spread out onto a selection plate. After air-drying the plates in a laminar hood, they were incubated at 30°C for two or three days.

2.2.3. Gene disruption in *C. glabrata* strains

The ORFs in *C. glabrata* were disrupted by homologous recombination using a cassette containing the nourseothricin acetyltransferase (*NAT1*) gene, which provides resistance to nourseothricin. This cassette was flanked by 5'- and 3'-UTR regions of the target gene (schematic in appendix 8.4). To initiate this process, the 5'- and 3'-UTRs (about 600 bps) of the target gene were amplified from wild-type genomic DNA. Simultaneously, the *NAT1* gene was amplified from a plasmid with one-half of each of the 5'- and 3'-UTR fused to it. 300–350 bp complementary regions were shared by the two *NAT1*-amplified fragments. After co-transforming the fused PCR products into the wild-type strain, and the transformants were plated onto YPD. Following a 12–16 hour incubation at 30°C to promote homologous recombination between the *NAT1* fragments and the genomic loci, cells were replica-plated onto YPD plates supplemented with 200 mg/ml nourseothricin. These plates were then incubated for an additional 24 hours at 30°C. Colonies resistant to nourseothricin were isolated, and PCR was used to test for gene disruption caused by homologous recombination.

To obtain double deletions, the parental single-deletion strain was subjected to transformation with a plasmid carrying the FLP flippase gene. Transformants exhibiting uracil prototrophy in the CAA medium was selected. The *NAT1* resistance cassette excision is made possible by the flippase enzyme at FRT (Flippase recognition target) sites flanking the *NAT1* gene. The transformants that were sensitive to nourseothricin were isolated and subjected to curing of the FLP flippase-encoding plasmid after four to five passages in a non-selective YPD medium. This was further confirmed in CAA plates for the loss of URA. Cells were then transformed using a linearized segment of the second target gene, which carried the sequence 5'-untranslated region (5'-UTR)-*NAT1*cassette-3'-untranslated region (3'-UTR), and had been PCR amplified from the genomic DNA of a single deletion strain. Colonies that were resistant to nourseothricin were isolated, and a PCR was used to check for target gene disruption.

For complementation assays, the genes for *CgSLX5* (1.52 kb), *CgSLX8* (633bps), and *CgULS1* (4.22 kb) were amplified by PCR from wild-type genomic DNA using the high-fidelity Vent polymerase. Then, at the BamHI-XhoI restriction enzyme sites, these genes were cloned into the pGRB2.2 plasmid, which is located downstream of the PGK1 promoter. The recombinant

plasmids were then transformed into the appropriate deletion mutants for the complementation assay, and the phenotypes were scored.

2.2.4. Plate-based growth assay

C. glabrata wild type and deletion strains were grown in YPD/CAA medium at 30°C until reaching an O.D of 1.0 at A₆₀₀. Then 10-fold serial dilutions were made in 96 well plates. 2-3µl of diluted cell suspensions were spotted onto appropriate plates and incubated for 2-4 days at the required temperatures to evaluate the cell growth.

2.2.5. Biofilm formation

Biofilm formation was performed on a flat-bottomed 24-well polystyrene plate. YPD-grown *C. glabrata* cells in the exponential phase were added to the plate and incubated at 37°C with mild rotation (75 rpm) for 90 minutes. Wells were then washed twice using PBS, followed by the addition of 1ml of RPMI-1640 complete media to each well. The plate was then incubated at 37°C with 75 rpm, for another 24 hours to promote biofilm formation. The next day, 500µl of used media was pipetted out and fresh 500µl RPMI containing 10% FBS was added. The plate was further incubated for 48 to 72 hours. During incubation, every day 500µl of fresh media was replaced to provide continuous nutrients for the growing biofilm. After 72 hours, the plate was washed with PBS to eliminate all the planktonic cells. Cells were then collected in PBS, pelleted down, and stored at -80°C for RNA isolation. XTT assay was used to quantify biofilm formation in wild type and various deletion strains of *C. glabrata*, after 72 hours of biofilm formation, the plate was washed thrice with PBS to remove unattached cells. 100µl of 1x XTT solution was added, and the plate was incubated in the dark for one hour. Absorbance at 492nm was measured, RPMI-1640 complete medium serving as a negative control, and the absorbance of the *Candida*-grown wells was subtracted from this value.

2.2.6. Yeast two-hybrid assay

Yeast two-hybrid assay was used to test and confirm CgUlp2-CgSUMO interaction. The ‘bait’ which is denoted by *CgULP2* and ‘prey’, represented by *CgSMT3* were cloned in the pGBDU-C1 and pGAD-C1 vectors respectively. The integrity of both constructs was confirmed by sequencing. The constructs were also checked and evaluated for their GAL4 self-activation

possibilities. Both the constructs containing ‘bait’ and ‘prey’ plasmids were then co-transformed in a reporter strain. The reporter strain (PJ694-A) used in the study is equipped with *HIS3* and *ADE2* as reporter genes. Transformants were then selectively cultured on SC-Leu-Ura medium plates and subsequently patched onto SC-Leu-Ura-His and SC-Leu-Ura-Ade plates to confirm the interaction between CgUlp2 and CgSmt3.

2.3. PROTEIN AND RELATED BIOCHEMICAL TECHNIQUES

2.3.1. Whole cell protein extracts from yeast cells by Trichloroacetic acid (TCA)

The TCA method of protein precipitation was used to extract the proteins. *C. glabrata* cells were cultured in the appropriate medium until they reached the exponential phase. Cultures were spun down at 3000 rpm for 5 minutes and glass beads were added to the cell pellet along with 200µl of 20% TCA, and the mixture was vortexed at a high speed for three minutes to lyse the cells. Lysates were collected in a new 1.5 ml Eppendorf tube. Then, two washes of 5% TCA were applied to the tube containing glass beads, followed by three minutes of vortexing, and the lysates were pooled and centrifuged for 10 minutes at 13000 rpm. As a final step, 200µl of Laemmli buffer was used to resuspend the pellet after the supernatant was removed. Then a drop of 2M Tris (unpHed) was added to the buffer till it turned blue to neutralize the acid. To denature proteins, the extracts in Laemmli buffer were boiled at 95°C for five minutes, followed by centrifugation at 3000 rpm for five minutes. The supernatant was then carefully collected in fresh Eppendorf tubes. Protein quality was assessed by running 8-15% SDS-PAGE followed by Coomassie brilliant blue staining for at least an hour and then destaining.

Composition of solutions used:

4X SDS loading dye (Laemmli buffer): 200 mM Tris-HCl pH 6.8, 8% SDS w/v, 40% Glycerol v/v, 6 mM Bromophenol Blue, 4 % β-mercaptoethanol

Coomassie staining solution: Coomassie Brilliant Blue-250- 0.25 g, Methanol-45 ml, Glacial acetic acid (GAA)-10 ml, Water- 45 ml

destaining solution: Methanol-45 ml, Glacial acetic acid-5 ml, Water- 50 ml

2.3.2. SDS- PAGE and Western blot

A standard protocol was followed for casting the gels for SDS-PAGE. A 4% stacking gel was used, and depending on the molecular weight of the targeted protein being examined, the resolving gel ranged from 8 to 15%. Electrophoresis was carried out at 75–80V for the stacking gel and 100–120V once the proteins entered the resolving gel. To monitor the migration, the samples were loaded with pre-stained molecular weight markers. Western blotting was performed using a conventional semi-dry transfer technique to the PVDF membrane after SDS-PAGE. 5% skim milk powder was then used to block the membrane for one hour with. Following blocking, it was incubated with α -flag primary antibody (Sigma, 1:10,000) for 14-16 hours. Further, the membrane was washed thrice with TBST for 10 minutes each before being exposed to a mouse HRP-conjugated secondary antibody (Abcam, 1:20,000) for 1 hour. Following three TBST washes, the blot was developed using Biorad western ECL substrate i.e., luminol and peroxide reagent, and imaged using the ChemiDoc Imaging system to detect the signal through chemiluminescence. Blots were further reprobed with α -tubulin for the loading control.

Ponceau Staining solution was used to stain PVDF membranes to assess the total protein as well as examine the transfer of proteins to the membrane.

Composition of Ponceau Staining solution (100 mL): Ponceau S -100 mg, Water- 95 ml, Glacial acetic acid-5 ml.

2.3.3. Quantification of the western blot

ImageJ/FIJI's Gel Analyzer option was used to perform a densitometric analysis of the chemiluminescence readout following a western blot. The loading control for protein expression, which is typically an antibody to a housekeeping gene-expressed protein, was used to normalize protein expression. Western blots from at least three separate experiments were quantified for protein expression.

2.3.4. Protein expression and purification

We cloned *CgULP2* and *CgSMT3* in both pET28a and pGEX4T1 vectors, which have 6x-Histidine and Glutathione S-transferase (GST) tags. For protein expression, the recombinant plasmids were then transformed into the BL21(DE3)RIL bacterial strain. A single colony was then inoculated in an appropriate volume of LB broth containing the necessary antibiotics and was incubated for 14–16 hours at 37°C and 180 rpm. One percent of the primary culture was then transferred to a secondary culture and incubated at 37°C until it reached an O.D-0.6. Then 1 ml of culture was set aside in a 1.5 ml microfuge tube for further use as a control (uninduced culture) and the remaining culture was induced to overexpress the target recombinant protein using 0.4 mM IPTG. Both induced and uninduced cultures were incubated at 22°C and 180 rpm for 16 hours. Following induction, 1 ml of each induced and uninduced culture was placed in a 1.5 ml microfuge tube and centrifuged at room temperature for one minute at 13,000 rpm. The resulting pellet was resuspended in 100 μ l of 1x SDS loading dye and vortexed for one minute at high speed. The samples were boiled at 95°C for 10 minutes. Afterward, protein expression was checked by analyzing both induced and uninduced samples using 8-15% SDS-PAGE with a molecular mass ladder, followed by Coomassie Blue G-250 staining.

Following protein expression analysis, we lysed the cell pellet in lysis buffer, sonicated it for 3 minutes with a 3-second 'on' and 7-second 'off' cycle. The lysate was then centrifuged at 15,000 rpm for an hour to obtain the protein supernatant. The supernatant containing the protein was then incubated with Ni-NTA or glutathione resin for two hours at 4°C. Following incubation, the flow through was discarded, and the resin-bound proteins were washed with wash buffer to remove impurities. Elution of the bound proteins was carried out using elution buffer. The quality of the affinity-purified proteins was assessed using 8–15% SDS-PAGE with an unstained protein marker, followed by Coomassie Blue G-250 staining.

Buffers for purification of 6xHIS-tagged proteins:

Lysis buffer- 25 mM Tris (pH7.5), 350 mM NaCl, 1 mM β ME, 5 mM Imidazole, 0.1% IGEPAL CA-630, and 1 mM PMSF

Wash buffer- 25 mM Tris (pH7.5), 250 mM NaCl, 1 mM β ME, and 50 mM Imidazole

Elution buffer- 25 mM Tris (pH7.5), 250 mM NaCl, 1 mM β ME, 1 mM EDTA, 10% glycerol, and 300 mM of imidazole

Buffers for purification of GST-tagged proteins:

Lysis buffer- 25 mM Tris (pH7.5), 350 mM NaCl, 1 mM β ME, 0.1% IGEPAL CA-630, and 1 mM PMSF

Wash buffer- 25 mM Tris (pH7.5), 250 mM NaCl, and 1 mM β ME

Elution buffer- 25 mM Tris (pH 7.5), 250 mM NaCl, 1 mM β ME, 1 mM EDTA, 10% glycerol, and 10 mM reduced glutathione.

Gel filtration, or size exclusion chromatography, was carried out using SEC buffer containing 25 mM Tris-HCl, pH 7.5, 250 mM NaCl, 1 mM EDTA, 2% glycerol, and 1 mM DTT. This step aimed to eliminate the non-specific proteins that were detected after affinity purification. To separate the purified proteins, approximately 500 μ l of the protein sample at a concentration of 2–3 mg/ml was loaded onto a Superdex 200 10/300 GL column (GE Healthcare) that had been pre-equilibrated with the SEC buffer. The purified proteins were separated into fractions, which were then analyzed using 8-15% SDS-PAGE to visualize the protein of interest. The purified proteins obtained from this process were used for the biophysical and functional characterization of the CgUlp2 protein.

2.3.5. Invitro pull-down assay

Invitro pull-down was carried out using Ni-NTA resin beads. Initially, pellets from 1-litre cultures each of bacteria expressing two distinct proteins with two different tags (6xHIS and GST) were co-lysed, combining the cell pellets in 20ml of lysis buffer for each culture. After sonication, the cell lysate was spun at 15,000 rpm for an hour at 4°C. The supernatant was separated and kept for binding with an equilibrated 1ml Ni-NTA column for 2-3 hours at 4°C. Following the incubation, the flow through was removed, and the resin was thoroughly washed with 40 ml of wash buffer to remove nonspecifically bound proteins. Then the bound proteins were eluted from the resin using 1 ml of elution buffer containing 300 mM imidazole (3 times). To test for the interaction of the two proteins, the eluted proteins were separated by SDS-PAGE, followed by western blot analysis using both α -GST and α -His antibodies.

2.3.6. CgUlp2 binding assay

We transformed various constructs of CgUlp2 protein and constructs of various lengths of linear SUMO chains in BL21(DE3)RIL bacterial strain which were expressed and purified as described in the sections above. To investigate the interaction, we incubated 100µg of 1x-SUMO, 2x-SUMO, and 6x-SUMO protein individually, as well as a combination of linear poly-SUMO chains containing these proteins, with 10µl CgUlp2 protein-coupled glutathione resin in 100µl of PBS buffer at 4°C for two hours. Following the incubation period, the beads were subjected to three washes with 200 µl of PBS each to remove any nonspecifically bound proteins. Subsequently, proteins bound to the resin were eluted by boiling beads at 95°C in 50µl of 1x SDS loading dye. The eluted proteins, as well as the input and flow-through samples, were analyzed using Coomassie staining on a 12% SDS-PAGE gel to visualize the protein interactions.

2.3.7. CgUlp2 protease activity

For testing the protease activity of CgUlp2, WT and SIM mutant of CgUlp2 protein was expressed and purified from bacteria. Additionally, 2x-SUMO and 6x-SUMO substrates were purified from bacteria for use in the assay. In this experiment, 100 µg of each protein construct, whether it was in dimer or hexamer, was mixed with 50 µl of PBS containing 1 mM DTT and 1 µg of either CgUlp2 (WT) or its SIM mutant variant. The reactions were conducted at room temperature, and aliquots of 10 µl were taken at 0, 15, 30, and 60 minute interval after the addition of protein construct. These aliquots were subjected to SDS-PAGE analysis by loading them onto a 12% SDS-PAGE gel. Visualization of the proteins was achieved using Coomassie staining.

2.3.8. Pull down of SUMOylated proteins

To isolate proteins SUMOylated in vivo from *C. glabrata* extracts, a protocol was derived using SUMO-interacting motifs. This method was adapted from M.S. Rodriguez lab ¹¹⁸. SUMO Interacting Motif was cloned tandemly in the pET28a bacterial expression vector, which has a 6xHIS tag at the N-terminus. Initially, we annealed the forward and reverse primers that contained sequences for 2 SIM peptides and utilized them as a template for PCR. In the first PCR round, the forward primer incorporated Nde1, and the reverse primer included a BamH1 site. Following successful PCR amplification, indicative of the presence of the 2 SIM peptide, the

product was digested with Nde1 and BamH1 for direct cloning into the pET28a vector. After confirming the presence of the 2 SIM peptide in the pET28a vector through sequencing, this construct was employed as a template to introduce an additional 2 SIM peptide. This step involved using a forward primer with a BamH1 site and a reverse primer with a Sac1 site. The presence of the resulting 4 SIM peptide was confirmed through sequencing, and the 4 SIM-pET28a construct served as the template for the subsequent round. In the final PCR round, a forward primer with a Sac1 site and a reverse primer with a HindIII site was utilized to generate the next set of 4 SIM peptides. The successful cloning resulted in the tandem incorporation of 8 SIM peptides into the pET28a vector. Following the transformation of these motifs in the BL21(DE3)RIL bacterial strain, they were expressed and purified using the previously described Ni-NTA affinity purification method.

To employ the SIM motif as affinity traps for the pull-down of SUMOylated protein, *C. glabrata* yeast strains containing 3xFLAG-tagged SUMO were cultured at 30°C until they reached O.D-1.4. The cells were then collected by centrifugation at 3000 rpm for 5 minutes at 4°C. The cell pellet was promptly snap-frozen in liquid nitrogen and kept at -80°C until needed. The pellet was first mixed with freshly made, chilled lysis buffer containing protease inhibitors, and the tube was then put in the ice for two minutes. Next, the cell suspension was moved to a 2 mL microfuge tube that had been chilled beforehand and had been filled to about 500 µl with glass beads. A bead beater was used to lyse the cells, with six cycles of a 30-second pulse and a 1-minute ice rest in between to prevent the lysate from overheating. The lysate was spun for 40 minutes at 4°C at 13000 rpm. After collecting the supernatant, the pH of the lysate was assessed, and if it measured below 8, 1 M Tris (unadjusted pH) was employed to bring it to the desired level. A total of 5% of the whole cell lysate was utilized as input; it was then boiled with 1x SDS loading dye and kept at -20°C until needed.

After pre-washing 100µl of Ni-NTA SIM immobilized beads in lysis buffer free of protease inhibitors, the lysate was incubated for 6 hours at 4°C in an end-over-end rotator. The tube was spun at 1000 rpm for five minutes at 4°C to collect the beads. The beads were washed thrice with wash buffer at 1000rpm for two minutes at 4°C. For elution of potentially SUMOylated proteins, beads were incubated in an elution buffer in an end-over-end rotator at 4°C for 10 minutes. The eluates were mixed with 2xSDS-loading dye and subjected to boiling at 95°C for 5 minutes to

denature the proteins. The proteins were then separated on a 10% SDS-PAGE and western blots with anti-FLAG antibody were done to visualize the enrichment of SUMOylated proteins.

Composition of the buffers used:

Lysis buffer- 25 mM Tris (pH7.5), 250 mM NaCl, 0.1% IGEPAL CA-630, 25 mM NEM, and 1x protease inhibitor (complete mini, EDTA-free)

Wash buffer- 25 mM Tris (pH7.5), and 250 mM NaCl

Elution buffer- 25 mM Tris (pH7.5), 250 mM NaCl, and 300 mM of imidazole

Precautions:

All the buffers should be freshly prepared and pre-chilled before use.

All the steps should be performed on ice or 4°C.

2.4. CELL CULTURE

2.4.1. Preparation of THP-1 Macrophage monolayer

Human monocyte, THP-1 cells were seeded in a 25mm culture flask in RPMI-1640 medium supplemented with 10% FBS, 2 mM glutamine, and an antibiotic cocktail, and kept at 37°C in a humidified 5% CO₂. Cells were allowed to grow until a monolayer was formed. Cell density was maintained at 2.5×10^5 cells/ml by counting with a hemocytometer and 1µl of 160mM PMA (prepared in DMSO) was added to 10ml THP-1 cell suspension, resulting in a final concentration of 16nM to stop the cells from further division and to differentiate them into macrophages. After 12 hours of incubation, the old medium containing PMA was replaced with fresh prewarmed RPMI-1640 complete medium. The cells were then allowed to recover for another 12 hours under the same incubation conditions. These macrophage cells were checked under an inverted microscope for their flattened, spindle-shaped, and adherent morphology.

2.4.2. Infection of *C. glabrata* in THP-1 cells

C. glabrata strains were cultured in liquid YPD media at 30°C with 180 rpm of agitation. A cell density of 2.5×10^6 cells/ml was used to infect the cells, maintaining a multiplicity of infection of 1:10. *C. glabrata* cells were co-cultured with THP-1 macrophages for 2 hours, followed by gently washing several times (3-6 times) using 1 ml of sterile PBS to ensure the complete

elimination of the non-phagocytosed yeast pathogen. Cells were then incubated for 8, 12, and 24 hours to examine the *C. glabrata* cell survival/proliferation.

To quantify internalized yeast, macrophage internalized *C. glabrata* cells were washed and lysed using 1ml of sterile water for 2-3 minutes in the culture plate. Cells were scraped softly and cell lysate was collected. The cells were then serially diluted to 100 folds and 100µl of it was plated on a solid YPD medium followed by incubation at 30°C for 1-2 days. Colonies that appeared on YPD plates, were counted manually, and CFU (colony forming units) was calculated by multiplying with the dilution factor.

2.5. TANDEM MASS TAG (TMT)-BASED QUANTITATIVE TOTAL PROTEOMICS ANALYSIS

2.5.1. Sample preparation

For proteome analysis, exponential phase YPD grown *C. glabrata* cells were pelleted at 3000 rpm, washed once with MiliQ, and suspended in a lysis buffer composed of 50 mM HEPES pH 8.2, 8 M Urea, 50 mM NaCl, and EDTA-free protease inhibitors. Cell lysis was achieved using a Bead-beater in microfuge tubes for six cycles of 30-second duration each, with 1-minute interval between cycles to prevent lysate overheating. Lysates were cleared by centrifugation at 15,000 rpm for 30 minutes at 4°C. The protein concentration was then determined using the Lowry method and 200µg of proteins were run on a 10% SDS-PAGE to check the protein quality which was then sent to Mass Spectrometry (MS) facilities, Institute of Bioinformatics, Bangalore, India. At the MS facility, proteins were acetone precipitated, alkylated, and digested with trypsin.

2.5.2. TMT labeling and mass spectrometry

For TMT labeling, the 12 channels from the TMT pro-16 plex kit were used for labeling the respective samples. Each TMT channel reagent was solubilized in 200µl of anhydrous acetonitrile and allowed to incubate for 5 minutes. Then, 40 µl of respective TMT reagent was added to 80µg of each of the 12 sample peptide mix (dissolved in 40mM TEABC buffer), incubated for 60 mins at RT (after mixing) and the labeling reaction was stopped by adding 5% methylamine solution. An aliquot (5µl) of each of the labeled samples was quenched by adding 8 µl of 5% hydroxylamine, pooled, dried, reconstituted in 0.1% TFA, cleaned up using C18 stage

tip, dried and re-dissolved in LCMS grade water (containing 0.1% TFA and 3% acetonitrile) for labeling efficiency check. After the C18 stage tip clean-up, the sample was dried and dissolved in water (containing 0.1% TFA and 3% acetonitrile) for LCMS analysis to check the labeling efficiency. The labeling efficiency (>95 %) and reporter ion intensity normalization were performed. After the labeling efficiency check, the reaction was quenched using 5% hydroxylamine (for 15 min, at RT). Based on the reporter ion intensity values, each of the labeled samples was pooled, dried, and fractionated using basic pH Reverse phase HPLC into 96 fractions and concatenated into 12 fractions. The final LCMS run was performed (in triplicates) after dissolving the (desalted) 12 fractions in water (containing 0.1% TFA and 3% acetonitrile) and loading 1µg equivalent peptide to the mass spectrometer.

The LCMS analysis was performed using Q Exactive HF-X (Thermo Scientific, Bremen, Germany) interfaced with Dionex Ultimate 3000 nanoflow liquid chromatography system. The acquired mass spectrometry data was analyzed against *C. glabrata* reference proteome (downloaded from the UniProt database) using the SEQUEST search algorithm on the Proteome Discoverer platform (version 2.4, Thermo Scientific). Data was filtered with a 0.01% false discovery rate (FDR) based on the decoy search.

2.5.3. Analysis of gene ontology

The corresponding systematic ORF (CAGL IDs) of *C. glabrata* were achieved from the UniProt database using the accession numbers obtained from the proteins identified through mass spectrometry analysis. These proteins were then mapped to CAGL IDs in the Candida Genome Database. Subsequently, functional enrichment analysis, encompassing biological processes, cellular components, and molecular functions, was conducted using the DAVID tools. The resulting data were visualized through graphs generated using GraphPad Prism and SRplot software.

2.6. Imaging mitochondria in *C. glabrata* cells by confocal microscopy

To study the mitochondrial morphology and reactive oxygen species (mtROS) in wild-type and deletion strains of *C. glabrata*, MitoTracker Green dye (Invitrogen, M7514) and MitoSOX Red (Invitrogen, M36008) were used respectively. Exponential phase cells grown in YPD medium (1.0 A600) were harvested, and the pellet was washed thrice with 1x PBS to get rid of any

remaining YPD medium. After that, cells were treated with 200 nM MitoTracker Green and 1 μ M MitoSOX Red in 250 μ l PBS and allowed to incubate for 20 minutes at 30°C. Following incubation, cells were rinsed with PBS and resuspended in PBS without MitoTracker Green and MitoSOX Red.

Cells resuspended in PBS were placed on a 35mm cover glass bottom dish that had been coated with 0.1% solution of concanavalin A to facilitate imaging. Images were captured using a Leica TCS SP8 confocal microscope equipped with an HC PL APO CS2 63x/1.40 OIL objective. The microscopy data was processed and analyzed using Fiji Software.

2.7. COMPUTER-AIDED DRUG DESIGN

2.7.1 Homology modeling

The crystal structure of the catalytic domain of Ulp2 (PDB Id: 5LNB) and the Smt3 protein (PDB Id: 1EUV) of *S. cerevisiae* were obtained from the Protein Data Bank (PDB). These structures served as a template for homology modeling of the *C. glabrata* proteins Ulp2 and Smt3 using SWISS MODEL. CgUlp2 protein has a sequence identity of 58.57 percent and 96 percent sequence coverage whereas CgSmt3 protein has a sequence identity of 87.88 percent and 92 percent sequence coverage with *S. cerevisiae* counterparts. The models were refined and their energy was minimized using GalaxyWeb and PROCHECK. On PDBsum, the models were evaluated for quality and rated using Ramachandran plots. The identification of key catalytic triad residues in the model was determined through a sequence alignment with those of *S. cerevisiae* and *H. sapiens*.

2.7.2 Protein-protein docking

Protein-protein docking was conducted utilizing the homology-modeled catalytic domain of CgUlp2 and CgSmt3 protein. The ClusPro 2.0 web server generated 22 docked models, which were subsequently ranked based on their weighted scores. From these, the top 10 ranking docked complexes were manually visualized. Models demonstrating direct interactions between CD_CgUlp2 and CgSmt3 via SUMO interacting motif were selected for active site prediction in order to perform pharmacophore-based virtual screening.

2.7.3 Determination of active site on the protein

The active site on CD_CgUlp2-CgSmt3 protein was determined using the DoGSiteScorer application¹¹⁹. It estimates various physicochemical characteristics and predicts active site pockets on the protein. It returns a druggability score between 1-0. Pockets showing the highest druggability score were chosen as active site pockets.

2.7.4 Pharmacophore mapping and virtual screening for ligands

In order to design a suitable ligand, that can fit into the given active site pocket, along with the location and its residues, we need to know the binding nature of the active site pocket. The pharmacophore map of protein gives us the required molecular definitions and structural features, of the pocket. A ligand complementing such features can be virtually screened which will act as a ligand.

The pharmacophore map for the given protein was designed using the ‘Phase’ module of Schrodinger suite¹²⁰. Molecular features in terms of placement of aromatic ring, hydrogen bond density electron density are determined. Coordinates of these pharmacophore features were used to screen for ligands using the Pharmit website¹²¹. It screened ligands from ChEMBL32, Molport, ChemDiv, ChemSpace, MCULE, PubChem, ZINC, LabNetwork, NCI open repository, and 2000 FDA drugs. Lead molecules were screened out of thousands of molecules based on RMSD score (less than 0.5) and MM/GBSA score (less than 0.002).

2.7.5 Protein-ligand docking

Molecular docking for all top 10 ligands was performed with CD_CgUlp2-CgSmt3 protein using the Flare suite of CressetUk software (<https://www.cresset-group.com/software/flare/>). Protein was prepared by removing excess water molecules and neutralizing ions, following molecular docking at a pre-determined receptor site. Top 10 hits were shortlisted based on docking scores (less than -9.0).

2.7.6 Binding energy calculations

After docking, relative binding energies between ligand and protein are calculated by Average Molecular mechanics generalized by the Born surface area (MM/GBSA) technique. MM/GBSA

calculations in Schrodinger (Prime, version 2.1, Schrodinger, LLC, New York 2022) were used for these calculations¹²². The binding energy is given by:

$$\Delta G_{\text{bind}} = \Delta G_{\text{pL}} - (\Delta G_{\text{p}} + \Delta G_{\text{L}})$$

here, Protein(P), and ligand (L) form a complex (PL). Protein-ligand complexes from molecular docking were used for the calculation of MM/GBSA. Local optimization feature was used to minimize protein-ligand poses and the OPLS-2005 force field was used in the generalized -Born/Surface Area continuum solvent model for the calculation of the energies of complexes. Complex giving the lowest MM/GBSA score were further shortlisted.

2.7.7 Molecular dynamics simulation

Molecular dynamics (MD) simulation for 10ns was performed for the top 4 hits using the GROMACS simulation program. MD simulation for protein complex (CD_CgUlp2-CgSmt3) and top 3 protein-ligand complexes for 10ns in TIP3P water using CHARMM36 force field was performed. The system was solvated in a truncated octahedral boxes using TIP3P water, with the protein centered within the simulation container, ensuring a minimum distance to the box area of 1 nm to satisfy the minimum image convention. For neutralization of the overall system, NaCl ions were delivered, followed by minimization steps of 5000 using the Steepest Descent algorithm, achieving convergence within the force threshold of 1000 KJ⁻¹ nm⁻¹ to resolve any steric conflict. NVT and NPT ensembles were employed for 100ps and 1000ps at 300K temperature to equilibrate the system and ensure the system is entirely converged for production. The Particle Mesh Ewald (PME) technique was used to compute electrostatic interactions and forces. Finally, production was executed for 10ns. The generated output was further processed for the calculation of RMSD.

2.8. STATISTICAL ANALYSIS

Using GraphPad Prism software, the data was analyzed using the student's t-test and one-way analysis of variance (ANOVA) with Tukey's test. A statistical significant threshold was set at a p-value of ≤ 0.05 .

Chapter 3 Perturbation of SUMOylation affects the homeostasis of proteins essential for the establishment of successful infection

3.1 INTRODUCTION

Earlier work in our laboratory has established the role of SUMOylation machinery, particularly the *CgULP2* gene, for the pathogenicity of *Candida glabrata*. Deletion of deSUMOylase *ULP2* in *C. glabrata* was shown to be defective in infection and reduces virulence in mice models as well ^{90,116}. Our biochemical studies indicated that loss of *CgULP2* reduced the accumulation of polySUMOylated proteins. We had reasoned that the accumulation of polySUMOylated proteins in *CgULP2* deletion may have led to protein degradation via the SUMO-dependent ubiquitin-mediated protein degradation. This led to our hypothesis that *ULP2* reduces the pathogenicity of the *C. glabrata* due to perturbed protein homeostasis and that is mediated through the STUbL pathway.

In the budding yeast *S. cerevisiae*, accumulation of higher molecular weight polySUMOylated proteins was detected in strains lacking the heterodimer *SLX5* and *SLX8*. These strains also showed sensitivity towards DNA-damaging agents ^{76,113}. They are known as STUbLs because they ubiquitinate the SUMOylated substrate and are found in *Drosophila*, *Schizosaccharomyces pombe*, and mammals. In a SUMO-independent mechanism, the Slx5-Slx8 complex is found to ubiquitinate the target proteins, but SUMOylation can also stimulate the ubiquitylation of some proteins ¹²³. Additionally, damaged DNA in *S. cerevisiae* is shunted to the nuclear pore and repaired through a process involving Nup84 and Slx5/Slx8, highlighting the functional involvement of STUbL in DNA maintenance and repair ¹²⁴.

ULS1 on the other hand is not much explored as STUbL in yeast but the presence of multiple SIM motifs on it suggests its possible role as STUbL. Growth defects were observed in the cells lacking *ULS1*. In comparison to single mutants of *slx5Δ* and *uls1Δ*, double mutant strains of *uls1Δslx5Δ* displayed a greater accumulation of SUMOylated proteins. Furthermore, studies have shown the physical and genetic interaction of Uls1 with Slx5-Slx8, where the loss of Uls1 resulted in a decrease in the SUMOylation of Slx5 ^{111,125}. It is also known that human STUbL, RNF4, depleted cells are sensitive to DNA damage and also showed an accumulation of stress granules (SG particles) in the cytoplasm ¹²⁶.

STUbLs have not been studied in pathogenic fungi, including *C. glabrata*. We have identified STUbLs in various pathogenic fungi using a computational approach and have experimentally interrogated the importance of STUbLs in the normal physiology and pathogenesis of *C. glabrata*.

3.2 RESULTS

Identification of SUMO-targeted ubiquitin ligases in *Candida glabrata*

In our previous work, we identified the homologs of the components of SUMOylation pathway in fungi⁹⁰. Here, we identified the SUMO-targeted ubiquitin ligases (STUbLs) pathway across fungi. To identify orthologs in selected fungi, we retrieved the sequences of STUbL proteins, namely Slx5, Slx8, and Uls1, in *Saccharomyces cerevisiae* from the Saccharomyces Genome Database (SGD). Fungal organisms representing the five major phyla (Ascomycota, Basidiomycota, Chytridiomycota, Mucoromycota, and Microsporidia) were selected for analysis. For each protein, homologs were obtained by performing BLASTp against the NCBI nr database restricted to each of the organisms considered¹²⁷. The top hits (RefSeq hits) obtained in the BLASTp analysis were then assessed by using reciprocal BLAST analysis (BLASTp restricted to *S. cerevisiae* S288c sequences) to identify true orthologs. Only the hits that returned the *Saccharomyces cerevisiae* query protein as the top hit were considered true orthologs. The sequences that did not give the *S. cerevisiae* protein in the rBLAST were excluded. In some cases where no orthologs were identified using *S. cerevisiae*, we used sequences from *S. pombe*, or *C. albicans* to identify orthologs.

This pathway is highly conserved in fungi and homologs of Slx5, Slx8, and Uls1 proteins could be identified. Slx5 and Slx8 were identified in a few organisms using *S. cerevisiae* sequences. For some fungal organisms, Slx8 orthologs could be detected using *C. albicans* and *S. pombe*. Few of them were found to have multiple Slx8 orthologs with *S. commune* having 4 orthologs, *C. dubliniensis* having 3 orthologs, and *L. maculans*, *C. graminicola*, *C. albicans*, *C. tropicalis*, *C. orthopsilosis*, *K. phaffii*, *C. neoformans* and *P. graminis* having 2 orthologs each (Table 5).

A single Uls1 ortholog is present in most of the organisms except in *B. cinerea*, *S. pombe*, *S. punctatus* which have two orthologs. In Microsporidia, Slx8 orthologs were identified in *E. intestinalis*, *E. hellem* and *O. colligate* and two orthologs of Slx8 were detected in *E. intestinalis* and *O. colligate* using *C. albicans* sequence. Interestingly, though Uls1 orthologs are more widespread across fungi, no ortholog could be identified in any of the Microsporidia shortlisted in our study. To summarise, all fungi have at least one and often both of the STUbLs, Uls1 and Slx8. Slx5 seems to be less conserved than Slx8 and Uls1 and is restricted to a few classes only (Table 5).

To further characterize the SUMO-targeted ubiquitin ligases (STUbLs) in *C. glabrata*, we compared homologies between the *S. cerevisiae* and *C. glabrata* proteins. Table 6 displays their percent similarity across the complete protein sequence. Uls1 is the most conserved of them, having a 60% similarity between *C. glabrata* and *S. cerevisiae* (Table 6). Both the Slx5 and Slx8 share over 45% protein sequence similarity between *C. glabrata* and *S. cerevisiae*, respectively. Additionally, STUbLs have conserved RING domains. Slx5 and Slx8 lack the "SNF2-like translocase" domain, which Uls1, a large protein with 1408 amino acids, has in addition to the SIM motif and RING domain (Figure 5).

Table 5. Orthologs of SUMO targeted Ubiquitin Ligases (STUbLs) across fungi.

Phylum	Class	Fungal Organisms	Slx5	Slx8	Uls1
Ascomycota	Eurotiomycetes	<i>Aspergillus fischeri</i>			
Ascomycota	Eurotiomycetes	<i>Aspergillus nidulans</i>			
Ascomycota	Eurotiomycetes	<i>Microsporum canis</i>			
Ascomycota	Dothideomycetes	<i>Leptosphaeria maculans</i>			
Ascomycota	Dothideomycetes	<i>Parastagonospora nodorum</i>			
Ascomycota	Leotiomycetes	<i>Botrytis cinerea</i>			
Ascomycota	Leotiomycetes	<i>Sclerotinia sclerotiorum</i>			
Ascomycota	Sordariomycetes	<i>Fusarium graminearum</i>			
Ascomycota	Sordariomycetes	<i>Colletotrichum graminicola</i>			
Ascomycota	Sordariomycetes	<i>Chaetomium globosum</i>			
Ascomycota	Sordariomycetes	<i>Thielavia terrestris</i>			
Ascomycota	Sordariomycetes	<i>Neurospora crassa</i>			
Ascomycota	Sordariomycetes	<i>Magnaporthe oryzae</i>			
Ascomycota	Pezizomycetes	<i>Tuber melanosporum</i>			
Ascomycota	Saccharomycetes	<i>Candida albicans</i>			
Ascomycota	Saccharomycetes	<i>Candida dubliniensis</i>			
Ascomycota	Saccharomycetes	<i>Candida tropicalis</i>			
Ascomycota	Saccharomycetes	<i>Candida parapsilosis</i>			
Ascomycota	Saccharomycetes	<i>Candida orthopsilosis</i>			
Ascomycota	Saccharomycetes	<i>Candida auris</i>			
Ascomycota	Saccharomycetes	<i>Candida glabrata</i>			
Ascomycota	Saccharomycetes	<i>Saccharomyces cerevisiae</i>			
Ascomycota	Saccharomycetes	<i>Zygosaccharomyces rouxii</i>			
Ascomycota	Saccharomycetes	<i>Kluyveromyces lactis</i>			
Ascomycota	Saccharomycetes	<i>Eremothecium gossypii</i>			
Ascomycota	Saccharomycetes	<i>Komagataella phaffii</i>			
Ascomycota	Schizosaccharomycetes	<i>Schizosaccharomyces pombe</i>			
Basidiomycota	Agaricomycetes	<i>Trametes versicolor</i>			
Basidiomycota	Agaricomycetes	<i>Schizophyllum commune</i>			
Basidiomycota	Agaricomycetes	<i>Agaricus bisporus</i>			
Basidiomycota	Tremellomycetes	<i>Cryptococcus neoformans</i>			
Basidiomycota	Ustilaginomycetes	<i>Ustilago maydis</i>			
Basidiomycota	Pucciniomycetes	<i>Puccinia graminis</i>			
Mucoromycota	Glomeromycetes	<i>Rhizophagus irregularis</i>			
Chytridiomycota	Chytridiomycetes	<i>Spizellomyces punctatus</i>			
Chytridiomycota	Chytridiomycetes	<i>Batrachochytrium dendrobatidis</i>			
Microsporidia	Microsporidia	<i>Encephalitozoon intestinalis</i>			
Microsporidia	Microsporidia	<i>Encephalitozoon cuniculi</i>			
Microsporidia	Microsporidia	<i>Encephalitozoon hellem</i>			
Microsporidia	Ordosporidae	<i>Ordospora colligata</i>			
Microsporidia	Nosematidae	<i>Nosema ceranae</i>			

The organisms that are pathogenic to animals are in red font, pathogenic to plants are in green font and those that are pathogenic to both animals and plants are in yellow font. The presence

and absence of orthologs identified for proteins are shown. Light green-filled rectangles represent the presence of a single ortholog and red-filled rectangles represent the absence of an ortholog. Likewise, dark green filled rectangles represent the presence of 2 orthologs, light blue represents 3 orthologs, and dark blue represents 4 orthologs.

Table 6. Orthologs of the *S. cerevisiae* STUbL proteins Slx5, Slx8, Uls1 in *Candida glabrata*.

<i>Saccharomyces cerevisiae</i>	<i>Candida glabrata</i>	% similarity	% Identity
SLX5 (619) RING domain : 527-604 SIM : 24-28, 93-97, 116-119, 155-159, 476-479	CAGL0K01881g (506) RING domain : 431-460 SIM : 10-13, 34-38, 140-143, 309-312	49.4	35.3
SLX8 (274) RING domain : 206-250 SIM : 178-181	CAGL0G03553g (210) RING domain : 146-194 SIM : 24-27, 124-127	48.6	34.4
ULS1 (1619) RING domain : 1330-1386 SIM : 7-10, 371-374, 470-473, 540-543 SNF2 : 956-1157 Helicase C : 1428-1605	CAGL0G09493g (1408) RING domain : 1118-1176 SIM : 216-219, 253-256, 836-839, 1325-1328, 1368-1371 SNF2 : 736-1070 Helicase C : 1232-1348	60.0	46.5

The *Saccharomyces* Genome Database was used to collect *S. cerevisiae* proteins, and Blastp was used to determine which *C. glabrata* proteins served as orthologs. Pfam was used to search the protein sequences for annotated domains. The EMBOSS Stretcher (pairwise sequence alignment) tool was used to calculate the percentages of identity and similarity.

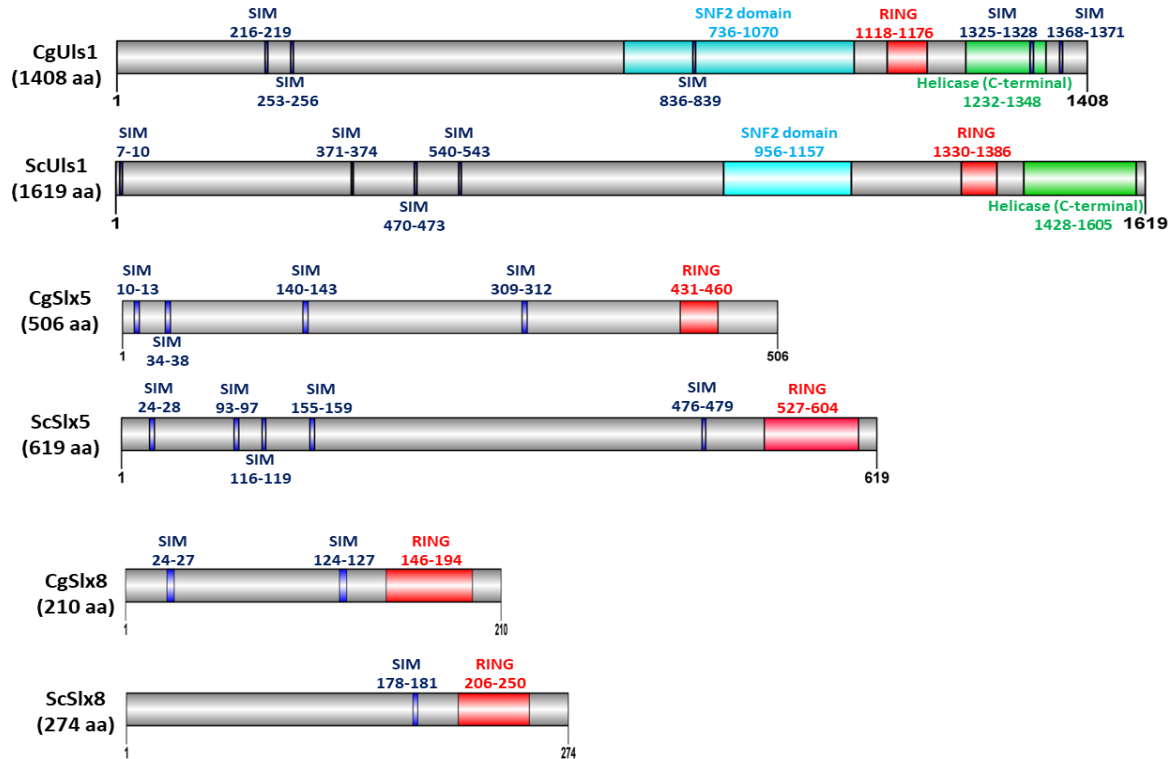


Figure 5. Schematic representation of orthologs of the *S. cerevisiae* STUbL proteins Slx5, Slx8, Uls1 in *Candida glabrata*. Using IBS 1.0, maps of proteins and their domains were produced.

STUbLs in the physiology and pathology of *C. glabrata*

We generated STUbL-deficient strains of *C. glabrata* to study the impact of STUbL disruption on the physiology and pathogenicity of the organism. Using a fusion PCR and homologous recombination-based method, we were able to create deletion strains for the *CgSLX5*, *CgSLX8*, and *CgULS1* genes. In order to better understand inter-STUbL interactions and STUbL interaction with *CgULP2* in the pathobiology of *C. glabrata*, we also created double deletion strains of STUbLs and STUbLs with *CgULP2*. In this context, while we could create all combinations of these double mutants, we could not obtain *Cgslx8ΔCguls1Δ* double mutant despite several attempts. We believe that the loss of both the conserved STUbLs may be lethal to the cell. As shown in Table 5, all fungi have at least one of these two STUbLs, underscoring the importance of this pathway for survival.

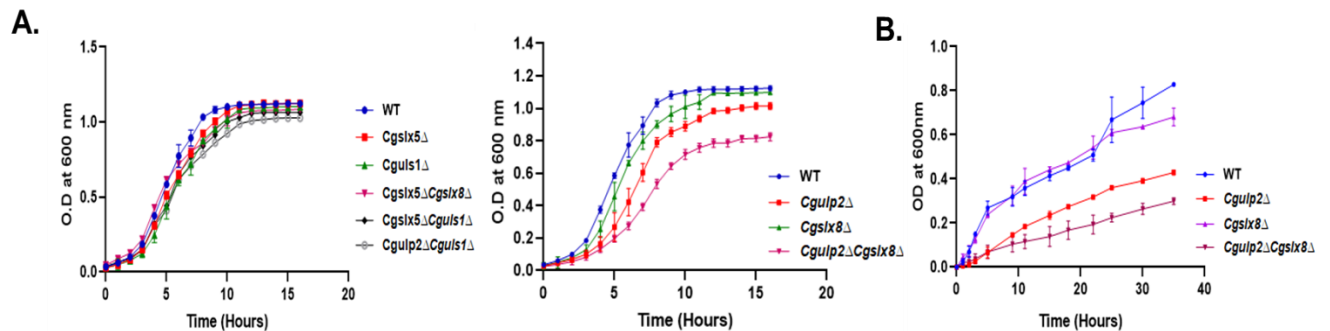
We first performed the phenotypic characterization of the STUbL mutants in *C. glabrata* to study the role of SUMO-targeted ubiquitin ligases in cell physiology. Growth analysis of single deletion strains of *Cgulp2* Δ , *Cgslx5* Δ , *Cgslx8* Δ , and *Cguls1* Δ , as well as double deletion strains of *Cgslx5* $\Delta*Cgslx8* Δ , *Cgslx5* $\Delta*Cguls1* Δ , *Cgulp2* $\Delta*Cgslx8* Δ , and *Cgulp2* Δ *Cguls1* Δ , confirmed that the mutant strains of *Cgulp2* Δ grew slower than the wild-type (WT). In addition to that, *Cgulp2* Δ *Cgslx8* Δ double mutant grew much slower as compared to WT and *Cgulp2* Δ in both YPD and RPMI complete medium even at conducive temperatures (Figure 6A-B). All other strains did not have any growth defects in these conditions.$$$

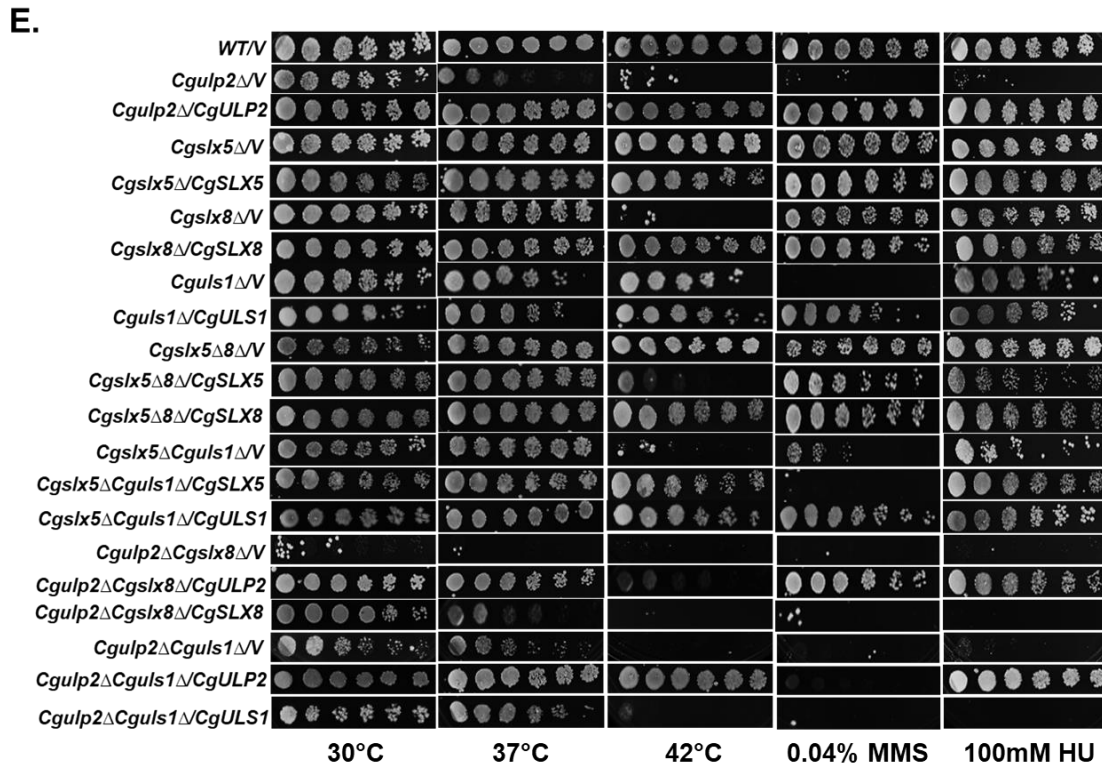
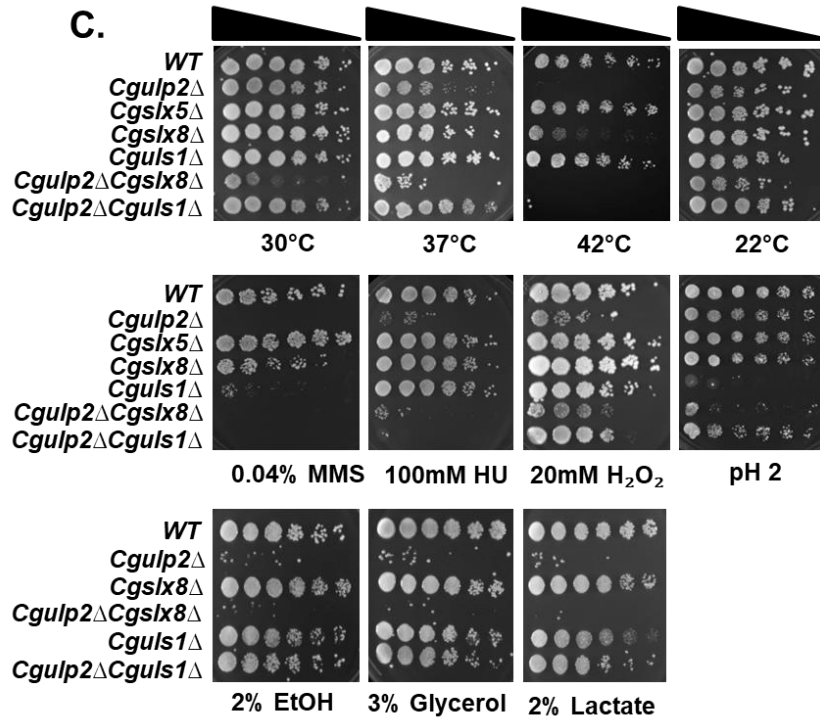
Earlier studies have shown that *Cgulp2* Δ mutant grew slowly at 42°C and was vulnerable to the DNA-alkylating agent methyl methanesulfonate (MMS), replication fork staller hydroxyurea, and oxidative stress-inducing agent hydrogen peroxide (Gujjula et al., 2016). In this study, compared to WT cells, *Cguls1* Δ showed more sensitivity to DNA-damaging stress conditions (0.04% MMS) and *Cgslx8* Δ was sensitive to a higher temperature (42°C) which could be rescued by ectopic expression of *CgULS1* and *CgSLX8*, respectively (Figure 6C-E). Contrary to both the mutants, the growth of the *Cgslx5* Δ mutant remains unaffected under diverse stress conditions. In addition, both *Cgslx5* Δ *Cguls1* Δ and *Cgulp2* Δ *Cguls1* Δ double mutants were temperature-sensitive for growth at 42°C and were more sensitive to 0.04% MMS when compared with WT but similar to the single *Cguls1* Δ (Figure 6D). Interestingly, *Cgulp2* Δ *Cguls1* Δ double mutants suppressed the phenotype of *Cgulp2* Δ single mutant in oxidative stress (20 mM H₂O₂), non-fermentable carbon sources (2% ethanol, 3% glycerol, 2% lactate, and 2% sodium acetate) and DNA damage (0.03% MMS) respectively. *Cgulp2* Δ *Cgslx8* Δ grows poorly even at 30°C compared to either single mutants while at low temperature (22°C), it appears to grow better than at 30°C. But it still grows poorly compared to all other strains at 22°C (Figure 6C-D). *Cguls1* Δ , *Cgslx5* Δ *Cgslx8* Δ , *Cgslx5* Δ *Cguls1* Δ , and *Cgulp2* Δ *Cgslx8* Δ were sensitive to acidic pH as well (Figure 6D).

S. cerevisiae strains with *slx5* Δ and *slx8* Δ deletion are sensitive to cell membrane stress (0.03% SDS)¹²⁸. However, unlike *S. cerevisiae*, neither the single nor the double mutants of STUbLs of *C. glabrata* were sensitive to SDS. The phenotypes of increased sensitivity of single and double deletion strains of SUMO-targeted ubiquitin ligases to various stress conditions were rescued by ectopic expression of either *CgULP2* and STUbL genes, indicating,

indeed, the phenotypes observed are due to loss of STUbLs (Figure 6E). In summary, these data show that the loss of *slx5* has minimal effect on the various conditions tested here; *slx8Δ* exacerbates multiple phenotypes of *ulp2Δ* and *uls1Δ* suppresses the sensitivity of *ulp2Δ* to several stress conditions.

As the loss of STUbLs had differential sensitivities to various stress conditions tested here, it is possible that they are specifically required to combat these conditions. Therefore, we analyzed the expression of the SUMO protease *CgULP2* and STUbL genes under thermal stress (42°C), DNA-alkylating agent methyl methanesulfonate (0.04% MMS), and replication fork staller hydroxyurea (100mM HU). In the aforementioned stress conditions, the lack of *CgULP2* caused significant growth anomalies and we found that the expression of *CgULP2* was increased in wild-type cells under these conditions (Figure 6F). Similarly, *CgSLX8* was upregulated at high temperatures and *CgULS1* was upregulated when exposed to MMS, again conditions in which loss of these genes showed poor growth. *CgSLX5*, *CgSLX8*, and *CgULS1* genes were down-regulated in response to hydroxyurea treatment and no phenotype was seen upon treatment in deletion of these genes. (Figure 6F). These findings collectively suggest that specific SUMO-targeted ubiquitin ligases are essential for resistance to heat and DNA damage.





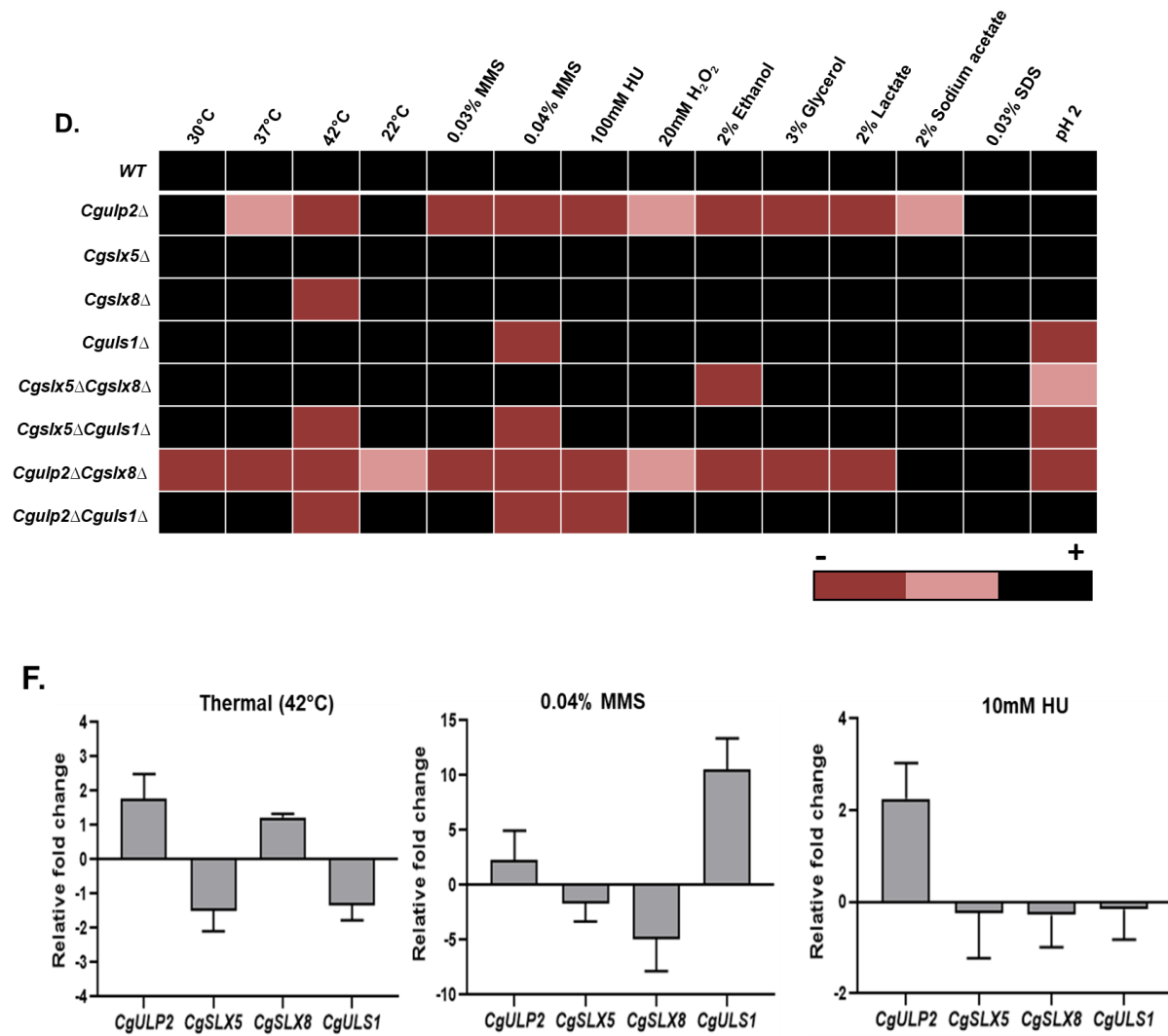


Figure 6. STUbL genes are crucial in the maintenance of cellular homeostasis. Analysis of growth of STUbL deletion strains. Overnight grown cultures of indicated *C. glabrata* strains were inoculated in (A) YPD and (B) RPMI complete medium to an initial A600 of 0.1. Absorbance at 600nm was measured. Data represent the mean of triplicates. C. Sensitivity of the STUbL deletion strains under various stress conditions. 3μl of ten-fold serial dilutions of overnight grown cultures were spotted on indicated plates and images were taken after 2-4 days of incubation. D. Heat map illustrating cell growth of STUbL mutants in the presence of diverse stress-causing agents. The sensitivity of STUbL mutants of *C. glabrata* to various treatments is represented as a heat map with black color (not sensitive; full growth) and different shades of red color (dark red indicates a high level of sensitivity and light red indicates a low level of sensitivity). E. Complementation assay of STUbL mutants where strains were transformed with

plasmids encoding the indicated genes and plated on appropriate plates **F**. Expression of SUMO protease and STUbL genes under selected stress conditions, namely, 42°C, 0.04% MMS, 10mM HU, was examined by performing qRT-PCR. Data was normalized with untreated wild-type cells. Data represents the mean and standard deviation of three independent experiments.

Biofilm formation by *C. glabrata* on the biotic (body tissue) and abiotic (plastic and medical devices) surfaces are major virulence factors ¹²⁹. We therefore tested the ability of these mutants to form biofilm and compared them with wild-type strains. Interestingly, all single mutants of STUbLs had reduced capacity to form biofilms, and the double mutants of *Cgulp2ΔCgslx8Δ* were more compromised than either single mutant (Figure 7A). *Cgulp2ΔCguls1Δ* on the other hand formed a biofilm similar to that of WT. This follows the same trend we have observed for stress conditions tested and discussed above that *Cgulp2ΔCguls1Δ* tends to mitigate the effects of *Cgulp2Δ*.

Further, to assess if these phenotypes indicated the requirement of these gene products for biofilm formation, we checked the expression of deSUMOylating enzyme *CgULP2* and STUbL (*CgSLX5*, *CgSLX8*, and *CgULS1*) genes in biofilms. *CgULS1* was found to be highly upregulated which was followed by *CgULP2* (Figure 7B). This observation was in correlation with the biofilm formation assay where a single mutant of *Cgulp2Δ*, *Cgslx5Δ*, *Cgslx8Δ*, and *Cguls1Δ* showed reduced biofilm formation, suggesting that deSUMOylase *CgULP2* and STUbLs function is important for biofilm formation.

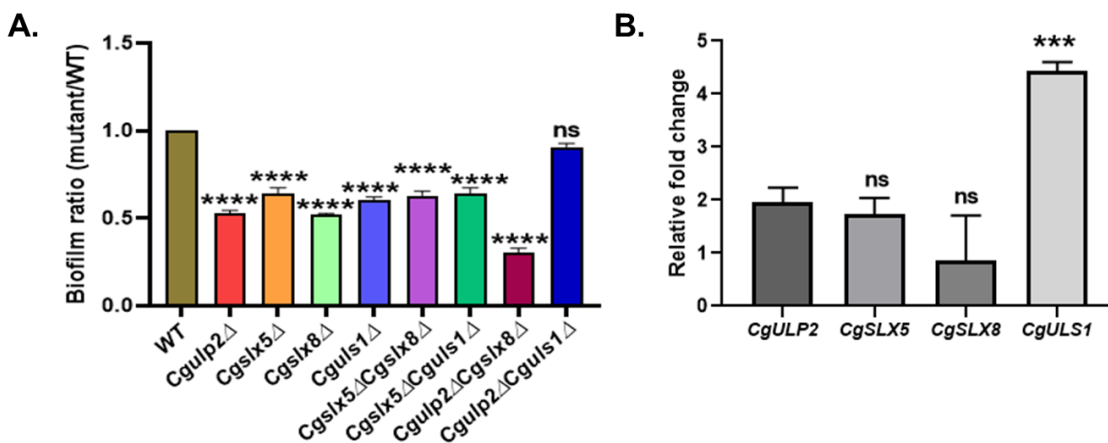


Figure 7. Biofilm in *C. glabrata* mutants. **A.** Biofilm formation was recorded for *C. glabrata* mutants vs wild type using XTT assay. Data represent the mean and SD of three independent experiments and significance was calculated with respect to WT (****, $p \leq 0.0001$; ns, non-significant, two-tailed unpaired Student's t-test). **B.** Biofilm formation from the wild-type cells was used for RNA isolation and cDNA conversion. RT-PCR was then performed to check the expression level of SUMO protease *CgULP2* and STUbL genes. The experiment was done in triplicate and the mean was plotted. The error bar shows the standard deviation of the mean and statistical significance was obtained using one-way ANOVA (***, $p < 0.0005$).

STUbLs are essential for the survival of *C. glabrata* within the macrophage

The studies described above suggest a crucial role for STUbLs in the physiology of *C. glabrata*. We wanted to ask now if survival in host cells also required STUbL function. To study this, we investigated the survival of STUbL mutants in macrophages using the colony-forming unit (CFU) assay. In parallel, we also investigated growth in the RPMI medium, which is closer to the human physiologic milieu than YPD. Except for *Cgulp2* Δ and *Cgulp2* Δ *Cgslx8* Δ , mutants were replicating as like WT when grown in YPD as expected. In contrast, growth in RPMI medium was 6 to 8 fold lower than in YPD medium for *Cgulp2* Δ and all the STUbL single and double mutants as compared to the wild type (Figure 8A-D).

To test for survival in macrophages, cultured human THP-1 macrophages were infected with equal numbers of wild-type, *Cgulp2* Δ , and STUbL mutants. Internalized *C. glabrata* were then extracted from the macrophages at the indicated time points and plated on a YPD solid medium to obtain the live cell count. Based on our previous studies, we know that after 8 hours, *C. glabrata* cells start to divide and multiply in numbers and this was captured in the WT infections in this experiment as well. However, we observed that the survival and replication of all the internalized STUbL mutants of *C. glabrata* were much less than the wild type (Figure 8E-F). The reason for the reduced propagation of *C. glabrata* cells in macrophages might be due to the cell's inability to grow in culture conditions or vulnerability towards oxidative stress.

The above data indicate that *CgULP2* and STUbLs may be required for replication in the macrophages. So we tested if these genes were induced upon infection, which could be an indication of their importance in these conditions. The expression of deSUMOylating enzyme

CgULP2 along with STUbL genes *CgSLX5*, *CgSLX8*, and *CgULS1* was measured by qRT-PCR at different time points after adding yeast cells to macrophage culture. Figure 8G shows the time course of changes in expression during the infection condition. As can be seen, the expression of *CgULP2* is upregulated as early as 2 hours of infection, which remained high up to 24 hours. Importantly, expression of *CgSLX5*, *CgSLX8*, and *CgULS1* was also found to be induced; while the *CgSLX5* and *CgSLX8* transcripts show a modest increase, *CgULS1* had higher levels of expression (Figure 8G). Notably, these findings are also corroborated with the prior CFU assay, which revealed that a single mutant of *Cgulp2* Δ , *Cgslx5* Δ , *Cgslx8* Δ , and *Cguls1* Δ had reduced intracellular proliferation and had a low CFU count (Figure 8E-F). This outcome indicates the key role played by *CgULP2* and STUbLs during infection. In other words, it supports the notion that *CgULP2*, *CgSLX5*, *CgSLX8*, and *CgULS1* are involved in the survival and proliferation of the pathogen in the host cells.

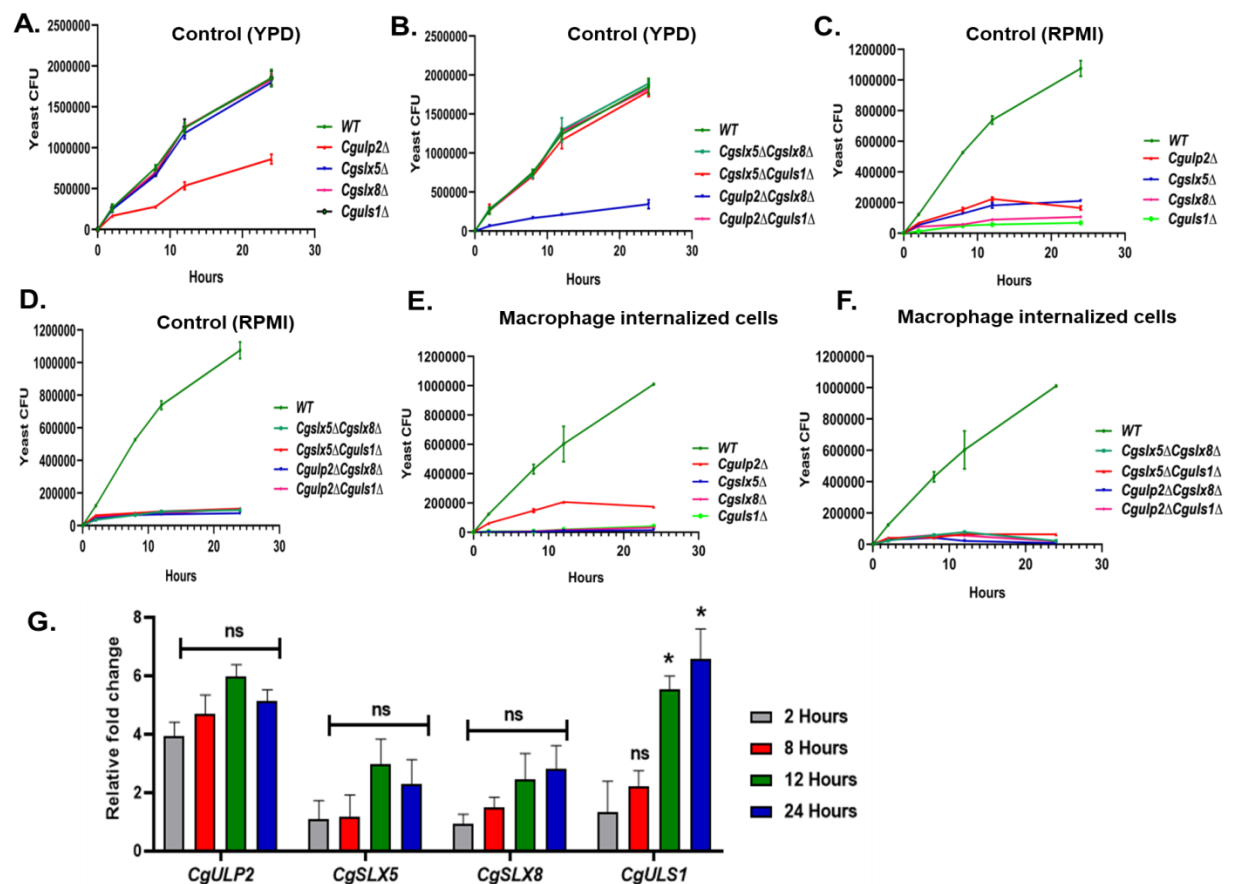


Figure 8. Reduced survival and replication inside macrophage by STUbL mutants of *C. glabrata*. **A, B, C, and D.** CFU assay was used to assess cell proliferation of the indicated *C. glabrata* strains in YPD and RPMI-1640 medium containing 10% FBS (fetal bovine serum). **E.** and **F.** 2.5×10^6 *C. glabrata* strains were added to phorbol 12-myristate 13-acetate-differentiated THP1 cells in a 24-well plate, and non-phagocytosed *C. glabrata* cells were removed after 2 hours. Intracellular yeast cells were recovered at the indicated time intervals by lysing THP-1 cells in water and plating appropriate dilutions of lysates on the YPD medium. After 24 - 48 hours of incubation at 30 °C, yeast colonies were counted. Data represents the mean and SD of three independent experiments. **G.** Expression of STUbL genes in internalized *C. glabrata* cells by qRT-PCR. RNA was extracted from *C. glabrata* isolated from macrophages at the indicated time intervals, cDNA prepared and expression of *CgULP2* and STUbLs was measured. Data represent the mean and standard deviation of three independent experiments (*, $p \leq 0.01$; ns, non-significant, two-tailed unpaired Student's t-test).

STUbL mutants in *C. glabrata* have differential SUMOylation pattern

Loss of function of CgUlp2 was earlier shown to reduce the amount of SUMOylated proteins⁹⁰. We had reasoned that accumulation of polySUMOylated protein led to increased degradation of proteins via the SUMO-targeted Ubiquitin ligases, and that is why lower levels of SUMOylated protein were detected. Therefore, we hypothesized that double deletion strain of both *Cgulp2* and STUbLs will lead to the retention of higher levels of polySUMOylated proteins. In order to determine the SUMO proteome in each of the STUbL mutants, we used an anti-FLAG antibody to perform western blots on whole cell lysates from the *C. glabrata* STUbL mutants augmented with dual tagged CgSmt3 protein (carrying His6 and FLAG epitopes at the N-terminus). The lack of higher molecular weight SUMOylated proteins in the *Cgulp2* Δ mutant was confirmed (Figure 9). Whereas in the case of STUbL single mutants, only *Cgslx5* Δ was found to have higher molecular weight SUMOylated protein (Figure 9). In principle, STUbL deletion strains should accumulate higher molecular weight polySUMOylated proteins as polySUMOylated proteins cannot be degraded⁷⁹. The absence of such a prominent accumulation of polySUMOylated proteins suggests that in the absence of one of the STUbLs, another might compensate. This could be the reason for the absence of polySUMOylated proteins in the *Cgslx8* Δ and *Cguls1* Δ single mutants. Interestingly, accumulation of polySUMOylated

proteins was seen in the double mutants of *Cgulp2ΔCguls1Δ*, and *Cgslx5ΔCguls1Δ* (Figure 9, Lane 7 and 9). This suggests that Uls1 could be the primary ubiquitin-ligase for many of the SUMOylated proteins that escape deSUMOylation in the absence of CgUlp2. Of note, we have demonstrated that *Cgulp2ΔCguls1Δ* double mutant was able to restore *Cgulp2Δ* mutant growth under a variety of stress conditions (Figure 6C-D) and interpret the appearance of these SUMOylated proteins as a reason for the restoration of growth in the double mutant. Importantly, the *Cgulp2ΔCgslx8Δ*, which had exacerbated the *Cgulp2Δ* phenotypes, not only grew very slowly, but it also had the least amount of SUMOylated protein, suggesting enhanced degradation of proteins.

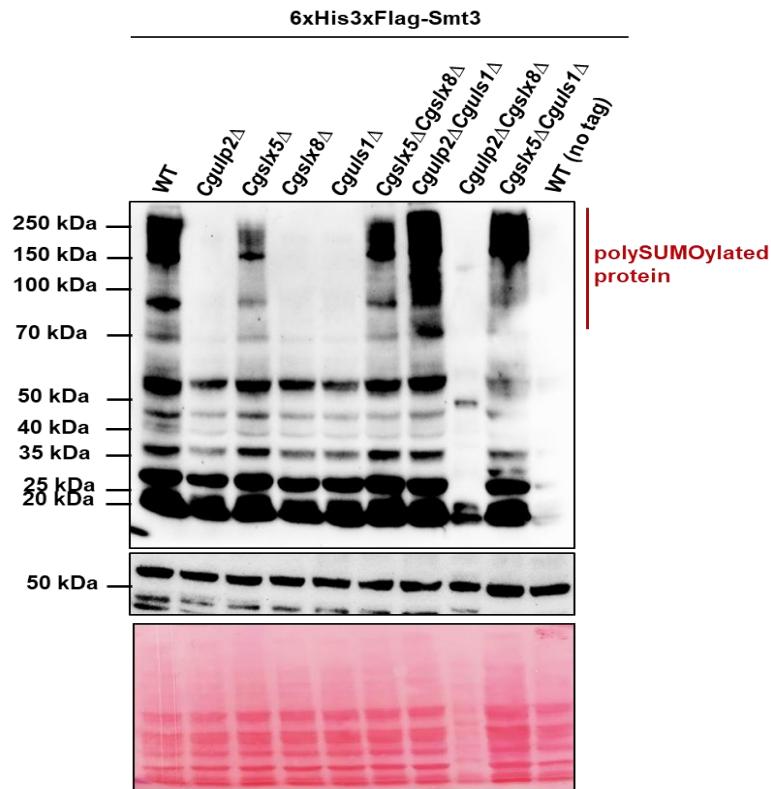


Figure 9. Differential SUMOylation pattern was shown by STUbL mutants of *C. glabrata*.

Strains were grown in a YPD medium and an equal number of cells were taken for whole-cell extracts. Tubulin was used as a loading control. Blot stained with Ponceau S to show the level of protein degradation in *Cgulp2ΔCgslx8Δ* double mutant in comparison to WT and other mutants.

Each of these enzymes affects growth phenotypes and polySUMOylated products differently. Based on these observations, we reason that because the polySUMOylated proteins are not

degraded when *Cguls1* is lost in the *Cgulp2Δ* mutants, the *Cgulp2Δ* growth phenotype is suppressed. On the other hand, *Cgulp2Cgslx8Δ* cells lose more protein as seen in the western blot, and get sicker (Figure 9). Based on this and the observation that single mutants of STUbLs do not accumulate polySUMOylated proteins, we hypothesize that *Cgslx8* deletion possibly upregulates CgUls1 activity and leads to increased protein degradation. An alternative explanation is that CgUls1 is the main STUbL, however, several essential proteins required for strong growth may be degraded in the *Cgslx8Δ* mutant leading to compromised growth in this double mutant.

CgUls1 protein is upregulated in STUbL mutants

To test whether CgUls1 is upregulated in *Cgslx8* deletion, we created a construct of dual-tagged 6xHIS-3xFLAG-*CgULS1* (HF-*CgULS1*) under its promoter and confirmed the expression of CgUls1 protein in the *Cguls1Δ* mutant by western blot using an α -flag antibody. As shown in Figure 7C, *Cguls1Δ* is sensitive to DNA-damaging agents (0.04%MMS). We tested the ability of HF-*CgULS1* to complement the MMS sensitivity of *Cguls1Δ* mutant and we found that expression of HF-*CgULS1* restored the phenotype of the *Cguls1Δ* mutant (Figure 10A).

When we checked the level of expression of CgUls1 protein in WT, *Cgulp2Δ*, *Cguls1Δ*, *Cgslx8Δ*, and *Cgulp2ΔCgslx8Δ* deletion strain, a two-fold higher expression of CgUls1 protein in *Cgulp2ΔCgslx8Δ* double deletion mutant was seen in comparison with wild type and *Cgulp2Δ*, *Cgslx8Δ* single mutants (Figure 10B-C). Further, to test if this increased levels of protein is a result of increased transcription, we tested the expression of the *CgULS1* gene in these deletion strains. We observed that RNA levels of *CgULS1* in *Cgulp2ΔCgslx8Δ* were higher than in *Cgulp2Δ* and *Cgslx8Δ* single mutants (Figure 10D). Together, these findings show that there is increased CgUls1 protein (and potentially increased activity) in the *Cgulp2ΔCgslx8Δ* mutant and this leads to elevated loss of proteins.

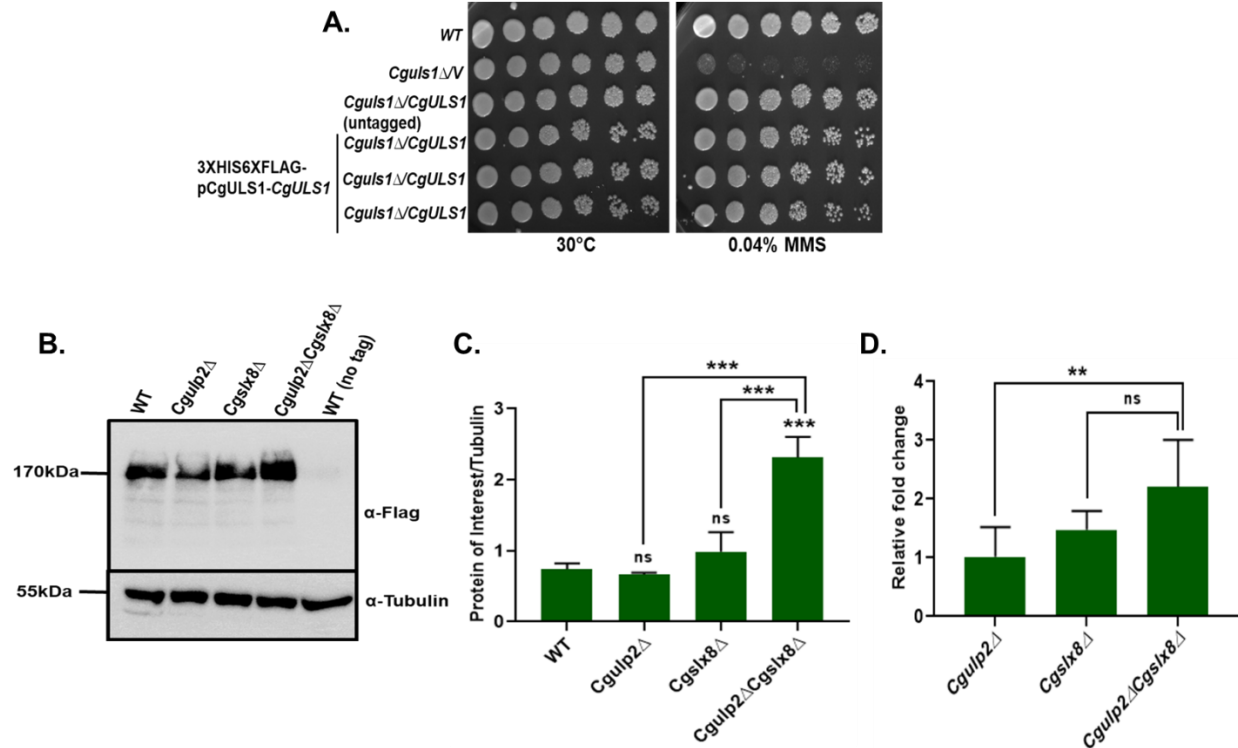
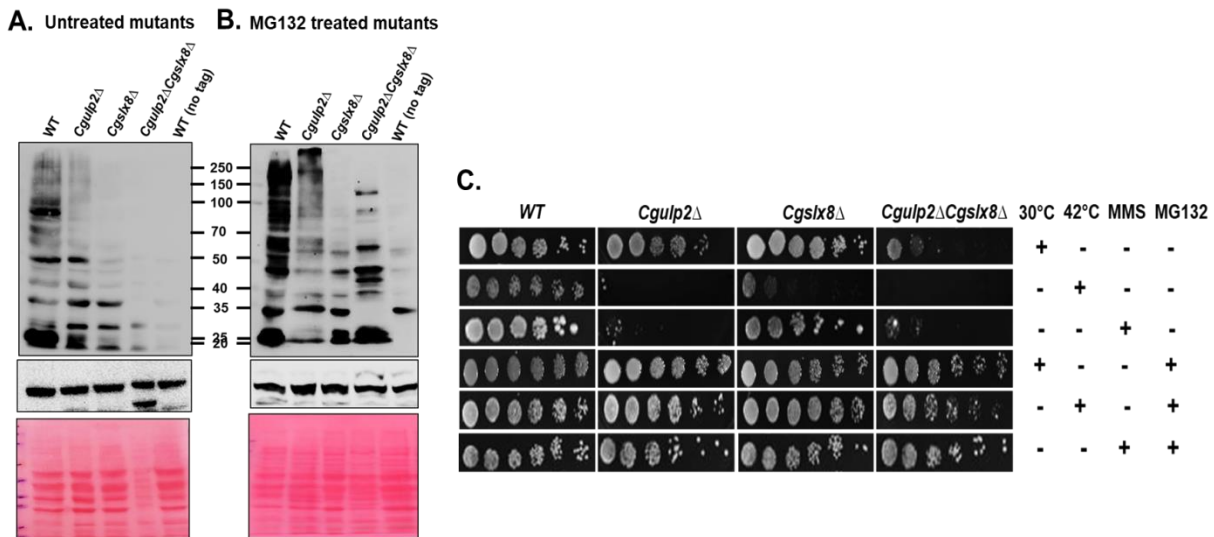


Figure 10. Upregulation of CgUls1 protein in STUbL mutant. **A.** Complementation of *Cguls1Δ* mutant with HF-CgUls1 was tested by growth at 30°C and in 0.04% MMS. 3μl of 10-fold serial dilutions of cultures of *C. glabrata* strains were spotted on indicated plates. Images were taken after 2-3 days of incubation **B.** Western blot analysis with anti-FLAG antibody to check the expression of CgUls1 protein in the STUbL mutant strains. Tubulin was used as a loading control. **C.** Quantification of the western blot. **D.** RNA was isolated from YPD-grown wild-type, *Cgulp2Δ*, *Cgslx8Δ*, and *Cgulp2ΔCgslx8Δ* cells and qRT-PCR was performed to check the expression of the *CgULS1* gene. Data represents the mean and standard deviation of three independent experiments. Statistical significance was obtained using one-way ANOVA (***: $p \leq 0.0005$; **, $p < 0.005$; ns: non-significant).

Loss of proteins and reduced growth of STUbL mutants is due to enhanced proteasomal degradation

We examined the SUMOylation pattern in *Cgulp2Δ*, *Cgslx8Δ*, and *Cgulp2ΔCgslx8Δ* cells treated with the proteasome inhibitor MG132 to test the possibility that an increase in the accumulation of polySUMOylated proteins in these mutants results in their degradation by the ubiquitin-

proteasome pathway. To test this, we used the proteasome inhibitor, MG132. Whole-cell protein extracts were prepared from cultures that were treated with MG132 for 2 hours and tested for the presence of SUMOylated proteins by western blots. Higher molecular weight proteins could be detected in the MG132-treated *Cgulp2* Δ single mutants (Figure 11B, Lane 2) as compared to the untreated mutant strain (Figure 11A, Lane 2) indicating that the degradation of polySUMOylated proteins was prevented. Fewer changes were seen in the *Cgslx8* Δ consistent with the phenotypes seen for this mutant. Most interestingly, in the double mutant of *Cgulp2* Δ *Cgslx8* Δ , with MG132, higher molecular weight proteins appeared suggesting prevention of degradation of proteins (Figure 11B, Lane 4) compared to the untreated mutant (Figure 11A, Lane 4) and we could also recover more protein from this strain when treated with MG132. Furthermore, the reappearance of higher molecular weight proteins is accompanied by the restoration of growth of these mutants (Figure 11C-G). Also, MG132 treatment could overcome the thermal and MMS sensitivity of the *Cgulp2* Δ , *Cgslx8* Δ , and *Cguls1* Δ single mutant (Figure 11C-G). These findings strongly imply that the reason for slow growth at 30°C and increased stress sensitivity of *Cgulp2* Δ *Cgslx8* Δ double mutant was due to excess proteasomal degradation. Together these results support our hypothesis that loss of Ulp2 leads to the accumulation of increased polySUMOylated proteins that are degraded by proteasome via the STUbL pathway.



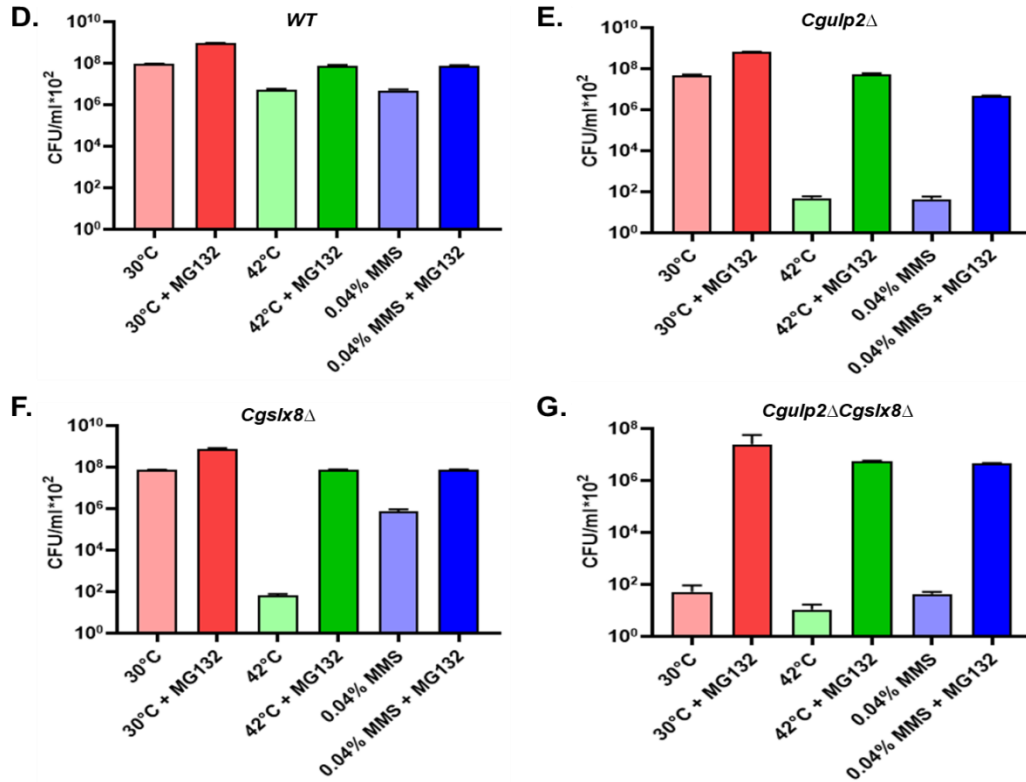


Figure 11. Loss of proteins and reduced growth of STUbL mutants is due to enhanced proteasomal degradation. *C. glabrata* strains were grown overnight in YNB medium at 30°C and 180 rpm with 0.1% proline serving as the only nitrogen source. After cell harvesting, a fresh medium containing 0.003% SDS was used to adjust the A600 to 0.5, and the cells were then grown for 4 hours. **A.** Cells without MG132 treatment were used as a control. **B.** Cultures were placed in a 2 ml microcentrifuge tube with 1 ml of YNB-proline media and 200μM MG132, and they were then incubated for two hours at 30°C and 180 rpm. Whole-cell extracts were prepared and analyzed by western blot using α-Flag antibody. Tubulin was used as a loading control. Blots stained with Ponceau S are shown for comparison of total proteins in *Cgulp2*Δ*Cgslx8*Δ in treated versus untreated cells. **C.** MMS (0.045%) was added to the media and grown for 3 hours after MG132 treatment. Similarly, thermal treatment was given to the cultures for 3 hours. After that, cultures were serially diluted 10-fold, and a 2 μl volume was spotted on the YPD medium. Images of the plates were taken following 2-3 days of incubation at desired temperatures. **D-G.** Quantification of growth of STUbL mutants in various stress conditions with and without MG132 treatment. Data represents the mean and SD from at least three independent experiments.

3.3 SUMMARY

We have identified STUbLs in multiple pathogenic fungi including *C. glabrata* using sequence comparisons. We created deletions of the STUbL components in *C. glabrata* and examined the effect on both the physiology and pathogenesis of *C. glabrata*. In the absence of STUbLs, this pathogen showed reduced biofilm formation and survival inside macrophages. Loss of STUbLs reduced the capacity of *C. glabrata* to combat stress. Additionally, we have shown here that, CgUls1 activity is increased in the strain lacking both deSUMOylating enzyme *CgUlp2* and a STUbL *Cgslx8*, resulting in enhanced protein degradation. This study also establishes that loss of protein homeostasis is the key reason for stress sensitivity observed in the STUbL mutants. Overall, we demonstrate here that STUbLs are important modulators of protein homeostasis and along with CgUlp2 play a vital role in *C. glabrata* pathogenesis.

Chapter 4 Identification of targets of deSUMOylase CgUlp2 and STUbLs

4.1 INTRODUCTION

The results shown in Chapter 3 indicate that the loss of a single STUbL has minimal effect on growth and the capacity of the organism to combat stress. However, when the deSUMOylase is also inactive, there is an excess accumulation of polySUMOylated proteins, which activates the STUbL pathway leading to the degradation of a large number of the polySUMOylated proteins. This affects the overall protein homeostasis. In order to determine if proteins that are part of critical growth pathways are particularly degraded in the STUbL double mutant (*Cgulp2ΔCgslx8Δ*) and restored in *Cgulp2ΔCguls1Δ*, we conducted tandem mass tag (TMT) labeled quantitative mass spectrophotometry analyses.

4.2 RESULTS

Identification of global proteome of *Cgulp2Δ*, *Cgslx8Δ*, *Cguls1Δ*, *Cgulp2ΔCgslx8Δ* and *Cgulp2ΔCguls1Δ* deletion strains in comparison with wild type

We analyzed the proteins of log-phase grown cells of WT, *Cgulp2Δ*, *Cgslx8Δ*, *Cguls1Δ* single mutants, *Cgulp2ΔCgslx8Δ*, and *Cgulp2ΔCguls1Δ* double mutants using a tandem mass tag (TMT) labeled quantitative mass spectrophotometry. The samples were sent to the Institute of Bioinformatics (IoB) in Bangalore for analysis by mass spectrometry. Through the TMT-labeled mass spectrometry, a comprehensive dataset of 3281 proteins was obtained. Following Principal Component Analysis (PCA), 2194 proteins were retained, excluding those exhibiting variations between biological replicates and those not consistently present across all mutants. Based on the abundance value of the proteins, we calculated the p-value for each mutant by using MeV_4.9.0 software and took a threshold of 0.05 for a significant p-value. We identified 107, 87, 148, 277, and 300 proteins that were significantly upregulated, and 90, 130, 241, 184, and 134 proteins were significantly downregulated in *Cgulp2Δ*, *Cgslx8Δ*, *Cguls1Δ* single mutants, *Cgulp2ΔCgslx8Δ*, and *Cgulp2ΔCguls1Δ* double mutants respectively. All the significantly upregulated and downregulated proteins for each mutant are shown in Appendix 9.6.

We used the DAVID database to perform Gene Ontology (GO) to identify the pathways of the upregulated and downregulated proteins for each mutant in comparison with WT. The pathways were then represented as horizontal bar graphs using SR plot software (Figure 12). We have seen proteins from multiple pathways that were differentially affected in these mutants. These include components of metabolic processes, biosynthetic processes, intracellular transport, ribosomal biogenesis, translation, proteasomal protein-mediated degradation, and many more.

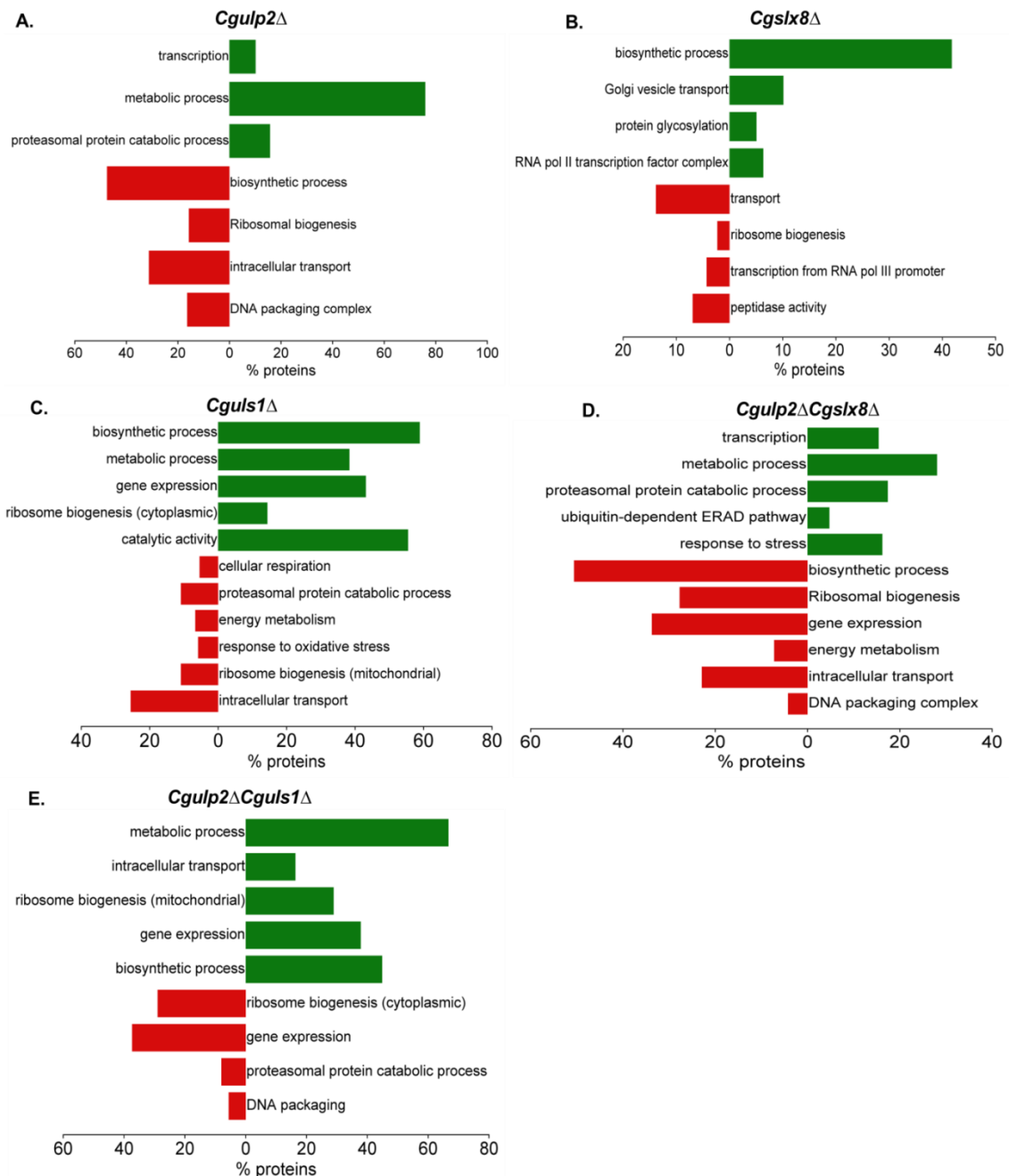
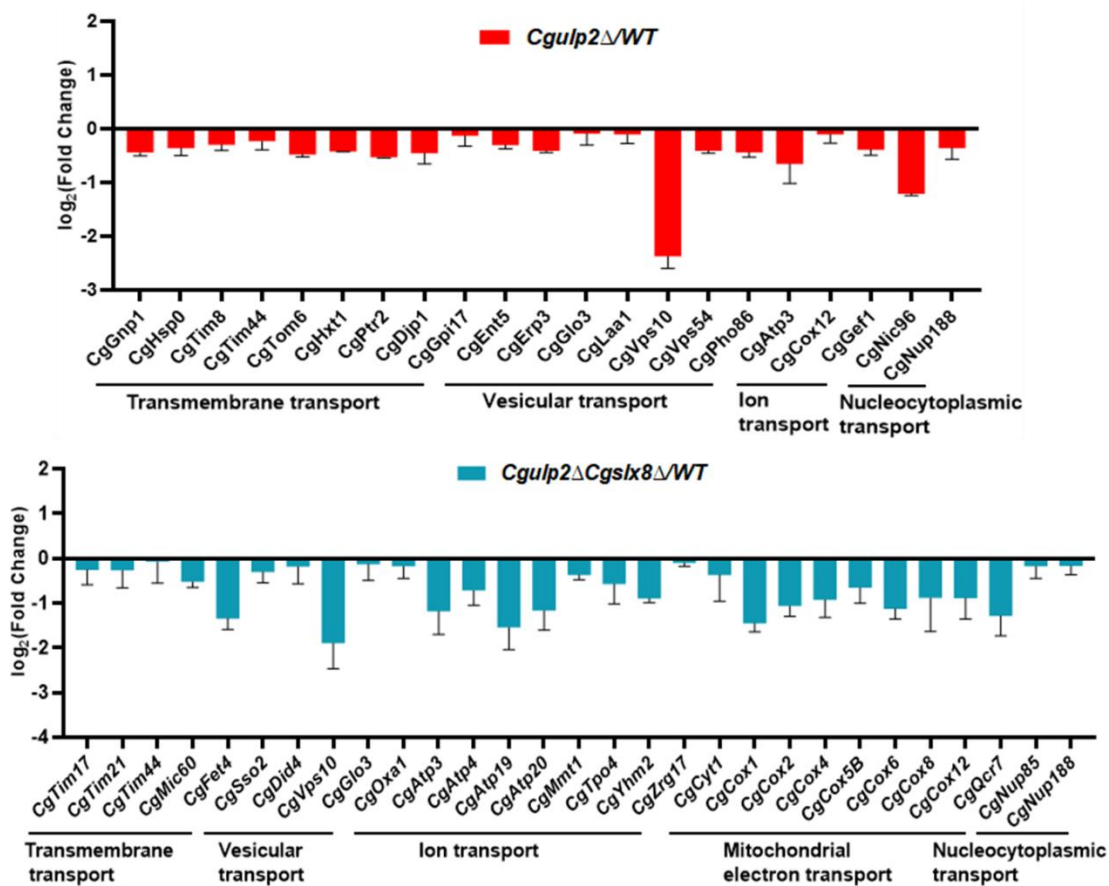


Figure 12. Pathways that are differentially affected in each mutant. The horizontal bar graph shows the pathways in A) *Cguld2Δ*, B) *Cgslx8Δ*, C) *Cguls1Δ* D) *Cguld2ΔCgslx8Δ*, and E) *Cguld2ΔCguls1Δ* mutant in comparison to WT. The green bar represents pathways with proteins that are higher and the dark red bar represents those that are lower than wild type.

Analyzing the data from proteomics suggested some key pathways that were particularly affected and also differentially affected in the mutants. It also could potentially provide a reason for the differential phenotypes observed. In this chapter, we describe some key observations made and a few follow-up experiments to validate these observations.

a) Intracellular transport: In our study, proteins involved in intracellular transport, particularly mitochondrial electron transport, transmembrane transport, and other ion transport were downregulated in *Cguld2Δ*, *Cgslx8Δ*, *Cguls1Δ*, and *Cguld2ΔCgslx8Δ*, but upregulated in *Cguld2ΔCguls1Δ* double mutant (Figure 12). Figure 13 shows some of these proteins.



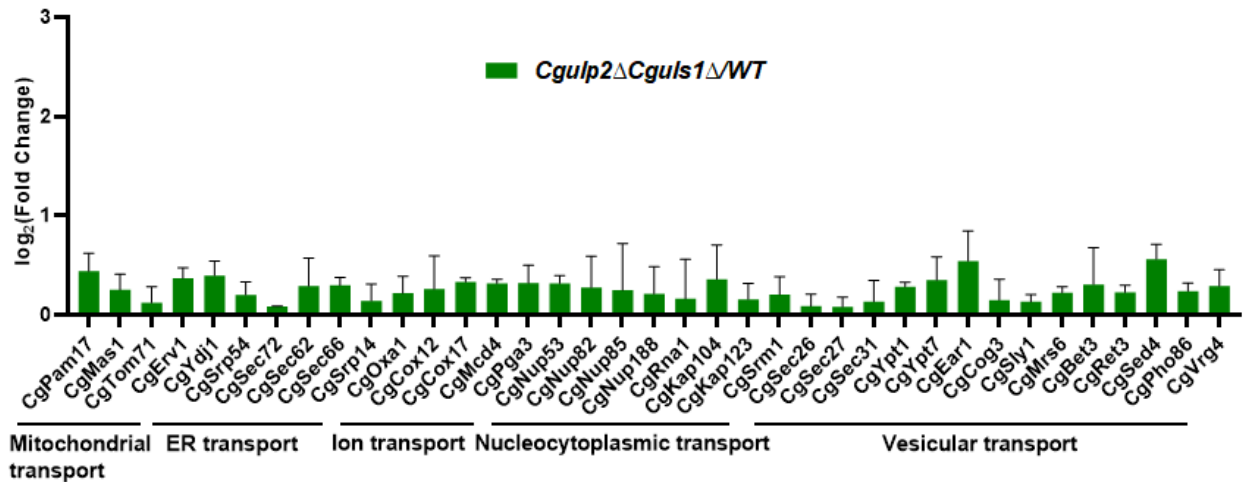


Figure 13. Graph shows the proteins involved in various transport that were downregulated in *Cgulg2Δ*, *Cgulg2ΔCgslx8Δ*, and upregulated in *Cgulg2ΔCguls1Δ* mutant. The error bar represents the log₂ standard deviation.

b) Ribosomal biogenesis and translation: Ribosomes are required for protein synthesis and therefore are essential for cellular survival, growth, and proliferation. Ribosome biogenesis includes ribosomal RNA synthesis and processing, ribosomal protein assembly, transport to the cytoplasm, and ribosomal subunit association. A few cytoplasmic and mitochondrial proteins involved in biogenesis were found to be downregulated in the *Cgulg2Δ* and *Cgslx8Δ* single mutant. In *Cguls1Δ*, we found that while the mitochondrial ribosomal proteins were reduced, there was an increase in cytoplasmic ribosome subunit levels (Figure 14). However, in the *Cgulg2ΔCgslx8Δ* double mutant, ribosomal subunits of both the cytoplasmic and mitochondrial translation were low. This could lead to an overall lower translation and could contribute to the reduced protein levels seen in this mutant. Although the *Cgulg2ΔCguls1Δ* double mutant had increased levels of mitochondrial ribosomal proteins of both large and small subunits, the majority of cytoplasmic ribosomal proteins were lower than wild type (Figure 14). This suggests that perhaps mitochondrial translation is required for suppressing the *Cgulg2Δ* single mutant phenotype by the *Cgulg2ΔCguls1Δ* double mutant.

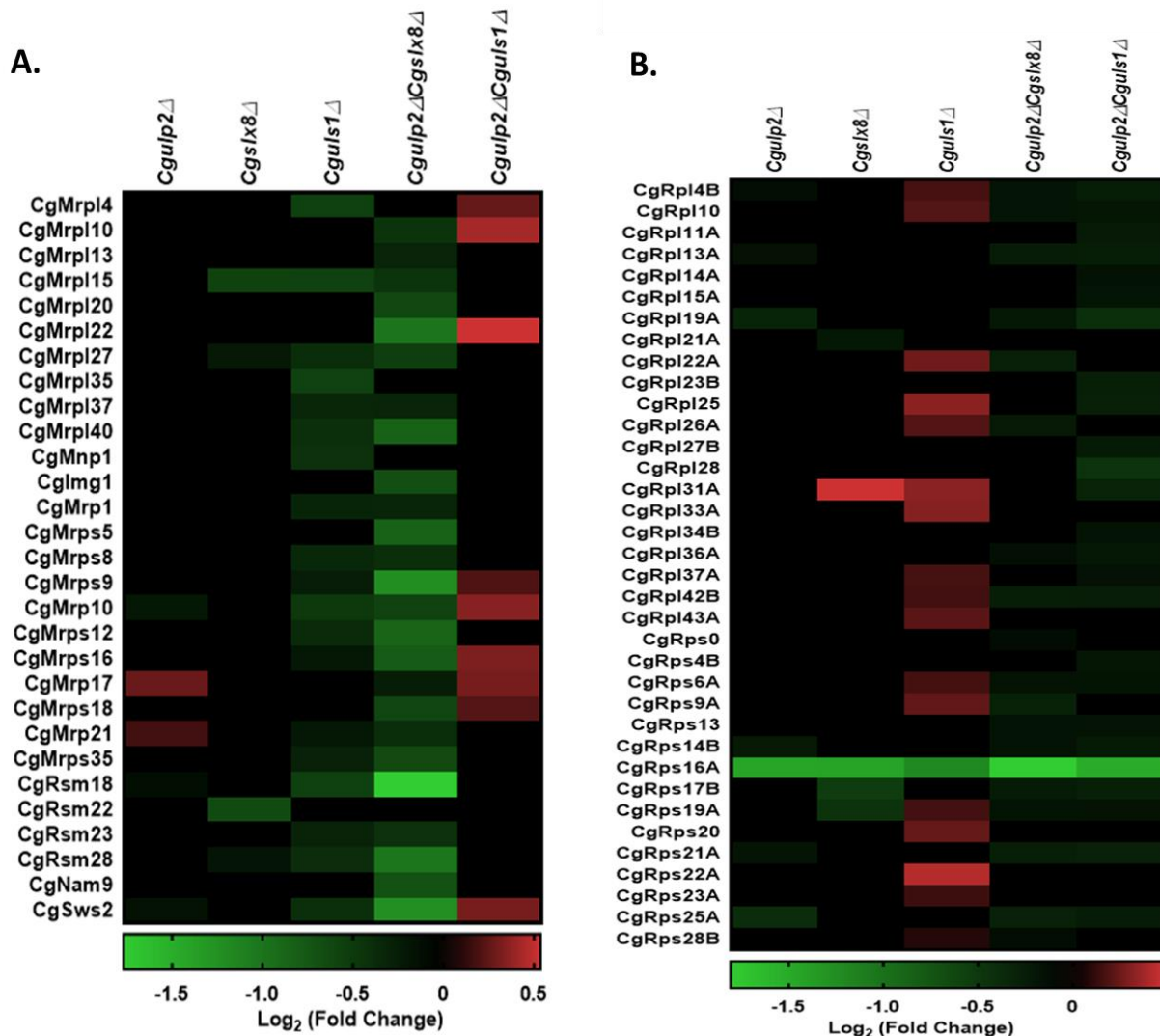


Figure 14. Heat map illustrating the differences in protein levels of ribosomal proteins. A. Heat map of mitochondrial ribosomal proteins and **B.** cytoplasmic ribosomal proteins of large and small subunits in *Cgulp2*Δ, *Cgslx8*Δ, *Cguls1*Δ single mutants, *Cgulp2*Δ*Cgslx8*Δ and *Cgulp2*Δ*Cguls1*Δ double mutant in comparison with WT. Heat maps were generated for the increased (different shades of red color) and decreased (different shades of green color) levels proteins for each mutant compared to the wild type.

c) Proteasomal mediated ubiquitin-dependent protein catabolic process: The massive protein complex known as the proteasome uses metabolic energy to break down intracellular proteins. The ubiquitin-proteasome system (UPS) is responsible for regulating a wide array of fundamental physiological functions by targeting short-lived regulatory or structurally abnormal

proteins for degradation. These functions encompass crucial processes such as signal transduction, cell cycle progression, cell death, metabolism, immunological responses, protein quality control, and development. The two subcomplexes that make up the proteasome are the catalytic core particle (CP), also referred to as the 20S proteasome, and one or more terminal 19S regulatory particle(s) (RP), which act as proteasome activators ¹³⁰. In this study, the *Cguld2Δ* single mutant was found to have elevated levels of a small number of proteins that belong to both subcomplexes. The majority of the proteins in the 26S proteasomal complex and those involved in endoplasmic reticulum degradation (ERAD) which include CgKar2, CgCdc48, CgHlj1, CgHrd3, CgJem1, CgUbr1, and CgUfd1 were also substantially elevated in the *Cguld2ΔCgslx8Δ* double mutant (Figure 15), suggesting that this increased protein degradation components could be responsible for the significantly elevated levels of protein degradation occurring in this double mutant. The proteasome-mediated ubiquitin degradation pathway proteins were reduced in *Cguls1Δ* and *Cguld2ΔCguls1Δ* mutants (Figure 15) and this correlates with increased protein levels in these mutants. In summary, the decreased translation and increased degradation collectively contribute to reduced protein levels in the *Cguld2ΔCgslx8Δ* double mutant.

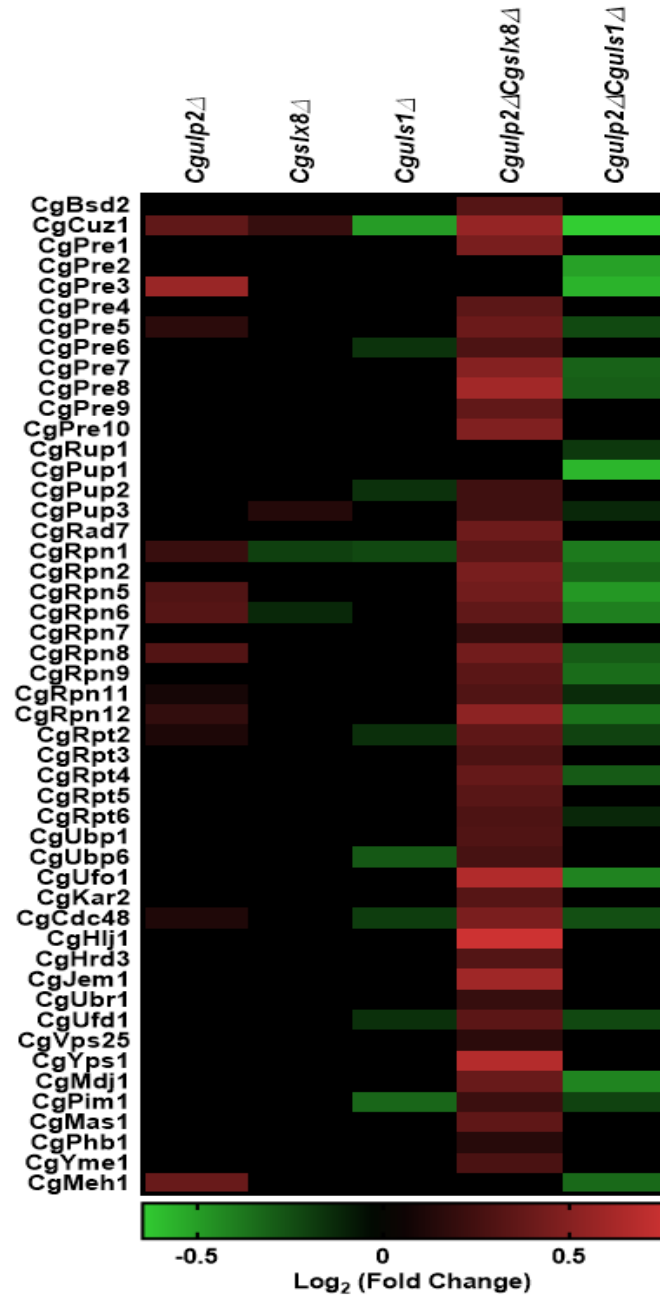


Figure 15. Heat map illustrating the altered levels of components of proteasome-mediated ubiquitin-dependent pathway in *Cgulp2*Δ, *Cgslx8*Δ, *Cguls1*Δ single mutants, *Cgulp2*Δ*Cgslx8*Δ and *Cgulp2*Δ*Cguls1*Δ double mutant in comparison with WT. A heat map of the elevated (different shades of red color) and diminished (different shades of green color) proteins for each mutant.

Purine biosynthetic pathway is compromised in *Cgulp2ΔCgslx8Δ*

The process of biosynthesis involves multiple steps that are catalyzed by enzymes to transform substrates into increasingly complex products. These include biosynthesis of lipids, nitrogen compounds, amino acids, nucleotides, secondary metabolites, and more. The biosynthetic pathways were differentially affected in *Cgulp2Δ*, *Cgslx8Δ*, *Cguls1Δ*, *Cgulp2ΔCgslx8Δ*, and *Cgulp2ΔCguls1Δ* mutants. One key observation was that components of the ATP biosynthesis process were reduced in *Cgulp2ΔCgslx8Δ* (Figure 17). It suggests that energy metabolism was impacted in these mutants and could be one contributing reason why *Cgulp2ΔCgslx8Δ* mutant cells might become sick at 30°C.

Among the biosynthetic gene products that were reduced in the *Cgulp2ΔCgslx8Δ*, we noticed that there were several enzymes of the de novo purine biosynthetic pathway (Figure 16A, B). Purine nucleotide biosynthesis, as shown in Figure 16A, begins with 5-phosphoribosyl-1-pyrophosphate (PRPP), which is then converted through a sequence of reactions into inosine 5'-monophosphate (IMP). After that, IMP may go through various processes that produce either GMP or AMP, which are subsequently transformed into ADP and GDP, respectively ^{131,132}. Based on the mass spectrometry data, the *Cgulp2ΔCgslx8Δ* double mutant had reduced levels of most of the proteins involved in this biosynthesis. These included CgAde57 which was also found to be elevated in the *Cgulp2ΔCguls1Δ* double mutant and reduced in the *Cgulp2Δ* and *Cgulp2ΔCgslx8Δ* double mutant (Figure 16B). Since this change in CgAde57 reflected the altered growth phenotypes observed, we investigated whether *CgADE57* overexpression could restore the *Cgulp2ΔCgslx8Δ* double mutant growth at 30°C. In order to accomplish this, we constructed a plasmid encoding *CgADE57* gene in the pGRB2.2 *C. glabrata* expression vector (carrying 3x-FLAG at the N-terminus), and we used an α -flag antibody to confirm the expression of CgAdeE57 protein in the WT, *Cgulp2Δ*, *Cgslx8Δ*, *Cguls1Δ*, *Cgulp2ΔCgslx8Δ*, and *Cgulp2ΔCguls1Δ* mutants through western blot. All these mutants showed the same levels of expression of the ectopically expressed CgAde57 protein (Figure 16C). In comparison to controls, the overexpression of the *CgADE57* gene resulted in some degree of improved growth in the cells of the WT and mutants at 30°C and 42°C (Figure 16D). Significantly, we found that overexpressing the *CgADE57* gene allowed the *Cgulp2ΔCgslx8Δ* double mutant to partially

recover its growth at 30°C and 42°C (Figure 16D-F) suggesting reduced levels of this protein could be a major contribution to the poor growth of the mutant.

Since these results suggested that the purine biosynthesis pathway is particularly compromised, we further tested this idea using inhibitors of this pathway. If some strains are already compromised in this biosynthetic pathway, they will be more sensitive to inhibitors of this pathway. We used three inhibitors- azaserine, pemetrexed, and isatin that inhibit specific enzymes of the purine biosynthesis pathway to examine growth inhibitions in WT and mutants. The initial, rate-limiting step involves the conversion of PPRP to PRA, which is catalyzed by PRPP amidotransferase. azaserine, an antifungal agent, inhibits this step^{131,132}. An anticancer drug called pemetrexed prevents GAR transformylase from converting GAR to FGAR in the third step. The process by which the enzyme AIR carboxylase converts AIR to CAIR is inhibited by isatin, an antifungal agent. In this study, we observed 40-50% inhibition and reduced growth in WT cells upon the addition of 2mM azaserine, 6mM pemetrexed, and 4mM isatin in comparison to vehicle control. 80% inhibition was seen in the case of *Cgulp2Δ* and *Cgulp2ΔCgslx8Δ* mutants when treated with 2 mM azaserine, and 55% inhibition was observed with 6 mM pemetrexed and 4 mM isatin. In contrast, the *Cgslx8Δ*, *Cguls1Δ*, and *Cgulp2ΔCguls1Δ* mutants showed similar levels of inhibitions to those of WT (Figure 16G-J). When these inhibitors were added, we found that both *Cgulp2Δ* and *Cgulp2ΔCgslx8Δ* were more sensitive to the drug. The doubling time of the WT, *Cgslx8Δ*, *Cguls1Δ*, and *Cgulp2ΔCguls1Δ* mutants ranged between 2-3 hours, whereas the *Cgulp2Δ* and *Cgulp2ΔCgslx8Δ* mutants displayed a prolonged doubling time of around 9-10 hours, especially in the presence of 2mM azaserine. This could be the reason for the slow growth exhibited by *Cgulp2Δ* and *Cgulp2ΔCgslx8Δ* mutants in the presence of the inhibitors. Altogether, this data suggests that purine nucleotide biosynthesis was one of the key factors for the compromised growth of *Cgulp2ΔCgslx8Δ* mutant, and cell growth was restored in *Cgulp2ΔCguls1Δ* double mutant.

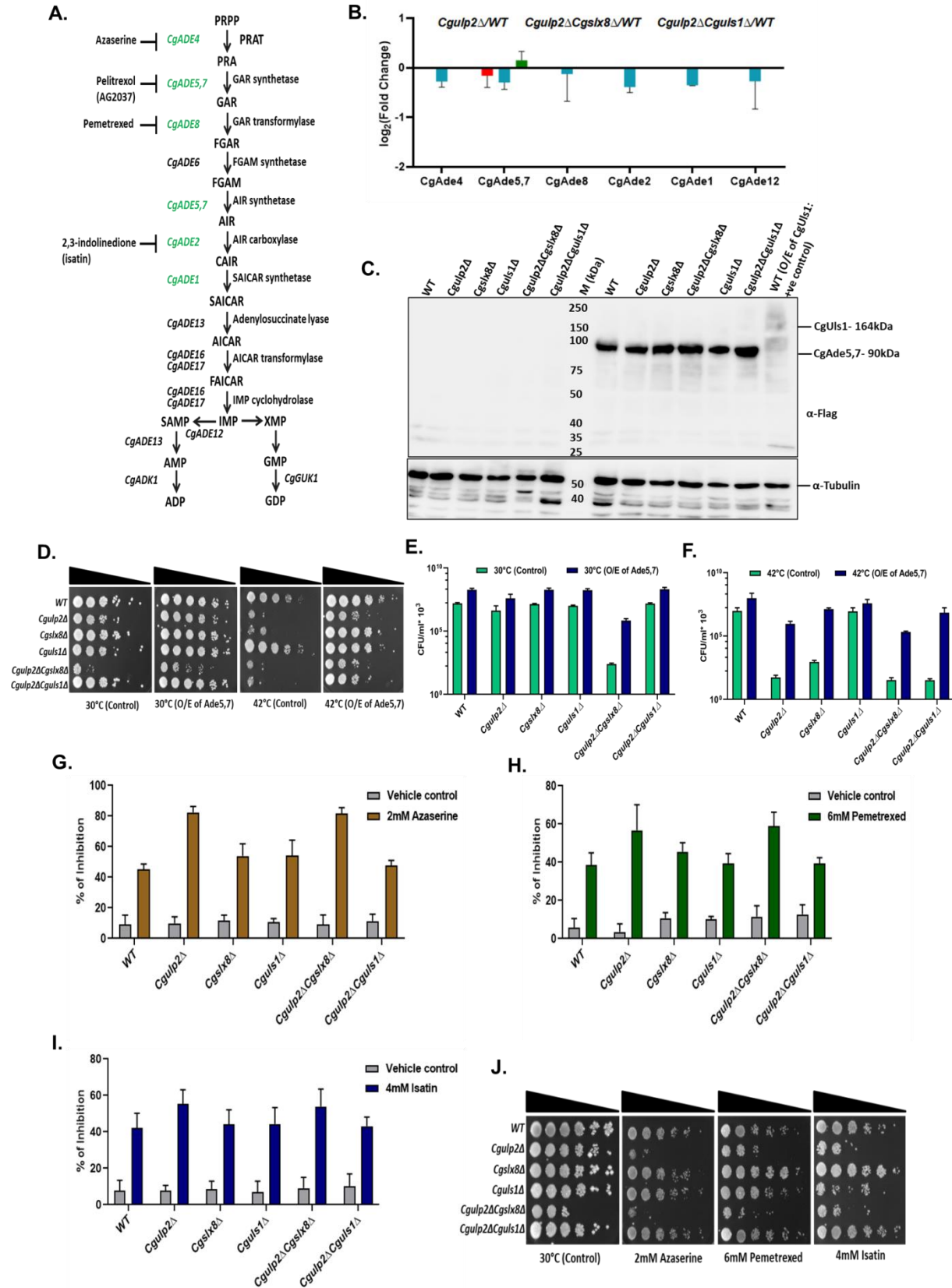


Figure 16. Loss of proteins and reduced growth of *Cguld2ΔCgslx8Δ* double mutant is due to the altered proteins involved in the purine biosynthetic pathway. **A.** de novo pathway of purine nucleotide biosynthesis. Proteins labeled in green color were displayed in mass spectrometry data. A few inhibitors (Azaserine, pemetrexed, and isatin) that were carried out in this study were mentioned in a particular reaction step. Abbreviation: PRPP- 5-phosphoribosyl-1- pyrophosphate, PRAT- phosphoribosylamidotransferase, PRA- phosphoribosylamine, GAR- glycineamide ribonucleotide, FGAR- N-formylglycinamide ribonucleotide, FGAM- N-formylglycinamidine ribonucleotide, AIR- 5-aminoimidazole ribonucleotide, CAIR- AIR carboxamide, SAICAR- N-succino-5-aminoimidazole-4-carboxamide ribonucleotide, AICAR- aminoimidazole-4-carboxamide ribonucleotide, FAICAR- 5-formyl-AICAR, IMP, SAMP- adenylosuccinate, AMP- adenosine monophosphate, ADP- adenosine diphosphate, XMP- xanthosine monophosphate, GMP- guanosine monophosphate, GDP- guanosine diphosphate, ADK1-adenylate kinase 1, GUK1- guanylate kinase 1. **B.** Bar graph shows the upregulation and downregulation of the proteins involved in this pathway for *Cguld2Δ*, *Cguld2ΔCgslx8Δ*, and *Cguld2ΔCguls1Δ*. The error bar represents the log₂ standard deviation. **C.** Western blot was performed to check the expression of CgAde57 protein in WT and mutants by using an α-flag antibody. Before the protein marker, there are negative controls of WT and mutants (no tag). Overexpression of CgUls1 protein in WT cells acts as a positive control. Tubulin is used as a loading control. **D.** Overexpression of *CgADE57* in STUbL mutants. To test the growth upon overexpressing *CgADE57*, a serial dilution-spotting assay was used. 3 μl of 10-fold serial dilutions of exponential phase (1.0 A600) grown cultures of *C. glabrata* strains were spotted in media lacking uracil. Plate images were taken after 1-3 days of incubation at 30°C and 42°C, respectively. **E** and **F.** Bar graphs showed the quantification of cell growth of each mutant upon overexpressing the *CgADE57* gene. **G-I.** Inhibition assay in STUbL mutants. Overnight grown cultures of indicated *C. glabrata* strains were inoculated in YPD medium to an initial A600 of 0.2. 100μl of *C. glabrata* cell suspension were added to the wells. Azaserine, Pemetrexed, and Isatin inhibitors were added in the concentration of 2mM, 6mM, and 4mM to the cell suspension, and the final volume of 200ul for each well was maintained by adding YPD-rich media. YPD medium and cell suspension (100ul) without inhibitors were used as a control. Inoculum was incubated at 30°C for 24-48 hr with 300 rpm agitation. Absorbance at 600nm was measured at the relevant time intervals. Data represent the mean of triplicates and the error bar represents the

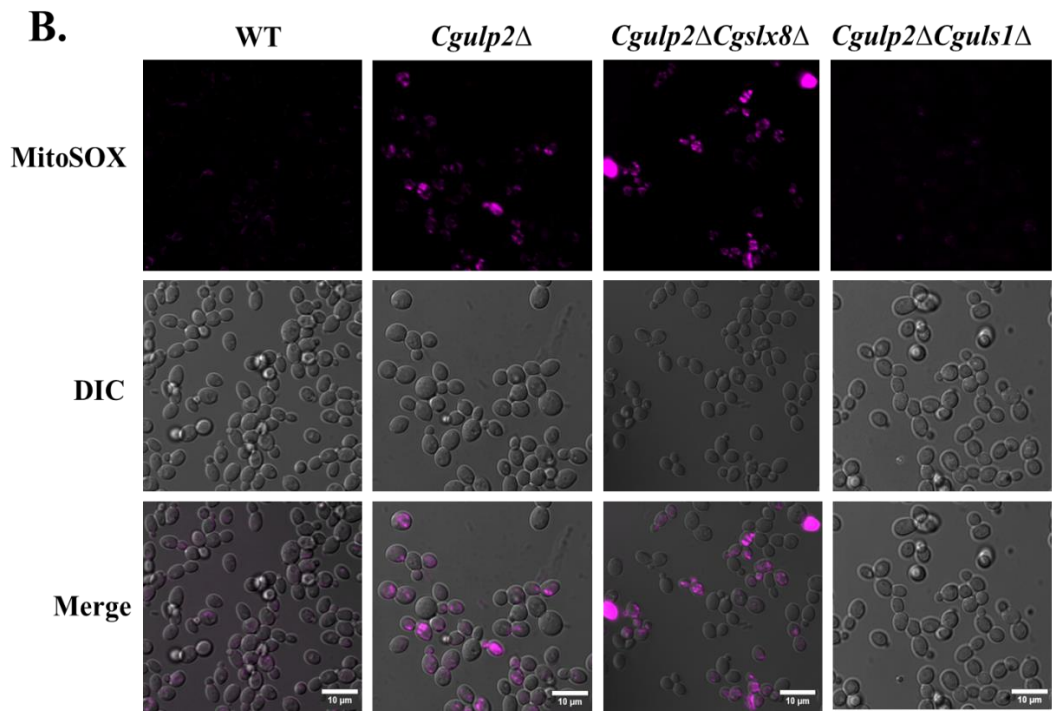
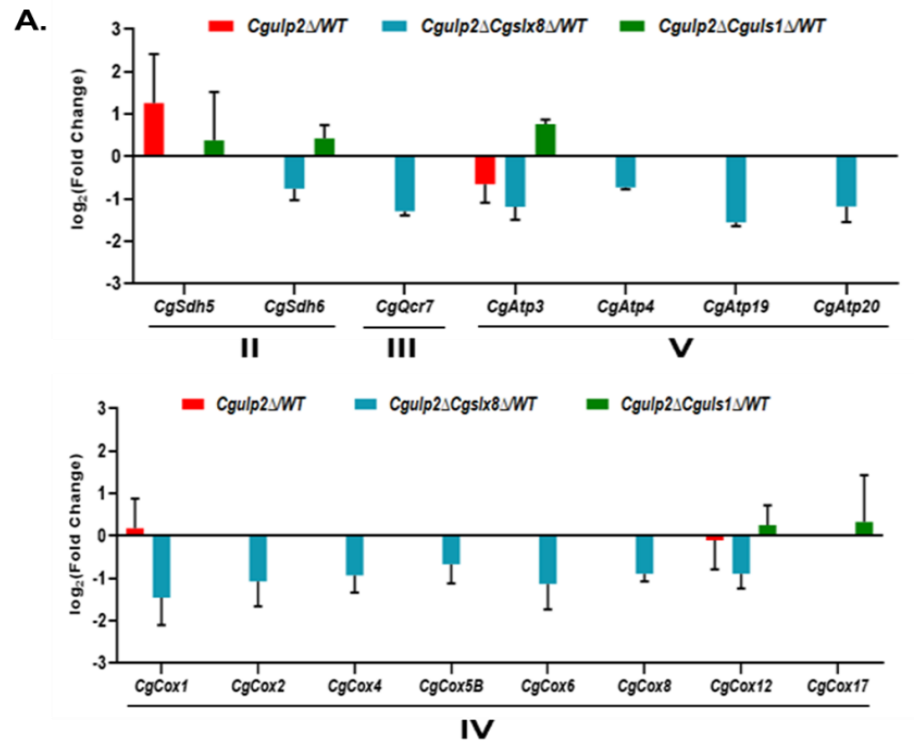
standard deviation. **J.** Plate assay shows the inhibition of cell growth of WT and mutants at 30°C by adding 2mM azaserine, 6mM pemetrexed, and 4mM isatin. 2 µl of 5-fold serial dilutions of logarithmic phase grown culture of WT and mutants were spotted on YPD medium. Plate images were captured after 48 hours of incubation at 30°C.

Mitochondria function is compromised in *Cgulp2Δ* and *Cgulp2ΔCgslx8Δ*

The electron transport chain (ETC) complex was another pathway that was critically affected by *Cgulp2ΔCgslx8Δ* double mutant as complex II, III, IV, and V were highly reduced in *Cgulp2ΔCgslx8Δ* suggesting mitochondrial metabolism was severely affected. In the case of *Cgulp2Δ*, there was no clear down-regulation, with few components being present in larger or lesser levels than the wild type. In addition, proteins associated with these complexes were either upregulated or up to the level of WT in the *Cgulp2ΔCguls1Δ* double mutant (Figure 17A).

Dysregulation of mitochondria has several consequences such as ATP depletion, ROS generation, oxidative stress, etc. Also, *Cgulp2Δ* and *Cgulp2ΔCgslx8Δ* were shown to have reduced growth in non-fermentable carbon sources like 2% ethanol, 3% glycerol, and 2% lactate, and *Cgulp2ΔCguls1Δ* appeared to suppress the defects seen in the single mutant *Cgulp2Δ* (Chapter 3, Figure 6D).

To test if these mutants had compromised mitochondrial function, we examined mitochondrial morphology and reactive oxygen species in the mutants. The wild-type cells exhibited a tubular mitochondrial morphology, while *Cgulp2ΔCgslx8Δ*, and *Cgulp2Δ* displayed fragmented mitochondria. Interestingly, *Cgulp2ΔCguls1Δ* retained tubular mitochondria similar to the wild-type cells (Figure 17C). To assess reactive oxygen species (ROS) levels, we stained WT and *C. glabrata* mutants with MitoSOX, a mitochondria superoxide indicator. *Cgulp2ΔCgslx8Δ* cells showed elevated ROS levels, with a stronger fluorescent signal followed by *Cgulp2Δ* mutant. *Cgulp2ΔCguls1Δ* and WT had no or very little amount of ROS (Figure 17B-C). Further to validate that the increased signal in *Cgulp2Δ* and *Cgulp2ΔCgslx8Δ* cells is due to enhanced mtROS production, we treated these cells with N-acetylcysteine (NAC), an antioxidant known to scavenge ROS. NAC treatment significantly decreased fluorescent signals in both *Cgulp2Δ* and *Cgulp2ΔCgslx8Δ* cells compared to untreated cells (Figure 17C). These data together suggest a compromised mitochondrial function in *Cgulp2ΔCgslx8Δ* and *Cgulp2Δ*.



C.

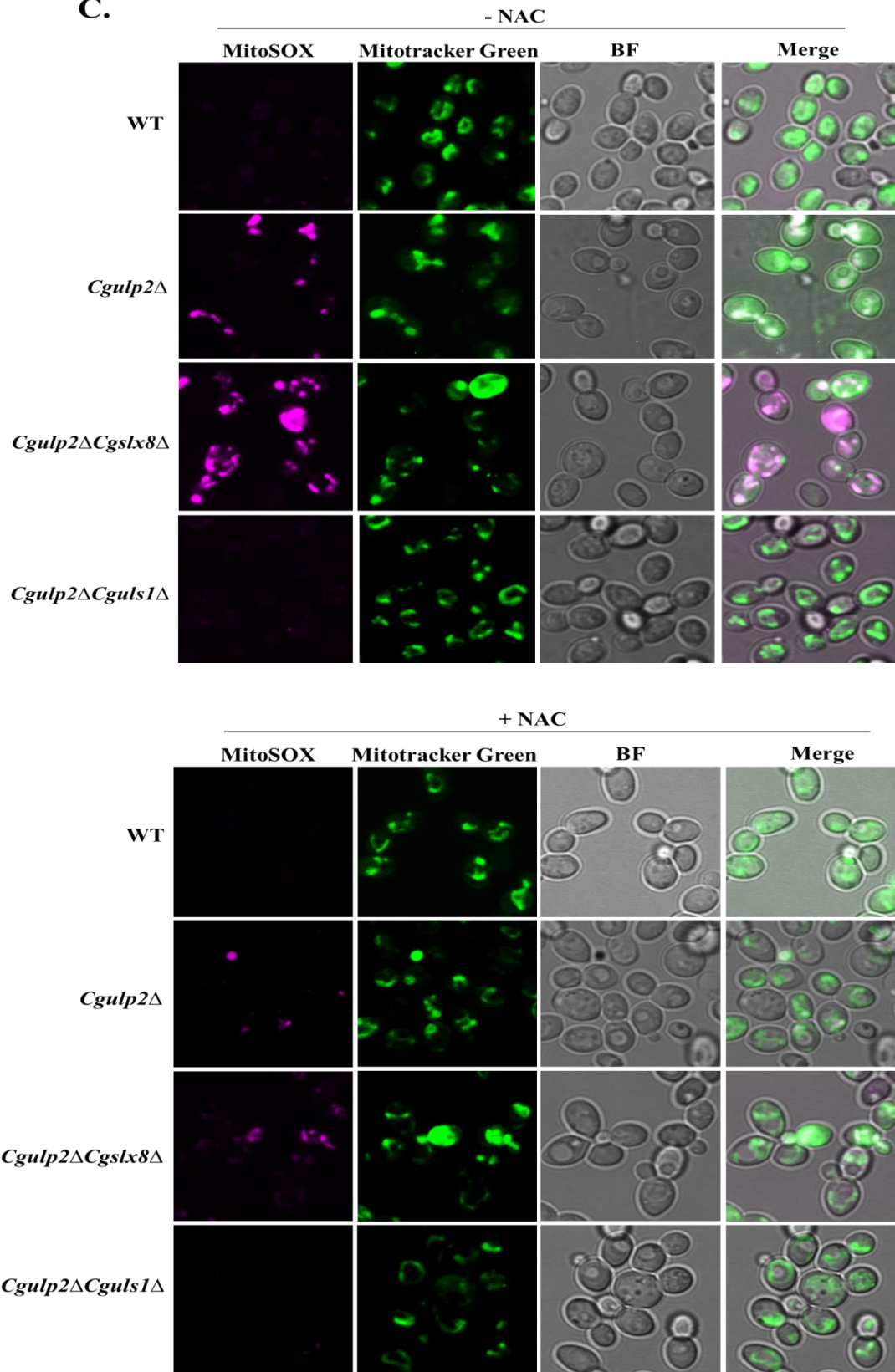


Figure 17. Mitochondrial functions were affected in *Cgulp2Δ* and *Cgulp2ΔCgslx8Δ*. **A.** Bar graph shows the upregulation and downregulation of the proteins involved in the mitochondrial complexes of the ETC pathway for *Cgulp2Δ*, *Cgulp2ΔCgslx8Δ*, and *Cgulp2ΔCguls1Δ*. The error bar represents the standard deviation. **B.** Confocal microscopic images of the WT, *Cgulp2Δ*, *Cgulp2ΔCgslx8Δ*, and *Cgulp2ΔCguls1Δ* that showed the level of ROS generation. Scale bar- 10 μm. **C.** Cells were grown in YPD medium with (+ NAC) or without 10mM NAC (- NAC) at 30°C for 12 hours and then stained with MitoTracker Green and MitoSOX. Images were captured in confocal microscopy. Scale bar- 5 μm

4.3 SUMMARY

From the quantitative mass spectrometry, we have identified multiple pathways that were differentially affected in the mutants of *C. glabrata* (Figure 12). Pathways such as intracellular transport, biosynthetic process, ribosomal biogenesis, mitochondrial, and proteasomal pathways were severely affected in *Cgulp2ΔCgslx8Δ*. However, upon validating the pathways experimentally that were directly correlating with the phenotypes of the mutants, the de novo pathway of purine biosynthesis was found to be one of the key factors for the slow growth of *Cgulp2ΔCgslx8Δ* double mutant. In addition, mitochondrial function was compromised in *Cgulp2Δ* and *Cgulp2ΔCgslx8Δ* double mutant. Both these pathways were partly restored to WT levels (or higher in some cases) in the *Cgulp2ΔCguls1Δ* double mutant. These molecular data correlate with the phenotypic observations presented in the previous chapter. While these two pathways could contribute significantly, based on the proteomics data, it is clear that multiple pathways are compromised and could also contribute to the phenotype.

The major findings from Chapters 3 and 4 are schematically summarized in Figure 18. In the presence of CgUlp2, SUMO chains are removed from the target protein whereas, in its absence, polySUMOylated proteins were accumulated and targeted for protein degradation via STUbL CgUls1. This is a key contributor to the homeostasis of the target proteins. As more polySUMOylated protein accumulates in the absence of CgUlp2, due to the lack of CgUls1, it is not degraded. The double mutant *Cgulp2ΔCgslx8Δ* exhibits an increased level of protein degradation possibly because of upregulated activity of Uls1. Following MG132 treatment, degradation of proteins was prevented and SUMOylated proteins appeared in *Cgulp2Δ* and *Cgulp2ΔCgslx8Δ* mutants and so did the improved growth. Based on the experimental validation

of quantitative mass spectrometry data, ubiquitin-proteasome pathway, purine nucleotide biosynthesis pathway, and mitochondrial function were found to be significantly impacted in *Cgulp2ΔCgslx8Δ* double mutant. This was followed by the *Cgulp2Δ* single mutant. We propose that these are the primary causes of the increased protein loss and slow growth of *Cgulp2ΔCgslx8Δ* cells at 30°C. On the other hand, the level of the components of these pathways was opposite in *Cgulp2ΔCguls1Δ* double mutant. The ubiquitin-proteasome pathway had decreased level whereas purine nucleotide biosynthesis pathway and mitochondrial function had increased level due to which restoration of growth and accumulation of polySUMOylated protein was seen in *Cgulp2ΔCguls1Δ* mutant.

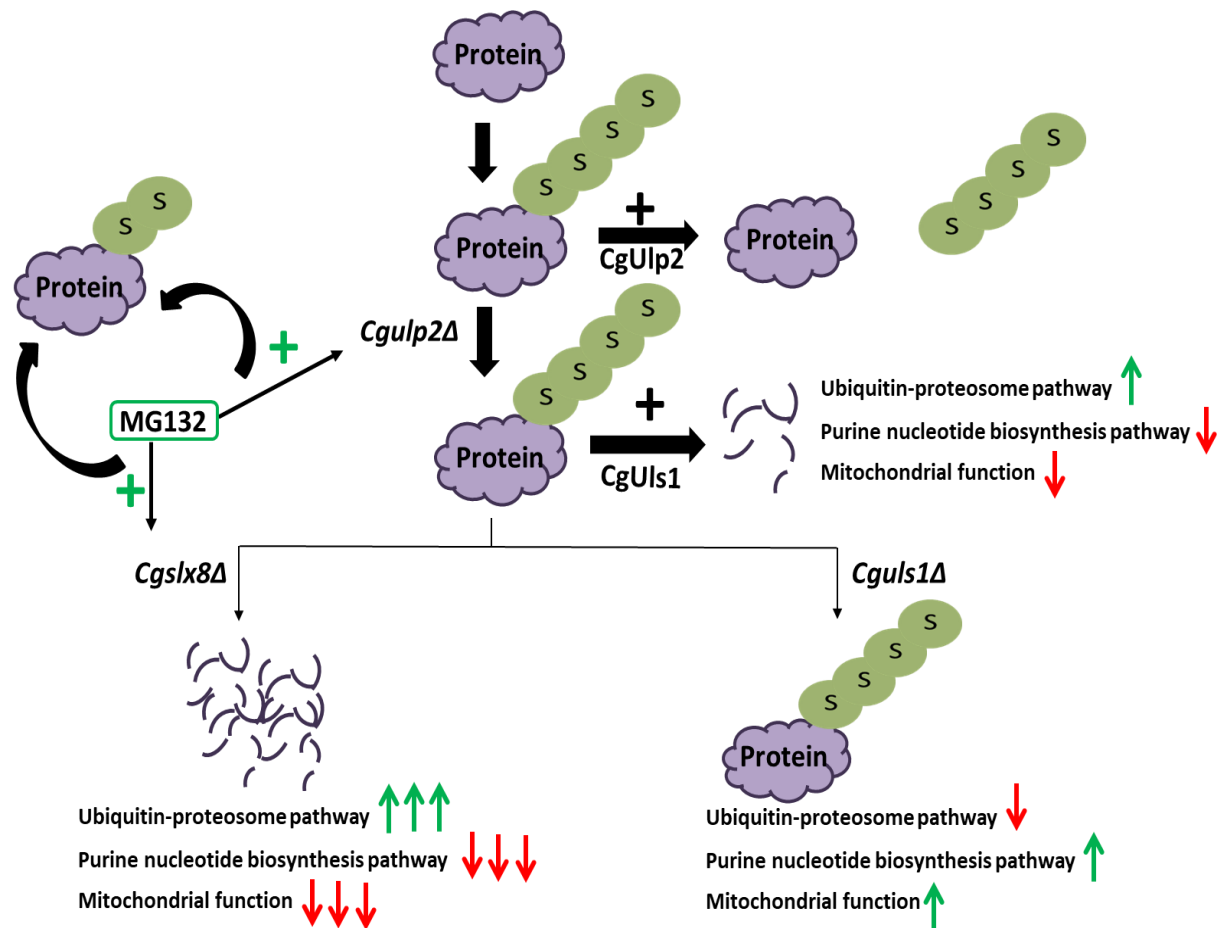


Figure 18. Molecular basis for the phenotypes of loss of deSUMOylase CgUlp2 and STUbLs in *C. glabrata*. Description in text.

Chapter 5 Establishment of a method to enrich for SUMOylated proteins

5.1 INTRODUCTION

In the majority of pathogenic fungi investigated, the SUMO pathway is found to be indispensable for either survival or virulence. In *Candida glabrata*, for instance, the gene encoding for SUMO, *CgSMT3*, is essential and disruption of SUMOylation leads to increases stress sensitivity and diminished virulence in the pathogenic fungi^{41,90,116}. Of note, deletion of *Cgulg2* results in decreased cell survival and reduced adhesion, underscoring the significance of SUMOylation in fungal physiology and pathogenesis. In Chapter 3, we have shown that STUbL mutants have reduced cell survivability under various stress conditions. So we aim to identify the target proteins that are SUMOylated and whose SUMOylation or levels in the cell affect the response of *C. glabrata* to these stress conditions. In order to do this, we have to pull down the SUMOylated proteins and do mass spectrometry to identify the targets.

5.2 RESULTS

For the pull-down protocol for SUMOylated proteins, there are multiple methods available for SUMOylated proteins in *S. cerevisiae* and in mammalian cell lines. We tried several of these, including methods previously published in our laboratory for *S. cerevisiae*⁵¹. However, while most worked well in *S. cerevisiae*, they did not work efficiently with *C. glabrata*. After several attempts with different modifications to the procedure, we set out to develop an alternate method which is described below.

We standardized SUMO proteome isolation in *C. glabrata* using tandem SUMO interacting motifs (SIM), inspired by a similar method developed in the laboratory of M.S. Rodriguez¹¹⁸. The protocol for SIM-based enrichment of SUMOylated proteins is schematically presented in Figure 19. SIM consists of a sequence comprising four hydrophobic amino acids surrounded by acidic amino acids. SIM was identified in the Uls1 protein of *S. cerevisiae*. By sequence alignment of the Uls1 proteins from *S. cerevisiae* and *C. glabrata*, we identified the putative sequence of SIM as “VVIVD” spanning amino acid 1325 to 1329 in *C. glabrata* (Figure 20A). At first, we cloned 8 SIM tandemly in the pET28a bacterial expression vector, and subsequently

proceeded with the expression and purification of the tandem SIMs using Ni-NTA affinity purification. We could detect a 20kDa peptide representing the tandem SIM motif (Figure 20B-E). Then the Ni-NTA column bound SIM was incubated with the *C. glabrata* cell extracts for 6 hours. It was then washed several times to remove non-specifically bound proteins and eluted with 300mM imidazole to pull SUMOylated proteins along with the 8xSIM peptide and analyzed by western blot using anti-FLAG antibody (Figure 21).

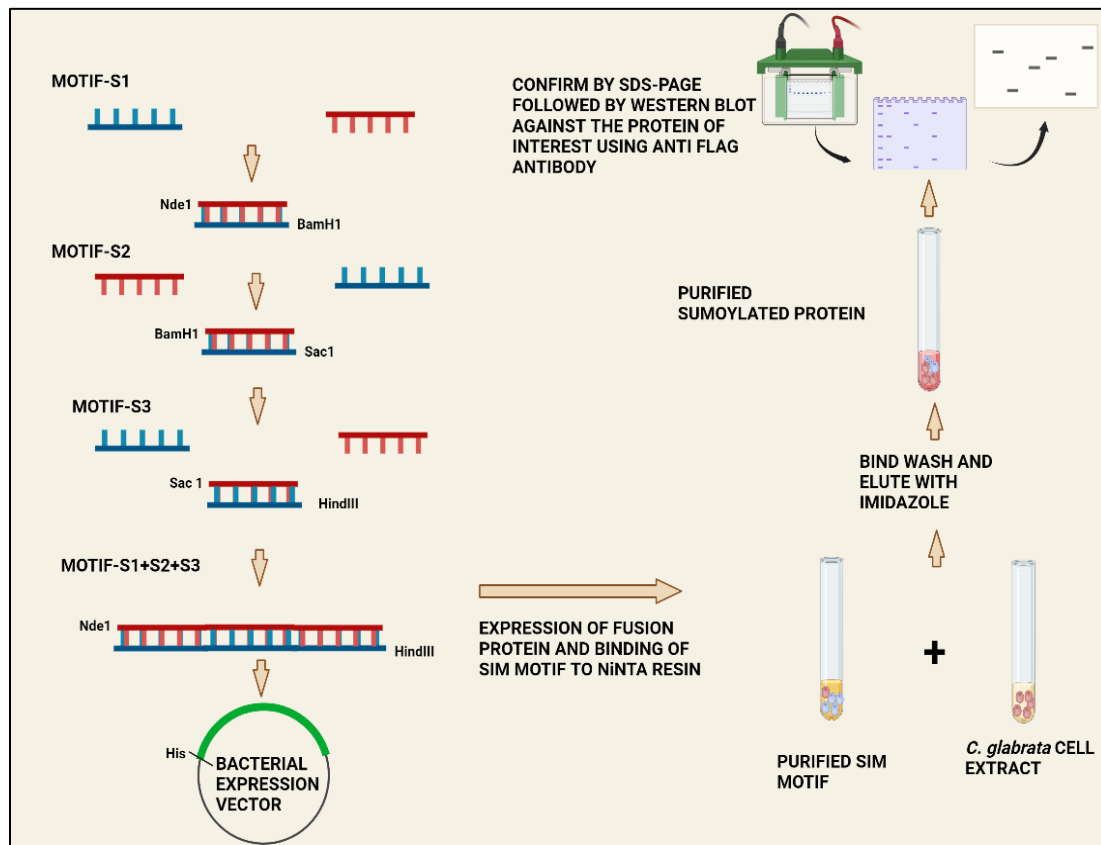


Figure 19. Schematic representation of SIM-based SUMOylated protein enrichment in *C. glabrata*.

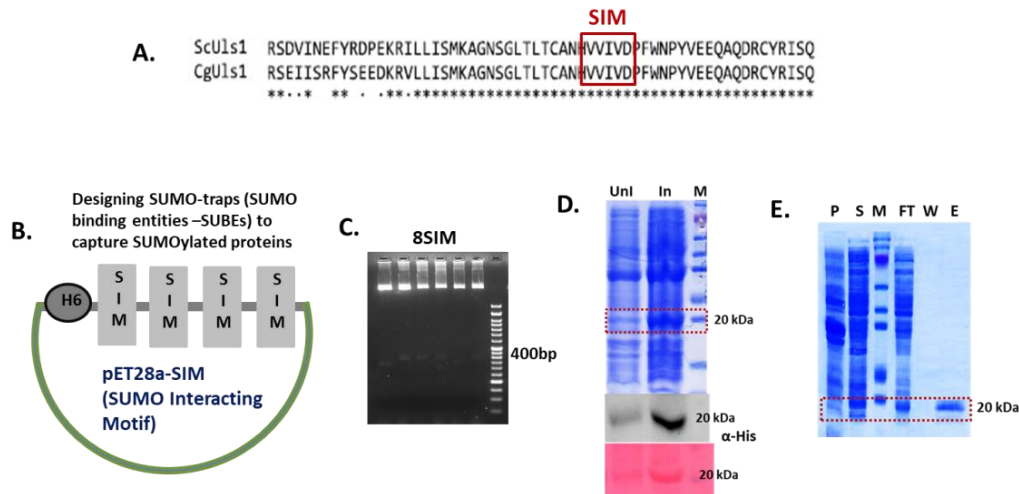


Figure 20. Purification of SUMO-interacting motif (SIM) in *C. glabrata*. **A.** Putative sequence of SIM in *C. glabrata* by sequence alignment of *ULS1* of *S. cerevisiae* and *C. glabrata*. **B.** Schematic representation of SIM cloned tandemly in the bacterial expression vector (pET28a). **C.** 1% agarose gel shows the SIM cloned in the pET28a vector. **D.** Protein expression of SIM motifs and **E.** purification of SIM motifs. UnI-Uninduced protein, In-induced protein, P and S-pellet, and supernatant after sonication, FT- Flow through, W- Wash after binding of protein to Ni-NTA beads, and E- purified protein elution.

In objective 3, we have shown that loss of *Cguls1* suppresses the growth phenotype of *Cguls1* mutant due to accumulation/lack of degradation of the polySUMOylated proteins in the absence of *Cguls1*. Therefore, we performed the above-mentioned protocol in dual-tagged *CgSmt3* expressed in wildtype, *Cguls1Δ*, *Cguls1Δ* single mutant, and *Cguls1ΔCguls1Δ* double mutant strain. Also, a wild-type strain lacking dual-tagged *CgSmt3* served as a negative control to check the specificity of SUMO enrichment. Notably, slower-moving bands were absent in this strain, suggesting that the proteins eluted from this column are likely to be specific. When we performed a pull-down assay with these mutants, as expected, higher molecular weight SUMOylated protein bands (above 25kDa) were observed in the wild type strain and also several prominent polySUMOylated protein bands in *Cguls1ΔCguls1Δ* double mutant compared with negative control when probed with anti-FLAG antibody (Figure 21). In the absence of a deSUMOylase, we were unable to detect any higher molecular weight (potentially polySUMOylated) proteins as in the western blots

performed with whole cell extracts earlier. Together, these data establish that tandem SIM motifs could be a viable means of enriching SUMOylated proteins in *C. glabrata*.

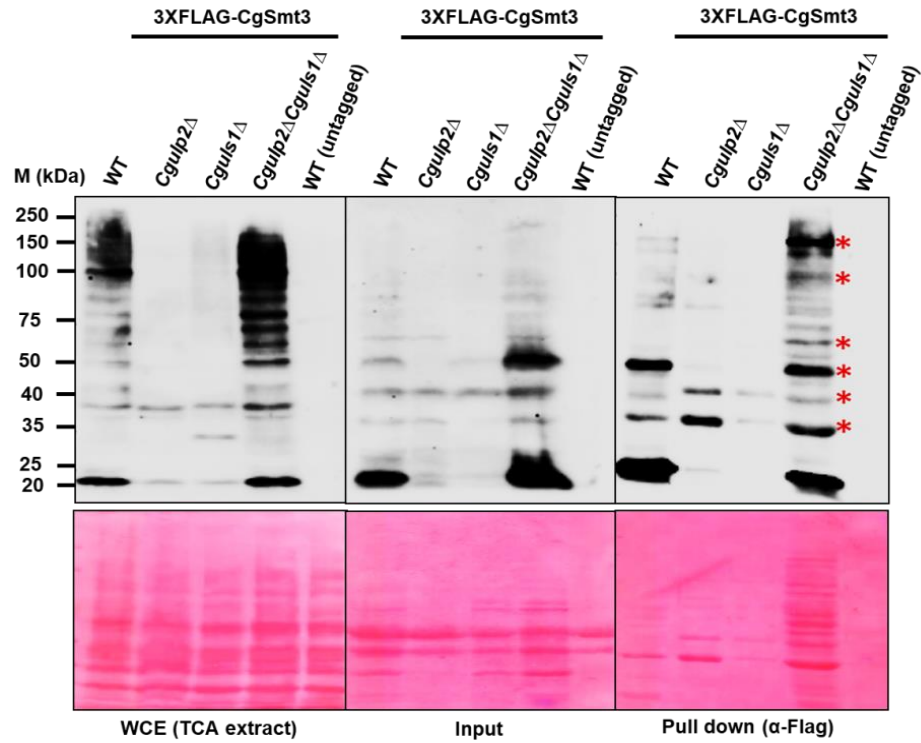


Figure 21. Enrichment of SUMOylated proteins in SUMO protease and STUbL mutants in *C. glabrata*. * indicates SUMOylated proteins.

5.3 SUMMARY

We have established tandem SUMO-interacting motifs (SIMs) based enrichment of SUMOylated proteins in *C. glabrata*. This can be used to determine the SUMOylation targets that impact infections in this organism.

Chapter 6 Determination of atomic resolution structure of key enzymes in protein SUMOylation to design drugs that target them

6.1 INTRODUCTION

In yeast *S. cerevisiae*, both Ulp1 and Ulp2 are responsible for removing monoSUMOylation from proteins, while Ulp2 specifically targets the removal SUMO from polySUMOylated target proteins^{82,116}. As a result, this enzymatic activity ensures the reversibility of protein SUMOylation and maintains a pool of free SUMO within the cell to continue the SUMOylation cycle. Ulp1 is also involved in the initial step of SUMOylation, maturing SUMO precursors. In our laboratory, previous and current work has shown these pathways are likely to be conserved in *C. glabrata* as both *ULP1* and *SMT3* are essential in both yeasts. Perturbations to the SUMO pathway affect the pathobiology of *Candida glabrata*. Ulp2 exhibits high selectivity as a deSUMOylase, targeting specific protein complexes involved in crucial cellular processes such as DNA replication, ribosomal DNA maintenance, and chromosomal segregation in *S. cerevisiae*. Consequently, our primary objective in studying SUMOylation in *C. glabrata* is to explore the potential for targeting this pathway for intervention since perturbing SUMOylation will adversely affect several essential processes. Given the limited availability of drugs inhibiting SUMOylation (e.g., Ginkgolic acid), our aim is to design inhibitors targeting this pathway.

In chapters 3 and 4, we have established that protein homeostasis via deSUMOylase CgUlp2 is a critical factor and also identified downstream pathway, so we planned to target Ulp2 as loss of Ulp2 has been shown to affect the virulence of multiple pathogenic fungi. In addition, the components of the SUMO pathway in various fungi were compared with humans to identify the least conserved protein that will define the better target.

6.2 RESULTS

SUMO protease CgUlp2 was identified as least conserved with human

Based on sequence homology, we have identified the orthologs of components of the SUMOylation pathway in multiple pathogenic fungi and identified the level of conservation of the proteins involved. We found that many proteins of this pathway share a very high level of similarity with human orthologs, with Ubc9 sharing maximum similarity. The full length of Siz1,

Ulp1, and Ulp2 proteins share the least similarity (Table 7A). On comparing the catalytic domains, the SUMO proteases Ulp1 and Ulp2 share lesser similarity with their human counterparts (Table 7B). Among them, the SUMO protease Ulp2 orthologs exhibit the lowest similarity with the human protein, making it most suitable for a potential drug target. **Peptidase C48**, spanning amino acid residues 450-675, represents the catalytic domain in the CgUlp2 protein.

A.

SUMO pathway genes	<i>Saccharomyces cerevisiae</i>	<i>Candida glabrata</i>	<i>Candida albicans</i>	<i>Cryptococcus neoformans</i>	<i>Magnaporthe oryzae</i>	<i>Aspergillus nidulans</i>	<i>Candida parapsilosis</i>
<i>SMT3</i>	57.4	54.7	51.6	48.1	50.0	51.7	39.2
<i>AOS1</i>	52.8	50.4	46.0	48.5	43.7	47.0	46.5
<i>UBA2</i>	50.4	51.0	51.5	51.6	51.7	54.3	50.0
<i>UBC9</i>	69.9	70.8	51.5	75.1	73.3	67.0	68.0
<i>MMS21</i>	40.1	40.7	40.3	43.6	36.6	32.8	-
<i>SIZ1</i>	24.6	27.4	17.5	26.1	39.5	31.2	21.9
<i>ULP1</i>	39.3	40.5	47.4	46.0	23.6	30.8	44.0
<i>ULP2</i>	38.9	38.7	38.2	34.1	37.9	36.4	36.8

B.

<i>Candida glabrata</i>	<i>Homo sapiens</i>	Full length (% similarity)	Catalytic Domain (% similarity)
<i>CgSIZ1</i>	<i>PIAS1</i>	39.7	58.6
<i>CgSIZ1</i>	<i>PIAS2</i>	39.1	57.9
<i>CgSIZ1</i>	<i>PIAS3</i>	38.6	56.1
<i>CgSIZ1</i>	<i>PIAS4</i>	33.3	60.3
<i>CgSIZ1</i>	<i>ZMIZ1</i>	35.5	56.1
<i>CgSIZ1</i>	<i>ZMIZ2</i>	35.6	56.1
<i>CgULP1</i>	<i>SEN1</i>	41.0	49.1
<i>CgULP1</i>	<i>SEN2</i>	40.2	47.9
<i>CgULP1</i>	<i>SEN3</i>	38.8	45.0
<i>CgULP1</i>	<i>SEN5</i>	39.8	47.9
<i>CgULP2</i>	<i>SEN6</i>	37.5	31.1
<i>CgULP2</i>	<i>SEN7</i>	38.7	32.0

Table 7. SUMO protease CgUlp2 showed the least conservation with its human homolog. A. Comparison of the percentage similarity of full-length *H. sapiens* ortholog with the orthologs identified in selected fungi. **B.** Comparison of the percentage similarity of the full-length protein and only the catalytic domains of the SIZ2, ULP1, and ULP2 orthologs in *C. glabrata* and *H. sapiens*. CgUlp2 is highlighted with a red color box.

Full-length Ulp2 structure is not available at high resolution in any organism. Therefore, our objective is to express and purify significant quantities of CgUlp2 for structural studies.

Expression and purification of CgUlp2 protein

CgUlp2 protein was cloned in the pET28a vector followed by checking the expression of the CgUlp2 protein. We observed a low level of expression of pET28a encoded CgUlp2 (105kDa) protein (Data not shown). Since our attempts to improve the expression were unsuccessful, we cloned, expressed, and purified parts of CgUlp2 protein in the pET28a vector (Figure 22A). We generated constructs for the N-terminal domain (NTD), catalytic domain (CD), and C-terminal

domain (CTD) encompassing amino acid residues 1-478, 405-710, and 590-917, respectively (Figure 22A) in pET28a vector and expressed in BL21(DE3)RIL bacterial strain. Further, the full length and the domains of CgUlp2 protein were purified using Ni-NTA resin and we found that these constructs were affinity purified with non-specific protein bands. Also, the full length of CgUlp2 protein had a very low yield upon purification (Figure 22B).

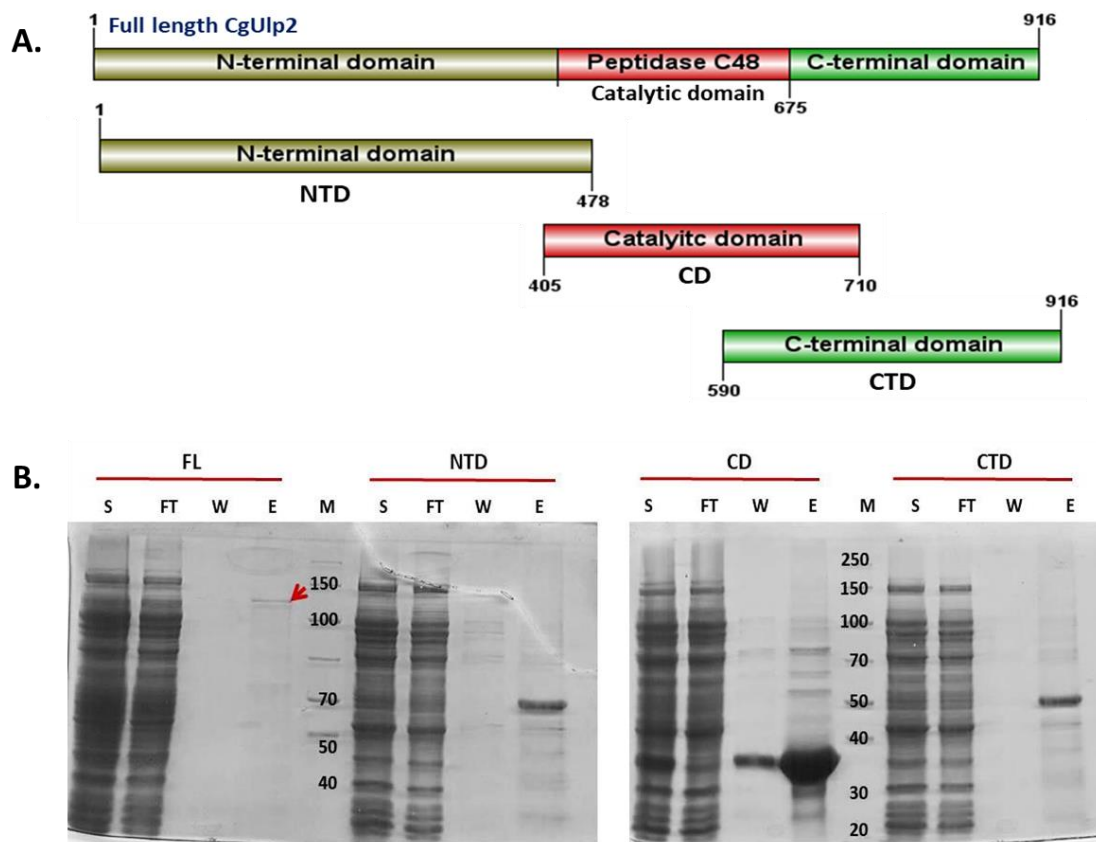


Figure 22. Purification of the CgUlp2 protein. **A.** Schematic representation of full-length CgUlp2 showing three individual domains: N-terminal domain (NTD-50kDa), Catalytic domain (CD-37kDa), and C-terminal domain (CTD-50kDa). **B.** Affinity purification of the CgUlp2 protein. S- supernatant form after sonication, FT- Flow through, W- Wash after binding of protein to Ni-NTA beads, E- purified protein elution, and M-protein marker.

To remove the non-specific proteins that were observed after affinity purification of the full length and the smaller fragments of CgUlp2 protein, size exclusion chromatography (gel filtration) was performed. The full length, NTD, and CTD of the CgUlp2 protein were observed

to form soluble aggregates, indicating a potential higher oligomeric state or both the NTD and CTD contain a significant amount of unstructured amino acid residues.

Secondary structure prediction of CgUlp2 protein

The PDBsum server was used to predict the secondary structures of the CgUlp2 SUMO protease. The analysis revealed that the N- and C-terminal domains of the CgUlp2 protein consist of unstructured residues, with the C-terminal domain exhibiting significant loop regions. In contrast, the catalytic domain of the CgUlp2 protein predominantly contains both α -helices and β -sheets. Altogether, the absence of a well-defined secondary structure in the N- and C-terminal domains posed challenges in the efficient purification of the full length and these domains. (Figure 23).

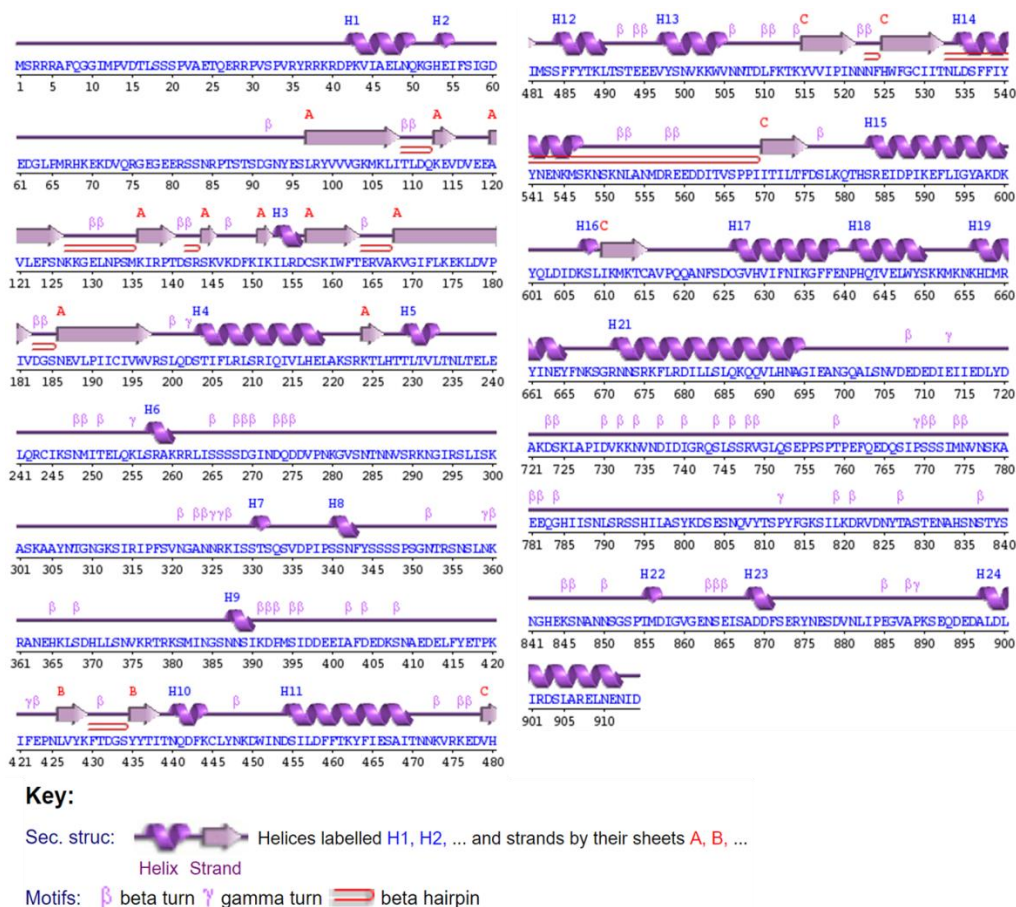


Figure 23. Diagrammatic representation of the CgUlp2 protein secondary structure as shown in PDBsum.

Crystallization of the catalytic domain of CgUlp2 protein

Our major goal is to obtain the crystals of the CgUlp2 protein for structural studies. To that end, since purification of full-length, N- and C- terminal domains of the CgUlp2 protein have low yield and soluble aggregates, we focused on the catalytic domain of this protein. The catalytic domain of the CgUlp2 protein was successfully purified (Figure 24A-B). This protein was concentrated to 12 mg/ml and utilized in experiments to crystallize. The preliminary screening was carried out using the sitting-drop vapor-diffusion technique. Microcrystals were developed in a variety of conditions (Figure 24C). Unfortunately, we didn't get good crystals of this protein upon optimization in various buffer conditions for X-ray diffraction to obtain a high-resolution structure of the protein.

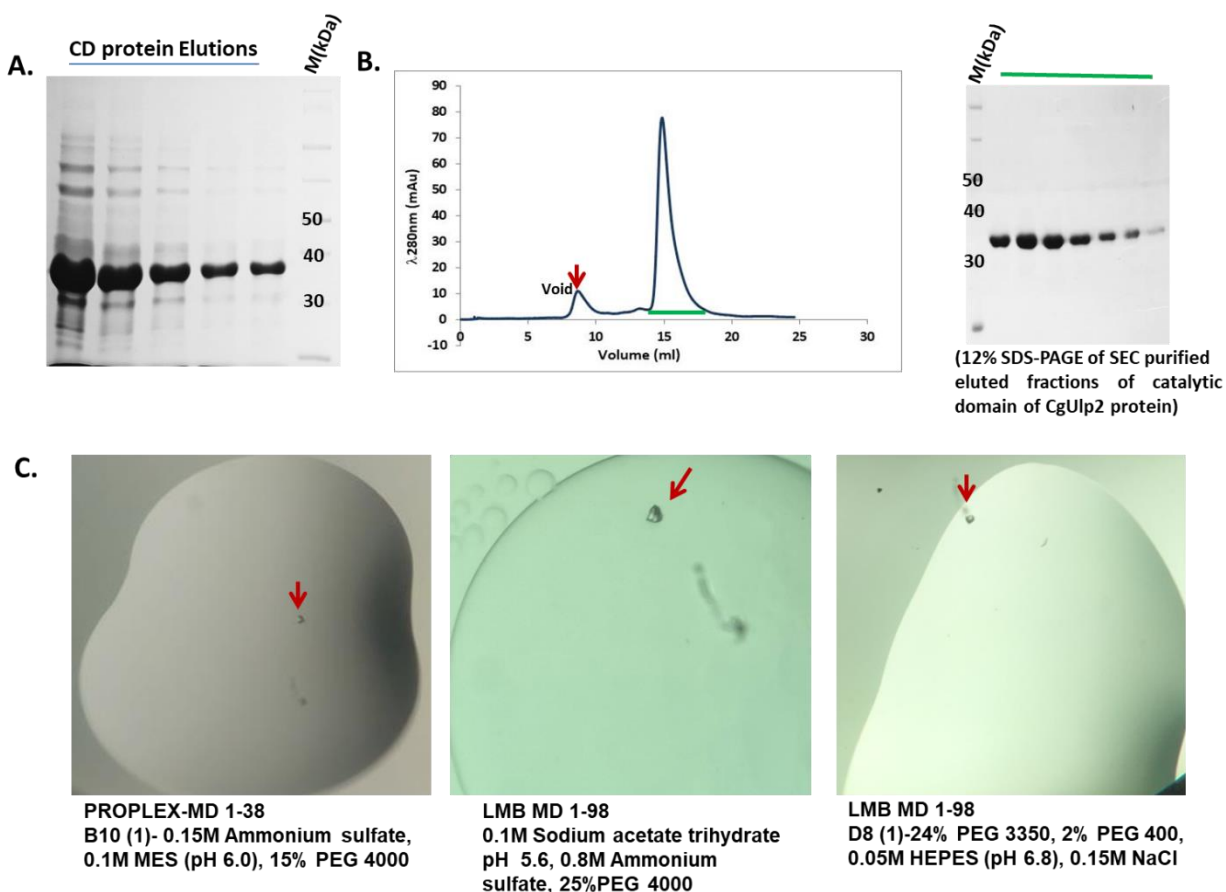


Figure 24. Purification and crystallization screening of the catalytic domain of CgUlp2 protein. **A.** 12% SDS-PAGE of the affinity purification of CD_CgUlp2 protein. **B.** Gel filtration

chromatographic elution profile of CD_CgUlp2 protein. C. Catalytic domain of CgUlp2 protein's crystal hits were shown in the above-mentioned buffer conditions.

Biophysical characterization of the SUMO protease CgUlp2 protein

1. Oligomeric state

SEC-MALS (Size exclusion chromatography-multiangle light scattering) was used to determine the oligomerization of CD_CgUlp2, with the column coupled to both multiangle and dynamic light scattering detectors. Multiangle light scattering facilitated the determination of weight-averaged molar masses, while dynamic light scattering provided information on translational diffusion coefficients (DT). The analysis revealed an average molecular weight of 36.2 kDa for the catalytic domain of the CgUlp2 protein, indicating that CD_CgUlp2 exists as a monomeric protein (Figure 25).

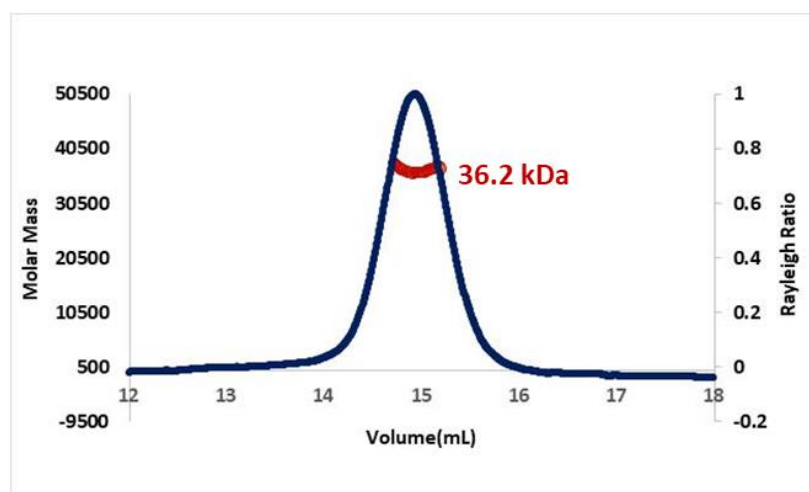


Figure 25. SEC-MALS profile showing the molecular weight of catalytic domain of CgUlp2 protein.

2. Steady-state fluorescence measurements

A Cary Eclipse fluorescence spectrometer was used to assess the intrinsic fluorescence of the protein. The protein solution, with a concentration of 0.2mg/ml, was subjected to excitation at 295 nm, and the emission was measured within the wavelength range of 310-400 nm at 25°C . Both the excitation and emission slit widths were set to 10.0 nm. The spectra were recorded at a rate of 100 nm/min. The signal created by the buffer solution was used to remove background

emission. The CD_CgUlp2 protein intrinsic fluorescence spectrum had a maximum of 347 nm, indicating that tryptophan (W) residues were exposed to the surrounding environment (Figure 26A).

Further to know if there are any buried tryptophan residues, we performed the fluorescence experiments under the denaturing conditions. In the first case, we used denaturants on the CD_CgUlp2 protein. The CD_CgUlp2 protein samples, purified via SEC, were prepared at a concentration of 0.2 mg/ml and incubated with diverse concentrations of denaturants (ranging from 1.0 to 5.0 M for GdnHCl and from 0 to 6.0 M for urea) at pH 7.0 and 25 °C for a duration of 24 hours. In this analysis, fluorescence data obtained from scans of the native CD_CgUlp2, as well as those denatured with GdnHCl and urea, were used. In fluorescence measurements, the peak values observed for the CD_CgUlp2 protein in the presence of different denaturant concentrations were relatively similar to those of the native protein (Figure 26B-C).

In the second case, we used pH-induced denaturation on the CD_CgUlp2 protein. SEC purified CD_CgUlp2 protein samples were subjected to treatment in different pH buffers for 24 hours at 25°C. Buffers ranging from Glycine-HCl (pH 1-3), sodium acetate (pH 4), citrate-phosphate buffer (pH 5), potassium phosphate buffer (pH 6-7), Tris-HCl (pH 8-9), and glycine-NaOH (pH 10-12) were utilized at a concentration of 100 mM. Blank samples containing only the respective pH buffers without the protein were used to rectify the values. After 24 hours, the intrinsic fluorescence spectrum of CD_CgUlp2 protein was more or less identical to native protein in the pH range of 5.0–9.0, albeit with a slight left shift in fluorescence intensity observed at extreme acidic and alkaline pH values. This left shift of the intrinsic fluorescence spectrum indicates protein compactness. As a result, the protein was stable in the pH range of 5.0 to 9.0. (Figure 26D-E). Collectively, these findings suggest that the tryptophan residues located within the catalytic domain of CgUlp2 SUMO protease are exposed on the protein surface and exhibit stability across a range of ambient pH levels.

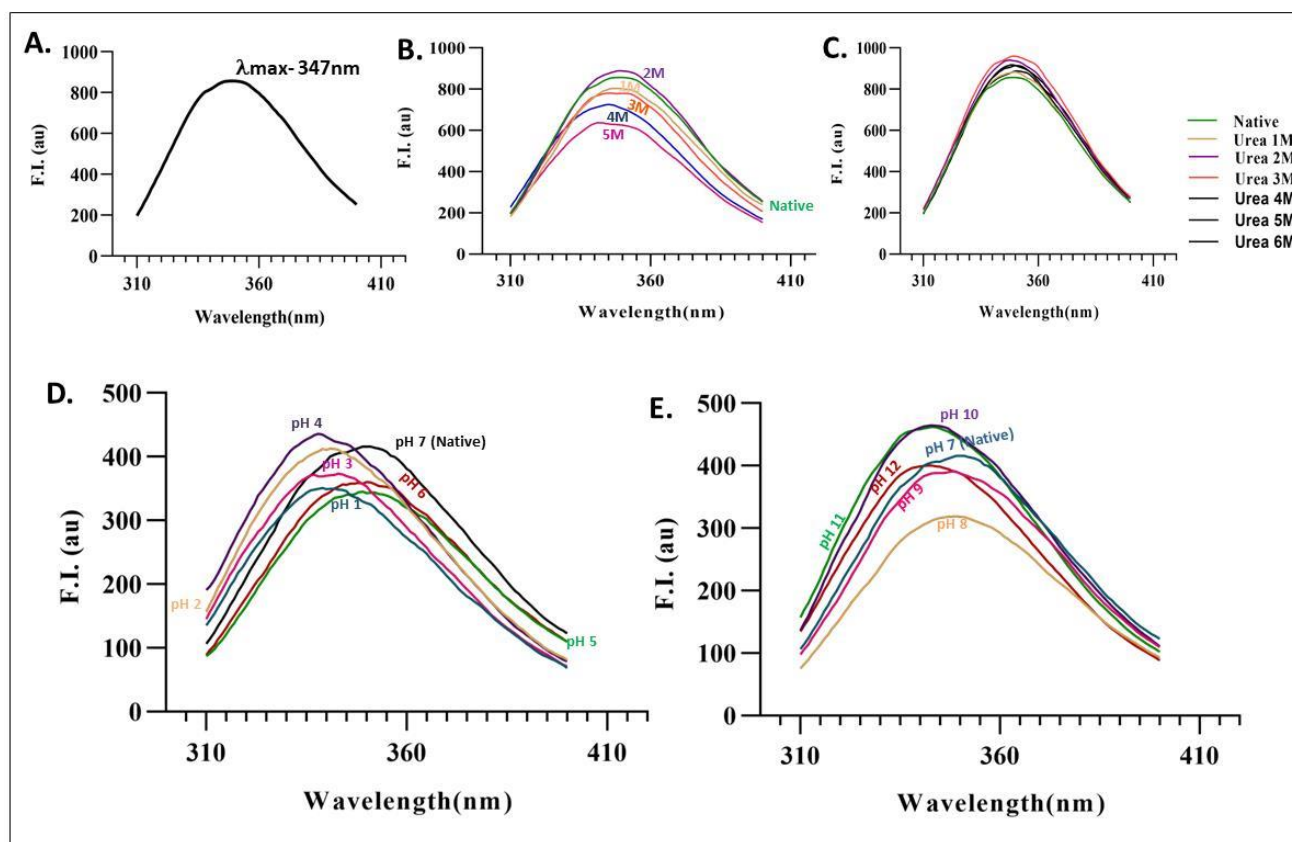


Figure 26. Fluorescence spectrum of the catalytic domain of CgUlp2. A. Tryptophan environment in native condition, B-C. GdnHCl and Urea mediated denaturation profile, D-E. pH-induced denaturation profile.

Secondary structure characterization by Circular dichroism (CD) spectroscopy

Far-UV CD spectra of the purified catalytic domain of CgUlp2 protein were recorded using a Jasco J-815-150S spectropolarimeter linked to a PTC343 Peltier unit circulating water bath set at 28°C. Spectra were acquired in a rectangular quartz cell with a 1mm path length over the range of 195-250 nm at a scan speed of 100 nm/min, a response time of 1 sec, and a slit width of 1 nm. A protein concentration of 0.3 mg/ml was maintained for all of the samples, and the average of

three scanned spectra was used to create each spectrum.. CDPro software was used to calculate the relative amount of various secondary structural elements.

The far-UV CD spectrum of the native catalytic domain of the CgUlp2 protein exhibited negative ellipticity minima at 209 nm with a shoulder at 218 nm, along with positive maxima at 198 (Fig. 27A). The secondary structure components were estimated as 58.3 percent α -helix, 7.5 percent β -sheet, 17.7 percent turns, and 16.5 percent unordered using the CONTINLL tool of CDPro software.

During thermal denaturation, far-UV CD spectroscopy revealed a gradual decrease in negative ellipticity, indicating changes in secondary structure. T_m values of 45.8°C ($R^2 = 0.972$) and 46.1°C ($R^2 = 0.978$) were calculated using a sigmoidal fit analysis of the change in ellipticity at 209 and 218 nm (Fig. 27B). At and above 45°C, there was a significant alteration that resulted in the loss of structure. Proteins with an isodichroic point of about 203 nm also imply a two-state population (α -helix, random coil) (Fig. 27C). In summary, the catalytic domain of the CgUlp2 protein exhibited the presence of both the secondary structures, namely, α -helix, and β -sheet, with α -helix being the predominant form. However, these secondary structures were diminished under elevated temperatures.

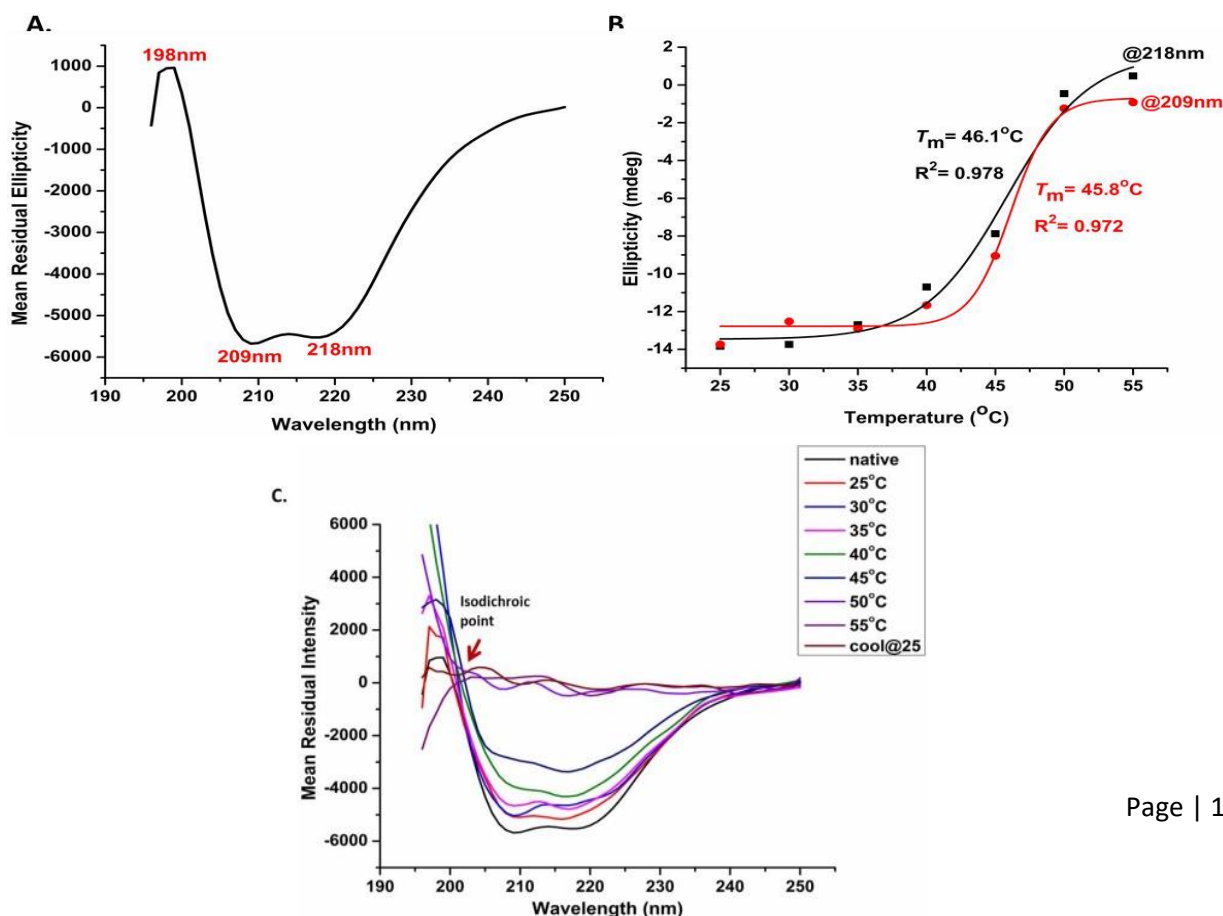


Figure 27. Secondary structure characterization of the catalytic domain of CgUlp2 protein (A) Far-UV CD spectrum (300 µg/ml at pH 7.5), (B) Sigmoidal fit analysis of the change in ellipticity at 209 nm and 218nm following thermal denaturation at pH 7.5, (C) Far-UV CD spectra of the catalytic domain (0.3 mg/ml) incubated at various temperatures for 5 minutes.

Homology model of the catalytic domain of Ulp2 (CD_Ulp2) protein of various pathogenic fungi

ScUlp2 and HsUlp2 catalytic domains are currently the only crystal structures available in the protein data bank (PDB). Due to limited sequence similarity between the catalytic domains of CgUlp2 and HsUlp2 (PDB Id: 3EAY), which was only 29%, template 3EAY could not be used. Therefore, the catalytic domain of *S. cerevisiae* Ulp2 (PDB Id: 5LNB) with a higher sequence identity of 58.57 percent and 96 percent sequence coverage, was selected as the template for building a homology model of the catalytic domain of the CgUlp2 protein using SWISS-MODEL. The resulting models were subjected to energy minimization and refinement processes using GalaxyWeb and PROCHECK.

The quality of the models was assessed using PDBsum, which evaluated them based on Ramachandran plots. PDBsum was used to examine secondary structural elements of the homology models that were created. The model demonstrates that the CgUlp2 SUMO protease catalytic domain has both secondary structures (helices and sheets), with α -helices in the majority (Figure 28A). The Ramachandran plot analysis indicated that the resulting model conformed to the requirements for favored and allowed regions (Figure 28B, Table 8).

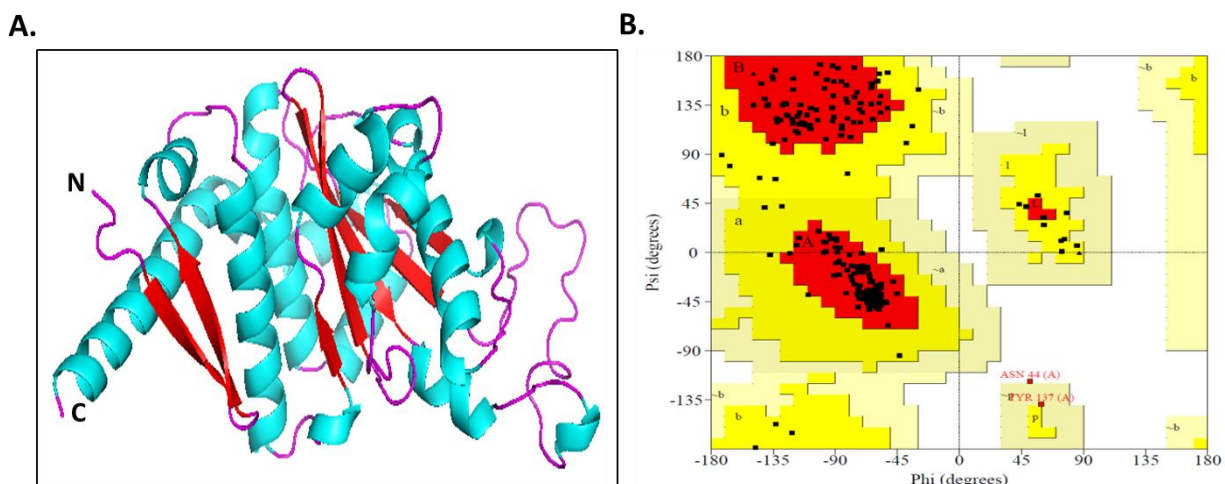


Figure 28. Homology model of CD_CgUlp2 protein. (A) Cartoon representation of the homology model of CgUlp2 SUMO protease catalytic domain built using *S. cerevisiae* Ulp2 protein (PDB ID: 5LNB) as a template. (B) Ramachandran plot for model validation.

Table 8. Ramachandran plot statistics for the homology model of CD_CgUlp2 protein (after refinement)

	No. of Residues	Percentage
Most favored + additionally allowed regions	211	99
Generously allowed regions	2	0.9
Disallowed regions	0	0

Furthermore, using ScUlp2 as a template, we performed homology modeling for the catalytic domain of Ulp2 proteins from several fungal species (for example, *C. albicans* (CaUlp2), *C. auris* (CauUlp2), *A. nidulans* (AnUlp2) and *C. neoformans* (CnUlp2)). After energy minimization and refining, the models were validated by Ramachandran plot (RCP). With *C. glabrata*, the catalytic domain of Ulp2 protein of *C. albicans* and *C. auris* has both secondary structures (helices and sheets) with 99.2% RCP. With an additional large unstructured region, *A. nidulans* and *C. neoformans* catalytic domains have α -helices and β -sheets with 96.5% and 91.8% RCP (Figure 29A). To identify pan-antifungal targets, we superimposed the catalytic domain of *C. glabrata* (CgUlp2), *C. albicans* (CaUlp2), *C. auris* (CauUlp2), *A. nidulans*

(AnUlp2) and *C. neoformans* (CnUlp2) with *S. cerevisiae* (CD_ScUlp2) and *Homo sapiens* (CD_HsUlp2). We observed that α -helices and β -sheets of all these fungal species overlapped with CD_ScUlp2 with the exception that CD_ScUlp2 α -helices at the C-terminus were missing in CD_CnUlp2 (Figure 29B). While there was conservation among the fungal proteins, the β -sheets of these organisms did not overlap with CD_HsUlp2 and had less similarity with the human protein (Figure 29C). Since the overlap with human Ulp2 is much less based on homology models, the catalytic domain of the Ulp2 protein could serve as a non-toxic antifungal target.

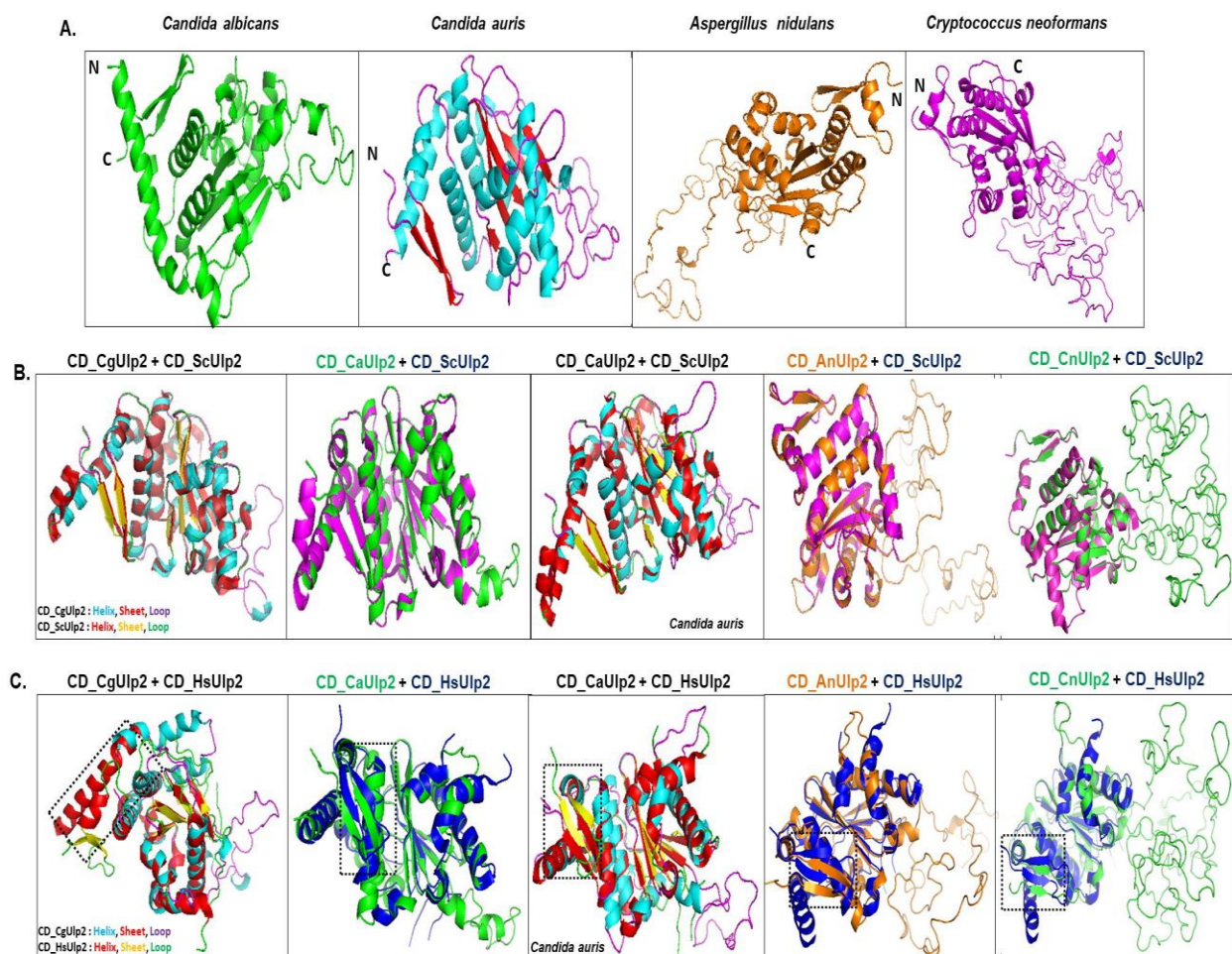


Figure 29. Homology modeling of the catalytic domain of Ulp2 protein of various pathogenic fungi. **A.** Cartoon representation of homology models of Ulp2 SUMO protease catalytic domain of *C. albicans* (CaUlp2), *C. auris* (CauUlp2), *A. nidulans* (AnUlp2) and *C. neoformans* (CnUlp2) using *S. cerevisiae* Ulp2 protein as a template. **B.** Superimposition of

CD_Ulp2 protein of various pathogenic fungi with CD_ScUlp2. **C.** Superimposition of CD_Ulp2 protein of various pathogenic fungi with CD_HsUlp2. Non-overlapping β -sheets among the catalytic domain of CgUlp2, CaUlp2, CauUlp2, AnUlp2, and CnUlp2 are represented by a black dotted box.

CgUlp2 protein has conserved catalytic triad residues

Based on sequence alignment and active center similarities with the catalytic domain of Ulp2 protein for both the *S. cerevisiae* (PDB Id: 5LNB) and *H. sapiens* (PDB Id: 3EAY), key catalytic residues were identified for CgUlp2 protein. We identified His525, Asp576, and Cys626 as the potential catalytic triad residues by aligning the sequences of ScUlp2, CgUlp2, and HsUlp2 proteins (Figure 30A). In the CD_CgUlp2 model, these residues were found in β -sheet (His525), loop (Asp576), and α -helix (Cys626) respectively (Figure 30B). To validate the conserved catalytic triad, we mutated Cys to Ser at position 626 by site-directed mutagenesis. Complementation with the C626S mutant of CgUlp2 showed that it was similar to *Cgulf2* Δ single mutant under various stress conditions (Figure 30C). This indicates that the C626S could be catalytically inactive. We further confirmed this by checking the SUMOylation pattern of this mutant. We used an α -FLAG antibody to perform a western blot on whole cell lysates from the *C. glabrata* WT, *Cgulf2* Δ , *Cgulf2* Δ /*CgULP2*, and *Cgulf2* Δ /*CgULP2*(C626S) augmented with dual tagged CgSmt3 protein (carrying 6xHis and FLAG epitopes at the N-terminus). Higher molecular weight SUMOylated proteins were not observed after the cysteine was changed to serine, which was similar to the *Cgulf2* Δ single mutant (Figure 30D). Altogether, these results show His525, Asp576, and Cys626 as the conserved catalytic triad residues in the CgUlp2 protein.

CgUlp2 interacted with CgSmt3 (SUMO) protein directly via SUMO-interacting motifs

As fungi are eukaryotic organisms, it is difficult to identify a drug target because the protein we are targeting must not be present in the host or be sufficiently different from the pathogen protein. As CgUlp2 had the least similarity to the human ortholog among the SUMO pathway enzymes, we hypothesized that it could be a potential antifungal target. We would like to develop SUMOylation inhibitors as a potential antifungal target. We hypothesized that the interaction of the SUMO of polySUMOylated protein with Ulp2 would be essential for Ulp2 function. As a result, we investigated whether Ulp2 and SUMO interact. To do so, we performed the molecular docking of both the catalytic domain of the CgUlp2 and CgSmt3 proteins. A homology model of the CgSmt3 protein was created using the SWISS-MODEL, with a sequence identity of 87.88 percent and 92 percent sequence coverage, utilizing the *S. cerevisiae* Smt3 protein as a template. Using GalaxyWeb and PROCHECK, the model was subjected to energy minimization and refining. Using the Cluspro 2.0 server, we then performed protein-protein interactions for both of the aforementioned proteins and revealed that CD_CgUlp2 and CgSmt3 were primarily interacting hydrophobically at the interfacial regions (Figure 31A). Additionally, we have shown that CD_CgUlp2 ILE 165 (the first amino acid residue of the SUMO-interacting motif) interacts with CgSmt3 ARG 79, and GLU 102 and that CD_CgUlp2 M208, K209, T210, and K248 (the helices) interact hydrophobically with CgSmt3 ILE104 (the loop) (Figure 31B).

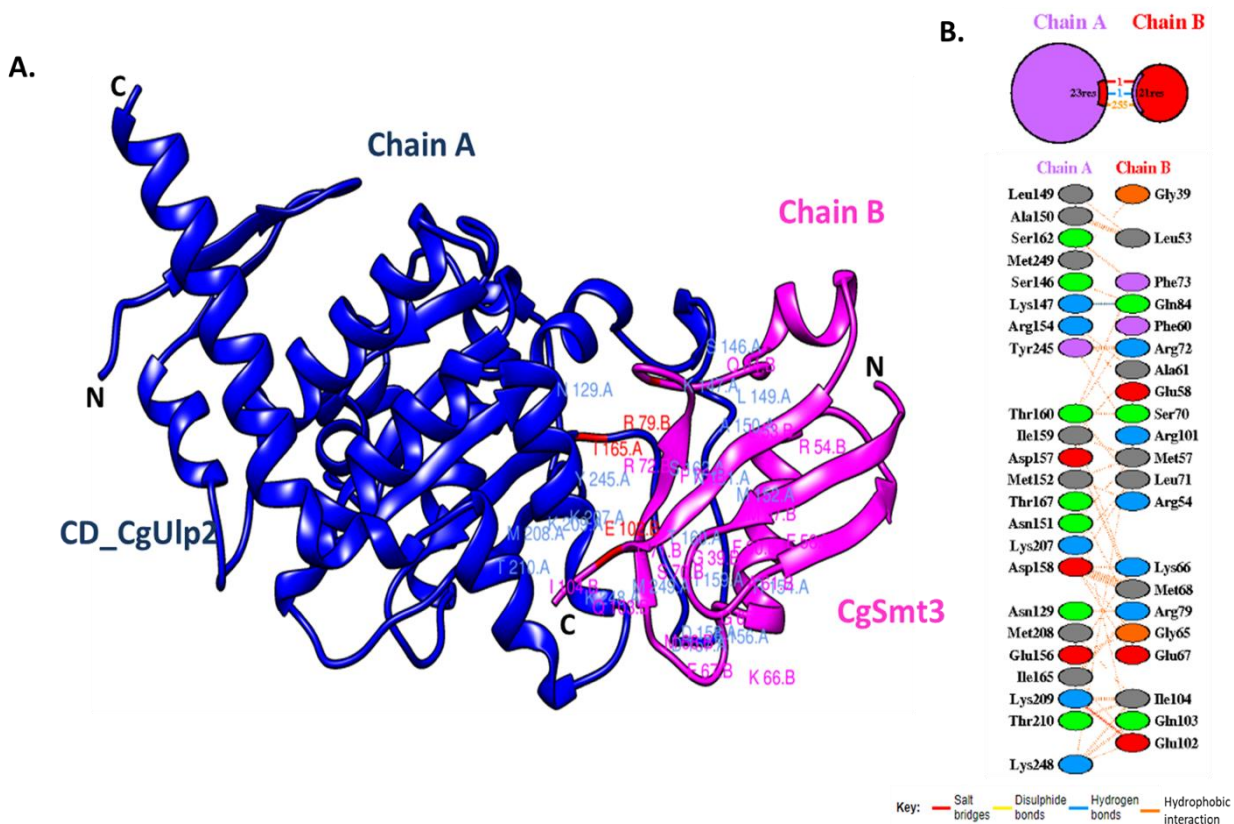


Figure 31. Interaction studies of SUMO protease CgUlp2 and SUMO protein. A. CD_CgUlp2 (Chain A) protein was docked with CgSmt3 (Chain B) protein by using ClusPro 2.0 to identify the interaction between these proteins. Chimera was used to generate the model. **B.** Diagrammatic illustration showed the amino acid residues of CD_CgUlp2 that interacted with the CgSmt3 protein.

To validate the interaction between both proteins, we used yeast two-hybrid (Y2H) studies to show that this interaction happens physically. Based on the fact that eukaryotic transcriptional activators have two domains—the DNA binding domain (DBD) and the activating domain (AD)—the yeast two-hybrid system was developed. In this test, two proteins of interest (X and Y) are fused to the DNA binding and activating domains of GAL4. Here, the "bait" was DBD- fused to domains of CgUlp2, and the "prey" was GAD-CgSmt3. A functional GAL4 transcription factor is produced when the two proteins physically bind, causing the reporter gene to be expressed under the control of the GAL4 promoter. In our study, we found that all three domains of the CgUlp2 protein interacted physically with the CgSmt3 protein (Figure 32A).

Further, we wanted to see if this interaction was direct through in vitro pull-down experiments. We cloned domains of the CgUlp2 protein and CgSmt3 protein in bacterial expression vectors and these proteins were expressed well (Figure 32B). We cotransformed *E. coli* cells for CTD_CgUlp2 and CgSmt3. We colysed the pellets from two cultures for CD_CgUlp2 and NTD_CgUlp2 pull-down experiments with CgSmt3. The cell lysates were then purified using Ni-NTA resins. When we eluted His-tagged protein (NTD_CgUlp2, CgSmt3) using imidazole then GST tagged proteins (CgSmt3, CD_CgUlp2, and CTD_CgUlp2) was also present in elution fraction which shows that CgUlp2 domains do interact with CgSmt3 directly. Western blot confirms the invitro pull-down results using α -GST and α -His antibodies (Figure 32C).

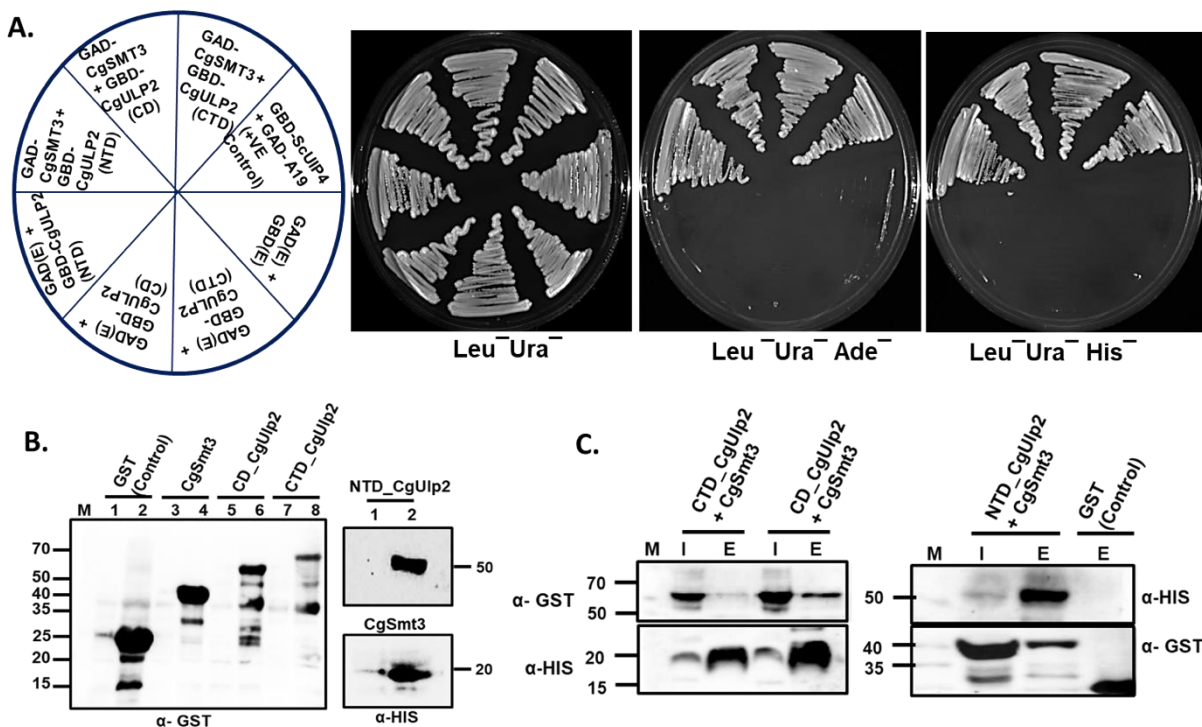


Figure 32. Interaction of domains of CgUlp2 protein with CgSmt3 protein. **A.** Domains of CgUlp2 protein were shown to have interaction with CgSmt3 protein physically by using a Yeast-two hybrid assay. **B.** Western blot analyses to show the expression of CgUlp2 domains and CgSmt3 protein constructs in BL21(DE3)-RIL bacterial expression strain using α -HIS and α -GST antibodies. Lane M: Protein marker (kDa), 1, 3, 5, and 7: Uninduced samples and 2, 4, 6, and 8: Induced samples. **C.** Western blot analyses showing the direct interaction of domains of CgUlp2 and CgSmt3 protein by invitro pull down using α -HIS and α -GST antibody. Lane M, I, and E: Protein marker, Input, and Eluate.

Further, we wanted to test the possibility that the interaction between CgUlp2 domains and CgSmt3 protein was due to the putative SUMO-interacting motif (SIM) present in the CgUlp2 protein. SIM motifs have been previously identified in the C-terminal domain of the Ulp2 protein in *Saccharomyces cerevisiae*¹³³. We predicted SIM motifs (amino acid residues in N-terminal- IICI, and IQIV; in catalytic domain- IITI, and in the C-terminal domain- IEII) in the CgUlp2 protein using the GPS-SUMO server (Figure 33A). Now, if the predicted SIM motifs in these domains are crucial for the interaction of CgUlp2 with SUMO protein, the interaction with SUMO would be compromised. For this, we successfully generated SIM mutated catalytic domain (I569K) and C-terminal domain (I712T) of CgUlp2 protein which was expressed well under IPTG induction. We performed an in vitro pull-down assay with either Ni-NTA or GST resin beads and found that the SIM mutant of both the domains did not interact with CgSmt3 anymore (Figure 33B-C). It lost the ability to bind to SUMO following the mutation, suggesting that the predicted SIM is essential for binding of CgUlp2 and CgSmt3 protein and also the putative SIMs were actual SIMs present in the Ulp2 protein of *C. glabrata*.

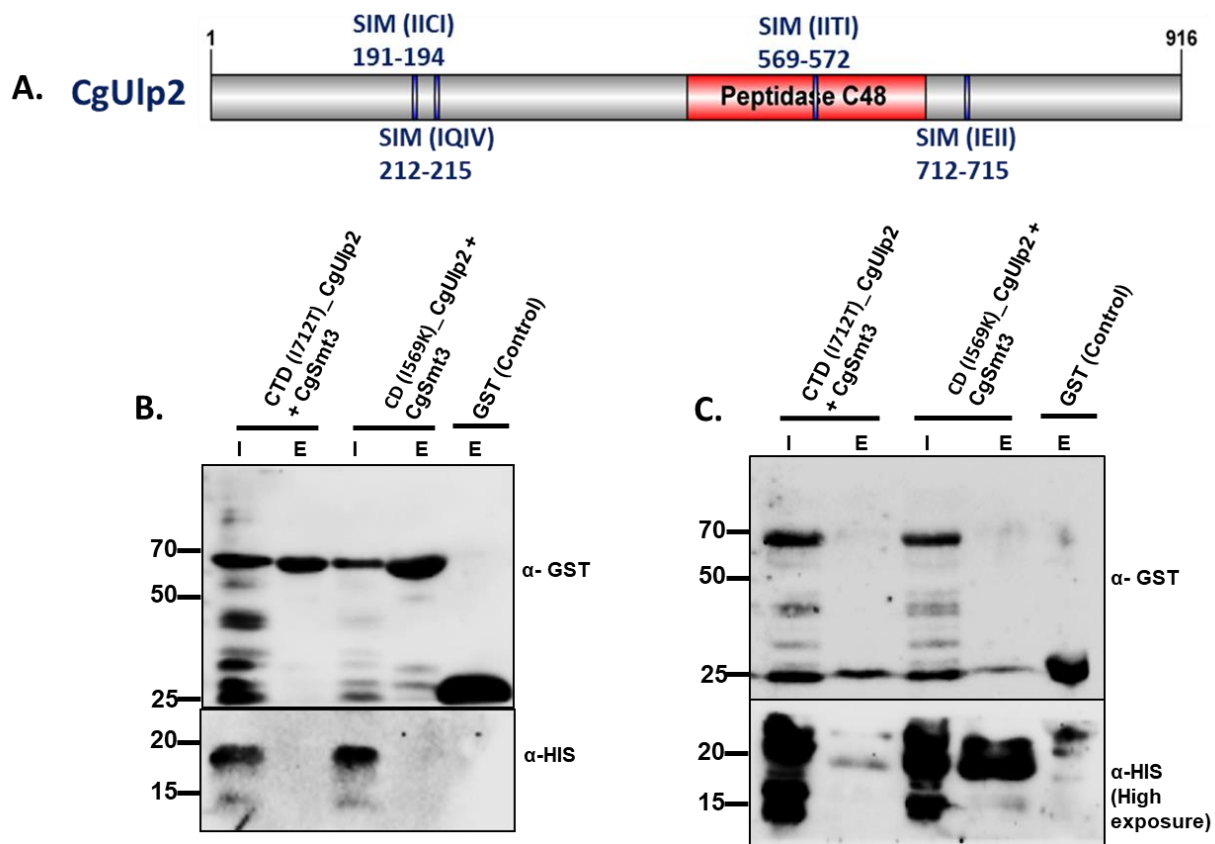


Figure 33. A. CgUlp2 interacted with CgSmt3 by SUMO-interacting motifs. Schematic representation of CgUlp2 protein showing putative SUMO-interacting motifs (SIMs) using GPS-SUMO server. **B-C.** Invitro pull-down was performed either with Glutathione (left side) or Ni-NTA resin beads (right side). Western blot was performed using the α -HIS and α -GST antibodies revealing that the SIM mutant of the CgUlp2 protein did not interact with the CgSmt3 protein. Lane M, I, and E: Protein marker, Input, and Eluate.

Functional characterization of SUMO-CgUlp2 interaction

1. Longer linear SUMO chains have a greater affinity towards the binding of the CgUlp2 protein.

As CgUlp2 targeted polySUMOylated substrates, we then checked the interaction between CgUlp2 protein (WT and SIM mutant) and linear SUMO chains of various lengths (1x-SUMO from *C. glabrata*, 2x-SUMO, and 6x-SUMO from *S. cerevisiae*) of *S. cerevisiae* by performing the binding assay. All the constructs were expressed and purified from bacteria. When the full length of CgUlp2 protein was exposed to a combination of different lengths of linear SUMO chains, we found that it selectively and tightly interacted with 6x-SUMO proteins but not with 2x-SUMO or 1x-SUMO (Figure 34B). This protein was weakly attached to 2x-SUMO while performing with each linear SUMO chain separately (Figure 34A). A similar finding was made with the catalytic domain of CgUlp2 protein (WT). However, this was not the case for the CD_CgUlp2 SIM mutant as it didn't bind to the linear SUMO chains (Figure 34C-D). This indicates that the CgUlp2 protein has a greater affinity to bind to larger linear SUMO chains.

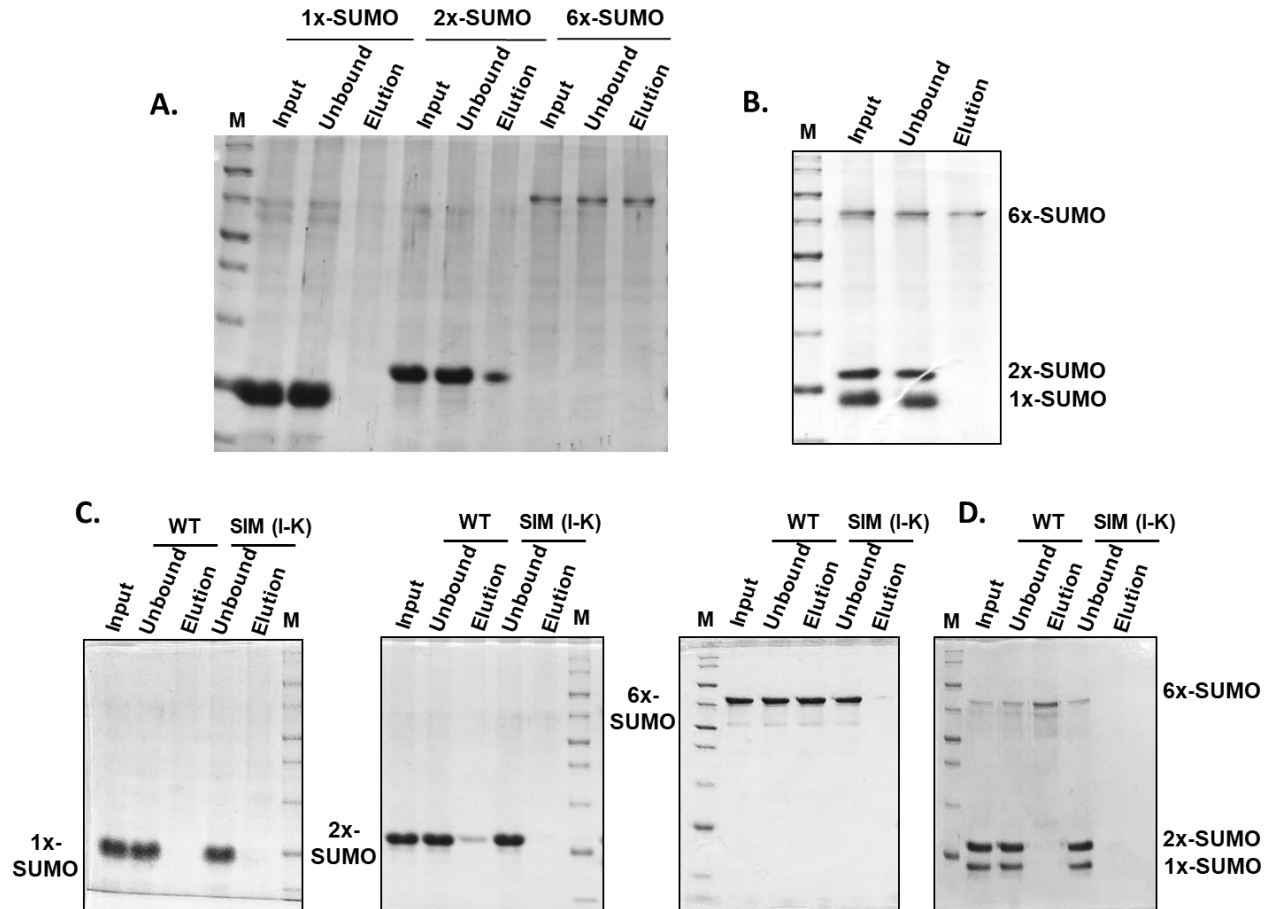


Figure 34. Longer linear SUMO chains are the preferred binding partners of the CgUlp2 protein. Pulldown assay is used to determine if the linear SUMO chains bind to **(A)** the full length of SUMO protease CgUlp2 individually or **(B)** in combination, **(C)** WT or SIM mutant of the catalytic domain of CgUlp2 protein individually or **(D)** in combination. **M**- protein marker

2. Enhanced SUMO protease activity induced by the SUMO interacting motif of the CgUlp2 protein

We investigated the effect of full-length and CD_CgUlp2 SIM in modulating Ulp2 SUMO protease activity in vitro to understand the function of CgUlp2 SIM. To do this, we expressed and purified both the full-length (WT) and CD_CgUlp2 (comprising amino acid residues 400-750) variants, including either the WT or SIM mutant, from bacterial sources. Additionally, recombinant 2x-SUMO and 6x-SUMO were purified from bacteria and employed in the assays (Figure 35A). Subsequently, 100 µg of each of these proteins (EDTA-free), whether it be a dimer or hexamer, was added to 50 µl of PBS containing 1 mM DTT and 1 µg of full-length (WT) and

CD_CgUlp2 (WT or SIM mutant) at room temperature. At specified time intervals (0, 15, 30, and 60 minutes) subsequent to the initial addition of each construct, 10 μ l aliquots of the reaction were taken for SDS-PAGE analysis. Each fraction was loaded onto a 12% SDS-PAGE gel and subjected to Coomassie staining for visualization. We found that CgUlp2 SUMO protease activity for all the linear SUMO substrates is drastically decreased by the SIM mutant. However, in the case of WT, the impact appeared more pronounced as the SUMO chain length increased. This suggest that CgUlp2 SIM enhances its affinity for longer SUMO chains, enabling it to preferentially cleave such substrates (Figure 35B-C). Thus, our results indicate a preference of the CgUlp2 protein for cleaving longer SUMO chains in vitro.

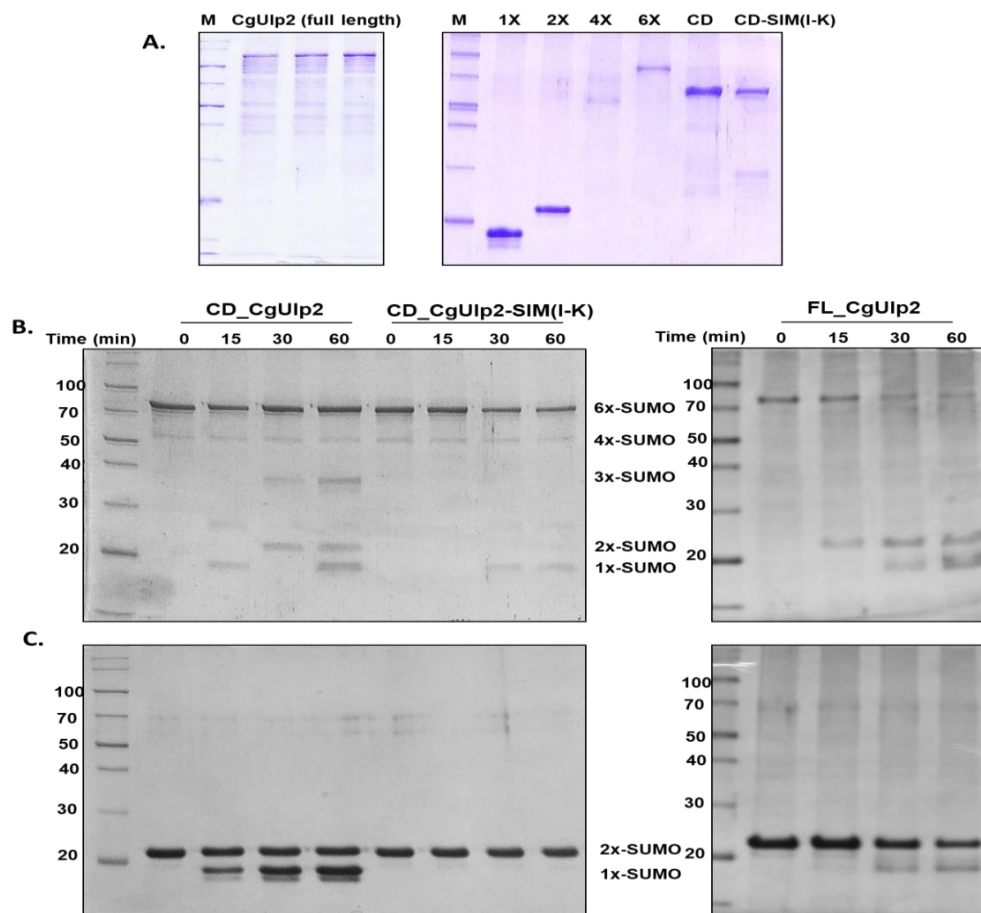


Figure 35. The SUMO protease activity of CgUlp2 targeting linear SUMO chains is encouraged by the CgUlp2 SIM. **A.** purified full-length (WT), CD (WT and SIM mutant) of CgUlp2 proteins stained with Coomassie. **B-C.** CgUlp2 enzyme cleavage of 2x, and 6x-SUMO (WT and SIM mutant).

Structure of the SUMO protease CgUlp2 full length with 6x-SUMO protein complex revealed by Negative stain electron microscopy (EM)

As we were getting microcrystals of the catalytic domain of CgUlp2 protein, we could not pursue the X-ray crystallography. In Figure 34, we have shown that the CgUlp2 protein binds tightly to 6x-SUMO and also this protein has 4 SUMO-interacting motifs. So, intending to solve full-length CgUlp2 protein with the 6x-SUMO complex structure to gain insight into interaction, we analyzed the structure of this complex using electron microscopy. Initially, we purified the CgUlp2●6xSUMO protein complex, confirming the presence of both proteins in the eluted fraction through 10% SDS-PAGE (Figure 36A). Subsequently, the purified protein complex at a concentration of 0.05 mg/mL was adsorbed on EM grids and stained with 2% uranyl acetate. Using approximately 4473 selected particles, single-particle analysis was conducted using CryoSPARC. Figure 36B shows the representative micrograph of purified CgUlp2●6xSUMO complex stained with 2% uranyl acetate. The 2D class averages obtained from this analysis revealed the “globular structures” of the complex (Figure 36C). These 2D classes were further utilized for ab initio 3D reconstruction and refinement, resulting in the generation of the density map (Figure 36D). We obtained the homology models of the CgUlp2 protein using the SWISS model and 6xSUMO using PHYRE and then built a complex model. This modeled complex was subsequently docked into the EM density map (Figure 36E), demonstrating an excellent fit within the density and adopting a compact ‘globular’ shape within it. Overall, the architecture of the CgUlp2●6xSUMO complex exhibited a compact globular structure.

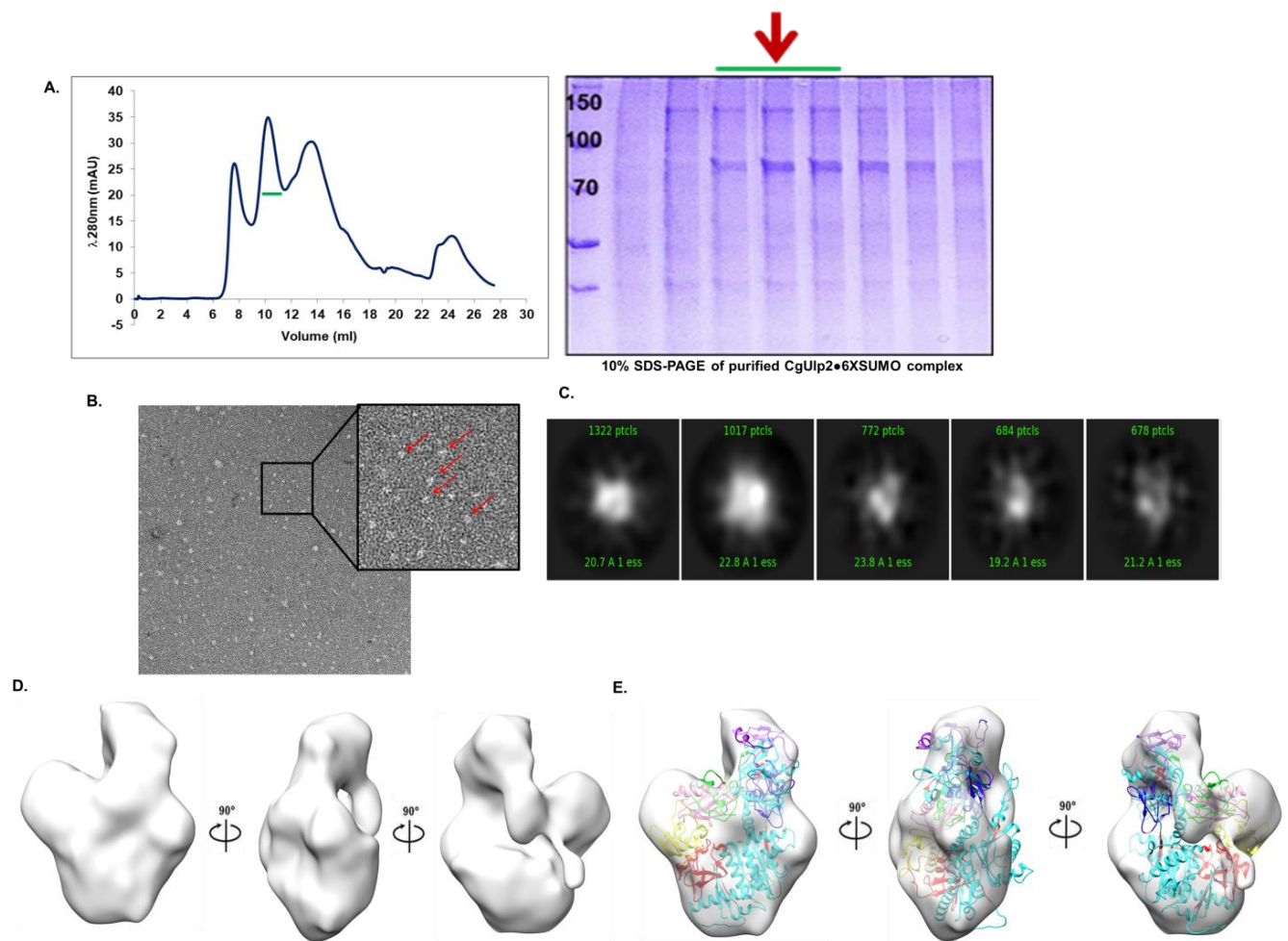


Figure 36. Negative staining EM analysis of CgUlp2•6xSUMO complex complex. **A.** Gel filtration profile of the CgUlp2•6xSUMO complex followed by 10% SDS-PAGE that showed the purified complex. The fraction of the protein complex indicated in the red arrow was used for the negative staining. **B.** A representative micrograph of purified CgUlp2•6xSUMO complex stained with 2% uranyl acetate. Representative particles used for the analysis are shown with the red arrow. **C.** Representative 2D class averages. **D.** 3D density map obtained for the CgUlp2•6xSUMO complex. **E.** The density map is fitted with modeled structures of the complex.

Pharmacophore-based virtual screening of CD_CgUlp2-CgSmt3 protein complex for small molecule inhibitors (ligands)

To identify inhibitors that will disrupt the interaction of CD_CgUlp2 and CgSmt3 protein, we have taken an approach of pharmacophore-based virtual screening of this complex. To do this, we used the CD_CgUlp2-CgSmt3 protein complex (shown in Figure 31A) to predict the active site residues of this complex.

Determination of active site on the protein

The active site with the highest druggability for -CD_CgUlp2_CgSmt3 was identified precisely at the docking interface of CD_CgUlp2 and CgSmt3 protein. The amino acid residues that were involved in the active site prediction were mentioned as follows- CD_CgUlp2 (Chain A): 45, 173, 214, 216, 221, 222, 224, 225, 228, 229, 231, 232, 233, 235, 236, 240, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 273 and CgSmt3 (Chain B): 33, 37, 39, 40, 41, 24, 43, 44, 45, 46 (Figure 37), with druggability score of 0.80.

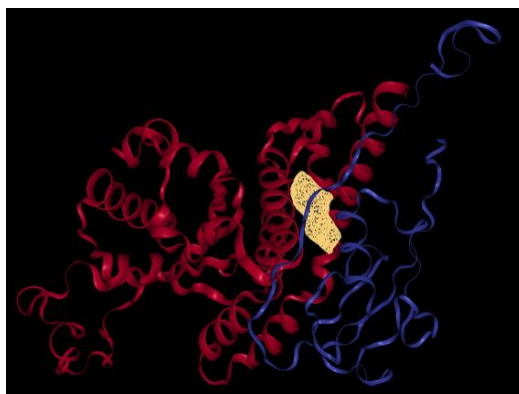


Figure 37. Active site of the CD_CgUlp2_CgSmt3 protein complex. CD_CgUlp2 protein is represented in red color and CgSmt3 protein is represented in blue color along with the docking site shown in the beige color electron cloud.

Virtual screening for ligands with pharmacophore map and binding energy calculations

The pharmacophore map for the CD_CgUlp2_CgSmt3 protein complex was derived using the ‘Phase’ module of Schrodinger suite ¹²⁰. 7 molecular features were determined for active site on CD_CgUlp2-CgSmt3, consisting of three aromatic rings (R25,R20 and R21), two sites with low

electron (N14 and N16) density and two sites with maximum electron density (D11 and D13) (Figure 38).

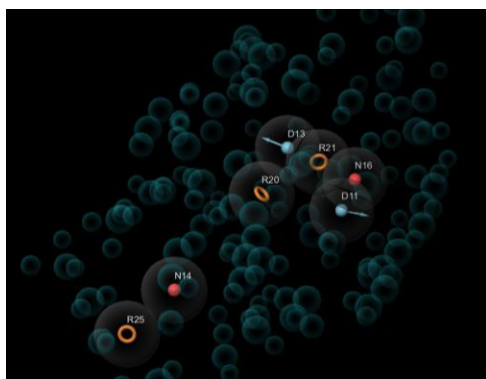


Figure 38. Pharmacophore map of CD_CgUlp2-CgSmt3 protein complex. Protein in the background shows electron density in blue bubbles, with pharmacophore features (R25, R20, R21, N14, N16, D11, and D13).

The Pharmit website was used to search for ligands using the coordinates of these pharmacophore features (listed in Table 9). It used open repositories from NCI, ChEMBL32, Molport, ChemDiv, ChemSpace, MCULE, PubChem, ZINC, and LabNetwork to filter ligands. From thousands of molecules, lead molecules were selected using the RMSD and MM/GBSA scores (Table 10). Apart from this, an independent screening from 2000 FDA drugs was done using a Phase module from Schrodinger, and the top 10 molecules that showed $MM/GBSA \leq 0.002$ were carried forward for molecular docking with protein.

Table 9. Coordinates (X, Y, and Z) of pharmacophores for the CD_CgUlp2-CgSmt3 protein complex.

Pharmacophore	X	Y	Z
R25	27.56	19.09	61.89
R21	14.67	27.75	61.12
R20	17.63	25.03	60.56
N14	24.04	20.73	61.74
N16	14.78	30.10	63.50
D11	16.35	29.14	63.98
D13	15.56	27.53	59.18

Molecular docking

Using the Flare suite of CressetUk software, the CD_CgUlp2-CgSmt3 protein complex was used for molecular docking with the ligands that were selected from the FDA database as well as the small ligand database. The top 20 hits were shortlisted based on docking scores ≤ -9.0 (Tables 10-11). Out of all these, the top 3 molecules: CHEMBL3936817 (small ligand) from the small ligand database and silymarin and honokiol from FDA screening were selected, as they showed good binding with protein complex.

Table 10. Top hits of lead molecules identified through virtual screening of the CD_CgUlp2-CgSmt3 protein from the small molecules database.

S.No	ID	Source	RMSD	Docking score	MMGBSA
1	CHEMBL1950571	CHEMBL	0.73	-9.891	0.0936113
2	CHEMBL4761817	CHEMBL	0.79	-9.358	-0.018152
3	CHEMBL344827	CHEMBL	0.63	-9.232	-0.02565
4	CHEMBL3936817	CHEMBL	0.621	-9.772	-0.002182
5	MCULE-3584742176	MCULE	0.70	-8.734	-0.000987
6	MCULE-8451610057	MCULE	0.85	-8.724	-0.016537
7	MCULE-1667318895	MCULE	0.76	-8.724	-0.003371
8	MCULE-1330778436	Zinc	0.72	-8.712	-0.0008812
9	ZINC8902579	Zinc	0.79	-8.494	-0.001586
10	CHEMBL7557647	CHEMBL	0.826	-9.156	-0.023766

Table 11. Top hits of lead molecules identified through virtual screening of the CD_CgUlp2-CgSmt3 protein from the FDA database.

S.no	Ligand molecule	Docking Score	MMGBSA
1	Tizanidine hydrochloride	-9.963	-0.018152
2	Silymarin	-9.579	-0.02565
3	Silibinin	-9.465	-0.002182
4	Chlorquinaldol	-9.452	-0.000987
5	Cisatracurium besylate	-9.401	-0.016537
6	Bromfenac sodium	-9.288	-0.003371
7	Broxyquinoline	-9.191	0.0008812
8	Guanabenz acetate	-9.075	-0.001586
9	Ritonavir	-9.051	-0.023766
10	Honokiol	-10.042	-0.0008817

We studied the protein-ligand interaction between CD_CgUlp2-CgSmt3 and small ligand (CHEMBL3936817), silymarin, and honokiol. 2D analysis of protein complex with small ligand was shown to have hydrogen bond, electrostatic and hydrophobic interaction with the complex (Figure 39A). With silymarin, it showed hydrogen bonds with Glu260, and Ser265, electrostatic interactions at Lys264, and Glu260, and hydrophobic interactions at Lys264 (Figure 39B). Similarly, with honokiol, it displayed hydrogen bonds with Asn263, Glu42, and Gly232, hydrophobic interactions with Pro214 and Lys264 (Figure 39C). Table 12 shows the details of the interaction of CD_CgUlp2-CgSmt3 with these 3 molecules. Furthermore, 3D analysis of the CD_CgUlp2-CgSmt3 protein complex with all the ligands revealed that the ligand placement was in between the binding site of CD_CgUlp2 (chain A- green) and CgSmt3 (chain B-blue) protein (Figure 39D-F).

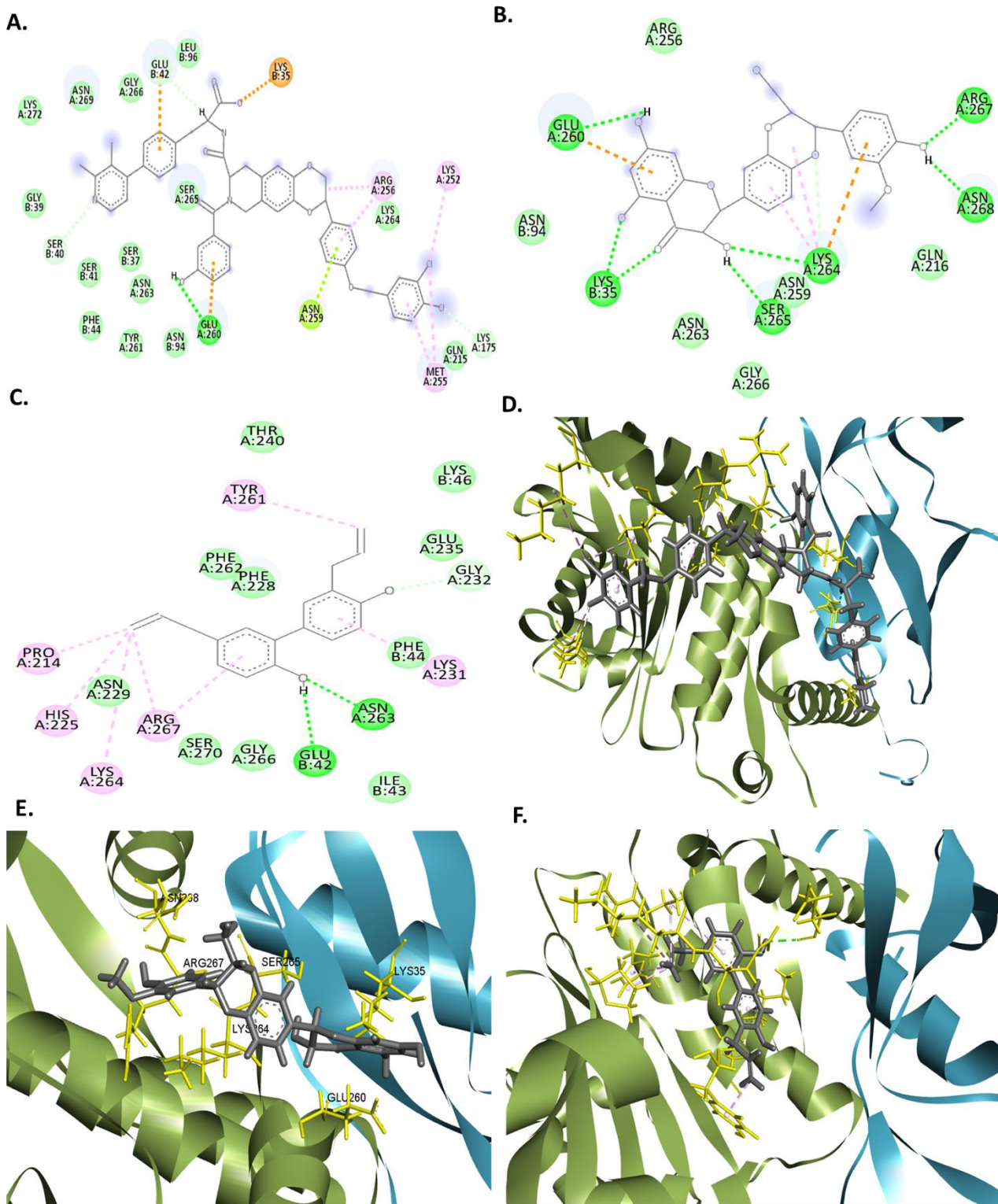


Figure 39. Molecular docking of a CD_CgUlp2-CgSmt3 protein complex with small ligand, silymarin, and honokiol. A-C. 2D images of CD-CgUlp2-CgSmt3 complex with small ligand,

silymarin, and honokiol. **D-F.** 3D images showing CD_CgUlp2 (green) and CgSmt3 (blue) complex with small ligand, silymarin, and honokiol, silymarin (grey). Active site interacting residues (yellow) of CD_CgUlp2- complex interacting with ligand.

Table 12. List of the amino acid residues of CD_CgUlp2 (chain A) and CgSmt3 (chain B) protein complex interacted with lead molecules along with the distance and type of interaction.

CD_CgUlp2-CgSmt3 protein complex with small ligand (CHEMBL3936817)			CD_CgUlp2-CgSmt3 protein complex with silymarin			CD_CgUlp2-CgSmt3 protein complex with honokiol		
Protein complex residues	Distance	Interaction type	Protein complex residues	Distance	Interaction type	Protein complex residues	Distance	Interaction type
B:LYS35	4.72361	Electrostatic	A:LYS264	4.89388	Electrostatic	A:ASN263	1.86	Hydrogen
A:GLU260	1.8272	Hydrogen	A:GLU260	3.32107	Electrostatic	B:GLU42	2.4	Hydrogen
A:LYS175	2.5415	Hydrogen	A:LYS264	2.58325	Hydrogen	A:GLY232	1.78	Hydrogen
B:SER40	2.98157	Hydrogen	A:ARG267	2.02626	Hydrogen	A:PRO214	3.345	Hydrophobic
A:GLU260	2.59799	Hydrogen	A:ARG267	1.99519	Hydrogen	A:LYS264	5	Hydrophobic
B:GLU42	3.7665	Electrostatic	B:LYS35	1.99174	Hydrogen	A:ARG267	4.41	Hydrophobic
A:ASN259	3.24863	Electrostatic	B:LYS35	2.07006	Hydrogen	A:HIS225	4.32	Hydrophobic
A:LYS175	4.06837	Hydrophobic	A:SER265	2.11711	Hydrogen	A:TYR261	3.71	Hydrophobic
A:LYS252	4.20676	Hydrophobic	A:ASN268	1.85512	Hydrogen	A:LYS231	4.413	Hydrophobic
A:MET255	4.8719	Hydrophobic	A:GLU260	3.06052	Hydrogen	A:ARG267	4.99	Hydrophobic
A:ARG256	4.02391	Hydrophobic	A:LYS264	3.07085	Hydrogen			
A:MET255	4.80113	Hydrophobic	A:LYS264	4.70904	Hydrophobic			
			A:LYS264	4.21527	Hydrophobic			
			A:LYS264	4.19883	Hydrophobic			

Molecular Dynamics (MD) Simulation of CD_CgUlp2-CgSmt3 protein complex with and without ligands

To determine the conformational changes and stability, MD simulation on the CD_CgUlp2-CgSmt3 protein complex was carried out for 10 nanoseconds. It took 1.4 ns to reach equilibrium and showed an average RMSD of 0.33 Å. This protein complex showed good stability throughout the time (Figure 40).

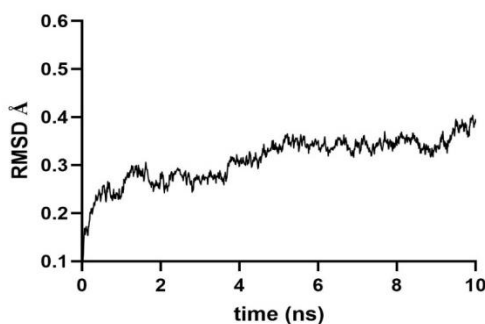


Figure 40. MD simulation of CD_CgUlp2-CgSmt3 protein complex

Further MD simulations were performed with protein complex and lead molecules for 10ns each. The generated trajectories were examined by calculating RMSD and thus, generated RMSD plots.

RMSD of the small ligand (CHEMBL3936817) shown in Figure 41A was more stable, but the average RMSD was 13.6 Å when compared to CD_CgUlp2-CgSmt3 protein complex. This indicates the protein complex was highly unstable with this ligand. In the case of silymarin, RMSD was very high with an average of 0.78 Å as compared to that of a protein complex (0.33 Å). The plot shows that there is no stability throughout the period of 10ns as there is a sharp disturbance with high peaks at 7ns and between 2ns to 4ns (Figure 41B). But with honokiol, RMSD was close to that of the protein complex, but it showed no uniformity and did not seem to equilibrate throughout the 10ns time period. This may indicate certain instability with the complex (Figure 41C). Though the average RMSD is 0.34 Å, which is close to the RMSD of protein, the inability of the complex to stabilize is a concern. Overall, this result demonstrated that the small ligand (CHEMBL3936817) was extremely unstable, followed by silymarin and

honokiol when compared to the CD_CgUlp2-CgSmt3 protein complex, suggesting that these molecules interfere with the interaction between CD_CgUlp2 and CgSmt3 protein.

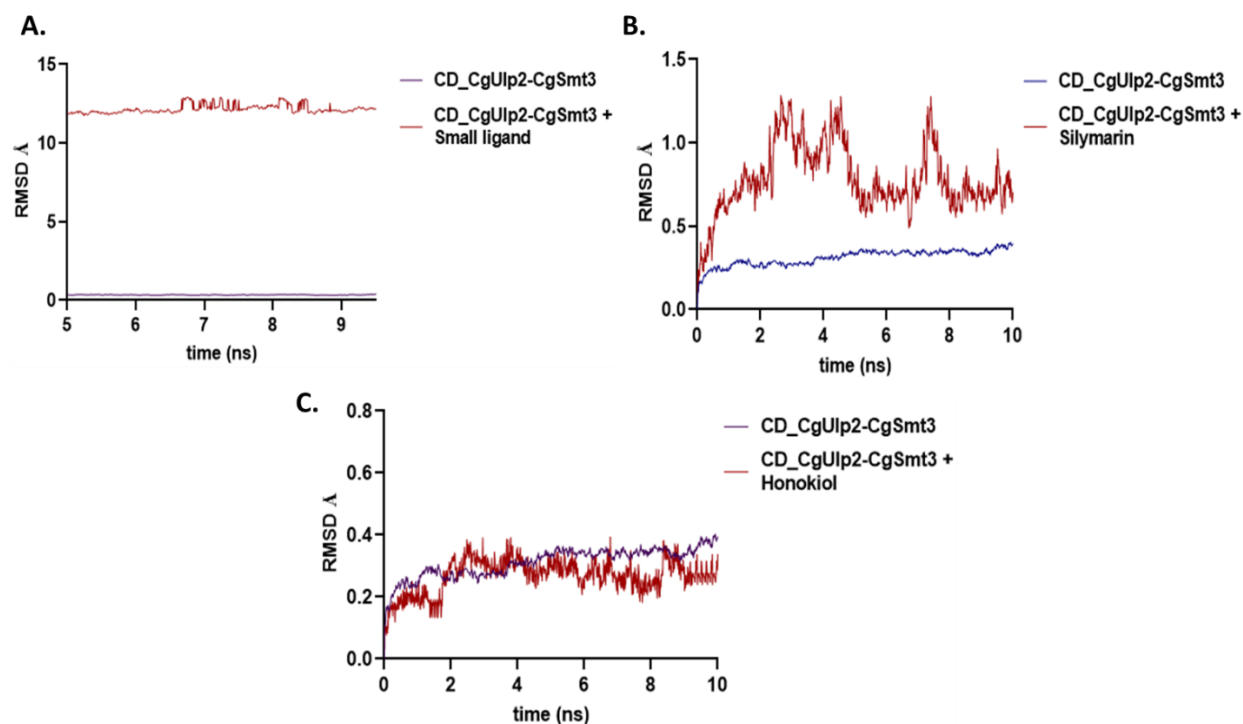


Figure 41. MD simulation of CD_CgUlp2-CgSmt3 protein complex with 3 lead molecules. A-C. RMSD plots of CD_CgUlp2-CgSmt3 (black) with small ligand (A), silymarin (B), and honokiol (C).

6.3 SUMMARY

We conclude this objective with a few key points that are mentioned below.

- The full length, N-terminal domain (NTD), and C-terminal domain (CTD) of CgUlp2 protein were found to be soluble aggregates as it has a large number of unstructured amino acid residues in both NTD and CTD of CgUlp2 protein.
- The catalytic domain of CgUlp2 is monomeric.
- Analysis of the far-UV CD spectrum of the native catalytic domain of CgUlp2 protein revealed the presence of both α -helices and β -sheet, with α -helices constituting approximately 58.3 % of the secondary structure.
- In comparing homology models, the catalytic domain of Ulp2 protein may serve as an antifungal target because it has the least similarities to human proteins.
- Through sequence alignment of ScUlp2, CgUlp2, and HsUlp2 protein, we identified His525, Asps-576, and Cys626 as the putative active site residues forming the catalytic triad. Additionally, mutating Cys626 to serine (C626S) in the full-length CgUlp2 protein resulted in catalytic inactivation.
- In vitro, pull-down analysis showed that CgUlp2 protein interacts directly with CgSmt3 protein through SUMO interacting motifs.

In addition, we have experimentally shown the physical interaction between the domains of the CgUlp2 protein and CgSmt3 using the yeast two-hybrid assay (Y2H). Furthermore, we have established a screening platform based on the Y2H system for identifying small molecules capable of inhibiting Ulp2. These inhibitors aim to disrupt the interaction between the SUMO protease CgUlp2 and the SUMO protein CgSmt3. Based on the pharmacophore-based virtual screening of CD_CgUlp2-CgSmt3 protein complex, 3 lead molecules (small ligand-CHEMBL3936817, silymarin, and honokiol) were identified that disrupt the interaction of CgUlp2 and CgSmt3 protein. Furthermore, we established that CgUlp2 interacts with 6xSUMO in vitro, and this forms a stable complex. So, we are currently attempting to decipher the structure of the CgUlp2•6xSUMO complex in order to gain a better understanding of the interaction between these proteins and to better define the target

for drug discovery. This will make it possible to design molecules that can interfere with the SUMO-deSUMOylase interaction.

CHAPTER 7 DISCUSSION

Fungal infections are a global health problem. Estimates say over 150 million severe cases of fungal infections occur worldwide, resulting in approximately 1.7 million deaths per year¹³⁴. A key reason for deaths is that many fungi are tolerant to very high levels of antifungals or, sometimes, entirely resistant to antifungals, leading to increased mortality levels. This led WHO to release a first-ever Fungal priority pathogens list (FFPL), highlighting the importance of fungal infections and antifungal resistance (ISBN 978-92-4-006024-1). *Candida glabrata* is listed as a high-priority pathogen due to its innate resistance to antifungals and as one of the key causative agents of candidiasis. Currently, available antifungals fall into three main categories: azoles, echinocandins, and polyenes. All these drugs target either the cell wall or cell membrane. Therefore our project started with the idea of exploring new targets for developing antifungals.

There is limited research on drugs that target post-translational modifications, but such drugs could be highly effective. So, our investigation focused on SUMOylation, a type of post-translational modification, in order to find new targets. SUMOylation of proteins serves as a crucial regulator in a variety of cellular activities, encompassing gene transcription, cell cycle control, DNA replication, and repair. SUMO protein, belonging to the family of ubiquitin-like modifiers, is present in all eukaryotic organisms, from yeast to humans. SUMO is covalently attached to other proteins, modifying their functions. Notably, SUMOylation is a reversible phenomenon, with deSUMOylating enzymes responsible for removing SUMO from target proteins. Apart from monoSUMOylation which alters protein properties, polySUMOylation, the sequential addition of SUMO molecules to target proteins, holds significance in maintaining protein homeostasis. These polySUMOylated proteins undergo ubiquitination facilitated by a group of ubiquitin ligases and are subsequently directed for proteasomal degradation, a process known as SUMO-targeted ubiquitin ligases (STUbLs). This method of controlling protein homeostasis (proteostasis) is key to several physiological processes including DNA repair, and cell cycle regulation.

Previous studies conducted in our lab have demonstrated the critical role of the SUMOylation machinery in *C. glabrata*. In particular, the *CgULP2* gene, which is a deSUMOylase with specificity to both mono and polySUMO, was shown to be particularly critical for pathogenicity and was speculated to have the potential to be a promising target for the development of drugs against *C. glabrata*^{90,116}.

In this thesis, we have explored the idea that Ulp2 contributed to pathogenicity via its capacity to deSUMOylate polySUMOylated proteins and thus protect proteins from being polyubiquitinated and degraded. This degradation of polySUMOylated protein is attributed to the STUbL pathway. So, in the first part of the work, we identified the STUbL pathway across the fungal kingdom, focusing on pathogenic fungi. We found that the pathway is conserved and all fungi have at least one STUbL. Focusing on *C. glabrata*, we identified that like *S. cerevisiae*, *C. glabrata* has three STUbLs, namely, Slx5, Slx8, and Uls1. In order to test the significance of these STUbLs in *C. glabrata* physiology and pathology, we generated deletions of these, and deletions in combination with the Ulp2. Our studies show that while loss of these STUbLs leads to reduced capacity to infect and survive in macrophages, proliferate in RPMI, or form biofilms, they are not particularly affected by stress conditions in YPD. This suggests that the STUbL pathway might be particularly important in the host or in conditions mimicking the host environment.

Our genetic and biochemical investigations of these mutants revealed that loss of CgUlp2 indeed leads to loss of proteins, potentially activating the STUbL pathway. Interestingly, we found that *Cgslx8* loss in the background of *Cgulp2* loss was particularly detrimental and the double delete strains were very sick and sensitive to several forms of stress. They also had elevated protein degradation. On the contrary, the *Cgulp2Cguls1* double deletes could rescue some of the stress sensitivities of the *Cgulp2* loss suggesting that Uls1 could probably be the main STUbL for the Ulp2 targets. This provided us with a genetic handle to investigate the targets of STUbLs that were involved in pathogenicity.

We identified downstream pathways that were affected in the mutants of STUbLs and CgUlp2 by performing quantitative mass spectrometry. The analysis unveiled a significant impact on the ubiquitin-proteasome pathway, ribosomal biogenesis, purine nucleotide biosynthesis pathway, and mitochondrial function in the *Cgulp2ΔCgslx8Δ* double mutant. This effect was more

pronounced than in the *Cgulp2Δ* single mutant. The combination of reduced protein synthesis, enhanced proteasomal pathway, and potentially upregulated CgUls1 collectively contributes to the increased protein loss and slow growth of *Cgulp2ΔCgslx8Δ* cells at 30°C. Conversely, the *Cgulp2ΔCguls1Δ* double mutant exhibited opposite trends, with a decreased level in the ubiquitin-proteasome pathway and an increased level or up to the level of wild-type in the purine nucleotide biosynthesis pathway and mitochondrial function. These changes correlated with the restored growth and accumulation of polySUMOylated protein in the *Cgulp2ΔCguls1Δ* mutant. Our study underscores the importance of STUbLs as essential regulators of protein homeostasis. Along with CgULP2, they play a pivotal role in the pathophysiology of *C. glabrata*. Further work on the pathways that are particularly affected would provide a better understanding of the processes involved in establishing a successful infection.

We worked towards establishing methodologies to identify SUMOylated proteins in order to establish the causal relationship and also identify the precise targets to get a better understanding of the process of pathogenicity. We found it quite challenging to establish a method to enrich/isolate SUMOylated proteins. Despite having had considerable success in identifying SUMOylated proteins in *S. cerevisiae*, we found those methods were not efficient in *C. glabrata*. Following multiple efforts, we successfully developed a technique for identifying targets of SUMOylation that play a role in contributing the pathogenicity of *C. glabrata*, employing tandem SUMO-interacting motifs (SIMs). This method can now be used to determine the SUMOylation targets influencing infection in this organism.

This work was initiated with the intention of identifying new targets for antifungals. We zeroed in on CgUlp2 as a potential strong candidate as loss of *Cgulp2* compromises the pathogenicity properties. Also, the protein sequence is least conserved with respect to humans among the SUMO pathway components. We aimed to purify the protein and derive atomic resolution structures to design drugs that can target this pathway by rational design. Despite attempts to purify large quantities of CgUlp2 for high-resolution structural studies, soluble purified CgUlp2 protein proved elusive due to numerous unstructured regions in N- and C-terminal domains. Crystallization efforts focused on the catalytic domain of the CgUlp2 protein were unsuccessful for X-ray diffraction after several attempts. Meanwhile, we characterized the protein and performed a homology model of the catalytic domain of the CgUlp2 protein. This domain was

identified as monomeric, displaying both α -helix and β -sheet secondary structures, with α -helix predominance. Furthermore, we identified His525, Asp576, and Cys626 as the conserved catalytic triad residues in the CgUlp2 protein.

Since Ulp2 activity would be dependent on recognizing polySUMOylated protein, we investigated the potential for Ulp2 and SUMO to interact. Our findings highlight a direct interaction between CgUlp2 and CgSmt3 (SUMO) proteins, facilitated by SUMO interacting motifs. Further, our results demonstrate the ability of CgUlp2 to interact with polySUMOylated (6xSUMO) substrates, forming a stable complex. To gain insights into this interaction, we aimed to elucidate the structure of the full-length CgUlp2 with the 6xSUMO. We conducted negative staining of the CgUlp2-6xSUMO protein complex, and subsequent data processing using CryoSPARC unveiled its overall architecture as a compact globular structure. In future, elucidation of this structure will allow the designing of drug molecules that target fungal-specific features of this structure thus avoiding toxicity to humans. Since the structure of this complex has not been established in any system, it will also provide valuable information on the mechanistic aspects of this interaction.

In parallel, using computational approaches including homology modeling of the catalytic domain of CgUlp2 and CgSmt3 proteins we conducted molecular docking studies to understand the interaction between these proteins. Our analysis revealed that the interaction between the two proteins was predominantly through hydrophobic interactions at the interfacial regions. Subsequently, we proceeded with the pharmacophore-based virtual screening of the CD_CgUlp2-CgSmt3 protein complex to identify lead molecules capable of interfering with their interaction. Through this virtual screening, we successfully identified three promising lead molecules: small ligand-CHEMBL3936817, silymarin, and honokiol. Our next step involves conducting preliminary assays to evaluate the efficacy of these molecules in disrupting the interaction between CD_CgUlp2 and CgSmt3 proteins, thereby testing their potential as novel drugs against fungal infections.

FUTURE PROSPECTS

Our ongoing efforts involve elucidating the structure of the CgUlp2•6xSUMO complex using cryo-electron microscopy. A comprehensive understanding of this complex structure will enable the development of compounds capable of disrupting the SUMO-deSUMOylase interaction. Once the structure of this complex is determined, a comparative analysis with the human Ulp2 will be conducted to identify differences, facilitating effective drug targets. Notably, STUbL mutants impede pathogen replication within macrophages, reducing survivability, indicating their potential as targets for designing antifungal drugs.

The mass spectrometric analysis has provided us with multiple pathways that are particularly affected in these mutants. This data can be used to study the role of these pathways in pathogenesis, providing us with a molecular understanding of the mechanisms involved in establishing a successful infection. The method established for identifying SUMOylated proteins can now be used for specifically interrogating which proteins in the pathways identified are direct targets of SUMO-mediated protein homeostasis. Additionally, our identification of downstream pathways for both CgUlp2 and STUbLs opens avenues for developing combinatorial drugs, enhancing their effectiveness as targets. In sum, the work presented in this thesis has opened several avenues to better understand the pathogenesis mechanisms of *Candida glabrata* with a view to develop much-needed new antifungals.

APPENDIX

9.1 LIST OF PLASMIDS USED IN THE STUDY

Plasmid no.	Description of the plasmid	Source
CKM 225	pGEX-4T1, Bacterial expression vector having GST tag	Lab collection
CKM 289	pGBDU-C1, Yeast two-hybrid bait vector	Lab collection
CKM 292	pGAD-C1, Yeast two-hybrid prey vector	Lab collection
CKM 294	pBS, pBluescript vector for blunt-end cloning (blue-white screening)	Lab collection
CKM 364	pGRB2.2, a CEN-ARS plasmid of <i>C. glabrata</i> carrying <i>S. cerevisiae</i> URA3 as a selection marker. MCS sites are flanked by <i>S. cerevisiae</i> PGK1 promoter at one end and by 3' UTR of HIS3 at the other end.	Dr. Rupinder Kaur (CDFD)
CKM 379	<i>CgSMT3</i> cloned in pGRB2.2 as BamH1/Xho1 fragment	Lab collection
CKM 382	<i>CgULP2</i> cloned in pGRB2.2 as BamH1/Sal1 fragment	Lab collection
CKM 391	<i>CgULP2</i> cloned in pBEVY-L as BamH1/Sal1 fragment	Lab collection
CKM 402	pCN-PDC1, a high expression promoter with nat1 gene	Dr. Rupinder Kaur (CDFD)
CKM 405	6XHIS3XFLAG tagged <i>CgSMT3</i> at the N-terminus of pCN-PDC1vector (6XHIS3xFLAG in Xba1/BamH1, <i>CgSMT3</i> in BamH1/Xho1)	Lab collection
CKM 431	6XHIS3XFLAG tagged <i>CgULP2</i> in pGRB2.2 (6XHIS3xFLAG in Xba1/BamH1, <i>CgULP2</i> in BamH1/Sal1 site)	Lab collection
CKM 468	6XHIS3XFLAG tagged <i>CgSMT3</i> in pGRB2.2 (6XHIS3xFLAG in Xba1/BamH1, <i>CgSMT3</i> in BamH1/Xho1 site)	Lab collection
CKM 476	3XFLAG tagged <i>CgSMT3</i> in pGRB2.2 (3XFLAG in Xba1/BamH1, <i>CgSMT3</i> in BamH1/Xho1 site)	Lab collection
CKM 661	Full length <i>CgULP2</i> subcloned in BamH1/Sal1 site of pET28a from CKM 391	This study
CKM 686	NAT cassette cloned in pCR2.1 plasmid	Dr. Rupinder Kaur (CDFD)

CKM 687	<i>FLP1</i> , <i>FLP1</i> containing plasmid under <i>EPA1</i> promoter to excise NAT cassette	Dr. Rupinder Kaur (CDFD)
CKM 689	C-terminal domain (CTD) of <i>CgULP2</i> cloned in EcoRV digested pBS vector	This study
CKM 690	Catalytic domain (CD) of <i>CgULP2</i> cloned in EcoRV digested pBS vector	This study
CKM 691	<i>CTD_CgULP2</i> subcloned in BamH1/Sal1 site of pGEX4T1 from CKM 689	This study
CKM 692	<i>CD_CgULP2</i> subcloned in BamH1/Sal1 site of pGEX4T1 from CKM 690	This study
CKM 693	N-terminal domain (NTD) of <i>CgULP2</i> cloned in pET28a as BamH1/Sal1 fragment	This study
CKM 694	<i>CD_CgULP2</i> subcloned in BamH1/Sal1 site of pET28a from CKM 690	This study
CKM 695	<i>CTD_CgULP2</i> subcloned in BamH1/Sal1 site of pET28a from CKM 689	This study
CKM 717	<i>CgSLX5</i> (1.5 kb) cloned in EcoRV digested pBS vector	This study
CKM 718	<i>CgSLX8</i> (633 bps) cloned in EcoRV digested pBS vector	This study
CKM 719	<i>CgSLX5</i> subcloned in BamHI/XhoI site of pGRB2.2 from CKM 717	This study
CKM 720	<i>CgSLX8</i> subcloned in BamHI/XhoI site of pGRB2.2 from CKM 718	This study
CKM 721	<i>CgSMT3</i> cloned in pGAD-C1 as BamH1/Pst1 fragment	This study
CKM 733	<i>CgULS1</i> (4.2 kb) cloned in EcoRV digested pBS vector	This study
CKM 737	<i>CgSMT3</i> subcloned in BamH1/Xho1 site of pET28a from CKM 379	This study
CKM 738	<i>CgULP2</i> (C626S), Cysteine residue was replaced with serine at the position of 626 in the full length of <i>CgULP2</i> that was cloned in pGRB2.2	This study
CKM 741	<i>CgULS1</i> subcloned in BamHI/XhoI site of pGRB2.2 from	This study

	CKM 733	
CKM 742	<i>CgSMT3</i> subcloned in BamH1/Xho1 site of pGEX4T1 from CKM 379	This study
CKM 743	SIM (72 bps)-pET28a, 72 bps of SIM motif directly cloned in pET28a with Nde1/BamH1 sites	This study
CKM 744	SIM (154 bps)-pET28a, 154 bps of SIM motif cloned in BamH1/Sac1 site of pET28a using SIM (72 bps) as template	This study
CKM 745	SIM (308 bps)-pET28a, 308 bps of SIM motif cloned in Sac1/HindIII site of pET28a using SIM (154 bps) as a template	This study
CKM 754	<i>CD_CgULP2(I569K)</i> -pGEX4T1, Isoleucine was replaced with lysine at the position of 569 in the catalytic domain of <i>CgULP2</i> that was cloned in pGEX4T1	This study
CKM 755	<i>CTD_CgULP2(I712T)</i> -pGEX4T1, Isoleucine was replaced with threonine at the position of 712 in the C-terminal domain of <i>CgULP2</i> that was cloned in pGEX4T1	This study
CKM 758	6XHIS3XFLAG tagged <i>CgULS1</i> under its native promoter (500 bps) in pGRB2.2	This study
CKM 793	<i>CgADE5,7</i> cloned in EcoRV digested pBS vector	This study
CKM 794	3XFLAG- <i>CgADE5,7</i> - pGRB2.2, Replace <i>CgSMT3</i> from CKM 476 and cloned <i>CgADE5,7</i> with BamHI/XhoI sites in frame with 3XFLAG in pGRB2.2 vector	This study
CKM 726	<i>CD_CgULP2</i> cloned in BamH1/Sal1 site of pGBD-C1	This study
CKM 728	<i>CTD_CgULP2</i> cloned in BamH1/Sal1 site of pGBD-C1	This study
CKM 736	<i>NTD_CgULP2</i> cloned in BamH1/Sal1 site of pGBD-C1	This study
CKM 806	pET28a, Bacterial expression vector having His tag	Lab collection

9.2 LIST OF PRIMERS USED IN THE STUDY

Primer sequences	Description
For generation of deletion strains	
TATCCTCTCTGCCTGCAATG	<i>CgSLX5</i> 5'UTR Forward
gcgtcgacctgcagcgtacgTCTGTCTCCTCTGGGGCTAA	<i>CgSLX5</i> 5'UTR Reverse
cgacgggtgtcgggtctcgtagGGGAGTGTCCACGACAACA	<i>CgSLX5</i> 3'UTR Forward
AGGCACATTCCACAATCTCC	<i>CgSLX5</i> 3'UTR Reverse
CGAACAAGAGCGATGACAGA	<i>CgSLX5</i> Internal check Forward
CCCGATTCTTAAGTCAACTCC	<i>CgSLX5</i> Internal check Reverse
GATCGGACATCCCGTCTTTA	<i>CgSLX5</i> 5' Integration check Forward
GGATCCATGCATAGCGAAAC	<i>CgSLX5</i> 3' Integration check Reverse
TCCTGACAAGAGCTGGACCT	<i>CgSLX8</i> 5'UTR Forward
gcgtcgacctgcagcgtacg CCACCTCGCACACATAATTG	<i>CgSLX8</i> 5'UTR Reverse
cgacgggtgtcgggtctcgtagTGTCCGTTTTTTCAGGGATTC	<i>CgSLX8</i> 3'UTR Forward
ATGTGTGGGAAAGGGACAGA	<i>CgSLX8</i> 3'UTR Reverse
AGGTCTGGTGGAAACCAAGTG	<i>CgSLX8</i> 5' Integration check Forward
GGCACAAGAAGGGCATTTT	<i>CgSLX8</i> 3' Integration check Reverse
AAGCTGGACAACATTGATTGC	<i>CgULS1</i> 5'UTR Forward
gcgtcgacctgcagcgtacgGCAGGGTCCTATAGTGACAACC	<i>CgULS1</i> 5'UTR Reverse
cgacgggtgtcgggtctcgtagTGAGATTCATGAAAGCATTGTT	<i>CgULS1</i> 3'UTR Forward
CATTTCAAAGAATTTTGGATGC	<i>CgULS1</i> 3'UTR Reverse
TTGACGGTGTTCCTATTTTGG	<i>CgULS1</i> Internal check Forward
TTCCTTTTGGACCGCTTCTA	<i>CgULS1</i> Internal check

	Reverse
AACCAATGGAAAGCAGATGG	<i>CgULS1</i> 5' Integration check Forward
TCCTTGTGCACCACTAGAAGG	<i>CgULS1</i> 3' Integration check Reverse
For amplification of <i>nat1</i> cassette for <i>C. glabrata</i> gene disruption	
CGTACGCTGCAGGTCGACGCcttccgctgctaggcgc	5' NAT half Forward
TCTGTTCCAACCAGAATAAG	5' NAT half Reverse
GTCTACTACTTTGGATGATACTGC	3' NAT half Forward
ctacgagaccgacaccgtcgGGCCGCTGACGAAGT	3' NAT half Reverse
TGCGCACGTCAAGACTGTCAAGG	5' Integration check Forward
TGTGAATGCTGGTCGCTATACTGC	3' Integration check Forward
For gene cloning	
AATGGATCCATGAAACGGGATGGT	<i>CgSLX5</i> Forward
GCGCTCGAGTTAGAAATATACTTCTCTCAT	<i>CgSLX5</i> Reverse
GCCGGATCCATGGCTAAATCTGAG	<i>CgSLX8</i> Forward
GAGCTCGAGTCAGGTCTTCTTCTG	<i>CgSLX8</i> Reverse
GCGGGATCCATGCCAAAAGTTACA	<i>CgULS1</i> Forward
GCGCTCGAGTCATAATGAATTTAGTCCG	<i>CgULS1</i> Reverse
GGCGAGCTCGTTAATCACAAGTCAT	<i>CgULS1</i> Promoter Forward
AATTCTAGATTGCCATCTGCGATGG	<i>CgULS1</i> Promoter Reverse
GGCGGATCCATGTTGAACATTCTAG	<i>CgADE5,7</i> Gene Forward
GGA CT CGAGTTAGTATAGGTTGCTGG	<i>CgADE5,7</i> Gene Reverse
TAGTGGATCCATGTCTGACACTAACG	<i>CgSMT3</i> Forward
TAGTCTCGAGCTAGTACGCACCACCG	<i>CgSMT3</i> Reverse
GGATCCAATAGTATGTCGCGGAG	<i>CgULP2</i> (Full length) Forward
GTCGACCTAATGAGTATCGATATTTTC	<i>CgULP2</i> (Full length)

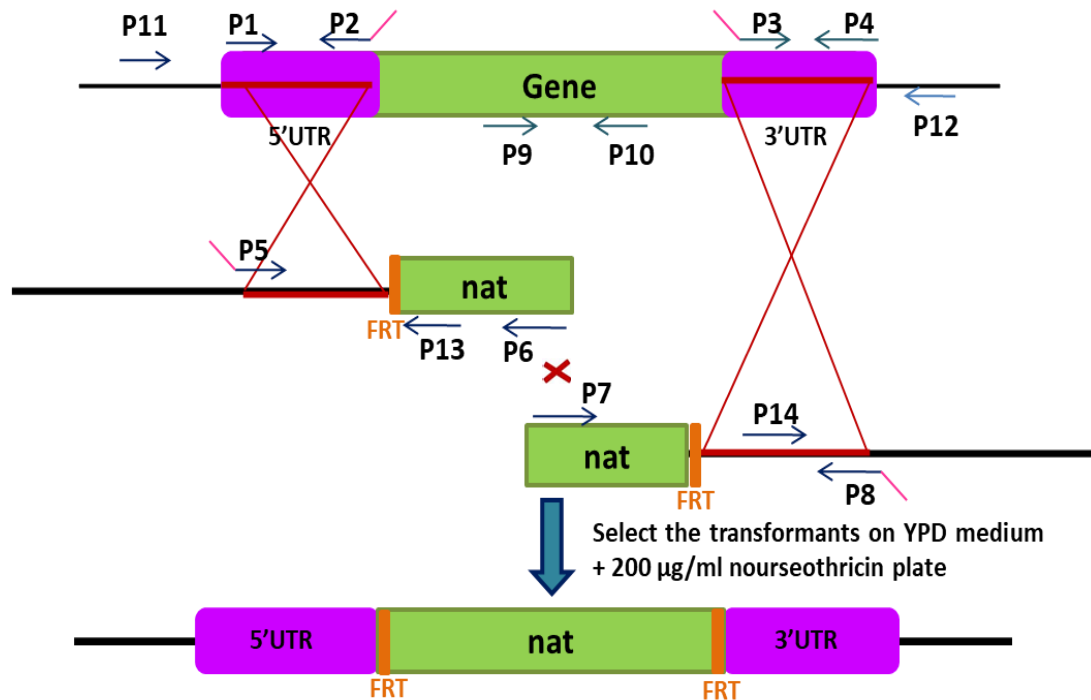
	Reverse
AATGGATCCATGTCGCGGAGG	<i>CgULP2</i> (N-terminal domain) Forward
AATGTCGACCATCCTCTTTCCGCA	<i>CgULP2</i> (N-terminal domain) Reverse
AGTGGATCCATGGAGGACAAAAGC	<i>CgULP2</i> (Catalytic domain) Forward
AGTGTGACAATGCTTGTCCATTGG	<i>CgULP2</i> (Catalytic domain) Reverse
AATGGATCCATGACGCATAGCAGAGAA	<i>CgULP2</i> (C-terminal domain) Forward
CGGGTCGACCTAATGAGTATCGATATTTTC	<i>CgULP2</i> (C-terminal domain) Reverse
For cloning of tandem SUMO-Interacting Motifs (SIM)	
CGGCATATGATGGTAGTGATCGTAGACGG	SIM 1, 2 Forward
ATAGGATCCCCACCGCCTCCTCC	SIM 1, 2 Reverse
CGGGGATCCGTAGTGATCGTAGACGG	SIM 3, 4 Forward
ATAGAGCTCCCCACCGCCTCCTCC	SIM 3, 4 Reverse
CGGGAGCTCGTAGTGATCGTAGACGG	SIM 5-8 Forward
ATAAAGCTTCTACCCACCGCCTCCTCC	SIM 5-8 Reverse
For qRT-PCR	
AAGGCGGTATAATGCCTGTG	<i>CgULP2</i> Forward
CTCACCTTCACCACGCTGTA	<i>CgULP2</i> Reverse
GTGGTGGACGAGGAGGATAA	<i>CgSLX5</i> Forward
GCACTAAATTCCCACCCAGA	<i>CgSLX5</i> Reverse
GACACCCGAGACCATCAACT	<i>CgSLX8</i> Forward
GAACAAGCATTTCGCAACAGA	<i>CgSLX8</i> Reverse
GGCAAATGAGCTCAAAAAGC	<i>CgULS1</i> Forward
CTTTAGCGGCCTGTGTCTTC	<i>CgULS1</i> Reverse
GTAACATCGTTATGTCCGGTGGTAC	<i>CgACT1</i> Forward
CCAAGATAGAACCACCAATCCAGAC	<i>CgACT1</i> Reverse

For Site-directed mutagenesis	
CAGGCAAACCTTTAGTGATTCTGGTGTCCATGTGATTTTC	CgULP2(C626S) Forward
GAAAATCACATGGACACCAGAATCACTAAAGTTTGCCTG	CgULP2(C626S) Reverse
CAGTTAGTCCACCAAAAATTACGATACTCACGTTTGATTC	CD (I569K) Forward
GAATCAAACGTGAGTATCGTAATTTTTGGTGGACTAACTG	CD (I569K) Reverse
GAAGATGAGGATACTGAAATAATTGAAGATTTATAT	CTD (I712T) Forward
ATATAAATCTTCAATTATTTTCAGTATCCTCATCTTC	CTD (I712T) Reverse

9.3 LIST OF STRAINS USED IN THE STUDY

Yeast strain	Genotype	Reference
YRK 19	<i>ura3Δ::Tn903 G418^R</i> (WT Ura- strain)	Dr. Rupinder Kaur (CDFD)
YRK 971	<i>YRK 19 except Cgulf2Δ::nat1</i>	Dr. Rupinder Kaur (CDFD)
KRC 6	<i>Smt3::hyg/CKM405</i>	Lab collection
KRC 30	<i>YRK 19 except Cgslx8Δ::nat1</i>	This study
KRC 33	<i>YRK 19 except Cguls1Δ::nat1</i>	This study
KRC 36	<i>YRK 19 except Cgulf2Δ::nat1/CKM 687</i>	This study
KRC 42	<i>YRK 19 except Cgulf2ΔCguls1Δ::nat1</i>	This study
KRC 45	<i>YRK 19 except Cgslx5Δ::nat1</i>	This study
KRC 46	<i>YRK 19 except Cgslx5ΔCguls1Δ::nat1</i>	This study
KRC 47	<i>YRK 19 except Cgslx5ΔCgslx8Δ::nat1</i>	This study
KRC 50	<i>YRK 19 except Cgslx5Δ::nat1/CKM 687</i>	This study
KRC 53	<i>YRK 19 except Cgulf2ΔCgslx8Δ::nat1</i>	This study

9.4 SCHEMATIC REPRESENTATION OF SINGLE DELETION STRAINS GENERATED FOR THIS STUDY



Full-length single deletion knockout of STUbLs (*CgSLX5*, *CgSLX8*, and *CgULS1*) was carried out by employing a homologous recombination-based strategy using a cassette encoding the nourseothricin acetyltransferase (*NAT1*) gene.

The primers used for amplifying the deletion cassette using *NAT* as the template and for screening the deletion strains are listed in Appendix 8.2.

9.5 DETAILS OF RECOMBINANT DNA CONSTRUCTS GENERATED FOR THIS STUDY

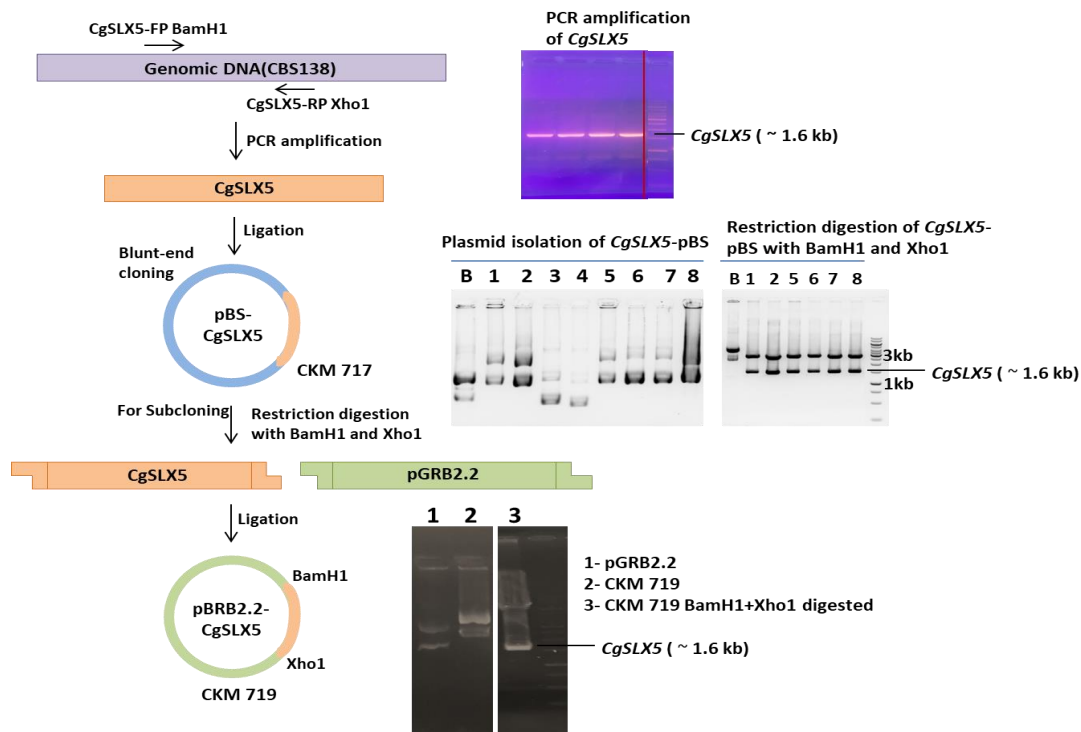
Generating *CgSLX5* in pGRB2.2 (CKM 719)

In order to clone *CgSLX5* in pGRB2.2, genomic DNA from YRK19 (*C. glabrata* WT strain-CBS138) was used as a template for amplifying the *CgSLX5* gene. The following primers were used for the amplification:

CgSLX5 FP Forward primer from START codon of *CgSLX5* with BamH1 site
AATGGATCCATGAAACGGGATGGT

CgSLX5 RP Reverse primer from up to STOP codon of *CgSLX5* with Xho1 site
GCGCTCGAGTTAGAAATATACTTCTCTCAT

The ~1.6 kb PCR product obtained was ligated into pBlueScript vector. This clone was digested with BamH1 and Xho1 restriction enzymes to generate the fragment for ligation into pGRB2.2 vector. The clone was confirmed by restriction digestion. This clone (CKM 719) was used for the complementation assay (Refer to **Figure 6E**).



Schematic showing cloning generation of CKM 719

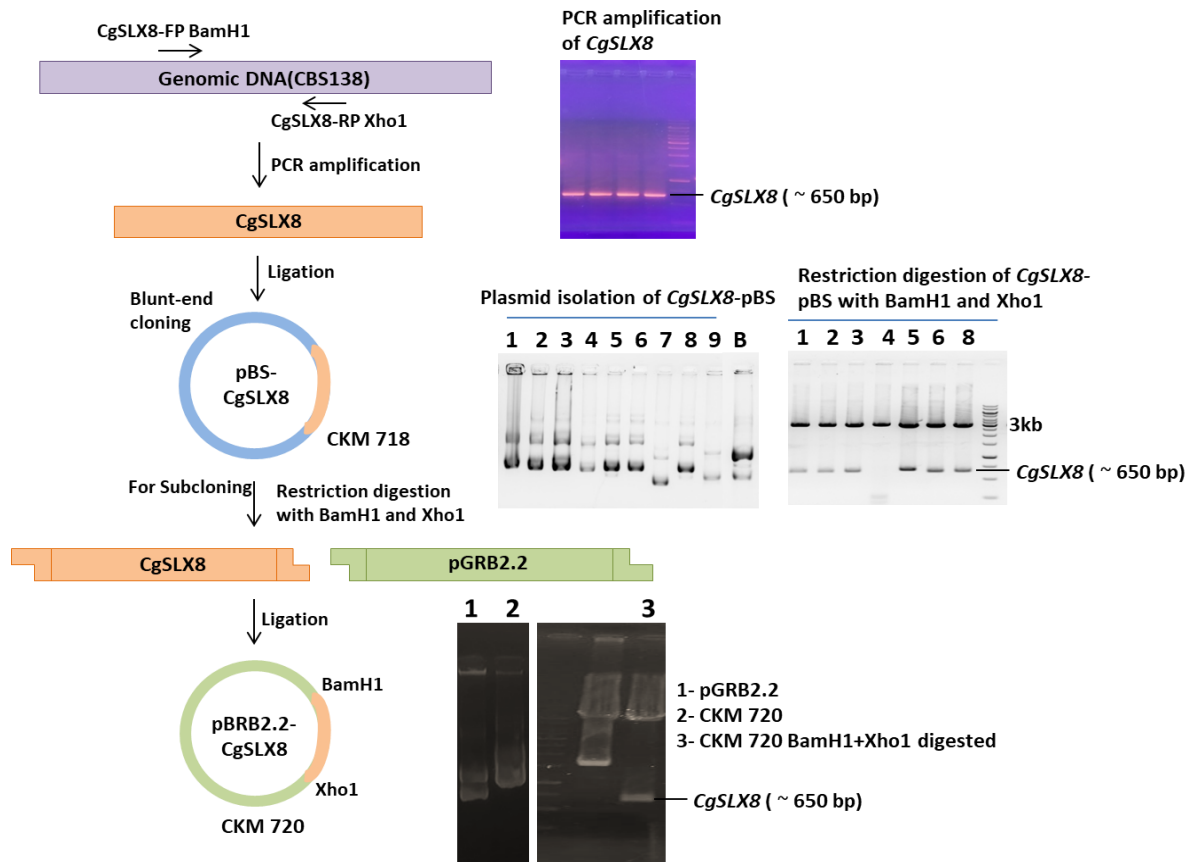
Generating *CgSLX8* in pGRB2.2 (CKM 720)

To clone *CgSLX8* into pGRB2.2, genomic DNA from YRK19 (*C. glabrata* WT strain-CBS138) was used as a template for amplifying the *CgSLX8* gene. The following primers were employed for the amplification:

CgSLX8 FP Forward primer from START codon of *CgSLX8* with BamH1 site
GCCGGATCCATGGCTAAATCTGAG

CgSLX8 RP Reverse primer from upto STOP codon of *CgSLX8* with Xho1 site
GAGCTCGAGTCAGGTCTTCTTCTG

The ~650 bp PCR product obtained was ligated into pBlueScript vector. This clone was digested with BamH1 and Xho1 restriction enzymes to generate the fragment for ligation into pGRB2.2 vector. The clone was confirmed by restriction digestion. This clone (CKM 720) was used for the complementation assay (Refer to **Figure 6E**).



Schematic showing cloning generation of CKM 719

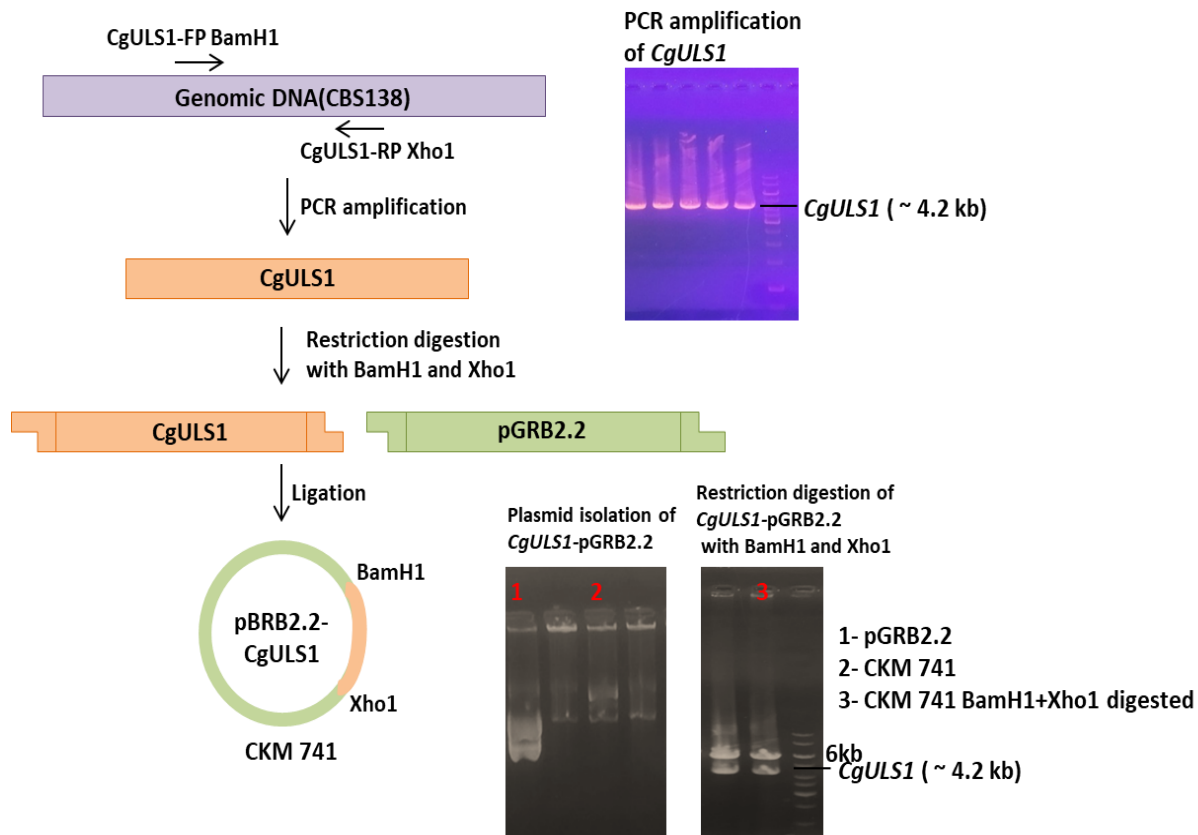
Generating *CgULS1* in pGRB2.2 (CKM 741)

In order to clone *CgULS1* in pGRB2.2, genomic DNA from YRK19 (*C. glabrata* WT strain- CBS138) was used as a template for amplifying the *CgULS1* gene. The following primers were used for the amplification:

CgULS1 FP Forward primer from START codon of *CgULS1* with BamH1 site
GCGGGATCCATGCCAAAAGTTACA

CgULS1 RP Reverse primer from upto STOP codon of *CgULS1* with Xho1 site
GCGCTCGAGTCATAATGAATTTAGTCCG

The ~4.2 kb PCR product was obtained and digested with BamH1 and Xho1 restriction enzymes to ligate in to pGRB2.2 vector. The clone was confirmed by restriction digestion. This clone (CKM 741) was used for the complementation assay (Refer to **Figure 6E**).

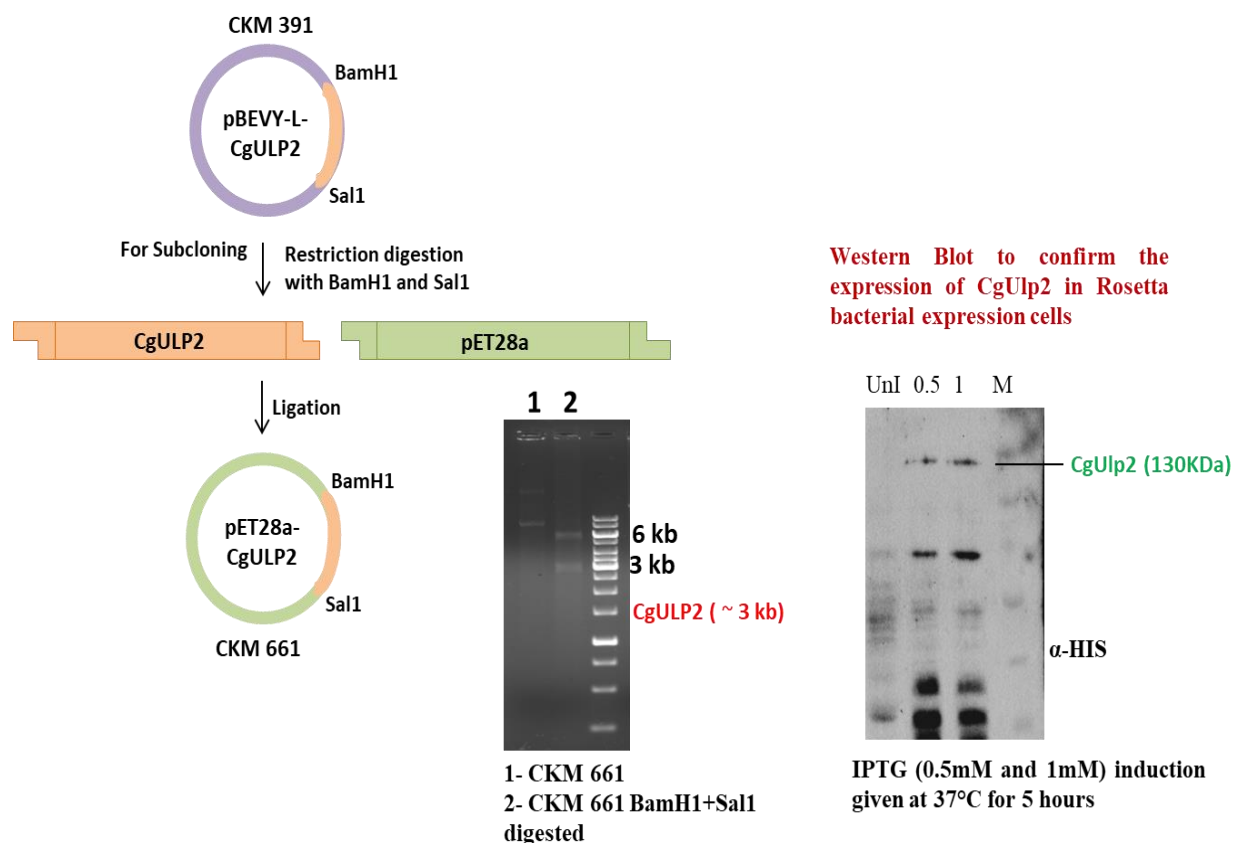


Schematic showing cloning generation of CKM 741

Generating *CgULP2* in pET28a (CKM 661)

The *CgULP2* gene was subcloned from the CKM 391 (*CgULP2*-pBEVY-L). *CgULP2* was digested with BamH1 and Sal1 restriction enzymes to generate ~3kb fragment for ligation into pET28a vector. The clone was confirmed by restriction digestion and sequencing.

The *CgULP2* protein was expressed in the Rosetta bacterial expression strain using IPTG concentrations of 0.5 mM and 1 mM at 37°C for 5 hours. The expression of *CgULP2* protein was confirmed by using an α -HIS antibody. Purification of the *CgULP2* protein was attempted (Refer to **Figure 22B**).



Schematic showing cloning generation of CKM 661

Generating N-terminal domain (NTD) of the *CgULP2* in pET28a (CKM 693)

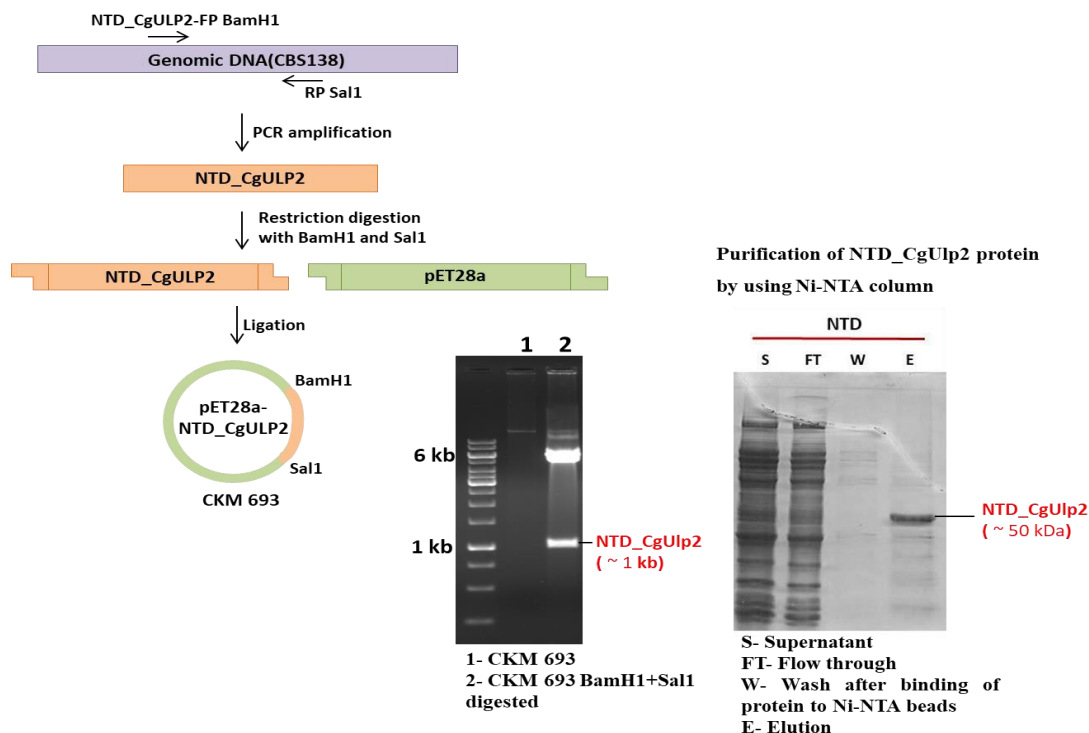
In order to clone NTD_*CgULP2* in pET28a, genomic DNA from YRK19 (*C. glabrata* WT strain-CBS138) was used as a template for amplifying the NTD_*CgULP2* gene. The following primers were used for the amplification:

NTD_*CgULP2* FP Forward primer from START codon of NTD_*CgULP2* with BamH1 site
AATGGATCCATGTCGCGGAGG

NTD_*CgULP2* RP Reverse primer from up to STOP codon of NTD_*CgULP2* with Sal1 site
AATGTCGACCATCCTCTTTCCGCA

The ~1kb PCR product was obtained and was digested with BamH1 and Sal1 restriction enzymes to ligate into pET28a vector. The clone was confirmed by restriction digestion and sequencing.

The NTD_*CgULP2* protein was expressed in the BL21(DE3)-RIL bacterial expression strain using an IPTG concentration of 0.4 mM at 22°C for 16 hours. The expression of NTD_*CgULP2* protein was confirmed by using α -HIS antibody (Refer to **Figure 32B**). Purification of the NTD_*CgULP2* protein was attempted.



Schematic showing cloning generation of CKM 693

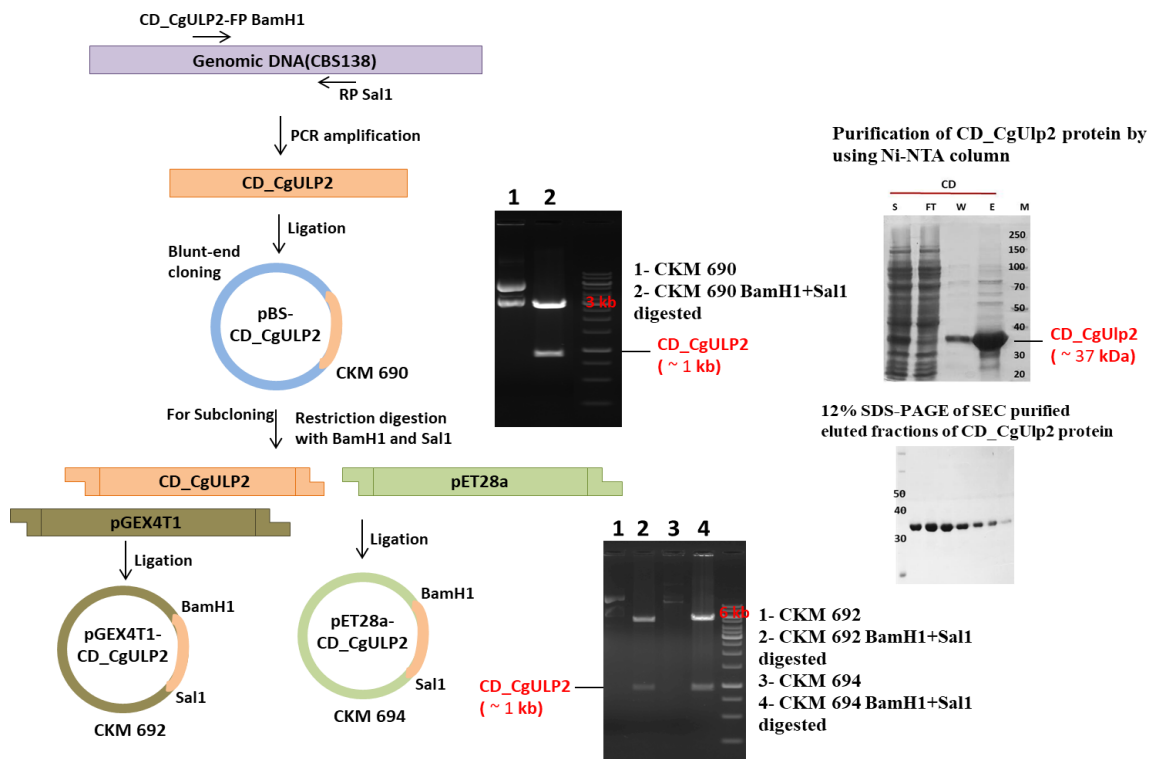
Generating catalytic domain (CD) of the *CgULP2* in pGEX4T1 (CKM 692) and pET28a (CKM 694)

In order to clone CD_*CgULP2* in pGEX4T1 and pET28a, genomic DNA from YRK19 (*C. glabrata* WT strain-CBS138) was used as template for amplifying the CD_*CgULP2* gene. The following primers were used for the amplification:

CD_*CgULP2* FP Forward primer from START codon of CD_*CgULP2* with BamH1 site
AGTGGATCCATGGAGGACAAAAGC

CD_*CgULP2* RP Reverse primer from up to STOP codon of CD_*CgULP2* with Sal1 site
AGTGTGCGACAATGCTTGTCCATTGG

The ~1kb PCR product obtained was ligated into pBlueScript vector. This clone was digested with BamH1 and Sal1 restriction enzymes to ligate into pGEX4T1 and pET28a vector. The clone was confirmed by restriction digestion and sequencing. The CD_*CgULP2* protein was expressed in the BL21(DE3)-RIL bacterial expression strain using an IPTG concentration of 0.4 mM at 22°C for 16 hours. The expression of CD_*CgULP2* protein was confirmed by using α -GST antibody (Refer to **Figure 32B**). CD_*CgULP2* protein was purified well (Figure 24A).



Schematic showing cloning generation of CKM 692, and CKM 694

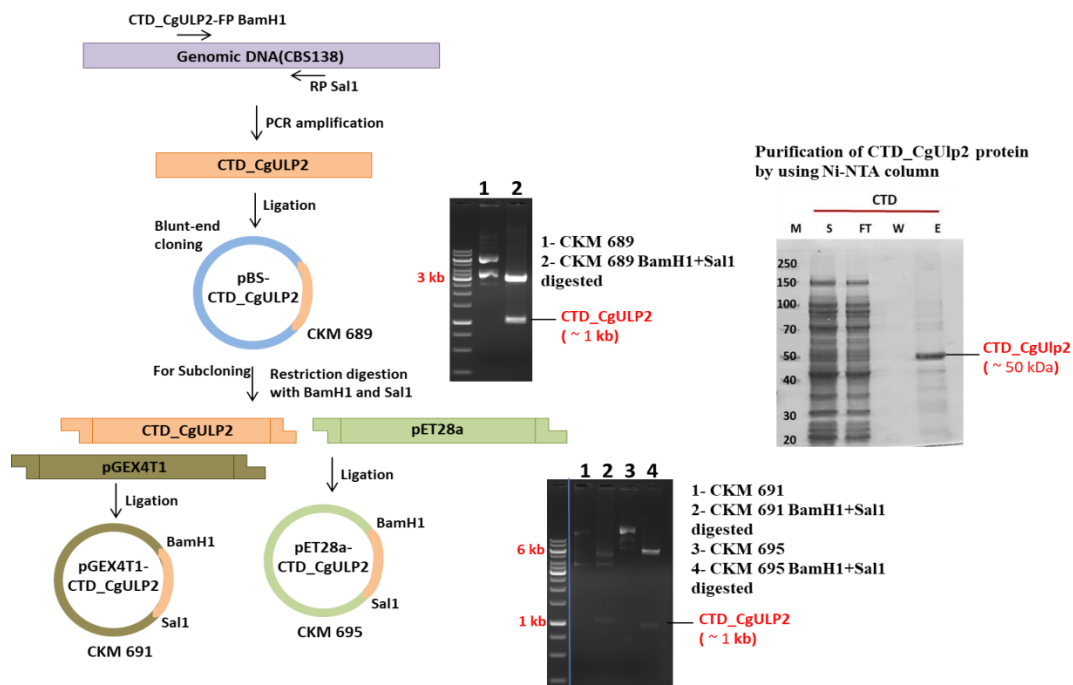
Generating C-terminal domain (CTD) of the *CgULP2* in pGEX4T1 (CKM 691) and pET28a (CKM 695)

In order to clone CTD_*CgULP2* in pGEX4T1 and pET28a, genomic DNA from YRK19 (*C. glabrata* WT strain-CBS138) was used as a template for amplifying the CTD_*CgULP2* gene. The following primers were used for the amplification:

CTD_*CgULP2* FP Forward primer from START codon of CTD_*CgULP2* with BamH1 site
AATGGATCCATGACGCATAGCAGAGAA

CTD_*CgULP2* RP Reverse primer from upto STOP codon of CTD_*CgULP2* with Sal1 site
CGGGTCGACCTAATGAGTATCGATATTTTC

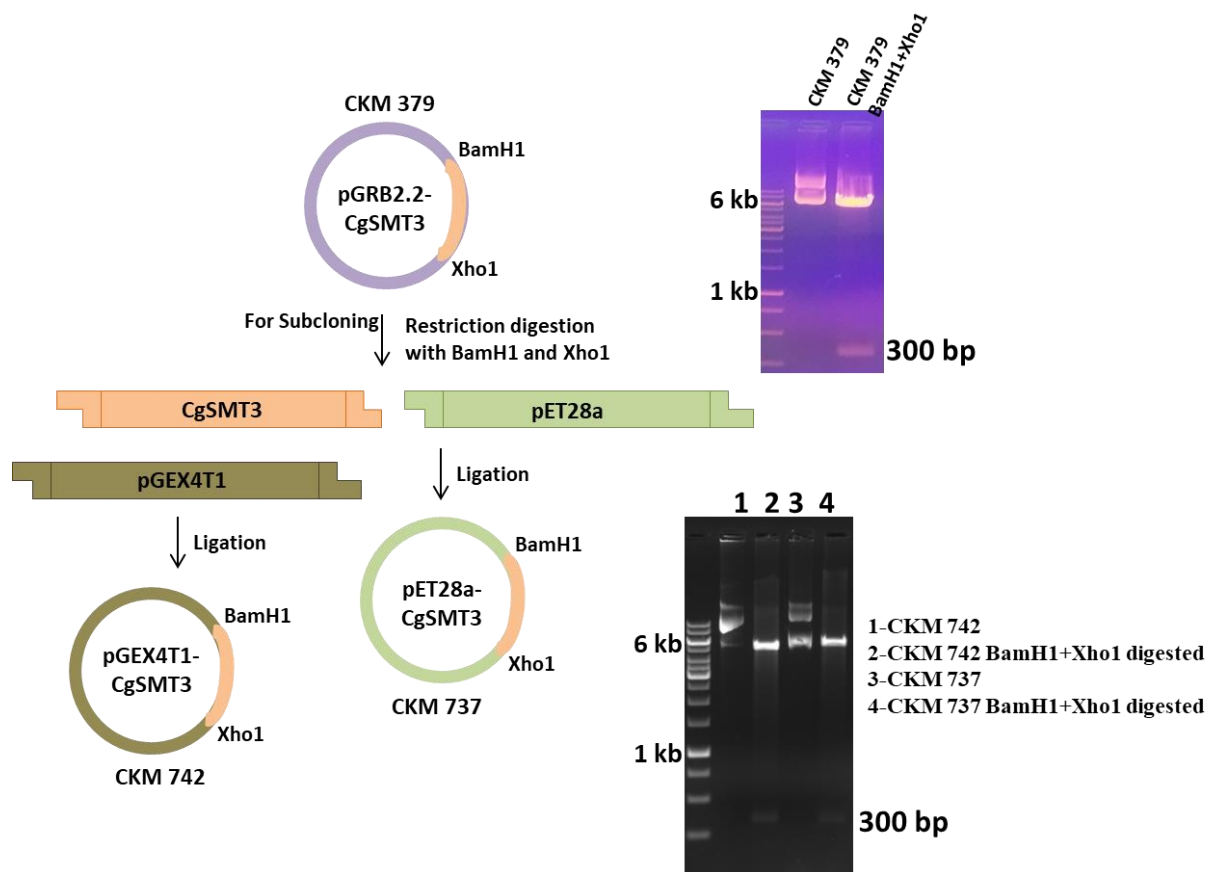
The ~1kb PCR product obtained was ligated into pBlueScript vector. This clone was digested with BamH1 and Sal1 restriction enzymes to ligate into pGEX4T1 and pET28a vector. The clone was confirmed by restriction digestion and sequencing. The CTD_*CgULP2* protein was expressed in the BL21(DE3)-RIL bacterial expression strain using an IPTG concentration of 0.4 mM at 22°C for 16 hours. The expression of CTD_*CgULP2* protein was confirmed by using α -GST antibody (Refer to **Figure 32B**).



Schematic showing cloning generation of CKM 691, and CKM 695

Generating *CgSMT3* in pGEX4T1 (CKM 742) and pET28a (CKM 737)

The *CgSMT3* gene was subcloned from the CKM 379 (*CgSMT3*-pGRB2.2). *CgSMT3* was digested with BamH1 and Xho1 restriction enzymes to generate ~300 bp fragment for ligation into pGEX4T1 and pET28a vector. The clone was confirmed by restriction digestion and the expression of *CgSMT3* protein was confirmed by western blot using α -HIS and α -GST antibodies (Refer to **Figure 32B**).



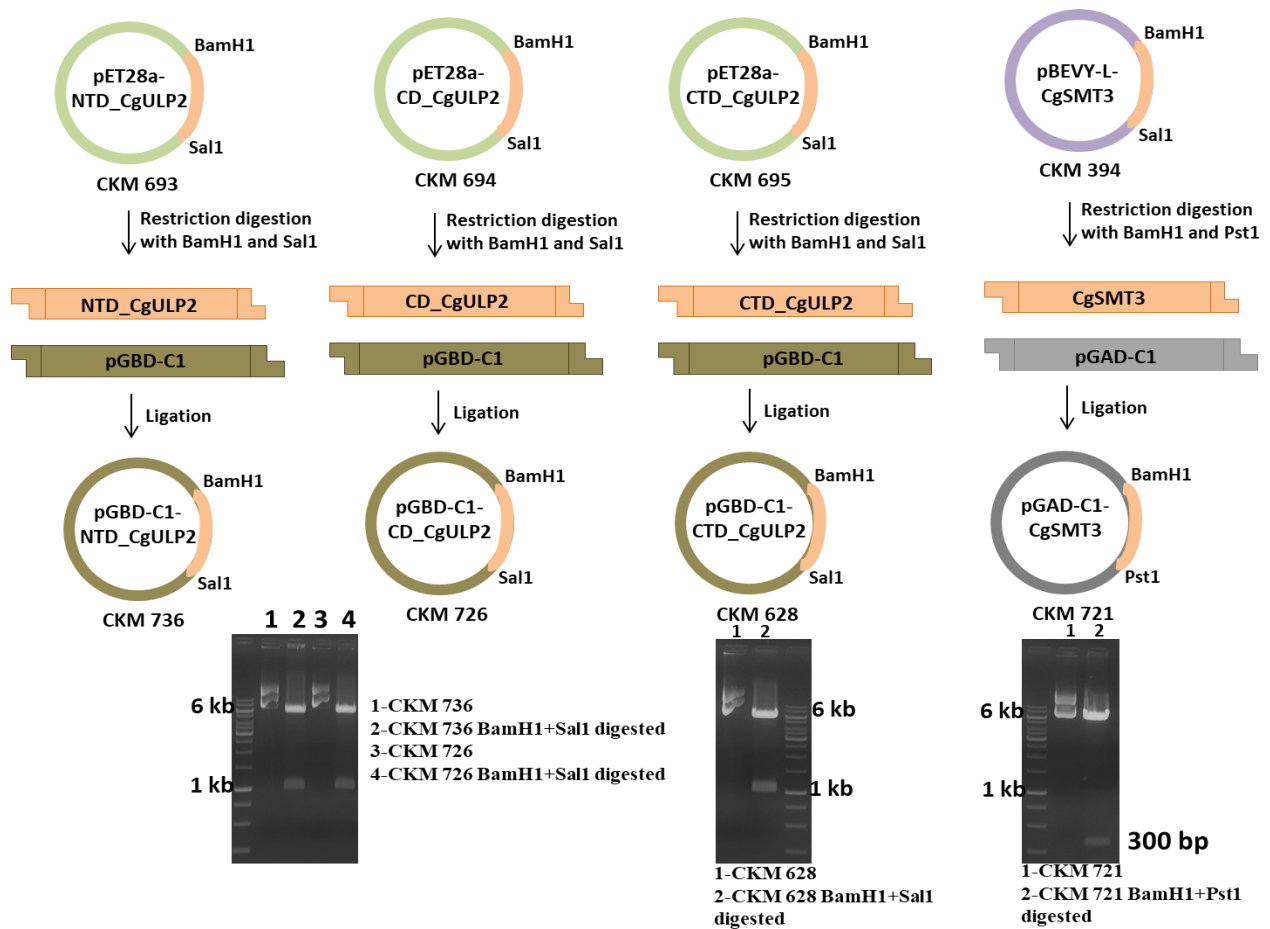
Schematic showing cloning generation of CKM 742, and CKM 737

Generating *CgULP2* in pGBDU-C1 and *CgSMT3* in pGAD-C1 for Yeast-two hybrid assay

N-terminal, catalytic domain, and C-terminal domain of the *CgULP2* cloned in pGBDU-C1 vector was used as bait and *CgSMT3* cloned in pGAD-C1 was used as prey for yeast two-hybrid screen. The respective gene was subcloned from the CKM 693 (NTD_*CgULP2*- pET28a), 694 (CD_*CgULP2*- pET28a), 695 (CTD_*CgULP2*- pET28a) and 394 (*CgSMT3*- pBEVY-L)

All the domains of the *CgULP2* were digested with BamH1 and Sal1 restriction enzymes to generate ~1 kb fragment for ligation into pGBDU-C1 vector. *CgSMT3* was digested with BamH1 and Pst1 restriction enzymes to generate ~300 bp fragments for ligation into pGAD-C1 vector. The clone was confirmed by restriction digestion.

Domains of *CgUlp2* protein interacted with *CgSmt3* protein physically (Refer to **Figure 32A**).



Schematic showing cloning generation of CKM 736, CKM 726, CKM 628, and CKM 721

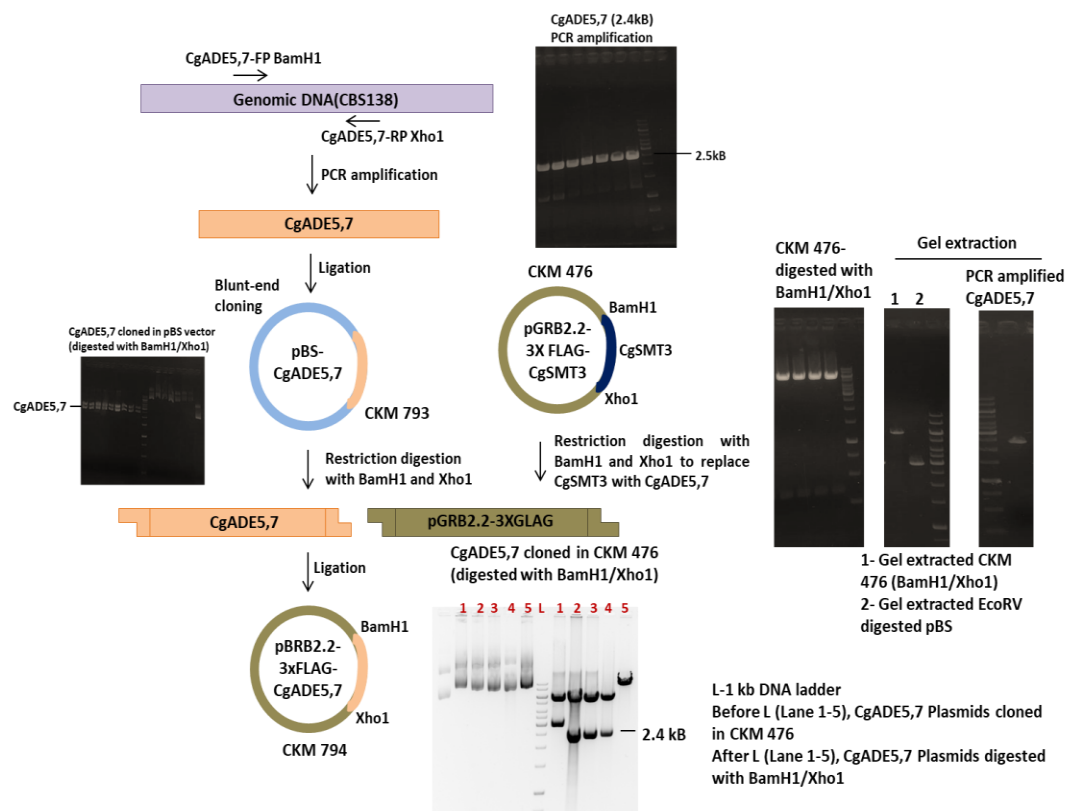
Generating *CgADE5,7* in 3xFLAG-pGRB2.2 (CKM 794)

In order to clone *CgADE5,7* with a 3xFLAG tag into pGRB2.2, genomic DNA from YRK19 (*C. glabrata* WT strain-CBS138) was used as a template for amplifying the *CgADE5,7* gene. The following primers were used for the amplification:

CgADE5,7 FP Forward primer from START codon of *CgADE5,7* with BamH1 site
GGCGGATCCATGTTGAACATTCTAG

CgADE5,7 RP Reverse primer from up to STOP codon of *CgADE5,7* with Xho1 site
GGACTCGAGTTAGTATAGGTTGCTGG

The ~2.4 kb product obtained was ligated into pBlueScript vector. This clone was digested with BamH1 and Xho1 restriction enzymes to generate the fragment for ligation into 3xFLAG-tagged pGRB2.2 vector. The clone was confirmed by restriction digestion. The expression of *CgADE5,7* protein was confirmed by western blot using an α -FLAG antibody (Refer to **Figure 16C**).



Schematic showing cloning generation of CKM 794

9.6 LISTS OF SIGNIFICANT PROTEINS IDENTIFIED BY QUANTITATIVE MASS SPECTROMETRY FOR EACH MUTANT

A list of 107 significantly upregulated proteins and 90 significantly downregulated proteins were identified in the *Cg*ulp2 Δ mutant in comparison with the wild type.

<i>Cg</i> ulp2 Δ			
Upregulated Proteins		Downregulated Proteins	
Protein name	log2 fold change	Protein name	log2 fold change
CgCca1	1.357	CgGlo3	-0.089
CgCdc48	0.114	CgLaa1	-0.104
CgAcl4	0.325	CgHpr1	-0.302
CgAdh6	0.715	CgPho86	-0.439
CgAro3	0.172	CgHsp10	-0.361
CgAsk10	0.101	CgNic96	-1.211
CgCat2	0.088	CgEnt5	-0.303
CgCdc10	0.105	CgSky1	-0.203
CgCog1	0.311	CgTom6	-0.478
CgCox1	0.180	CgVps54	-0.411
CgCpr6	0.174	CgMge1	-0.203
CgCuz1	0.356	CgRho1	-0.165
CgCys3	0.197	CgErp3	-0.409
CgDnf1	0.088	CgMtr2	-0.090
CgDph2	0.211	CgNup188	-0.359
CgDph6	0.374	CgGnp1	-0.444
CgEcm15	0.108	CgAfg3	-0.471
CgEfm1	0.156	CgTim8	-0.298
CgEnp2	0.334	CgTim44	-0.232
CgFmn1	0.161	CgDjp1	-0.450
CgFub1	0.311	CgVps10	-2.374
CgGcn1	0.062	CgSod1	-0.416

CgHam1	0.160	CgTif6	-0.397
CgHch1	0.161	CgGef1	-0.388
CgHom3	0.243	CgDnf2	-0.140
CgKrr1	0.196	CgMmt1	-0.322
CgLat1	0.122	CgGln1	-0.169
CgLcb4	0.309	CgCsg2	-0.241
CgLsg1	0.165	CgHtb1	-0.324
CgMas1	0.300	CgIxr1	-0.356
CgMed1	0.263	CgHmo1	-0.312
CgMeh1	0.385	CgMcm6	-0.099
CgMet12	0.262	CgPat1	-0.309
CgMet18	0.171	CgHtz1	-0.339
CgMeu1	0.139	CgHhf2	-0.331
CgMrp17	0.273	CgSmt3	-0.380
CgMrp21	0.167	CgAsk1	-0.291
CgMsh2	0.327	CgHhoA	-0.250
CgMtc2	0.342	CgCin8	-0.162
CgNar1	0.168	CgSmc1	-0.300
CgNat1	0.103	CgHtb2	-0.324
CgNnt1	0.417	CgFpr1	-0.144
CgNop15	0.964	CgShm2	-0.393
CgNus1	0.146	CgGcd11	-0.082
CgNut2	0.114	CgNet1	-0.175
CgPan1	0.201	CgGpi17	-0.128
CgPbr1	0.216	CgTps2	-0.350
CgPop3	0.162	CgHir2	-0.457
CgPre3	0.566	CgAde5,7	-0.160
CgPrp45	0.434	CgTfb5	-0.190
CgPrt1	0.148	CgAdk1	-0.105
CgPse1	0.149	CgYHR020W	-0.112
CgPus7	0.143	CgPsa1	-0.367

CgRet1	0.337	CgFlc1	-0.137
CgRet3	0.110	CgFcy2	-0.499
CgRio2	0.250	CgHxt1	-0.424
CgRna1	0.171	CgPtr2	-0.528
CgRpa34	0.229	CgAtp3	-0.651
CgRpn1	0.207	CgCus1	-0.367
CgRpn11	0.080	CgHsm3	-0.652
CgRpn12	0.185	CgSin4	-0.373
CgRpn5	0.293	CgTef1	-0.116
CgRpn6	0.315	CgHal5	-0.188
CgRpn8	0.304	CgClu1	-0.331
CgRpt2	0.106	CgPin4	-0.302
CgSdh5	0.333	CgBem1	-0.144
CgSdt1	0.213	CgChz1	-0.195
CgSec5	0.047	CgPom33	-0.239
CgSec72	0.090	CgYbp1	-0.087
CgSfl1	0.254	CgRps14B	-0.231
CgSis2	1.036	CgRpl19A	-0.323
CgSrb6	0.326	CgRps16A	-1.449
CgSrp14	0.164	CgRps21A	-0.187
CgSto1	0.098	CgRpl4B	-0.122
CgSua7	0.453	CgRpl13A	-0.152
CgSup45	0.140	CgRps25A	-0.197
CgTaf9	0.174	CgTup1	-0.487
CgTfb4	0.235	CgSws2	-0.155
CgTml25	0.256	CgMrp10	-0.006
CgTmt1	0.430	CgRsm18	-0.128
CgTom71	0.173	CgMrm1	-0.143
CgTrp3	0.272	CgMae1	-0.171
CgUtp9	0.369	CgMrx3	-0.170
CgUtr1	0.268	CgUsa1	-0.957

CgVps75	0.157	CgHpc2	-0.574
CgYar1	0.170	CgEcm21	-0.557
CgYdj1	0.179	CgFet4	-0.484
CgYhm2	0.460	CgSlk19	-0.272
CgYJR015W	0.217	CgCox12	-0.000
CgYKR011C	0.178		
CgYKR018C	0.301		
CgYLR287C	0.174		
CgYML082W	0.284		
CgYMR099C	0.048		
CgYpk3	0.213		
CgYps1	0.389		
CgCwp1.1	0.755		
CgEcm4	0.224		
CgExg1	0.430		
CgGas1	0.179		
CgGsh1	0.134		
CgLap3	0.149		
CgLrs4	0.429		
CgPir3	1.041		
CgPre5	0.156		
CgTrx3	0.410		

A list of 87 significantly upregulated proteins and 130 significantly downregulated proteins were identified in the *Cgslx8Δ* mutant in comparison with the wild type.

<i>Cgslx8Δ</i>					
Upregulated Proteins		Downregulated Proteins			
Protein name	log2 fold change	Protein name	log2 fold change	Protein name	log2 fold change
CgLap3	0.068	CgVps10	-2.310	CgYDR415C	-0.287
CgVig9	0.074	CgGcn5	-1.744	CgMss116	-0.286
CgRet3	0.081	CgRps16A	-1.438	CgApe3	-0.280
CgRvb1	0.082	CgEsp1	-1.415	CgPex5	-0.273
CgOye2	0.108	CgAtp3	-1.223	CgMcp2	-0.270
CgOla1	0.109	CgArg5,6	-1.120	CgSsa3	-0.260
CgCdc60	0.111	CgPpz2	-1.028	CgMam3	-0.257
CgCdc10	0.112	CgTog1	-1.011	CgUtp5	-0.256
CgPup3	0.131	CgMrx3	-0.989	CgSky1	-0.253
CgSec26	0.137	CgHpc2	-0.939	CgClu1	-0.249
CgSec17	0.144	CgCtk2	-0.932	CgRmd1	-0.248
CgRer1	0.148	CgSec3	-0.916	CgYNL320W	-0.246
CgGas1	0.150	CgEcm21	-0.865	CgYLR126C	-0.244
CgFpt1	0.153	CgMak10	-0.823	CgGar1	-0.228
CgHom3	0.153	CgSit1	-0.823	CgBig1	-0.225
CgSec4	0.155	CgHsm3	-0.807	CgYro2	-0.223
CgRps0B	0.155	CgSrc1	-0.783	CgVph2	-0.220
CgNat1	0.157	CgRtn1	-0.779	CgMrpL27	-0.214
CgPmi40	0.157	CgTrm5	-0.774	CgMic26	-0.210
CgTao3	0.160	CgRfc4	-0.745	CgAco1	-0.206
CgTfb4	0.161	CgKin4	-0.736	CgRpl21A	-0.205
CgUfo1	0.168	CgHxt2	-0.728	CgRpn1	-0.204
CgGrx8	0.171	CgTfb6	-0.720	CgSnq2	-0.197
CgRps20	0.173	CgDug3	-0.706	CgCaf20	-0.195

CgKar2	0.174	CgCti6	-0.694	CgOtu1	-0.194
CgSrp14	0.176	CgMix17	-0.688	CgCdc33	-0.191
CgTub3	0.176	CgHal5	-0.684	CgRsm28	-0.183
CgCcp1	0.177	CgErg7	-0.666	CgUrb2	-0.180
CgCdc12	0.178	CgRsm22	-0.653	CgSec13	-0.174
CgFmn1	0.179	CgSdh6	-0.646	CgSwh1	-0.173
CgArc15	0.181	CgMhp1	-0.646	CgLsb3	-0.162
CgKre2	0.184	CgPep8	-0.644	CgYpt1	-0.137
CgExg2	0.186	CgFzo1	-0.643	CgBil1	-0.136
CgPbr1	0.188	CgMsf1	-0.642	CgTim44	-0.134
CgCdc8	0.194	CgSmt3	-0.628	CgZrg17	-0.133
CgCuz1	0.200	CgUsa1	-0.627	CgRpt6	-0.128
CgOst3	0.202	CgYOR283W	-0.607	CgDid4	-0.124
CgSub2	0.202	CgMrpl15	-0.576	CgTim21	-0.119
CgEpt1	0.212	CgBck1	-0.571	CgVhs3	-0.108
CgRpa34	0.217	CgPex23B	-0.567	CgMbf1	-0.098
CgLap2	0.217	CgShq1	-0.562	CgYbp1	-0.095
CgYps1	0.218	CgDog2	-0.558	CgPtc5	-0.080
CgPyp1	0.220	CgFet4	-0.546	CgYMR099C	-0.062
CgVma2	0.221	CgTfb5	-0.546		
CgRnr1	0.221	CgRps17B	-0.542		
CgDpm1	0.222	CgHnt2	-0.538		
CgAcs2	0.225	CgYGR237C	-0.538		
CgMed1	0.231	CgMiy1	-0.536		
CgMvd1	0.236	CgUra4	-0.535		
CgDph5	0.243	CgCpa2	-0.523		
CgHho1	0.251	CgRot2	-0.509		
CgSec7	0.266	CgYta12	-0.506		
CgYMR027W	0.268	CgDur1:2	-0.500		
CgCox1	0.270	CgLcb4	-0.493		
CgCpr1	0.271	CgMia40	-0.490		

CgRgt1	0.298	CgGip2	-0.485		
CgMet12	0.309	CgCgi121	-0.471		
CgHmg1	0.309	CgRpl19A	-0.451		
CgSdd2	0.311	CgSod1	-0.450		
CgCog1	0.335	CgBud16	-0.449		
CgVti1	0.335	CgErc1	-0.445		
CgMtd1	0.339	CgCmc1	-0.442		
CgAsk1	0.340	CgMic60	-0.420		
CgSw14	0.344	CgAdh6	-0.409		
CgErg5	0.362	CgPap1	-0.406		
CgGlm6	0.364	CgSfb3	-0.406		
CgYkt6	0.368	CgGsp1	-0.398		
CgLsm3	0.370	CgSpt15	-0.392		
CgTaf9	0.370	CgDnf2	-0.375		
CgRad51	0.382	CgPho81	-0.373		
CgEhd3	0.402	CgHir2	-0.370		
CgScd6	0.418	CgSdh8	-0.368		
CgDog2	0.419	CgCus1	-0.364		
CgCta1	0.419	CgGrr1	-0.351		
CgTom14	0.420	CgSte13	-0.345		
CgScs7	0.471	CgPtk1	-0.335		
CgRpl31A	0.476	CgPro2	-0.334		
CgPff1	0.525	CgMam33	-0.331		
CgTdh3	0.595	CgNab2	-0.328		
CgPil1	0.605	CgOsh7	-0.326		
CgEnt2	0.633	CgNar1	-0.324		
CgSrb6	0.646	CgTuf1	-0.324		
CgAqy1	1.078	CgRtp1	-0.319		
CgCca1	1.178	CgSfk1	-0.313		
CgUra3	1.933	CgSpn1	-0.311		
CgPck1	2.169	CgYey2	-0.293		

A list of 148 significantly upregulated proteins and 241 significantly downregulated proteins were identified in the *Cguls1Δ* mutant in comparison with the wild type.

<i>Cguls1Δ</i>					
Upregulated Proteins		Downregulated Proteins			
Protein name	log2 fold change	Protein name	log2 fold change	Protein name	log2 fold change
CgVas1	0.048	CgYbp1	-0.112	CgNup188	-0.458
CgCdc10	0.062	CgRts3	-0.220	CgTsl1	-0.178
CgRps28B	0.083	CgYBR137W	-0.394	CgSws2	-0.408
CgZpr1	0.153	CgGga1	-0.270	CgYJL068C	-0.289
CgSub2	0.158	CgBbc1	-0.340	CgAim22	-0.257
CgMft1	0.221	CgMam3	-0.270	CgAat2	-0.284
CgOla1	0.338	CgSdd3	-0.139	CgPim1	-0.324
CgMet12	0.413	CgPre6	-0.163	CgVps10	-2.900
CgHom2	0.222	CgErg7	-0.339	CgAim9	-0.287
CgAro4	0.421	CgMam33	-0.730	CgGip2	-0.391
CgCdc8	0.366	CgYro2	-0.291	CgRsm18	-0.558
CgCam1	0.169	CgMrm1	-0.211	CgTim44	-0.222
CgMnt3	0.113	CgCdc48	-0.193	CgVid28	-0.342
CgYBR242W	0.637	CgYDL124W	-0.129	CgYta12	-0.364
CgRps20	0.234	CgEcm21	-1.004	CgSdh1	-0.826
CgHts1	0.247	CgMpm1	-0.310	CgCox6	-0.166
CgMpd2	0.176	CgPep8	-0.343	CgHsh49	-0.224
CgWrs1	0.173	CgDcs1	-0.112	CgRsm23	-0.305
CgMcd4	0.182	CgCoq1	-0.287	CgMrps35	-0.292
CgGln4	0.128	CgSod1	-0.713	CgNif3	-0.657
CgBcp1	0.196	CgOsh2	-0.339	CgLaa1	-0.134
CgAro3	0.300	CgGre2	-0.799	CgHal5	-0.412
CgEpt1	0.153	CgSdh8	-0.309	CgVps35	-0.314
CgPyp1	0.175	CgTae1	-0.494	CgPtc2	-0.092

CgYML119W	0.221	CgOxa1	-0.477	CgCsg2	-0.319
CgVig9	0.112	CgYey2	-0.308	CgSec13	-0.176
CgEbp2	0.298	CgShm1	-0.206	CgPup2	-0.156
CgRpl43A	0.204	CgFmp52	-0.439	CgRps16A	-1.207
CgCyb5	0.413	CgRpn1	-0.229	CgAfg3	-0.459
CgSsh1	0.165	CgDid2	-0.162	CgPtk1	-0.334
CgUtp9	0.265	CgCmc1	-0.667	CgMsf1	-0.534
CgRps19A	0.152	CgAdd66	-0.156	CgCoa1	-0.615
CgYMR027W	0.188	CgMss51	-0.322	CgSte23	-0.168
CgOpi3	0.840	CgYMR196W	-0.683	CgGlr1	-0.312
CgRpl42B	0.150	CgDyn1	-0.480	CgMrp21	-0.203
CgRpp0	0.174	CgImg1	-0.313	CgMnp1	-0.429
CgKre2	0.206	CgMas1	-0.230	CgNam8	-0.252
CgSpe3	0.101	CgAtp4	-0.456	CgDpl1	-0.240
CgFes1	0.308	CgLeu2	-0.343	CgTuf1	-0.415
CgPcf11	0.122	CgRib4	-0.384	CgDnf2	-0.390
CgAsc1	0.193	CgMrx3	-0.574	CgZrg17	-0.245
CgYFR006W	0.211	CgNpy1	-0.440	CgMrps12	-0.377
CgSec4	0.146	CgFzo1	-0.593	CgMic60	-0.580
CgRpl25	0.320	CgCub1	-0.122	CgApe3	-0.358
CgRpa34	0.385	CgMrpl35	-0.568	CgPhm3	-0.231
CgPrp45	0.271	CgTom70	-0.452	CgTps2	-0.418
CgMsh3	0.192	CgMtc1	-0.219	CgRsm28	-0.394
CgRpc34	0.207	CgMrpl37	-0.328	CgSsa3	-0.564
CgPnc1	0.651	CgHxt2	-0.598	CgEsp1	-2.179
CgRet1	0.248	CgRpt2	-0.148	CgIdh1	-0.267
CgOye2	0.222	CgBpl1	-0.183	CgYLR345W	-0.250
CgArc1	0.219	CgBcy1	-0.293	CgPex23	-0.215
CgYar1	0.175	CgExg1	-0.355	CgEno1	-0.526
CgRsn1	0.553	CgDsk2	-0.327	CgYGR237C	-0.374
CgAro2	0.133	CgCus1	-0.310	CgMic26	-0.202

CgShm2	0.208	CgTpm2	-0.491	CgRmd9	-0.371
CgRpl22A	0.256	CgUsa1	-0.992	CgIrc24	-0.417
CgSui2	0.235	CgPdx1	-0.447	CgMss116	-0.308
CgDpm1	0.404	CgYKR070W	-0.610	CgMss4	-0.295
CgTef1	0.162	CgEcm4	-0.425	CgEde1	-0.287
CgScm4	0.619	CgImp2	-0.542	CgMrpl40	-0.412
CgMsh2	0.162	CgMrps16	-0.201	CgKsp1	-0.353
CgErg11	0.510	CgSte13	-0.443	CgCym1	-0.331
CgRps22A	0.417	CgDnf1	-0.065	CgMef1	-0.171
CgUra3	1.752	CgYPL113C	-0.474	CgNut1	-0.226
CgHht1	0.314	CgMcm6	-0.051	CgGtb1	-0.157
CgErg5	0.597	CgYMR099C	-0.047	CgHir1	-0.125
CgCta1	0.476	CgPex11	-0.254	CgTrx3	-0.304
CgThs1	0.109	CgHfd1	-0.213	CgAco1	-0.365
CgEfm7	0.622	CgTim21	-0.204	CgRad7	-0.214
CgExg2	0.090	CgPho88	-0.131	CgPho4	-0.346
CgRps6A	0.157	CgPex5	-0.423	CgYPR127W	-0.463
CgTaf9	0.299	CgHis4	-0.489	CgGcv1	-0.555
CgRna1	0.282	CgSam2	-0.311	CgMia40	-0.588
CgRpl33A	0.309	CgCuz1	-0.495	CgSdo1	-0.686
CgPff1	0.340	CgXrn1	-0.236	CgMtr2	-0.237
CgYDR391C	0.304	CgHpc2	-0.482	CgCpa2	-0.735
CgBos1	0.696	CgLpd1	-0.429	CgYJR096W	-0.546
CgCaf20	0.196	CgLip5	-0.554	CgMxr1	-0.195
CgPdr13	0.363	CgPhb1	-0.546	CgMsc3	-0.186
CgSup45	0.178	CgPan1	-0.088	CgMic10	-0.402
CgRps23A	0.139	CgMbf1	-0.110	CgMxr2	-0.482
CgMls1	1.268	CgSlc1	-0.350	CgNdi1	-0.247
CgNop15	0.820	CgGrr1	-0.251	CgCin8	-0.154
CgCnb1	0.238	CgPmc1	-0.458	CgMrpl27	-0.388
CgRpl10	0.191	CgTog1	-1.027	CgPre4	-0.174

CgSes1	0.164	CgTim8	-0.278	CgCmc2	-0.373
CgSec26	0.100	CgVma4	-0.170	CgShp1	-0.305
CgPrt1	0.168	CgAtp3	-1.279	CgPex23B	-0.348
CgKti12	0.292	CgCbp3	-0.368	CgMrp1	-0.311
CgTrm2	0.297	CgLsb3	-0.212	CgChc1	-0.184
CgYNL134C	0.317	CgTpo4	-0.813	CgSdh6	-0.689
CgMcm4	0.478	CgMdh1	-0.476	CgCox1	-0.153
CgSlx9	0.369	CgLcb4	-0.384		
CgCat2	0.094	CgIdh2	-0.304		
CgTop1	0.160	CgYGL082W	-0.354		
CgHom3	0.092	CgNam9	-0.383		
CgTdh3	1.286	CgPpa2	-0.412		
CgFaa1	0.193	CgYnk1	-0.317		
CgRex4	0.553	CgMrpl15	-0.567		
CgHmt1	0.105	CgGdi1	-0.362		
CgArf2	0.122	CgAro8	-0.116		
CgFap7	0.503	CgUbp6	-0.278		
CgErp5	0.157	CgCbf3D	-0.278		
CgPby1	0.292	CgSfk1	-0.249		
CgBap2	0.216	CgAro1	-0.093		
CgDys1	0.125	CgRcf2	-0.656		
CgPck1	3.277	CgMrps8	-0.348		
CgRps9A	0.286	CgHsp78	-0.347		
CgRli1	0.224	CgClu1	-0.389		
CgApt1	0.284	CgEht1	-0.120		
CgCct3	0.222	CgAlg2	-0.299		
CgNop6	0.265	CgDur1:2	-0.667		
CgCct7	0.083	CgBem1	-0.154		
CgKap123	0.240	CgYNL320W	-0.306		
CgAsk10	0.236	CgMrpl4	-0.557		
CgPil1	0.942	CgBig1	-0.170		

CgLap2	0.171	CgVhs3	-0.158		
CgGrs1	0.129	CgAim45	-0.388		
CgRpl26A	0.189	CgMrps9	-0.250		
CgGas1	0.220	CgYhb1	-0.554		
CgZuo1	0.177	CgGts1	-0.301		
CgErg8	0.428	CgIsn1	-0.301		
CgScs7	0.283	CgAim41	-0.437		
CgFyv7	0.279	CgGsf2	-0.295		
CgHam1	0.357	CgGcv2	-0.140		
CgLia1	0.647	CgOtu1	-0.260		
CgCpr1	0.216	CgSpc2	-0.167		
CgRnr4	0.221	CgRga1	-0.441		
CgMeu1	0.230	CgNvj2	-0.255		
CgGcn1	0.064	CgZrc1	-0.230		
CgMed1	0.180	CgPdb1	-0.563		
CgRpl31A	0.317	CgPyc2	-0.303		
CgNat1	0.244	CgYHR112C	-0.294		
CgPop2	0.118	CgYme1	-0.250		
CgGpn2	0.286	CgSto1	-0.118		
CgAcs2	0.261	CgHse1	-0.541		
CgFpt1	0.194	CgAtp20	-0.539		
CgDph5	0.281	CgRps5	-0.192		
CgRer1	0.149	CgMrp10	-0.494		
CgRpl4B	0.160	CgMix17	-0.920		
CgAqy1	1.397	CgLat1	-0.254		
CgDed81	0.153	CgTps1	-0.609		
CgCdc12	0.137	CgCpr6	-0.137		
CgAto3	1.442	CgPnp1	-0.178		
CgPmi40	0.135	CgPtc5	-0.095		
CgRpl37A	0.158	CgUfd1	-0.154		
CgCca1	1.356	CgZwf1	-0.419		

A list of 277 significantly upregulated proteins and 184 significantly downregulated proteins were identified in the *Cgulf2ΔCgslx8Δ* mutant in comparison with the wild type.

<i>Cgulf2ΔCgslx8Δ</i>					
Upregulated Proteins				Downregulated Proteins	
Protein name	log2 fold change	Protein name	log2 fold change	Protein name	log2 fold change
CgRfc3	0.062	CgPan6	0.382	CgAco1	-0.538
CgSto1	0.086	CgMdj1	0.383	CgAde2	-0.390
CgCmr1	0.088	CgPre5	0.394	CgAat2	-0.219
CgHcr1	0.098	CgRib4	0.395	CgAbf2	-0.302
CgCdc60	0.099	CgRds1	0.399	CgAch1	-0.573
CgRet2	0.111	CgRad7	0.401	CgAde1	-0.353
CgCop1	0.113	CgExg2	0.408	CgAde12	-0.274
CgTfg2	0.114	CgGpn2	0.410	CgAde3	-0.126
CgCys4	0.117	CgGsh1	0.410	CgAde4	-0.277
CgSly1	0.118	CgRpn5	0.417	CgAde5,7	-0.295
CgTom71	0.119	CgRpn8	0.423	CgAdp1	-0.157
CgCdc10	0.120	CgSrv2	0.426	CgApd1	-0.287
CgErp2	0.121	CgAqy1	0.429	CgApt1	-0.065
CgLsb3	0.121	CgEfm3	0.430	CgArg5,6	-0.648
CgSec7	0.122	CgCpr1	0.433	CgAsc1	-0.239
CgMxr1	0.124	CgCym1	0.435	CgAtp19	-1.546
CgYbp1	0.131	CgFmp27	0.435	CgAtp20	-1.174
CgCam1	0.132	CgVmr1	0.441	CgAtp3	-1.184
CgYMR155W	0.136	CgPfs2	0.441	CgAtp4	-0.721
CgPop2	0.136	CgRpn2	0.442	CgBat1	-0.235
CgTaf4	0.138	CgSnf1	0.443	CgBat2	-0.754
CgCpr6	0.139	CgMet18	0.443	CgCbp3	-0.121
CgPob3	0.140	CgZrt3	0.443	CgCcp1	-0.308
CgDst1	0.142	CgPre1	0.447	CgCcw22	-0.327

CgPhb1	0.142	CgUtp20	0.451	CgCit1	-0.519
CgInp51	0.144	CgTmt1	0.452	CgClu1	-0.487
CgInm1	0.146	CgYdc1	0.45	CgCox1	-1.451
CgRer1	0.146	CgCdc48	0.453	CgCox2	-1.066
CgRgd1	0.147	CgMcy1	0.455	CgCox4	-0.928
CgBsc6	0.151	CgCnb1	0.458	CgCox5B	-0.660
CgLcb1	0.155	CgSpt10	0.460	CgCox6	-1.134
CgVps25	0.156	CgRba50	0.468	CgCox8	-0.887
CgGbp2	0.159	CgPre10	0.473	CgCta1	-0.273
CgGyl1	0.162	CgDys1	0.475	CgCyt1	-0.373
CgYpt1	0.163	CgNnt1	0.475	CgDed81	-0.141
CgCne1	0.166	CgYML082W	0.476	CgDid4	-0.185
CgSpt5	0.166	CgZwf1	0.481	CgDsk2	-0.685
CgNut2	0.169	CgRib1	0.482	CgEcm21	-0.873
CgCsg2	0.171	CgPre7	0.492	CgEht1	-0.333
CgSte23	0.171	CgYop1	0.497	CgErg20	-0.163
CgSrp54	0.173	CgTrx3	0.499	CgEsp1	-2.868
CgSec4	0.174	CgSed4	0.505	CgFcy21	-0.581
CgYFR006W	0.175	CgYme2	0.515	CgFet4	-1.348
CgMeu1	0.177	CgCdc8	0.515	CgFhn1	-0.557
CgSgv1	0.177	CgSca7	0.516	CgFpr1	-0.168
CgOsm1	0.179	CgYcf1	0.518	CgFpt1	-0.236
CgSrp101	0.179	CgYDR248C	0.521	CgFzo1	-0.483
CgLap2	0.180	CgRpn12	0.522	CgGcd11	-0.157
CgHir1	0.181	CgInm2	0.524	CgGcv1	-0.839
CgHch1	0.186	CgYKR018C	0.525	CgGcv2	-0.701
CgCat2	0.188	CgCwp2	0.527	CgGip2	-0.291
CgRpn7	0.193	CgPyp1	0.530	CgGlm6	-0.269
CgTaf3	0.193	CgLys4	0.536	CgGln1	-0.303
CgEmc2	0.194	CgMbf1	0.538	CgGln4	-0.445
CgKes1	0.196	CgTmn2	0.538	CgGlo3	-0.130

CgPbr1	0.196	CgGsf2	0.549	CgHal5	-0.511
CgGas1	0.197	CgPam17	0.549	CgHir2	-0.366
CgUbr1	0.199	CgCuz1	0.550	CgHis4	-0.670
CgMrx18	0.200	CgSft2	0.556	CgHmt1	-0.200
CgCaj1	0.200	CgZta1	0.563	CgHrp1	-0.408
CgDpb4	0.202	CgNpy1	0.565	CgHsh49	-0.200
CgTfb5	0.202	CgCtf18	0.567	CgHtb2	-0.206
CgRpd3	0.202	CgShq1	0.578	CgIdh1	-0.254
CgNma1	0.203	CgMet30	0.586	CgImg1	-0.679
CgYGR130C	0.203	CgIfa38	0.586	CgKin4	-0.244
CgUbx4	0.203	CgSak1	0.587	CgLat1	-0.279
CgAos1	0.205	CgJem1	0.588	CgLip5	-0.584
CgDpm1	0.209	CgGrx8	0.594	CgLpd1	-0.292
CgInp51	0.209	CgPre8	0.600	CgLpx2	-0.249
CgKre2	0.212	CgRfs1	0.601	CgMae1	-0.501
CgClp1	0.213	CgMp65	0.627	CgMdh1	-0.380
CgCin8	0.214	CgScs7	0.630	CgMic10	-0.604
CgRpb2	0.215	CgOye2	0.633	CgMic60	-0.529
CgPim1	0.215	CgFmp52	0.645	CgMmt1	-0.371
CgMgm1	0.219	CgInp53	0.653	CgMrm1	-1.249
CgAro3	0.220	CgUfo1	0.655	CgMrp1	-0.321
CgXrn1	0.221	CgYps1	0.668	CgMrp10	-0.581
CgRet3	0.221	CgFub1	0.703	CgMrp17	-0.261
CgSec14	0.224	CgBos1	0.754	CgMrp21	-0.388
CgSte50	0.227	CgHlj1	0.762	CgMrpl10	-0.451
CgPlp1	0.228	CgNop15	0.778	CgMrpl13	-0.318
CgEug1	0.232	CgErv1	0.780	CgMrpl15	-0.451
CgVma2	0.232	CgSnq2	0.830	CgMrpl20	-0.524
CgPup2	0.232	CgPup1	0.865	CgMrpl22	-0.812
CgPup3	0.233	CgRex4	0.892	CgMrpl27	-0.539
CgSec24	0.236	CgApe1	0.896	CgMrpl37	-0.225

CgTaf7	0.236	CgPdr16	0.955	CgMrpl40	-0.845
CgYMR027W	0.236	CgAdh6	0.971	CgMrps12	-0.870
CgNpa3	0.238	CgHfd1	1.000	CgMrps16	-0.791
CgKel3	0.240	CgPir3	1.254	CgMrps18	-0.603
CgCak1	0.243	CgAdh6	1.302	CgMrps35	-0.645
CgYER152C	0.244	CgPdh1	1.369	CgMrps8	-0.413
CgMpd2	0.246	CgCca1	1.422	CgMrps9	-1.226
CgRnr4	0.246	CgYML131W	1.561	CgMrx3	-0.453
CgYMR099C	0.249	CgPup1	2.332	CgMsc3	-0.278
CgGlo4	0.250			CgMss51	-0.196
CgPff1	0.251			CgMtd1	-0.452
CgYDL124W	0.251			CgNam9	-0.715
CgAro1	0.252			CgNdi1	-0.270
CgBna3	0.252			CgNup85	-0.181
CgZds1	0.253			CgOxa1	-0.182
CgUbp6	0.261			CgPdb1	-0.697
CgSnf4	0.263			CgPds5	-0.366
CgIst3	0.264			CgPex23B	-0.338
CgReh1	0.266			CgPnc1	-0.327
CgSfl1	0.267			CgPyc2	-0.427
CgErg6	0.267			CgQcr7	-1.285
CgPrp45	0.268			CgRcf2	-0.650
CgYap3	0.271			CgRck2	-0.440
CgYme1	0.271			CgRod1	-0.497
CgTma23	0.272			CgRpl10	-0.190
CgPho88	0.275			CgRpl19A	-0.207
CgLap3	0.276			CgRpl22A	-0.189
CgGlr1	0.276			CgRpl26A	-0.232
CgArp2	0.277			CgRpl42B	-0.267
CgPre6	0.282			CgRpp0	-0.171
CgYDR391C	0.283			CgRpp1A	-0.224

CgRpt6	0.283			CgRps0	-0.106
CgRpt3	0.285			CgRps14B	-0.173
CgFun30	0.285			CgRps19A	-0.176
CgGas1	0.289			CgRps21	-0.279
CgSrm1	0.289			CgRps25A	-0.291
CgPtk2	0.290			CgRps9A	-0.301
CgHbs1	0.293			CgRsm18	-1.767
CgTml25	0.295			CgRsm23	-0.427
CgSec66	0.296			CgRsm28	-1.020
CgSsd1	0.296			CgScm4	-0.443
CgUbp1	0.296			CgSdh6	-0.751
CgRpn11	0.296			CgSdo1	-0.699
CgSec62	0.296			CgShm1	-0.425
CgSsl2	0.298			CgShm2	-0.507
CgMak3	0.299			CgSmt3	-0.462
CgPdr17	0.300			CgSrc1	-0.527
CgTfb4	0.302			CgSso2	-0.309
CgVrg4	0.303			CgSws2	-1.239
CgHrd3	0.306			CgTef1	-0.078
CgAge2	0.307			CgThr4	-0.052
CgSdt1`	0.310			CgTif3	-0.160
CgTaf2	0.310			CgTim17	-0.263
CgCub1	0.311			CgTim21	-0.273
CgBol2	0.313			CgTim44	-0.078
CgBsd2	0.314			CgTog1	-1.306
CgRvb2	0.317			CgTpo4	-0.579
CgKar2	0.318			CgTps2	-0.413
CgYar1	0.320			CgTrp4	-0.093
CgOrc5	0.321			CgTuf1	-0.286
CgRpt5	0.324			CgTup1	-0.093
CgKre5	0.324			CgTwf1	-0.964

CgCue4	0.324			CgUra4	-0.158
CgYPL247C	0.327			CgUsa1	-0.894
CgTcb3	0.327			CgUtp5	-0.166
CgPga3	0.327			CgVas1	-0.045
CgRpn1	0.329			CgVps10	-1.898
CgYnd1	0.330			CgYBR242W	-0.622
CgSec27	0.331			CgYhm2	-0.896
CgGpi12	0.333			CgYHR020W	-0.194
CgRpn9	0.333			CgYLR456W	-0.151
CgUfd1	0.334			CgYnk1	-0.291
CgCdc43	0.334			CgZrg17	-0.104
CgPre4	0.337			CgCox12	-0.891
CgRtp1	0.340			CgDur1:2	-0.726
CgEcm15	0.342			CgEno1	-0.470
CgCog1	0.345			CgGsp1	-0.373
CgRpt2	0.347			CgHtb2	-0.175
CgMas1	0.350			CgIdh2	-0.252
CgRsc3	0.350			CgOla1	-0.138
CgYPR127W	0.354			CgPho4	-0.291
CgRpn6	0.354			CgRpl13A	-0.251
CgCcr4	0.356			CgRpl36A	-0.139
CgPre9	0.358			CgRpl4B	-0.191
CgEfm1	0.358			CgRps13	-0.180
CgApe4	0.362			CgRps16	-0.206
CgHem12	0.363			CgRps16SA	-1.506
CgVma4	0.364			CgRps17B	-0.252
CgYBR137W	0.365			CgRps28B	-0.130
CgYDR476C	0.368			CgRps6A	-0.170
CgAto3	0.369			CgSdh1	-0.669
CgAim2	0.373			CgSsa3	-0.383
CgRpt4	0.374			CgTps1	-0.404

CgMed1	0.375			CgUbp3	-0.178
CgSmd2	0.377			CgVig9	-0.108
CgYIL108W	0.378			CgYhb1	-0.629

A list of 300 significantly upregulated proteins and 134 significantly downregulated proteins were identified in the *Cguls2ΔCguls1Δ* mutant in comparison with the wild type.

<i>Cguls2ΔCguls1Δ</i>					
Upregulated Proteins				Downregulated Proteins	
Protein name	log2 fold change	Protein name	log2 fold change	Protein name	log2 fold change
CgYMR099C	0.049	CgMdh1	0.275	CgVps10	-2.153
CgImd4	0.058	CgRpc34	0.277	CgRps16	-1.521
CgSec27	0.079	CgDis3	0.280	CgEsp1	-1.191
CgSec72	0.084	CgYta12	0.280	CgDsk2	-1.171
CgSec26	0.086	CgCdc24	0.280	CgTwf	-0.928
CgOsm1	0.089	CgCym1	0.281	CgUsa1	-0.911
CgPtc5	0.102	CgRrp8	0.281	CgYpi1	-0.772
CgArf2	0.103	CgRai1	0.282	CgFet4	-0.665
CgLcb1	0.103	CgPdr13	0.282	CgSmt3	-0.656
CgPhb1	0.107	CgYpt1	0.283	CgTps2	-0.619
CgCys4	0.114	CgBud22	0.285	CgSod1	-0.614
CgSui1	0.120	CgIdh1	0.287	CgSdo1	-0.584
CgBig1	0.121	CgVrg4	0.289	CgPsa1	-0.580
CgTom71	0.121	CgSec62	0.289	CgYMR196W	-0.565
CgTsh1	0.124	CgSfl1	0.290	CgLip5	-0.556
CgGcn1	0.126	CgUtp22	0.290	CgYBR238C	-0.536
CgMet12	0.127	CgHir1	0.291	CgTup1	-0.531
CgCwh43	0.127	CgLip1	0.293	CgIxr1	-0.471
CgSly1	0.131	CgInp51	0.293	CgFcy21	-0.470
CgSec31	0.132	CgSec66	0.295	CgRpl28	-0.456

CgLap2	0.136	CgSdt1	0.295	CgPnc1	-0.455
CgRvb1	0.136	CgRqc2	0.296	CgHtb2	-0.454
CgSrp14	0.140	CgMak3	0.297	CgClu1	-0.447
CgArc1	0.142	CgGly1	0.297	CgShm2	-0.440
CgNat1	0.145	CgPdr16	0.298	CgHmo1	-0.438
CgShs1	0.146	CgRio2	0.299	CgHis4	-0.433
CgHom3	0.149	CgYDR391C	0.301	CgGln1	-0.421
CgCog3	0.149	CgBet3	0.305	CgRpl19A	-0.419
CgCdc10	0.151	CgGsh1	0.305	CAGL0A02299g	-0.403
CgSla2	0.153	CgMrp17	0.305	CgDap1	-0.388
CgKap123	0.155	CgDph2	0.306	CgHtb2	-0.380
CgYMR027W	0.155	CgSmm1	0.308	CgSlf1	-0.360
CgRna1	0.160	CgYGR130C	0.315	CgMaf1	-0.354
CgUbx4	0.160	CgMcd4	0.316	CgCcw22	-0.338
CgHom2	0.162	CgNup53	0.316	CgSlc1	-0.331
CgMnp1	0.164	CgExg1	0.317	CgHem1	-0.321
CgTif35	0.167	CgPga3	0.318	CgTpm2	-0.321
CgTrp3	0.167	CgNip7	0.318	CgCta1	-0.318
CgCbp3	0.168	CgGpn3	0.320	CgBmt3	-0.318
CgNus1	0.169	CgCue4	0.320	CgMtd1	-0.317
CgMyo4	0.169	CgNar1	0.322	CgRpl31	-0.316
CgSnq2	0.171	CgBre1	0.324	CgPin4	-0.301
CgElp3	0.171	CgMed1	0.325	CgRps21	-0.299
CgPdx1	0.176	CgIml2	0.325	CgRps17B	-0.290
CgExg2	0.178	CgBmt2	0.325	CgHhoA	-0.289
CgCdc37	0.178	CgTaf7	0.326	CgPir5	-0.286
CgKin4	0.178	CgHsp78	0.327	CgRpl25	-0.284
CgMbf1	0.179	CgVps75	0.328	CgYGL082W	-0.281
CgMft1	0.180	CgPfs2	0.328	CgScm4	-0.281
CgMkt1	0.181	CgTaf3	0.329	CgRpl23B	-0.279
CgGas1	0.181	CgCox17	0.331	CgRpp1A	-0.271

CgRrt12	0.182	CgYJL193W	0.332	CgRpl4B	-0.268
CgYER152C	0.183	CgMix17	0.334	CgRpl13A	-0.265
CgCdc34	0.184	CgMeh1	0.338	CgRpl42B	-0.262
CgRer1	0.185	CgNop2	0.341	CgRps25A	-0.253
CgPpg1	0.185	CgMak21	0.344	CgYLR456W	-0.252
CgYDL124W	0.186	CgPet54	0.345	CgEpl26A	-0.251
CgGcd1	0.193	CgIst3	0.346	CgRpl27B	-0.243
CgAim2	0.193	CgSte50	0.348	CgRps14B	-0.242
CgSto1	0.194	CgYpt7	0.348	CgHal5	-0.240
CgTfg2	0.194	CgMis1	0.349	CgGlt1	-0.239
CgKel3	0.194	CgHpm1	0.349	CgRpl11A	-0.232
CgSgv1	0.196	CgNop6	0.349	CgRbl2	-0.229
CgTif5	0.197	CgRpc17	0.350	CgRps16	-0.220
CgYar1	0.199	CgHbs1	0.350	CgNam8	-0.211
CgMpd2	0.200	CgUtp23	0.350	CgTif3	-0.208
CgSrp54	0.200	CgPrp43	0.354	CgAdp1	-0.208
CgMrps9	0.201	CgKap104	0.359	CgVph2	-0.208
CgMet18	0.203	CgNar1	0.359	CgRpl36A	-0.206
CgSrm1	0.203	CgRet1	0.361	CgAsc1	-0.203
CgZpr1	0.203	CgApe4	0.361	CgRps4B	-0.199
CgRrp40	0.207	CgAcl4	0.365	CgFpr1	-0.199
CgMgm1	0.207	CgRpa135	0.366	CgRpl10	-0.198
CgPmt7	0.209	CgErc1	0.369	CgChz1	-0.196
CgGin4	0.209	CgUra6	0.369	CgRpl15A	-0.192
CgLsg1	0.210	CgTfb4	0.370	CgTup1	-0.191
CgErp2	0.210	CgSit1	0.371	CgReb1	-0.190
CgSnu71	0.211	CgUtp13	0.374	CgMnt3	-0.186
CgRmd9	0.211	CgEcm4	0.378	CgTim13	-0.184
CgTaf9	0.212	CgHrr25	0.380	CgAdk1	-0.181
CgCat2	0.214	CgSdh5	0.380	CgRpl34B	-0.179
CgNew1	0.215	CgCog1	0.381	CgRps6A	-0.176

CgSup45	0.215	CgRqc1	0.383	CgRps13	-0.170
CgOxa1	0.218	CgSsl2	0.390	CgRps19A	-0.167
CgGyl1	0.220	CgFub1	0.390	CgRpl14A	-0.163
CgMrps16	0.222	CgYdj1	0.395	CgVig9	-0.157
CgGpi12	0.222	CgUtp9	0.396	CgMam3	-0.157
CgHch1	0.222	CgBud16	0.396	CgRpl37A	-0.156
CgMrs6	0.223	CgLap3	0.402	CgAbf2	-0.154
CgYNL195C	0.224	CgNrd1	0.404	CgDur1:2	-0.150
CgNip1	0.224	CgYps1	0.411	CgRpp0	-0.149
CgRet3	0.225	CgNoc2	0.412	CgGcv1	-0.144
CgInp51	0.227	CgNop16	0.413	CgArc15	-0.143
CgYLR287C	0.230	CgFyv7	0.413	CgYHR020W	-0.136
CgNot3	0.230	CgLcb4	0.418	CgAcs2	-0.133
CgCam1	0.231	CgCnb1	0.419	CgGlo3	-0.130
CgRvb2	0.231	CgTma64	0.420	CgGsp1	-0.128
CgMsh6	0.233	CgNmd3	0.421	CgVsh3	-0.123
CgPrp45	0.233	CgRci37	0.421	CgEde1	-0.107
CgArt10	0.233	CgOaf1	0.422	CgApt1	-0.097
CgPob3	0.236	CgYML082W	0.424	CgToa2	-0.094
CgZta1	0.236	CgUtp11	0.424	CgGcd11	-0.083
CgYta7	0.236	CgNep1	0.424	CgTef1	-0.082
CgGcn20	0.238	CgPbr1	0.424	CgMcm6	-0.048
CgLat1	0.238	CgKri1	0.425	CgErp5	-0.039
CgPex5	0.239	CgYDR248C	0.425	CgDef1	-0.038
CgVma4	0.240	CgSdh6	0.426	CgCuz1	-0.647
CgZds1	0.241	CgUpa2	0.428	CgPre2	-0.509
CgVma3	0.244	CgMrpl10	0.429	CgPre3	-0.566
CgIls1	0.245	CgShq1	0.432	CgPre5	-0.232
CgClp1	0.246	CgTrx3	0.432	CgPre7	-0.311
CgCdc8	0.246	CgBol2	0.434	CgPre8	-0.299
CgCdc53	0.246	CgMrpl22	0.434	CgRup1	-0.177

CgBsc6	0.246	CgYhm2	0.435	CgPup1	-0.577
CgRpp1	0.246	CgGpx2	0.436	CgPup3	-0.125
CgTrp2	0.247	CgSua7	0.437	CgRpn1	-0.387
CgMeu1	0.248	CgPam17	0.438	CgRpn2	-0.321
CgNup85	0.249	CgDbp10	0.440	CgRpn5	-0.477
CgRpo31	0.250	CgGpn2	0.440	CgRpn6	-0.403
CgTrm44	0.250	CgMct1	0.452	CgRpn9	-0.343
CgMas1	0.251	CgCpr6	0.454	CgRpn8	-0.288
CgPus7	0.252	CgHis1	0.455	CgRpn11	-0.135
CgUra4	0.253	CgRrp12	0.461	CgRpn12	-0.361
CgDld2	0.253	CgDsd1	0.469	CgRpt2	-0.210
CgArg5,6	0.253	CgRex4	0.469	CgRpt4	-0.286
CgAos1	0.253	CgNog1	0.477	CgRpt6	-0.126
CgRcl1	0.254	CgAsf1	0.480	CgUfo1	-0.416
CgTml25	0.255	CgSlx9	0.481	CgUfd1	-0.228
CgEpt1	0.256	CgKgd1	0.486	CgCdc48	-0.251
CgMrpl4	0.262	CgSpt10	0.491	CgMeh1	-0.333
CgMtr2	0.263	CgMxr2	0.502	CgMdj1	-0.417
CgPse1	0.266	CgMrx12	0.502	CgPim1	-0.209
CgPsp2	0.269	CgEfm7	0.505		
CgCak1	0.269	CgBud21	0.510		
CgDpm1	0.269	CgNma111	0.521		
CgNpa3	0.270	CgLys12	0.527		
CgNcs6	0.271	CgAyr1	0.528		
CgTma23	0.273	CgTmn2	0.535		
CgNup82	0.273	CgAtp3	0.669		
CgPan1	0.274	CgRpc25	0.537		
CgSnf1	0.275	CgEar1	0.540		
CgTmt1	0.726	CgNap1	0.541		
CgNre1	0.819	CgLrs4	0.546		
CgAdh6	0.837	CgVti1	0.557		

CgNop15	1.038	CgAde5,7	0.150		
CgPir3	1.178	CgSed4	0.562		
CgCwp2	1.293	CgUtp20	0.580		
CgCha1	1.351	CgLys4	0.609		
CgCca1	1.531	CgPpz2	0.620		
CgUpt6	0.479				

9.7 LIST OF PUBLICATIONS

- **Gupta, D.;** Garapati, H. S.; Kakumanu, A. V. S.; Shukla, R.; Mishra, K. SUMOylation in Fungi: A Potential Target for Intervention. *Comput Struct Biotechnol J* **2020**, *18*, 3484–3493. <https://doi.org/10.1016/j.csbj.2020.10.037>.

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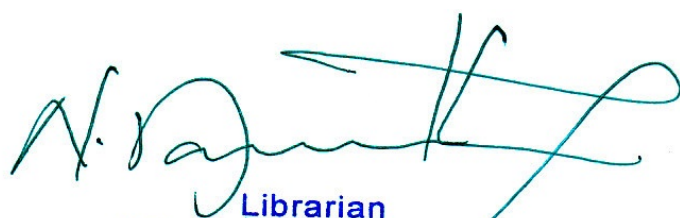
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