Synopsis of the thesis on

Probing Intricacies of Cell Cycle Progression and Morphogenesis in *Candida albicans* and Their Relation to Ubiquitin Mutations

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By

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To
The Registrar (Academic Section),
The M. S. University of Baroda,
Vadodara -390 002.

Subject: Submission of synopsis of the Ph.D. work entitled– "Probing Intricacies of Cell Cycle Progression and Morphogenesis in *Candida albicans* and Their Relation to Ubiquitin Mutations"

Respected Sir/Madam,

Kindly accept the synopsis of my Ph.D work entitled–**"Probing Intricacies of Cell Cycle Progression and Morphogenesis in *Candida albicans* and Their Relation to Ubiquitin Mutations"**.My date of registration is 15/07/2019 and registration no. is FOS/2158.

Thanking you,

Yours sincerely,

(Sandeep Kumar Dantuslia) (Prof. C. Ratna Prabha)
Guide

Head
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INTRODUCTION

Candida albicans is the most commonly found commensal organism in humans, which can undergo morphogenesis into an opportunistic pathogen. *Candida* species are naturally present in the skin, mouth, digestive tract, and urogenital systems of healthy people [1, 2]. They have evolved characteristics that enable them to infect and thrive in various environments while avoiding the host's immune response. Diseases caused by *Candida* infection are termed candidiasis and candidemia. Notably, a bloodstream infection by *C. albicans* is the fourth leading serious condition seen in those who are immunocompromised [1, 3, 4]. *C. albicans* can adopt yeast, pseudohyphal, and hyphal growth forms [5, 6]. *C. albicans* infections are showing increased resistance to current anti-fungal medications, an observation that emphasizes on the urgency to discover new treatment targets and develop innovative antibiotics. In eukaryotic cells, ubiquitination is crucial in regulating vital functions, that include cell cycling, transcription, translation, and coping with stress.

Ubiquitin (Ub) is a small protein, with seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) in its primary structure. The C-terminal glycine of ubiquitin is conjugated to lysine side chains of substrate proteins via sequential actions carried out by three enzymes, a process that requires ATP in the first step. Initially, ubiquitin is activated by an enzyme known as ubiquitin-activating enzyme (E1), which is associated with ATP hydrolysis. This activated ubiquitin is then bound to the enzyme's active site by forming a thioester bond between ubiquitin and E1. Subsequently, ubiquitin is transferred to the second enzyme, ubiquitin-conjugating enzyme (E2), where ubiquitin once again is bound by a thioester linkage. Lastly, the target substrate is identified and bound by the third enzyme, ubiquitin ligase (E3). In the final step, ubiquitin bound to E2 is either directly transferred to the substrate or initially to E3 and subsequently to the substrate. This series of events can be repeated between substrate-conjugated ubiquitin and free ubiquitin molecules, leading to the formation of a polyubiquitin chain. The lysine residues of ubiquitin used in polyubiquitin determine the topology of the branches and the resultant signaling. For instance, K48 linked polyubiquitin chain typically serves as a signal for the degradation of the substrate by the 26S proteasome.

Conformation of ubiquitin protein and its sequence showcase evolutionary conservation, with only about three alterations in its seventy-six amino acid residues from unicellular eukaryotes to both animals and plants [7-9]. In earlier studies, to understand the relationship between the conserved primary structure of ubiquitin and the multiple cellular

functions in which it serves as a posttranslational modification, mutants of ubiquitin were generated and examined in *Saccharomyces cerevisiae* [10-12]. Studies on the effects of ubiquitin mutations like UbEP42 and the four mutations UbS20F, UbA46S, UbL50P and UbI61T derived from it led to several interesting observations, in *S. cerevisiae*. Interestingly, the mutations UbEP42, UbL50P, and UbI61T negatively influenced the growth and various other functions in *S. cerevisiae* [13, 14]. The rich repository of information available on *S. cerevisiae* positions it as an excellent initial model for hypothesis testing before exploring similar queries in a more complicated yeast such as *C. albicans*, an opportunistic human pathogen [15, 16]. Both *C. albicans* and *S. cerevisiae* trace back to a common ancestor, which had existed over 300 million years ago. Notable parallels in the molecular details of cell cycling, mating behavior, metabolism, cell wall formation, and signaling processes have been thoroughly documented [3]. However, CUG codon of *S. cerevisiae* encodes Leucine (Leu), while the same codon encodes Serine (Ser) in *C. albicans*. To date little has been reported about ubiquitin regulation and its relation to morphological switching in *C. albicans*. Ubiquitin mutations studied in *S. cerevisiae* have been employed to gain insights into cell cycle dynamics and morphogenesis in *C. albicans*, especially concerning the transition from yeast to hyphal forms, an essential step in pathogenesis. However, the CUG codon in the gene at 56^{th} position was replaced by UUG to have Leucine (Leu) in its respective position. The BWP17 strain of *C. albicans* (ura3/ura3 his1Δ/his1Δ arg4Δ/arg4Δ), which is a derivative of CAI4 provided by Prof. Yue Wang from IMCB Singapore, was utilized to study the functional changes produced by ubiquitin mutations in cell cycle progression and morphogenesis.

The BWP17 of *C. albicans* strain lacks virulence and possesses a full complement of genes that express ubiquitin proteins. In *C. albicans*, ubiquitination is vital for pathogenesis [17]. This organism has two documented ubiquitin genes, UBI3 and UBI4 [18]. The UBI3 gene produces a fusion protein combining ubiquitin and a ribosomal protein, and it closely resembles the UBI3 gene found in *S. cerevisiae*. On the other hand, the UBI4 gene product is polyubiquitin with three sequential ubiquitin repeats without spacers. When this fusion protein undergoes post-translational processing, it yields ubiquitin monomers. Interestingly, the expression profiles of these genes from *C. albicans* align with those of the ubiquitin genes in *S. cerevisiae* [19, 20]. Additionally, in *C. albicans*, the Fkh2 mitotic transcription factor modulates the cyclin B level. This factor is analogous to the Phd, Sok2, and StuA transcription factors, which influence the activity of secretory aspartyl proteases in *Aspergillus* species. However, there are no reports on the role of Fkh2 in the regulation of

aspartyl protease activity under influence of ubiquitnation in *C. albicans.* A significant number of genes that typically see increased activity during hyphal growth and pathogenesis were observed to have decreased activity in the fkh2ΔΔ and fkh2(6A) mutants. This includes the SAP4 and SAP6 aspartyl protease genes, which were notably down regulated in the fkh2ΔΔ mutants [21-22]. Historical research has also shown that individuals with conditions like HIV, or cancer, or those undergoing radiotherapy for the head and neck tend to be more susceptible to candidiasis. This is possibly due to alterations in the pH of saliva of these patients.

Key question addressed

- ✓ **What would be the effect of ubiquitin mutations on cell cyclins in different forms and stages of *C. albicans* life cycle?**
- ✓ **What would be the effect of ubiquitin mutations on the secretory proteases associated with pathogenesis of *C. albicans*?**
- ✓ **What would be the effect of ubiquitin mutations on the transcription factors and transcription inhibitors of *C.albicans,* which are known to influence cell cycling and morphogenesis?**
- ✓ **Whether mutant forms of ubiquitin serve as substrates and form polyubiquitin chains, when there is a di- glycine deletion at the c- terminus?**

Proposed objective

Objective-1: To study the cell cycle progression and morphology switching in *C. albicans* expressing ubiquitin mutations.

Objective-2: Status of cyclins in the various stages of cell cycle in *C. albicans* expressing ubiquitin mutations.

Objective-3: To Study the regulation transcription factors involved in cell cycle and morphogenesis in *C. albicans* expressing ubiquitin mutations.

Objective 4: Generating di-glycine deletions of ubiquitin mutants to understand the mechanism of polyubiquitin chain formation in *C. albicans* expressing ubiquitin mutations.

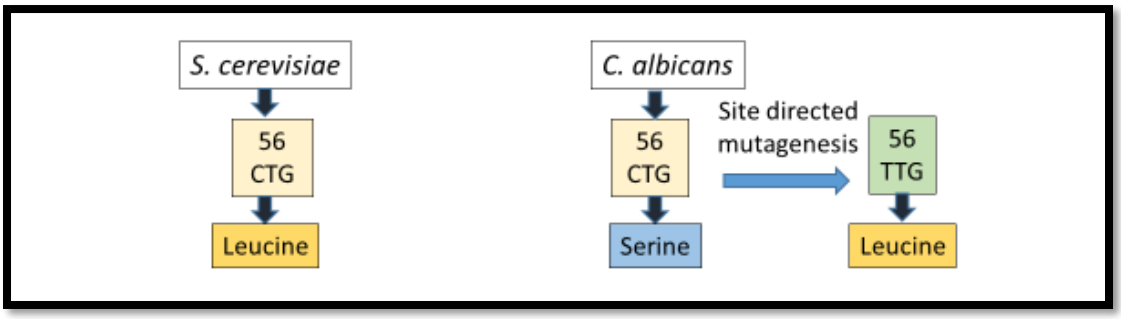
Objective-1: To study the cell cycle progression and morphology switching in *C. albicans* expressing ubiquitin mutations and to understand the effects of ubiquitin mutations on A) Thermotolerance B) Antibiotic Resistance C) Secretion of aspartyl protease activity.

1.1 Site-directed mutagenesis for generating mutant forms of ubiquitin gene & cloning in pRC2312 vector.

In the present study pRC2312 vector, with a constitutive promoter was used for cloning wild type ubiquitin gene and its mutant forms, which encode mutant proteins (UbEP42, UbS20F, UbA46S, UbL50P and UbI61T). The vector was provided by Prof. Richard D. Canon from University of Otago, New Zealand. As mentioned CUG codon encodes 'Serine' in *C. albicans*, instead of the desired 'Leucine'. Hence, site directed mutagenesis was performed to convert CTG to TTG nucleotide sequence at the 56th codon position in ubiquitin gene. The gene and its mutant forms were amplified by PCR and cloned into pRC2312 vector. Restriction digestion of the vector was used to establish successful cloning of ubiquitin wild type gene and its mutant forms and subsequently sequencing was used for confirmation.

The sequences of primers used are as follows:

Ub Forward primer: 5'	*GCTAAGCTTATGCAGATCTTCGTCAAGACG3'*
Ub Reverse mutagenic primer:	*5'GTTGTAATCAGACAACGTTCTACCGTCCTC3'*
Ub Reverse primer:	*5' GATGGATCCTCAACCACCTCTTAAGA3'*
Ub Forward mutagenic Primer	*5'GAGGACGGTAGAACGTTGTCTGATTACAAC3'*
Ub Forward primer:	*5' CTAAGCTTATGCAGATCTTCGTCAAGACG3'*
Ub Reverse mutagenic primer:	*5'GTTGTAATCAGACAACGTTCTACCGTCCTC3'*



1.2 Cloning of mutant forms of ubiquitin gene in pTET25M-NC vector and expression in BWP17 strain of *C. albicans* for functional analysis.

pRC2312 vectors with inserts of ubiquitin gene are double digested with *Hind III* and *Bam HI* restriction enzymes to release the wild type and each of the mutant forms of the gene with sticky ends. Double-digested fragments of the inserts are then ligated to pTET25M-NC vector for functional study in the BWP17 strain of *C. albicans*. The plasmid pTET25M-NC is an inducible vector with a doxycycline-inducible promoter and a 5' *gfp* tag. Genes of interest cloned in the vectors were confirmed by restriction digestion and sequencing. The genes for the wild type and all the mutant forms are successfully cloned in the pTET25MN-C vector under Tet (doxycycline) inducible promoter.

1.3 To check dosage dependent lethality & survival capacity of BWP17 strain of *C. albicans* cells expressing mutant forms of ubiquitin.

The proteins of ubiquitin wild type and mutant forms were expressed with GFP tag in the N-terminus. Further we analysed the dosage dependent lethality in BWP17 strain. The mutants UbEP42, UbL50P, and UbI61T exhibit lethality based on dosage at a low inducer concentration of 45 µg/ml. The other two mutant forms UbS20F and UbA46S were tolerated with no negative effects on survival.

1.4 To study the influence of stress on BWP17 strain of C. albicans cells expressing mutant forms of ubiquitin:

(A) Effect of ubiquitin mutations over thermotolerance

To study thermotolerance effect on the cells of *C. albicans* BWP17 strain transformed with wild type and mutant forms of ubiquitin. The expression of the wild type and mutant forms of

ubiquitin present on the plasmid vector was driven by doxycycline inducible TET1 promoter. Cells were allowed to grow in the presence of sublethal concentration of the inducer. Growth profile was monitored by measuring OD at 600 nm at regular intervals of 2 hours and plated in the presence of 45 μg/ml doxycycline. The plates were incubated at 30^0C, 40^0C and 55^0C for 2, 4, 8, 12 and 16 hrs., which was followed by 3-4 days of incubation at 30^0C. Percent Survival was calculated by counting colonies formed. Studies revealed that UbEP42, UbL50P and UbI61T displayed slow growth, reduced survival and impaired thermotolrence. At 55ºC after one hour of incubation complete loss of survival capacity was observed with no growth observed on the plates.

(B) Effect of ubiquitin mutations over antibiotic stress tolerance

C. albicans wild type cells cannot withstand high levels of antibiotic stress. In order to study the effect of ubiquitin mutations on antibiotic stress survival, 400 μg/ml cycloheximide, 1 μg/ml canavanine and 1 μg/ml tunicamycin were used. The results of antibiotic exposure reveal that cells expressing UbS20F and UbA46S exhibited no changes in their survival and behaved like the wild type. Notably, when exposed to varying dosages, transformants that express the lethal forms of ubiquitin mutations UbEP42, UbL50P, and UbI61T showed greater degree of sensitivity to antibiotics.

(C) Secretory Aspartyl protease activity at different pH values

Various studies in the past observed that under immunocompromised conditions, HIV patients and cancer patients undergoing head and neck radiotherapy become more susceptible to oral candidiasis. This may be due to changes in the pH of patients' saliva [27]. The present study addressed the regulation of aspartyl protease secretion under different pH conditions, with or without ubiquitin mutations in the background. The regulation of aspartyl protease secretion under four different pH conditions was investigated with cells expressing wild type and ubiquitin mutations. It was observed that the wild type and UbS20F and UbA46S secreted copious amounts of protease at pH values 6.3, 6.8, and 7.1. However, cells expressing UbEP42, UbL50P and UbI61T showed the presence of very low amounts of protease at the same pH values as above. Interestingly, at pH 5.9 cells expressing wild type and all five types of ubiquitin mutations under consideration here were found to secrete insignificantly low amounts of protease. Therefore, to understand the dependence of morphological changes in *C. albicans* over pH, cultures of *C. albicans* expressing wild type ubiquitin were grown under different pH with YPD medium and 10% bovine calf serum for 6

hr at 37°C. The cultures of BWP17 strain expressing wild type ubiquitin exhibited more defects in hyphal growth at pH 5.9 and the cells remained in yeast bud form and morphological switching to hyphal form failed to occur in spite of the growing in the presence of serum. While, the cells of BWP17 at the other pH conditions grew as both pseudohyphal and true hyphal filaments. Hence, it can be concluded that at acidic pH *C. albicans* finds it hard to penetrate host tissues due to low level of aspartyl protease secretion.

1.5 To check the morphogenesis from yeast to hyphal state under the effect of mutant ubiquitin proteins

C. albicans cells expressing ubiquitin mutations UbEP42, UbL50P and I61T no longer change their form from yeast to hyphae, which results in a loss of their infective capability. Confocal analysis results show that the mutation UbEP42 and its segregated single mutations UbL50P and UbI61T hampered yeast to hyphal transition and cells showed signs of budding with constriction occurring at the site of septation under serum induced conditions.

1.5.1. To check the chitin deposition on C. albicans cell wall under the effect of mutant ubiquitin proteins

In *C. albicans*, the PKC and HOG MAP kinase cascades and the Ca^{2+}/calcineurin pathway regulates *CHS* gene expression and chitin synthesis in response to cell wall stresses. Coordinated synthesis of chitin also requires the localization of the enzymes to be regulated throughout the cell cycle [28]. Therefore, investigation of accumulation of chitin on *C. albicans* cells was examined by staining them with Calcofluor white fluorochrome subsequent to the expression of a ubiquitin mutations. Uneven staining on the cell wall was noticed, suggesting varying thicknesses of the chitin layer on the yeast cells expressing UBEP42, UbL50P, and UbI61T. Additionally, the confocal microscopic images captured of the mutants UBEP42, UbL50P, and UbI61T showed the formation of cell aggregate, while the rest including wild type showed separate cells.

1.5.2. To study β-glucan exposure and cell surface of *C. albicans* under the effect of mutant ubiquitin proteins

It has been previously reported that the presence of β-glucan is influenced by the CEK1-mediated MAPK pathway. Moreover, Hsp90 and the proteasome impact morphogenesis via the cAMP-PKA pathway in *C. albicans* [30]. For analyzing whether there

is any change in β-glucan exposure on cell surface due to ubiquitin mutations, Congo red fluorochrome staining was done. The stain binds with (1,3)-β-glucan, causing the fungal cell wall to emit a red fluorescence when viewed under a microscope. Interestingly, it was discovered that yeast cells with UbEP42, UbL50P, and UbI61T mutations displayed a significantly increased β-glucan content in their wall, when compared to cells expressing wild type, UbS20F and UbA46S. There are numerous studies on the effect of compounds like the azole derivative drugs in exposing the β-glucan layer. In the present study, β-glucan exposure was observed after overexpression of ubiquitin mutations even the absence of azole drugs. Further SEM technique was used to visualize the cell wall surface. The *C. albicans* cell wall in the control culture was clearly smooth and uniform. Overexpression of UbEP42 by adding inducer doxycycline 45 μg/mL displayed a disturbance in the integrity of the fungal cell wall. The extent of β-glucan exposure was less in UbL50P and UbI61T mutations.

Objective-2: Status of cyclins in the various stages of Cell cycle in *C. albicans* expressing ubiquitin mutations.

2.1 Cell cycle analysis of yeast and hyphal forms of *C. albicans* expressing ubiquitin mutations.

To assess the impact of ubiquitin mutations on cell cycle, FACS analyses were done with the wild type and five ubiquitin mutants in the presence and absence of serum. Initially, in yeast state in the absence of serum FACS analysis was performed. BWP17 and BWP17/UbWt cells served as positive controls. The findings show that yeast cells UbEP42, UbL50P, and UbI61T were halted in the G0/G1 phase, resulting in a delayed transition into the S phase. Conversely, BWP17/UbS20F and BWP17/UbA46S progressed through the S phase in a manner similar to the positive controls. This hints at possible disruption of interaction between yeast cyclins and Cdc28 complex in the background of the mutant ubiquitin. Further, from the western blot analysis of Cdc28 it was found UbEP42, UbL50P and UbI61T were causing decline in protein level of Cdc28 protein kinase, leading to G1 phase arrest and delayed entry into S phase.

2.2 Studies on cyclins at transcript and protein levels associated with the yeast and hyphal forms of *C. albicans* while expressing ubiquitin mutations.

It was reported earlier that decrease of Cdc28 level causes hyphal formation [28]. We have already observed that dosage dependent lethal mutations of ubiquitin hamper yeast to

hyphal transition. However, the effect ubiquitin mutations over the levels of Cdc28 and cyclins have not been investigated. During the growth phase of yeast, cell shape closely correlates with the progression of the cell cycle due to the synchronized function of the Cdc28 and cyclin complexes in C. albicans. In the G1 phase of the C. albicans cell cycle, the G1 cyclins, Ccn1 and Cln3, are produced. When these cyclins bind with Cdc28, they facilitate apical growth and bud stretching **[23,24]** As the cell cycle moves forward, the G1 cyclins break down and are succeeded by the G2 cyclins, Clb2 and Clb4. These cyclins then connect with Cdc28, inhibiting polarized growth and promoting even bud growth **[25].** This harmonized transition between bud stretching and its suppression results in the creation of fully distinct, oval-shaped mother cell and daughter buds. Consequently. Any imbalance in the synthesis or breakdown of cyclins can lead to significant alterations in cell shape, as observed by Berman in 2006[26]. The mRNA levels Cdc28 and cell cycle stages regulatory cyclins were monitored in cells expressing wild type and mutant forms of ubiquitin. It was observed that G1 cyclins Ccn1 and Cln3 were not degraded in UbEP42, UbL50P and UbI61T, instead they were upregulated and not replaced by the G2 cyclins. Since, the G2 cyclins Clb2 and Clb4 bind to Cdc28 and suppress polarized growth. In our study we also found the G2 cyclins transcript Clb2 levels upregulated and hyphal specific gene Hgc1 downregulated. Further Western blot analysis results show that the mutations UbEP42 and its segregated mutations UbL50P and I61T produced negative effects on polyubiquitination with Lys63 and Lys48 linkages. The G_0/G_1 arrest observed with the three mutants could be due to impairment in the ubiquitin mediated degradation pathway by ubiquitin mutants.

Objective-3: To Study the transcription factor regulation in *C. albicans* expressing ubiquitin mutations.

3.1 Real time PCR analysis for the detection of the levels of transcription factors and transcription inhibitors in *C. albicans* expressing ubiquitin mutations (Efg1p Nrg1p, Rbf1p, Rim101p, Fkh2p and Tec1p)

It is evident that both the cell cycle and proteome make up play key roles in the filamentation of *C. albicans*, and from the previous results it is known that ubiquitin mutants affect morphogenesis and cell cycling. Secretory aspartyl protease (SAP) secretion is a necessary step in the establishment of filamentous form of *C. albicans* in the tissues. SAP secretion was found to be affected in *C. albicans* expressing UbEP42, UbL50P and UbI61T from zymography studies. Further, in the background of ubiquitin mutations the status of the transcription factors governing morphogenesis are not known. In this study, the mRNA levels

of the transcription activators and transcription inhibitors were examined to understand the influence of ubiquitin mutations on the regulation of morphogenesis of *C. albicans*. It was reported in the literature that the transcription factor FkH2 is involved in the secretion of SAP. Hence, the mRNA level of FkH2 was monitored and found to be downregulated in UbEP42, UbL50P and UbI61T. Consequently, expression of hyphal-specific genes such as HGC1 were affected. Further, certin virulence associated transcriptional activators and repressors were selected for this study on the basis of literature, namely Efg1p Nrg1p, Rbf1p, Rim101p and Tec1p [29]. Earlier studies have indicated that for hyphal growth, the morphogenetic inhibitor Nrg1 needs to be downregulated and degraded by the kinase Sok1. This process is hindered by the transcription reppressor Cup9. However, when conditions promote filamentous growth, Cup9 is broken down by the ubiquitin ligase (E3) Ubr1, which alleviates Sok1's inhibition, leading to Nrg1's degradation and subsequently, hyphal growth [23]. The findings in the presence of ubiquitin mutations show a significant increase in Nrg1 transcript levels in UbEP42, UbL50P and UbI61T, suggesting that its upregulation keeps the cells predominantly in their yeast form, negatively impacting their ability to transition into hyphal structures in serum environment.

Earlier studies reported that *C. albicans* undergoes filamentous growth when exposed to various environmental and host-specific triggers. These signals are processed through intricate and often overlapping signaling pathways, notably the cyclic AMP protein kinase pathway, which plays a pivotal role in filamentation in response to serum [23]. Within this pathway, protein kinase A (PKA) is believed to modify the transcription factor Efg1, triggering a series of genetic responses that control morphogenesis. While Efg1 is crucial for this specific filamentation process dependent on the cAMP-PKA pathway, it is not necessary for all morphological responses. Here, upon expression of ubiquitin mutations UBEP42, UBL50P, and UBI61T, the transcript levels of Efg1 diminished. This suggests potential disruption of the central cAMP PKA signaling pathway by these mutations.

To understand the effect of ubiquitin mutations the levels of four more hyphal-associated transcription factors Rim101p, Tec1, Stp1 and Rbf1 were examined in the presence of serum. Rim101 alkaline responsive transcription regulator, which plays an important role in cell wall assembly. Tec1 and Rbf1 regulate hyphal development. Stp1 plays a crucial role in nutrient acquisition. Reduced levels of Rim101p, Tec1 and Rbf1 with the mutations UBEP42, UBL50P and UBI61T. However, the levels of Stp1, which activates transcription of genes required for extracellular proteolysis and absorption of resultant peptides did not seem to

follow any particular trend, unlike the other transcription factors in response to ubiquitin mutations, showing Stp1 levels in UbI61T comparable to that of wild type.

In C. albicans, ubiquitin-mediated proteolysis has been implicated in morphological transitions, stress response, and virulence factor regulation [31,32]. By elucidating the roles of ubiquitin in biological response pathways and metabolic processes, present study aims to broaden the understanding of virulence of C. albicans and identify novel therapeutic targets to combat fungal infections. Here ubiquitin mutations were employed to unravel the role of ubiquitin regulation in the molecular mechanisms of pathogenesis of C. albicans. Proteomics approach offers a comprehensive view of cellular protein dynamics and has been instrumental in clarifying the effects of genetic perturbations on the proteome [33].

Earlier studies from our laboratory pointed out that certain mutations of ubiquitin namely UbEP42, UbL50P, and UbI61T interfere with the cell cycle, antibiotic stress, thermotolerance, polyubiquitin chain formation, morphogenesis and virulence of *C. albicans* [34]. Hence, the same mutations were chosen here to find the differences in protein profile to pinpoint the biological and metabolic changes that contribute to morphogenesis and virulence. The combination of genetic manipulation of ubiquitin and proteomics helped in understanding the influence of ubiquitin-dependent processes over cellular physiology in *C. albicans*. UbEP42, UbL50P, and UbI61T mutants showed significant changes in protein expression. 61 individual proteins were commonly downregulated due to overexpression of the ubiquitin mutants. These downregulated proteins play crucial roles in metabolism, stress response, protein folding, and cell wall synthesis. Enzymes involved in metabolic pathways were downregulated, suggesting disruptions in fatty acid, amino acid, and carbohydrate metabolism. The downregulation of stress response proteins indicates compromised cellular stress coping mechanisms. Proteins involved in cell wall synthesis were downregulated, potentially compromising cell wall integrity and virulence. Dysregulation of these proteins could lead to increased susceptibility to host immune responses and antifungal treatments. Downregulation of molecular chaperones suggests impairment in protein folding and quality control mechanisms. Dysregulation of ubiquitin-mediated proteolysis pathways could lead to the accumulation of misfolded proteins and cellular stress. Decreased levels of forkhead transcription factor 2 may regulate the secretion of aspartyl proteases, impacting virulence. Further investigations are needed to elucidate the role of ubiquitination in protease secretion.

Downregulated proteins impact crucial pathways such as carbohydrate metabolism, stress response, and cell wall synthesis. Dysregulation of these pathways contributes to observed phenotypic changes in C. albicans. Common upregulated proteins play roles in cell cycle

regulation, ubiquitin signaling, and stress tolerance. String analysis revealed interactions among these proteins, suggesting compensatory mechanisms in response to ubiquitin mutant overexpression, twelve common upregulated proteins were identified among the ubiquitin mutants. These proteins are involved in modulating the cell cycle, ubiquitin signaling, and stress response.

Objective 4: Generating di-glycine deletions of ubiquitin mutants to understand the mechanism of polyubiquitin chain formation in *C. albicans* expressing ubiquitin mutations.

4.1 Deletion of the codons di-glycine at the C-terminus of the wild type and all mutant forms of ubiquitin by site directed mutagenesis, cloning them in pTET25-MCH vector, and transformation into BWP17 strain respectively.

As mentioned in the Introduction, the incoming ubiquitin molecule's C-terminal carboxylate is attached to the side chain amino group of lysine residue of the substrate or the last ubiquitin molecule in the growing polyubiquitin chain through an isopeptide bond. It was observed previously that the mutants were not affecting the formation of stand-alone polyubiquitin chains free of any substrate. On the contrary, the formation of substrate bound K48 and K63-linked polyubiquitins were affected. Ubiquitin mutants with di-glycine deletion at C-terminus of ubiquitin were employed to find whether the lethal-mutants of ubiquitin are bound to the substrate either at the first position preventing polyubiquitin chain formation or to previously attached ubiquitin terminating the process of elongation abruptly or both. Alternatively, do they act as substrates and a polyubiquitin chain is built over the lysine side chains of the mutant forms of ubiquitin. The ubiquitin molecules with diglycine deletion cannot bind to substrate, but can act as substrates for polyubiquitin chain formation. To understand polyubiquitination in depth the strategy of diglycine deletion was employed here. The di-glycine deletions were generated using PCR, and cloned into pTET25 M-CH vector. The diglycine deletions of the gene in wildtype and mutant forms in the vector are confirmed by restriction digestion and gene sequencing.

4.2 Western blot of individual cell lysates and determination of polyubiquitin chain formation with different linkages.

Western blot analysis of cell lysates from C. albicans expressing ubiquitin mutants lacking the di-glycine motif UbEP42GG-, UbL50GG- and UbI61TGG-) showed a notable absence of

polyubiquitin chains, suggesting that our generated ubiquitin mutant having di- glycine motif at the C- terminal play a pivotal role in impairment in polyubiquitin chain formation in c. albicans that caused to lethality, further it also suggested that individual ubiquitin mutants as a monomer not aggregated in proteome pool and may be degraded through proteasomal machinery. However interestingly we observe that our ubiquitin mutants with di-glycine motif conjugated with substrates not degraded and show aggregation of the ubiquitination pool. further, we align the Di-glycine deleted ubiquitin mutants structure model generated from trRosetta. Structural alignment was done through the RPSB PDB Mol* 3D viewer. further we investigate to check whether there is any difference in Helix, strand and Coil no. in 2D structure through trRosetta. We don't find any such difference. all the 2D structures having same no. of Helix, Strand, and Coil.

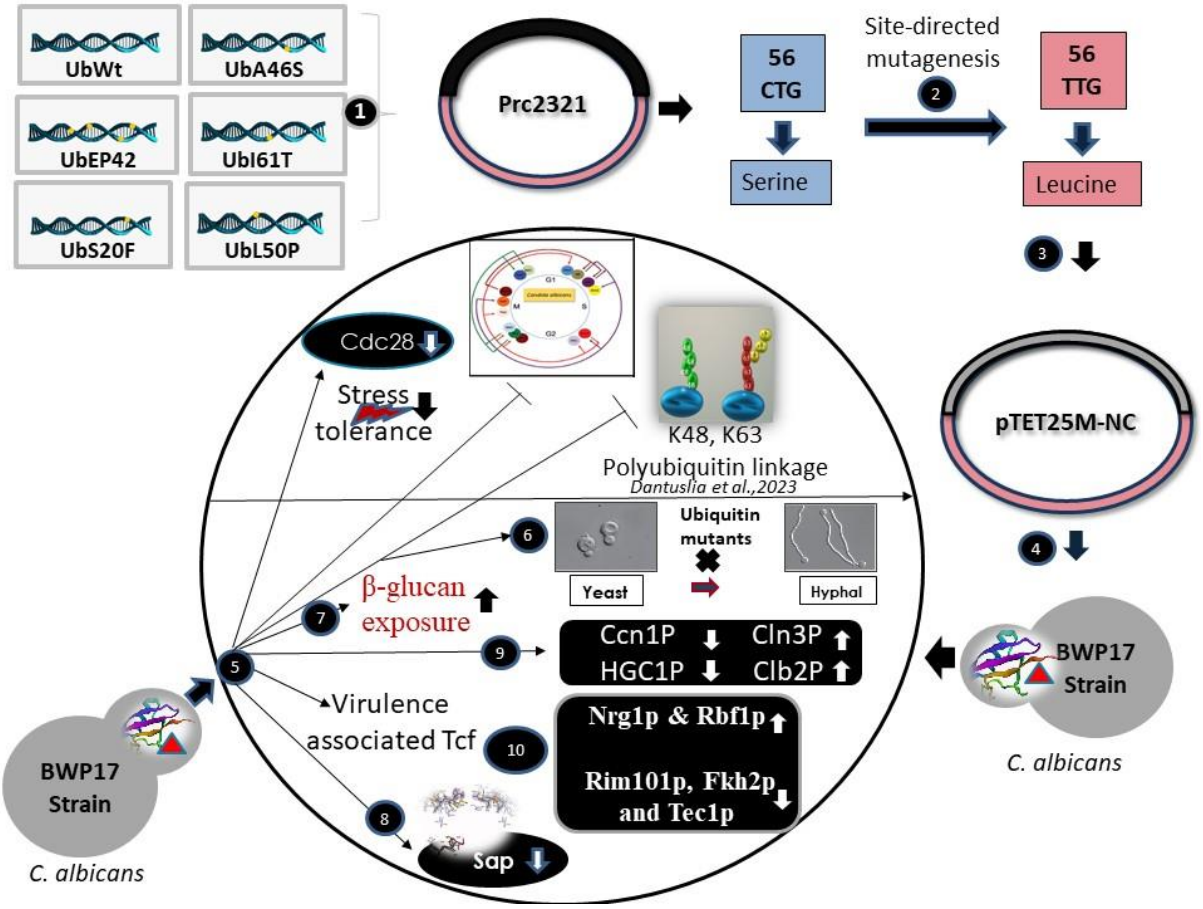
Conclusion:

The present study suggests that under the influence of mutant forms of ubiquitin - namely UbEP42, UbL50P, and UbI61T in C. albicans, leads to decreased viability, slower growth rates, prolonged doubling times, disrupted cell surface. Stress tolerance studies using translational inhibitor antibiotics suggest that UbEP42, UbL50P, and UbI61T expressing cells could not withstand antibiotic stress. Further the mutations affected morphogenesis from yeast to hyphal form. These lethal mutants hamper G1 to S transition during cell cycle because of: (i) alteration in the levels of Cdk, (ii) upregulation of G1 cyclins Ccn1 and Cln3 and (iii) decreased expression of the G2 cyclins Clb2 and Clb4, which bind to Cdk (Cdc28) and suppress polarized growth. These effects could be due to the impairment of the protein degradation pathway caused by ubiquitin mutants UbEP42, UbL50P and UbI61T. Usually, reduction in Cdc28 levels leads to formation of hyphae. However, in the case of the mutants UbEP42, Ub L50P, and UbI61T, yeast to hyphal transition did not occur. Interestingly, these mutants show elevated expression of the bud-specific gene NRg1, which could be seen as the possible reason for continuing in yeast form. The decline in the formation of polyubiquitin chains with K48 linkage indicates the breakdown of UPS-mediated degradation, most likely at the stage of ubiquitination. Experimental analysis suggested that pH also plays an important role in yeast-to-hyphal transitions by affecting the Fkh2 transcription factor that is involved in virulence-associated secretory aspartyl protease secretion. C. albicans cultures

grown in the presence serum show a ready transformation to hyphal form, which is seen associated with the upregulation of Fkh2. On the other hand, ubiquitin mutants suppress the expression of Fkh2 in C. albicans grown in serum conditions. Further, it was reported in the literature that low levels of cyclins lead to alteration in the phosphorylation of Fkh2, preventing it from activating the expression of hyphal-specific genes such as HGC1. It was observed in the present study that ubiquitin mutations led to decreased expression of HGC1. Interestingly it was observed that under ubiquitin mutant conditions of UBEP42, UbL50P and UbI61T the cells contain significantly higher β-glucan in their walls and uneven chitin deposition on the outer surface of the cell wall compared to wild type cells and UBS20F, UbA46S mutants. Proteomic analysis of Candida albicans exposed to dosage-dependent lethal ubiquitin mutants (UbEP42, UbL50P, and UbI61T) revealed downregulation of key proteins involved in metabolism and stress response. This led to alterations in proteins related to cell cycle regulation, stress tolerance, viability, ubiquitin chain formation, cell wall synthesis, protein quality control, and virulence factor secretion. These findings illuminate the molecular mechanisms behind observed phenotypic changes in C. albicans. Further research on the interactions of known proteins and roles of unidentified proteins will enhance our understanding of fungal ubiquitin signaling and its implications for C. albicans biology and virulence. Overall, these insights highlight the consequences of ubiquitin dysregulation on the molecular landscape of C. albicans pathogenicity. Our study explored polyubiquitination profiles in Candida albicans, focusing on UbWT and dosage-dependent lethal mutants UbEP42, UbL50P, and UbI61T. Deleting the di-glycine motif in mutants led to impaired polyubiquitin chain assembly, crucial for cellular functions. Western blot analysis of UbEP42 revealed decreased K48 and K63 chain linkages in mutants lacking the di-glycine motif, impacting substrate recognition and degradation pathways. Structural analysis highlighted the importance of the di-glycine motif in maintaining ubiquitin's regulatory functions. Overall, our findings underscore the critical role of the di-glycine motif in ubiquitin-mediated processes, offering potential targets for therapeutic interventions against fungal infections.

In conclusion, by probing with ubiquitin mutants the study sheds light on the impact of ubiquitination over intricate molecular mechanisms determining cell morphology, cell cycle progression, protease secretion, and the composition of the yeast cell wall in Candida albicans, Di-glycine motifs importance in our generated mutants and proteomics analysis finding upregulated and downregulated protein turnover and its role in biological and metabolic pathway under mutants Further research is needed to fully understand the

molecular details of these effects and their potential implications for fungal virulence and pathogenesis.



The Working model for present study. Proteomics and Di- glycine deletion data are not shown in the figure.

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1. **2nd Best Poster Award" (CPBC G.N. Ramachandran 2023), held at The M. S. University of Baroda, Vadodara, Gujarat, India on 3rd - 4th March 2023.**
2. **Best Talk Award (Myco Talk 2022 December)**

Poster/Oral Presentations:

1. Sandeep Kumar Dantuslia, Bhoomi Prajapati, C. Ratna Prabha 'Impact of UbEP42 quadruple ubiquitin mutant and its single derivatives On *Candida albicans* morphogenesis, Aspartyl Protease Secretion, and Cell Cycle at International Conference on "Celebrating Proteins on the Birth Centenary of Dr. G. N. Ramachandran" (CPBC G.N. Ramachandran 2023), held at The M. S. University of Baroda, Vadodara, Gujarat, India on 3rd - 4th March 2023.

2. Bhoomi Prajapati, Sandeep Kumar Dantuslia, C. Ratna Prabha 'Studies on the impact of ubiquitin mutations in candidiasis mice model at International Conference on "Celebrating Proteins on the Birth Centenary of Dr. G. N. Ramachandran" (CPBC G.N. Ramachandran 2023), held at The M. S. University of Baroda, Vadodara, Gujarat, India on 3rd - 4th March 2023.
3. Doshi Ankita, Sandeep Kumar Dantuslia, C. Ratna Prabha 'Morphological switching is disabled in candida albicans by Ub EP42 and its segregated single mutations at International Conference on 'Proteins, miRNA and Exosomes in Health and Diseases' held at The M. S. University of Baroda, Vadodara, Gujarat, India on 11th - 13th December 2018.

- **Organised Webinar**
- **Mycotalks: Petra Bacher and Clarissa Nobile protective immunity and inflammation by fungus-reactive T helper cells [25, August 2022]**
- **Other activities:**
- **Attended Biotechnica Protein Engineering Workshop**
- **CSIR UGC NET-JRF Qualified 2019**
- **DBT JRF Qualified in 2019**
- **GATE Qualified in 2019**
- **Attended Online Cold Spring Harbor Laboratory Conference in 2021**
- **Attended National and International conferences and seminars organized by Deprtment of Biochemistry, Faculty of Science, The M.S. University of Baroda**

Date: Signature of the candidate

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