Chapter IV

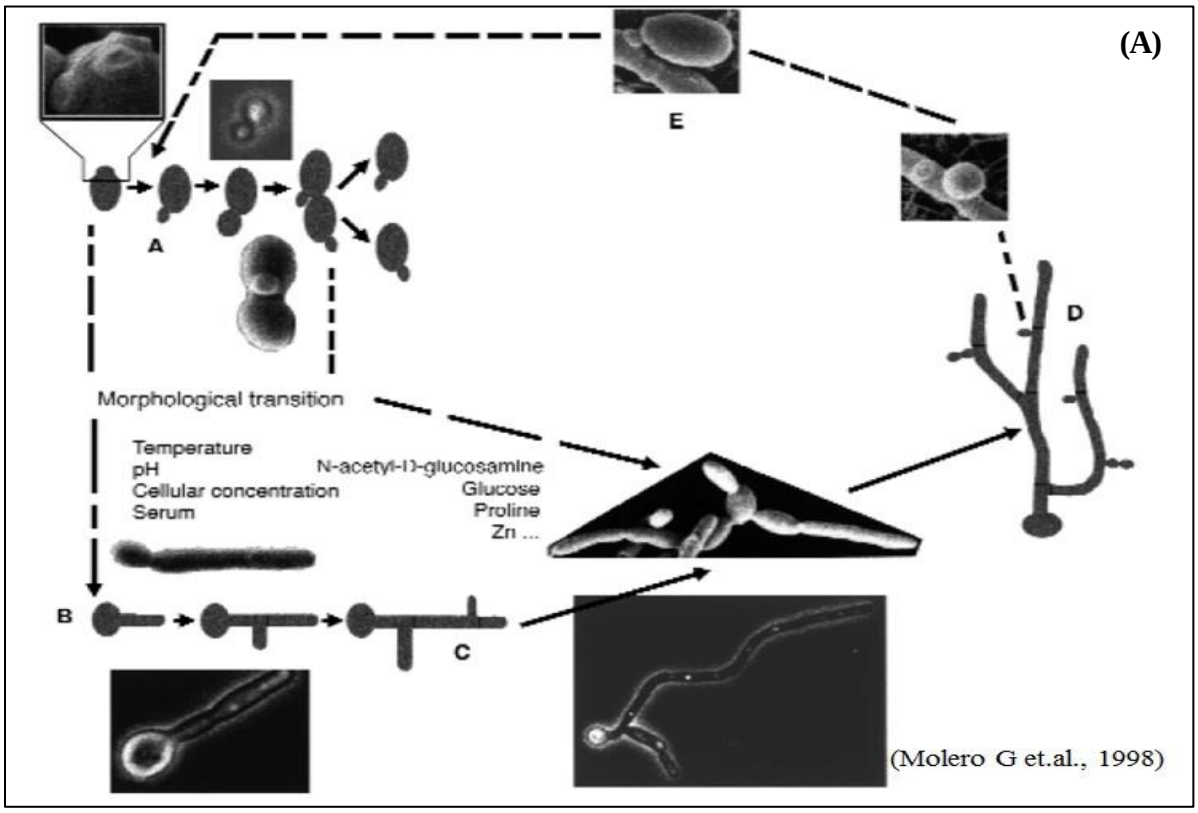
Molecular effects of ubiquitin mutants on growth and propagation of *Candida albicans*

4 Molecular effects of ubiquitin mutants on growth and propagation of Candida albicans

4.1 INTRODUCTION

Candida albicans is the most prevalent, classical, opportunistic pathogen and commensal of humans, known to be residing harmlessly in approximately 50% of individuals. This yeast occupies divergent niches such as skin, oral cavity, gastrointestinal and urogenital tracts of healthy individuals (Odds, 1988; Brown, 2002). Diseases caused by *Candida* sp. are known as candidiasis and candidemia, depending on the organs infected and the severity of infection. *C. albicans* is responsible for the fourth most common life-threatening, nosocomial bloodstream infections in immunocompromised patients (Odds, 1988; Runke, 2002; Kabir et.al., 2012). Recent survey conducted by Centers for Disease Control and Prevention (CDC) and world health organisation (WHO) reported incidence of 250,000 to 400,000 deaths per annum worldwide due to candidiasis and candidemia (Brown et.al., 2012; Kullberg et.al., 2015).

An important aspect of its biology is it can grow in yeast, pseudohyphal and hyphal forms (Chandra et al., 2001; Nobile & Mitchell, 2005; Nobile et al., 2006) (Figure 4.1). The hyphal form of *C. albicans* undergoes fragmentation intermittently giving rise to new mycelia and yeast forms (Chandra et al., 2001; Nobile & Mitchell, 2005; Nobile et al., 2006) The adaptive traits of *C. albicans* accumulated evolutionarily enable it to infect and survive in diverse niches by escaping the surveillance of host immune system. A variety of virulence factors and structural attributes are responsible for establishing *C. albicans* as an opportunistic pathogen (Figure 4.2). Anti-fungal drugs used for treating *C. albicans* infections are becoming ineffective due to increase in resistant organisms. Therefore, there is a need for inventing novel antibiotics and strategies to combat *C. albicans* infections successfully.



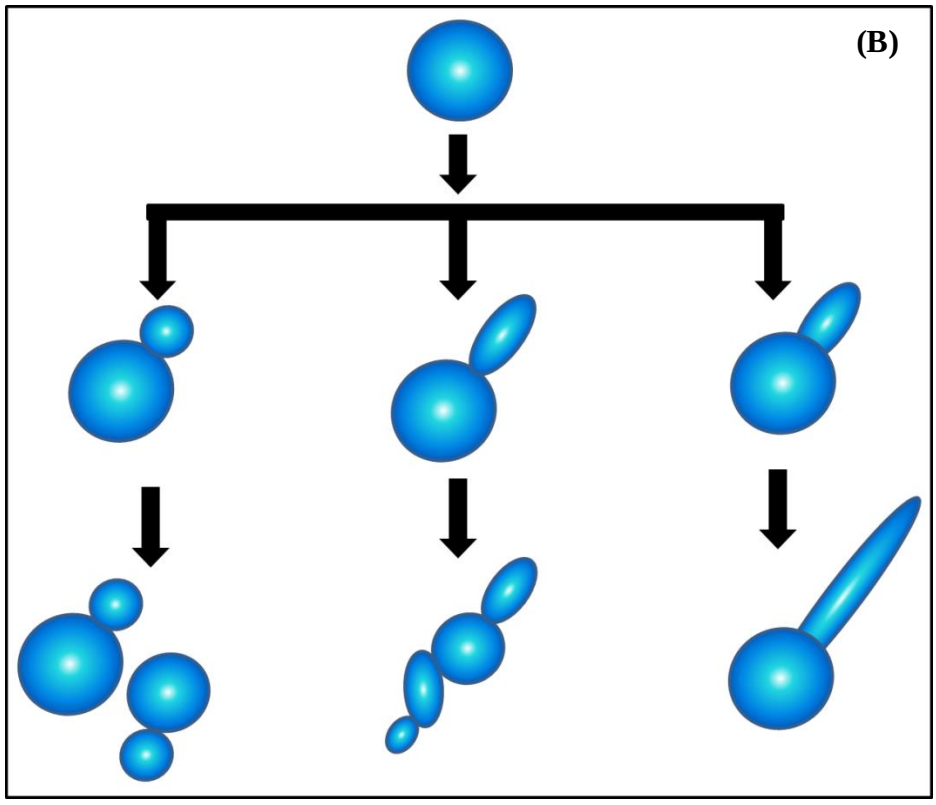


Figure 4.1: (A) life cycle of *C. albicans* (B) morphogenesis in *C. albicans* from yeast to

pseudohyphae and hyphae.

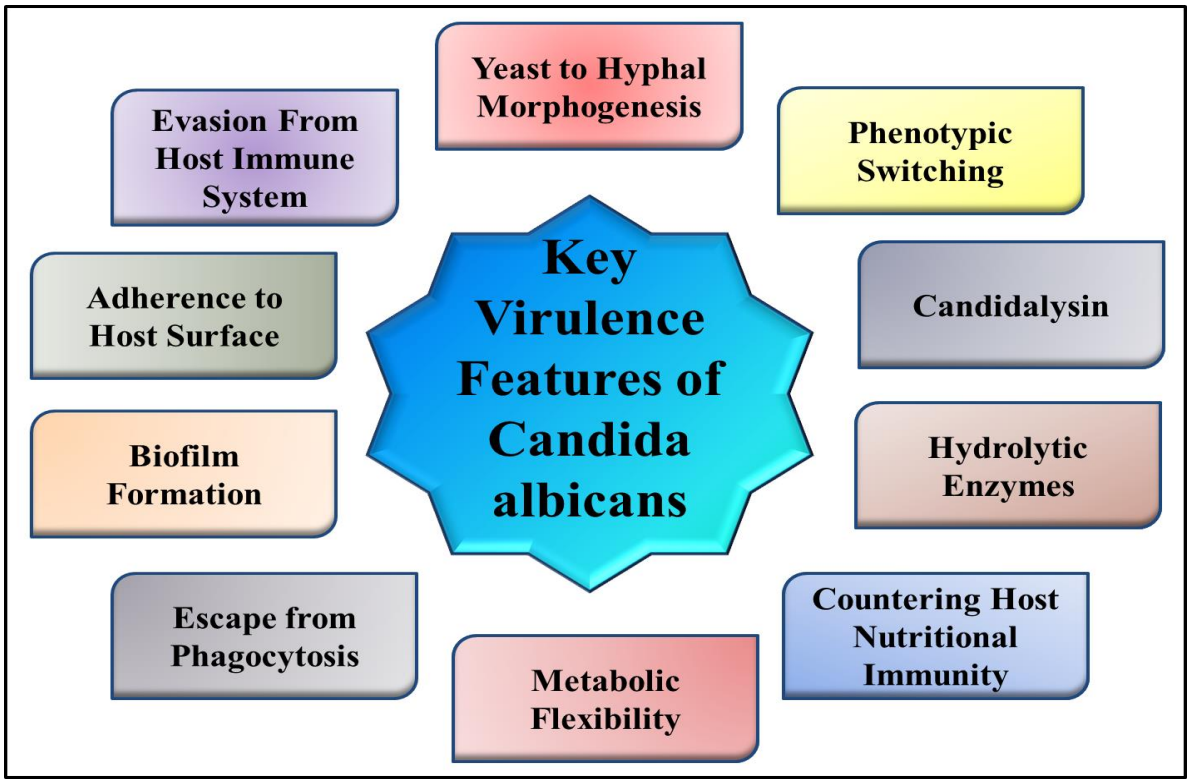


Figure 4.2: Pictorial representation of key virulence factors of *C. albicans*.

Ubiquitination plays important role regulating various biological aspects of a eukaryotic cell like cell cycle progression, transcription, translation, metabolism, signal transduction and others, contributing to successful survival of the organism. The protein sequence and structure of ubiquitin are the best examples of evolutionary conservation with only three or fewer replacements in amino acid residues from yeast to animals and plants (Schlesinger and Goldstein, 1975; Gavilanes et al., 1982; Dworkin-Rastl et al., 1984; Ozkaynak et al., 1984; Wiborg et al., 1985; Vierstra et al., 1986). Unravelling the importance of conserved residues in ubiquitin has been the focus of our laboratory. In order to meet this objective, several mutants of ubiquitin have been generated to study their role in the maintenance of its structure and functions (Misra et.al., 2009; Prabha et al., 2010; Mishra et.al., 2011; Sharma et.al., 2011; Sharma et.al., 2015). Dosage dependent lethal mutation of ubiquitin UbEP42 and the four single mutations segregated from it have been studied for their effects in *S. cerevisiae*. The mutations UbEP42, UbL50P and UbI61T produced negative effects on the growth and other functionalities in *S. cerevisiae* (Doshi et.al., 2014, Doshi et.

al., 2017). The mutations were introduced into *C. albicans* to check if similar effects can be observed with the opportunistic pathogen as it shared common ancestry with *S. cerevisiae* over 300 million years ago. The similarities between the two organisms in vital processes like cycle progression, mating, metabolism, cell-wall biosynthesis, signalling pathways have been well established (Kabir et.al., 2012). In addition, availability of a wealthy database of information on *S. cerevisiae* makes it ideal model system for preliminary testing of hypotheses before addressing the same questions in *C. albicans* (Berbee et.al., 2000; Heckman et.al., 2001; Asleson et.al., 2001). To study the functional alterations observed earlier in *S. cerevisiae* due to mutant forms of ubiquitin, we have used in *C. albicans* CAI4 strain which is avirulent and contains a complete set of ubiquitin protein expressing genes. Ubiquitination plays crucial role in the pathogenesis of *C. albicans* (Leach et al., 2011). Two ubiquitin genes, *UBI3* and *UBI4*, have been described in this organism (Roig and Gozalbo, 2002). Here, the *UBI3* gene encodes a fusion protein in which ubiquitin is fused to ribosomal polypeptide and it is highly homologous to *UBI3* gene of *S. cerevisiae*. *UBI4* gene encodes polyubiquitin containing three ubiquitin repeats in a head-to-tail spacerless arrangement. Post-translational cleavage of this natural fusion protein generates monomers of ubiquitin. However, expression pattern of these genes is similar to the ubiquitin genes of *S. cerevisiae* (Sepulveda et. al., 1996; Planta et. al., 1997; Lopez et. al., 1999; Roig et. al., 2000). Plasmid pUB221 carrying mutant forms of ubiquitin genes, was used to overexpress the mutant proteins and functional studies were carried out.

Results we observed in *C. albicans* are similar to *S.cerevisiae* model system. The mutants UbEP42, UbL50P and UbI61T are showing dosage dependent lethality at low inducer concentration as compared to *S.cerevisiae*. These mutants slower down the growth rate, arrest cells in G1 phase and leading to low survival of cells. Also the cells showed lowered tolerance towards heat and antibiotic stresses. The phenotypes of cells expressing UbS20F and UbA46S remain unaltered during all functional studies and behaved as wild type strain. Interestingly, all five transformants expressing ubiquitin mutants have lost their ability to switch their morphology from yeast to hyphal, in turn losing their infectivity.

4.2 MATERIALS AND METHODS

4.2.1 Strains and media

The *C. albicans* strain, CAI4 has been used in the study. CAI4 (ura3A : :

imm434/ura3A : : imm434) is a ura3- derivative of Ca5314 and was a gift from Alistair J P Brown, UK. It is avirulent strain and uridine auxotroph (Fonzi and Irwin, 1993). It harbours full set of ubiquitin coding genes. CAI4 was grown in YPD (1% w/v, yeast extract; 2 % w/v, peptone; 2 % w/v, dextrose) supplemented with 25 mg/litre uridine for maintenance. Host strain, transformants which received ubiquitin gen of wild type and mutant forms have been referred as CAI4, CAI4/UbWt, CAI4/UbEP42, CAI4/UbS20F, CAI4/UbA46S, CAI4/UbL50P and CAI4/UbI61T. The transformed strains were grown in synthetic dextrose (SD) medium containing 0.67% Hi-media yeast nitrogen base and 2% glucose as carbon source with uracil (20 mg L^{-1}) as supplement. Cultures were grown at 30°C with shaking at 200 rpm.

4.2.2 Experimental procedures

Mutated forms of ubiquitin gene were cloned in pUB221 (Ellison and Hochstrasser, 1991). The description of pUB221 and insertion of mutated forms of ubiquitin gene, namely UbEP42, UbS20F, UbA46S, UbL50P and UbI61T used here have been given in **Chapter 2.**

Plasmids with inserts of mutant forms of ubiquitin gene were transformed in *C. albicans* cells using "lazy bones" method (Burke, 2000). To study the effect of ubiquitin mutants, cells were subjected to varying concentration of $CuSO_4$ i.e. 0 µM, 10 µM, 25 µM, 50 µM, 75 µM where $CuSO_4$ acts as an inducer. Cultures were allowed to grow at 30°C with shaking at 200 rpm and growth was monitored.

The cultures of CAI4 and its transformants expressing wild type and mutant forms of ubiquitin were grown and their growth kinetics was studied using the conditions described in **Chapter 2**. Sub-lethal concentration of inducer was decided based on the lethality pattern seen during overexpression study. 10 µM copper sulphate was used for over expressing ubiquitin variants. Growth pattern was assessed by harvesting cells at regular intervals of two hours and optical density was recorded at 600nm. Exponential phase of the culture was considered to calculate the generation time by using the formula:

$$\textbf{Doubling Time} = t/g$$

Where $g = [\log_{10} (N_t/N_0)]/ 0.3$, N_0 = Number of cells or OD at start, N_t = Number of cells or

OD at end and t = time cultured (in Hours).

C. albicans CAI4 and its transformant cells were tested for survival capacity. Cultures grown to mid-log phase were diluted four-fold and to equal cell density. Then the cells were plated on synthetic dextrose minimal media with 25 mg/litre uridine as per requirement. Plates were incubated at 30°C for 3-4 days and colonies were counted.

Wild type *C. albicans* cells and their transformants were grown till log phase and prepared for cell cycle analysis with propidium iodide staining, as described in **Chapter 2**. The cell populations in different stages of cell cycle were recorded using flow cytometer BD FACS Aria II. The experiment was repeated in three independent sets and the percentage population of cells G0/G1, S and G2/M phase were studied.

Cdc28 protein kinase level in *C. albicans* CAI4 cells and their transformants was estimated with anti-Cdc28 antibody (Santa cruz, Cat No.: sc-28550) and with Goat anti Rabbit IgG HRP Conjugate (GeNei, Cat No.: HPO3). Blot was developed by ECL (Amersham).

Effect of ubiquitin variants on survival of *C. albicans* under antibiotic stress was carried out using plate assays as per standard protocol (Hanna et.al., 2003; Bor et.al., 2016). CAI4 cells and their transformants were plated on SD agar plates containing antibiotic and 10 µM copper sulphate. The antibiotics used in this study were cycloheximide (4 µg ml^{-1}), tunicamycin (1 µg ml^{-1}), canavanine (1 µg ml^{-1}). Plates were incubated for 5-6 days at 30^0C. Colonies were counted.

Effect of ubiquitin variants on survival of *C. albicans* under thermal stress was compared with that of CAI4 cells. Mid-log phase cultures of *C. albicans* CAI4 and its transformants were serially diluted and plated on SD selection plates containing 10 µM copper sulphate. The plates were incubated for time periods of 0, 4, 8 and 12 h at 40°C and shifted to permissible temperature of 30°C. After five to six days the effect of ubiquitin mutants under heat stress was assessed by counting number of colonies. The experiment was repeated in three independent sets and Mean ± SEM values have been presented in the form of a graph.

Thermotolerance of *C. albicans* cells expressing mutant forms of ubiquitin were

carried out as per the method reported earlier (Zeuthen & Howard, 1989). Briefly, cultures were grown at 30^0C till log phase (OD at 600nm = 0.6). After a short incubation of cells at supraoptimal temperature (45^0C, 30 min.), cells were shifted to 55^0C, which is lethal temperature for *C. albicans*. Samples were withdrawn at regular intervals, washed in distilled water and resuspended in SD medium prewarmed to 55^0C such that the cell densities remain same in all samples. The cells were plated on SD agar plates containing 10 µM copper sulphate. Colonies were counted after 5-7 days of incubation. The experiment was repeated in three independent sets and Mean ± SEM values are presented in the form of graph.

Incorporation of mutant forms of ubiquitin into polyubiquitin chains was monitored using myc-tagged ubiquitin. The cloned mutants are tagged as the vector used in the study carries c-myc tag which was utilized to test their incorporation in to formation of polyubiquitin chain. Mutant and wild type ubiquitin transformed C. albicans cells were grown till log phase at 30° C in the medium containing 10 µM CuSO4. Cells were harvested, washed by phosphate buffer saline and lysed by sonication. Protein concentration was calculated and equal quantity of protein was loaded in 17% SDS-PAGE. Proteins were transferred on nitrocellulose membrane and were blocked by blocking buffer (5% PBS-MT) for one hour. The membrane was probed overnight with anti-c-myc-antibody conjugated to peroxidise (Roche, Cat No.: 11814150001, 1:1000 dultion). The blot was developed with enhanced luminol in ECL kit (Amersham).

Formation of polyubiquitin chains with K48 and K63 linkages were monitored in CAI4 cells and their transformants using western blotting. After transferring protein bands and blocking, the nitrocellulose membranes were probed with Anti-Ubiquitin Lys63 specific clone HWA4C4 (Merk Millipore, Cat No.: 05-1313, Mouse monoclonal antibody) and K48 linkage specific polyubiquitin antibody (CST, Cat No. 4289) overnight at 4^0C. The blots were developed using ECL kit (Amersham). Details of the methods have been given in **Chapter 2.**

To study the colony structure of CAI4 and its transformants, the cultures were plated on YPD plates containing 10 µM copper sulphate. Plates were incubated for 3 days at 30°C and for another 3 days at 37°C. Images of colonies were captured using a Carl Zeiss laser scanning microscope.

To compare the hyphae forming potential of transformants of CAI4 strain of *C. albicans* and its transformants, cultures of cells were inoculated freshly in YPD liquid medium and incubated overnight at 37°C. Hyphal growth was induced by incubating cells with either YPD containing 10% fetal calf serum or proline-containing media. CAI4 strain was used as positive control (Leberer et. al., 1997; Heilmann et. al., 2011). For microscopy, cells were harvested, washed twice in phosphate buffered saline, pH 7.4. The cells were suspended in 1X PBS containing mounting medium. The images were captured using a Carl Zeiss laser scanning microscope, with 710 by 63x objective at a magnification of 630x.

4.3 RESULTS

4.3.1 Overexpression of UbEP42, UbL50P and UbI61T causes lethality in *C. albicans*

The mutant ubiquitin genes cloned into pUB221 under copper sulphate inducible *CUP1*promoter were transformed into CAI4 strain of *C. albicans*. Overexpression of the mutants was regulated by adding different concentration of inducer $CuSO_4$ i.e. 0 µM, 10 µM, 50 µM and growth was monitored. With increased $CuSO_4$, the cultures expressing UbEP42, UbL50P and UbI61T showed dosage dependent lethality due to elevated expression of mutant forms of ubiquitin, even under permissive conditions. In contrast UbS20F and UbA46S had grown well similar to wild type cells (Figure 4.3). CAI4 strain and UbWt transformed CAI4 strains were used as positive controls for the study. Based on the results we have chosen sublethal concentration of the inducer i.e. 10 µM, to study other processes at molecular level.

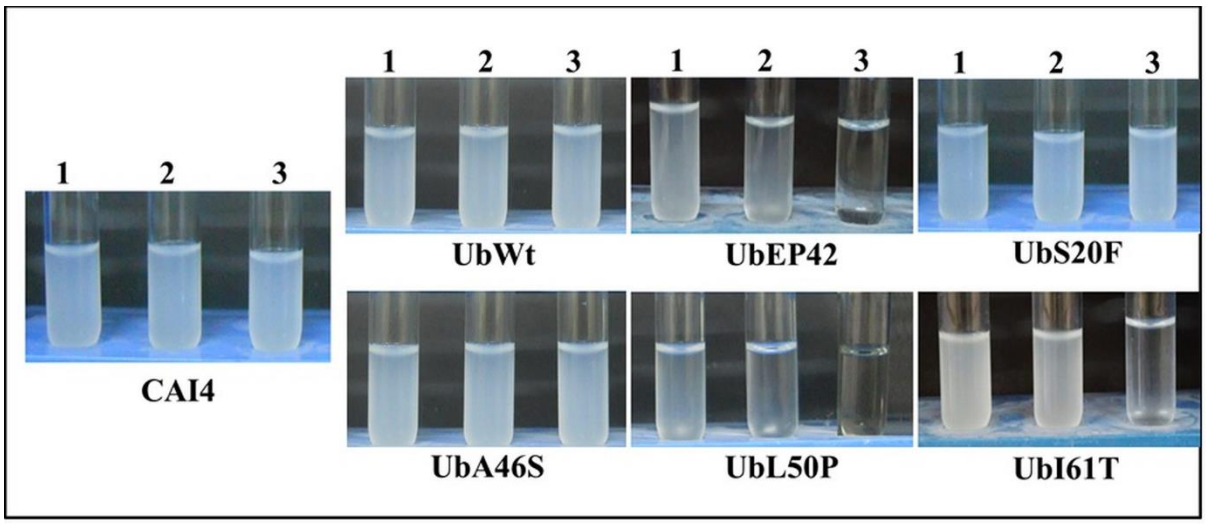


Figure 4.3: Effect of overexpression of UbEP42 and its single mutant derivatives on *C. albicans*. CAI4 and its transformats were grown in the presence varying concentrations of $CuSO_4$. 1, 2, 3 correspond to 0 µM, 10 µM and 50 µM of $CuSO_4$ respectively. CAI4 and CAI4 transformed with the gene for UbWt were positive controls. Cells expressing UbEP42, UbL50P and UbI61T produced dosage dependent lethality at permissive conditions and those expressing other mutants, UbS20F and UbA46S did not show any phenotypic changes and behaved as wild type ubiquitin expressing cells.

4.3.2 *C. albicans* cells expressing UbEP42, UbL50P and UbI61T show slow growth phenotype and reduced survival

Sublethal concentraion of inducer was used to overexpress the mutant forms of ubiquitin in the transformants of *C.albicans* cells and the growth was monitored. Result shows slow growth with CAI4/UbEP42, CAI4/UbL50P and CAI4/UbI61T (Figure 4.4(A)). Moreover, generation time of the above mentioned lethal mutants was found to be approximately double (Figure 4.4(B)). Viability of cells expressing lethal mutations was found to decline as these mutants might interefere in several biological functions (Figure 4.4(C)). CAI4/UbS20F and CAI4/UbA46S grew at similar rates as CAI4 and CAI4/UbWt and generation period, % viability also remained unaffected.

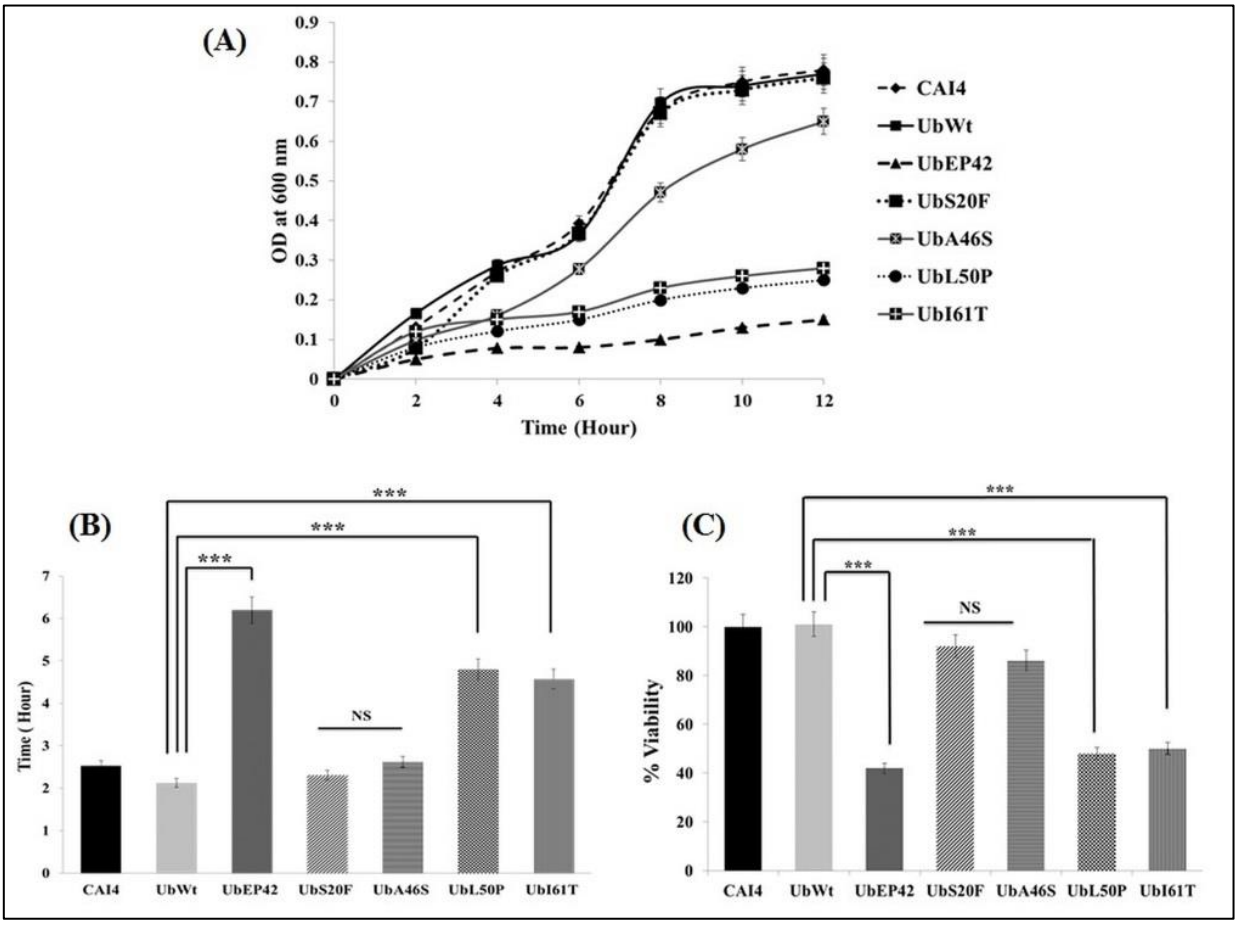


Figure 4.4: Influence of ubiquitin protein mutants on growth profile and viability of *C. albicans* cells. (A) Cells were transformed with genes for wild type and mutant forms of ubiquitin whose expression was driven by copper inducible *CUP1* promoter. Cells were allowed to grow in the presence of sub lethal concentration of inducer. Growth profile was monitored by measuring OD at 600nm at regular intervals of 2 hours. Studies revealed that CAI4/UbEP42, CAI4/UbL50P and CAI4/UbI61T displayed slow growth. (B) Generation time was found to be increased in CAI4/UbEP42, CAI4/UbL50P and CAI4/UbI61T. (C) The transformants were plated on SD plates in the presence of inducer. After incubation colonies were counted. Results show decreased percentage survival of cells expressing lethal mutants. Results represented Mean ± SE. (n = 3, P* = <0.001, NS = non-significant).**

4.3.3 UbEP42, UbL50P and UbI61T impair cell cycle progression in *C. albicans*

To study the effect of expression of ubiquitin mutations on cell cycle progression the log phase cultures of CAI4 cells and their transformants were subjected to FACS analysis. CAI4 and CAI4/UbWt cells were used as positive controls. Results indicate that CAI4/UbEP42, CAI4/UbL50P and CAI4/UbI61T cells were arrested in G0/G1 phase of the cell cycle and hence had delayed entry into S phase. On the contrary, CAI4/UbS20F and CAI4/UbA46S were able to move through S phase similar to positive controls (Figure 4.5(A)).

4.3.4 Diminished expression of Cdc28 protein kinase in cells expressing UbEP42, UbL50P and UbI61T

In yeast, Cdc28 protein kinase or cyclin dependent kinase (Cdk) plays major role in G1 to S phase transition. Western blot analysis showed decrease in Cdc28 levels due to the overexpression of UbEP42, UbL50P and UbI61T. On the contrary, expression of UbS20F and UbA46S did not cause any noticable change in Cdc28 protein kinase expression pattern from positive controls (Figure 4.5 (B)).

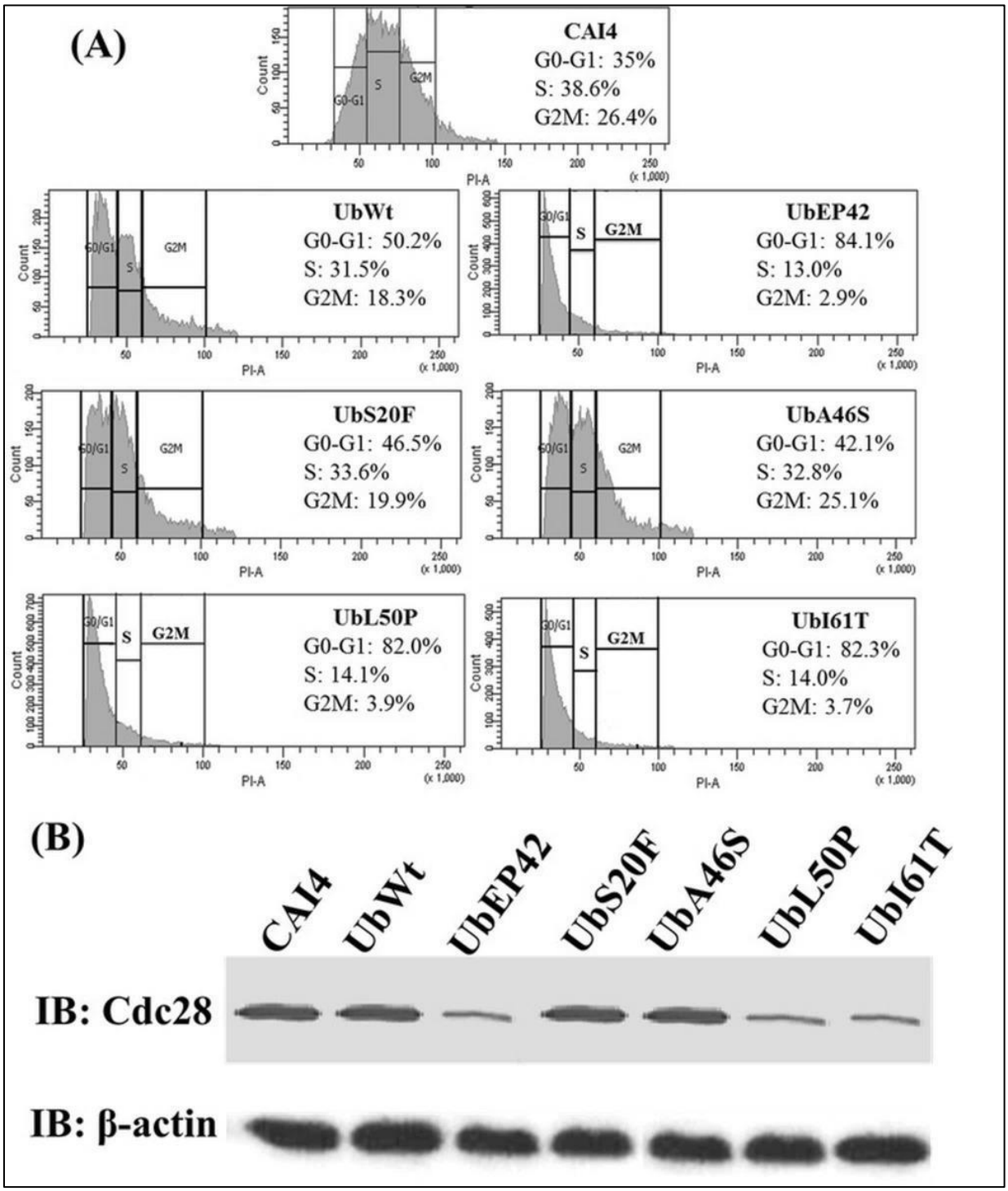


Figure 4.5: The effect of expression of ubiquitin mutants on cell cycle progression. (A) FACS analysis of CAI4 strain and CAI4 transformants. CAI4/UbEP42, CAI4/UbL50P and CAI4/UbI61T expressing cells showed delayed G0/G1 phase, whereas CAI4/UbS20F and CAI4/UbA46S could progress through G0/G1 phase, similar 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 mutant forms of ubiquitin. Results clearly display diminished level of Cdc28 protein kinase in UbEP42, UbL50P and UbI61T expressing cells.

4.3.5 UbEP42, UbL50P and UbI61T reduce thermotolerance of host cells and impair heat stress withstanding capacity

The influence of UbEP42 and its derivatives on the functional efficacy of ubiquitin produced from host cell genome was tested under heat stress conditions by using the method commonly used with *S. cerevisiae* cells. *C. albicans* CAI4 strain and its transformants were exposed to 40°C for varying time periods and were returned to 30°C to allow normal growth. Under stress conditions increased demand for ubiquitin is met by the *UBI4* gene in the genome of *C. albicans*, which encodes polyubiquitin. Interstingly, transformants of *C. albicans* expressing UbEP42, UbL50P and UbI61T could not withstand heat stress. Being a pathogenic yeast *C. albicans* has greater thermotolerance compared to free living *S. cerevisiae*. Therefore to check the effect of the mutations the cells grown at 30°C were exposed initially to 45°C for 30min. and 55°C for varying time periods. The mutants expressing UbEP42, UbL50P and UbI61T exhibited lowered thermotolerance capacity. However, transformants expressing UbS20F and UbA46S could show endurance similar to the two positive controls *C. albicans* namely, CAI4 and CAI4/UbWt (Figure 4.6).

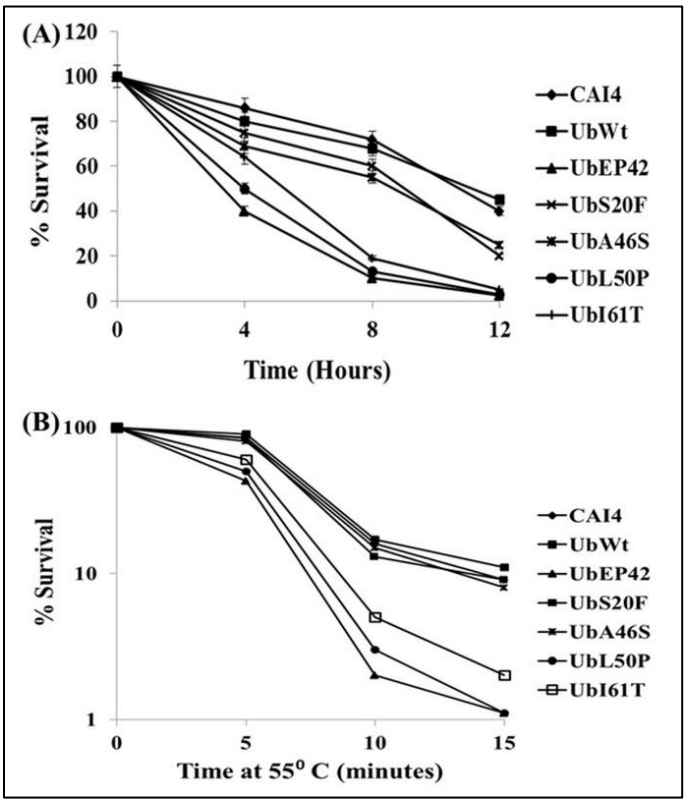


Figure 4.6: Effect of ubiquitin variants on the survival of *C. albicans* cells subjected to heat stress and thermotolerence test. (A) The transformants were equalised and plated in presence of inducer 10 µM $CuSO_4$. Plates were incubated at 40^0 C for 4, 8 and 12 hours and brought back to 30^0 C followed by 3-4 days of incubation. % survival was calculated by counting colonies formed. (B) CAI4 strain containing mutant ubiquitin was grown till log phase and shifted from supraoptimal temperature to 55^0 C. For both the studies, the results showed that UbEP42, UbL50P and UbI61T had reduced thermotolerance and decreased resistance towards heat stress. Results represented in Mean ± SE. (n = 3, P* = <0.001, NS = non-significant).**

4.3.6 UbEP42, UbL50P and UbI61T reduce resistance towards antibiotic stress

To study the effect of expression of mutant ubiquitins on survival of *C. albicans* in presence of antibiotics, the cells of CAI4 strain and their transformants were allowed to grow on plates containing cycloheximide, canavanine and tunicamycin (Wimalasena et al., 2008). Results obtained were similar to heat stress response i.e. CAI4/UbEP42, CAI4/UbL50P and CAI4/UbI61T failed to withstand antibiotic stress as their viability was reduced considerably.

Other two transformants, CAI4/UbS20F and CAI4/UbA46S showed viability closer to positive controls in the presence of all three antibiotics (Figure 4.7).

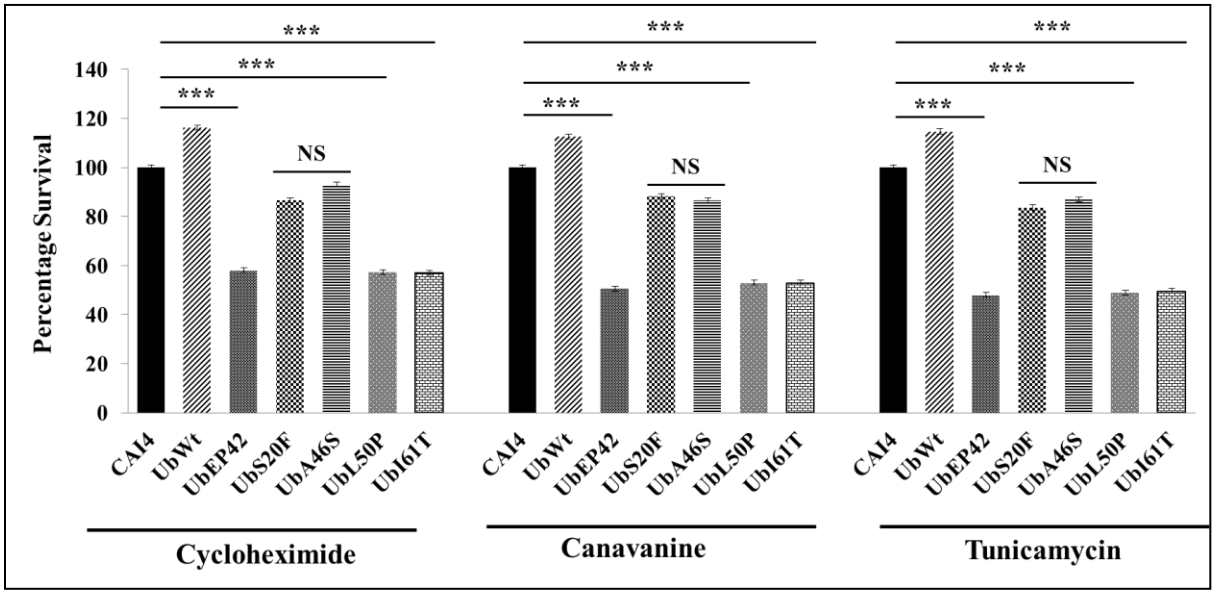


Figure 4.7: Effects of mutant ubiquitin protein on the survival of *C. albicans* under antibiotic stress. Collectively, results depict that UbEP42, UbL50P and UbI61T could not withstand antibiotic stress. UbS20F and UbA46S cause marginal decrease in viability. Results represented in Mean ± SE. (n = 3, P* = <0.001, NS = non-significant).**

4.3.7 Expression of UbEP42, UbL50P and UbI61T results in impairment of K63 and K48 linked polyubiquitinations

Ubiquitin monomer consists of seven lysine residues and all of them participate in polyubiquitination. Branching pattern of polyubiquitin chain changes depending on the lysine residue participating in isopeptide formation, which serves as a signal for regulation of varied functions. For instance, polyubiquitin chains with K48 linkage on substrate proteins drive them towards degradation by proteasomes and polyubiquitin chains with K63 linkage serves variety of purposes including endocytosis, lysosomal degradation, various signal transductions, kinase activation and DNA repair, which in the later case is partly determined by the substrate and its function. Immunoblotting was carried out with anti-K48 antibody and anti-K63 antibody to study the effect of mutant ubiquitins on polyubiquitin chain formation and to check efficacy of mutant ubiquitins to participate in K48 and K63

linked polyubquitination. Results revealed that mutants expressing UbEP42, UbL50P and UbI61T showed impairment in polyubiquitination with both K48 and K63 linkages, whereas UbS20F and UbA46S did not show any significant influence over polyubiquitin chain formation (Figure 4.8).

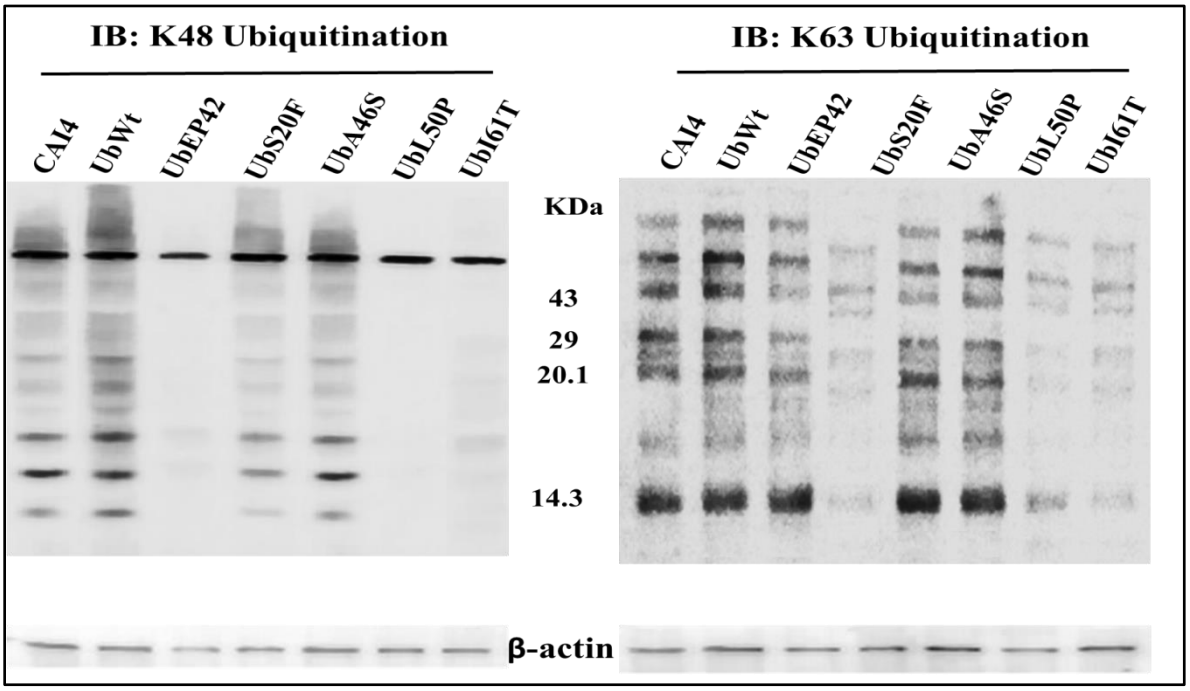


Figure 4.8: Western blot analysis showing polyubiquitination profile of K63 and K48 linkages of *C. albicans* cells expressing mutant forms of ubiquitin. UbEP42, UbL50P and UbI61T displayed impairment in polyubiquitination with both K63 and K48 linkages. The polyubiquitination profile of UbS20F and UbA46S did not show significant changes with K63 and K48 linkages.

4.3.8 Mutant ubiquitin proteins interfere with morphogenesis of yeast form to hyphae

Pathogenic phenotype of *C. albicans* is associated with switching of its morphology from yeast form to hyphae. Various reports suggest that ubiquitination contributes to the virulence of this organism (Leach et.al., 2011; da Silva Dantas et.al., 2016). In order to study the virulence of *C. albicans* cells expressing mutant ubiquitin proteins, hyphae formation capacity of yeast form was observed. *C. albicans* cells and their transformants were grown in the presence of serum, which induces hyphae formation at elevated temperature (37^0C). Only CAI4 and CAI4/UbWt cells showed yeast to hyphae

morphological transformation in serum at 37°C. CAI4/UbEP42, CAI4/UbL50P and CAI4/UbI61T exhibited formation of peudohyphae upon overexpression in serum induced condition and showed lysis after 1-2 hrs. CAI4/UbS20F and CAI4/UbA46S continued in yeast from. However, at 37° C in the absence of mutant protein overexpression all the mutants grew as yeast forms. Colony morphology was monitored. The colony structure of the CAI4/UbEP42 and CAI4/UbL50P showed fried-egg phenotype, although hyphae formation did not occur in these two colonies. The appearance of the colonies CAI4/UbS20F, CAI4/UbA46S and CAI4/UbI61T was normal. So collectively, the results suggest that none of the ubiquitin mutants described here have the ability to infect host cells (Figure 4.9).

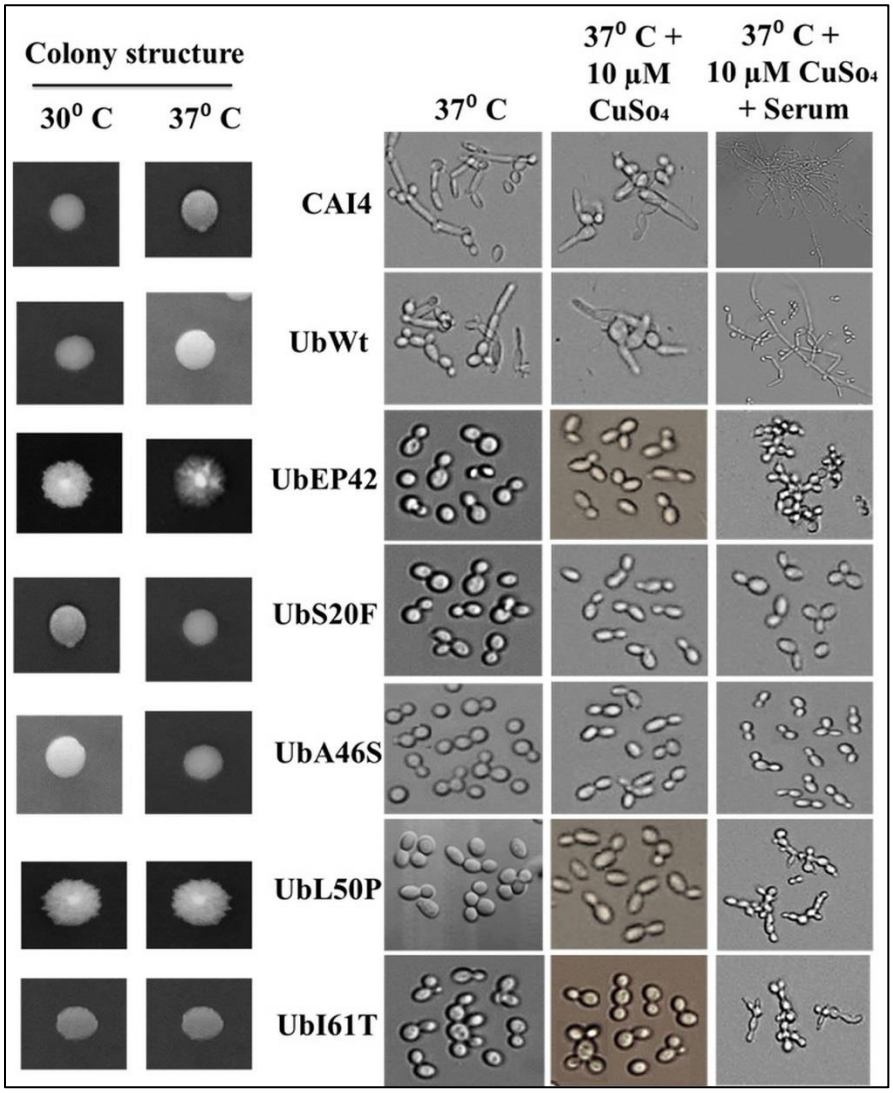


Figure 4.9: Initiation and maintenance of hyphal development in *C. albicans* CAI4 cells

and their transformants. CAI4 and CAI4 transformed with UbWt switched from yeast form to hyphae, while none of the transformants expressing mutant forms of ubiquitin could undergo yeast to hyphae morphogenesis, in serum induced media.

Transformants were plated on YPD pates and incubated at 30^0 C and 37^0 C to study colony morphology and filamentous growth. UbEP42 and UbL50P transformants were shown to have fried-egg (FE) colony phenotype. UbS20F, UbA46S and Ub I61T shown colony morphology as of CAI4 strain and Wild type transformed strain.

4.4 DISCUSSION

Post-translational modifications such as ubiquitination play key role in regulating major events in the cell such as growth, differentiation, cellular adaptations and development. Ubiquitination is one of the well-studied post-translational modifications. It contributes to cell via proteolytic as well as non proteolytic mechanisms to maintain steady state levels of proteins required for cell cycle progression, DNA repair, transcriptional and translational regulations, stress responses, protein trafficking, genome stability and many more.

Mutational approach was utilized by various groups in order to study the significance of conserved residues in ubiquitin. For example, Glu16 and Glu18 present on the acidic patch of the ubiquitin are essential for electrostatic interactions with the basic histone proteins (Debelouchina et.al., 2017). K63R mutant of ubiquitin renders cells more sensitive towards translational inhibitors and hampers ribosomal functions *in vivo* and *in vitro* (Spence et.al., 2000). Arginines present at 42, 54, 72 and 74 positions take part in E1 catalyzed hydrolysis of ATP and release of PPi (Burch & Hass, 1994). Seven lysine residues of ubiquitin participate in ubiquitination important for modulating many cellular processes (Chau et.al., 1989; Johnson et.al., 1992; Finley et.al., 1994; Spence et.al., 1995; Johnson et.al., 1995; Baboshina et.al., 1996). The surface residues present in the two hydrophobic clusters participate in vegetative growth, proteasome recognition and/or endocytosis and residues near the tail region participate in ubiquitin conjugation and deubiquitination (Sloper-Mould et.al., 2001). In our laboratory mutants were generated for Gln2, Glu64 and Ser65, which form parallel β-bulge in ubiquitin to study their importance in the context of its structure and functions (Mishra et.al., 2009; Mishra et.al., 2011; Sharma et.al., 2011). Later

on the importance of phosphorylation of ubiquitin at Ser65 by PINK1 protein kinase was reported another group (Wauer et.al., 2015a & b). Mutants of ubiquitin were also generated in our laboratory using random mutagenesis approach and screened in stress hypersensitive SUB60 strain of *S. cerevisiae*. Mutant UBEP42 isolated in the study has four amino acid residue replacements namely, S20F, A46S, L50P and I61T in its sequence. It has exhibited dosage dependent lethality. These four mutations were separated into single mutant derivatives. Structural characterization of the five mutant forms of ubiquitin revealed that UbEP42, UbL50P and UbI61T have distorted structure with less α-helix and more of β-sheet content (Prabha et.al., 2010; Doshi et. al., 2014; Doshi et.al., 2017). These mutant forms were found to hamper functions such as cell cycle progression, stress responses, protein sorting of carboxypeptidase S into lysosomes, lysosomal degradation of uracil permease, polyubiquitination with Lys63 and Lys48 linkages and protein degradation by UFD pathway in SUB60 strain of *S. cerevisiae*. The other two mutant forms UbS20F and UbA46S did not show any significant differences with respect to structure and function.

The effects of mutant forms of ubiquitin were studied here in *C. albicans* considering the pathogenicity associated with it and the evolutionary ancestry shared between *S. cerevisiae* and *C. albicans*. In *C. albicans* expression of UbEP42, UbL50P and UbI61T caused decline in viability along with slow growth kinetics and increased doubling time, which are identical to the results obtained with *S. cerevisiae* (Doshi et al., 2014; Doshi et.al., 2017). The results with transformants expressing UbS20F and UbA46S were similar to those obtained with UbWt expressing cells in *C. albicans*. The results of FACS analysis showed that cells expressing UbEP42, UbL50P and UbI61T were arrested in G0/G1 phase, delaying entry into S phase of cell cycle, once again similar to *S. cerevisiae* cells (Doshi et.al., 2014; Doshi et.al., 2017). The association of evolutionarily conserved Cdc28 protein kinase with G1 cyclins initiates START event for transition from G1 phase to S phase. *C. albicans* and *S. cerevisiae* share the same mechanism at START (Sherlock et.al., 1994). In *S. cerevisiae* cyclin dependent kinase Cdc28 is the central player of the cell cycle and therefore it is highly regulated (Mendenhall and Hodge, 1998). Since there is only one cyclin dependent kinase present both in *S. cerevisiae* and *C. albicans*, the enzyme associates with all the cyclins from G1 phase to B phase. G1-cyclin, Cln3 activated Cdc28 protein kinase triggers start-specific transcription, mediates cell size specific control and also allows expression of Cln1 and Cln2, which in turn help to move cell through G1 phase to S phase transition (Cvrcková and

Nasmyth, 1993). It was also observed that overexpression of 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. Further, ubiquitination determines regulation, activation and degradation of cyclins and their inhibitors during cell cycle (Cross, 1988; Chang et. al., 1990; Cross, 1990; Basco et al., 1995). Hence, functionally impaired variants of ubiquitin, UbEP42, UbL50P and UbI61T may interfere with and possibly inhibit ubiquitination of cyclin inhibitors leading to G1 phase arrest.

UBI4 expression in *C. albicans* is enhanced in response to various stress conditions similar to *S.cerevisiae* (Finley et.al., 1987; Finley et.al., 1991; Mager et.al., 1993; Riog et.al., 2000; Roig and Gozalbo, 2002; Leach et al., 2011). As *C. albicans* can cause pathogenesis, it has been evolutionarily tuned to a variety of host niches and relatively resistant to physiological stresses compared to *S.cerevisiae* (Jamieson et al., 1996; Nikolaou et al., 2009). Besides, factors that promote virulence in *C. albicans* also confer adaptability to a variety of metabolic and stress conditions even in the absence of direct contact with host. *C. albicans* cells expressing the mutations UbEP42, UbL50P and UbI61T could not withstand heat stress, antibiotic stress and showed diminished thermal tolerance. Under stress conditions misfolded, truncated and unfolded proteins accumulate and the degradation of which depends on functional ubiquitin proteasome system (UPS). Decline in the formation of polyubiquitin chains with K48 linkage indicates breakdown of UPS mediated degradation, most likely at the stage of ubiquitination. On the other hand UbS20F and UbA46S were able to survive all the stress conditions similar to wild type CAI4 and CAI4/UbWt. This finding goes hand in hand with the results obtained in the model organism *S. cerevisiae* (Doshi et.al., 2014; Doshi et.al., 2017). Besides, K63 linked ubiquitination of L28 subunit of ribosome confers increased resistance to antibiotics (Spence et.al., 2000; Hanna et.al., 2003). It is clear from earlier studies that the structural changes caused due to amino acid replacements in UbEP42, UbL50P and UbI61T affect their functions.

Any of the seven lysine residues in ubiquitin are utilized for the purpose polyubiquitination giving rise to various topologies, which in turn act as signals for carrying out different functions (Komander, 2009; Komander et.al., 2012; Sadowski et.al., 2012). Here, mutant forms of ubiquitin were expressed in *C. albicans* CAI4, where in *UBI4* gene is intact. In spite of optimal ubiquitin levels in the cell UbEP42, UbL50P and UbI61T produced

drastic effects, which made the cells incapable of overcoming the stress conditions. Moreover, ubiquitination profiles of UbEP42, UbL50P and UbI61T showed decline in the formation of both K48 and K63 linked chains on substrate proteins. At this stage, we can venture a guess that the mutant forms of ubiquitin could bind and inhibit either the enzymes of ubiquitination pathway or prevent further extension of polyubiquitin chains after being added to growing ubiquitin chain on substrates. It is well known that when polyubiquitin chain is synthesized enzymes sample different regions of donor ubiquitin and acceptor ubiquitin. It is possible that under normal conditions when there is no stress, the ubiquitin expressed from host system is contributing to survival of the organisms at sublethal levels of expression of UbEP42, UbL50P and UbI61T. The status of only two of the linkages was probed here and it is difficult to comment whether they can participate in other linkages or not.

The ability to switch between yeast, psedohyphal and hyphal forms acts as major virulence determinant in *Candida albicans* (Homann et al., 2009). The signalling pathways responsible for morphology switching occur through activation of either CaEfg1 transcription factor by cAMP and Protein kinase A (PKA) or targeting CaCph1 transcription factor by mitogen activated protein kinase (MAPK) which is also used to transduce the mating pheromone response (Whiteway, 2000; Liu, 2001; Brown, 2002). Overexpression of the mutant forms UbEP42, UbL50P and UbI61T at permissive conditions made *C. albicans* adopt yeast type morphology. At 37^0C, these mutants formed pseudohyphae in serum induced condition. Studies with the mutations in *S.cerevisiae* established that the protein level of Fus3, the MAPK which drives mating-pheromone induced response, has been found to be increased (Doshi et al., 2014; 2017). Cascade of events of the MAPK pathway results in the activation of Fus3, which further activates Far1, leading to G1 phase arrest due to inhibition of Cdc28-Cln2 or Cdc28-Cln3 complexes (Elion, 1993; Saito, 2010). Moreover, protein expression of transcription factors Efg1p, Nrg1p, Rbf1p, Rim101p, Fkh2p and Tec1p, which are responsible for morphology switching, was reported to be decreased in the cells upon Cdc28 depletion (Umeyama et.al., 2006). CaCDC4 of *C. albicans* is homologue of F-box protein component of the SCF^{CDC4} ubiquitin ligase of *S.cerevisiae*. Hyphae formation observed with Ca*CDC4*$^{-/-}$ suggests that the dimorphic switch between yeast and hyphal forms is regulated by ubiquitin-mediated proteolysis (Atir-Lande et.al., 2005). The above observations imply that decrease in Cdc28 protein levels in the mutants expressing UbEP42,

UbL50P and UbI61T may be responsible for the morphological changes.

In conclusion, structural studies of mutant ubiquitin UbEP42, UbL50P and UbI61T revealed that these mutations caused distortions in the structure of ubiquitin (Doshi et.al, 2017). Therefore, structural changes in ubiquitin may hamper interactions of these mutant forms of ubiquitin with other proteins leading to functional aberrations and poor survival of *C. albicans*. UbS20F and UbA46S produced milder effects on most functions tested in *C. albicans*, as the structure of ubiquitin is largely intact. However, all five mutations prevent morphogenesis of hyphae from the yeast form in *C. albicans*, which is required for its pathogenicity.