Functional Characterization of UbEP42 Derived Double Mutants in *Saccharomyces cerevisiae*

2.1 Introduction

Mutations fuel the process of evolution. Some mutations are detrimental to the organism, and are eliminated over time. Others confer advantages on the organism by helping it survive better in its environment, and thus lead to evolution of the organism. But there are some proteins that resist any mutation that changes their amino acid sequence. These proteins remain unchanged over many millions of years across species, and their resistance to change indicates that much of their amino acid sequence is involved either in ensuring their structural stability or in the performance of their functions. Ubiquitin is one such protein. Ubiquitin is one of the most conserved proteins in eukaryotes. It differs by just three amino acids between yeast, plants and humans (Gavialnes et al., 1975, Watson et al., 1978, Schlesinger et al., 1975 and Schlesinger and Goldsteiner., 1975, Wilkinson et al., 1986, Vierstra et al., 1986).

This high level of conservation suggests that virtually every amino acid in ubiquitin has either structural or functional significance. Consequently, most mutations in ubiquitin gene over millions of years have been removed through natural selection. The functional significance of these amino acids could be either in protein degradation (Ciechanover et al., 1980 and Hershko et al.,1980), or in the other functions that ubiquitin is involved in (Wilkinson et al., 2000, Pickart et al., 2001 and Shih et al.,200). Therefore, it appears that a large number of constraints have been imposed on the amino acid sequence of ubiquitin through the folding and stability requirements of the molecule, as well as through the requirement of interactions with other proteins for functional performance.

There is a natural variant of ubiquitin found in the virus *Autografa californica*, which shows only 75% sequence similarity to normal ubiquitin. This ubiquitin variant competitively inhibits the formation of polyubiquitin chains (Hass et al., 1996). This means that ubiquitin variants could be used as competitive inhibitors of polyubiquitin chain formation, in the treatment diseases resulting from ubiquitination or ubiquitin mediated degradation of important proteins by normal ubiquitin. In *Saccharomyces cerevisiae*, ubiquitin is expressed by four genes, namely *UBI1*, *UBI2*, *UBI3* and *UBI4* (Varshavsky et al., 1987). Out of these, *UBI1*, *UBI2* and *UBI3* are expressed constitutively, and are used for normal functioning of a cell. *UBI4* is expressed only in stress conditions and is used by a cell to overcome the stress. *UBI4* encodes a polyubiquitin precursor protein, which is basically a translational fusion of five ubiquitin molecules (Ozkaynak et al., 1984 and 1987).

The null phenotype of ubiquitin, in which all four ubiquitin genes are deleted, is lethal. But *Saccharomyces cerevisiae* can survive if just *UBI4* gene is deleted, while the other three genes are intact and functional. However, this *ubi4* mutant is hypersensitive to stresses like UV exposure, high temperature, amino acid analogues and nutrient starvation (Finley et al., 1987). The previous chapter described studying the importance of β bulge residues in ubiquitin by changing them using site directed mutagenesis. However, this approach has its limitations. It can only be applied on residues that are known to be functionally and structurally relevant. There might be other residues or combinations thereof, whose structural or functional relevance is unknown. Further, if one is looking for a particular functional effect it cannot be achieved by site directed approach.

A powerful method to find out such residues is random mutagenesis. Random mutagenesis is also used in protein engineering to develop altered versions of natural proteins, that have novel properties that can be useful from commercial and healthcare point of view. Due to its random nature and ability to scan a large number of mutations in a small amount of time, random mutagenesis can reveal relevance of the residues and relationships between different residues of a protein, that could not have been predicted through an empirical study of the protein's structure. Random mutagenesis can be carried out either through error prone PCR, or by exposing the cells to mutagens like UV rays and mutagenic chemicals, or by using a mutator strain.

Mutator strains are those microbial strains in which certain key genes involved in DNA repair have been knocked out. This significantly increases the mutation rate of these strains. The most popular mutator strain is *E. coli* XL1- red, in which three genes involved in DNA repair, namely Mut S, MutT and MutL, have been deleted. As a result, the random mutation rate of this strain is approximately 5000 times higher than the wild type. Random mutagenesis using a mutator strain is also simpler than error prone PCR approach. However, a mutator strain suffers mutation throughout its genome instead of causing random mutations specifically in the gene of interest, making it less efficient. Random mutagenesis through UV exposure or chemical mutagens also has this same limitation of specificity. Error prone PCR, on the other hand, can specifically cause random mutagenesis in the gene of interest, without affecting the rest of the genome. Still another method to carry out random mutagenesis is DNA shuffling, but it cannot be used on ubiquitin due to the small size of the gene.

Error prone PCR is a variant of PCR method in which the rate of error of the DNA polymerase is increased (Arnheim, 1992). This can be done by biasing the concentrations of dNTPs, so that the dNTP with higher than the required concentration will have a high probability of being wrongly incorporated during DNA replication. Another way is to play with the concentration of Mg^{+2} ions, as they are needed as cofactors by DNA polymerase, or to add Mn^{+2} ions. Once a PCR amplicon containing random mutations is obtained, its ends are digested with restriction enzymes, it is separated using electrophoresis and then ligated into a plasmid vector that can be transformed into the host cell. The transformed cells are then screened or selected for the phenotype of interest.

The random ubiquitin mutants generated through this process were transformed into SUB60, which is a strain of *S. cerevisiae* that lacks the *UBI4* gene, and is therefore hypersensitive to stress conditions like high temperature. SUB60 cells thus transformed with random ubiquitin mutants were then screened for their failure to complement the stress hypersensitive phenotype of SUB60. This process led to the isolation and identification of UbEP42 mutant, a ubiquitin mutant that did not just fail to complement the stress hypersensitive phenotype, but the expression of which was also detrimental in absence of stress in a dosage dependent manner, suggesting that it competed with the wild type ubiquitin constitutively expressed from *UBI1*, *UBI2* and *UBI3* genes (Prabha et al., 2010).

Sequencing of the UbEP42 gene subsequently revealed that it contained seven base substitutions. Out of these seven base substitutions, three mutations resulted in synonymous codons. Hence, they do not cause any amino acid substitution in the protein. But the remaining four base substitutions cause amino acid substitutions S20F, A46S, L50P and I61T. Functional characterisation of the SUB60 cells expressing UbEP42 was carried out in order to understand the reason for dosage dependent lethality. Reduced levels of the protein kinase Cdc28 in these cells prolonged generation time and delayed S phase. The cells also became increasingly sensitive to antibiotics and heat stress (Doshi et al., 2014). To gauge the contribution of each of these four substitutions to the lethality of UbEP42 mutations, they were first studied singly, by introducing each of them separately into ubiquitin gene. This led to the creation of four single ubiquitin mutants, namely UbS20F, UbA46S, UbL50P and UbI61T (Doshi et al. 2017). It was observed that the influence of UbS20F and UbA46S over the cells was insignificant with respect to the above functions. However, UbL50P and UbI61T produced lethal effects like the parent mutation UbEP42 (Doshi et al., 2017).

It was shown with several proteins that have double mutations in their sequence, may produce varied effects in different proteins (Mildvan et al., 1992; Mildvan, 2004). The effects may be additive, partially additive, synergistic, antagonistic or they may be structurally compensatory and show no effect at all (Sharma et al., 2021). Double mutations have been introduced into the ubiquitin gene in various combinations to study their effects on the functions of ubiquitin. These include the six double mutants resulting from UbEP42, namely S20F-A46S, S20F-L50P, S20F-I61T, A46S-L50P, A46S-I61T and L50P-I61T. Previously the above double mutants were studied in our laboratory with respect to their impact on the survival of the yeast when they are over expressed, as well as their impact on the growth curve of yeast, generation time, cell viability, heat stress and sensitivity to cycloheximide. Among the six double mutants, S20F-A46S and A46S-I61T were found to be the least detrimental mutants. The only aspect that was negatively impacted by S20F-A46S was the generation time, which increased upon overexpression of the mutant ubiquitin gene by $CuSO_4$, while A46S-I61T did not bear any effect on any function, even under induced expression. The other four double mutants were found to be detrimental. While the S20F-I61T substitution prolonged the generation time upon overexpression, other functions tested remained unaffected. S20F-L50P, A46S-L50P and L50P-I61T showed negative effect over all the functions mentioned above.

In this work, the effects of all these double mutants have been studied over many other functions of the ubiquitin proteasome system, in order to better understand the extent to which each of the double mutants affect the functioning of ubiquitin, and what their significance is. The effects produced by the double mutants derived from UbEP42 on UFD pathway, proteasomal degradation of Cdc28, lysosomal degradation of uracil permease, endosomal sorting of carboxypeptidase S, K48 linked polyubiquitination, and sensitivity to antibiotics canavanine, G418, gentamycin, hygromycin B and tunicamycin have been studied here.

2.2 Materials and Methods

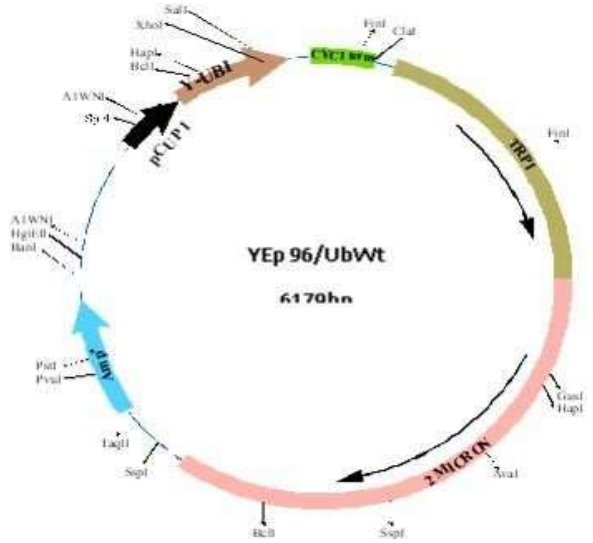
2.2.1 Yeast Strains, Media and Plasmids

Two *Saccharomyces cerevisiae* strains have been used in this study, namely SUB62 (Mata, lys2-801leu2-3,2-112 ura3-52 his3-Δ200 trp1-1) and SUB60 (Mata, lys2-801, leu2-3,112,

ura3-52, his3-Δ200, trp1-1, ubi4-Δ2::LEU2) (Finley et al., 1987, 1994). The cells were cultured at 30°C at 200 r.p.m. The media used for growing yeast are Synthetic Dextrose (SD) medium, composed of 0.67% yeast nitrogen base (without amino acids) from Hi-media and 2% glucose as carbon source, and Yeast Peptone Dextrose (YPD) medium, composed of 2% peptone (w/v), 2% dextrose (w/v), 1% yeast extract (w/v) and 2% agar (w/v). The supplements added to SD medium for selection are 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}), depending on requirements of experiments (Finley et al.,1994). SD is a minimal medium, while YPD is a rich medium.

2.2.2 Plasmids

YEp96 (Yeast Expression plasmid 96) was used to express the genes for wild type and mutant forms of ubiquitin (Fig. 2.1) (Finley et al., 1994). YEp96 is a high copy number plasmid that uses TRP1 as a selection marker and replicates in both *Escherichia coli* and *S. cerevisiae*, which enables it to be used as a shuttle vector. The ubiquitin gene that is cloned in YEp96 is under the control of *CUP1* promoter, which can be induced by $CuSO_4$. YEp96 plasmids in which wild type and *UbEP42* ubiquitin genes have been cloned are designated YEp96/UbWt and YEp96/UbEP42, respectively. And YEp96 plasmids in which ubiquitin double mutants derived from *UbEP42* have been cloned are designated Yep96/UbS20F-A46S, YEp96/UbS20F-L50P, YEp96/UbS20F-I61T, YEp96/UbA46S-L50P, YEp96/UbA46S-I61T and YEp96/UbL50P-I61T. As per the protocols standardized in our laboratory, the cells were overexpressed with 100 mM $CuSO_4$ for shorter durations, and with 25 mM $CuSO_4$ for longer duration,to avoid the cytotoxic effects of induced proteins. SUB60 cells transformed with YEp96/UbWt, YEp96/UbEP42, YEp96/UbS20F-A46S, YEp96/UbS20F-L50P, YEp96/UbS20F-I61T, YEp96/UbA46S-L50P, YEp96/UbA46S-I61T and YEp96/UbL50P-I61T were cultured in SD medium at 30°C, and growth was estimated by measuring OD at 600 nm (OD_{600}).



GAATTC ATTATGC AGATCT…….Yeast-UB……..GGT GGT TGA GGTACC

*Eco R*I Met *Bgl* II Gly Gly *Kpn* I

Figure 2.1: Yep96/UbWt plasmid carrying the gene for wild type ubiquitin (UbWt). It is 6179 base pairs long. The restriction sites present in the DNA sequence are indicated in the figure.

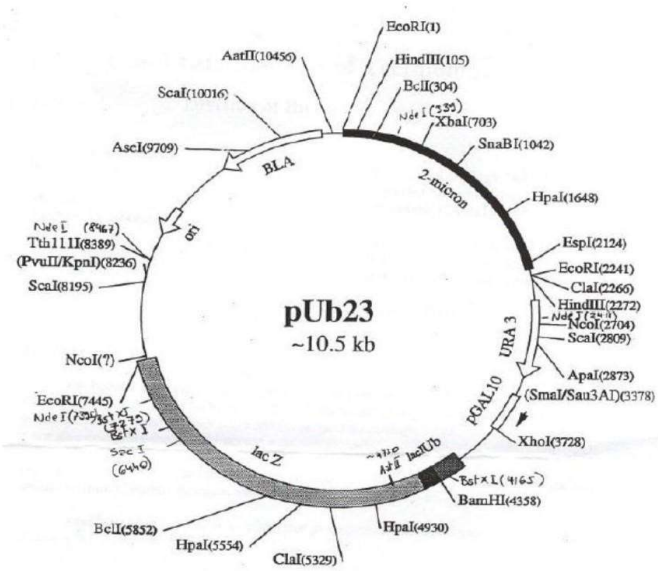


Figure 2.2: Plasmid pUb23 map showing the position of genes, promoters and restriction sites.

2.2.3 Antibiotic Sensitivity Test

Antibiotic sensitivity test has been conducted to test the complementation potential of UbEP42 derived double mutants (Hanna et al., 2003). SUB60 cells were transformed with YEp96/UbWt YEp96/UbS20F-A46S, YEp96/UbS20F-L50P, YEp96/UbS20F-I61T, YEp96/UbA46S-L50P, YEp96/UbA46S-I61T and YEp96/UbL50P-I61T, and were grown up to an OD_{600} of 0.2. At this point, four-fold dilutions of cultures were prepared, and each dilution was spotted on SD agar. Concentrations of the antibiotics were canavanine 2 µg/ml, G-418 50 µg/ml, gentamicin 80 µg/ml, hygromycin B 40 µg/ml and tunicamycin 0.75 µg/ml. Three plates were prepared in the combinations of two for each antibiotic, one without $CuSO_4$ and antibiotic, for negative control, and one with 25 µM $CuSO_4$, for induction. The growth of the mutants was compared to that of SUB62 and untransformed SUB60 after incubation at 30 °C for ten days.

2.2.4 K-48 linked polyubiquitination assay

SUB60 cells were transformed with YEp96/UbWt, YEp96/UbS20F-A46S, YEp96/UbS20F-L50P, YEp96/UbS20F-I61T, YEp96/UbA46S-L50P, YEp96/UbA46S-I61T and YEp96/UbL50P-I61T, and then cultured in YPD at 30°C, up till OD_{600} of 0.6. At this stage, cells were pelleted down by centrifugation, given normal saline washes, and then suspended in normal saline. The OD of the cells was then equalized and to prevent protein degradation, protease inhibitor cocktail was added to this suspension. This was followed by lysing of cells through sonication. Quantification of the total protein in the lysate was carried out using Folin Lowry method, and accordingly, the lysate samples, each containing 50 µg of protein, were subjected to SDS-PAGE using 15% sodium dodecyl sulphate polyacrylamide gel and electrophoresis.

The gel was then used in western blotting using nitrocellulose membrane, followed by Ponceau staining of the blot to ensure that equal protein concentrations were used in loading samples. The blot was given PBS washes, followed by incubation in blocking buffer containing 5% (w/v) non-fat dry milk in PBS-T for 1 h, in order to block non-specific antibody binding. This was followed by incubation of the blot for 16 hours in PBS-MT (PBS + Milk Powder + Tween 20) containing antibody specific for K-48 linked polyubiquitin. After incubation, the blot was given PBST (Phosphate Buffer Saline + Tween 20) washes, followed by incubation

in PBSMT containing secondary antibody (raised in rabbit). This was followed by PBST washes, followed by PBS washes to remove tween 20. Lastly, the bands corresponding to K-48 linked polyubiquitin were observed using ECL solution (Horseradish peroxidase and H_2O_2) (Bio-Rad Clarity).

2.2.5 Lysosomal Degradation of Uracil Permease

SUB60 cells were transformed with YEp96/UbWt, YEp96/UbS20F-A46S, YEp96/UbS20F-L50P, YEp96/UbS20F-I61T, YEp96/UbA46S-L50P, YEp96/UbA46S-I61T and YEp96/UbL50P-I61T, and were cultured till log phase. This was followed by treatment with 100 µg/ml of cycloheximide for 20 minutes to inhibit the synthesis of uracil permease. Analysis of lysosomal degradation of the uracil permease synthesised before cycloheximide treatment was done using western blotting, involving antibody specific for uracil permease, generously gifted to us by Rosine Haguenauer-Tsapis from Institut Jacques Monod, France. The protocol for cell lysis and western blot is same as that described in case of K48 linked polyubiquitination assay.

2.2.6 Cdc28 Assay

CDK1 is the cyclin dependent kinase, that acts as Cdc28, that is the master regulator of cell cycle in yeast, and it is expressed by the *CDC28* gene. The levels of Cdc28 in cells were analysed by western blot, using antibody specific for Cdc28. SUB60 cells were transformed with YEp96/UbWt, YEp96/UbS20F-A46S, YEp96/UbS20F-L50P, YEp96/UbS20F-I61T, YEp96/UbA46S-L50P, YEp96/UbA46S-I61T and YEp96/UbL50P-I61T. The protocols for culturing of cells, cell lysis and western blotting are same as that described in the case of K48 linked polyubiquitination assay.

2.2.7 Ubiquitin fusion degradation (UFD) Pathway

In order to study the degradation of substrate proteins through UFD pathway, untransformed SUB60 cells and SUB60 cells transformed with YEp96/UbWt, YEp96/UbS20F-A46S, YEp96/UbS20F-L50P, YEp96/UbS20F-I61T, YEp96/UbA46S-L50P, YEp96/UbA46S-I61T and YEp96/UbL50P-I61T, were cotransformed with pUb23 (Fig. 2.2). pUb23 plasmid contains the gene that encodes ubiquitin-β-galactosidase fusion protein (Ub-X-βgal), and the plasmid uses URA3 as a selection marker (Bachmair et al., 1986; Baker and Board, 1991). The experiment involved using two different sets of cells. In one set pUB23 contained the gene fusion encoding Ub-M-βgal (M- methionine) fusion protein, the ubiquitin tag of which is removed by ubiquitin C-terminal hydrolases. Since methionine is a stabilizing amino acid residue according to N-end rule pathway the reporter protein β-galactosidase is expected to remain stable (Johnson et al., 1992; Varshavsky, 1996). In the second set, pUB23 contained the gene fusion encoding Ub-P-βgal (P- proline) fusion protein, which is useful for studying UFD pathway (Johnson et al., 1992, 1995).

Analysis of the degradation of β-galactosidase through UFD pathway was carried out using western blot and anti-β-galactosidase antibody. The protocol for western blotting is the same as that described in the case of K48 linked polyubiquitination assay. The ubiquitin-β-galactosidase gene in pUb23 is under the control of *GAL10* promoter, which is inducible by galactose. For the constitutive expression of Ub-X-βgal (X can be Methionine or Proline as the case may be), the cells were cultured to mid log phase at 30°C in synthetic galactose medium. 100 μM $CuSO_4$ was then added to these mid log phase cultures, in order to express UbWt and UbEP42 derived double mutants of ubiquitin.

2.2.8 Confocal Microscopy for Endosomal Sorting of GFP-CPS Fusion Protein

SUB60 cells were transformed with YEp96/UbWt, YEp96/UbS20F-A46S, YEp96/UbS20F-L50P, YEp96/UbS20F-I61T, YEp96/UbA46S-L50P, YEp96/UbA46S-I61T and YEp96/UbL50P-I61T. These cells were subsequently transformed with pGOGFP426, a

plasmid expressing fusion of GFP and carboxypeptidase S (GFP-CPS), and grown at 30°C in SD medium, up to OD_{600} of 0.8. At this stage, cells were pelleted down by centrifugation and resuspended in YPD medium. This was followed by addition of FM4-64 dye, from a stock solution of 16 µM DMSO, up to a final concentration of 20 µM. This was followed by incubation of the cells in a stack incubator at 30°C for 15 minutes. At the end of the incubation period, cells were pelleted down by centrifugation at 700g for 3 minutes at 4°C, followed by resuspension in YPD medium. Cells from this suspension were then deposited on slide for observation under confocal microscope (Zeiss LSM 700).

2.3 Results

2.3.1 Sensitivity to Translational Inhibitors

It is known that ubiquitination is involved in regulating protein synthesis. For example, L28 is a ribosomal protein that undergoes K-63 linked polyuniquitination. This makes yeast more resistant to certain translational inhibitors (Spence et al. 2000). Many other ribosomal proteins are also known to be substrates for ubiquitination. Previous studies in our laboratory have shown that UbEP42 mutant, as well as UbEP42 derived double mutants S20F-L50P, S20F-I61T, A46S-L50P and L50P-I61T are more sensitive towards cycloheximide than wild type cells. Hence, in this work, the sensitivity of UbEP42 derived double mutants towards translational inhibitors Canavanine, G-418, Gentamycin and Hygromycin B has also been tested. In addition, the sensitivity of the double mutants to tunicamycin, which inhibits synthesis of glycoproteins, has also been tested.

The results of the experiments show that UbEP42 derived double mutants S20F-A46S, A46S-I61T and L50P-I61T are more sensitive to canavanine than wild type cells (Fig. 2.3). None of the double mutants appear to be more sensitive to G-418 than wild type cells (Fig 2.4). Double mutants S20F-A46S, A46S-I61T and L50P-I61T are more sensitive to Hygromycin B than wild type cells (Fig 2.5). Gentamicin acts on prokaryotic cells, as it acts on 30S subunit of the bacterial ribosome. But a previous study has shown that a mutation in Rsp5p ubiquitin ligase makes yeast sensitive to gentamicin (Kwapisz et al., 2005). Hence, in this work, the

sensitivity of the UbEP42 derived double mutants towards gentamicin was also tested. However, none of the double mutants have been found to render host cells more sensitive to gentamicin than wild type cells (Fig. 2.6). None of the double mutants have been found to be more sensitive to tunicamycin than wild type cells either (Fig. 2.7).

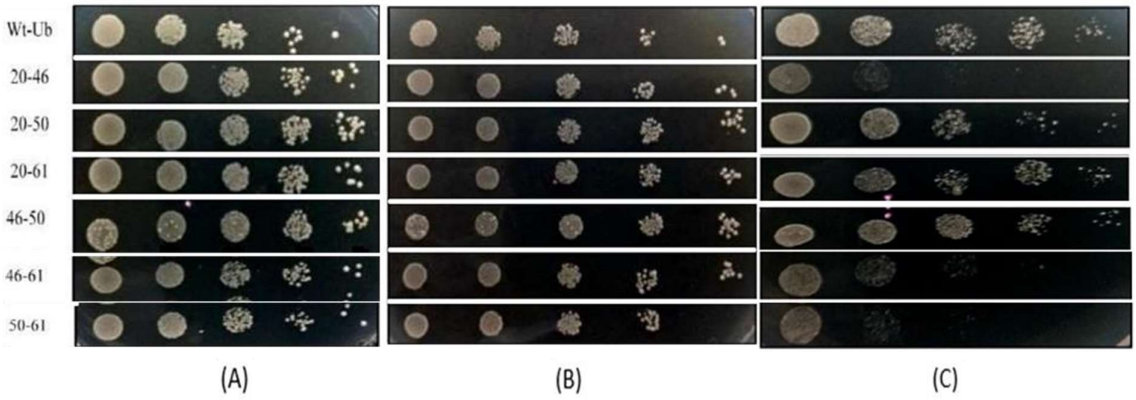


Figure 2.3: Drop test for testing sensitivity to canavanine of SUB60 transformed with YEp96/UbWt, YEp96/UbS20F-A46S, YEp96/UbS20F-L50P, YEp96/UbS20F-I61T, YEp96/UbA46S-L50P, YEp96/UbA46S-I61T and YEp96/UbL50P-I61T in SD medium in (A) without inducer and without canavanine, (B) with 25 µM $CuSO_4$ without canavanine and (C) with 25 µM $CuSO_4$ and in presence of 2 µg/ml canavanine.

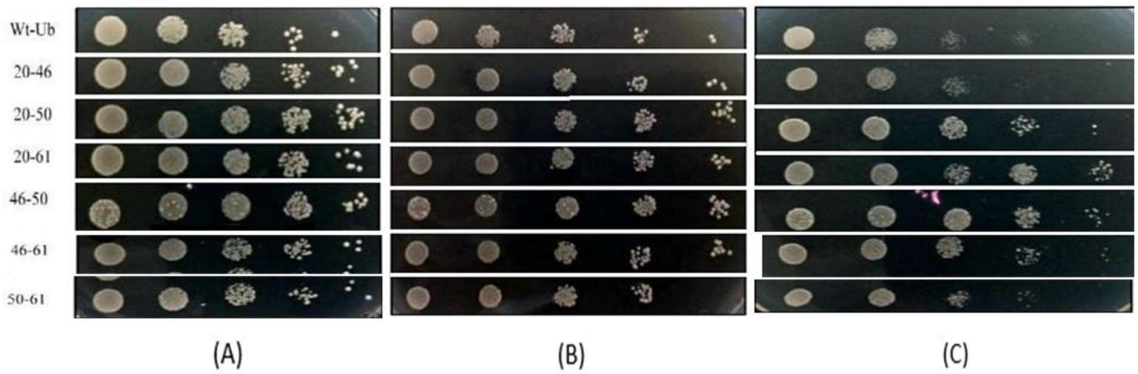


Figure 2.4: Drop test for testing sensitivity to G-418 of SUB60 transformed with YEp96/UbWt, Yep96/UbS20F-A46S, YEp96/UbS20F-L50P, YEp96/UbS20F-I61T, YEp96/UbA46S-L50P, YEp96/UbA46S-I61T and YEp96/UbL50P-I61T in SD medium in (A) without inducer and without G-418, (B) with 25 µM $CuSO_4$ without G-418 and (C) with 25 µM $CuSO_4$ and in presence of 50 µg/ml G-418.

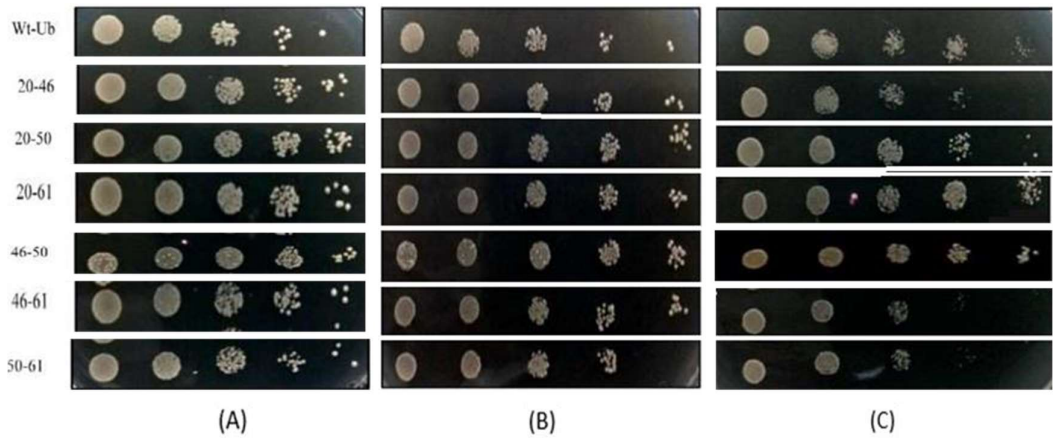


Figure 2.5: Drop test for testing sensitivity to hygromycin B of SUB60 transformed with YEp96/UbWt, YEp96/UbS20F-A46S, YEp96/UbS20F-L50P, YEp96/UbS20F-I61T, YEp96/UbA46S-L50P, YEp96/UbA46S-I61T and YEp96/UbL50P-I61T in SD medium in (A) without inducer and without hygromycin B, (B) with 25 µM $CuSO_4$ without hygromycin B and (C) with 25 µM $CuSO_4$ and in presence of 40 µg/ml hygromycin B.

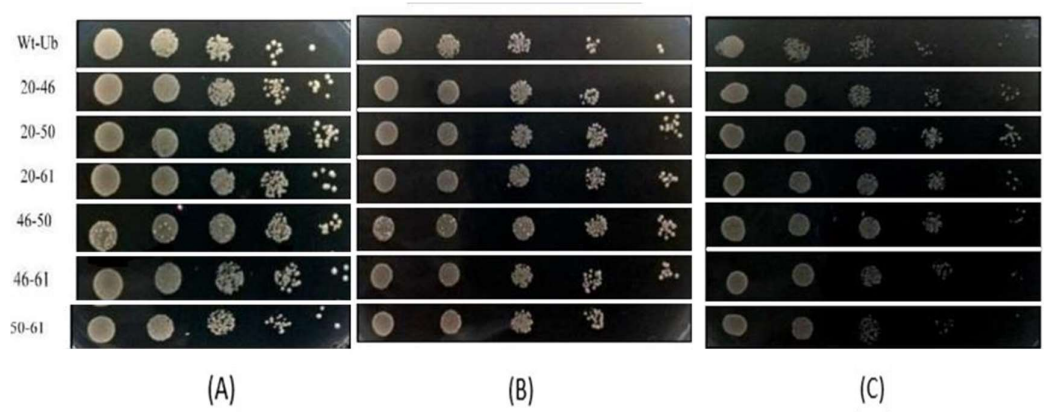


Figure 2.6: Drop test for testing sensitivity to gentamicin of SUB60 transformed with YEp96/UbWt, YEp96/UbS20F-A46S, YEp96/UbS20F-L50P, YEp96/UbS20F-I61T, YEp96/UbA46S-L50P, YEp96/UbA46S-I61T and YEp96/UbL50P-I61T in SD medium in (A) without inducer and without gentamicin, (B) with 25 µM $CuSO_4$ without gentamicin and (C) with 25 µM $CuSO_4$ and in presence of 80 µg/ml gentamicin.

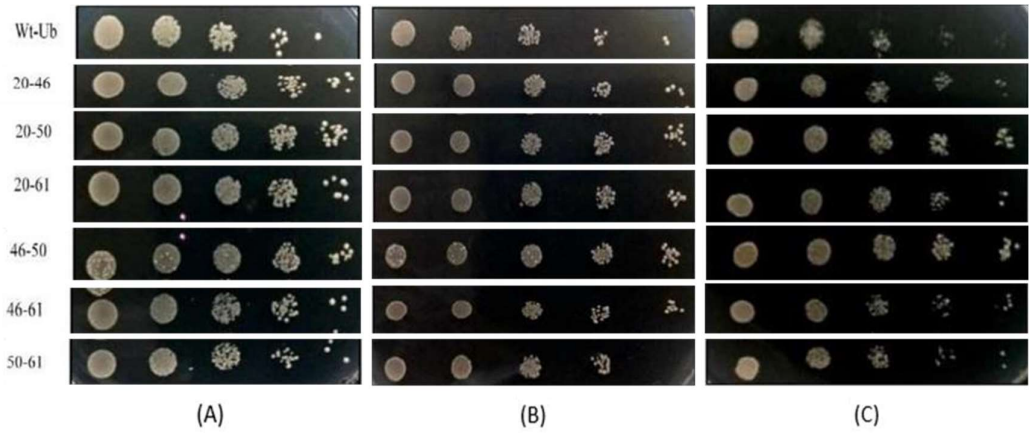


Figure 2.7: Drop test for testing sensitivity to tunicamycin of SUB60 transformed with YEp96/UbWt, YEp96/UbS20F-A46S, YEp96/UbS20F-L50P, YEp96/UbS20F-I61T, YEp96/UbA46S-L50P, YEp96/UbA46S-I61T and YEp96/UbL50P-I61T in SD medium in (A) without inducer and without tunicamycin, (B) with 25 µM $CuSO_4$ without tunicamycin and (C) with 25 µM $CuSO_4$ and in presence of 0.75 µg/ml tunicamycin.

2.3.2 Protein degradation through UFD Pathway

Ubiquitin fusion degradation (UFD) pathway is responsible for the proteasomal degradation of substrate proteins which are fused to the C-terminus of ubiquitin and have proline as their N-terminal residue. To study the degradation of the UFD substrates, the gene for a reporter protein (*lac Z* gene for β-galactosidase) is fused to the 3'-end of ubiquitin gene, and this fusion is expressed. The N-terminal residue of β-galactosidase is proline. Another such construct with the N-terminal residue of β-galactosidase being methionine is employed as a control. The efficiency with which deubiquitinating enzymes cleave off ubiquitin fused to the protein depends on which amino acid residue is present at the N terminal of the substrate protein. Out of all residues, methionine is the most favourable, enabling the deubiquitinating enzymes to cleave off ubiquitin very easily. Proline, on the other hand, is not recognized and the fusion is not cleaved by the deubiquitinating enzymes. In this case, instead of deubiquitinating enzymes cleaving off ubiquitin from the fusion protein, the ubiquitin N terminally fused to the substrate protein is further ubiquitinated to produce a K48 linked polyubiquitin chain. This causes proteasomal degradation of the substrate protein.

In order to study the effects of UbEP42 derived double mutants on the UFD pathway, SUB60 cells were transformed with a chimeric gene that encodes a ubiquitin-β-galactosidase fusion protein. These SUB60 cells had previously been transformed with YEp96 plasmids expressing wild type ubiquitin or UbEP42 derived double mutants of ubiquitin. Two different sets of SUB60 cells were prepared for the experiment. In one set, the chimeric gene expressed Ub-M-βgal (M- methionine) fusion protein. And in the other set, the chimeric gene expressed Ub-P-βgal (P- proline) fusion protein. The cells were induced using 100μM of $CuSO_4$ during culturing, in order to over-express the wild type or UbEP42 double mutant ubiquitin genes from the YEp96 plasmids. These cells were later lysed, and the lysates were subjected to western blotting using antibody specific for β-galactosidase. The results show that in case of S20F-I61T, A46S-L50P and L50P-I61T double mutants, the intensity of the bands of Ub-P-βgal remains similar to the bands of Ub-M-βgal (Fig. 2.8). This observation suggests that these three double mutants have affected the degradation of β-galactosidase fused to ubiquitin, and consequently, the UFD pathway.

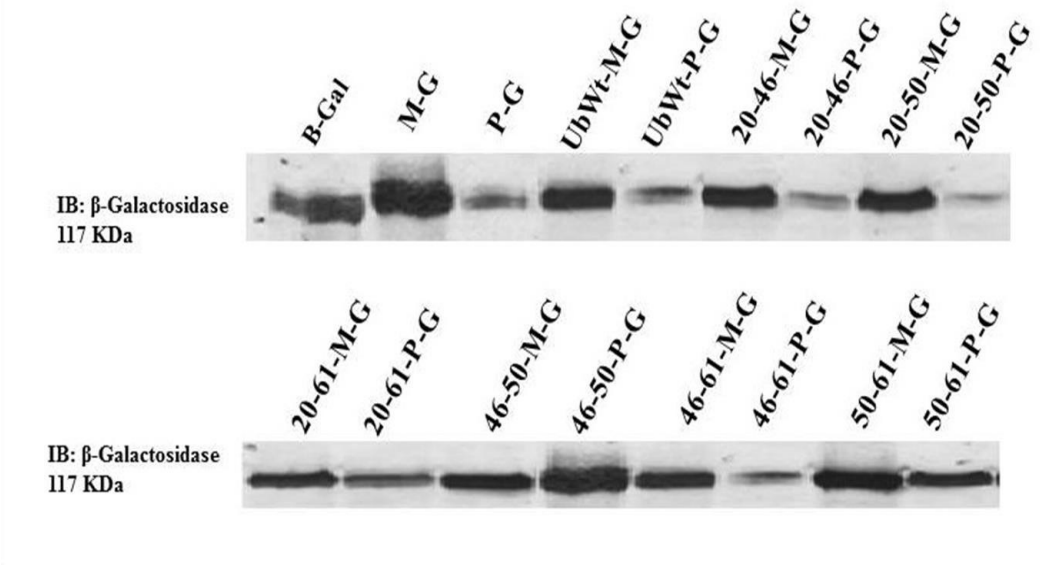


Figure 2.8: Effect of UbEP42 derived double mutations on UFD pathway in SUB60 cells. The S20F-I61T, A46S-L50P and L50P-I61T affected protein degradation by UFD pathway.

2.3.3 Cdc28 levels, effect on cell cycle

It is known that ubiquitin proteasome system is involved in cell cycle. The proteins involved in one stage of cell cycle are proteasomally degraded to allow proteins of the next stage to work. This leads to transition from one stage of cell cycle to the next. Hence, in this work, the effect of UbEP42 derived double mutants on the function of ubiquitin system in cell cycle has been studied. CDK1 (Cyclin Dependent Kinase 1) is a serine/threonine kinase that plays a role in the regulation of cell cycle. CDK1 is produced by the *cdc28* gene in *Saccharomyces cerevisiae*. In order to study the effects of UbEP42 derived double mutations on the levels of Cdc28 *in vivo*, SUB60 cells expressing the UbEP42 derived double mutants were subjected to western blotting involving antibody specific towards Cdc28. The results show that three double mutants, namely S20F-I61T, A46S-L50P and L50P-I61T, have less Cdc28 levels than the wild type cells (Fig. 2.9). These results show that the above three double mutants could exert a detrimental effect on the cell cycle.

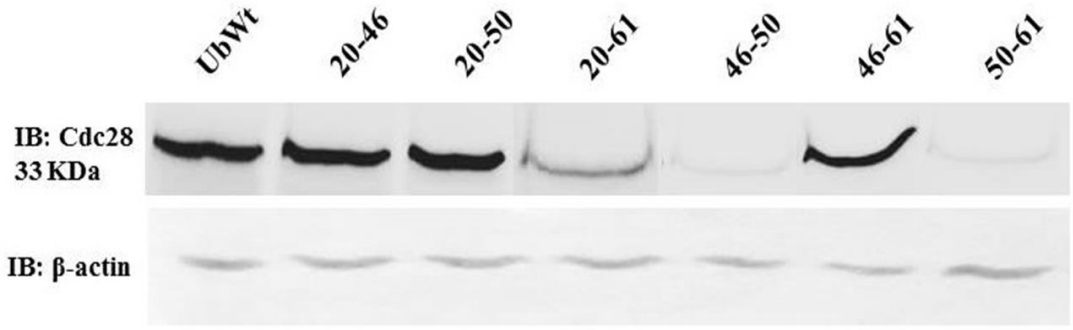


Figure 2.9: Effect of UbEP42 derived double mutations on Cdc28 levels in SUB60 cells. Cells expressing the double mutants of ubiquitin, UbS20F-UbI61T, UbA46S-L50P and UbL50P-I61T have lower levels of Cdc28 protein kinase.

2.3.4 Effect on lysosomal protein degradation studied by monitoring uracil permease levels

Membrane proteins are degraded in the lysosome. Previous studies have shown that ubiquitin is involved not only in the proteasomal degradation, but also in lysosomal degradation of proteins (Dupre et al. 2001; Blondel, Morvan et al. 2004). Hence, in this work, it was investigated whether or not UbEP42 double mutations affect the lysosomal degradation of proteins. To do this, YEp96 plasmids carrying genes for wild type ubiquitin and UbEP42 derived double mutants were transformed into SUB60 cells. These cells were then subjected to western blotting using antibody specific for uracil permease.

Uracil permease is an integral membrane protein that transports uracil across the plasma membrane of a cell. Before being lysed, the cells were incubated in 100 µg/ml cycloheximide for 20 minutes, in order to prevent further protein synthesis of uracil permease. Two different western blots were then carried out using the lysates. In one western blot, lysates of cells lysed immediately after cycloheximide treatment were used, and in the other western blot, cells lysed after an incubation of 90 minutes after cycloheximide treatment were used. The 90-minute incubation was done to allow the lysosomal degradation of uracil permease that was synthesised before inhibition of protein synthesis by cycloheximide treatment. The results of these experiments suggest that double mutations S20F-I61T, A46S-L50P and L50P-I61T have a detrimental effect on the lysosomal degradation of uracil permease, as the uracil permease bands do not lose intensity in their case even in the cells lysed after 90-minute incubation (Fig. 2.10).

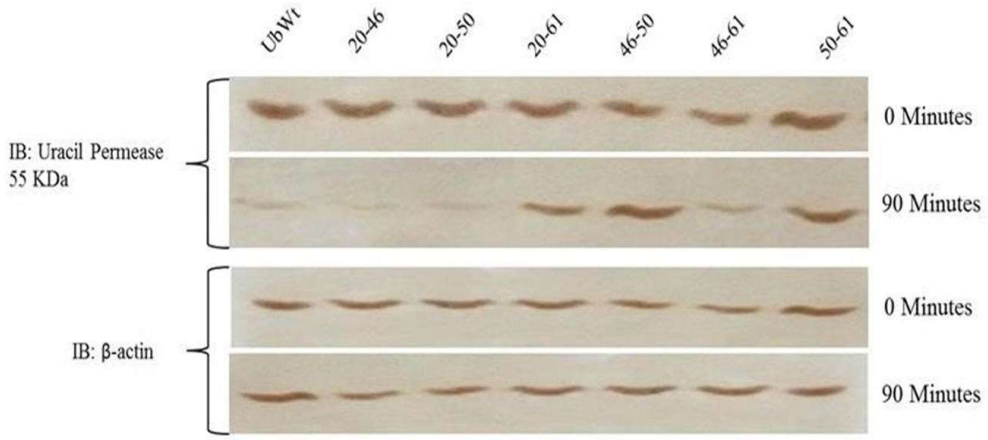


Figure 2.10: Effect of UbEP42 derived double mutations on lysosomal degradation of uracil permease in SUB60 cells.

2.3.5 Endosomal sorting of Carboxypeptidase S

Carboxypeptidase S (CPS) is a single pass membrane protein that is present in the membrane of vacuoles in *S. cerevisiae*. As its name suggests, carboxypeptidase S is a proteolytic enzyme that hydrolyses the C terminal residues of proteins. Carboxypeptidase S is also known to be a cargo of multivesicular bodies (MVBs), and monoubiquitination as well as K-63 linked polyubiquitination are known to be involved in sorting of membrane proteins like carboxypeptidase S through MVBs (Emr et al. 2001). Specifically, K-63 linked polyubiquitination is believed to be involved in internalizing membrane proteins through the ESCRT pathway, while monoubiquitination seems to be involved in internalization of membrane proteins, as well as their sorting into MVBs after internalization.

Since the UbEP42 derived double mutations have been shown to affect various functions of ubiquitin system in yeast, their effect on endosomal sorting of carboxypeptidase S has also been studied in this work. For this purpose, SUB60 cells were transformed with a plasmid pGOGFP426 bearing gene fusion of carboxypeptidase S and GFP (GFP-CPS). These SUB60 cells had earlier been transformed with YEp96/UbWt or YEp96 expressing the UbEP42 derived double mutants. The cells were then cultured at 30°C in SD medium up till log phase, treated with FM464 dye and fixed for confocal microscopy. FM464 is a dye that

emits red fluorescence and specifically stains vacuolar membrane (Thomas et al. 1995). The sorting of GFP-CPS and its localization to vacuole was studied by observing the yellow fluorescence that results from the colocalization of green fluorescence emitting GFP-CPS, and red fluorescence emitting FM464 in vacuolar membrane and MVBs. The results show that the transformants of SUB60 cells expressing UbS20F-L50P, UbS20F-I61T, UbA46S-L50P, and UbL50P-I61T, showed failure of sorting of CPS into endosomes, while the ubiquitin mutations UbS20F-A46S and UbA46S-I61T had no effect over sorting of CPS in the cells expressing them, and CPS levels were comparable to UbWt control (Fig. 2.11).

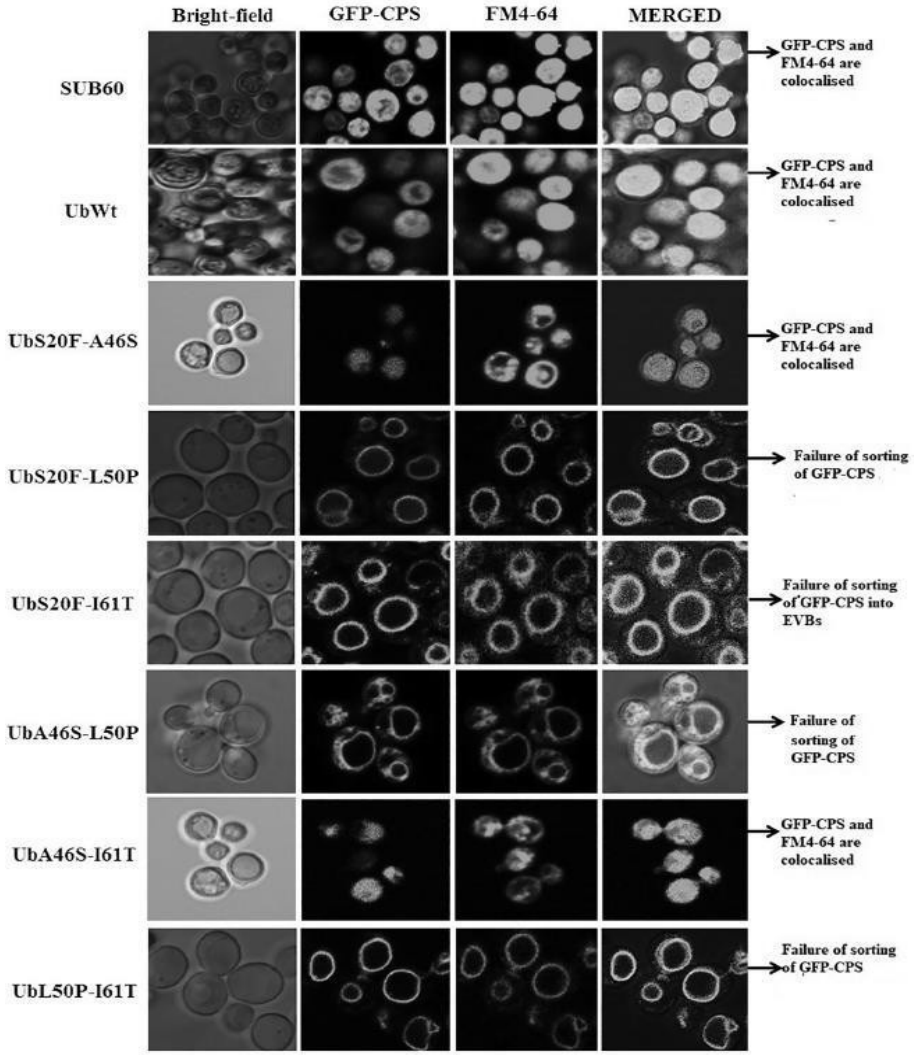


Figure 2.11: Confocal microscopy of SUB60 cells transformed with YEp96 expressing wild type ubiquitin and the UbEP42 derived double mutants. (A) bright field, (B) RFP filter (FM4-64), (C) GFP filter (GFP), (D) merging of (B) and (C) for observing colocalization. It can be seen from the images that the transformants of SUB60 cells expressing UbS20F-I61T, UbA46S-L50P, UbL50P-I61T showed failure of sorting of CPS into endosomes, while sorting remains unaffected in the cells expressing UbS20F-A46S, UbS20F-L50P and UbA46S-I61T and is comparable to UbWt control.

2.3.6 Effect on K-48 linked polyubiquitination

It is possible that the reason why UbEP42 derived double mutations affect various functions of ubiquitin is that these mutations might prevent the formation of K-48 linked polyubiquitin chains. To find out if this is indeed the case, YEp96 plasmids expressing wild type ubiquitin or UbEP42 derived double mutants were transformed into SUB60 cells. These cells were then lysed and subjected to western blotting using K-48 linkage specific polyubiquitin antibody. This approach stains all the proteins in the blot that are tagged with polyubiquitin chains containing K-48 linkage. Results of these experiments indicate that double mutants S20F-I61T, A46S-L50P and L50P-I61T exert a detrimental effect on K-48 linked polyubiquitination of proteins, as the band intensity in their lysates is less than wild type cell lysates (Fig. 2.12). This blocking of K-48 linked polyubiquitin chain formation could be the reason behind the detrimental effect of these double mutations on other functions studied. This is further supported by the observation that these three are also the only double mutants that exert detrimental effects on UFD pathway, Cdc28 functioning and lysosomal degradation of uracil permease.

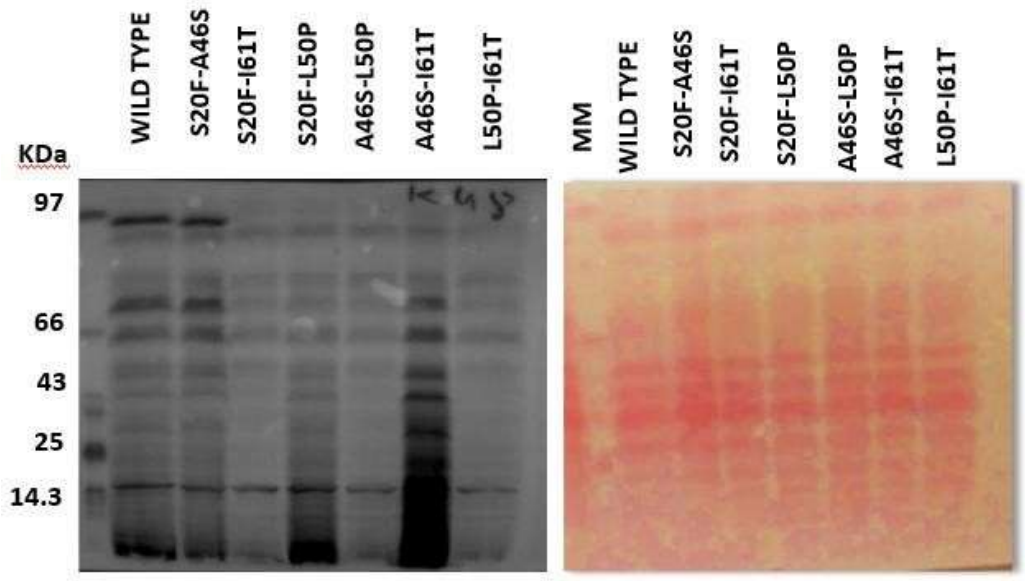


Figure 2.12: Effect of UbEP42 derived double mutations on K-48 linked polyubiquitination. Left: Western blotting carried out with K-48 linkage specific polyubiquitin antibody. Cells expressing UbS20F-A46S, UbS20F-L50P, UbA46S-I61T showed polyubiquitination to the same extent as the cells expressing UbWt. In contrast, in the cells expressing UbS20F-I61T, UbA46S-L50P, UbL50P-I61T the K-48 linked polyubiquitination is much reduced. Right: Ponceau staining of the same SDS-PAGE gel before western blotting.

2.4 Discussion

It was observed with many proteins that the effect of the double mutations may be additive, partially additive, synergistic and antagonistic or may have no effect at all. The structural relationship present between the two residues constituting the double mutation determine the kind of effect seen with a given protein. Besides revealing how the two residues contribute to the protein structure and function, they provide insight into the structural details of a protein and their functional consequences. Ubiquitin with 96% of its residues conserved presents an interesting case for studies with double mutations.

The two most detrimental single substitutions, namely L50P and I61T observed earlier (Doshi et al., 2017), also exhibit their effect when they are present along with other substitutions in the double mutants. One of the least detrimental of the six double mutants generated from

UbEP42, is S20F-A46S. The effects of this double mutant are very mild as it does not contain either L50P or I61T substitutions. Results from this work, when viewed along with those from previous studies in our laboratory, show that although S20F-A46S confers sensitivity to hygromycin, G418 and canavanine and leads to increase in generation time under overexpression, it does not increase sensitivity to cycloheximide, tunicamycin and gentamicin, shows complementation in case of heat stress, Cdc28 levels, lysosomal degradation of uracil permease, UFD pathway, and K48 linked polyubiquitination. It is also non-lethal under overexpression, and has no effect on the endosomal sorting of carboxypeptidase S.

The most detrimental of all six double mutants is L50P-I61T, which contains both the lethal substitutions, namely L50P and I61T. L50P-I61T does not increase sensitivity to tunicamycin and gentamicin, while affecting the endosomal sorting of CPS. But it increases sensitivity to cycloheximide hygromycin and canavanine. It also renders host cells even more sensitive to heat stress than untransformed SUB60 (suggesting that it competitively inhibits the functioning of wild type ubiquitin in SUB60), leads to increase in generation time, slows down growth, and has a negative effect on lysosomal degradation of uracil permease, UFD pathway, Cdc28 levels, and K48 linked polyubiquitination.

The next two most detrimental double mutants, after L50P-I61T, are S20F-I61T and A46S-L50P. It is believed that the effects of these two double mutants are so detrimental because A46S-L50P contains the L50P substitution, and S20F-I61T contains the I61T substitution, both of which are lethal even when they are present in isolation. These results reinforce the previous findings from studies on the UbEP42 derived single mutants in our lab, which show that L50P and I61T are the most detrimental of the four substitutions found in the UbEP42 mutant, and likely contribute to the bulk of the detrimental effects shown by UbEP42.

The compensatory effect shown by A46S against the detrimental I61T substitution is apparent from the observation that even though A46S-I61T contains I61T, A46S-I61T does not increase generation time of yeast or its sensitivity to G418. This compensatory effect is particularly intriguing, as it involves substituting a hydrophobic amino acid, namely, alanine with serine which is not just hydrophilic but differs significantly from alanine structurally. In spite of not containing either L50P or I61T, S20F-A46S increases generation time. But otherwise, S20F-A46S is a benign substitution. This might be due to the fact that despite being hydrophobic, A46 is a surface residue, and not buried in the core of ubiquitin. So, its

substitution with a hydrophilic residue like serine might not cause any structural instability to ubiquitin.

All the antibiotics used in the above experiments, except tunicamycin, are translational inhibitors. But they use different modes of action to inhibit translation. Cycloheximide inhibits the translocation of tRNA and mRNA relative to the ribosome. Canavanine structurally mimics L arginine, which causes the cells to incorporate it instead of L arginine in the growing polypeptide chain during protein synthesis. G-418 inhibits the elongation phase of translation. Gentamicin acts on bacteria, by increasing the error rate of translation, by binding to a part of small ribosomal subunit that discriminates between correct and incorrect codon-anticodon base pairs. Hygromycin prevents the translocation of tRNA to P site by stabilizing its binding to A site of ribosome. Tunicamycin is actually not a single antibiotic, but a mixture of homologous nucleoside antibiotics. It is not a translational inhibitor. Instead, it prevents N linked glycosylation during glycoprotein synthesis. This leads to arrest of cell cycle in G1 phase.

Canavanine seems to be the most detrimental of all the antibiotics used in these experiments. Four out of the six double mutants, namely S20F-A46S, A46S-L50P, A46S-I61T and L50P-I61T made cells more sensitive to canavanine than wild type cells. Out of these four, L50P-I61T and A46S-L50P are two of the most detrimental of all six double mutations overall. On the other hand, none of the double mutations seem to increase the sensitivity of yeast towards tunicamycin and gentamycin. Interestingly, both A46S-L50P and L50P-I61T contain L50P substitution and lead to increase in sensitivity of yeast to canavanine. However, S20F-L50P, also contains L50P, and yet does not increase sensitivity to canavanine. On the other hand, as mentioned before, all double mutants that contain L50P substitution increase sensitivity of yeast to cycloheximide. Hence, it appears that the molecular mechanisms behind the increased sensitivity of yeast containing UbEP42 derived double mutations to different translational inhibitors are complicated, and further investigation is needed to unravel them.

There are three possible reasons why the UbEP42 derived double mutants are detrimental to yeast. One possible reason is that L50P and I61T substitutions structurally alter ubiquitin, which impedes its normal functioning. On the other hand, S20F and A46S substitutions somehow seem to compensate for the said structural alteration. A46S substitution could contribute to structural stability of ubiquitin by replacing a surface hydrophobic residue with a hydrophilic one. Another possible reason is that these substitutions do not structurally alter ubiquitin, but prevent its recognition by other proteins. If this is indeed the case, then the

substitutions might prevent ubiquitin from being recognized either by one or more of the enzymes that are involved in the ubiquitination pathway, or by other proteins, outside the ubiquitination pathway.

Of the four residues that are substituted in the UbEP42 mutation, L50 and I61 are buried residues while S20 and A46 are surface residues (Prabha et al., 2010). Being buried in the core of ubiquitin, it's unlikely that L50 and I61 are directly involved in recognition of ubiquitin by other proteins. Results from previous studies in our laboratory showed that L50P and I61T substitutions structurally alter ubiquitin. And as mentioned above, the most detrimental of the four substitutions involve L50 and I61 residues (Doshi et al. 2017). Hence, the L50P-I61T, S20F-I61T and A46S-L50P double mutants most likely exert their detrimental effects through the structural alteration of ubiquitin.

Since the effects of S20F and A46S are not significantly harmful, it seems that despite being surface residues, they are not necessary for recognition of ubiquitin by target interacting proteins. This is also suggested from a previous study, in which all the surface residues of ubiquitin that are essential for life in yeast were identified, and they did not include either S20 or A46 (Sloper-Mould et al. 2001). Also, as mentioned before, the fact that A46 is a surface residue despite being hydrophobic might also be the reason why substitution of A46 with S, a hydrophilic residue more suited to the water exposed surface of ubiquitin, is not too detrimental.

Another interesting observation is that K48 linked polyubiquitination is affected by L50P-I61T, S20F-I61T and A46S-L50P substitutions. Further, K63R substitution, which prevents K-63 linked polyubiquitination, makes yeast sensitive to cycloheximide compared to wild type cells (Spence et al., 2000), similarly the L50P-I61T, S20F-I61T and A46S-L50P substitutions make it more sensitive to cycloheximide than wild type cells. These observations suggest that the L50P-I61T, S20F-I61T and A46S-L50P and S20F-L50P substitutions are not as detrimental to monoubiquitination and K63 linked polyubiquitination as they are to K48 linked polyubiquitination.

UbEP42 derived double mutants fail to make yeast sensitive to gentamicin. Gentamicin is a translational inhibitor that is known to act on prokaryotes and not eukaryotes. Specifically, when there is correct codon anticodon base pairing, adenosines 1492 and 1493 of the 16S rRNA of the 30S subunit of the bacterial ribosome establish two nonspecific hydrogen bonds with the codon-anticodon pair, stabilizing it. On the other hand, when there is incorrect codon-anticodon

base pairing, steric hinderance prevents these hydrogen bonds from forming. Thus, an incorrect codon-anticodon pair is less stable than a correct one, making it more likely to break off before the wrong amino acid is incorporated into the growing polypeptide chain. When gentamicin binds to the 16S rRNA, it stabilizes the incorrect codon-anticodon pair, increasing the chances of incorporation of wrong amino acids into the growing polypeptide chain.

Interestingly, while this mechanism dictates that gentamicin should act only on the prokaryotic ribosome, a previous study has found that mutation in the Rsp5 ubiquitin ligase makes yeast sensitive to gentamicin as well (Kwapisz et al., 2005). The mechanism behind this phenomenon is yet to be deciphered, and it is not clear at present, whether gentamicin in the yeast carrying the Rsp5 mutation binds to the same or different site. In this work, as mentioned before, the yeast cells carrying the UbEP42 derived double mutations were tested to see if these mutations also make yeast sensitive to gentamicin like the Rsp5 mutation does. The results show that there is no increase in sensitivity of UbEP42 derived double mutants to gentamicin compared to wild type cells. In fact, since even untransformed SUB60 cells grow as well as wild type cells in presence of gentamicin (which is not the case with cycloheximide, G418 and hygromycin), it shows that the inability of gentamicin to act on eukaryotes is not in any way changed by the UbEP42 derived double mutations.

The Rsp5 ubiquitin ligase is involved in the process of endocytosis and sorting of membrane proteins into vacuole or lysosome. The more detrimental of the UbEP42 derived double mutations behave like the way mutation in Rsp5 does (Kwapisz et al., 2005). Given this observation, it is not surprising that UbEP42 derived double mutations L50P-I61T, S20F-I61T and A46S-L50P negatively impact endocytosis and sorting of membrane proteins into vacuole/lysosome, as observed in the case of carboxypeptidase S and the lysosomal or vacuolar degradation of membrane proteins as observed with uracil permease.

In conclusion, L50P-I61T, S20F-I61T and A46S-L50P, the three double mutants that are the most detrimental to cells, have potential applications in healthcare that should be explored. Results show that SUB60 transformed with A46S-L50P and L50P-I61T is even more sensitive to heat stress than untransformed SUB60. This suggests that ubiquitin containing either of these two double mutations can inhibit the functioning of wild type ubiquitin that is constitutively expressed from the *UBI1*, *UBI2* and *UBI3* genes in SUB60. A46S-L50P and L50P-I61T double mutants could therefore come handy in dealing with diseases that are caused by an over-expression of wild type ubiquitin in cells.