# Chapter II

# Functional characterization of ubiquitin mutations in *Saccharomyces cerevisiae*

# 2 Functional characterization of ubiquitin mutations in *Saccharomyces cerevisiae*

## 2.1 INTRODUCTION

Ubiquitin is one of the highly conserved proteins in eukaryotic cells, playing central role in maintaining homeostasis of cell. To understand the structural and functional significance of the conserved residues *in vitro* evolution of ubiquitin gene was carried out and the mutated copies of ubiquitin gene were introduced into *S. cerevisiae* cells. Mutants showing interesting phenotypes were isolated. One of them was a dosage dependent lethal mutation UbEP42, carrying four amino acid substitutions, namely S20F, A46S, L50P and I61T (Figure 2.1) (Prabha et al., 2010).

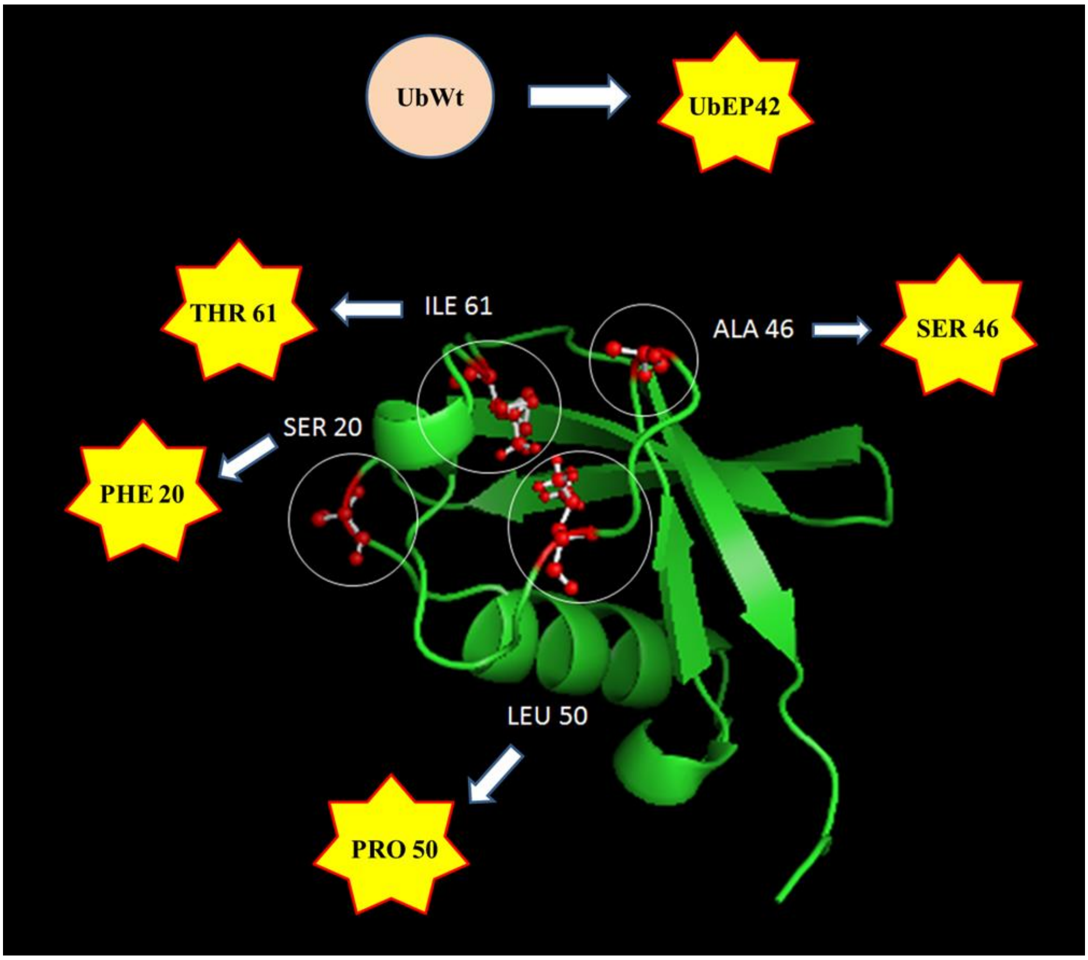



**Figure 2.1: Cartoon structure of UbWt and UbEP42 showing original and substituted**

**amino acid residues.**

Expression of UbEP42 in SUB60 cells led to dosage dependent lethality (Figure 2.2), slow growth, ultimately increasing the doubling time of cells (Figure 2.3). UbEP42 was incorporated into polyubiquitin chains. Cells expressing UbEP42 showed reduced degradation of substrates of UFD (ubiquitin fusion degradation) pathway and displayed heightened sensitivity towards translational inhibitor, cycloheximide. SUB60 cells expressing UbEP42 could not withstand elevated temperature (Figure 2.4) (Pradeep Mishra’s thesis, 2009).

Mutations present in UbEP42 were dissected out into single mutants UbS20F, UbA46S, UbL50P and UbI61T and studied to understand their individual effects on growth rate, doubling time, stress survival. UbL50P and UbI61T expressing SUB60 cells displayed phenotypic lethality similar to UbEP42 (Figure 2.2, 2.3 and 2.4). For instance, slow growth rate, increased doubling time, increased sensitivity towards antibiotic stress and heat stress. However, the expression of UbS20F and UbA46S did not have any influence over SUB60 cells (Mrinal Sharma’s thesis, 2013). In the present study UbEP42 and its single mutant derivatives were studied further for their effects at molecular level.

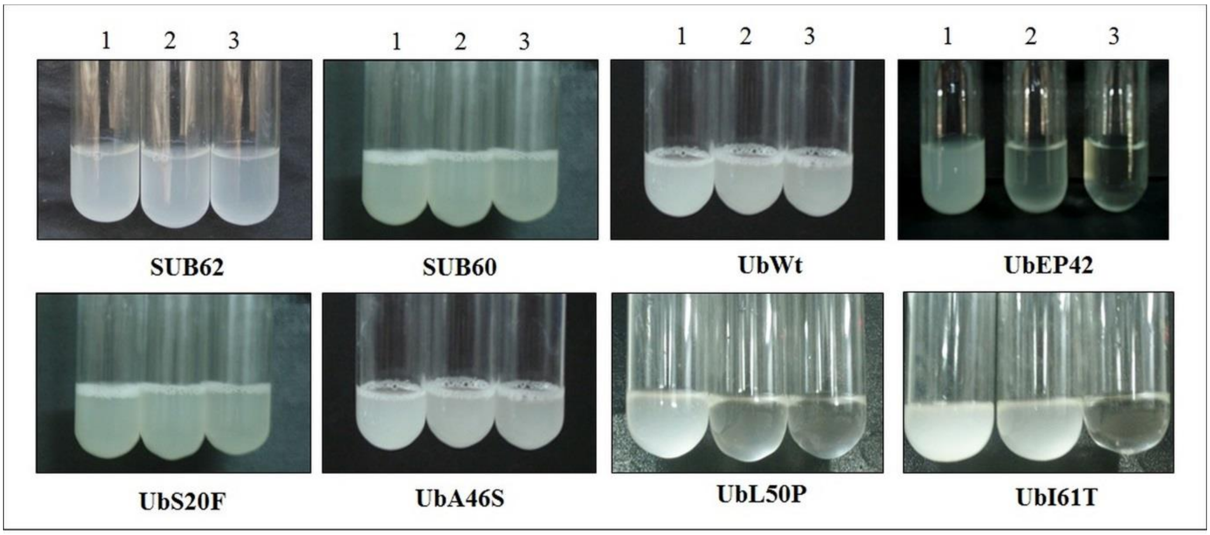



**Figure 2.2: Effect of overexpression of ubiquitin variants on SUB60 cells. Effect of the expression of single mutations derived from UbEP42 on the growth of SUB60 strain of *S. cerevisiae*. 1, 2 and 3 correspond to 0 µM, 100 µM and 200 µM of $CuSO_4$. SUB62, SUB60 and SUB60 expressing ubiquitin wild type gene extrachromosomally (UbWt)**

**were used as controls. The three mutant forms of ubiquitin UbEP42, UbL50P and UbI61T confer dosage dependent lethality on host SUB60 cells, while UbS20F and UbA46S had no effect.**

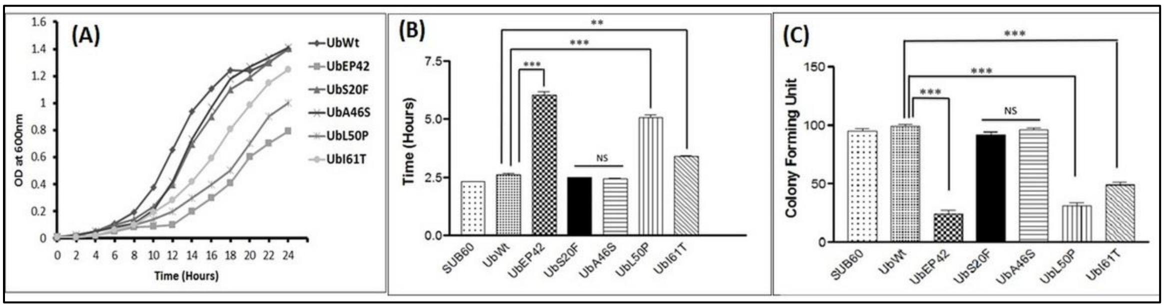



**Figure 2.3: Ubiquitin variants affect growth pattern of SUB60 cells. (A) Growth pattern of SUB60 cells expressing mutant forms and wild type of ubiquitin gene. (B) Generation time of SUB60 transformants expressing UbWt, UbEP42 and its single mutant derivatives. (C) Percentage survival rate of cells expressing mutant forms of ubiquitin. UbEP42, UbL50P and UbI61T impaired growth, increasing the generation time. Survival of these mutants was also affected. The generation time values were represented as Mean ± SE. p*** <0.001 and p = NS represents non-significance and the experiment was repeated in three independent sets.**

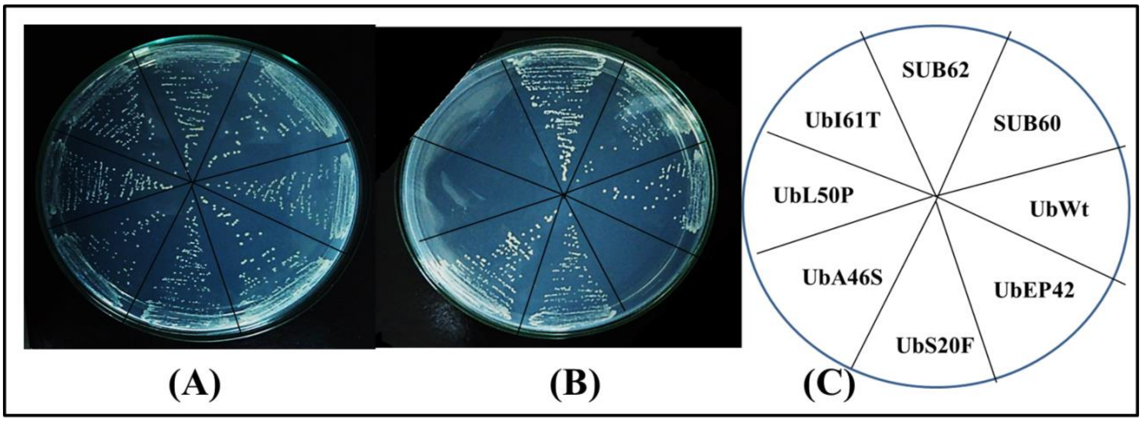



**Figure 2.4: Effect of expression of mutations on the survival of SUB60 cells upon exposure to heat stress. The cells were exposed to 40°C to induce heat stress and brought back to 30°C. UbEP42, UbL50P and UbI61T negatively affected the survival of SUB60 cells. UbS20F and UbA46S complemented better than all other single mutants. SUB60 and SUB60 transformed by YEp96/UbWt were used as controls.**

## 2.2 MATERIALS AND METHODS

### 2.2.1 Strains, media and plasmids

*S. cerevisiae* strains used in the study were SUB62 (*MATa lys2-801 leu2-3,112 ura3-52 his3- Δ 200 tr1-1*), and SUB60 (*MATa ubi4- Δ2::LEU2 lys2-801 leu2-3,112 ura3-52 his3-Δ200 tr1-1*) (gift from Prof. Daniel Finley). SUB60 is *ubi4* deletion mutant lacking *UBI4* polyubiquitin gene and was used for studying the effects of expressing the single mutations of ubiquitin *in vivo* (Finley et al., 1987). SUB62, which is a wild type strain for all four ubiquitin genes, was used as a positive control. *S. cerevisiae* cultures were grown in synthetic dextrose (SD) medium containing 0.67% Hi-media yeast nitrogen base and 2% glucose or 4% galactose as carbon source. Media were supplemented with histidine (20 mg $L^{-1}$), lysine (30 mg $L^{-1}$), uracil (20 mg $L^{-1}$), leucine (100 mg $L^{-1}$) and tryptophan (20 mg $L^{-1}$) as per the experimental requirement. Cultures were grown at 30°C at 200 rpm. *Escherichia coli* DH5α was used to amplify plasmid which harbours mutant forms of ubiquitin gene and was cultured at 37°C on LB medium from Hi-media. Transformants were selected in the presence of 100 µg $ml^{-1}$ of ampicillin.

### 2.2.2 Cell cycle analysis

From the previous results of our laboratory with UbEP42 and single mutant derivatives, it was established that expression of UbEP42, UbL50P and UbI61T slowed down growth rate of SUB60 cells, increased doubling time. Cell cycle analysis was carried out in order to study the detailes of cell cycle phases to elucidate at which phase ubiquitin variants are hindering cell cycle progression.

#### 2.2.2.1 Analysis by confocal microscopy

The cultures of SUB62 cells, SUB60 cells and transformants of SUB60 cells expressing UbWt, UbEP42 and single mutant derivatives were grown to mid-log phase to approximately same OD at 600nm. Cells were stained with DAPI and washed twice with phosphate buffed saline pH7.0. The cultures were observed using confocal microscopy. The images were captured by Carl Zeiss LSM (laser scanning microscope) 710 by 63X (Oil immersion) objective at a magnification of 630X with DAPI filter. Excitation and emission wavelengths were 403 nm and 430 nm respectively. Experiment was repeated with three independent sets.

#### 2.2.2.2 Analysis by flow cytometry

SUB60 transformants carrying wild type and mutant ubiquitin genes were grown till log phase. Cells were harvested and washed with phosphate buffered saline (PBS), pH 8.0 (137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$), fixed in 70% ethanol for 30 min and washed with PBS once again. Then the cells were stained with propidium iodide at a final concentration of 10 µg $ml^{-1}$ and incubated at room temperature for 30 min. Once again the cells were given a PBS wash. Cells were treated with RNase to ensure staining of DNA alone. SUB62, SUB60 carrying wild type gene for ubiquitin (UbWt) on the plasmid YEp96 were used as positive controls and SUB60 as negative control. Results were recorded on flow cytometer BD FACS Aria II. The experiment was repeated in three independent sets and the percentage of cells in G0/G1, S and G2/M phase were plotted. The values given in the graph represent mean ± SEM.

#### 2.2.2.3 Cell proliferation by BrdU assay

To study proliferation of cells expressing mutant forms of ubiquitin BrdU assay was performed as described by Lengronne et al., 2001. Thymidine kinase (TK) plays key role in DNA synthesis by *de novo* pathway. Yeast cells lack thymidine kinase (TK) and therefore synthesize dTMP by thymidylate synthatase. Hence, incorporation of BrdU which is a convenient tool to study cell cycle progression in eukaryotes and cannot be utilized with yeast system. As an alternative, Schwob's group utilized yeast cell expressing *herpes simplex TK* gene under control of constitutive *GDP* promoter leaving *de novo* pathway unaltered.

SUB60 cells transformed with wild type and mutant forms of ubiquitin gene were co-transformed with ESD422 vector containing *HSV-TK* gene driven by *GPD* promoter for BrdU incorporation (Kind gift from Prof. Schwon E., Institute of Molecular Genetics, France). Cells were grown in the presence of 100µM $CuSO_4$ inducer to overexpress mutant ubiquitin protein. Cells were arrested at G1 phase by incubating with 2 µg/ml of α-factor for 2.5 hours followed by releasing in to S phase by adding 50µg/ml pronase. BrdU was added 30 minutes prior to release of cells. BrdU assay was carried out according manufacturer's instructions. Briefly, cells were harvested at regular time intervals after addition of BrdU and probed with anti BrdU antibody and HRP-linked secondary antibody. Cells were treated with TMB substrate and OD was recorded at 650nm. Experiment was repeated in three independent sets and the OD values were presented as mean±SEM.

### 2.2.3 Levels of Cdc28 protein kinase and Fus3 MAPK in presence of mutant ubiquitin

Cdc28 protein kinases, Fus3 MAPK are involved in cell cycle progression. According to previous studies, cell cycle is getting hindered at G1 phase of cell cycle. So this study was carried to check its mRNA levels, protein levels and efficacy to degrade through proteasome mediated degradation. Along with this, CDC37, a chaperon and CLN3 mRNA transcript levels were also checked.

#### 2.2.3.1 Detection of protein levels of Cdc28 protein kinase and Fus3 MAPK by western blot analysis

SUB60 cells harbouring mutant ubiquitin as well as wild type ubiquitin carrying plasmid were grown till log phase in presence of 100 µM $CuSO_4$. Cells were harvested, washed with phosphate buffer saline followed by sonication in order to lyse it. The lysate were clarified by centrifuging cells at high speed. Protein was quantified by Folin-Lowry method and equal concentration of protein was loaded in 15% SDS-PAGE. Separated proteins were transferred to nitrocellulose membrane. This was followed by probing the membrane overnight at $4^oC$ with primary antibody anti-Cdc28 antibody (Santa cruz, Cat No.: sc-28550) for Cdc28 protein kinase, anti Erk1/2 antibody (CST, Cat No.: 9102) for Fus3 MAPK. The membrane was then probed with Goat anti Rabbit IgG HRP Conjugate (GeNei, Cat No.: HPO3). After that the membrane was subjected to washing with PBS-T six times and with PBS twice. Blot was developed by ECL western Blotting Detection Kit (Amersham Biosciences).

#### 2.2.3.2 Determination of mRNA levels of *CDC28* protein kinase, *FUS3* MAPK, *CDC37* chaperon and *CLN3* G1 cyclin

**RNA extraction and cDNA synthesis:** SUB60 cells harbouring mutant ubiquitin as well as wild type ubiquitin carrying plasmid were grown till log phase in presence of 100 µM $CuSO_4$. Cells were harvested and total RNA was isolated by TRIZOL method. RNA integrity was verified by 1.5% agarose gel electrophoresis and RNA yield was determined spectrophotometrically at 260/280 nm. cDNA was synthesized using 1 µg of total RNA by Thermo Scientific VersoTM cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

The expression of *CDC28*, *FUS3* and *ACT1* transcripts levels in SUB60 cells, and SUB60 expressing UbWt, UbEP42, UbS20F, UBA46S, UbL50P and UbI61T were measured by amplifying above mentioned genes by polymerase chain reaction (PCR) using gene specific primers (Eurofins) as shown in table 2.1. Expression of *ACT1* gene was used as a reference. Densitometry analysis was carried out to measure transcript levels. Transcript level of SUB60 expressing UbWt from plasmid was considered as positive control and SUB60 as negative control.

| Primer | Oligonucleotide sequence | Amplicon size |
|---|---|---|
| **CDC28 FR** | 5' – CCAAGGTCAAAGAGTAGTCG - 3' | 228 |
| **CDC28 RE** | 5' - GGT TGG TCC TTT GGA ATA CC - 3' | |
| **FUS3 FR** | 5' – TATATCCAGTGACTTCCA - 3' | 248 |
| **FUS RE** | 5' - TGT AGA CCT CAT TGA AGT - 3' | |
| **ACT1 FR** | 5' – AAGGTATCATGGTCGGTATGG - 3' | 282 |
| **ACT1 RE** | 5' - AAA CGT AGA AGG CTG GAA CG - 3' | |
| **CDC37 FR** | 5' - CTA AGC CAC TGG AAG CAA CT - 3' | 260 |
| **CDC37 RE** | 5' - GAT ACT CAA AGG CCT TCA TC - 3' | |
| **CLN3 FR** | 5' - CGA CCT CGA CTT TGT TCC TT - 3' | 261 |
| **CLN3 RE** | 5' - GAA TGT TGC CGA CTG ACA GA - 3' | |

**Table 2.1: Oligonucleotide sequence of genes to study mRNA transcript level.**

**2.2.3.3 Determination of proteasomal degradation of Cdc28 protein kinase and Fus3 MAPK by western blot analysis**

This experiment was carried out to study if Cdc28 protein kinase and Fus3 MAPK are degraded by proteasomes. SUB60 cells and their transformants expressing wild type and mutant forms of ubiquitin were grown till log phase in the presence of 100 µM $CuSO_4$ in order to overexpress ubiquitin protein. Later, they were grown for another 4 hours in the presence and absence of 50 µM proteasome inhibitor, MG132. Cell lysates were prepared and immunoblotting was carried out as described above.

### 2.2.4 Effects of ubiquitin variants on ROS level

Reactive oxygen species (ROS) generated inside SUB60 cells and their transformants expressing wild type and mutant forms of ubiquitin were monitored as described by Aleksandra et.al. (2014). Cultures of SUB60 and its transformants were grown till they reached to 0.6 OD. SUB60 cells were treated with 1mM $H_2O_2$ to stimulate ROS generation. This was taken as positive control and SUB60 cells without staining as negative control. Cells from the cultures were harvested at log phase and washed with PBS, pH 7.0. Cells were stained with 2'7'-dichlorohydrofluorescein diacetate (DCFDA) and washed with PBS. The levels of reactive oxygen species were estimated using Flow cytometer BD FACS Aria II. The values are represented as mean±SE. The experiment was repeated in triplicate.

### 2.2.5 Consequence Of ubiquitin mutants on lysosomal degradation of uracil permease

**Detection by Western blot analysis:** Uracil permease is a membrane protein which follows lysosomal degradation pathway. Its degradation is carried out by Rsp5 dependent K63 linked ubiquitination. To check the efficiency of ubiquitin mutants for mediating uracil permease degradation though lysosomal pathway, experiment was designed as described below. The SUB60 cells containing UbWt and mutant ubiquitin expressing Yep vectors were cotransformed with YEp352fF (2µ *URA3 FUR4*) which carries the *FUR4* gene under the control of its endogenous promoter. SUB60 cells carrying UbWt and ubiquitin variants were grown till log phase at 30°C at 200 rpm. The overexpression of transformed gene was carried out by adding 100 µM $CuSo_4$. At 0.6 OD, cells were treated with 100 µg/ml of cycloheximide in order to inhibit protein synthesis. To accurately measure degradation of uracil permease, protein synthesis is blocked. Cells were harvested at 0 min and 120 min. Cell extract was prepared and uracil permease content was determined by immunoblot using an antiserum to the last 10 residues of the permease (C-terminal antipeptide antiserum, kind gift from Prof. Finley). The membrane was then probed with Goat anti Rabbit IgG HRP Conjugate (GeNei, Cat No.: HPO3). After that the membrane was subjected to washing with PBS-T six times and with PBS twice. Blot was developed by ECL western Blotting Detection Kit (Amersham Biosciences).

**Fluorimetric detection:** To study the accumulation of uracil permease in the mutant ubiquitin protein expressing cells, fluorescent spectroscopy was utilized. SUB60 cells were co-transformed with mutant ubiquitin and GFP tagged uracil permease coding vectors. Cells were treated as described above and harvested at regular intervals of 0 min, 30 min, 60 min, 90 min and 120 min. Intensity of GFP was measured at 509 nm using F-7000 Fluorescence Spectrophotometer. Data represented as Mean±SE.

### 2.2.6 Effect of ubiquitin mutations on trafficking of carboxypeptidase S protein

#### 2.2.6.1 Construction of GFP - Carboxypeptidase S (CPS) chimeric protein

Trafficking of carboxypeptidase S from cytoplasm to MVBs in the cell is mediated by K63 linked ubiquitination. To test the effect of mutations on intracellular protein trafficking GFP tagged CPS was constructed. The plasmid pG045 carrying the gene for carboxy peptidase S was a gift from Prof. Greg Odorizzi, MCD Biology, University of Colorado, Boulder. The gene for carboxy peptidase S was cloned in pUG46. Vector map of pUG46 is shown in figure 2.5. pUG46 expresses yEGFP gene under *ADH* promoter. *URA3* was the selection marker.

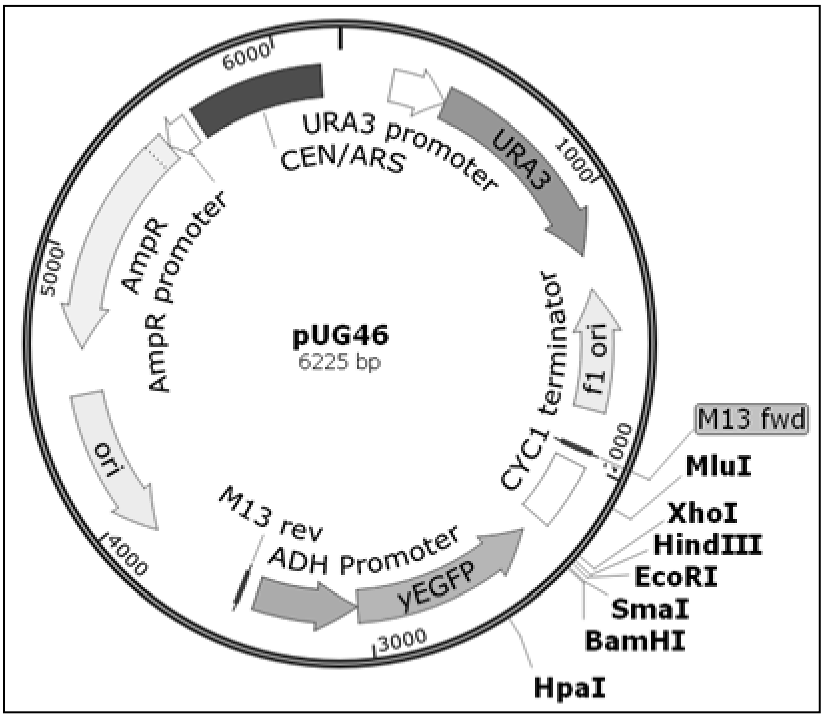


**Figure 2.5: Vector map of pUG46.**

Carboxy peptidase S gene was PCR amplified using CPS FR (5'AATACAGGATCCATGAT CGCCTTACCAGTAGAG-3') and CPS RE (5'AGCGGGGAATTCTTAAGCGTATTCGTT AACATT-3'). The amplicon was cloned between *Bam*HI and *Eco*RI of pUG46. Clones were confirmed RE mapping, sequencing and EGFP fluorescence was monitored under microscope.

#### 2.2.6.2 Monitoring localization of carboxypeptidase S by confocal microscopy

To study the localization of carboxypeptidase S in the yeast cells, confocal microscopy was carried out. Untransformed SUB60 control and SUB60 cells transformed with plasmids carrying wild type and mutant forms of ubiquitin gene were grown till exponential phase at 30°C. Cells were harvested and washed with culture media. Cells were resuspended in 500 µl culture media and added with FM4-64 (Molecular probe, Invitrogen) endosome tracker dye to a final concentration of 8 µM. The cells were incubated at 30°C in water bath for 30 minutes in dark. After spinning down the cells were resuspended in 4 ml of culture media and grown for 90 min at 30°C. After that cells were harvested and washed twice with PBS. Cells resuspended in PBS were observed under confocal microscope for fluorescence. The localization of GFP fused CPS in the cell was observed using GFP filter and the localization of FM4-64 was monitored with Cy3 filter. The images were captured by Carl Zeiss LSM (laser scanning microscope) 710 by 63X (Oil immersion) objective.

### 2.2.7 Influence of variants of ubiquitin on different antibiotic stress

YEp96 vectors carrying wild type and mutated ubiquitin genes were transformed into stress hypersensitive *ubi4* mutant SUB60 strain. Antibiotic complementation study of ubiquitin variants was carried out using plate assays as per standard protocol (Spence et al., 2000; Hanna et al., 2003). SUB60 transformants harbouring YEp96 plasmids with genes for wild type as well as mutant ubiquitin were grown at 30$^0$C until their optical density values reached 0.2 and the cultures were subjected to successive fourfold serial dilutions and spotted on SD agar plates. Cells were induced with 25 µM copper sulphate in the presence of antibiotic. The concentration of antibiotics used in this study were geneticin (75 µg ml$^{-1}$), tunicamycin (1 µg ml$^{-1}$), gentamycin (90 µg ml$^{-1}$), canavanine (1 µg ml$^{-1}$ ) and hygromycin

(80 μg $ml^{-1}$). Plates were incubated for 5-6 days at $30^{0}$C to assess the complementation potential of the mutant ubiquitin under antibiotic stress by examining growth.

### 2.2.8 *In Vivo* Polyubiquitin chain formation assay

#### 2.2.8.1 Cloning of mutant ubiquitin in yeast expression vector

Incorporation of mutant forms of ubiquitin into polyubiquitin chains was tested using the method described by Ellison and Hochstrasser (1991). Plasmid pUB221 carries c-myc tagged wild type ubiquitin gene and *URA3* as selection marker (Figure 2.6). The gene for UbWt was replaced by ubiquitin gene carrying mutations.

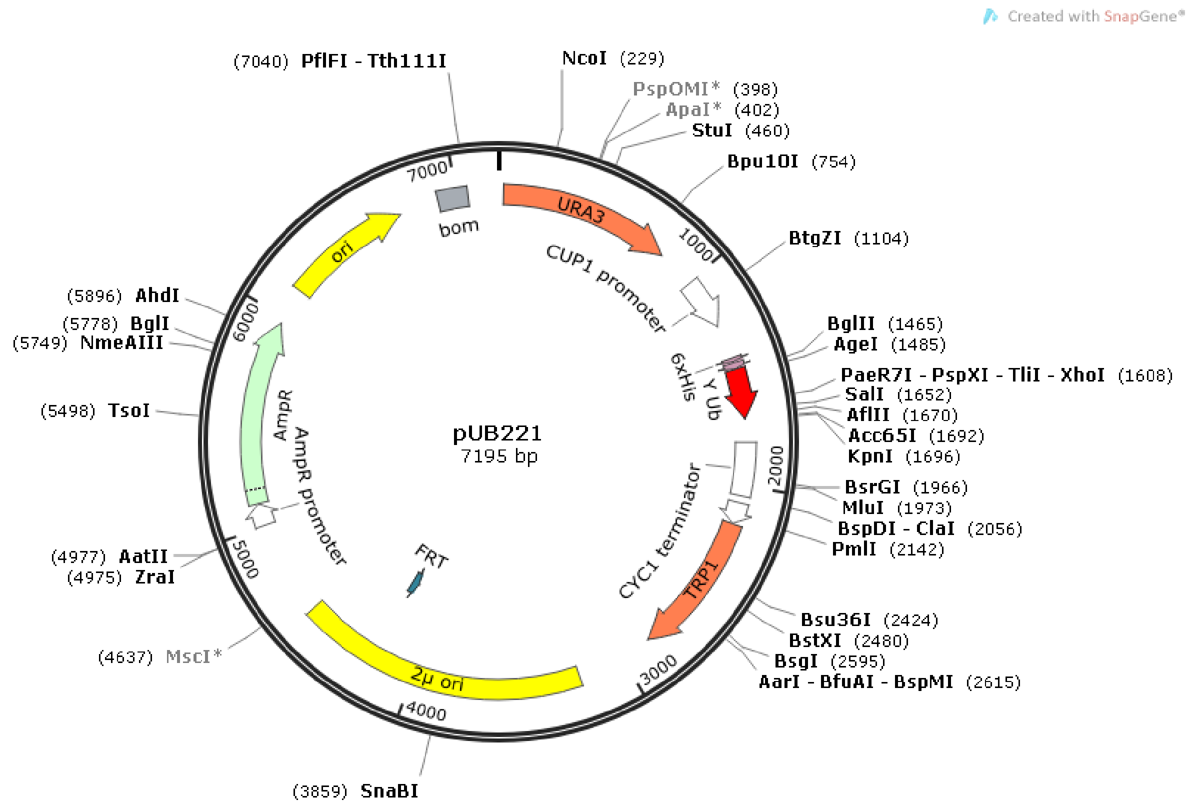



**Figure 2.6: Vector map of pUB221.**

Mutants namely UbS20F, UbA46S, UbL50P and UbI61T were PCR amplified from YEp vectors using gene specific primers UBFR and UBRE. The PCR amplicon was digested and cloned between *Bgl*II and *Kpn*I restriction sites. Clones were confirmed by RE mapping and sequencing.

#### 2.2.8.2 Study of polyubiquitin chain formation by western blot

Plasmid pUB221 carrying wild type ubiquitin gene and plasmids carrying single mutants of ubiquitin gene were transformed in SUB60 strain. SUB60 transformants were grown to midlog phase ($OD_{600}$=0.6) in the presence of 100 µM $CuSO_4$. The cells were pelleted and washed twice with distilled water, suspended in normal saline and treated with gel loading buffer. Equal amounts of protein were loaded in each well on 17% SDS-PAGE. Proteins were transferred on PVDF 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) from Roche at 1/1000 dilution followed by washing membrane with PBS-T and PBS several time. The blot was developed with enhanced luminol in ECL kit (Amersham).

### 2.2.9 Incorporation of variants in Lys63 and Lys48 linked chain formation by western blot analysis

Yep96 vector carrying the inserts of *UbWt*, *UbEP42* and its single mutant derivatives were transformed into SUB60 strain of *S. cerevisiae*. Transformed ubiquitin genes were overexpressed with the help of an inducer, 100 µM $CuSO_4$. To study the formation of K63 and K48 linked polyubiquitin chains, the method described above was followed up to blocking, which was followed by probing 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 $4^{0}$C respectively followed by probing it with HRP-tagged secondary antibody. Six washes were given to membrane with PBS – Tween20 solution and followed by two washes of PBS. The blot was developed as in the above case using ECL kit (Amersham).

### 2.2.10 Degradation of substrate ubiquitin fusions by N-end rule and UFD pathways

β-galactosidase assay was performed with cultures expressing ubiquitin mutants using the method described earlier for estimating the levels of Ub-Pro-β galactosidase and Ub-Met-β galactosidase (Johnson et al., 1995; Varshavsky, 1996; Doshi et al., 2014). Briefly, the transformants of SUB60 with YEp96 carrying genes for UbS20F, UbA46S, UbL50P and UbI61T were cotransformed with pUB23. Plasmid pUB23 is a shuttle vector with *URA3* as selection marker, carries ubiquitin β-galactosidase gene fusion (Ub-X-bgal) under galactose inducible *GAL10* promoter Figure 2.7 (Bachmair et al., 1986). The two variants of pUB23 namely pUB23/Met and pUB23/Pro have codons for Met and Pro in place of X respectively.

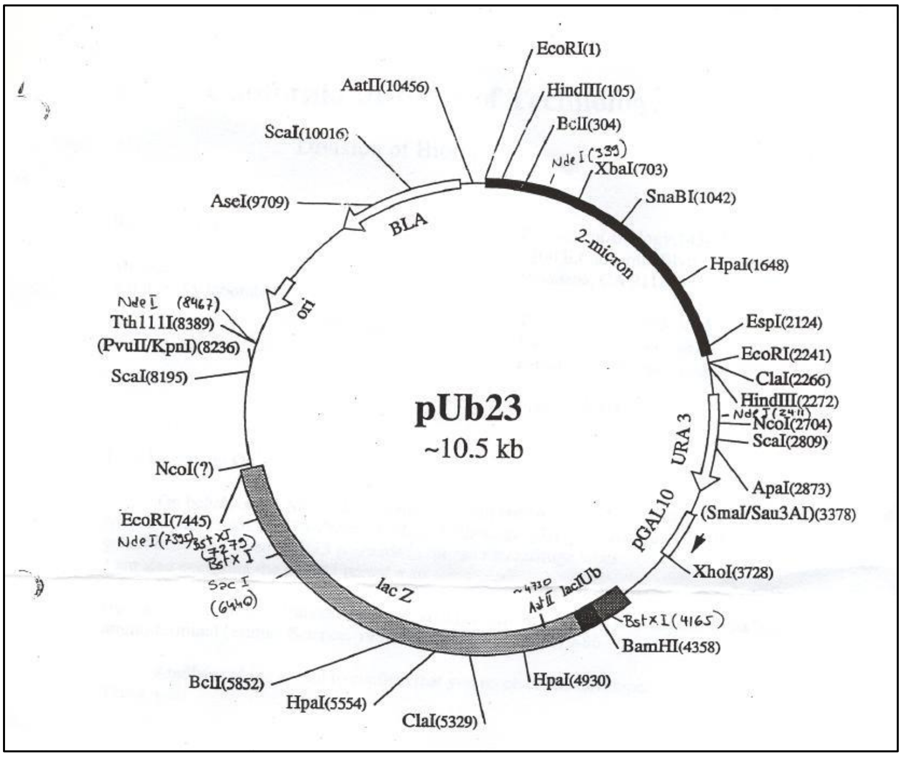



**Figure 2.7: The vector map of pUB23.**

The ubiquitin synthesized by YEp vectors were utilized to build polyubiquitin chain. Based on the N-terminal residue of substrate protein it will either be stabilized or it will be degraded through proteasomal degradation pathway i.e. if the N-terminal residue is Met, attached ubiquitin monomer will be detached by deubiquitinating enzyme and stabilize the substrate. On other hand if Pro is the N-terminal residue, deubiquitinating enzyme cannot cleave ubiquitin monomer and as a result polyubiquitin chain will form which ultimately drags the substrate for degradation. The experiment was carried out to check the potency of ubiquitin mutant's involvement in N-end rule degradation pathway.

### 2.2.10.1 Enzyme assay

Cells were grown till mid-log phase at 30°C and in synthetic galactose media, were added with 100 µM $CuSO_4$. β-Galactosidase was assayed to measure protein stability using o-nitrophenyl thiogalactoside (ONPG) substrate as described by Baker et.al., 1991 and protein concentration was estimated using Folin-Lowry method. pUB23 transformants of SUB60 and

SUB60 cells with YEp96/UbWt were used as controls, while SUB60 cells with YEp96/UbEP42 was used as reference. The enzyme assay was repeated in three independent sets and the activity of β-galactosidase was expressed in nanomoles of ONPG converted per minute per milligram protein. Mean ± SEM values are presented in the graphs.

## 2.3 RESULTS

### 2.3.1 Effects of ubiquitin variants on cell cycle progression.

In the budding yeast, the cell size and the bud size vary according to different phases of cell cycle. The cells which are in G0/G1 phase are mostly round in shape and possess small bud initiation, S phase cell contains cells with large or growing bud whereas G2/M phase cells have large bud and cell size is also larger. Based on these criteria, cells were classified into different stages of cell cycle and counted. Results suggests that UbEP42, UbL50P and UbI61T displayed more number of cells in G0/G1 phase with small bud and quite large mother cell. UbS20F and UbA46S expressing cells appeared healthy, with large bud and behaved as UbWt expressing cells (Figure 2.8). SUB62, SUB60 and UbWt expressing SUB60 cells were considered as controls.

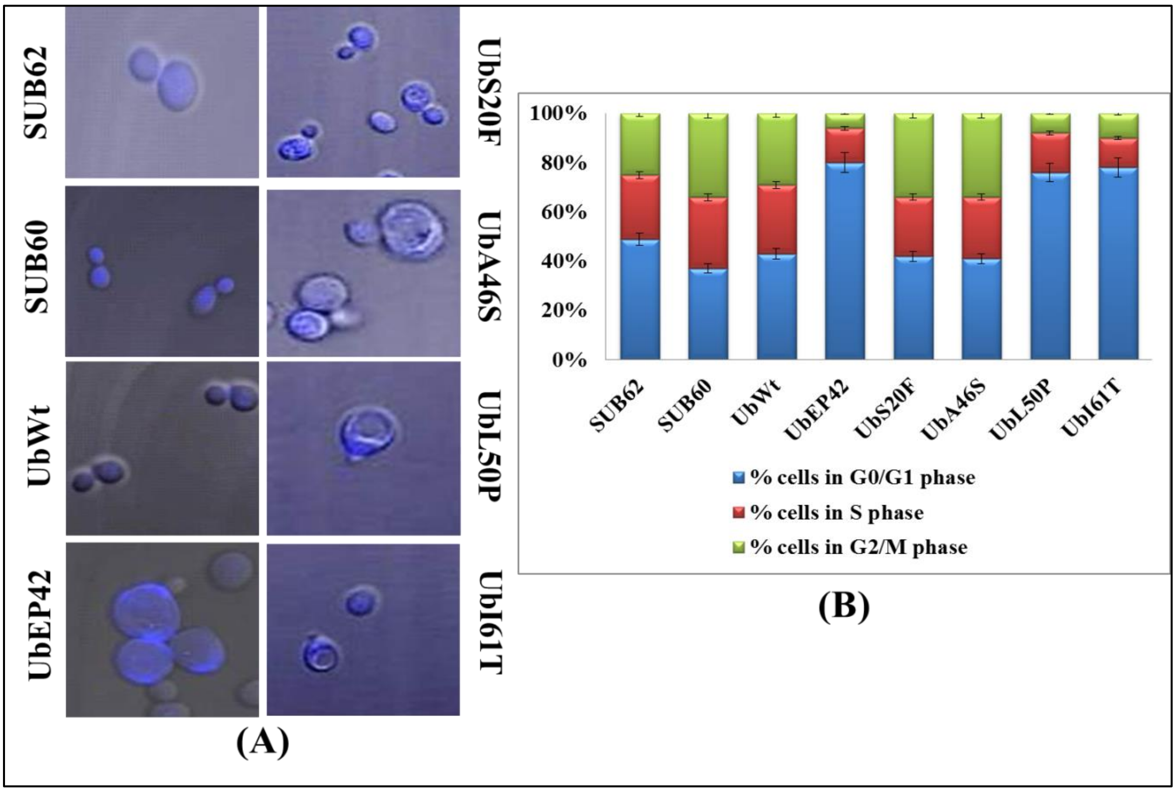


**Figure 2.8: Confocal images showing influence of ubiquitin variants on budding and cell cycle. Based on the bud size cells were counted in different cell cycle phases. (A) confocal images of DAPI stained SUB60 cells expressing ubiquitin variants (B) graph showing percentage cells found in G0/G1, S and G2/M cell cycle phases. Graph represents values in Mean±SE.**

FACS analysis of the cultures of UbEP42 and single mutant derivatives of UbEP42 was carried out to confirm the above results with cell cycle progression (Figure 2.9(A)). SUB62 cells and the transformants of SUB60 cells expressing UbWt were used as positive controls, SUB60 cells were used as negative control. The results indicate that the transformants expressing UbEP42, UbL50P and UbI61T were held up in G1 phase and showed delayed entry into S phase leading to a reduction in growth (Figure 2.9(B)). On the contrary UbS20F and UbA46S expressing SUB60 cells remained unaffected.

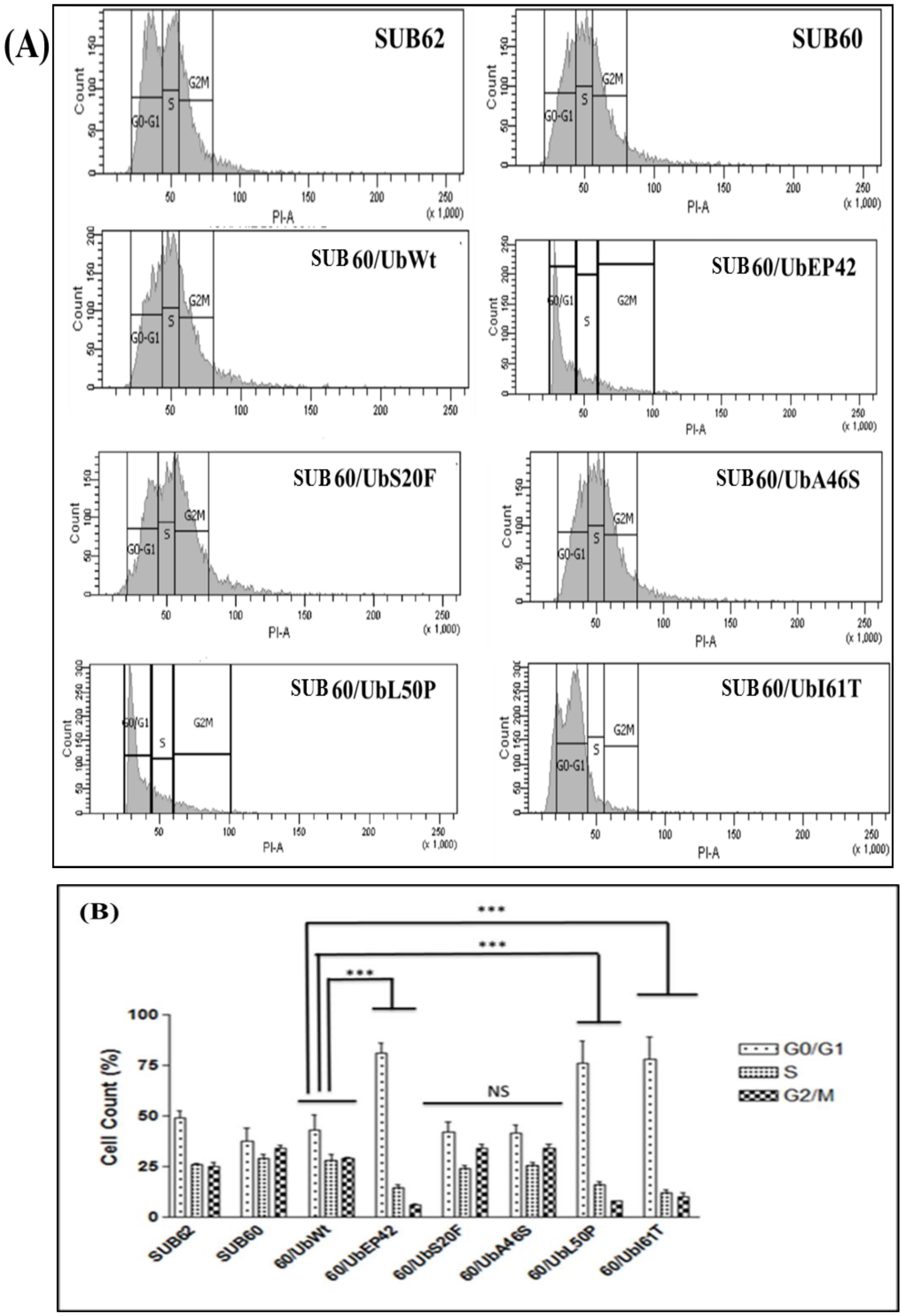



**Figure 2.9: Consequence of ubiquitin mutants on cell cycle progression. (A) FACS analysis showing cell cycle progression of mutant expressing SUB60 cells. (B) Bar graph displays percentage count of cells in G0/G1, S and G2/M phase of cell cycle based on FACS analysis. Results represented in Mean±SE. (n = 3, P*** = <0.001, NS = non-**

**significant).**

From the FACS analysis, it was evident that the mutants expressing UbEP42, UbL50P and UbI61T displayed delayed entry in S phase lowering the growth of SUB60 cells. Protein levels of Cdc28 protein kinase and Fus3 MAPK which play key roles in cell cycle progression were affected by these three mutations. BrdU cell proliferation assay was carried out to study ability of UbEP42, UbL50P and UbI61T expressing cells to enter into and progress through S phase of cell cycle. Here incorporation of BrdU was monitored in order to study START of replication and progressing through S phase. Results show that the mutants expressing UbEP42, UbL50P and UbI61T were unable enter into S phase whereas SUB60, SUB60 transformed with UbWt, UbS20F and UbA46S were able to progress through S phase (Figure 2.10).

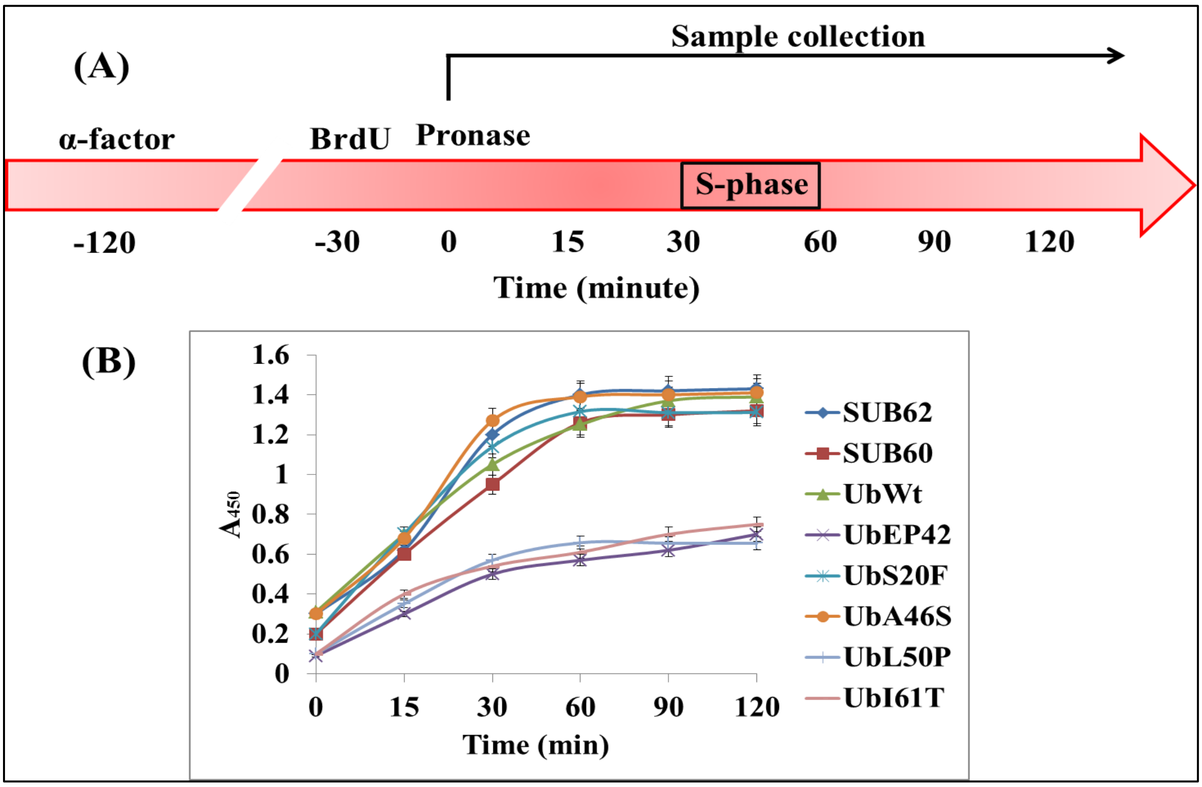



**Figure 2.10: BrdU cell proliferation assay. (A) Schematic presentation of experiment (B) It displays cell proliferation ability of ubiquitin variants expressing SUB60 cells along with SUB60 and UbWt.**

### 2.3.2 Levels of Cdc28 protein kinase and Fus3 MAPK in presence of mutant ubiquitin

Cdc28 protein kinase or cyclin dependent kinase (Cdk) plays a crucial role in cell cycle progression. To gain further insight into the results of FACS analysis, Cdc28 protein kinase levels were analyzed in the UbEP42 and its single mutant derivatives. Cells expressing the two mutants UbL50P and UbI61T, which showed delayed entry into S phase have also showed reduced levels of Cdc28 protein kinase, similar to UbEP42 (Figure 2.11). Cells expressing the other two mutants UbS20F and UbA46S did not show any alteration in their Cdc28 protein kinase levels with respect to control cells, which was in agreement with their normal cell cycling behaviour.

Fus3 is a mitogen activated protein kinase (MAPK), which is known to arrest cells in G1 phase. Since the cells expressing UbEP42, UbL50P and UbI61T were delayed in G1 phase, we wanted to determine if Fus3 had any role in it. The results established that the levels of Fus3 were elevated in SUB60 transformants expressing UbEP42, UbL50P and UbI61T, while the levels of the protein in cells expressing UbS20F and UbA46S matched the levels seen in SUB60 cells and SUB60 transformants expressing UbWt, which was in line with the results of FACS analysis (Figure 2.11).

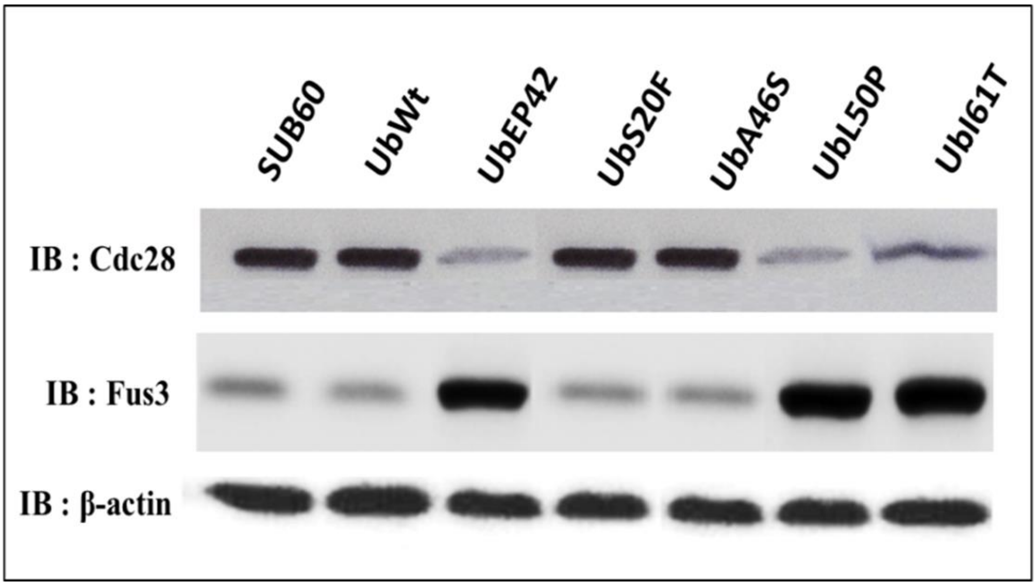



**Figure 2.11: Western blot showing the levels of Cdc28 protein kinase and Fus3 MAPK in SUB60 cells and its transformants expressing UbWt, UbEP42 and single mutants of ubiquitin. Expression of UbEP42, UbL50P and UbI61T caused decrease in the level of**

**Cdc28 protein kinase and increase in Fus3 MAPK protein levels in SUB60 cells.**

In order to see if the expression of UbEP42, UbL50P and UbI61T negatively influence the expression of Cdc28 protein kinase and Fus3 at genetic level, we monitored the mRNA levels of the two proteins in SUB60 cells, their transformants expressing UbWt, UbEP42 and the single mutant derivatives of UbEP42. The results show that the mutant ubiquitins bear no effect over the expression of the proteins at mRNA level (Figure 2.12).

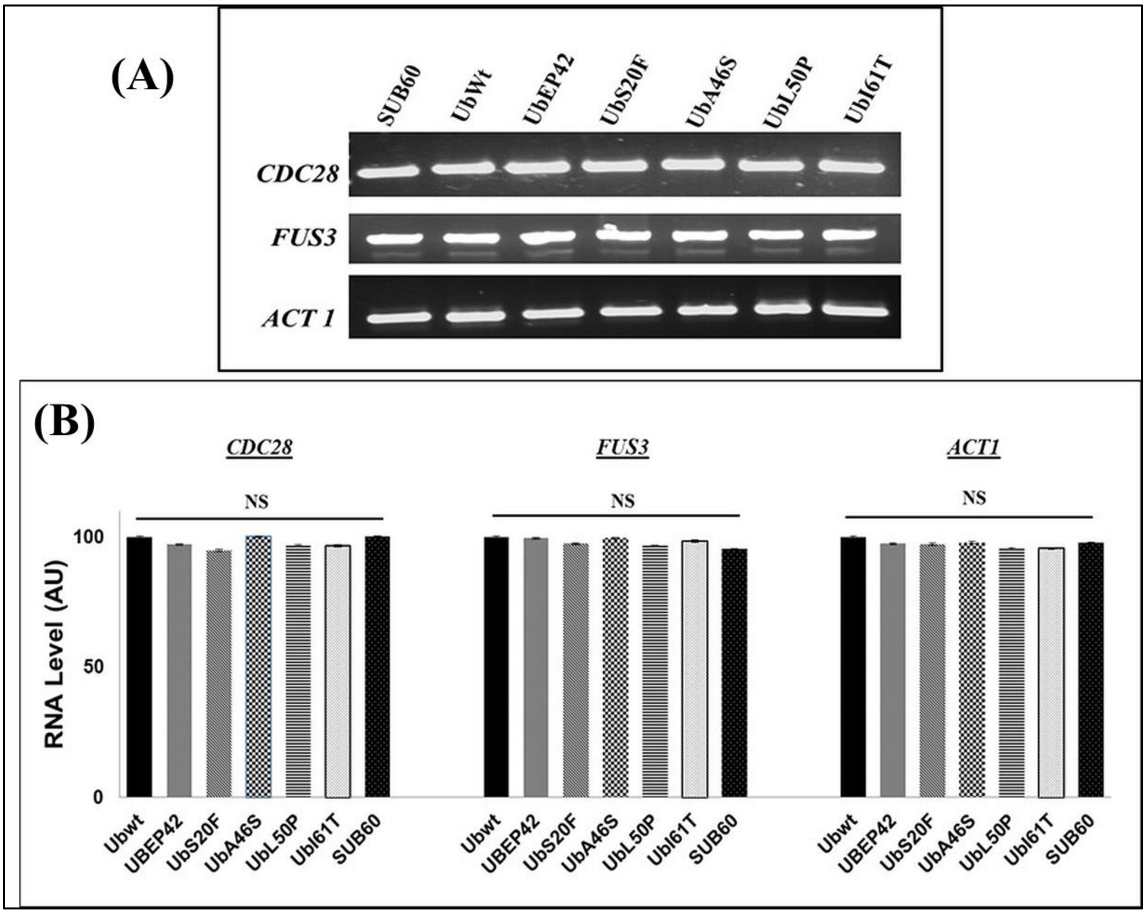



**Figure 2.12: Monitoring mRNA levels of *CDC28* protein kinase and *FUS3* MAPK. (A) The northern blots show that the mRNA levels of *CDC28* protein kinase and *FUS3* MAPK. (B) Densitometry analysis of *CDC28* protein kinase and *FUS3* MAPK. Experiments repeated in three independent sets and values represent mean±SE.**

So far there is no evidence in literature on the degradation of Cdc28 protein kinase and Fus3 MAPK. In order to test if the proteins are degraded by proteasomes, SUB60 cells, their transformants expressing UbWt, UbEP42 and the single mutant derivatives of UbEP42 treated with proteasome inhibitor, MG132 were subjected to western blot analysis. Cultures of the above strains grown in the absence of MG132 were used as controls. From the results it is evident that Cdc28 protein kinase and Fus3 are not degraded by proteasomes, as MG132 treatment had no effect over the levels of these proteins (Figure 2.13).

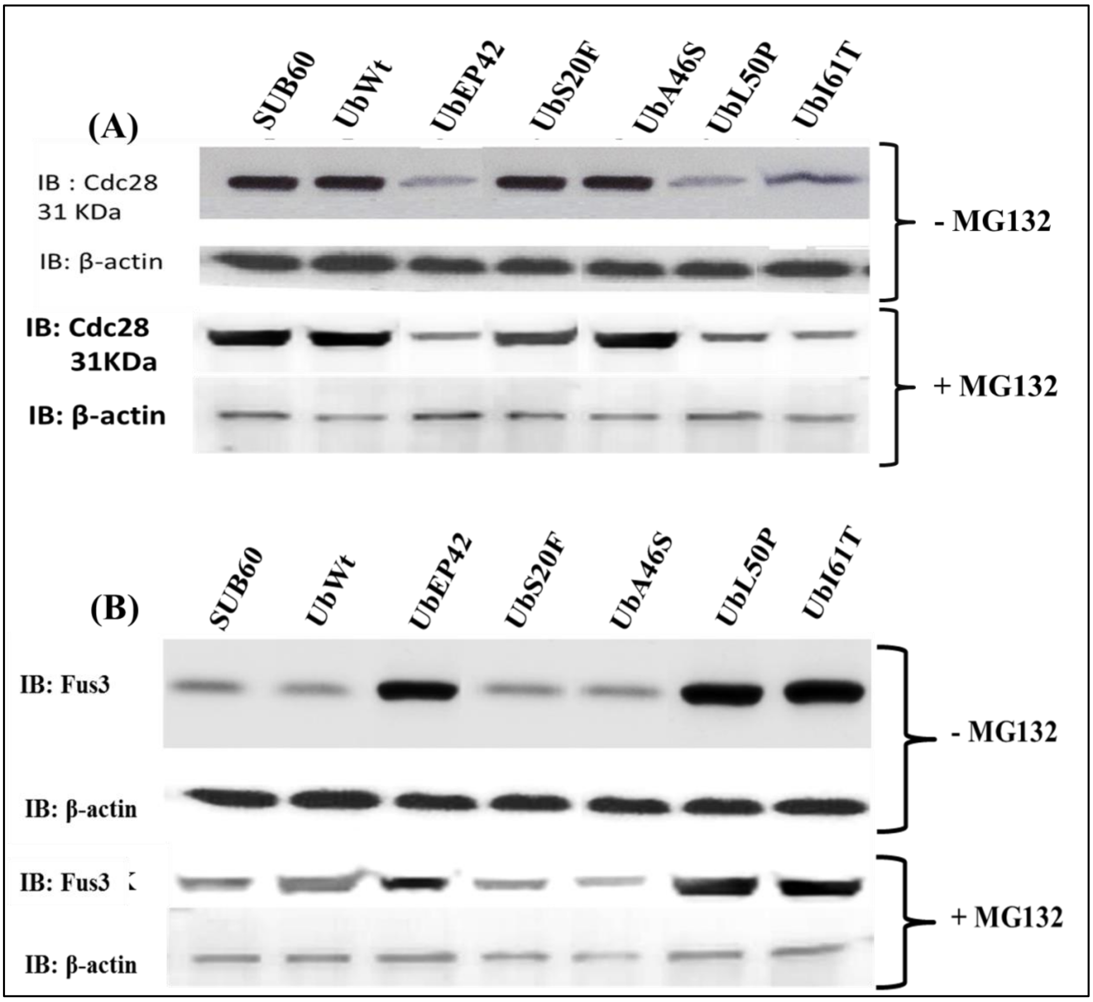



**Figure 2.13: Protein levels of Cdc28 and Fus3 in presence and absence of proteasome inhibitor. (A) Cdc28 protein kinase and (B) Fus3 MAPK protein levels were monitored in the cells expressing SUB60 cells, UbWt, UbEP42 and single mutant derivatives in presence and absence of proteasomal inhibitor MG132. SUB60 cells and UbWt were used as controls. Inhibition of proteasomes by MG132 established that these proteins are not degraded by proteasome.**

G1 cyclin, Cln3 regulate cell cycle progression by interacting with Cdc28 protein kinase and the molecular chaperone Cdc37 binds to nascent chain of Cdc28 protein kinase and stabilizes it (Tyers et al., 1992; Mandal et al., 2007). Cyclin-dependent protein kinase, Cdc28 plays central role in cell cycle regulation. G1 cyclins Cln1, Cln2 and Cln3 together with Cdc28 are required for DNA replication, duplication of the spindle pole body and bud emergence. When a cell reaches its critical size, replication of DNA takes place by an event known as START. G1 cyclin, Cln3 activates Clnl and Cln2 at START, which can then stimulate their own accumulation by means of a positive feedback loop and also activate another pair of S phase cyclins, Clb5 and Clb6. Cdc37 is a molecular chaperone that functions in association with Hsp90 to promote folding of protein kinases, transcription factors and many other clients by stabilizing or maintaining them in a folding competent conformation (Millson et al., 2005; Zhao et al., 2005; Kimura et al., 1997). Cdc28 protein kinase and Fus3 MAPK are stabilized by Cdc37 chaperone. The mRNA levels of *CDC37* and *CLN3* were analyzed to check the effect of ubiquitin variants on their transcription. Results indicated no significant differences in presence of all ubiquitin mutants, suggesting that these variants are not hampering mRNA expression of either protein (Figure 2.14).

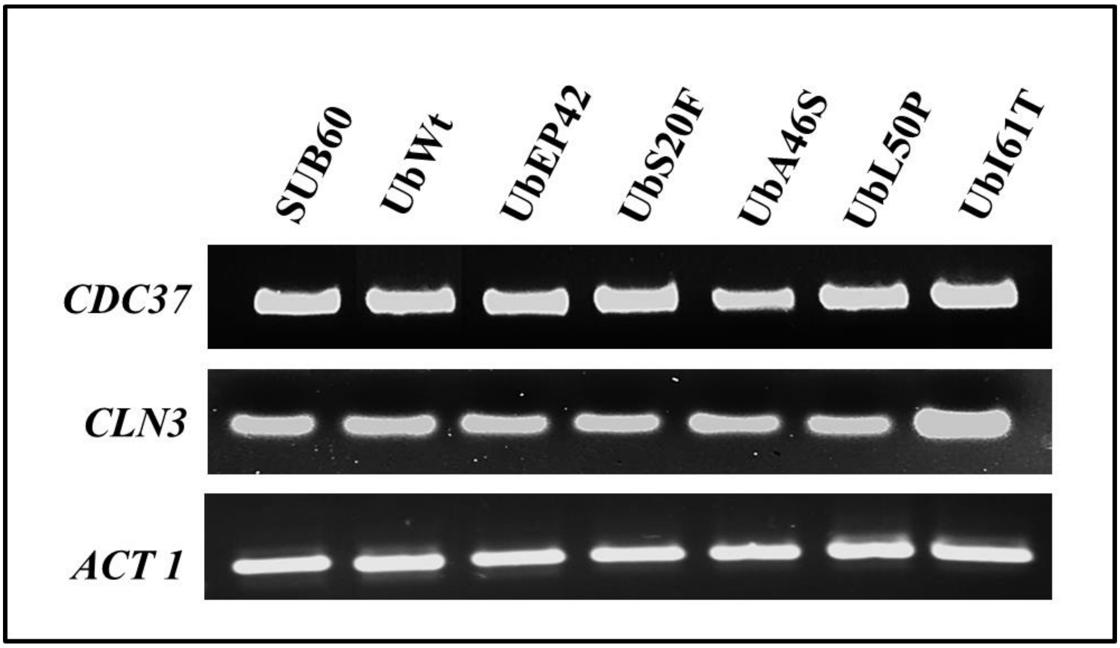



**Figure 2.14: Monitoring mRNA levels of *CDC37* and *CLN3*. *ACT1* used as internal control.**

### 2.3.3 Influence of ubiquitin variants on the production of reactive oxygen species in SUB60 cells

Reactive oxygen species play active role in cell physiology. Endogenous ROS at low levels are required for cell cycle progression and redox signalling pathways. The results of confocal microscopy, FACS analysis established cell cycle arrest in G0/G1 phase in cells expressing UbEP42, UbL50P and UbI61T. This experiment was carried out to check the effect of mutant ubiquitins on ROS levels. Results suggests that in SUB60 cells expressing UbEP42, UbL50P and UbI61T the ROS levels were considerably lowered as compared to UbWt, UbS20F and UbA46S expressing SUB60 cells. Even SUB60 cells showed higher level of ROS than these three lethal mutants (Figure 2.15).

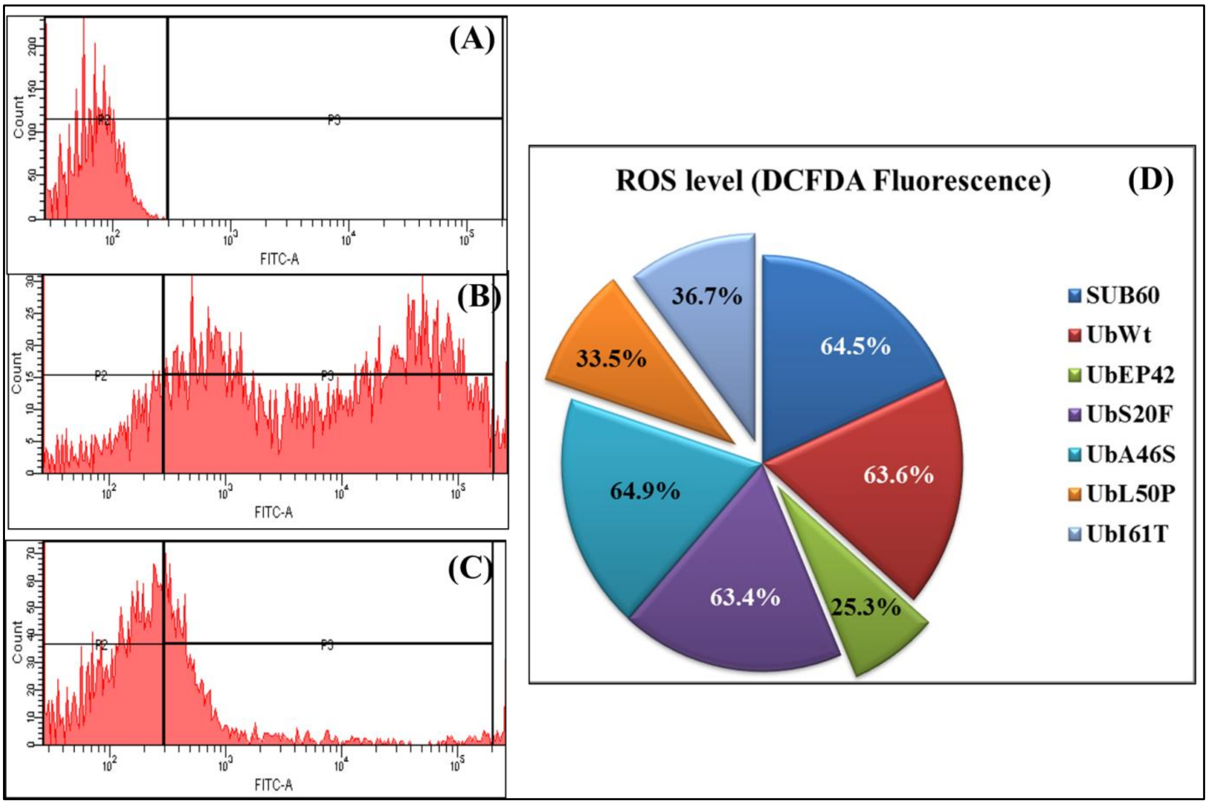



**Figure 2.15: Levels of reactive oxygen species in presence of mutant ubiquitin in SUB60 cells. (A) negative control which does not show DCFDA fluorescence (B) representative FACS image of SUB60 cells, SUB60 expressing UbWt, UbS20F and UbA46S (C) representative FACS image of SUB60 cells expressing UbEP42, UbL50P and UbI61T (D) graphical representation showing percentage of ROS levels in SUB60 cells**

**expressing wild type as well as mutant ubiquitin protein.**

### 2.3.4 Consequence of ubiquitin mutants on lysosomal degradation of uracil permease

Uracil permease is a membrane protein, which is earmarked for degradation by polyubiquitin chains with K63 linkage. Subsequently, the protein undergoes endocytosis and degradation in multivesicular bodies (Galan et al., 1996). Transformants expressing UbL50P and UbI61T show undiminished levels of uracil permease similar to UbEP42, while the transformants with UbS20F and UbA46S showed degradation of uracil prermease similar to SUB60 expressing UbWt (Figure 2.16). Hence, the results suggest that UbEP42, UbL50P and UbI61T cannot participate in the formation of ubiquitin chains with K63 linkage.

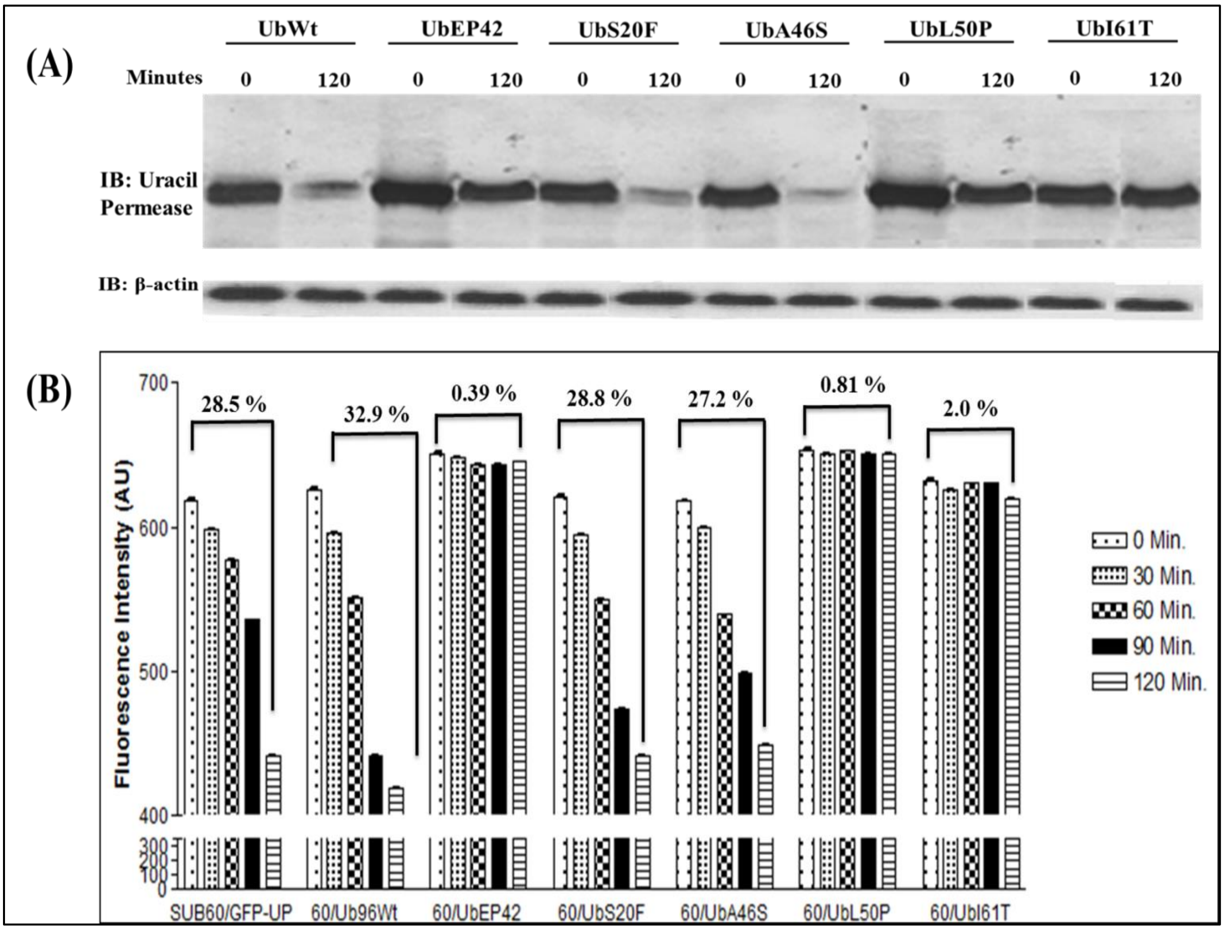



**Figure 2.16: Effect of mutant forms of ubiquitin on lysosomal degradation of uracil permease. (A) Western blot showing changes in the levels of uracil permease in SUB60 cells expressing UbWt and mutant ubiquitins Lanes 1 to 12 show SUB60 transformants**

**expressing UbWt, UbEP42, UbS20F, UbA46S, UbL50P and UbI61T after being exposed to inducer for 0 min. and 120 min. respectively (B) Fluorescence spectroscopy of UbWt and mutant ubiquitin transformed SUB60 cells to monitor accumulation of EGFP tagged uracil permease at regular intervals of 30 min.**

### 2.3.5 Effect of ubiquitin mutations on trafficking of carboxypeptidase S protein

Carboxypeptidase S is a resident protease present in multivesicular bodies, the equivalent of lysosomes in higher eukaryotes. It is transported across the membrane of multivesicular bodies only after ubiquitinated by ubiquitin ligase Rsp5, which is known to participate in the formation of polyubiquitin chains with K63 linkage. The sorting of carboxypeptidase S was monitored by expressing it with a green fluorescent protein (GFP) tag (Figure 2.17) and monitoring its co-localization with a multivesicular body specific dye FM4-64 (Life Technologies, Molecular Probes). The images of laser scanning microscopy establish that cells expressing UbEP42, UbL50P and UbI61T mutations show dramatic decline in the sorting of Carboxypeptidase S, unlike cells expressing UbS20F and UbA46S, which display sorting similar to cells expressing UbWt (Figure 2.18).

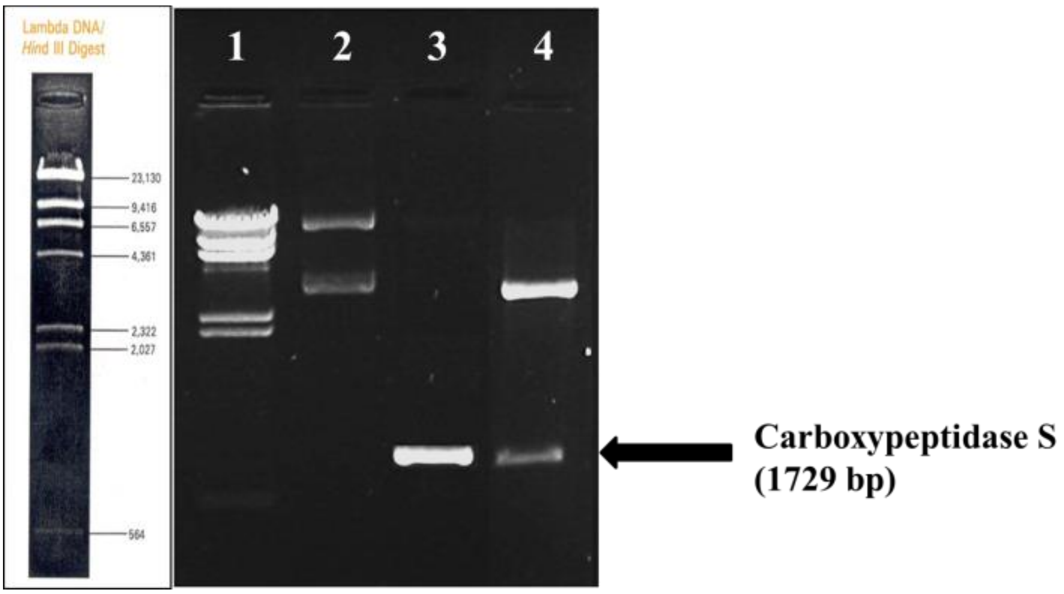



**Figure 2.17: 1% agarose gel showing clone confirmation of GFP tagged carboxypeptidase S. lane 1 shows lambda DNA marker, Lane 2 shows the plasmid pUG46 containing GFP-CPS insert, Lane 3 shows PCR amplicon of carboxypeptidase S,**

**Lane 4 displays inset release of Carboxypeptidase S upon double digestion with cloning restriction enzymes *Eco*RI and *Bam*HI.**

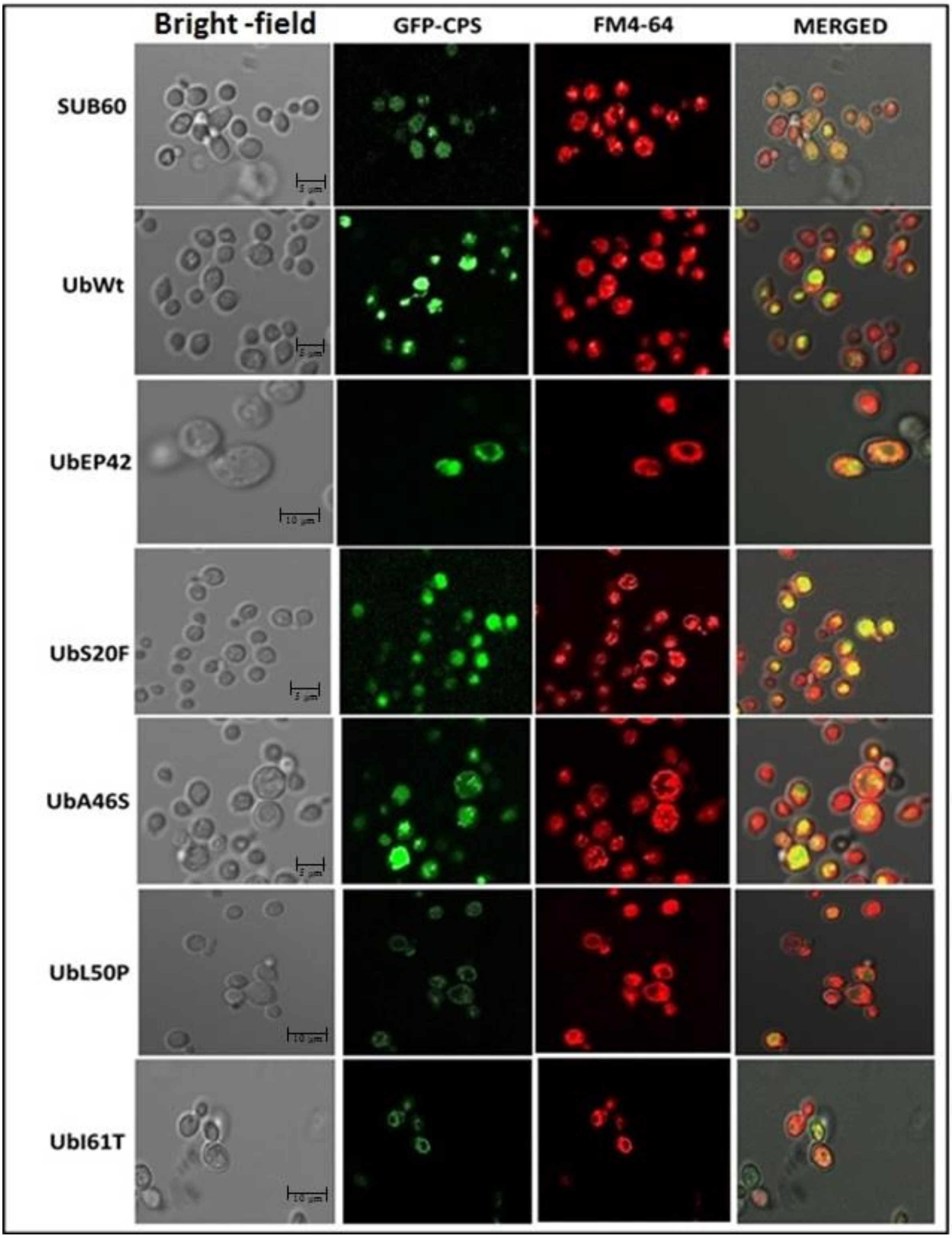


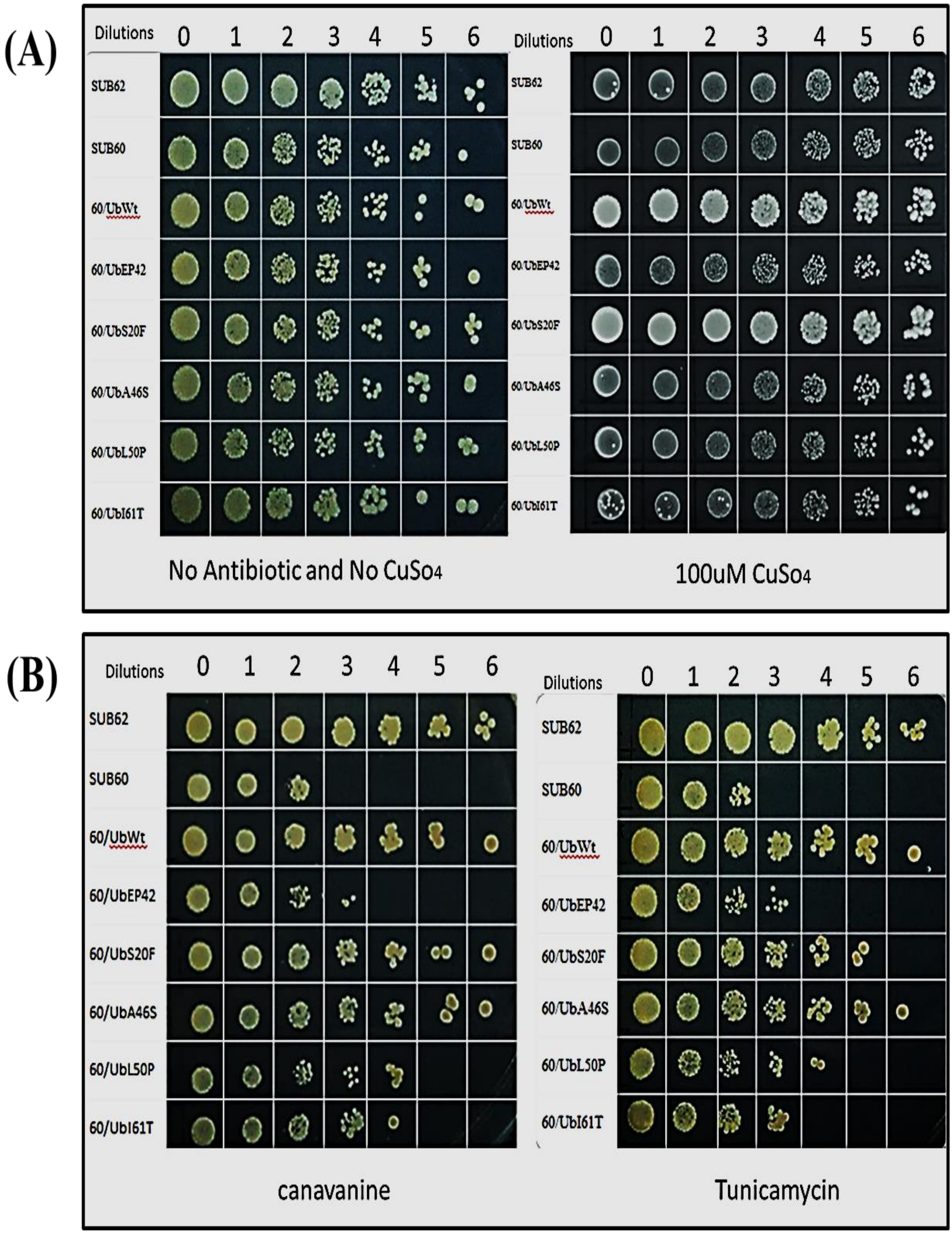
**Figure 2.18: Effect of expression of UbWt, UbEP42, UbS20F, UbA46S, UbL50P and UbI61T on the sorting of carboxypeptidase S (CPS) monitored using confocal microscopy. The first lane shows bright field images, while the second and third monitor localization of chimeric protein of GFP-CPS and the endosomal tracker dye (FM4-64) respectively. The fourth lane shows overlay of images of second and third lanes.**

### 2.3.6 Influence of ubiquitin variants on complementation ability to withstand antibiotic stress by various antibiotics

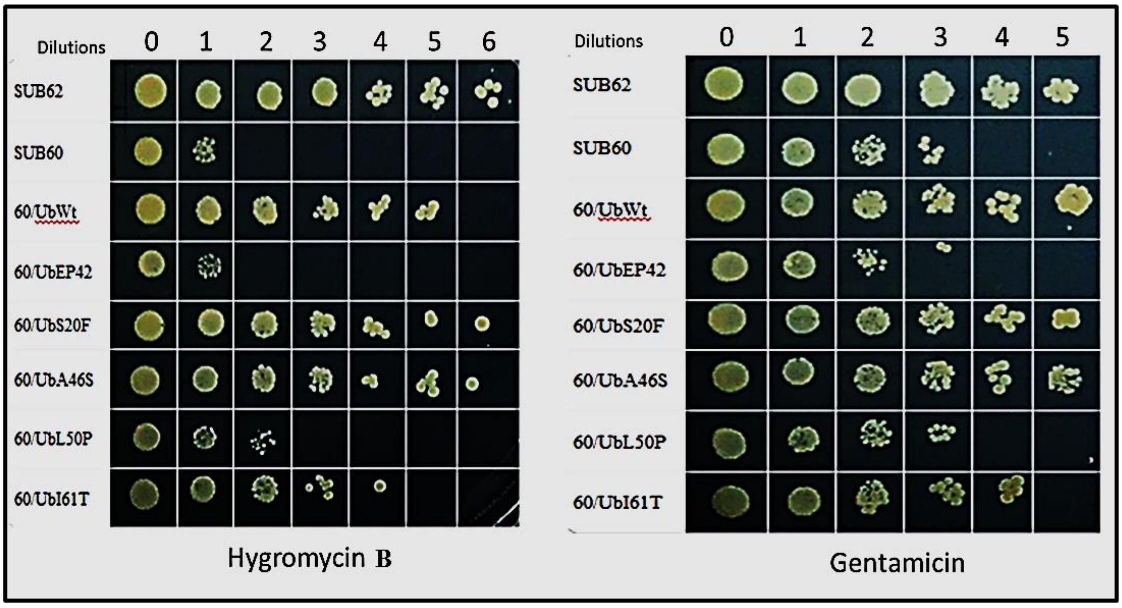
In the presence of translational antibiotics truncated proteins are accumulated, which are degraded by proteasomes through ubiquitin dependent mechanism. Hence, insufficiency of ubiquitin resulting from ubiquitin gene deletion in SUB60 cells increases their sensitivity to antibiotics (Hanna et al., 2003). Moreover, polyubiquitin chains with K63 branching on L28 subunit make ribosomes more resistant to translational inhibitors (Spence et al., 2000). SUB60 and SUB60 tranformants expressing wild type and mutant ubiquitin genes were exposed to antibiotic stress. Translational inhibitors cycloheximide, hygromycin, canavanine, gentamicin and genticin were used in the study. Transformants expressing the two single mutants UbL50P and UbI61T showed lower tolerance similar to UbEP42. Transformants expressing the other two mutations UbS20F and UbA46S behaved similar to the cells expressing UbWt.

Tunicamycin blocks N-acetyl glycosylation, causes cell cycle arrest and induces unfolded protein response. SUB60 and SUB60 tranformants expressing wild type and mutant ubiquitin genes were exposed to tunicamycin. Results showed that mutants UbS20F, UbA46S could partially overcome the deleterious effect of tunicamycin, while the other three mutants UbEP42, UbL50P and UbI61T became hypersensitive to tunicamycin and could not survive, establishing functional impairment of ubiquitin due to the mutations. Similar effect was observed with ubiquitin variants which were exposed to arginine analogue, canavanine (Figure 2.19(B)).

Other antibiotics act as protein synthesis inhibitors. For example, hygromycin B inhibits polypeptide synthesis by stabilizing tRNA-ribosomal acceptor site, thereby inhibiting translocation. Gentamicin interrupts protein synthesis by binding irreversibly to the 30S subunit of the bacterial ribosome. Geneticin (G-418) interrupt polypeptide synthesis at elongation step and Cycloheximide is also a translational inhibitor. Cells expressing UbEP42, UbL50P and UbI61T became more sensitive to the antibiotics. UbS20F and UbA46S complemented well similar to UbWt expressing SUB60 cells (Figure 2.19(C) & (D)) Cycloheximide was used as reference (Mrinal Sharma's thesis, 2013).


(A)
Dilutions
0
1
2
3
4
5
6
SUB62
SUB60
60/UbWt
60/UbEP42
60/UbS20F
60/UbA46S
60/UbL50P
60/UbI61T
No Antibiotic and No CuSo4
100uM CuSo4
(B)
canavanine
Tunicamycin

**(C)**




**(D)**

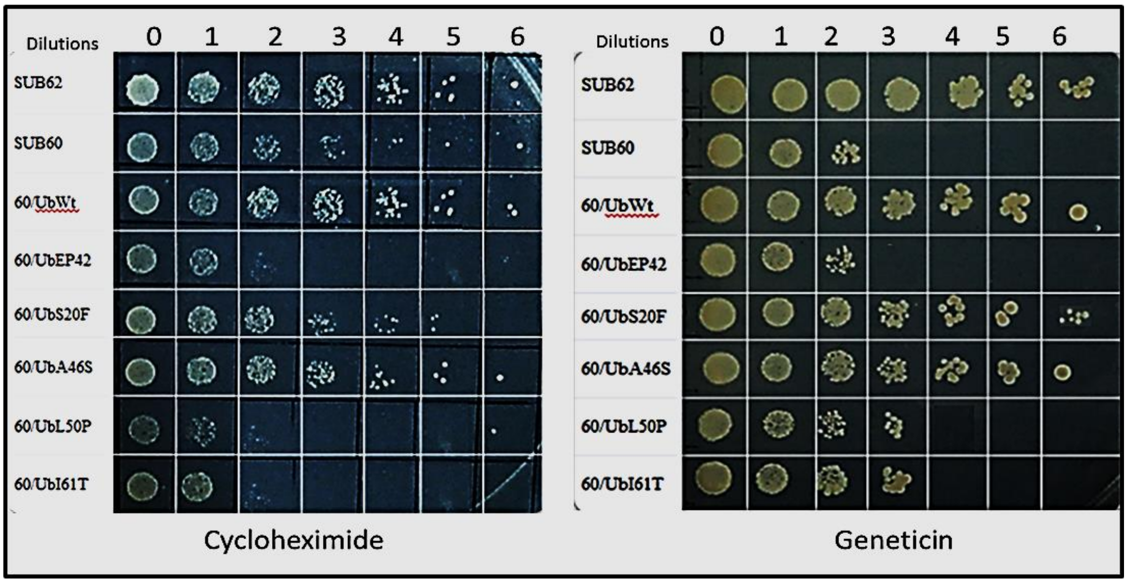



**Figure 2.19: Antibiotic stress complementation of *Saccharomyces cerevisiae* (SUB60). Antibiotic resistance ability of SUB62, SUB60, SUB60 cells transformed with UbWt, UbEP42 and it single mutant derivatives. The SUB62, SUB60 and SUB60 transformed with UbWt was taken as control. (A) Wild type and mutant ubiquitin expressing SUB60 cells in presence and absence of an inducer (B) in presence of canavanine and tunicamycin (C) in presence of hygromycin B and gentamicin (D) with geniticin and cycloheximide.**

### 2.3.7 *In vivo* polyubiquitin chain formation assay

As it became evident from various studies, when proteins are conjugated with the first molecule of ubiquitin, ubiquitinating enzymes bind the unstructured C-terminal tail of ubiquitin and transfer it to the protein substrate. On the other hand while building a polyubiquitin chain, an isopeptide linkage is synthesized between the lysine side chain of substrate conjugated ubiquitin (acceptor) and the C-terminal tail of the incoming ubiquitin (donor). In the cellular pool of SUB60 transformants there are two types of ubiquitins namely, UbWt and the mutant form. In the wake of this information, we hypothesized that the lethal effects seen with three of the mutants UbEP42, UbL50P and UbI61T could be due to any of the two possibilities. First, the three mutant forms cannot be recognized and employed for ubiquitination by the enzymes. In that case, under normal conditions the cells expressing UbEP42, UbL50P or UbI61T would not show any lethality and would behave similar to SUB60 cells. However, the results rule out the first possibility. Second, after successful incorporation into polyubiquitin chains, due to their distorted structure they fail to interact with ubiquitinating enzymes and other ubiquitin binding proteins. To test which one of the two reasons could be responsible for the observed functional impairment and lethality, polyubiquitin chains were examined for the presence of mutant forms of ubiquitin. Mutant forms of ubiquitin were expressed as chimeric fusions of myc-tag (Figure 2.20). pUb221 shuttle vector was used to prepare myc tagged ubiquitin cassette wherein the expression of mutant ubiquitin was modulated by the *CUPI* inducible promoter. Immunoblotting using anti-myc antibodies established that UbEP42 and its single mutant derivatives were capable of participating in the polyubiquitin chain assembly (Figure 2.21), suggesting the second possibility as the reason behind the functional aberration of UbEP42, UbL50P and IbI61T.

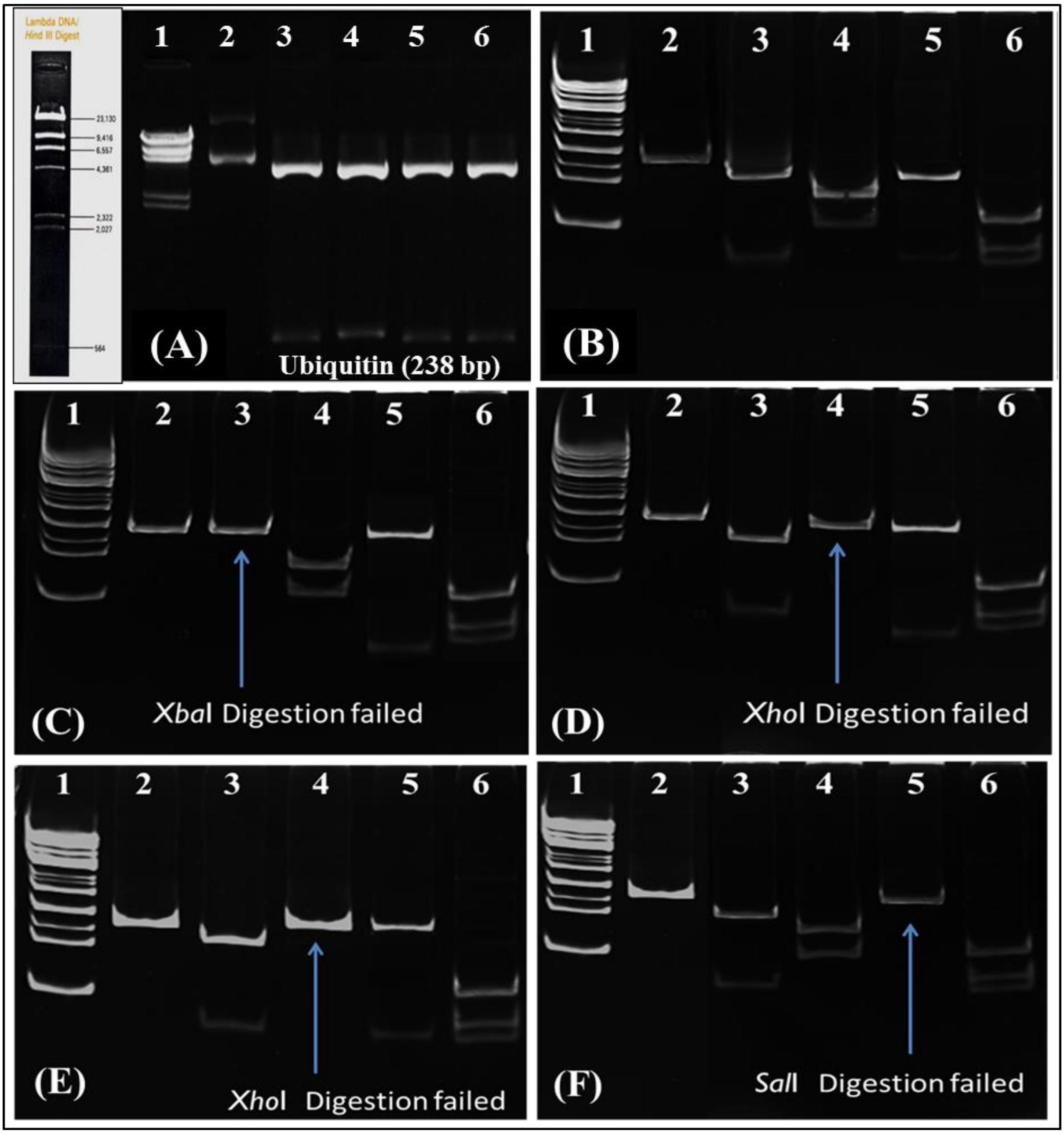



**Figure 2.20: Confirmation of cloning with RE digestions. (A) 1% agarose gel showing insert release of ubiquitin mutants over digestion with *Bgl*II and *Kpn*I restriction enzymes. Lane 1 shows lambda DNA marker, Lane 2 shows representative undigested plasmid, Lanes 3-6 display insert release of UbS20F, UbA46S, UbL50P and UbI61T. (B, C, D, E and F) 15% DNA PAGE showing restriction enzyme mapping of UbWt, UbS20F, UbA46S, UbL50P and UbI61T respectively. Synthetic ubiquitin was used to generate mutants in order to utilize restriction sites present in it to confirm replacement of amino acid residue at primary level. UbWt gets digested by all three RE i.e. *Xba*I,**

***Xho*I and *Sal*I. UbS20F lacks *Xba*I RE site so it remains undigested. UbA46S and UbL50P do not contain *Xho*I RE site and UbI61T lacks *Sal*I RE site.**

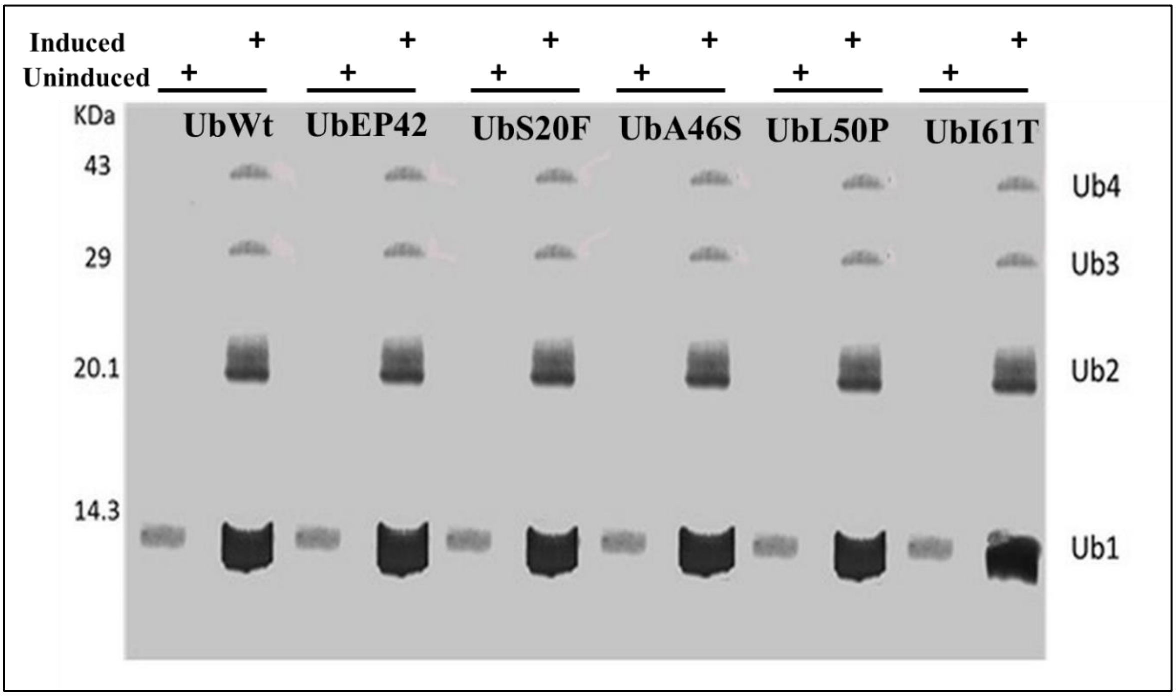



**Figure 2.21: Polyubiquitination profile of mutant forms of ubiquitin expressing SUB60 cells. Lane 1-12 shows UbWt, UbEP42, UbS20F, UbA46S, UbL50P and UbI61T in presence and absence of 100µM $CuSO_4$, which is an inducer.**

### 2.3.8 Incorporation of variants in K63 and K48 linked chain formation

Lethal mutants UbEP42, UbL50P and UbI61T displayed functional defeccts in the studies. Moreover, ubiquitin variants probably may not participate in polyubiquitin chain formation was also ruled out. Ubiquitin contains seven lysine residues. Since all of them are known to participate in polyubiquitin chains and the topology of polyubiquitin chain depends on the lysine linkages, which are synthesized by different E2 and E3 combinations. Polyubiquitin chain topology and nature of the substrate play important roles in carrying out various cellular functions to maintain cell in homeostatic condition. It is established that substrates having Lys48 linked polyubiquitin chain are known to undergo proteasomal degradation. Lys63 linked polyubiquitinated substrates are involved in protein trafficking, protein kinase activation, translation, DNA repair etc. Here, we decided to see if incorporation into polyubiquitination with K63 (Lys63) and K48 (Lys48) linkage is normal, as it signals many regulatory functions. Interestingly UbEP42, UbL50P and UbI61T which showed impairment in regulatory functions were also defective in the formation of polyubiquitin with both K48 and K63 linkages (Figure 2.22).

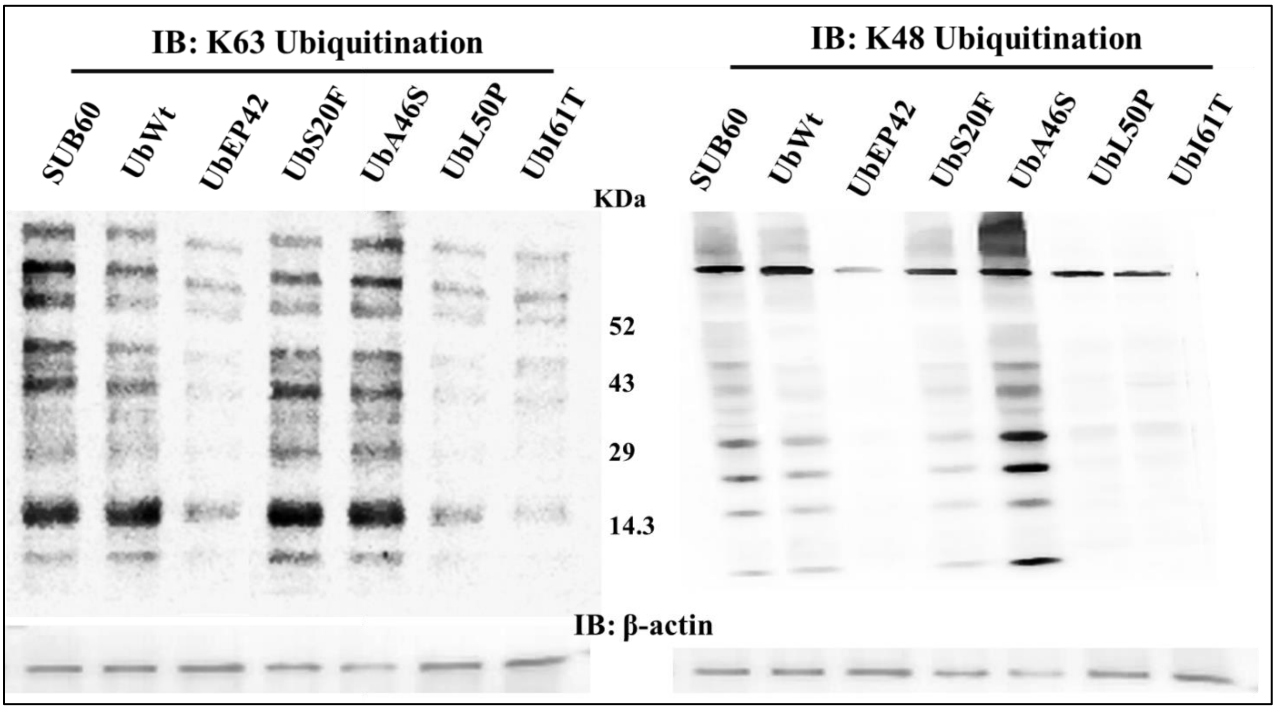



**Figure 2.22: Western blot analysis showing K63 and K48 linked polyubiquitnation profile in ubiquitin wild type and mutants expressing SUB60 cells. Lanes 1-7 show SUB60, SUB60 cells expressing UbWt, UbEP42, UbS20F, UbA46S, UbL50P and UbI61T.**

### 2.3.9 Degradation of substrate ubiquitin fusions by N-end rule and UFD pathways

Metabolic stability of protein is determined by its N-terminal residue known as N-degron, which act as degradation signal by destabilizing the protein Proteins with destabilizing residue at their N-terminals undergo ubiquitination, which results in enhancement of their degradation (Varshavsky et al., 1996).

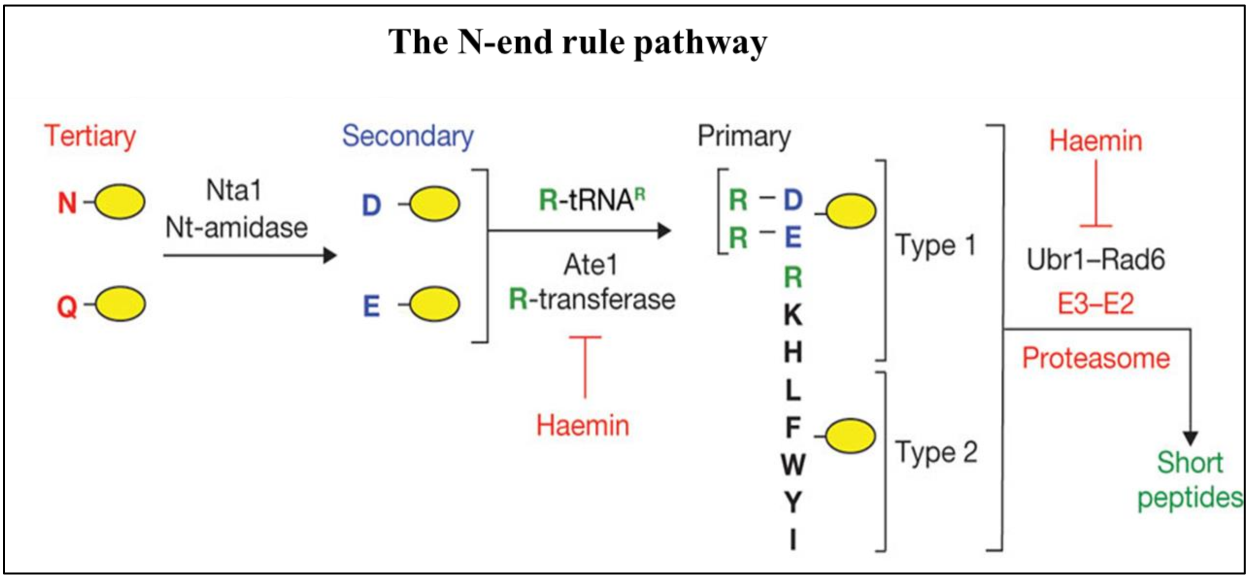



**Figure 2.23: The N-end rule pathway showing primary, secondary and tertiary destabilizing residues and their components.**

Ubiquitin fusions of proteins which have proline (Pro) as the first residue undergo polyubiquitination and degradation by proteasomes through UFD pathway [45]. In contrast, ubiquitin fusions of proteins which have methionine (Met) as their first residue are freed from ubiquitin tag by ubiquitin C-terminal hydrolases (Varshavsky, 1996). Two variants of plasmid pUB23 expressing the fusions of Ub-Met-β galactosidase which is stabilizing residue, the most destabilizing residue, Ub-Pro-β galactosidase, tertiary destabilizing residues i.e. Ub-Asn-β galactosidase and Ub-Gln-β galactosidase, secondary destabilizing residue Ub-Glu-β galactosidase, primary destabilizing residues i.e. Ub-Leu-β galactosidase and Ub-Arg-β galactosidase were cotransformed into SUB60 cells carrying YEp96 plasmid with inserts for wild type and mutant forms of ubiquitin. The results of western blot analysis and enzyme assay indicate that the cells expressing UbEP42, UbL50P and UbI61T failed to show degradation of Ub-Pro-β galactosidase by UFD pathway. Transformants expressing UbS20F and UbA46S behaved similar to the ones expressing UbWt and showed degradation. However, expression of mutant ubiquitins did not have any effect over the release of Met-β galactosidase from Ub-Met-β galactosidase. Ubiquitin in the fusion protein could be readily removed by ubiquitin C-terminal hydrolases as the ubiquitin is same as UbWt (Figure 2.24(A), (B) & (C)). Similar effect was observed with primary destabilizing residues i.e. Ub-Leu-β galactosidase and Ub-Arg-β galactosidase. Degradation of ubiquitin fusion protein found to be unsuccessful in SUB60 cells expressing UbEP42, UbL50P and UbI61T whereas

UbS20F and UbA46S were capable of degradation protein like UbWt expressing SUB60 cells (Figure 2.24 (D) & (E)).

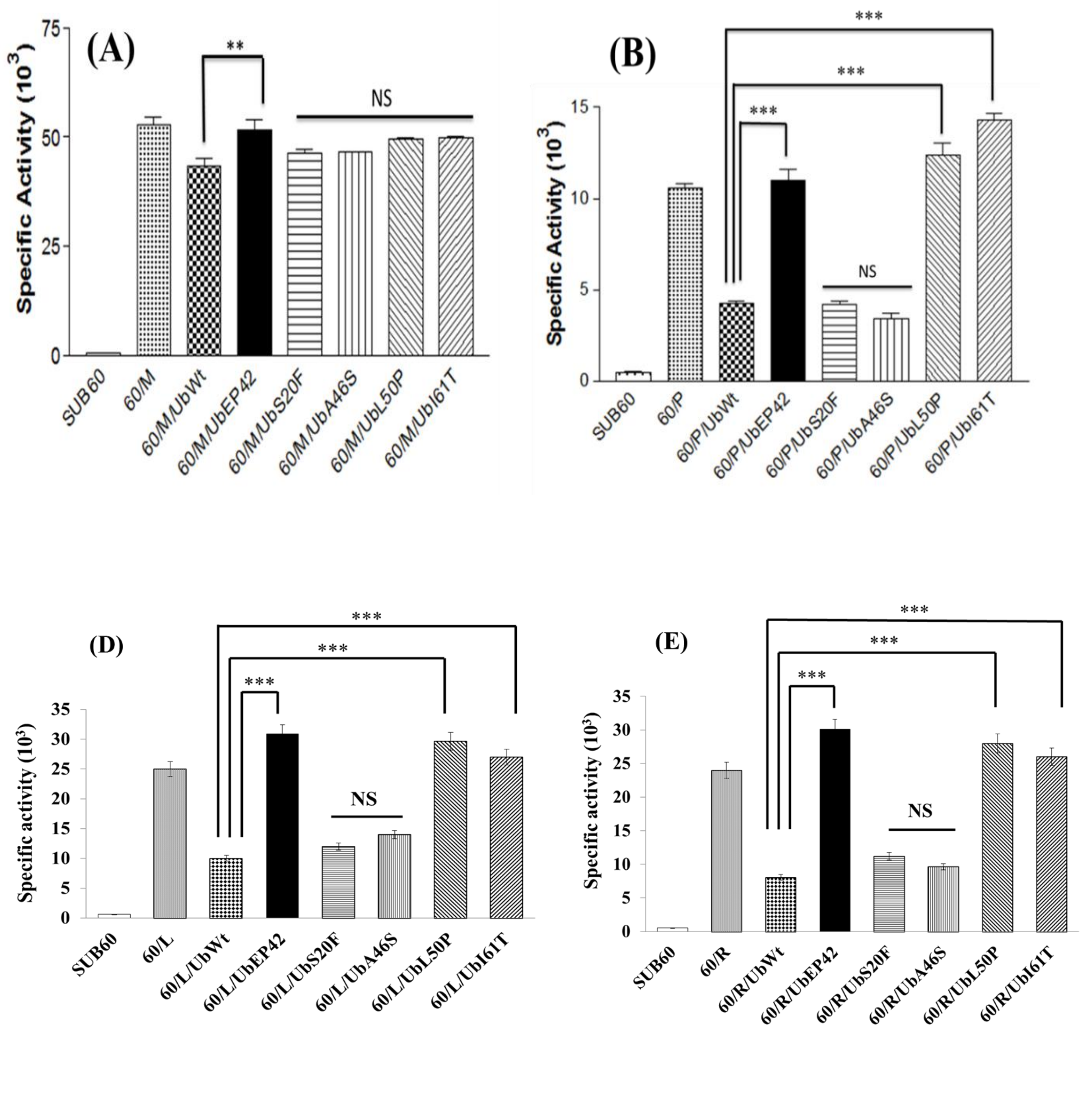


**Figure 2.24: Effect of UbWt, UbEP42 and its single mutant derivatives on the half-life of proteins in SUB60 cells. SUB60 were co-transformed by plasmid pUb23 expressing Ub-β-galactosidase fusion with different N-terminal residues. SUB60 and SUB60 cells expressing UbWt were considered as controls. (A & B) Bar graphs showing degradation levels of β-galactosidase fused with Met(M) and Pro(P) respectively (C) western blot showing degradation of Ub-Met-β-galactosidase and Ub-Pro-β-galactosidase. Lane 1 shows SUB60 cells transformed with pUB23 vector, Lane 2, 4, 6, 8, 10, 12 shows Ub-Met-β-galactosidase degradation and Lane 3, 5, 7, 9, 11, 13 shows Ub-Pro-β-galactosidase degradation by UbWt, UbEP42, UbS20F, UbA46S, UbL50P and UbI61T respectively (D & E) Bar graphs showing degradation levels of β-galactosidase fused with Leu(L) and Arg(R) respectively**

## 2.4 DISCUSSION

Ubiquitin is a highly conserved multifunctional protein. Interestingly enough, being devoid of any enzyme activity, ubiquitin executes its functions through covalent and noncovalent interactions with other proteins (Varshavsky, 2012). Studies on mutants of ubiquitin contribute to the understanding eukaryotic cell biology in many ways (Mishra et al., 2011; Mishra et al., 2015; Sharma and Prabha, 2011; Sharma and Prabha, 2015; Prabha et al., 2010; Doshi et al., 2014; Doshi et al., 2017). Firstly, depending on how crucial they are mutations of different residues, may knock out all functions or produce impairment of selective functions among an assortment of functions executed by ubiquitin. Hence, every mutation need not be lethal under the conditions selected. Secondly, such selective impairment associated with a particular mutation, throws light on the structural and functional role of the residue mutated and helps in identification of the partner proteins. Third and the most important aspect is if the function affected has any bearing on the actual cause of a disease, elucidating its molecular mechanism would pave way for developing therapeutics.

All seven lysine residues of ubiquitin take part in ubiquitination. Similarly, mutations in the C-terminus interfere with the myriad functions of ubiquitin, because it is crucial for ubiquitin conjugation (Ecker et al., 1987; Burch and Haas, 1994; Beal et al., 1996). The hydrophobic patch on the surface of ubiquitin formed by Leu8, Ile44 and Val70 is essential for proteasomal degradation (Beal et al., 1996), while the second hydrophobic patch constituting Phe4 and its adjacent residues is required additionally for endosomal protein degradation (Shih et al., 2000). Any mutations in the two hydrophobic patches as well as the C-terminus have been shown to be detrimental for vegetative growth of yeast (Sloper-Mould et al., 2001). The importance of three conserved residues of parallel β-bulge Gln2, Glu64 and Ser65 has been studied by us in relation to both structure and function of ubiquitin by creating Q2N, E64G and S65D substitutions and their combinations (Mishra et al., 2009; Mishra et al., 2011; Sharma and Prabha, 2001). Subsequently, point mutations of ubiquitin were generated and their effect on growth rate of yeast was investigated using bulk competition and deep sequencing methods (Wang et al., 2006). Some of the results with β-bulge residues which were reported earlier by us were confirmed by Roscoe et al. (2013) using another strain of *S. cerevisiae*. Ubiquitination system recognizes the free C-terminal tail and residue Arg72 of donor ubiquitin for conjugation. During polyubiquitination surface residues adjacent to specific lysines of the acceptor ubiquitin are crucial for the process of ubiquitination (Wang et al., 2006). The topological features of ubiquitin and its surface residues especially those surrounding lysines determine the docking site for enzymes and binding partners during ubiquitination, ubiquitin mediated signalling and protein degradation.

Previously, random mutants of ubiquitin were generated in our laboratory to gain insights in relation to the functional roles of conserved residues and one of the mutants UbEP42 was dosage dependent lethal phenotype with four single mutations (Prabha et al., 2010; Doshi et al., 2014; Doshi et al., 2017). In the present study, the constituent mutations were singled out from UbEP42, namely UbS20F, UbA46S, UbL50P and UbI61T and were scrutinized along with UbEP42 for various functions to answer the questions on consequences in relation to various functions involving different types of polyubiquitin linkages.

The results established that changes in growth profile of the three mutants expressing UbEP42, UbL50P and UbI61T were associated with decrease in the levels of Cdc28 protein kinase (cyclin dependent kinase or Cdk). Cdc28 protein kinase is the only cyclin dependent kinase present in *S. cerevisiae* (Forsburg and Nurse, 1991; Bloom and Cross, 2007), which is activated by interacting with both G1 phase and B type cyclins, as the cell progresses through various stages of cell cycle (Mendenhall et al., 1998). The activity of Cdk is regulated by variation in the levels of different cyclins, Cdk inhibitors, Cdk activators. After serving their function, cyclins and other regulatory molecules of Cdk are removed by ubiquitin mediated degradation, signaled by polyubiquitin chains with K48 linkage. However, there are no reports in literature on the lowering of Cdk protein levels.

On the other hand, histone H2B undergoes monoubiquitination by RAD6-BRE1 to enhance the transcription of certain genes necessary for entering cell cycle (Osley, 2004). RAD6-BRE1 pathway is also involved in the transcription of cyclin genes. Hence, unhindered supply of the free ubiquitin is a prerequisite for entering cell cycle. Doa1 shows strong interaction with Cdc28 at genetic level (Zimmermann et al., 2011). Ample supply of free ubiquitin, therefore, is necessary for the execution of both proteolytic and nonproteolytic functions through ubiquitination to regulate the levels and activities of the proteins crucial for cell cycling. We assume that functionally defective ubiquitins UbEP42, UbL50P and UbI61T disturb the levels of cyclins and other important proteins, thereby causing reduction in Cdk levels.

Fus3 is a member of mating-pheromone response cascade of mitogen activated protein kinase (MAPK) pathway. Fus3 protein of yeast is a homologue of ERK1/2 of higher eukaryotes. The Gβγ subunits of pheromone activated G-protein, along with Cdc42 activate Ste20 leading to sequential activation of Ste11, Ste7 and finally Fus3 (Elion et al., 1993). Activated Fus3 arrests the cells in G1 phase by activating Far1, which is an inhibitor of Cdc28-Cln2 and Cdc28-Cln3 complex (Elion et al., 1993), besides causing cell fusion with mating partner (Saito, 2010). However, Ste7 activator kinase of Fus3 is regulated through ubiquitination and deubiquitination catalyzed by $SCF^{Cdc4}$ and Ubp3 respectively (Osley, 2004). The polyubiquitin chains formed on Ste7 are of K48 linkage. In the present case the cells expressing UbEP42, UbL50P and UbI61T were arrested in G1 phase. However, the protein levels of Cdc28 protein kinase and Fus3 MAPK were found to be altered in SUB60 cells expressing lethal mutants of ubiquitin (Doshi et.al., 2014; Doshi et.al., 2017). The kinases Cdc28 and Fus3 are not degraded by proteasomes. Moreover, mRNA levels of *CDC28* and *FUS3* were unaffected by UbEP42, UbL50P and UbI61T expression and similar to UbWt expressing SUB60 cells. From the above studies it is clear that the difference seen with Cdc28 and Fus3 in cells expressing UbEP42, UbL50P and UbI61T is posttranslational.

The results of BrdU assay confirmed that cell cycle halts at G0/G1 phase leading to delayed entry of cells in S phase. This means that the replication START may not be occurring properly. G1 cyclins Cln1, Cln2 and Cln3 in association with Cdc28 protein kinase carry out replication. For initiating replication expression of Cln3-Cdc28 is required at START (Tyers M. et.al., 1992). Other important molecule is Cdc37, a molecular cochaperone. Cdc37 promotes association of chaperone Hsp90 with protein kinases to maintain their stability and functionality. Silencing of Cdc37 interferes with the maturation of kinases (Smith JR. et al., 2008). The Cdc37-1 mutant arrests cells at START with low levels of Cdc28 protein (Farrell A. and Morgan DO., 2000). However, the results displayed no significant variation in mRNA transcript levels of *CDC37* and *CLN3* suggesting that the levels of these proteins are altered either at translational level or posttranslationally in presence of UbEP42, UbL50P and UbI61T.

There are many reports in literature that reactive oxygen species are involved in many cellular processes. Low endogenous ROS play important role in redox signalling pathways whereas high ROS levels cause severe damage to cells. ROS has been reported to stimulate cell cycle progression as an intrinsic signal (Burdon, 1995; Sauer et al., 2001). During cell cycle progression, it was observed that ROS levels increase for a given cell size and mitochondrial mass. Antioxidant treatment of cells leads to cell cycle arrest at late G1 phase and failure in accumulation of cyclin A protein, leading to failure of initiation of DNA synthesis (Havens et al., 2006). Our results with SUB60 cells expressing three mutants viz. UbEP42, UbL50P and UbI61T displayed significantly low levels of ROS, which may be contributing to cell cycle arrest at G1 phase.

The membrane protein uracil permease undergoes degradation in multivesicular bodies after being tagged by K63 linked polyubiquitin chains (Blondel et al., 2004). Carboxypeptidase S (CPS) is resident protease of multivesicular bodies (MVB) and it is synthesized in cytoplasm and then transported to MVB. Prior to transport CPS undergoes K63 linked polyubiquitination by Rsp5 ubiquitin ligase. The results of both the studies showed that the lethal mutants UbEP42, UbL50P and UbI61T hampered both functions by accumulating uracil permease and hindering CPS protein trafficking respectively, which may be due to the inability of the mutants to form K63 linked polyubiquitin chains efficiently.

Translational antibiotics lead to synthesis of truncated proteins. Denatured and truncated proteins produced under stress conditions are tagged with K48 linked polyubiquitin chains and driven for degradation by proteasomes. SUB60 cells with their *ubi4* deletion are more sensitive to stress conditions. Wild type ubiquitin expressed from plasmids can complement SUB60 cells making them more resistant to stress. Among the mutants tested UbEP42, UbL50P and UbI61T failed to complement SUB60 cells under antibiotic stress. Moreover, tunicamycin induces endoplasmic reticulum associated stress, leading to unfolded protein response and rising the level of proteins tagged with K11 linked polyubiquitin chains (Blondel et al., 2004). Cells expressing UbEP42, UbL50P and UbI61T showed reduced tolerance towards tunicamycin.

K63 linked polyubiquitin on ribosomal subunit L28 is essential for withstanding antibiotic stress (Spence et al., 2000; Hanna et al., 2003). Further, It is well established that polyubiquitins linked through K48 recruit proteins for proteasomal degradation whereas polyubiquitins linked through K63 substrates serve various purposes such as kinase activation (Deng et. al., 2000), regulation of protein synthesis (Spence et. al., 2000), DNA repair (Hoege et. al., 2002; Hofmann and Pickart, 1999) and chromosome segregation (Vong et. al., 2005). Using linkage specific antibodies against both K48 and K63 polyubiquitin chains it was clearly established here that the mutant forms of ubiquitin cannot form either of these linkages efficiently and hence the mutants are functionally defective leading to all the adverse effects observed above.

The method for monitoring incorporation of ubiquitin into polyubiquitin chains was developed by Ellison and Hochstrasser, where myc-tagged ubiquitin is employed and anti myc antibody is used in western blot analysis (1991). The results with myc labelled mutants of ubiquitin and western blotting show that these three forms of ubiquitin could be successfully incorporated into polyubiquitin chains, as there was no difference observed in the intensity of bands between the lanes containing mutated ubiquitin and wild type ubiquitin. Accumulation of high molecular weight ubiquitinated proteins in western blots suggests that apparently these variants are not recognized as tags by proteasomes for protein degradation. Hence, we see two reasons for the band intensities of mutated ubiquitins in western blots with myc-tagged ubiquitin. Firstly, mutated ubiquitins are used as substrates and polyubiquitin chains are built on them. Alternatively, they are used as donors and added to growing polyubiquitin chains. For a donor ubiquitin the C-terminal tail (which is unstructured) is more important. And once mutant ubiquitin is added, it will not permit any further extension acting as cap. However, drawing conclusions on which E2 and E3 among the innumerable combinations are responsible for these chains and what is the linkage in the myc-tagged poyubquitins is not possible at this stage. Second reason for the observed intensity of bands is that myc-tagged ubiquitin and polyubiquitin chains are not degraded by proteasomes (Ellison and Hochstrasser, 1991). Polyubiquitin chains with myc-tagged ubiquitin with or without the mutant forms of ubiquitin studied here make dead end complexes.

Chimeric proteins fused to C-terminus of ubiquitin were employed by Varshavsky's group to study their stability in addition to N-end rule. β galactosidase was expressed as a C-terminal fusion of ubiquitin. All 20 amino acids were tested as N-terminal residues of β galactosidase in different constructs for their effect on the stability of the enzyme. Except Ubiquitin-Pro- β galactosidase all other constructs were cleaved by ubiquitin C-terminal processing enzymes to release β galactosidase. Those β galactosidase molecules with destabilizing N-terminal residues such as Arg and Leu undergo degradation after polyubiquitination, whereas β galactosidase with Met at N-terminus remains stable. Ubiquitin-Pro-β galactosidase is not processed by ubiquitin C-terminal processing enzymes instead polyubiquitin chain is built on N-terminal and the protein is degraded by ubiquitin fusion degradation (UFD) pathway. The substrate Ub-Pro-β galactosidase, Ub-Leu-β galactosidase and Ub-Arg-β galactosidase were accumulated in cells expressing UbL50P and UbI61T similar to UbEP42.The substrate β galactosidase in Ub-Met-β galactosidase was found to be stable. The substrates of ubiquitin fusion degradation (UFD) pathway are tagged with polyubiquitin chains of K29 linkage and K48 linkage by Ufd4 and Ufd2 respectively (Johnson et al., 1995). Deficiency of functional ubiquitin and defective K48 linkage formation could be one of the reasons for elevating half-life of the substrate protein of UFD pathway.

The results demonstrate that the two mutations UbS20F and UbA46S do not have any influence over the survival of yeast under laboratory conditions and under the conditions tested. However, conservation of Ser20 and Ala46 during evolution suggests they may confer selective advantage on the organism to thrive and compete under certain conditions in the environment outside the laboratory. In sharp contrast, the mutations UbEP42, UbL50P and UbI61T proved to be detrimental to survival of the organism. At molecular level, they not only debilitated the organism in relation to several functions, but also bore negative influence over activities involving different linkages in polyubiquitin chains. Wild type ubiquitin (UbWt) is also available in SUB60 transformants, which are expressing UbEP42, UbL50P or UbI61T and the ubiquitinating enzymes may bind UbWt or UbEP42/UbL50P/UbI61T. In the case where UbWt is transferred to substrate, the enzymes proceed for another round of conjugation and continue to do so till either polyubiquitin chain has reached its ideal length or they come across a road block in the form of UbEP42/UbL50P/UbI61T. If the ubiquitination system happens to encounter UbEP42, UbL50P or UbI61T during the conjugation of first molecule of ubiquitin, then the binding may lead to inhibition. If the enzymes are not inhibited and the C-terminal unstructured region can be recognized and bound by the enzymes without any difficulty and hence in such case, we assume that UbEP42, UbL50P and UbI61T are conjugated to substrates unhindered. If already conjugated to substrate, the distortion in the mutant ubiquitin structure around lysine residues does not allow recognition by ubiquitination system for adding another molecule of ubiquitin leading to chain termination, producing a ‘capping effect’. If UbEP42/UbL50P/UbI61T occurs as the last ubiquitin in the polyubiquitin of ideal length, once again distortion in structure acts as an impediment and wards off interactions to binding partners of polyubiquitin chain, adding a new dimension to the mechanisms of ubiquitin conjugation. However, the possibility that the distorted mutant ubiquitin are accepted as substrates on which polyubiquitins are built appears to be a strong possibility at this stage (Doshi et al., 2014; Doshi et al., 2017).

Presently defects in protein degradation are known to be the cause for many cancers and neuronal diseases (Schmidt and Finley, 2014) and methods based on protein degradation are being explored for developing new strategies to deal with them (Joshi et. al., 2015; Prabha et al., 2012). The knowledge generated in this study can be extrapolated to develop novel inhibitors and therapeutics to investigate and modulate eukaryotic cellular dynamics.

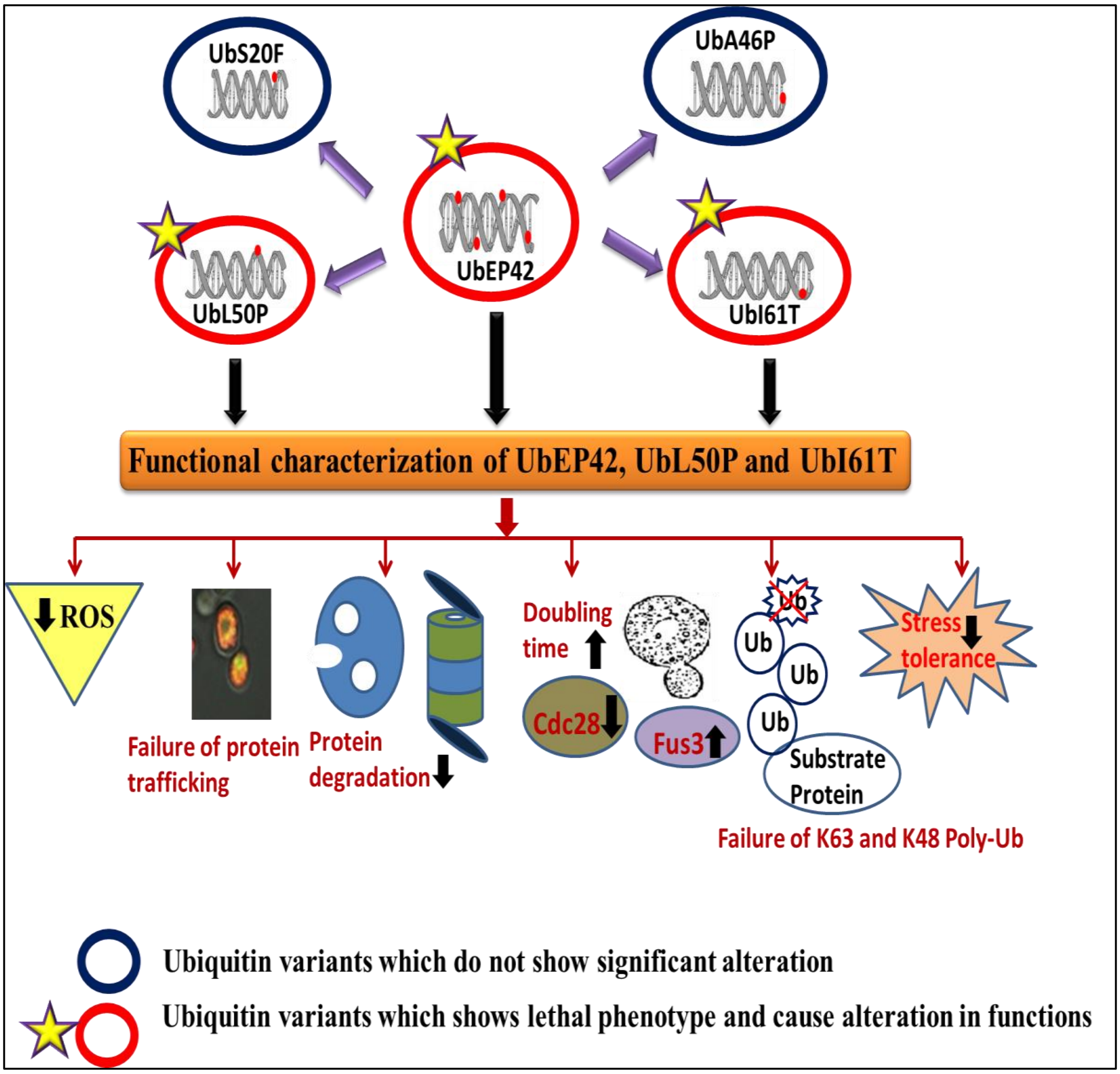



**Figure 2.25: Representative image showing cumulative effects of mutant forms of ubiquitin.**