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Mutations in the ubiquitin gene of *Saccharomyces cerevisiae* accompanied by divergent use of CUG codon affect morphogenesis in *Candida albicans*

Ankita Doshi, Sandeep Kumar Dantuslia & C Ratna Prabha*

Department of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara-390 002, Gujarat, India

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UbEP42, a mutant of ubiquitin constitutes four mutations, namely S20F, A46S, L50P, and I61T. Characterization of UbEP42 and the isolated mutations revealed that UbEP42, UbL50P, and UbI61T conferred dosage-dependent lethality on *S. cerevisiae*, while UbS20F and UbA46S produced no effect over survival. In the present study, opportunistic human pathogen *Candida albicans* has been employed here to investigate the influence of ubiquitin mutations of *S. cerevisiae* on morphogenesis, besides various ubiquitin-dependent functions. The codon for leucine 'CUG' in *S. cerevisiae* is read as serine in *C. albicans*. Hence the protein variants expressed from the *S. cerevisiae* gene are added with the prefix 'Sc'. ScUbEP42, ScUbL50P, and ScUbI61T had a negative influence over protein trafficking, lysosomal degradation of proteins, and polyubiquitination with K48 and K63 linkages in *C. albicans* as in *S. cerevisiae*. ScUbEP42 and all four derivative mutations impaired the morphogenesis of the yeast form into infective hyphal form. The impairment of morphogenesis of *C. albicans* by ubiquitin mutations is unprecedented and suggests a target pathway for future therapeutics.

Keywords: *Candida albicans*, Hyphae, Morphogenesis, Ubiquitin mutations, Yeast form

In humans, *Candida albicans* is one of the most common commensal organisms that become opportunistic pathogens under certain conditions. *C. albicans* usually found in the gastrointestinal, oral cavity urogenital tracts, and skin[1,2], causes candidiasis and candidemia, infecting blood and other organs of immunocompromised patients[3]. To become a pathogen, C. *albicans* undergoes morphogenesis from the non-infectious and commensal yeast and pseudo-hyphal forms to infectious hyphal form. The hyphal form of *C. albicans* breaks into fragments and generates new mycelia and yeast forms at regular intervals, ensuring its survival and propagation of infection within the human body[4]. These adaptive traits permit *C. albicans* to avoid the immune system of the host and establish infection in various organs and systems. Several changes in the biochemical and molecular makeup contribute to the morphogenesis of *C. albicans* from a commensal avirulent organism to an opportunistic pathogen. Developing new approaches to fight *C. albicans* infections has become the need of the hour in the face of increasing resistance to antifungal drugs used to treat *C. albicans* infections.

*Correspondence:
Phone: 91-265-2795594; 91-9327201349 (Mob);
Fax: 91-265-2795569
E-mail: chivukula_r@yahoo.com

Ubiquitination controls many basic functions of a eukaryotic cell, including regulation of cell cycle, transcription, protein metabolism, and signal transduction, while it regulates immune responses, differentiation, and development at the organismal level[5]. Only three or fewer amino acid replacements in the protein sequence of ubiquitin from yeast to animals or plants provide the strongest evidence for the conservation of its structure[6-11]. In our laboratory, ubiquitin mutant gene *UbEP42* was generated by error-prone PCR, and the mutations *UbS20F*, *UbA46S*, *UbL50P* and *UbI61T* were individually segregated from *UbEP42* to explore the role of specific residues to unravel the structure-function relationships in the protein[12-19]. One such mutation, known as UbEP42, has been widely studied for its effects on *S. cerevisiae*, and it exhibits a lethal phenotype depending on the dosage of expression[14-15]. Four different mutations make up UbEP42. UbEP42 and two of the single mutations present in it, UbL50P and UbI61T, have been shown to be unfavorable to the growth and other functions of *S. cerevisiae*, while the other two mutations UbS20F and UbA46S have no impact on the functions tested[14-16]. *C. albicans* and *S. cerevisiae* diverged from a common ancestor 300 million years ago. They show similarities in many biological processes, such as mating, cell cycle

progression, cell wall biosynthesis, metabolism, and signaling pathways[20]. The mutations present in the ubiquitin gene of *S. cerevisiae* have been introduced into *C. albicans* to understand the effects of the opportunistic pathogen.

However, a difference in codons between the two organisms poses a challenge. The 'CUG' codon is read as leucine, while it is read as serine in *C. albicans*. There is one CUG in all the mutant forms and the wild type of ubiquitin gene sequence of *S. cerevisiae*, which was transformed into *C. albicans*. *C. albicans* comprises only two ubiquitin genes, namely *UBI3* and *UBI4*. Even though the ubiquitination and deubiquitination mechanisms are similar to those of *S. cerevisiae*[21]. Ubiquitination plays a critical role in the pathogenesis of *C. albicans*[22]. The *UBI3* gene translates as a fusion protein of ubiquitin and ribosomal polypeptide, while the *UBI4* gene translates to form a polyubiquitin consisting of three tandem repeats of ubiquitin. Both the gene products are processed post-translationally to release free units of ubiquitin[21].

In the present work, we investigated the effects produced by the expression of mutant forms of ubiquitin of *S. cerevisiae* accompanied by divergent use of CUG codon in *C. albicans*. The results obtained with *C. albicans* were analogous to those with *S. cerevisiae* in previous studies[14-16]. Mutant forms of ubiquitin ScUbL50P and ScUbI61T, ScUbEP42 resulted in dose-dependent lethality in *C. albicans*, even at lower levels of expression when compared to *S. cerevisiae*. These mutant forms slowed and inhibited progression through the G1 phase, and exhibited decreased tolerance to antibiotics and heat. On the other hand, cells expressing ScUbS20F and ScUbA46S showed functional similarity to wildtype strain. Remarkably, all five transformants expressing mutant forms of ubiquitin block the transition from yeast to hyphae, a morphological shift associated with infectivity.

Materials and Methods

Candida albicans CAI4 strain (ura3A:: imm434/ura3A:: imm434) used in the study is a deletion mutant of ura3, a derivative of strain SC5314 (ATCC MYA-2876) and is wild type for ubiquitin coding genes. CAI4 is a uridine auxotroph and was provided by Alistair J. P. Brown, United Kingdom[23]. CAI4 was grown in YPD media containing 1% yeast extract, 2% peptone, 2% dextrose, and 25 mg/L uridine for routine maintenance. Wild type ubiquitin gene has the SGD ID: S000003962. The five mutants of the ubiquitin gene of *S. cerevisiae* encoding the proteins UbEP42, UbS20F, UbA46S, UbL50P, and UbI61T used in this study have the GenBank accession numbers MG977456, MG977457, MG977458, MG977459 and MG977460 respectively. Host strain CAI4 carries ubiquitin genes of the wild type. The wild type and mutant forms of ubiquitin gene of *S. cerevisiae* transformed into CAI4 strain of *C. albicans* are represented here as ScUbWt/CAI4, ScUbEP42/CAI4, ScUbS20F/CAI4, ScUbA46S/CAI4, ScUbL50P/CAI4, and ScUbI61T/CAI4. *C. albicans* strain and its transformants were grown on a synthetic dextrose (SD) minimal medium (made up of 0.67% Yeast nitrogen base with or without amino acid and as a carbon source 2% glucose with the supplement of 20 mg/L uracil) at 30°C, by shaking at 200 rpm. Plasmid pUB221[24], a shuttle vector with ubiquitin gene cloned under CUP1 promoter and carrying URA3 gene as a selection marker kindly provided by Daniel Finley. PUB221 carrying the wild type ubiquitin gene was replaced with the mutant forms of ubiquitin gene encoding UbEP42 and its derivatives, namely UbS20F, UbA46S, UbL50P or UbI61T[14,15].

In *C. albicans* cells, plasmids with wild type and mutant versions of the ubiquitin gene have been transformed by using the lazy bones method[25]. In order to investigate the effects of ubiquitin mutants, $CuSO_4$ concentration has been set at various levels, *i.e*. 0 μM, 10 μM and 50 μM, since $CuSO_4$ acts as an inducer over *CUP1* promoter. The growth profile of the cultures was monitored using the conditions defined earlier[14,15] and the generation times were calculated[17]. The sublethal concentration of the inducer found from the overexpression study was 10 μM $CuSO_4$ and it was used to express mutant ubiquitins. Survival assays were performed with CAI4 and its transformants. The mid-log phase of cultures was diluted (1:10) serially four times to attain equivalent cell number and the cells were then grown on SD media supplemented with 25 mg/L uridine and incubated at 30°C for a period of 3-4 days, and the resulting colonies were counted.

The methods followed for analyzing cell cycle progression, western blotting analysis for determining the levels of Cdc28 kinase, formation of polyubiquitin chains with K48 and K63 branching patterns, sensitivity to heat and antibiotic stresses were the same as previously described[15]. To test if the mutant forms of ubiquitin exert any influence over the formation of hyphae in the CAI4 strain of *C. albicans*,

fresh cultures were incubated overnight at 37°C in a YPD medium. Hyphal growth was induced by supplementing the culture medium with 10% FCS. The CAI4 strain was used as a positive control in the experiment[26]. For microscopic analysis, the cells were collected and washed with PBS at pH 7.4. They were suspended in 1X PBS and added to the mounting solution. The differences in morphology were observed by capturing images using a Zeiss laser scanning microscope, 710 using 63x objective with total magnification 630x.

Results

To determine the effects of overexpression of the mutants, the CAI4 strain of *C. albicans* and its transformants ScUbWt/CAI4, ScUbS20F/CAI4, ScUbA46S/CAI4, ScUbL50P/ CAI4, ScUbI61T/CAI4 and ScUbEP42/CAI4 were incubated with different concentrations of the inducer $CuSO_4$ (0 µM, 10 µM, 50 µM, 100 µM) and the growth was observed by monitoring OD at 600nm. The transformants expressing ScUbEP42/CAI4, ScUbL50P/CAI4, and ScUbI61T/CAI4 exhibited a dose-dependent decrease in viability, due to the overexpression of the mutant forms of ubiquitin. Notably, at 100 µM $CuSO_4$ the cultures did not exhibit any growth at all. In contrast, the cultures expressing ScUbS20F and ScUbA46S showed robust growth similar to the positive controls CAI4 and CAI4 transformants of wild-type ubiquitin gene ScUbWt/CAI4 (Fig. 1). On the basis of this result, the sublethal concentration of the inducer (at 10 µM $CuSO_4$) was taken to investigate other molecular processes.

The growth of the transformed cells was monitored in the presence of 10 µM $CuSO_4$ at 2 h intervals, and the optical density (OD) was measured at 600 nm. From the results, it is evident that the transformants ScUbEP42/CAI4, ScUbL50P/CAI4, and ScUbI61T/ CAI4 exhibited a decrease in growth rate in comparison with CAI4 (Fig. 2A). The generation time of these lethal mutants was approximately twice as long as that of the wild type control (Fig. 2B). Additionally, cells expressing lethal mutations cause decrease in cell viability, indicating interference with multiple biological functions (Fig. 2(C)). On the other hand, the growth rates of ScUbS20F/CAI4 and ScUbA46S/CAI4 were similar to the controls CAI4 and ScUbWT/CAI4, with no significant impact on the generation time or viability percentage of host cells.

Progression of the cell cycle was monitored in CAI4 and its transformants by analyzing the data generated by FACS. CAI4 cells expressing the ubiquitin mutations ScUbEP42, ScUbL50P, and ScUbI61T were paused

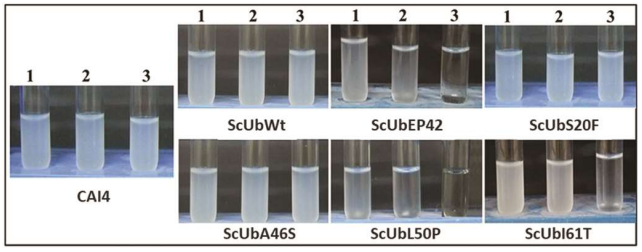

Fig. 1 — Effect of overexpression of ScUbEP42 and its single mutant derivatives on *C. albicans*. *C. albicans* CAI4 and its transformants were grown in the presence of varying concentrations of CuSO4. 1, 2, 3 correspond to 0 µM, 10 µM and 50 µM CuSO4 respectively. *C. albicans* CAI4 cells and CAI4 transformed with the gene for ScUbWt were positive controls. Cells expressing ScUbI61T produced dosage dependent lethality at permissive conditions and those expressing the other mutants, ScUbS20F and ScUbA46S did not show any lethal effects and behaved as wild-type ubiquitin-expressing cells

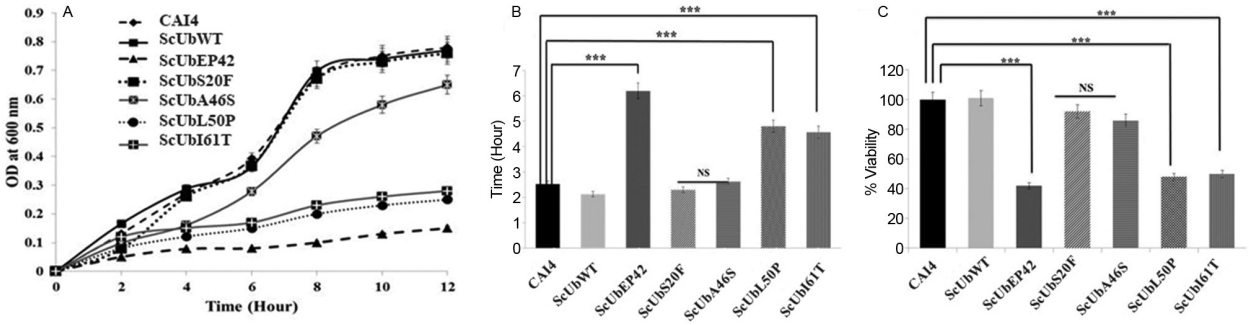

Fig. 2 — Influence of ubiquitin protein mutants on growth profile and viability of *C. albicans* cells. (A) Cells were transformed with genes for wild type and mutants of ubiquitin the expression of which was driven by copper inducible *CUP1* promoter. Cells were allowed to grow in the presence of sublethal concentration of inducer. Growth profile was monitored by measuring OD at 600nm at regular intervals of 2 hours. Studies revealed that ScUbL50P/CAI4 and ScUbI61T/CAI4 displayed slow growth. (B) Generation time was found to be increased in ScUbEP42/CAI4, ScUbL50P/CAI4, and ScUbI61T/CAI4. (C) The transformants were spread on SD plates in the presence of an inducer. After incubation colonies were counted. Results show decreased percentage of survival of cells expressing lethal mutants. Results represented Mean ± SE. (n = 3, P*** = <0.001, NS = non-significant)

during the G0/G1 phase of the cell cycle, consequently, they experienced a delay in entering the S phase. Conversely, ScUbS20F/CAI4 and ScUbA46S/CAI4 cells exhibited a progression through the S phase similar to the positive control cells, CAI4 and ScUbWt/CAI4, as depicted in (Fig. 3A).

G1 to S phase transition in both *S. cerevisiae* and *C. albicans* depends on the involvement of Cdc28/CDK protein kinase. Western blot analysis indicates that the levels of Cdc28 were reduced when ScUbEP42, ScUbL50P, and ScUbI61T were expressed. Conversely, the expression of ScUbS20F and ScUbA46S caused no significant alteration in the Cdc28 level compared to the positive controls (Fig. 3B).

The functional efficacy of ScUbEP42 and its derivatives was studied in the presence of heat stress. Specifically. Interestingly, *C. albicans* transformants expressing ScUbEP42, ScUbL50P, and ScUbI61T were unable to tolerate heat stress. The yeast form of *C. albicans* exhibits higher resistance to heat compared to the non-pathogenic *S. cerevisiae*. However, transformants expressing ScUbS20F and ScUbA46S exhibited similar endurance to heat stress as the positive controls CAI4 and ScUbWt/CAI4 (Fig. 4).

To study the survival under antibiotic stress the CAI4 strain and its transformants were grown in the presence of cycloheximide (4 µg/mL), tunicamycin (1 µg/mL), and canavanine (1 µg/mL). Similar to the response observed under heat stress, ScUbL50P/CAI4 and ScUbI61T/CAI4 showed significantly reduced viability in the presence of antibiotics. On the other hand, ScUbS20F/CAI4 and ScUbA46S/CAI4 transformants displayed viability comparable to the positive controls in the presence of all three antibiotics (Fig. 5).

Transformants expressing ScUbEP42, ScUbL50P, and ScUbI61T showed considerable reduction in polyubiquitination involving both K48 and K63 linkages. Conversely, the mutants ScUbS20F and ScUbA46S had minimal impact on the formation of polyubiquitin chains, as observed in (Fig. 6).

The influence of ubiquitin mutations on the *C. albicans* morphogenesis was investigated. Morphogenesis of *C. albicans* is associated with its transformation into a pathogen. Yeast forms of *C. albicans* transforms to hyphae and ubiquitination plays a role in the virulence of this organism[22,27]. The CAI4 were grown with 10% FCS (Fetal Calf Serum), which prompts the development of hyphae at 37°C temperature. Only the CAI4 and ScUbWt/CAI4 cells underwent the expected transformation from yeast to

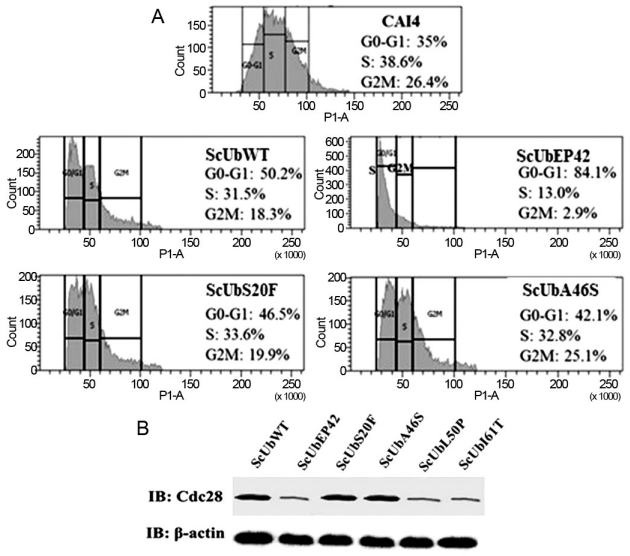

Fig. 3 — The effect of expression of ubiquitin mutants on cell cycle progression. (A) FACS analysis of CAI4 strain and CAI4 transformants. ScUbEP42/CAI4, ScUbL50P/CAI4 and ScUbA46S/CAI4 could progress through G0/G1 phase, similar to cells expressing wild type ubiquitin. Results represented in Mean ± SE (n = 3, P*** = <0.001, NS = non-significant). (B) Cdc28 protein kinase levels in CAI4 cells expressing wild type and mutants of ubiquitin. Results clearly display a diminished level of Cdc28 protein kinase in UbEP42, UbL50P and UbI61T expressing cells

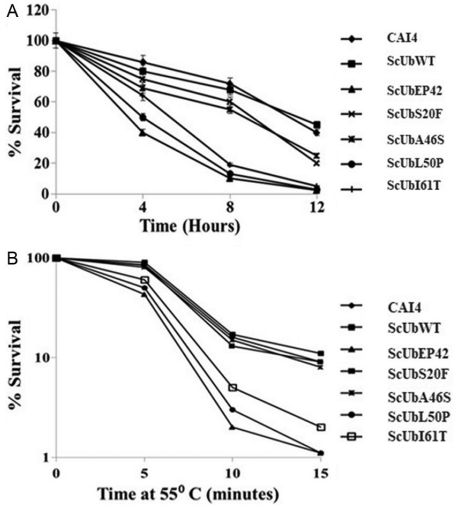

Fig. 4 — Effect of ubiquitin variants on the survival of *C. albicans* cells subjected to heat stress and thermotolerance test. (A) The transformants were equalized and plated in presence of inducer 10 µM CuSO4. Plates were incubated at 40°C for 4, 8 and 12 hours and brought back to 30º C followed by 3-4 days of incubation. % survival was calculated by counting colonies formed. (B) CAI4 strain containing mutant ubiquitin was grown till the log phase and shifted from supra optimal temperature to 55ºC. For both studies, the results showed that ScUbL50P and ScUbI61T had reduced thermotolerance and decreased resistance towards heat stress. Results represented in Mean ± SE. (n = 3, *P*** = <0.001, NS = non-significant)

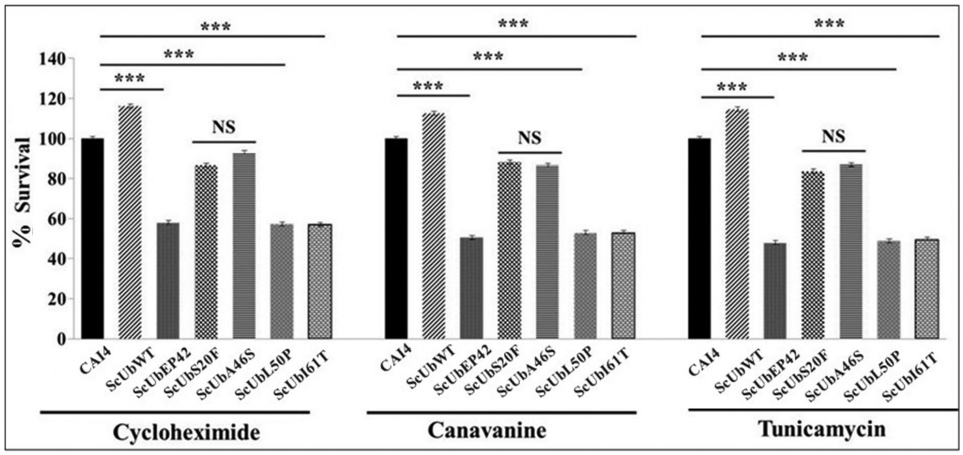

Fig. 5 — Effect of mutant ubiquitin protein on the survival of *C. albicans* under antibiotic stress. Collectively, results depict that ScUbEP42/CAI4 ScUbL50P/CAI4, and ScUbI61T/CAI4 could not withstand antibiotic stress. ScUbS20F/CAI4 and ScUbA46S/CAI4 cause a marginal decrease in viability. Results represented in Mean ± SE. (n = 3, *P**** = <0.001, NS = non-significant)

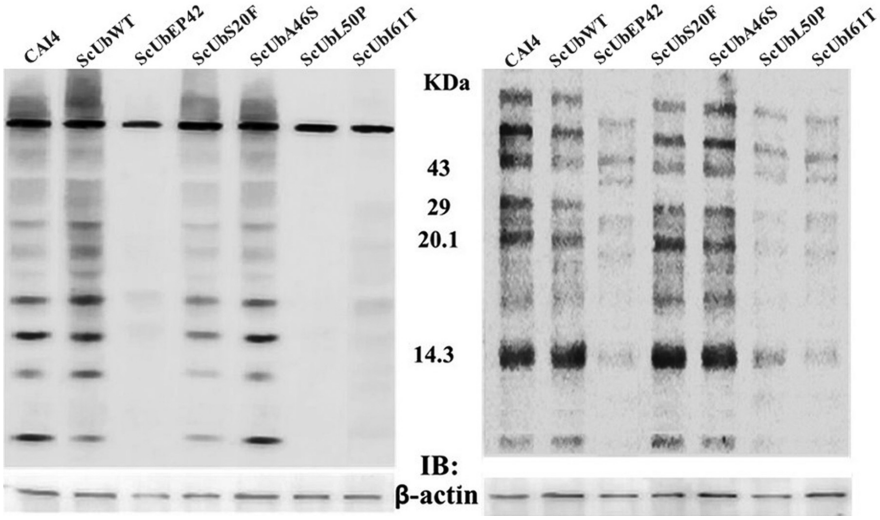

Fig. 6 — Western blot analysis showing polyubiquitination profile with K63 and K48 linkages of *C. albicans* cells expressing mutants of ubiquitin. ScUbEP42, ScUbL50P and ScUbI61T displayed impairment in polyubiquitination with both K63 and K48 linkages. The polyubiquitination profile of ScUbS20F and ScUbA46S did not show significant changes with K63 and K48 linkages

hyphae. On the other hand, ScUbEP42/CAI4, ScUbL50P/CAI4, and ScUbI61T/CAI4 exhibited the formation of pseudohyphae when overexpressed under serum-induced conditions, but subsequently underwent disintegration after 1-2 h. ScUbS20F/ CAI4 and ScUbA46S/CAI4, however, remained in the yeast form. Nevertheless, when the mutant proteins were not overexpressed, all mutants grew as yeast forms at 37°C. These findings indicate that all forms of ubiquitin mutants diminished the pathogen's potential to penetrance the host tissues (Fig. 7).

Discussion

Ubiquitin influences numerous cellular processes through the posttranslational modification known as ubiquitination[28]. Ubiquitination can be of two types, monoubiquitination and polyubiquitination. Ubiquitin containing seven lysine residues, is responsible for forming polyubiquitin chains, with different branching patterns.

However, the lack of enzymatic activity despite a remarkably conserved sequence raises questions about the importance of conserved residues in ubiquitin's diverse functions. Mutational approach was employed to understand the reasons behind the tight conservation of residues[29]. In our laboratory, we generated the mutations Q2N, E64G and S65D (of parallel β-bulge residue) in ubiquitin, to examine the effect on the structure and functions of ubiquitin in *S. cerevisiae*[12]. Another research group reported the

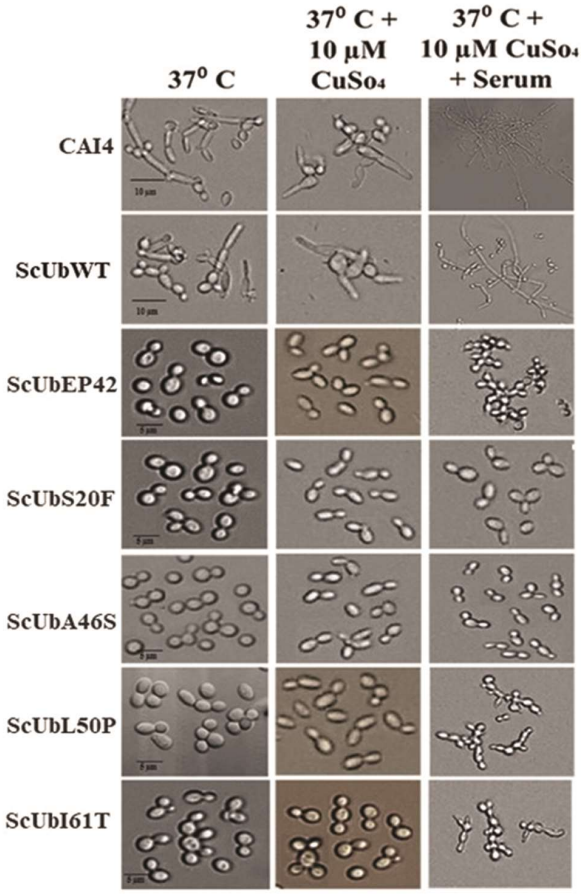

Fig. 7 — Initiation and maintenance of hyphal development in *C. albicans* CAI4 cells and their transformants. CAI4 and CAI4 transformed with ScUbWt switched from yeast form to hyphae, while none of the transformants expressing mutants of ubiquitin could undergo yeast to hyphae morphogenesis, in serum induced media

phosphorylation of S65 in ubiquitin by PINK1 in human neurons[30]. As mentioned here previously, we carried out in vitro evolution of ubiquitin by error-prone PCR in our laboratory and screened them in a stress-sensitive *S. cerevisiae* strain. Interestinlgy, UbEP42 mutant resulted from this experiment displayed dosage-dependent lethality[16]. UbEP42 carries amino acid substitutions at four different positions, namely S20F, A46S, L50P, and I61T. Subsequently, functional integrity of UbEP42 and all the single mutations was analyzed[14,15]. Structural analysis of the mutant proteins revealed that out of five three mutants of ubiquitin (UbEP42, UbL50P and UbI61T) retained the structures partially with increased β-sheet and reduced α-helix content[14-16]. Distorted structure of the mutants affected cell cycle progression, Lys63 linkage with polyubiquitination, stress responses, lysosomal protein degradation, protein sorting into MVBs, and

Table 1 — Details of substitution mutations when ubiquitin gene and its mutant forms of *S. cerevisiae* are expressed in *C. albicans*. Since, CUG codon of *S. cerevisae* is read as serine (S) instead of Leucine (L), in all the cases given below L56S substitution is present

S. No.	Name of the Protein of *S. cerevisiae*	Name of the Protein expressed in *C. albicans*	Amino acid substitutions in ubiquitin protein when the gene and its mutations from *S. cerevisiae* are expressed in *C. albicans*
1	UbWt	ScUbWt	L56S
2	UbEP42	ScUbEP42	S20F, A46S, L50P, I61T and L56S
3	UbS20F	ScUbS20F	S20F and L56S
4	UbA46S	ScUbA46S	A46S and L56S
5	UbL50P	ScUbL50P	L50P and L56S
6	UbI61T	SCUbI61T	I61T and L56S

UFD pathway for protein deprivation in the SUB60 strain of *S. cerevisiae*. The mutant forms UbS20F and UbA46S did not show any significant changes either in structure or in the functions studied. However, so far these mutations have not been reported to occur naturally, suggesting that these mutations are not advantageous for survival under certain conditions.

We decided to investigate the influence of these ubiquitin mutations on cellular functions and pathogenicity of *C. albicans*, considering its evolutionary relationship with *S. cerevisiae*. At this point, it is worth noting that the CUG codon is translated as serine in *C. albicans* instead of leucine. Additionally, there is a CUG codon in the ubiquitin gene that corresponds to the 56th position in the amino acid sequence.

In this study, ubiquitin and its mutants from *S. cerevisiae* were expressed in C. *albicans*. To avoid any ambiguity, we preferred to indicate their origin by adding the prefix 'Sc' (Table 1). Expression of ScUbEP42, ScUbL50P, and ScUbI61T resulted in decreased growth and viability in *C. albicans*, which were analogous to the findings in *S. cerevisiae*[14,15]. The results with ScUbS20F and ScUbA46S were similar to those of the wild-type ScUbWt in the CAI4 strain. CAI4 Cells show G0/G1 phase arrest and late entry into the S phase in *C. albicans* due to overexpression of ScUbEP42, ScUbL50P, and ScUbI61T, parallel to what was observed in *S. cerevisiae*. In the cell cycle, the interaction of G1 cyclins with Cdc28 protein kinase initiates G1 to S phase transition[31]. Being the only

cyclin-dependent kinase, Cdc28 exerts tight regulation over the cell cycle in both *S. cerevisiae* and *C. Albicans*[32]. Cdc28 interacts with both G1 phase and B phase cyclins. The activation of the G1-cyclin Cln3 initiates transcription that is specific to the START process, regulates cell size and enables the expression of Cln1 and Cln2. Consequently, Cln1 and Cln2 promote the cell's progression from the G1 phase to the S phase[33]. Overexpression of ScUbEP42, ScUbL50P, and ScUbI61T caused a depletion in Cdc28 level in *C. albicans*, resulting in delayed entry into the S phase due to G1 phase arrest, similar to what is observed in *S. cerevisiae*[19]. Ubiquitination plays a key role in the cell cycle by regulating the levels of cyclins and their inhibitors[34]. Therefore, functionally impaired variants of ubiquitin such as ScUbEP42, ScUbL50P, and ScUbI61T may restrict and conceivably they can interfere with the ubiquitination of cyclin inhibitors, leading to G1 phase arrest. UBI4 expression protected the cells and enhanced cell survival in *C. albicans* during stress conditions similar to *S. cerevisiae*[22]. Being a commensal and a pathogen *C. albicans* exhibits greater resistance to biological stresses compared to *S. cerevisiae*, as it evolved to survive in a variety of host environments to cause pathogenesis[28,35]. In our study ScUbEP42, ScUbL50P, and ScUbI61T showed reduced thermal tolerance and were unable to withstand antibiotic stress and heat stress. Presence of polyubiquitin chains with K63-linkages are found on ribosome L28 subunit. This polyubiquitination has been reported to confer increased resistance to antibiotics. In general, the potential to form K63 linked polyubiquitin chains showed a significant decrease in these mutants. The presence of functional ubiquitin is necessary for eliminating misfolded and truncated proteins generated during stressful conditions through the ubiquitin-proteasome system (UPS). Polyubiquitin chains with Lys48 (K48) branching are predominantly utilized to earmark defective proteins for protein degradation by UPS. Our findings indicate a reduction in the population of proteins connected by K48 linkage in the cells expressing ScUbEP42, ScUbL50P, and ScUbI61T in both *C. albicans* and *S. cerevisiae*. However, ScUbS20F and ScUbA46S survived under stress conditions similar to the wild type CAI4 and ScUbWt/CAI4. These results are in agreement with *S. cerevisiae* model organism[14,15]. The structural changes reported earlier in ScUbEP42, ScUbL50P, and ScUbI61T affect the functions of ubiquitin necessary for stress survival. Ubiquitin utilizes lysine residues for polyubiquitination, resulting in various topologies that act as signals for diverse functions[36-37]. In this study, ubiquitin mutant forms were expressed in the host *C. albicans* CAI4 strain, which has an intact UBI4 gene. Despite optimum levels of ubiquitin in the cell, ScUbEP42, ScUbL50P, and ScUbI61T had drastic effects that made the cells unable to overcome stress conditions, possibly due to inhibition at the level of polyubiquitin formation or proteasomal degradation. Only two linkages were probed in this study, and it is interesting to determine if these mutants can interfere with the formation of other types of lysine linkages. The ability to switch between yeast and hyphal forms is a critical factor associated with the virulence of *C. albicans*[38]. Overexpression of ScUbEP42, ScUbL50P, and ScUbI61T in *C. albicans* under permissive conditions results in the organism remaining in the yeast form. However, at an elevated temperature of 37°C and under serum-induced conditions, these mutants form pseudohyphae. The CaCDC4 gene in *C. albicans* corresponds to the SCFCDC4 ubiquitin ligase (F-box protein component) in *S. Cerevisiae*[39]. CaCDC4-/- regulates hyphae formation suggesting that yeast-to-hyphal transition is regulated by ubiquitin-proteasome system pathway[40]. The decline in Cdc28 protein levels in mutants expressing ScUbEP42, ScUbL50P, and ScUbI61T may contribute to the failure of morphogenesis. Although ScUbS20F and ScUbA46S have less pronounced effects on all the functions tested in our laboratory in *C. albicans*, their impact on morphogenesis suggests the involvement of ubiquitin ligases. This unprecedented observation of failure of morphogenesis explains why these mutations are not found naturally. The knowledge gained about the impairment of morphogenesis in *C. albicans* could be instrumental in developing therapeutics based on ubiquitination and UPS.

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Conflict of interest

All authors declare no conflict of interest.

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