# Functional Characterization of Q2N, E64G And S65D Single Mutants of Ubquitin in *Saccharomyces cerevisiae*

# 3.1 Introduction

Ubiquitin is used as a tag to mark cellular proteins for proteasomal degradation. The substrate proteins of ubiquitin come from diverse classes, like apoptotic factors, developmental regulators, cell cycle switches, anti-tumour proteins and others. Ubiquitin is 76 amino acids long, and its structure is a β grasp fold, which consists of a β sheet surrounding or "grasping an α helix (Vijay-Kumar et al., 1987). Ubiquitin is a highly conserved protein, differing in only three residues between yeast, plants and humans (Gavilanes et al., 1982; Watson et al., 1978; Schlesinger et al., 1975; Schlesinger and Goldsteiner, 1975). This high level of conservation suggests that many structural, folding and functional constraints have been imposed on the amino acid sequence of ubiquitin in the course of evolution. Hence, ubiquitin mutants are useful in understanding the structural and functional significance of the various conserved residues of the protein (Mishra et al., 2009; 2011; Prabha et al., 2010; Doshi et al., 2014; 2017; Sharma and Prabha, 2011; 2015; Sharma et al., 2021; Sloper-Mould et al., 2001; Roscoe et al., 2013; Lee et al., 2014).

Ubiquitin contains two β bulges, which partially overlap with β turns. The first β-bulge and the adjacent turn have been studied in detail (Chen et al., 2001; Searle et al., 1995; de Alba et al., 1996; Riemen and Waters, 2008). However, the second one, a G1 β bulge, has been topic of interest of our laboratory, as it possesses some interesting features (Mishra et al., 2009; 2011). This β bulge accompanies a type II turn, and occurs in a parallel β sheet. These features are very rare in proteins (Chan et al., 1993). E64 forms the first residue of the β bulge and the third residue of the adjacent, type II turn (Vijay-Kumar et al., 1987). This is unusual, because generally the first residue of a G1 β is a G. Presence of an E residue instead of G in this position leads to unusual Φ and Ψ angles, which do not fall in the permitted region for E in Ramachandran plot (Ramachandran, Ramakrishnan and Sasisekharan, 1963). In fact, these angles are observed generally with G (Chan et al., 1993). β bulges favour G in this position, as the percentage frequency of occurrence of G in this position of a β bulge is 11.3, while that of E in the same position is just 0.23 (Table 3.1). However, the E64 residue of ubiquitin is completely conserved in the eukaryotes indicates its relevance to ubiquitin's functioning (Table 3.2).

*Table 3.1*

*Occurrences of amino acid residues at different positions of β bulge. The residue that was present in wild type is shown in Red and the substitution is shown in Blue.*

| Amino acid | Position of amino acid in the β bulge | | | | | |
|---|---|---|---|---|---|---|
| | Position X | | Position 1 | | Position 2 | |
| | Faai/bulge | Naai | Faai/bulge | Naai | Faai/bulge | Naai |
| **I** | 0.53 | 5 | 0.00 | 0 | 0.21 | 2 |
| **F** | 0.17 | 1 | 0.17 | 1 | 0.52 | 3 |
| **V** | 0.76 | 10 | 0.00 | 0 | 0.54 | 7 |
| **L** | 0.8 | 8 | 0.00 | 0 | 0.80 | 8 |
| **W** | 0.57 | 1 | 1.13 | 2 | 0.57 | 1 |
| **M** | 0.00 | 0 | 0.00 | 0 | 0.40 | 1 |
| **A** | 0.81 | 6 | 0.00 | 0 | 0.54 | 4 |
| **G** | 1.0 | 8 | **11.30** | **84** | 0.54 | 4 |
| **C** | 2.3 | 6 | 0.39 | 1 | 0.00 | 0 |
| **Y** | 0.20 | 1 | 0.00 | 0 | 1.0 | 5 |
| **P** | 0.00 | 0 | 0.00 | 0 | 0.00 | 0 |
| **T** | 0.9 | 7 | 0.00 | 0 | 1.0 | 8 |
| **S** | 1.6 | 11 | 0.00 | 0 | **1.5** | **8** |
| **H** | 2.6 | 6 | 0.00 | 0 | 0.44 | 1 |
| **E** | 0.68 | 3 | **0.23** | **1** | 2.0 | 9 |
| **N** | **5.4** | **18** | 3.95 | 13 | 0.91 | 3 |
| **Q** | **0.34** | **1** | 0.34 | 1 | 3.1 | 9 |
| **D** | 3.5 | 15 | 0.71 | 3 | **2.4** | **10** |
| **K** | 0.17 | 1 | 0.34 | 2 | 2.6 | 15 |
| **R** | 0.25 | 1 | 0.25 | 1 | 2.7 | 11 |

| |
|---|
| **Faai/bulge - % frequency of occurrence of amino acid i in a bulge.** |
| **Naai - Total number of amino acid i found in a bulge.** |

Hence, to study the relevance of E64 residue of ubiquitin, E64G substitution was generated in our laboratory, using site directed mutagenesis. G was chosen to substitute E64 because as described above, G is found much more commonly than E in G1 β bulges in other proteins, and hence, substituting E64 with G was unlikely to cause any structural alteration of the G1 β bulge of ubiquitin. It is interesting to note that K63, the residue adjacent to E64, is involved in K63 linked polyubiquitination, which is involved in several non-proteasomal functions like membrane protein degradation, post replicative DNA repair, and UV induced DNA repair. Hence, the functional relevance of E64 might be connected to its proximity to the K63 residue. Another thing to note is that I61, another residue in close proximity to E64, is known to show slower slower protection in kinetic refolding experiments (Briggs and Roder, 1992). Besides, I61T is one of the four substitutions that make up the UbEP42 mutation generated through random mutagenesis in our lab (Prabha et al., 2010). The UbEP42 mutation, which is described in detail in chapter 3, is highly detrimental to yeast.

The residue in the second position of the G1 β bulge in ubiquitin is S65. It is also the last residue of a type II turn that accompanies this G1 β bulge (Vijay-Kumar et al., 1987). Just like E64, S65 also has a low percentage frequency of occurrence in G1 β bulges, with a value of 1.5 (Chan et al., 1993). On the other hand, D has a percentage frequency of occurrence of 2.4 in the same position of G1 β bulge in other proteins. The fact that just like E64, S65 is also highly conserved in the G1 β bulge of ubiquitin despite low percentage frequency of occurrence suggests that S65 too has functional relevance for ubiquitin. The fact that D is found with a higher percentage frequency of occurrence in this position of G1 β bulges than S means that substituting S65 with D in ubiquitin is unlikely to cause structural alteration of the G1 β bulge. Hence, to study the relevance of S65 in ubiquitin, S65D substitution was generated using site directed mutagenesis in our lab (Mishra et al., 2011).

The percentage frequency of occurrence of D residue in the second position of G1 β bulge is not the highest of all amino acids (Table 3.1). But D was still selected to replace S65 in ubiquitin because it is naturally found in this position of G1 β bulges in most of the structural homologues of ubiquitin (Jentsh et al., 2000). Table 3.2 shows the sequence comparison between ubiquitin and its homologues.

*Table 3.2*

*Partial comparison of the amino acid sequences of ubiquitin (UBI) and its structural homologues.*

| Ubiquitin & homologues | Position of the residue in the protein sequence | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | …. | 63 | 64 | 65 | 66 |
| UBI | M | Q | I | …. | K | E | S | T |
| NEDD8 | M | L | I | …. | L | G | G | S |
| RUB1 | M | I | V | …. | V | E | G | M |
| SMT3 | I | N | L | …. | D | E | D | T |
| SUMO1 | I | K | L | …. | E | E | D | T |
| SUMO2 | I | N | L | …. | D | G | D | V |
| SUMO3 | I | N | L | …. | D | G | D | Q |

The Q2 residue of ubiquitin, which is the second residue from its N terminal, forms hydrogen bonds with E64 and S65 residues. Q2, therefore, occupies the X position of the G1 β bulge of ubiquitin. The hydrogen bonds Q2 forms with E64 and S65 stabilize the G1 β bulge. Just like E64 and S65, Q2 shows total conservation in ubiquitin across species. However, the percentage frequency of occurrence of Q in G1 β bulges across proteins is just 0.34, while that of N in the same position is 5.4, which is the highest for any residue (Table 3.1). This shows that just like E64 and S65, Q2 plays an important role in the functioning of ubiquitin, which is why it is so highly conserved despite its low percentage frequency of occurrence in G1 β bulges. Since N is found with much more frequency in this position in G1 β bulges in the homologues of ubiquitin (Table 3.2), it was selected to substitute Q2 so that the substitution does not cause any structural alteration in the G1 β bulge in ubiquitin. Hence, substitution Q2N was generated using site directed mutagenesis in order to study the functional relevance of Q2 in ubiquitin.

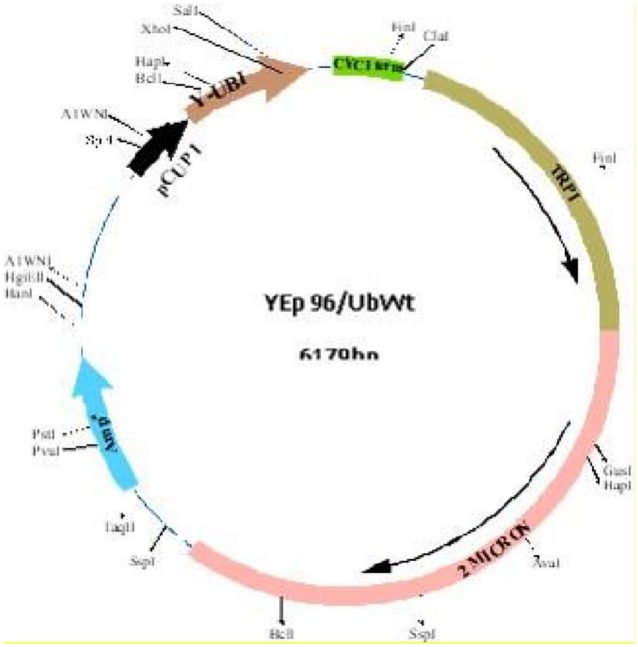
# 3.2 Materials and Methods

### 3.2.1 Yeast Strains and Media

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 and 200 rpm. 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) was added for plating. Additional 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 the requirements of the experiments (Finley et al.,1994). SD is a minimal medium, while YPD is a rich medium.

### 3.2.2 Plasmids

YEp96 (Yeast Expression Plasmid 96) was used to express the genes for wild type and mutant type ubiquitin (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* (Fig. 3.1). This enables it to be used as a shuttle vector. The ubiquitin gene that is cloned in YEp96 is under the control of CUP1, a $CuSO_4$ inducible promoter. YEp96 plasmids in which wild type ubiquitin gene has been cloned is designated as YEp96/UbWt. And YEp96 plasmids in which ubiquitin β bulge mutants have been cloned are designated YEp96/Q2N, YEp96/E64G and YEp96/S65D. As per the standardized protocol of our lab, the cells were overexpressed with 100 mM $CuSO_4$ for shorter durations, and with 25 mM $CuSO_4$ for longer duration, to avoid cytotoxic effects of $CuSO_4$. SUB60 cells transformed with YEp96/UbWt, YEp96/Q2N, YEp96/E64G and YEp96/S65D 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 3.1: Plasmid Yep96 carrying wild type ubiquitin gene under CUP1 promoter.*

### 3.2.3 Antibiotic Sensitivity Test

Antibiotic sensitivity test has been conducted to test the effect of β bulge mutations of ubiquitin on sensitivity of yeast towards translational inhibitors (Hanna et al., 2003). SUB60 cells were transformed with YEp96/UbWt, YEp96/Q2N, YEp96/E64G and YEp96/S65D, and were grown up to OD 0.2 as measured at 600 nm. At this point, three-fold dilutions of cultures were carried out, and each dilution was spotted on SD agar. Concentrations of the antibiotics were G418 50 µg/ml, gentamicin 80 µg/ml, and Hygromycin B 40 µg/ml. Two plates were prepared

for each antibiotic, one without $CuSO_4$, for negative control, and one with 25 µM $CuSO_4$, for induction. A positive control plate with $CuSO_4$ and without antibiotic was also prepared. The growth of the mutants was compared to that of SUB62 and untransformed SUB60 after incubation at 30°C for ten days.

### 3.2.4 K48 linked polyubiquitination assay

SUB60 cells were transformed with YEp96/UbWt, YEp96/Q2N, YEp96/E64G and YEp96/S65D, and cultured in YPD at 30°C, up till OD 0.6 at 600 nm. At this stage, cells were pelleted down by centrifugation, given normal saline washes, and then suspended in normal saline. The OD of 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. The lysate samples, each containing 50 µg of protein, were subjected to SDS-PAGE using 15% sodium dodecyl sulphate polyacrylamide gel.

The gel was then subjected to western blotting using nitrocellulose membrane. Ponceau staining of the blot was carried out to ensure equal protein concentration in all the 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 given 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).

### 3.2.5 Lysosomal Degradation of Uracil Permease

SUB60 cells were transformed with YEp96/UbWt, YEp96/Q2N, YEp96/E64G and YEp96/S65D, and were cultured till log phase. This was followed by treatment with 100 µg/ml

of cycloheximide for 20 minutes to halt the synthesis of uracil permease. Analysis of of uracil permease levels after 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. The protocol for cell lysis and western blot is same as that described in case of K48 linked polyubiquitination assay.

### 3.2.6 Cdc28 Assay

CDK1 is the cyclin dependent kinase 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, that involved antibody specific for Cdc28. SUB60 cells were transformed with YEp96/UbWt, YEp96/Q2N, YEp96/E64G and YEp96/S65D. The protocol for culturing of cells, cell lysis and western blotting is the same as that described in the case of K48 linked polyubiquitination assay.

### 3.2.7 Confocal Microscopy for Endosomal Sorting of GFP-CPS Fusion Protein

SUB60 cells were transformed with YEp96/UbWt, YEp96/Q2N, YEp96/E64G and YEp96/S65D. 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 0.8 as measured at 600 nm. 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 700 g 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).

## 3.3 Results

### 3.3.1 Effect of the β Bulge Mutations on Sensitivity to Translational Inhibitor

The G1 β bulge of ubiquitin is adjacent to the K63 residue, which plays a role in the regulation of protein synthesis. Specifically, a yeast large ribosomal subunit protein named L28 undergoes K63 linked polyubiquitination (Hanna et al., 2003; Spence et al., 2000). And K63R substitution in ubiquitin, which prevents K63 linked polyubiquitination, interestingly makes yeast more sensitive to cycloheximide and hygromycin compared to wild type cells. It also makes yeast more sensitive to anisomycin, G418 and rapamycin compared to wild type cells. Due to its close proximity to the K63 residue, it was hypothesized that the Q2N, E64G and S65D substitutions of the G1 β bulge of ubiquitin also affects the susceptibility of yeast to translational inhibitors.

Interestingly, as mentioned before, previous work in our laboratory showed that like the K63R substitution, the Q2N, E64G and S65D substitutions make yeast more sensitive to cycloheximide than wild type (Mishra et al., 2011). Hence, in this work, the sensitivity of yeast carrying these β bulge mutations has been studied against three more translational inhibitors, namely G418, hygromycin and gentamicin (Fig. 3.2). G418 and hygromycin were chosen because as mentioned before, in the study involving the K63R substitution, yeast carrying K63R substitution was found to have increased sensitivity to G418 and hygromycin. Gentamicin is normally not known to target eukaryotes, as it targets the 70s prokaryotic ribosome. However, a mutation in an E3 ligase named Rsp5 makes yeast sensitive to gentamicin. Hence, in this work the sensitivity of the β bulge substitutions was also tested against gentamicin.

The results of the experiments indicate that while the β bulge mutations do not increase the sensitivity of yeast towards gentamicin and hygromycin, while they do increase its sensitivity towards G418. In the drop test for gentamicin, it can be seen that even untransformed SUB60 cells, which are normally more sensitive to antibiotics than SUB62 cells (carrying all four ubiquitin genes in wild type configuration), and SUB60 cells transformed with YEp96/UbWT, are growing just as well. The result seen here can be expected as gentamicin does not have any negative effect over eukaryotic cells. This shows that the β bulge mutations

have no effect on sensitivity to gentamicin, unlike the Rsp5 mutation that makes yeast sensitive to gentamicin, an antibiotic that otherwise acts on prokaryotes.

On the other hand, untransformed SUB60 cells do not grow as well as SUB62 cells and SUB60 cells transformed with YEp96/UbWT. But SUB60 cells transformed with YEp96/Q2N, YEp96/E64G and YEp96/S65D grow just as well as SUB62 cells and SUB60 cells transformed with YEp96/UbWT in the presence of hygromycin. This shows that while hygromycin affects yeast, the β bulge mutations show complementation, making SUB60 cells carrying these mutations capable of surviving just as well as wild type cells in the presence of 70 µg/ml hygromycin. There are studies where hygromycin was used at 100 µg/ml (Spence et al., 2000), however this being the initial experiment lower concentration of the antibiotic was used as we were dealing with mutant forms of ubiquitin.

From the results it was very clear that the β bulge mutations fail to complement in case of G418. This can be seen in the figure showing results of G418 drop test, in which SUB60 cells transformed with YEp96/Q2N, YEp96/E64G and YEp96/S65D do not grow as well as SUB62 cells and SUB60 cells transformed with YEp96/UbWT. Interestingly, G418 acts on the elongation phase of protein synthesis. This is the same phase acted upon by cycloheximide, to which the β bulge mutants are also more sensitive than wild type cells. On the other hand, hygromycin acts by preventing the translocation of tRNA from A site to P site. This suggests that the G1 β bulge of ubiquitin might have a role to play in the elongation phase.

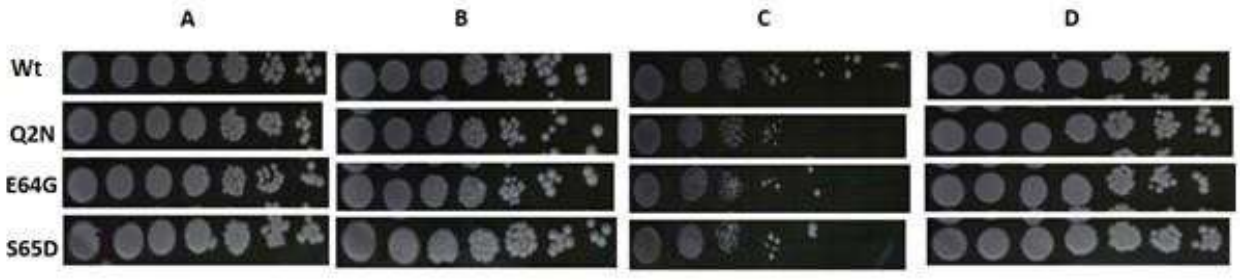



*Figure 3.2: Drop test for testing sensitivity to translational inhibitors, of SUB60 cells transformed with YEp96/UbWt, Yep96/UbQ2N, YEp96/UbE64G, and YEp96/UbS65D in SD medium under induction by (A) 25 µM $CuSO_4$, (B) 70 µg/ml hygromycin B, (C) 40 µg/ml G-418 and (D) 100 µg/ml gentamicin.*

### 3.3.2 Effect of the β Bulge Mutations on K48 Linked Polyubiquitination

The effect of Q2N, E664G and S65D substitutions on K48 linked polyubiquitination was tested through western blot involving antibody specific for the K48 linked polyubiquitin chain. This results in the bands of all the proteins in the lysate sample that are tagged with K48 linked polyubiquitin chains getting stained in the blot, producing a ladder pattern. Results of the blot show that the bands in lysates of SUB60 cells transformed with YEp96/Q2N and YEp96/E64G are markedly less intense than the bands in lysates of SUB60 cells transformed with YEp96/UbWT, although bands in the lysates of cells transformed with YEp96/S65D show intensities similar to the positive control (Fig. 3.3). This indicates that Q2N and E64G substitutions might have a mild affect over the K48 linked polyubiquitination of proteins, and by extension, their proteasomal degradation.

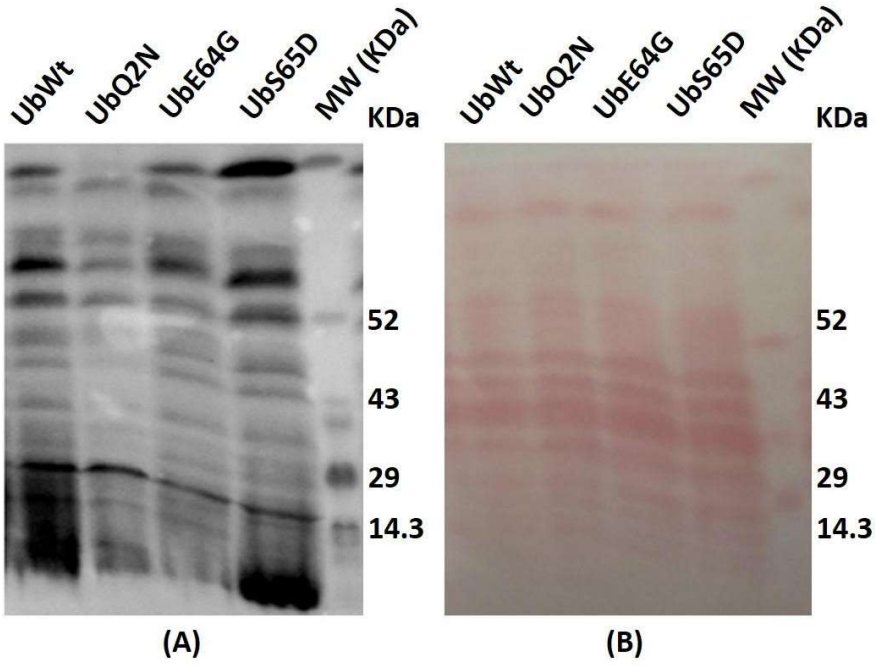



*Figure 3.3: (A) Western blot showing K-48 linked ubiquitination profile of SUB60 transformed with Yep96 plasmids expressing UbWt, UbQ2N, UbE64G and UbS65D. (B) Ponceau stain of the blot.*

### 3.3.3 Effect of the β Bulge Mutations on Lysosomal Degradation of Uracil Permease

While the cytosolic proteins of the cell can be degraded by the proteasome, the same is not true for membrane proteins. Instead, membrane proteins as well as endocytosed extracellular proteins, are degraded by the lysosome. Research established that ubiquitin also has a role to play in the lysosomal degradation of proteins (M.-O. Blondel et al., 2003). Specifically, a single ubiquitin tagged to the membrane protein might be enough to lead it to lysosomal degradation. To test if the β bulge mutations of ubiquitin have an effect on lysosomal degradation, the cell lysates of β bulge mutants and cells expressing wild type ubiquitin were analysed by monitoring uracil permease or Fur4p levels, since Fur4p is a membrane protein that is degraded in the lysosome. Western blotting analysis was carried out using an antibody specific for uracil permease (named Fur4p in yeast), gifted to us by Dr. Marie-Odile Blondel.

Before being lysed, the cells were first treated with cycloheximide for 20 minutes. This was done to inhibit protein synthesis and thus block the further production of uracil permease. After 90 minutes of treatment with cycloheximide, the cells were lysed and the lysates were analysed by western blot. The uracil permease produced by the cells before treatment with cycloheximide would be degraded during the 90-minute incubation period before the lysis of cells. Hence, if the β bulge mutations are inhibitory to the lysosomal degradation of uracil permease, the uracil permease bands in the lysates of β bulge mutants would be more intense than those in lysates of cells expressing wild type ubiquitin. However, results show no significant difference in band intensity, suggesting that the β bulge mutations are not detrimental to the lysosomal degradation of uracil permease (Fig. 3.4).

Interestingly, research also shows that Rsp5, a ubiquitin ligase mentioned above in context of gentamicin sensitivity, is also involved in lysosomal degradation. A previous study (Kwapisz et al., 2005) had found that mutation in Rsp5 makes yeast sensitive to gentamicin, even though gentamicin usually is specific for prokaryotes in its action. The finding in this work that β bulge mutations fail to make yeast sensitive to gentamicin agrees with the finding that they do not affect the lysosomal degradation of uracil permease, which also involves Rsp5. These two findings together suggest that Rsp5 mediated ubiquitination pathway might not be affected by the β bulge mutations.

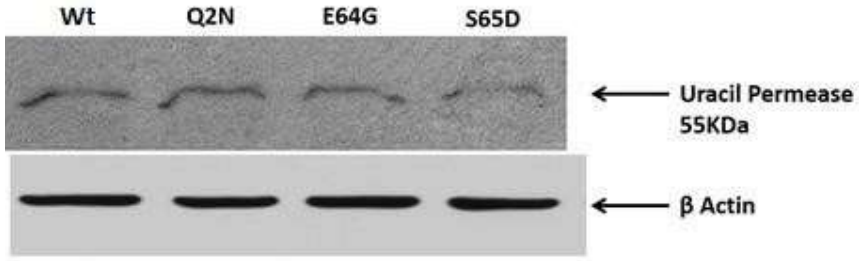



*Figure 3.4: Western blot showing uracil permease and beta actin in SUB60 transformed with Yep96 plasmids expressing UbWT, UbQ2N, UbE64G and UbS65D.*

### 3.3.4 Effect of the β Bulge Mutations on Cdc28 Levels

Cdc28 or CDK1 (cyclin dependent kinase 1) in yeast, a highly conserved serine/ threonine kinase that acts as the master regulator of yeast cell cycle, regulating the activity of other cell cycle regulators, including other kinases, through phosphorylation. These cell cycle regulators, including Cdc28, are known to undergo ubiquitination, allowing the ubiquitin system to regulate cell cycle. To test whether the β bulge mutations affect the levels of cdc28 in yeast, SUB60 cells carrying YEp96/UbWt, YEp96/Q2N, YEp96/E64G and YEp96/S65D were lysed and the lysates were subjected to western blotting using an antibody specific for Cdc28. The results of the blot show that there is no significant difference in band intensities of Cdc28 between lysates of any of the β bulge mutants and lysate of the wild type cells (Fig. 3.5). This suggests that the β bulge mutants do not affect the Cdc28 levels in yeast, and hence do not have an impact on the regulation of the yeast cell cycle.

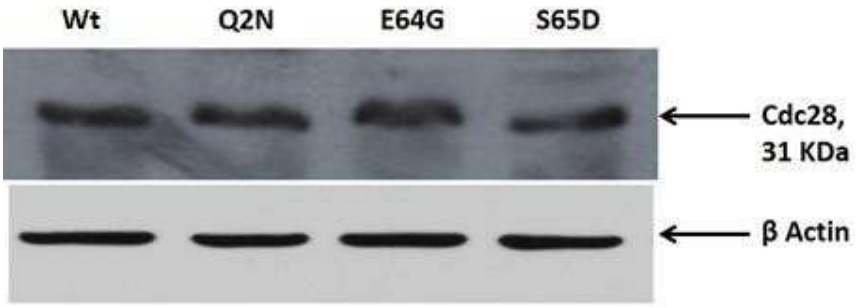



*Figure 3.5: Western blot showing Cdc28 and beta actin in SUB60 transformed with YEp96 plasmids expressing UbWT, UbQ2N, UbE64G and UbS65D.*

### 3.3.5 Effect of the β Bulge Mutations on Endosomal Sorting of GFP-CPS Fusion Protein

As mentioned before in the context of lysosomal degradation of uracil permease, ubiquitin system is involved in the endocytosis of membrane proteins in yeast and their subsequent degradation in the lysosome. Specifically, the Rsp5 ubiquitin ligase is believed to be responsible for the ubiquitination of membrane proteins, leading to their endocytosis, and Doa4p deubiquitinating enzyme is responsible for the deubiquitination of endocytosed membrane proteins prior to their degradation in the lysosome. Prior to degradation in the lysosome, the endosome containing membrane proteins undergoes invagination, producing multiple smaller vesicles within its lumen, all of them containing the endocytosed membrane proteins on their surface. These endosomes are called multivesicular bodies or MVBs (Li and Kane, 2009). MVBs then fuse with the lysosome/vacuole and release the smaller vesicles into it. MVBs are believed to aid in the lysosomal degradation of membrane proteins, as they increase the surface area on which the lysosome can act.

As mentioned before, in this work it was found that the β bulge mutations don't have an impact on the lysosomal degradation of uracil permease. However, it was important to see if the sorting of membrane proteins into lysosome/ vacuole is affected by β bulge mutations. For this, a different membrane protein, carboxypeptidase S (CPS) was chosen. As its name suggests, CPS is a lysosomal protease that degrades or hydrolyses the peptide backbone of substrate proteins from their C terminal end onwards. To study the sorting of CPS into the yeast vacuole or multivesicular body or the yeast equivalent of lysosome (Li and Kane, 2009), a plasmid pGFP-CPS, which is derived from the pGOGFP426 plasmid was used (Emr et al.,

1998). This plasmid expresses a fusion of CPS and GFP (GFP-CPS), enabling it to be observed in the cell through fluorescence microscopy.

SUB60 cells previously transformed with YEp96/UbWt, YEp96/Q2N, YEp96/E64G and YEp96/S65D were transformed with pGFP-CPS, and the GFP fluorescence was used as selection marker. These cells were then treated with FM4-64 dye, which intercalates with the plasma membrane, and then reaches endosomal, lysosomal and vacuolar membranes through endocytosis, and emits red fluorescence. The cells were then fixed and observed under confocal microscope at 100x magnification. The results indicate that the β bulge mutations do not have an impact on the sorting of CPS in the vacuole, as the GFP-CPS fusion protein colocalizes with the vacuolar membrane equally well in the β bulge mutants and wild type cells (Fig. 3.6). Hence, the sorting of proteins into lysosomes is not affected by β bulge mutants of ubiquitin.

When viewed alongside the results of the study of lysosomal degradation of uracil permease, it appears that the β bulge mutants do not affect either the sorting of yeast membrane proteins into the vacuole/ lysosome through endosomes, or their subsequent lysosomal/ vacuolar degradation. However, the Q2N and E64G might cause a general increase in endosomal sorting. This is because while there is no difference in overlapping of red fluorescence of FM4-64 dye and the green fluorescence of GFP-CPS between Q2N, E64G and wild type cells, the intensity of both the red and green fluorescence is higher in Q2N and E64G cells than in wild type cells. But the molecular mechanism behind this is as yet unclear, and further investigation is needed to test this hypothesis.

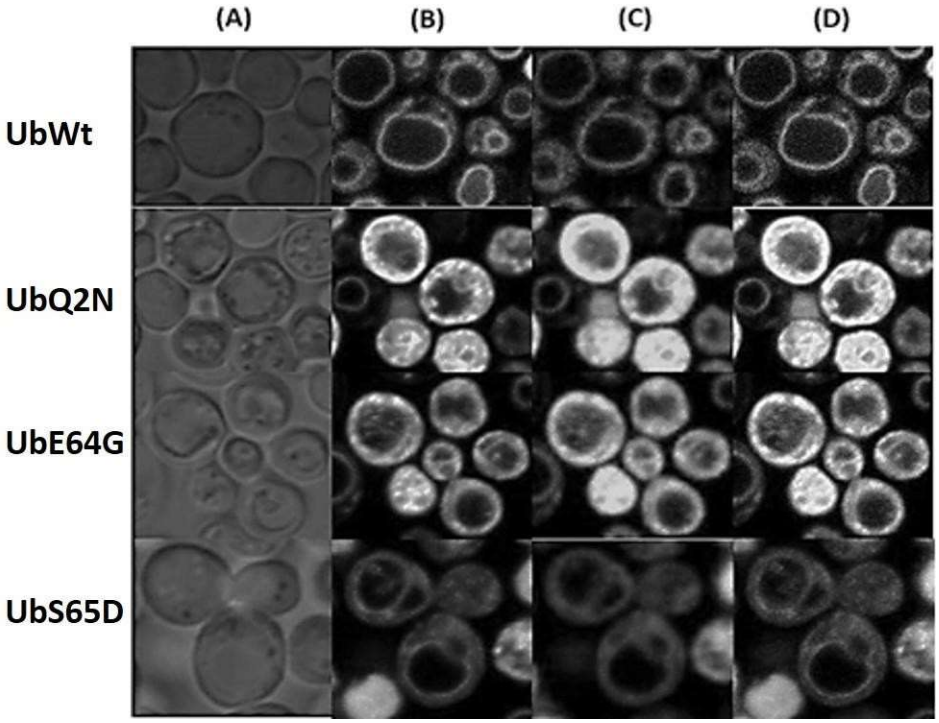



*Figure 3.6: Confocal microscopy of SUB60 cells transformed with YEp96 expressing wild type ubiquitin and the β bulge mutants. (A) bright field, (B) GFP filter (GFP), (C) RFP filter (FM4-64), (D) merging of (B) and (C) for observing colocalization.*

## 3.4 Discussion

Previous studies on the Q2N, E64G and S65D substitutions in our laboratory involved studying their impact on the structural and some functions of ubiquitin (Mishra et al., 2009; 2011). The structural characterisation in these studies involved fluorescence emission spectroscopy, far UV CD spectroscopy, and guanidine hydrochloride induced denaturation. The results of these studies showed that the β bulge mutations do not have an impact on the structural integrity of ubiquitin. This left only one possible reason behind the very high sequence conservation of the G1 β bulge in ubiquitin, despite it involving unfavourable residues. And this was that the β bulge residues are involved in some aspect of the functioning of ubiquitin. To probe the role of β bulge residues in functional aspects of ubiquitin, the impact of β bulge mutations on the growth curve of yeast, survival of yeast under heat stress, and sensitivity to cycloheximide were studied in our laboratory. Results showed that while the β

bulge mutations do not have an impact on the growth curve of yeast or its survival under heat stress.

The β bulge mutations do, however, make yeast more sensitive to cycloheximide. Since cycloheximide is a translational inhibitor, this suggests that the β bulge residues are involved in the regulation of protein synthesis. This is further lent credence by the fact that K63, the residue involved in K63 linked polyubiquitination, is adjacent to the G1 β bulge, and the finding that the L28 protein of the large subunit of yeast ribosome undergoes K63 linked polyubiquitination.

Further, K63R substitution, which prevents K63 linked polyubiquitination, makes yeast more sensitive to translational inhibitors G418, anisomycin and rapamycin. Based on these observations, in this work, the sensitivity of β bulge mutants towards translational inhibitors G418, hygromycin and gentamicin have been tested. Results of these experiments show that the β mutations make yeast more sensitive to G418, but not to gentamicin and hygromycin used at lower concentration, compared to wild type cells. G418 and cycloheximide both act on the same phase of protein synthesis, namely elongation phase, and both of them are more detrimental to the β bulge mutants than the wild type cells. Hygromycin on the other hand, acts by preventing the translocation of tRNA from A site to P site. Gentamicin acts by increasing the error rate of translation, or in other words, by stabilizing incorrect base pairing between codon and anticodon. And gentamicin too is not harmful to β bulge mutants, similar to wild type cells. These observations suggest confirm that the G1 β bulge residues in preventing the ubiquitination of ribosomal subunit and thereby producing a negative effect over protein synthesis in the presence of antibiotics. Interestingly, while the β bulge mutations make yeast more sensitive to both cycloheximide and G418 and produce an effect similar to the K63R mutation of ubiquitin (Spence et al., 2000). These observations suggest that the role of β bulge residues of ubiquitin have a direct influence over K63 linked polyubiquitination.

Gentamicin is a translational inhibitor that is known to act on prokaryotes and not on eukaryotes. Specifically, when there is correct codon anticodon base pairing, adenosines 1492 and 1493 of the 16S r RNA 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 r RNA, 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, the yeast cells carrying the β bulge mutations were tested to see if the β bulge mutations also make yeast sensitive to gentamicin like the Rsp5 mutation does. The results show that there is no increase in sensitivity of β bulge 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 β bulge mutations.

The Rsp5 ubiquitin ligase is involved in the process of endocytosis and sorting of membrane proteins into vacuole/lysosome. β bulge mutations fail to make yeast sensitive to gentamicin, like the way mutation in Rsp5 does, and Rsp5 is involved in endocytosis and endosomal sorting. Given this observation, it is not surprising that β bulge mutations do not have an impact, either on endocytosis or on sorting of membrane proteins into vacuole/lysosome, as observed in the case of carboxypeptidase S, or on the lysosomal/vacuolar degradation of these membrane proteins, as observed in the case of uracil permease.

Results of western blot involving antibody specific for K48 linked polyubiquitin chain show that Q2N and E64G substitutions have some negative effect on the K48 linked polyubiquitination, but the effect of S65D substitution does not have any influence over polyubiquitination. This is interesting, as the β bulge does not lie in the vicinity of the K48 residue, and yet K48 linked polyubiquitination seems to be affected by Q2N and E64G substitutions. However, as per previous studies in our laboratory, none of the three β bulge substitutions have an impact on the growth characteristics of yeast, or its survival under heat stress, in which misfolded and denatured proteins needing to be proteasomally degraded are produced in large amounts. It is known that polyubiquitination with K29 linkage and other linkages can mark proteins for degradation similarly to K48. Hence, the effect of the Q2N and E64G substitutions on K48 linked polyubiquitination might not be too detrimental.