Structural and Functional Consequences of Alteration in Ubiquitin

THESIS SUBMITTED TO

THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BIOCHEMISTRY

By

Mrinal Sharma

DEPARTMENT OF BIOCHEMISTRY

FACULTY OF SCIENCE

THE M. S. UNIVERSITY OF BARODA

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STATEMENT UNDER O. Ph. D 8/ (iii) OF THE M.S. UNIVERSITY OF BARODA, VADODARA.

This is to certify that the work presented in this thesis is original and was obtained from the studies undertaken by me under the supervision of **Dr. C. Ratna Prabha.**

Baroda **Mrinal Sharma**

Date: 30-10-2012

This is to certify that the work presented in the form of a thesis in fulfillment of the requirement for the award of the Ph.D. degree of The M.S. University of Baroda by **Mr. Mrinal Sharma** is his original work. The entire research work and the thesis have been built up under my supervision.

Dr. C. Ratna Prabha
Research supervisor
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This thesis is dedicated to my late father and my mother for their love, endless support and blessings and to my nephew Prit and niece Gudiya.

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I would like to end the acknowledgement by stating a quote from a renowned scientist.....

"the more I study science, the more I believe in God"

Albert Einstein

Mrinal Sharma

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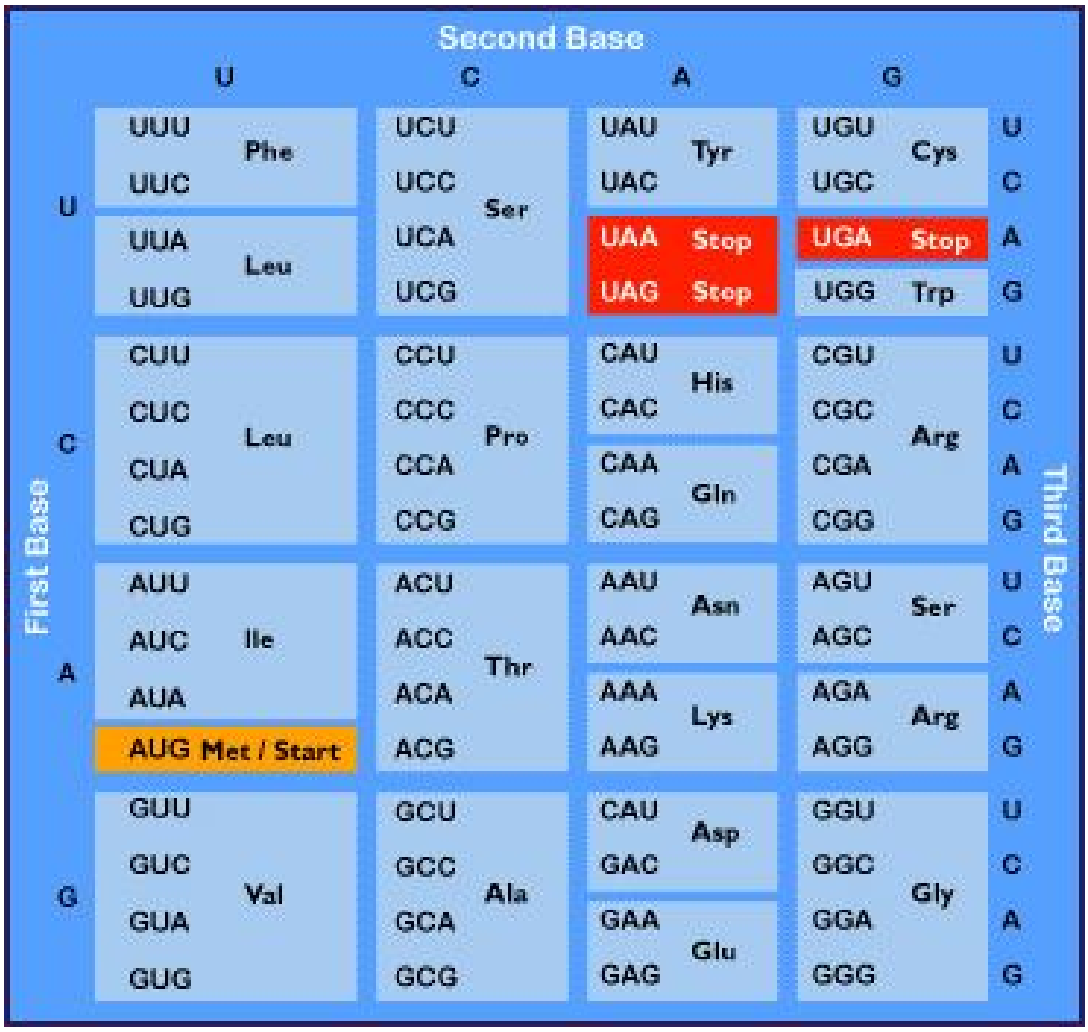
List of abbreviations:

Ub	Ubiquitin
UPS	Ubiquitin Proteasome System
SUMO	Small ubiquitin like modifier
CD	Circular dichroism
SELCON	Self consistent
SMP	Soluble + Membrane Protein
GdHCl	Guanidine hydrochloride
nm	nanometer
kDa	Kilodalton
SD	Selective media
LB	Luria Broth
IPTG	Isopropyl thio-ß -D-galactoside
PMSF	Pheny lmethyl sulfonyl fluoride
SDS	Sodium Dodecyl Sulphate
EDTA	Ethylene diamine tetra acetic acid
PEI	Polyetylene imine
IMAC	Immobilized metal affinity chromatography
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
SDM	Site Directed Mutagenesis

Codon Chart


Second Base
U
C
A
G
First Base
Third Base
UUU Phe
UUC
UUA Leu
UUG
UCU Ser
UCC
UCA
UCG
UAU Tyr
UAC
UAA Stop
UAG Stop
UGU Cys
UGC
UGA Stop
UGG Trp
CUU Leu
CUC
CUA
CUG
CCU Pro
CCC
CCA
CCG
CAU His
CAC
CAA Gln
CAG
CGU Arg
CGC
CGA
CGG
AUU Ile
AUC
AUA
AUG Met / Start
ACU Thr
ACC
ACA
ACG
AAU Asn
AAC
AAA Lys
AAG
AGU Ser
AGC
AGA Arg
AGG
GUU Val
GUC
GUA
GUG
GCU Ala
GCC
GCA
GCG
CAU Asp
GAC
GAA Glu
GAG
GGU Gly
GGC
GGA
GGG

UBIQUITIN GENE SEQUENCE

[YEAST SYNTHETIC]

Bgl II Hpa I

1 ATG CAG ATC TTC GTC AAG ACG TTA ACC GGT
30

Met Gln Ile Phe Val Lys Thr Leu Thr Gly
1 5 10

Xbal I

31 AAA ACC ATA ACT CTA GAA GTT GAA TCT TCC
60

Lys Thr Ile Thr Leu Glu Val Glu Ser Ser
11 15 20

61 GAT ACC ATC GAC AAC GTT AAG TCG AAA ATT
90

Asp Thr Ile Asp Asn Val Lys Ser Lys Ile
21 25 30

Bsm I

91 CAA GAC AAG GAA GGC ATT CCA CCT GAT CAA
120

Gln Asp Lys Glu Gly Ile Pro Pro Asp Gln
31 35 40

Xho I

121 CAA AGA TTG ATC TTT GCC GGT AAG CAG CTC
150

Gln Arg Leu Ile Phe Ala Gly Lys Gln Leu
41 45 50

Xho I

151 GAG GAC GGT AGA ACG CTG TCT GAT TAC AAC
180

Glu Asp Gly Arg Thr leu Ser Asp Tyr Asn
51 55 60

Sal I Afl II

181 ATT CAG AAG GAG TCG ACC TTA CAT CTT GTC
210

Ile Gln Lys Glu Ser Thr Leu His Leu Val
61 65 70

Afl II

211 TTA AGA CTA AGA GGT GGT TGA
231

Leu Arg Leu Arg Gly Gly End
71 75 76

CHAPTER 1

Introduction

1. INTRODUCTION

A thorough balance of protein synthesis and degradation is prerequisite for survival of any cell. Degradation of misfolded, damaged, or otherwise malfunctioning proteins is certainly of utmost significance for maintaining cellular homeostasis. Degradation of cellular proteins is a highly complex, controlled and tightly regulated process that plays major roles in a variety of basic cellular pathways during both cell life and cell death (Balch et al., 2008).

Ubiquitin is used by cells as a tag covalently attached to proteins in a polymeric form and marks them for degradation (Ciechanover et al., 1980). Ubiquitin along with the enzymes catalyzing ubiquitination, namely ubiquitin activating enzyme E1, ubiquitin conjugating enzyme E2, ubiquitin ligase E3 and the multi-subunit protease complex proteasome, which act in succession constitute the ubiquitin proteasome system (Varshavsky, 1997; Weissmann, 1997). The ubiquitin proteasome system regulates removal of many intracellular proteins, including those which control cell cycle progression, apoptosis, signal transduction and induction of the inflammatory response.

Subsequently many functions of ubiquitin other than its role in proteasomal degradation have been discovered, which include DNA repair, regulation of transcription and translation, cell signaling, endocytosis and autophagy. Hence, the role of ubiquitin in posttranslational modification in addition to the one in protein degrading system is gaining importance. Ubiquitination can therefore be compared to other post-translational modifications like phosphorylation, methylation and acetylation.

The essential components of ubiquitin structure, ubiquitination system along with its functions and UPS are described in the initial part of introduction. Failure of

UPS, different diseases associated with it and targeting UPS for drug design are highlighted in the later part of introduction.

1.1. The structure of ubiquitin

Ubiquitin is a small, globular and single domain protein. It is made up of 76 amino acid residues and has a molecular weight of 8565 Da. It also does not contain any cofactors. Because of these properties, it has been a good model system for the study of protein folding and stability. Its 3D structure was first determined using X ray crystallography (Vijay-Kumar et al., 1987; Vijay-Kumar et al., 1985). It contains five strands of β-sheet harbouring two parallel and three antiparallel strands, 3.5 turns of α helix and 3_{10} helix characterized by a particular fold denoted as SSHSSS (Fig. 1.1). The compact structure of ubiquitin has nine reverse turns and two β-bulges residing at N and C terminus of the protein (Vijay-Kumar et al., 1987; Vijay-Kumar et al., 1985).

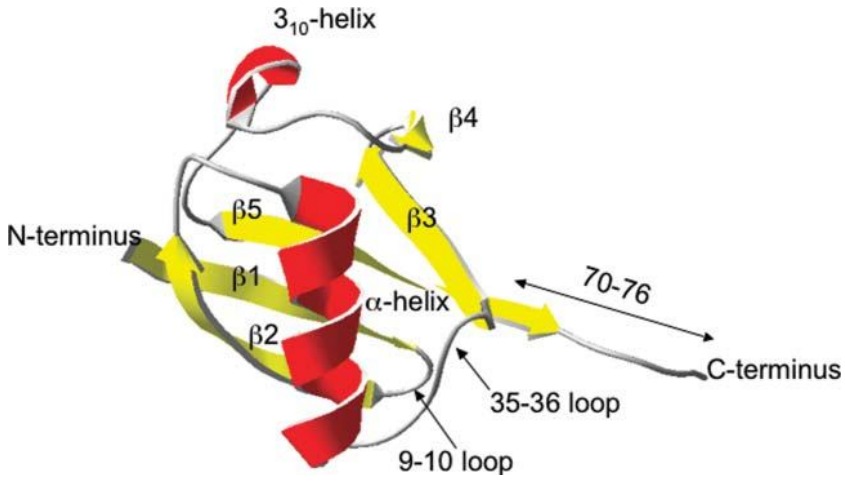


Fig. 1.1. Secondary and tertiary structure of ubiquitin from the 1.8 A° resolution. Shown in yellow are the five β-strands that make up the mixed parallel–anti-parallel β-sheet, shown in red are the major a-helix and the 3_{10}helix (Jackson, 2006).

The structure was found to be tightly packed and globular with β-grasp fold, in which mixed β-sheets pack against an α-helix to form highly stable hydrophobic core.

Due to this, ubiquitin is found to be stable from pH 1.2 to 8.5 and temperature from 23° to 80°C (Lenkinski et al., 1977). Its C- terminal end comprising LRLRGG amino acid residues protrudes from the core to interact with ubiquitin itself and other proteins.

1.1.1. β-bulges in ubiquitin

β-bulges are regions in a β sheet where two residues in one β strand form branched hydrogen bonds with a single residue of a second strand (Milner-White, 1987). The extra residues bulge out from the β strand, giving it the name β-bulge (Richardson et al., 1978). The two residues bulging out on the first strand are labelled "1" and "2", and the residue on the other strand is labelled "X" (Fig. 1.2).

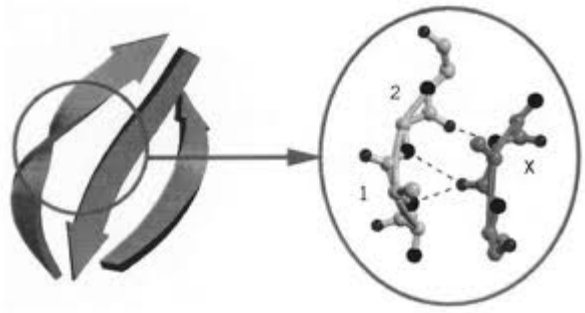


Fig. 1.2. Arrangement of β-bulge where one β-strand bulges out from the register (Axe et al., 1999)

Bulges are divided on the basis of conformation of the residues involved in the structure and the type of amino acid they have inside the bulge. The β-bulges are classified into five types, namely classic, G1, wide, bent and special types. Classic and wide include both parallel and antiparallel β bulges and G1 bulges are mostly antiparallel. G1 bulges are further classified into: (1) G1G type with glycine at position first of the bulge, (2) G1T type with glycine at first position and a Type I′ or Type II β turn between position 2 and any other residue, with glycine at position i+2 of turn (Verma et al., 2002). G1A type has any amino acid (excluding glycine) at first position (Chan et al., 1993).

G1 type β-bulge displays glycine in position 1and it has φ, ψ values of 85°, 0° which is favourable only for glycine (Vijay-Kumar et al., 1987) . Position 2 of G1 bulge is within the usual β region, but centred on φ = -90°, ψ = 150°. Gl type bulges are found within an interlocking structure in which the glycine in position 1 of the G1 bulge is also the required glycine in position 3 of a type-II tight turn (Vijay-Kumar et al., 1987).

Ubiquitin contains two β-bulges. The first G1 β-bulge is present at the N-terminal end of the protein on two antiparallel β-strands and involves Gly10 (1), Lys11 (2) and Thr 7 (N). It is present along with Type I turn. Two peptides covering the first β-bulge of ubiquitin were found to fold into β-hairpin conformation autonomously in aqueous solvent and also in methanol (Cox et al., 1993; Zerella et al., 1999) establishing the potential of first β-bulge of ubiquitin to act as an initiation site for its folding. Elimination of Gly from this turn results in loss of structure (Chen et al., 2001).(Cox et al., 1993)

Importance of strand-strand interaction in hairpin formation has been investigated by mutating Thr at 9^{th} position to Asp. The results underscored the role of charge-charge interaction in the stability of ubiquitin (Zerella et al., 2000).

The second G1 β-bulge is present between the C-Terminal end and the N-terminal region of protein on two parallel β-strands and involves Glu64 (1), Ser65 (2) and Gln2 (N). It is present along with a type II turn. Glu64 is the third and first residue in a type II turn and in parallel β-bulge respectively (Vijay-Kumar et al., 1987). Presence of Glu in 64^{th} position is unusual with Φ and Ψ angles which are not under the allowed region of Glu in Ramchandran plot. Generally the first residue in a parallel β bulge is a Gly (Chan et al., 1993). The reason for which nature selected Glu instead of Gly and conserved through evolution has been answered by replacing Glu at 64^{th} position by more preferred Gly. Replacement caused less helicity and increased

stability in the structure of ubiquitin (Mishra et al., 2009). Two more residue of second β bulge were also mutated to more preferred amino acid residues, which exhibited minor alteration in the structure and significantly affected the functionality in vivo (Mishra et al., 2011). Ile61 has been reported to show protection early on in kinetic refolding experiments (Briggs and Roder, 1992) and Lys63 has been reported to play an important role in DNA repair mechanism (Spence et al., 1995).

1.2. Stability of ubiquitin

Ubiquitin's valine, leucine, isoleucine, and methionine residues are buried in the hydrophobic core of the molecule, contributing to the extreme stability of ubiquitin (Thomas and Makhatadze, 2000). Site directed mutagenesis is successful in elucidating functions of many residues contributing to the stability of ubiquitin (Loladze et al., 2001). Makhatadze et al., in 2003 showed different salts increase the thermostability of ubiquitin through anion binding.

Amino acid replacement studies involving lysine to arginine substitutions have established that surface charges do not play a role in making ubiquitin stable (Makhatadze et al., 2003).

1.3. Folding of ubiquitin

A protein always folds so that it achieves the lowest possible energy (Anfinsen, 1973; Hagen, 2007). Theoretically a chain of 100 amino acids will take 10^{40} years to attain a thermodynamically stable native state if it has to run through all possible stable conformations one after another (Levinthal, 1968). Protein folding is often described by folding funnels and energy landscapes (Bryngelson and Wolynes, 1987; Dill, 1985). In contrast, protein folding in vivo takes several seconds to a few minutes. The rate and efficiency of folding of a protein in vivo can be attributed to assistance

of chaperones, post-translational modifications and various inter and intramolecular interactions.

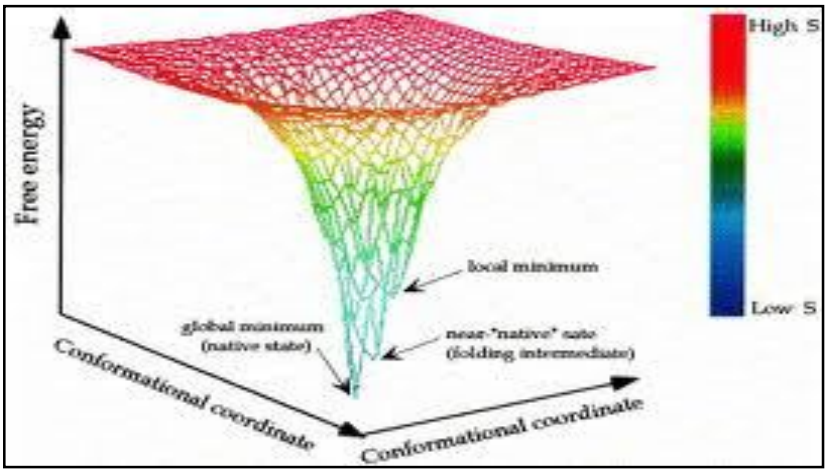


Fig. 1.3. Folding funnel representing various state of energy level (Se¨rgio T. Ferreira. and Felice., 2001)

Several models have been proposed to explain the process of protein folding, such as the Framework model (Karplus and Weaver, 1976) the diffusion collision model (Kim and Baldwin, 1982) and the hydrophobic collapse model (Chan and Dill, 1990).

Folding studies of ubiquitin have indicated that the N-terminal β-sheet and α-helix are formed early. Even though the C-terminal half of the protein shows slow folding kitetics, residues 59, 61, and the beginning of a 3_{10}-helix also exhibit protection in the early phase (Briggs and Roder, 1992). Studies on partially folded state of ubiquitin revealed formation of native secondary structural elements in the N-terminal half (Ub 1-21 and Ub 1-35). The C-terminal half (Ub 36-76) which has β strand character undergoes a transition to helical state (Brutscher et al., 1997; Harding et al., 1991; Stockman et al., 1993)

Current model for folding of ubiquitin proposes that the N-terminal portion of the protein (spanning residues 1-35) serves as an autonomously folding chassis,

governing the folding of rest of the protein through tertiary interactions (Zerella et al., 1999).

1.4. Ubiquitin – The highly conserved protein (Sequence Homology)

Among eukaryotes, the sequence ubiquitin protein is highly conserved (Gavilanes et al., 1982; Schlesinger and Goldstein, 1975; Watson et al., 1978). This can be due to its central role in the cellular metabolism. In the sequences of ubiquitin of yeast, human and oat (Vierstra, 1986) replacement of amino acids is seen in only three places. The sequences of yeast, plant and human ubiquitins are shown Fig. 1.4, highlighting the positions of the residues where there are differences.

YEAST UBIQUITIN PROTEIN SEQUENCE

MQIFVKTLTG KTITLEVE**S**S DTI**D**NVK**S**KI QDKEGIPPDQ QRLIFAGKQL
1 10 20 30 40 50
EDGRTLSDYN IQKESTLHLV LRLRGG
51 60 70 76

PLANT UBIQUITIN PROTEIN SEQUENCE

MQIFVKTLTG KTITLEVE**S**S DTI**D**NVK**A**KI QDKEGIPPDQ QRLIFAGKQL
1 10 20 30 40 50
EDGRTL**A**DYN IQKESTLHLV LRLRGG
51 60 70 76

HUMAN UBIQUITIN PROTEIN SEQUENCE

MQIFVKTLTG KTITLEVE**P**S DTI**E**NVK**A**KI QDKEGIPPDQ QRLIFAGKQL
1 10 20 30 40 50
EDGRTL**S**DYN IQKESTLHLV LRLRGG
51 60 70 76

Fig. 1.4. The amino acid sequences of yeast, plant and human ubiquitins. The residues which were changed have been highlighted

1.5. Ubiquitin gene family

In *Sacchromyces cerevisiae*, ubiquitin is expressed from four different genes namely *UBI1*, *UBI2*, *UBI3* and *UBI4*, as a natural translational fusion to either ubiquitin or other proteins. Ubiquitins encoded by *UBI1*, *UBI2* and *UBI3* are C-terminally fused to ribosomal subunit L40 and S31 respectively (Finley et al., 1987). *UBI4* is expressed as polymeric head to tail fusion containing five ubiquitin repeats (polyubiquitin). Ubiquitin C-terminal hydrolase cleaves the fusion proteins at the C-terminal end of ubiquitin, liberating free ribosomal proteins and individual ubiquitin monomers. During exponential phase all four ubiquitin genes are expressed while during stationary phase the expression of *UBI1* and *UBI2* is suppressed and expression of *UBI4* gene increases (Fig. 1.5). Polyubiquitin gene UBI4 is an important component of the stress response system and it helps the cell to cope up with stress. The 'heat shock box' element upstream of the coding region senses adverse conditions like various stresses including heat stress, nutritional stress (Ozkaynak et al., 1987), UV stress and antibiotic stress (Finley et al., 1994). The essential function of UBI4 is to provide more ubiquitin during stress conditions. Null phenotype of ubiquitin is lethal but *S. cerevisiae* can survive if the polyubiquitin gene *UBI4* is deleted from the system. The *UBI4* deletion mutant of *S. cerevisiae* becomes hypersensitive to stress conditions (Finley et al., 1987).

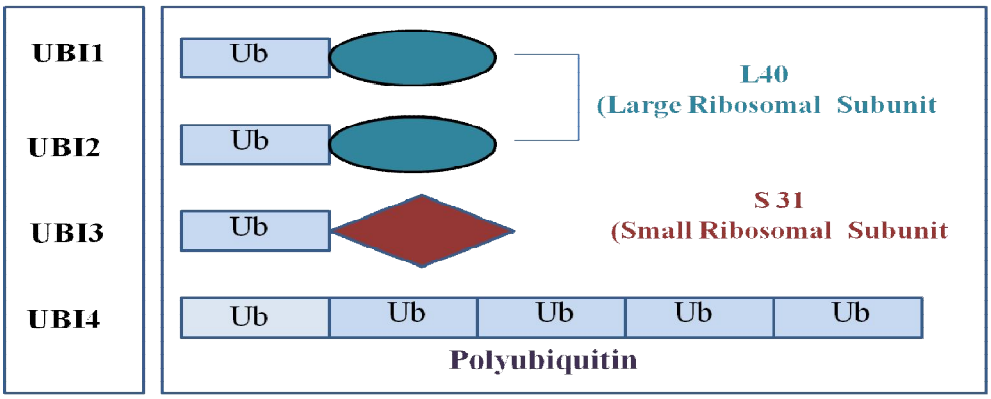
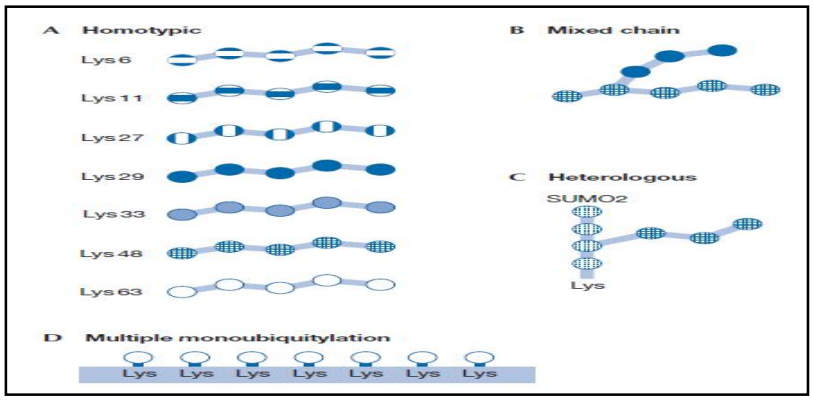


Fig. 1.5. Three ubiquitin precursors in *S. cerevisiae* ubiquitin fused to L40, S31 and itself (Finley et al., 1987)

1.6. Ubiquitin lysine linkage – The Molecular Signal

There are seven lysine residues in ubiquitin, namely K6, K11, K27, K29, K33, K48 and K63, all of which participate in ubiquitination (Johnson et al., 1995; Kim et al., 2007; Peng et al., 2003). Ub chains that form through Lys 48 or K48 serve as a tag for degradation of substrate protein (Hershko and Ciechanover, 1998). Different Ub linkages can be formed through Lys 6, Lys 11, Lys 27, Lys 29, Lys 33 and Lys 63 leading to a variety of conformations of Ub chain, which function as different molecular signals in the cell. Depending on the type of linkages Ub chains have been divided into several classes (Fig. 1.6). Ubiquitin with mixed linkages were also identified in a low proportion compared with conjugated mono Ub or different types of homotypic chains (Peng et al., 2003).

(I)



(Francisca E. Reyes Turcu. et al.)

Fig. 1.6. Polyubiquitin chains displaying different kinds of linkages

(I) shows presence of different lysine residues in ubiquitin, **(Francisca E. Reyes Turcu. et al.)** shows different classes of uniquitination (A) homotypic, showing only one kind of linkage (B) Mixed, showing multiple types of linkages (C) Heterologous,

involving ubiquitin and ubiquitin like proteins and (D) showing multiple monoubiquitinations (Fumiyo Ikeda and Dikic., 2008).

1.7. Role of different lysine linkages inside the cell

Lys48 linked chains are the mainly responsible for delivering proteins to proteasome and K48R mutation is lethal in yeast (Chau et al., 1989; Finley et al., 1994; Hochstrasser et al., 1991). Lys63 linked chains participates in DNA repair (Spence et al., 1995; Ulrich, 2002), the inflammatory response (Sun and Chen, 2004), protein trafficking (Hicke and Dunn, 2003) and ribosomal protein synthesis (Spence et al., 1995). Lys11 linked chains can signal proteasome degradation in vitro (Baboshina and Haas, 1996). Lys6 linked chains shows conjugation to tumor suppressor protein BRCA1 implicated in the pathogenesis of breast and ovarian cancer (Nishikawa et al., 2004). The functions of polyubiquitin chains are not fully understood (Kim et al., 2007).

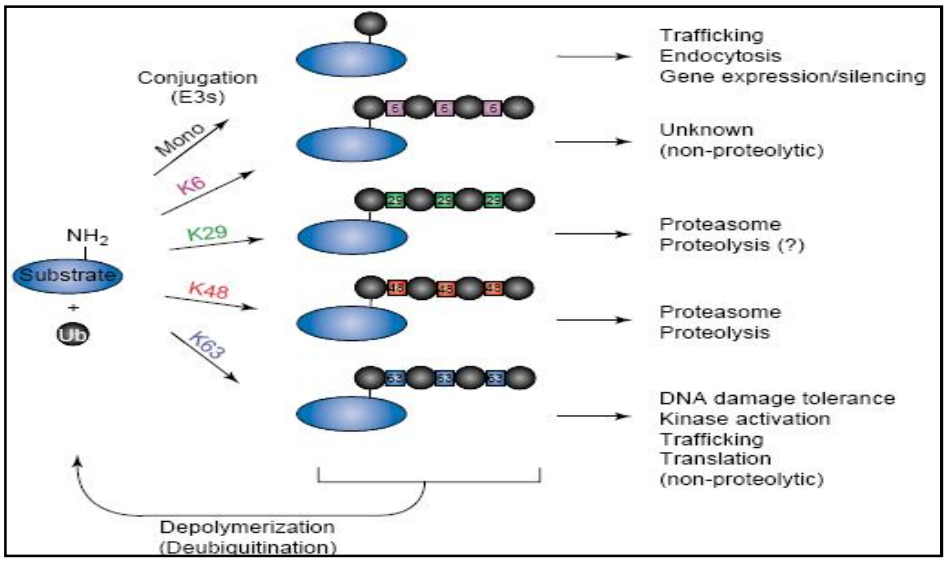


Fig. 1.7. Polyubiquitin chains through different ubiquitin lysine residues (Pickart and Fushman, 2004)

1.8. Ubiquitin Surface residues

Surface residues of ubiquitin that have been found to be essential for vegetative growth of yeast are concentrated in a hydrophobic patch containing L8, I44 and V70, a patch containing F4 and surrounding residues, and the C terminal tail region of ubiquitin (Fig. 1.8). While the I44 patch has been implicated in proteasomal degradation and endocytosis (Beal et al., 1996; Shih et al., 2000), F4 patch has been implicated in endocytosis but not in proteasomal degradation, and C terminal tail is involved in most functions of ubiquitin, because it covalently links ubiquitin to substrate proteins (Katherine E. et al., 2001).

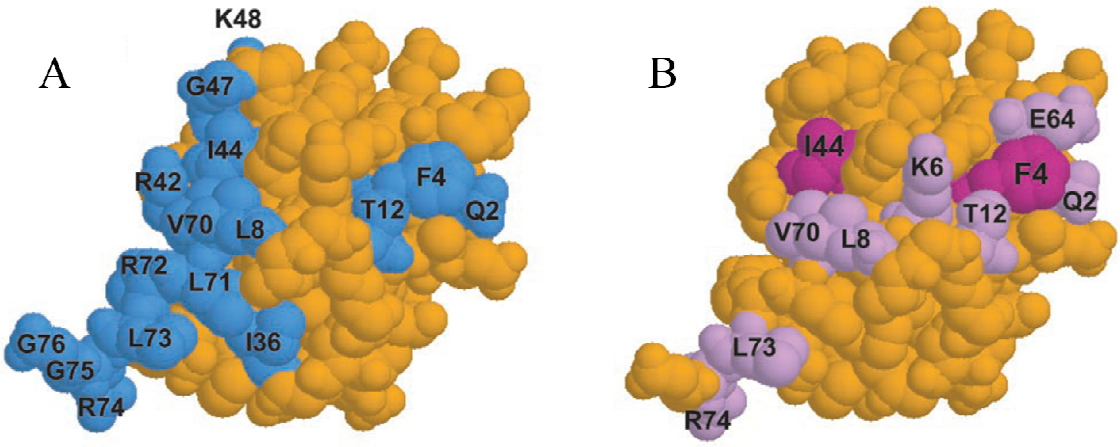


Fig. 1.8. Ubiquitin surface residues

(A) ubiquitin residues required for life in yeast. Essential ubiquitin amino acids are shown in *blue*. All essential residues are visible; none lie on the hidden surface. (B) Internalization information carried by the ubiquitin molecule. Primary residues required for internalization, Phe4 and Ile44, are shown in *magenta*; residues that play a minor role in endocytosis are shown in *pink* (Katherine E. et al., 2001).

1.9. Ubiquitin Binding Domains

Ubiquitin-binding domains (UBDs) recognise different parts of ubiquitin, and hence form an important part of proteins that interact with ubiquitin, and may be

responsible for high sequence conservation in ubiquitin (Hicke et al., 2005). UBDs are structurally diverse and have different biological functions.

The first ubiquitin-binding protein to be characterized was S5A/RPN10 subunit of the proteasome (Young et al., 1998). Nine UBDs have been characterised up to now (Wang et al., 2003). Mechanisms of action of UBDs are not clearly known, but their properties hint at their way of functioning *in vivo.*

1.10. Ubiquitin Proteasome System – Key Players of UPS

1.10.1. Ubiquitin

Ubiquitin is found ubiquitously among eukaryotes across species. The ubiquitination machinery uses ubiquitin as a post-translational modifier by covalently linking a lysine on target protein with the C terminal glycine of ubiquitin. C-terminal end of ubiquitin is activated by E1 enzyme as a preparation towards ubiquitination process.

1.10.1.1. Enzymes of Ubiquitination process

Ubiquitination serves as a molecular signal on target proteins for the execution of specified role. If single ubiquitin is attached to target protein, it is called **monoubiquitination** (Di Fiore et al., 2003; Haglund and Dikic, 2005; Hicke, 2001). If more than one ubiquitin is attached to the same protein at different places, it is called **multiubiquitination,** and if ubiquitin linked to substrate protein is further ubiquitinated, leading to the formation of polyubiquitin chains, it is called **polyubiquitination** (Haglund and Dikic, 2005; Welchman et al., 2005).

1.10.2. E1 (Ubiquitin activating enzyme)

E1 is a monomer of 110 kDa and its sequence has a weakly conserved 2 fold repeat (McGrath et al., 1991). It contains six domains which include adenylation domain, catalytic cysteine domain, four helix bundle and ubiquitin fold domain. All of them pack together to generate large canyon which at one end recruits a ubiquitin molecule.

E1 catalyzes the first step in the ubiquitination process of a target protein by activating C-terminal glycine of ubiquitin. This step requires Mg^{+2}, ATP and ubiquitin. Ubiquitin is converted to adenylate intitially, and subsequently transferred to the active site cysteine in the E1 (Haas and Rose, 1982). The thiol-linked ubiquitin is transferred to the next enzyme in the cascade, the E2 (Fig. 1.9).

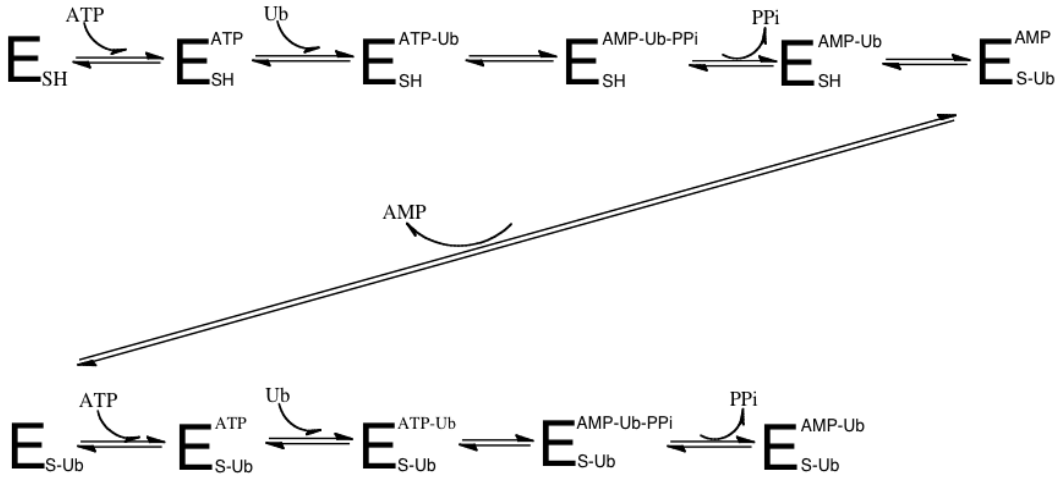


Fig. 1.9. The sequence of how the ubiquitin activating enzyme attaches to ATP and the ubiquitin substrate. It also shows how two ubiquitin substrates can be bound at one time (Haas and Rose, 1982).

1.10.3. E2 (ubiquitin conjugating enzyme)

E1 transfers ubiquitin to E2 by covalently linking C terminal glycine of the ubiquitin to a cystiene in E2. The diversity of E2s is much more compared to one or two E1s present in a cell.

The E2 enzymes have a 150 amino acids long core domain that is composed of four standard α helices, a four stranded antiparallel β sheet, and one 3_{10} helix, and is conserved across all E2s (Tyers and Jorgensen, 2000). Some of the residues surrounding the active site cysteine are very highly conserved (Cook et al., 1993; Jiang and Basavappa, 1999; Worthylake et al., 1998). In addition to the core domain, N or C terminal extensions are found in some, though not all E2s. It is believed that these extensions are responsible for interactions of these E2s with various E3s.

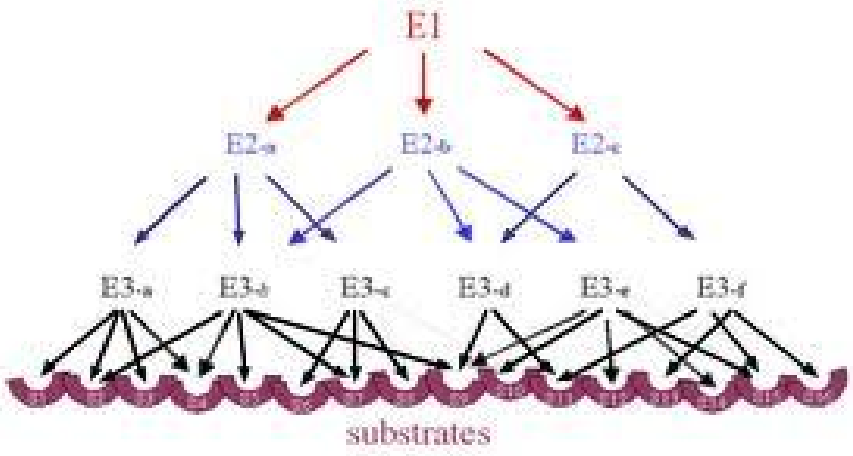


Fig. 1.10. Variety and substrate specificity of enzymes involved in ubiquitination process

1.10.4. E3 ubiquitin Ligase

Next enzyme in the ubiquitin proteasome cascade is E3 also known as ubiquitin ligase. E3s carry out the final step in ubiquitination, i.e. covalently linking C terminal glycine of ubiquitin taken from an E2, to a lysine on the substrate protein to

be ubiquitinated. E3 ligases are the most abundant and diverse proteins in a cell and are encoded by several hundred genes (Deshaies RJ, 2009). This diversity facilitates selective ubiquitination of proteins, consequently regulating cellular activities.

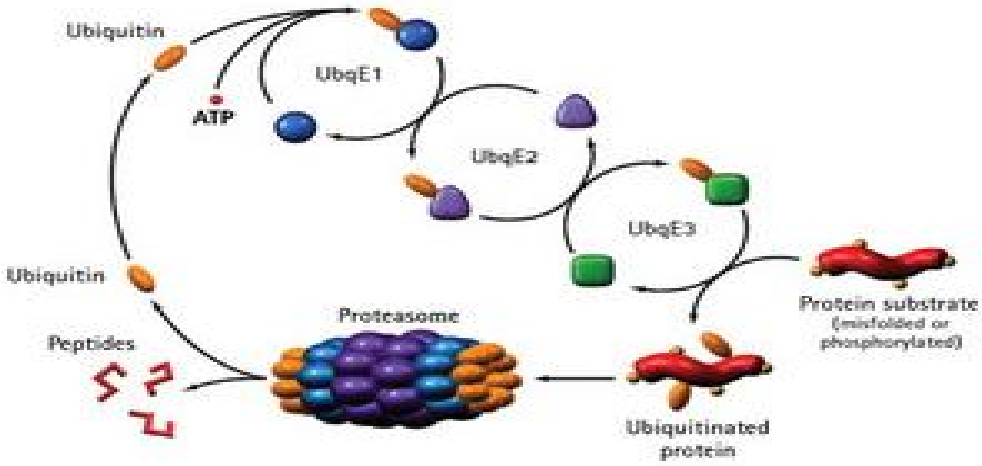


Fig. 1.11. Substrate recognition and transfer of ubiquitin to the substrate (Nalepa et al., 2006)

There are two main classes of ubiquitin E3s: the RING (really interesting new gene) (Borden, 2000) and HECT (homologous to E6-associated protein C terminus) domain families of E3s. The difference between HECT and RING domain family is that HECT E3s contain a conserved cysteine residue within the domain and accept ubiquitin from an E2 subsequently donating it to substrate protein. In contrast to HECT, RING domain serves as an adaptor between substrate and ubiquitin-loaded E2, binding both the proteins. Ubiquitin bound to E2 is directly transferred to substrate in this case.

HECT domain proteins contain a 350-amino acid residue sequence homologous to the C-terminal domain of the family E6-AP (E6-associated protein) (Huibregtse et al., 1995). This domain contains a conserved Cys residue, which interacts with the activated ubiquitin moiety transferred from E2 (Scheffner et al., 1995). The N-terminal domain, which varies among the different HECT domain proteins, is probably involved in specific substrate recognition.

The RING domain family of ubiquitin E3s is the largest class having near about 600 members. Within this family are multi-subunit cullin-RING ubiquitin E3s. In the characteristic RING sequence, four cysteines coordinate one Zn^{2+} ion and another Zn^{2+} ion is coordinated by three cysteines and a histidine. Hence the RING domain is structurally related to zinc finger domain. A well-known examples of RING E3s are SCF (Skp1/Cullin/Fbox) complex APC/C (anaphase-promoting complex/cyclosome).

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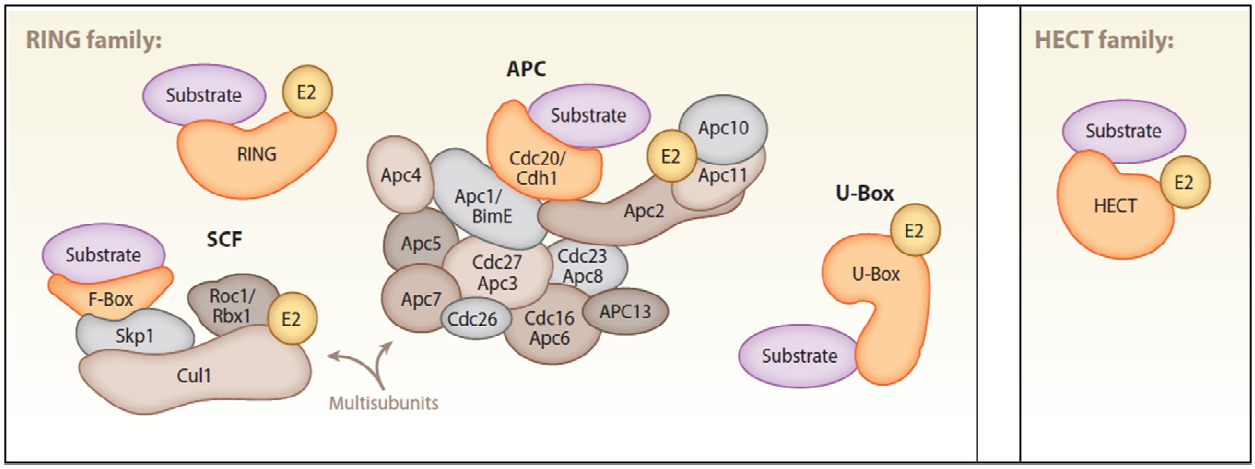


Fig. 1.12. Components of the ubiquitin E3 ligases - Ubiquitin E3s are divided into the RING (really interesting new gene) and HECT (homologous to E6-associated protein C terminus) domain families. RING domain includes the Skp1/Cullin/F-box (SCF) and anaphase-promoting complex/cyclosome (APC/C) complexes. U-Box proteins are a distinct set of RING-like ubiquitin E3s (Rotin and Kumar, 2009).

1.10.5. Proteasome – The Nano Machine

Proteasome is a cytoplasmic protease, which specifically degrades certain proteins instead of blindly targeting every protein encountered. It does this by specifically recognizing ubiquitinated proteins and degrading them. Besides the degradation of intact proteins, proteasome also degrades misfolded and denatured proteins that are formed during conditions of stress like high temperature, UV

exposure or starvation. Certain non-ubiquitinated proteins are also degraded by proteasomes with the help of other mechanisms.

Proteasome is composed of a 20S protein cylinder and two 19S lids (Peters, 1994). The active sites are situated inside the cylinder. Hence the lids are responsible for imparting specificity to proteasome as they mostly recognize ubiquitinated proteins.

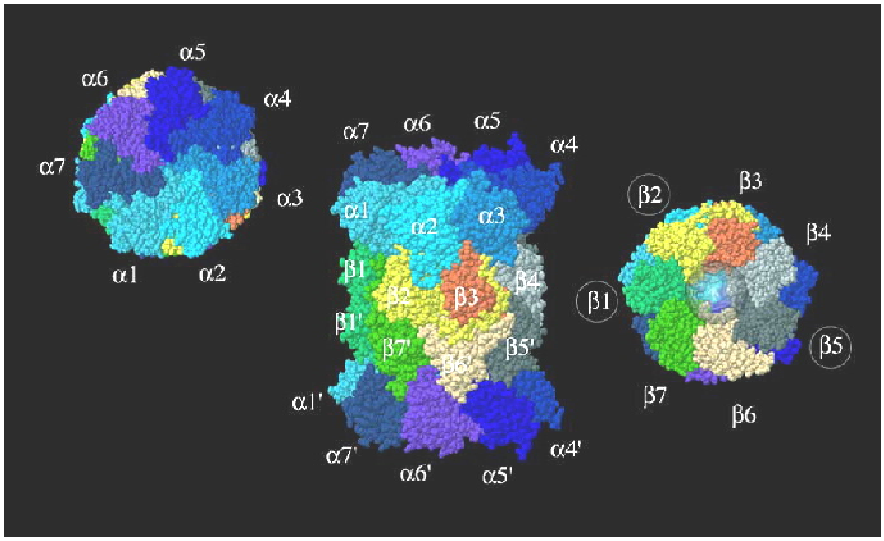


Fig. 1.13. Structure of the 20S core complex in α7β7 β7 α7 architecture (Dieter H. Wolf and Hilt., 2004)

The cylindrical part, which is called the 20S complex, is 15 nm long and 11 nm wide, and is made of two α rings and two β rings. The two α rings are joined together and make up the central portion of proteasome, while one β ring is situated on either end of the proteasome, attached to one α ring (Groll et al., 1997; Lowe et al., 1995). Both α and β rings consist of seven subunits each. Only three β subunits in each β ring have a proteolytic site. The three sites in each ring are different from each other, and are called β1, β2 and β5. β1 site cleaves near acidic residues, β2 site has trypsin like activity and β5 site has chymotrypsin like activity (Arendt and Hochstrasser, 1997; Heinemeyer et al., 1994). Proteins are degraded by these

proteolytic sites in the central chamber formed by β rings. Hence this chamber is called catalytic chamber.

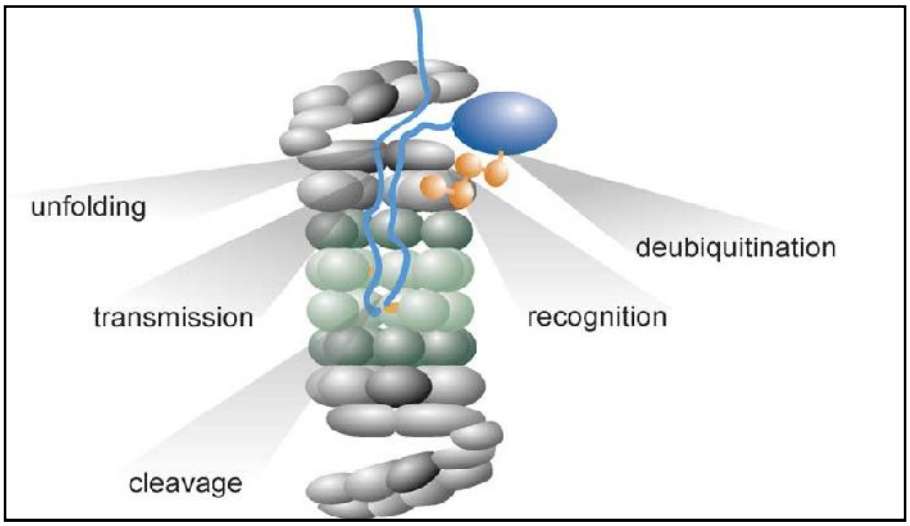


Fig. 1.14. Mechanisms executed by the proteasome (Dieter H. Wolf and Hilt., 2004)

Two 19S complexes are found in proteasome, one attached to either end of the 20S complex. These 19S complexes regulate the entry of proteins into proteasome. It is composed of two parts, namely the base, and the lid, and is made up of seventeen subunits (Peters, 1994). The base has eight subunits and is responsible for unfolding substrate proteins and inserting them into 20S complex. Six of the eight subunits in the base have ATPase activity, and provide the energy required for unfolding of the substrate proteins. These subunits are Rpt1 to Rpt6. Rpn1 and Rpn2 are the two non ATPase subunits. There are eight subunits in lid as well, which are Rpn3 and Rpn5 to Rpn11. Rpn10 is responsible for attaching the lid to the base (Glickman et al., 1998). The polyubiquitin tail must have at least 4-5 ubiquitins for it to be recognised by the 19S complex. There are only a few exceptional cases in which non- ubiquitinated proteins are recognised by the 19S complex, the best example of which is ornithine decarboxylase (Coffino, 2001; Hoyt and Coffino, 2004). Rpn10 (Deveraux et al.,

1995; Mayor et al., 2007) and Rpn13 (Husnjak et al., 2008) are responsible for binding to polyubiquitinated proteins.

1.10.5.1. Proteasome mediated degradation

For many short lived eukaryotic proteins, conjugation to ubiquitin is an obligatory step in their degradation. Ubiquitin is joined reversibly to proteins by covalent (isopeptide) linkage between the carboxyl-terminus of ubiquitin and lysine ε-amino groups of the acceptor proteins (Hochstrasser et al., 1999). For proteolytic substrates, assembly of ubiquitin chains on the protein is generally observed. Ubiquitinated proteins are in a dynamic state, subject to either further rounds of ubiquitin addition, ubiquitin removal by deubiquitinating enzymes, or degradation by a complex multicatalytic proteinase called the 26S proteasome (Fig. 1.16). The proteasome breaks down targeted substrates to short peptides but recycles the ubiquitin molecules.

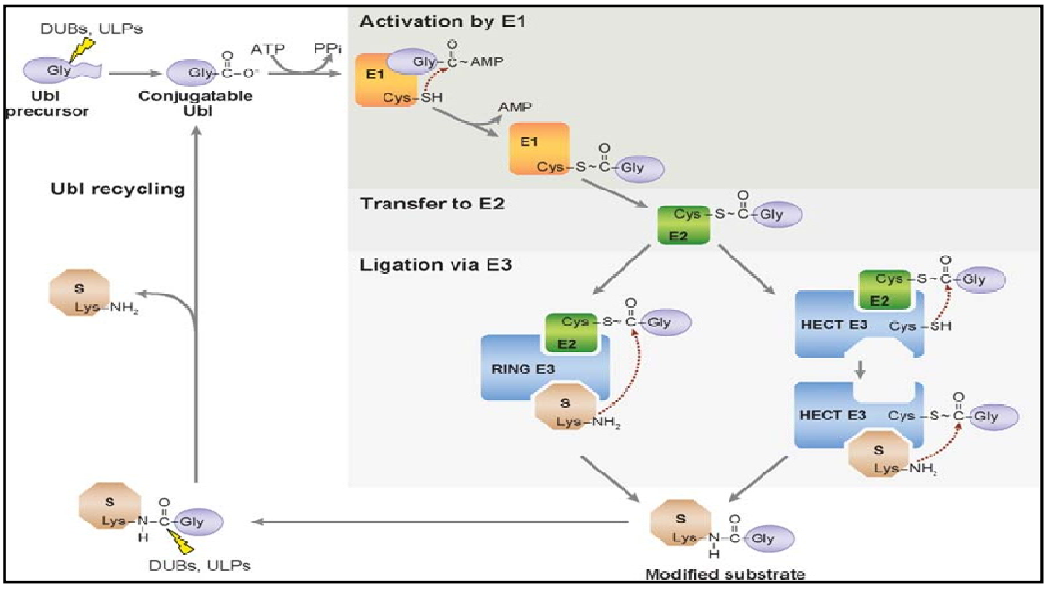


Fig. 1.15. The ubiquitin proteasome pathway (Proctor et al., 2007)

1.10.6. Deubiquitinating Enzymes (DUB'S) and their classes

Deubiquitinating enzymes are proteases that recognize the glycine residue of C-terminal end of ubiquitin and cleave it either from ubiquitin itself or from other target proteins. DUBs negatively regulate ubiquitination by cleaving ubiquitin from target proteins, maintain a pool of free ubiquitins by breaking down polyubiquitin chains (Amerik et al., 1997; Dayal et al., 2009). Besides this, polyubiquitins are also spontaneously produced by the ubiquitin system without being attached to a substrate protein. These free polyubiquitin chains are also broken down by DUBs. DUBs are also responsible for cleaving ubiquitin fused to ribosomal proteins. Five different families of DUBs, together containing around 100 DUBs are so far known in humans (Nijman et al., 2005).

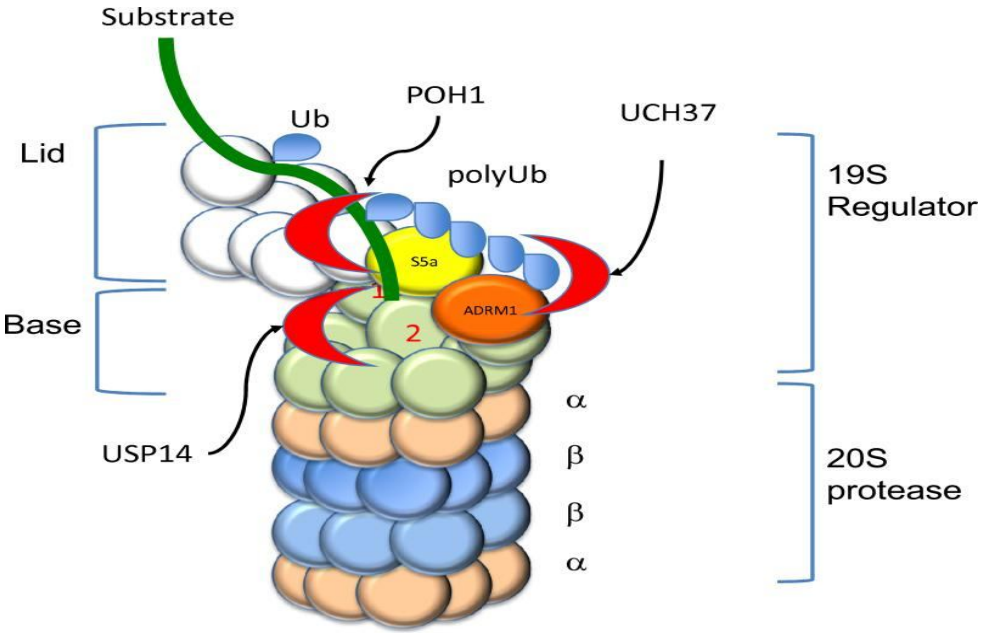


Fig. 1.16. **Proteasome bound deubiquitinating enzymes.** Deubiquitinating enzymes are indicated in red (POH1 and USP14), POH1 catalyzes the release of a polyubiquitin. RPN10 (yellow) binds the polyubiquitin chain and the distal end of the chain can be removed by the action of UCH37 bound to ADRM1 (orange). USP14 is bound to the proteasome via interactions with RPN1 (purple) and probably removes mono-ubiquitin attached to the substrate (Francisca E. Reyes Turcu. et al., 2009) .

1.10.7. Ubiquitin like modifiers (ULM'S)

Molecules other than ubiquitin were identified which mimic the structure and functions of ubiquitin to some extent hence called ubiquitin like proteins, molecules or modifiers (ULMs) (Welchman et al., 2005).

The family structural homology of ubiquitin-like protein modifiers (ULMs) is much more because of common 3D structure, the ubiquitin fold and a C-terminal protruding tail rather than the sequence homology. Hence, they are conjugated to proteins and function in "ubiquitin-like" manner. The attachment of ULMs to substrate protein changes its topology in such a way that it may facilitate or inhibit the binding of the protein to another molecule, affect enzymatic activity or sub-cellular localization and ultimately determine the half-life of the protein.

Ten different ULMs have been identified so far in mammals (Yamanaka et al., 2000). They are small Ub-like modifier, interferon stimulated gene 15 (ISG15), autophagy 8 (ATG 8) and neural precursor cell expressed, developmentally down regulated 8 (NEDD8). Ubls have a similar structural-fold to Ub, but use specific conjugation machineries and are recognized by distinct Ubl-binding domains. So far, SUMO2/3 and NEDD8 are the only Ubls known to participate in chain formation (Tatham et al., 2001; Xirodimas et al., 2004). Ubls are implicated in the regulation of many cellular processes such as gene transcription, signal transduction, autophagy and cell-cycle control (Kerscher et al., 2006) .

1.10.8. Non-proteasomal functions of ubiquitin

Besides the role played by ubiquitination in proteolysis, it performs several other functions such as protein kinase activation, DNA repair, regulation of chromatin dynamics through histone modification, vesicle trafficking and endocytic machinery.

For example, K29 or K33 linked polyubiquitin chains inhibit AMP-activated protein kinase and (AMPK)-related kinases (Al-Hakim et al., 2008).

1.10.8.1. Ubiquitination and replication

Proliferating cell nuclear antigen (PCNA) influences DNA polymerase recruitment to DNA during replication. Monoubiquitination of PCNA seems to shift recruitment preference from replicative to trans-lesion polymerases (Bienko et al., 2005; Kannouche et al., 2004), whereas UbK63 linked polyubiquitination signals error-free DNA repair (Friedberg et al., 2005) .

1.10.8.2. Ubiquitination as an endocytic signal

Mono-ubiquitination signals for the internalization of the membrane proteins in yeast and growth hormone receptor has been demonstrated in animal cells (Strous et al., 2004). Therefore, modulation of the endocytic internalization route by Ub could be important for signal transduction and receptor down-regulation. Ubiquitination also functions as a sorting signal at endosomes in the case of certain cargo proteins (Komada and Kitamura, 2005).

1.10.8.3. Ubiquitination involved in Transcription regulation

Ub-proteasome pathway is involved in transcription regulation as many transcription factors are unstable proteins that are degraded by UPS (Elsasser and Finley, 2005). In Most transcription factors, the degradation signal which is recognised by E3s overlaps closely with a transcriptional activation domain (TAD) (Dupre and Haguenauer-Tsapis, 2001).

1.10.8.4. Ubiquitin in Autophagic Degradation of Protein and organelle

Proteins and several other organelles have been found to be degraded following ubiquitin signalling. They are Mitophagy, Xenophagy, Ribophagy and others.

1.10.8.5. Ubiquitination involved in nonproteasomal removal of proteins

Protein misfolding results in the exposure of hydrophobic residues which are recognized by molecular chaperones of the heat shock protein (HSP) family, which bind to and shield exposed hydrophobic surfaces from the cytosol while promoting protein refolding (Goldberg, 2003). In addition, HSPs interact with Ub E3 ligases, such as CHIP and Parkin (Imai et al., 2002), which promote substrate polyubiquitination. These E3 ligases may play protective role by preventing accumulation of misfolded protein aggregates (Bjorkoy et al., 2005). Otherwise, the aggregates of proteins may inactivate the proteasome and mediate cytotoxicity. Thus, inhibition of the proteasome potently induces autophagy, which serves as a compensatory mechanism for degradation of accumulating polyubiquitinated misfolded proteins.

1.10.8.6. Ubiquitination in Selective Elimination of Ribosomes and mitochondria

Mechanism involving the selective removal of ribosomes is termed as ribophagy (Kraft et al., 2008). GFP labelling of ribosomal proteins have demonstrated that ribosomes are specifically targeted to the vacuole upon starvation. The process is dependent on functional autophagic machinery. Ub-specific protease, Ubp3, and an associated factor, Bre5 were found as proteins responsible for ribophagy.

Conjugation of monoUb/ Ub chains to an exposed mitochondrial protein is hypothesized to lead the mitochondrion towards autophagosome mediated degradation (Kirkin et al., 2009). There is an ongoing debate on the origin of E3 ligases which are responsible for the above ubiquitination as to they are residents of cytosol or mitochondrial membranes (Chu et al., 2009; Li et al., 2008).

1.10.8.7. Ubiquitination as Antibacterial Mechanism

Mechanism involving removal of intracellular pathogens is referred as xenophagy. Several medically relevant bacteria, including *Mycobacterium tubercuolosis*, *Salmonella enterica*, and *Listeria monocytogenes* are degraded by autophagy in vitro (Levine and Deretic, 2007).

Ub signalling found to be linked to xenophagy, Ub was recognized as a vital factor in plant resistance to bacterial pathogens (Nishimura and Somerville, 2002), and association with Ub chains was demonstrated for both gram-negative and gram-positive intracellular bacteria (Perrin et al., 2004).

1.10.9. The Role of Ubiquitin proteasome system in Diseases

Aberrations in the tightly regulated UPS pathway lead to the pathogenesis of many diseases, certain malignancies, and neurodegeneration. Some of the diseases which are linked to ubiquitin proteasomal machinery and the possible cure or drug development is mentioned below.

1.10.9.1. Cancer

Accelerated degradation of tumor suppressor proteins such as p53 and p27 are reported in malignant transformations. Mdm2, is the E3 ligase which binds and ubiquitinates the protein p53 (Michael and Oren, 2003) . A reduced level of p53 and overexpression of the Mdm2 or Hdm2 (human counterpart of Mdm2) have been found for many tumors carrying wild-type p53, such as neuroblastoma, acute lymphoblastic leukemia (ALL), melanomas, and colorectal, lung and breast carcinomas (Ciechanover and Schwartz, 2004; Sun, 2006). Many drugs have been formulated to target Mdm2 to inhibit its interaction with tumour suppressor proteins (p53 and p27).

1.10.9.2. Neurodegenerative diseases

Inability of timely removal and accumulation of ubiquitin conjugates and/ or inclusion bodies associated with ubiquitin proteasome system have been identified in a broad array of chronic neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (Hochstrasser et al., 1991) and Huntington's disease to name a few.

Recent finding have revealed one mutant form of ubiquitin known as [Ub(+1)], which is prevalent in the brain of AD patients (Van leeuwen et al., 1998). [Ub(+1)] is the result of frame shift mutation in the ubiquitin transcript which leads to extension of the molecule with 20 amino acid residues. [Ub(+1)] is an efficient substrate for polyubiquitination and its degradation is inhibited by 26S proteasomes, which results in the accumulation of the toxic proteins with neuro-pathological consequences. [Ub(+1)] was also described in other similar diseases and in other disorders such Down's syndrome (Van leeuwen et al., 1998) or supra-nuclear palsy (Fergusson et al., 2000), it is evident that it is not entirely specific to AD.

1.10.9.3. Immune and Inflammatory Response

Peptide epitopes presented to T-lymphocytes on class I MHC molecules are generated in the cytosol and processed by the ubiquitin-proteasome pathway (Kloetzel, 2001; Rock and Goldberg, 1999). Presentation of self antigen as non-self may be the potential cause that underlies the pathogenesis of autoimmune diseases.

A broad array of immune and inflammatory disorders is caused by activation of the immune system's central transcription factor NF-κB. Activation of the factor stimulates transcription of many cytokines, adhesion molecules, inflammatory response and stress proteins and immune system receptors. The factor is activated by the ubiquitin proteasome system's proteolytic mechanism by limited processing of the

precursor protein to yield the active subunit p50 and signal induced phosphorylation and subsequent degradation of the inhibitor IκB that enables translocation of the factor into the nucleus where it initiates specific transcriptional activity (Karin and Ben-Neriah, 2000).

1.10.10. Drug Development to Target Ubiquitin Proteasome System

Ubiquitin proteasome system plays a central role in a wide array of basic cellular processes, hence it is difficult to target and select cells which show abnormality. Inhibition of the proteasome may affect many processes non-specifically. Recent experimental evidence strongly suggests that such inhibition may be beneficial in certain pathologies, such as in cancer (Golab et al., 2000), asthma (Elliott et al., 1999), brain infarct (Phillips et al., 2000) and autoimmune encephalomyelitis.

In malignancies, the drugs may act by inhibiting the degradation of different cell cycle inhibitors, whereas in neuroprotection they may act via inhibiting activation of NF-κB, which elicits an inflammatory response. When accelerated degradation of a tumor suppressor results in exposure of cells to malignant transformation, selective inhibition of the recognition machinery can potentially reverse the malignant phenotype. Peptides that bind specifically to HPV-E6 and prevent its association with p53 can interfere with p53 targeting (Michael H. Glickman. and Ciechanover, 2001).

In autoimmune diseases, they may act by inhibiting presentation of self-peptides and by interfering with signal transduction cascades. Another approach of targeting may be the development of small molecules that are substrate specific and bind specific substrates or to their ancillary proteins rather than to an E3.

1.11. BRIEF INTRODUCTION TO CHAPTERS

Chapter 1 is an introduction ubiquitin and the enzymes, which are the key players of the ubiquitin proteasome system. Structure of ubiquitin, importance of its surface residues and ubiquitin binding domains in the interacting proteins have been discussed as ubiquitin accomplishes a variety of roles through protein-protein interactions, which explains the reason for its structural conservation. Beside proteasomal functions ubiquitin is also known to mark its presence in other non-proteasomal functions. A brief account on non-proteasomal functions of ubiquitin is presented here. Due to defective functioning of the UPS system, cells are prone to various diseases. Some of the diseases and the strategies for drug development which targeting UPS are also discussed. Central role of ubiquitin in cellular pathways and the need to throw light on high degree of conservation of its structure to its functional relevance are the main reasons to choose this protein in the current study.

Chapter 2 deals with generation of mutant ubiquitins, which are derivatives of dose dependent lethal mutant of ubiquitin UbEP42 and their functional characterization. UbEP42 a mutant form of ubiquitin generated earlier in our laboratory by error–prone PCR reaction harbours four amino acid substitutions (Prabha et al., 2010). In order to understand the importance of the residues which have undergone substitution, mutant forms of ubiquitin carrying single mutations and combinations of mutations were constructed using site directed mutagenesis. All mutant ubiquitins were analyzed for functional integrity in *UBI4* deletion strain SUB60 of *Saccharomyces cerevisiae*, which is hypersensitive to stress conditions. The SUB60 strain which fails to grow under stress, can withstand stress and behave like wild-type if ubiquitin is expressed extra-chromosomally from a plasmid. So if the mutant forms are expressed extra-chromosomally in the same strain in place of wild type ubiquitin and the cells can survive stress conditions, it can be concluded that the mutant forms retain their functionality. Following this line of logic, complementation studies were carried out

under heat stress and antibiotic stress. Prior to it the effect of over-expression of mutant ubiquitins on growth and doubling time have been studied to understand the impact of ubiquitin mutants on the normal functioning of the cells. Our results show that two of the mutations have compensatory effects over other two mutations which are drastically detrimental in their nature (Mrinal Sharma and C. Ratna Prabha, Manuscript under preparation).

Chapter 3 deals with the functional importance of residues in the parallel β-bulge of ubiquitin. This β-bulge is formed by Glu64, Ser65 and Gln2 residues. This structure of β-bulge complete with residue identities is totally conserved in all ubiquitins. Interestingly, it displays certain uncommon features. It was hypothesized that if the individual residues of the β-bulge are replaced with residues which have greater preference for the same secondary structure, then the structure would not be disturbed and whatever functional changes are observed will highlight the role of the replaced residues. Therefore, single mutants of the β-bulge were constructed and characterized earlier in our laboratory which in spite of minor structural changes showed significant functional differences (Mishra et al., 2009; Mishra et al., 2011). The above observations led us to the question of what would be the functional consequences if more than one residue is changed at once. Will the resultant mutations have effects which are synergistic in the same line of what was observed earlier or will they be compensatory? Thus, the chapter presents the generation of mutations in β-bulge in combinations and their functional characterization in *S. cerevisiae* by complementation analysis under various stresses. Our results establish that two of the mutations UbE64G-S65D and UbQ2N-E64G-S65D are severely debilitating compared to other two mutations UbQ2N-E64G and UbQ2N-S65D, implying that E64G and S65D together exert more debilitating effects than Q2N with either E64 or S65 (Sharma and Prabha, 2011; Mrinal Sharma, Ankita Doshi and C. Ratna Prabha, Oral presentation in Regional Science Congress held in Vadodara, September 15-16, 2012).

Chapter 4 presents the construction of dose dependant lethal ubiquitin UbEP42 and its derivative UbI61T in bacterial expression vector. This chapter describes the various steps of purification to achieve purified protein for structural studies. Secondary and tertiary structure characterization of the mutant has been carried out by circular dichroism and fluorescence spectroscopy respectively.

Chapter 5 describes the construction of double and triple mutants of second β-bulge of the ubiquitin in bacterial expression vector and their purification by affinity chromatography. Secondary and tertiary structures of the mutant proteins have been studied using circular dichroism and fluorescence spectroscopy respectively.

CHAPTER 2

Construction and functional assessment of derivatives of lethal ubiquitin in *Saccharomyces cerevisiae*

2.1. Introduction

The molecular evolution of proteins consists mainly of two steps: diversification and selection. Diversification and selection is a long course in evolution and it takes million of years. The diversification of molecules can be carried out artificially in vitro by introducing mutations, such as point mutations, deletions, insertions or recombination, into genes encoding proteins and selection of protein is mainly based on expression of gene for the desired functional characteristics. For example, the crystal structure of an ATP-binding protein revealed a novel fold (Prijambada et al., 1996) indicating that molecular evolution in vitro can yield proteins that have not been discovered by nature, but they exhibit function and folded structures. Molecular evolution can be used not only to improve or alter protein functions but also to elucidate the importance of the amino acid residues in sequence. Another example is a novel ATP-binding protein discovered from 6×10^{12} random sequences with a length of 80 amino acid residues using mRNA display (Keefe and Szostak, 2001). From these results, they postulated that approximately 1 in 10^{11} are functional in the entire protein sequence space, which is sufficiently high to be discovered by nature. Whether proteins with desired properties can be obtained by molecular evolution depends simply on the predefined structural landscape (Matsuura and Yomo, 2006).

Nevertheless, molecular evolution has proven to be a powerful strategy not only for generating proteins for various applications but also for increasing our understanding of proteins. Natural proteins, including mutants generated by various mutagenesis methods, cover significant region of the entire protein sequence space.

The most commonly used random mutagenesis method is **error prone PCR,** which introduces random mutation during PCR by reducing the fidelity of DNA polymerase. The fidelity of DNA polymerase can be reduced by adding Mn ions or playing with the concentration of Mg ions and biasing the dNTPs concentration. Use

of compromised DNA polymerase causes incorporation of incorrect nucleotides during PCR reaction yielding randomly mutated products. This random population of mutants are screened for desired phenotype after ligating them in suitable expression vector. The position and nature of the amino acid substitution can be known by comparing sequence of mutated gene with wild type gene.

Our laboratory has explored random mutagenesis method to generate mutants of ubiquitin with loss of function phenotype (Ratna Prabha. et al., 2010). Ubiquitin gene has been amplified under error prone conditions to have higher probability of mutations within the gene. Successive rounds of error prone PCR has generated mutants of ubiquitin gene. Among the many mutants obtained, one mutant namely UbEP42 revealed lethal phenotype when over-expressed in presence of inducer copper sulphate. Sequencing of the gene showed the presence of four mutations in the gene, which were novel (Table 2.1) (Ratna Prabha. et al., 2010).

Amino acid residue substitution in Ubiquitin	**Codon**	**Secondary structure involved**
S20F	TCC / TTC	3rd residue of a type I turn
A46S	GCC / TCC	2nd residue of type III turn
L50P	CTC / CCT	β-sheet
I61T	ATT / ACT	Between two turns in the turn rich region

Table 2.1. The positions of point mutation in UbEP42 ubiquitin variant, as revealed by gene sequencing

This chapter summarizes the functional studies of the four mutations in isolation and also in all possible combinations (Fig. 2.1). The mutants have been subjected to complementation and over-expression analysis.

2.2. Plan of work

Ubiquitin gene variants carrying the site directed mutations UbS20F, UbA46S, UbL50P and UbI61T were cloned in the shuttle vector YEp96**.** *UBI4* mutant of *Saccharomyces cerevisiae* (SUB60) was used to introduce these variants. Second line of experiment includes functional assessment of these above mentioned mutants and evaluation by phenotypic complementation (Fig. 2.1).

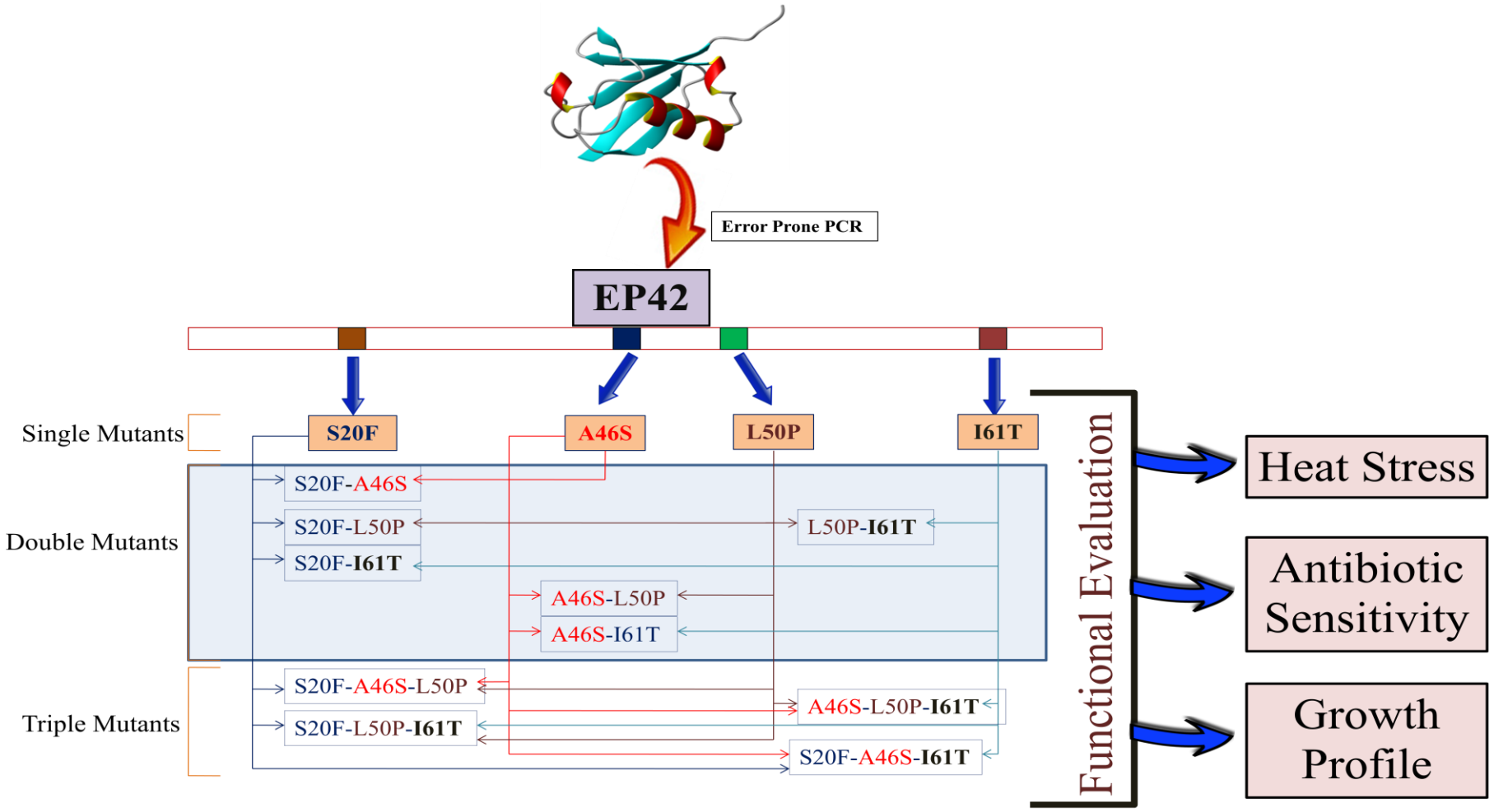


Fig. 2.1. Schematic representation of construction of single, double and triple mutants and their functional evaluation.

2.3. Material and methods

2.3.1. Strains, media and plasmids

2.3.1.1. Yeast strains, Media, Reagents

The *S. cerevisiae* strains used for the experiments are as follows- SUB62 (MATa lys2-801 leu2-3,112 ura3-52 his3-Δ200 trp 1-1) is a wild type ubiquitin strain and SUB60 (MATa ubi4-Δ2: LEU2 lys2-801 leu2-3,112 ura3-52 his3-Δ200 trp1-1) is a deletion mutant lacking UBI4 polyubiquitin gene (Finley et al., 1987). *S.cerevisiae* cultures were grown in synthetic dextrose (Hochstrasser et al.) medium containing 0.67% Hi-media yeast nitrogen base (without amino acid) and 2% glucose or 4% galactose as carbon source, histidine (20mg/liter), lysine (30mg/liter), uracil (20mg/liter), leucine(100mg/liter), tryptophan (20mg/litre). The cultures were grown at 30ºC at 200 rpm. *Escherichia coli* DH5α strain was used for all plasmid manipulations and grown at 37ºC at 200 rpm in nutrient rich Luria broth (Hi-media). Strain was maintained under a selection pressure of 50µg/ml of ampicillin.

2.3.1.2. Bacterial strains and media

E.coli DH5α (F, 80dlacZ M15, *endA*1, *recA*1, *hsdR*17 (r_k^-,m_k^+), *supE44*, *thi-1*, *gyrA96*, *relA1*, *(lacZYA-argF)U169*) strain was used for transforming recombinant DNA. Cultures were grown at 37°C at 200 rpm in Luria broth (Hi-media). 50µg/ml of ampicillin concentration was used for selection of plasmid. Bacterial transformation was done by $CaCl_2$ method. Plasmid isolation was done by standard alkaline lysis protocol (Sambrook, 2001).

2.3.1.3. Sequence Analysis

The mutation was confirmed with all the 14 mutants by sequencing of the mutant gene present in the plasmid YEp96 in a single forward reaction using primer sequence 5’ GCGCCGACATCATAACGGTTCTGGC 3’.

2.3.1.4. Plasmid construction in yeast expression vector YEp96

Yeast expression vector YEp96 (Fig. 2.2) carrying synthetic yeast ubiquitin gene under the inducible *CUP1* promoter was used for gene manipulation. YEp96, a shuttle vector between *E. coli* and *S. cerevisiae* was used to carry out functional evaluation of ubiquitin mutants. Ubiquitin gene variants generated by PCR were cloned into the *Bgl*II and *Kpn*I sites of YEp96. To facilitate screening procedure, restriction sites were created or destroyed adjacent to intended mutation. While manipulating the restriction sites in the sequence care was taken to avoid amino acid changes. Screening of mutations for S20F, A46S and L50P, I61T were done on the basis of loss of *Xba*I, *Xho*I and *Sal*I respectively.

The DNA sequence of the *Eco*RI -*Kpn*I insert in YEp96-UbWt is given below.

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

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

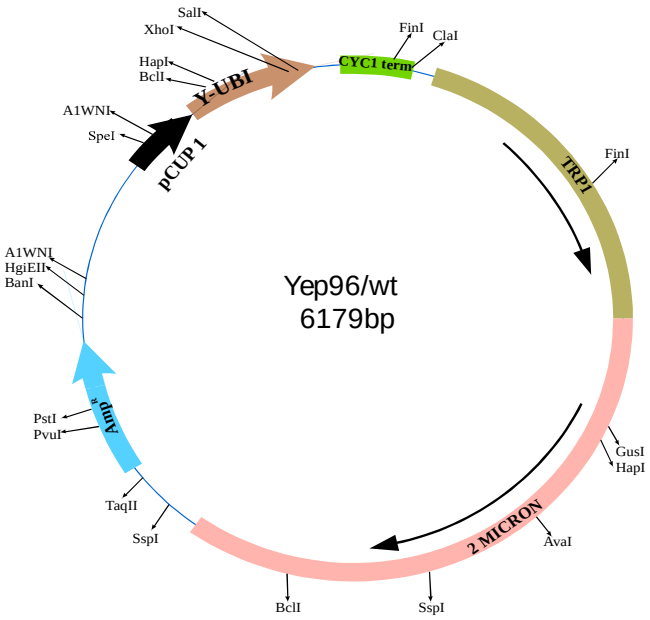


Fig. 2.2. Yeast expression vector Yep96/UbWt, expresses synthetic ubiquitin under CUP1 promoter (Finley et al., 1987)

2.3.1.5. Plasmid construction in yeast expression vector pUB221

Mutations of ubiquitin gene were carried out in plasmids pUB221 (Fig. 2.3) which expresses a c-myc tagged synthetic yeast ubiquitin gene under the *CUP1* promoter and is a shuttle vector between *E. coli* and *S. cerevisiae*. It possesses *URA3*, *TRP1* as selection marker and 2μ backbone in a YEp96. The peptide sequence of myc epitope in pUb221 yeast expression vector is EQKLISEEDL (Spence et al., 1995).

Ubiquitin gene amplicons generated by PCR were cloned into the *Bgl*II and *Kpn*I sites of pUB221. Screening of mutations for UbS20F, UbA46S, UbL50P and UbI61T were done with loss of *Xba*I, *Xho*I, and *Sal*I sites respectively.

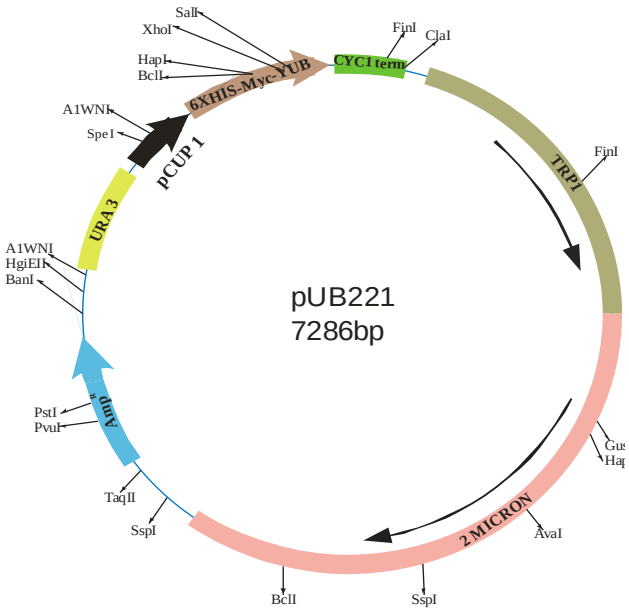


Fig. 2.3. Yeast expression vector pUB221/UbWt, expresses myc tagged ubiquitin under CUP1 promoter (Spence et al., 1995)

2.3.1.6. Primers used in the study

Four sets of mutagenic complementary primers were used in combination with non-mutagenic primers to get different sized amplicon from the Ubiquitin gene. In each case the design of primers included damage to a restriction site as a strategy to

ease the screening process for successful introduction of mutation. Sequences of the primers are given in Table 2.2.

Primer	**Sequence of the primer**
UB FR	5' ACA GAA TTC ATG CAG ATC TTC GTC AAG 3'
UB RE	5' GCC AAG CTT CGC TCA ACC 3'
UbEP 42-20FR	5' CC ATA ACT CTT GAA GTT GAA TCT TTC GAT ACC ATC GAC 3'
UbEP 42-20RE	5' GTC GAT GGT ATC GAA AGA TTC AAC TTC AAG AGT TAT GG 3'
UbEP 42-46FR	5' GA TTG ATC TTT TCC GGT AAG CAG CTG GAG GAC GGT AG 3'
UbEP 42-46RE	5' CT ACC GTC CTC CAG CTG CTT ACC GGA AAA GAT CAA TC 3'
UbEP 42-50FR	5' G ATC TTT GCC GGC AAG CAG CCT GAG GAC GGT AG 3'
UbEP 42-50RE	5' CT ACC GTC CTC AGG CTG CTT GCC GGC AAA GAT C 3'
UbEP 42-61FR	5' CT GAT TAC AAC ACT CAG AAG GAG TCC ACC TTA CAT CTT G 3'
UbEP42-61RE	5' C AAG ATG TAA GGT GGA CTC CTT CTG AGT GTT GTA ATC AG 3'

Table 2.2. List of primers used in recombinant PCR to generate various site directed mutants of ubiquitin (bases marked in green are conservative mutations which do not change the amino acid in the sequence, whereas bases marked in red are point mutations)

2.3.1.7. Cloning strategy used to generate mutant ubiquitins

Sr. No.	Single Mutant	Codon	Enzyme used for Cloning	Enzyme used for screening
1	S20F	TCC / TTC	*Bgl*II and *Kpn*I	*Xba*I
2	A46S	GCC / TCC	-----	*Xho*I
3	L50P	CTC / CCT	-----	*Xho*I
4	I61T	ATT / ACT	-----	*Sal*I
5	S20F- A46S	TCC / TTC and GCC / TCC	-----	*Xba*I and *Xho*I
6	S20F- L50P	TCC / TTC and CTC / CCT	-----	*Xba*I and *Xho*I
7	S20F-I61T	TCC / TTC and ATT / ACT	------	*Xba*I and *Sal*I
8	A46S -L50P	GCC / TCC and CTC / CCT	-----	*Xho*I
9	A46S-I61T	GCC / TCC and ATT / ACT	-----	*Xho*I and *Sal*I
10	L50P-I61T	CTC / CCT	-----	*Xho*I and *Sal*I

		and ATT / ACT		
11	S20F- A46S- L50P	TCC / TTC,	------	*Xba*I and *Xho*I
12	A46S -L50P- I61T	GCC / TCC	------	*Xho*I and *Sal*I
13	S20F- A46S- I61T	CTC / CCT	------	*Xba*I, *Xho*I and *Sal*I
14	S20F- L50P- I61T	ATT / ACT	-------	*Xba*I, *Xho*I and *Sal*I

Table 2.3. List of restriction enzymes and the sites of mutation in ubiquitin gene

2.3.2. Phenotype analysis

2.3.2.1. Growth Effects

The cultures of SUB60 cells exhibiting both wild type and mutant forms of ubiquitin were grown up to stationary phase and their growth profiles were compared with wild type strain for UBI4, SUB62 (Finley et al., 1994). To study the growth curve freshly grown culture were used as inoculum. Optical density was recorded at fixed interval of two hours at 600nm. Exponential phase of the culture was taken to calculate the generation time.

Doubling Time = t/g

$g = [\log_{10} (N_t/N_0)]/ 0.3$

N_t = Number of cells or OD at start

N_0 = Number of cells or OD at end

t = time cultured (in Hours)

2.3.2.2. Viability assay

SUB60 yeast transformants were allowed to grow up to log phase with optical density of the cultures at 600nm between 0.5 - 0.6 and four fold serial dilution was made followed by plating on SD selection media. Plates were incubated at permissible temperature 30°C and the colonies were counted.

2.3.2.3. Heat Stress Complementation

YEp96 vector carrying wild type and mutated ubiquitin gene was transformed in the stress hypersensitive UbI4 mutant SUB60 and was checked for complementation under various conditions of stress. SUB60 yeast cells can be complemented by wild type ubiquitin expressed extra-chromosomally and thus rescued from stress (Finley et al., 1987). SUB60 yeast transformants were grown up to log phase with optical density range between 0.5 to 0.6 and serial dilution of four fold was made and plated on SD selection plates were incubated at 40°C for variable time periods of 0, 4, 8, 12 and 16 hours followed by restoring to permissible temperature of 30°C and the colony count was made in order to verify the functional integrity of mutants.

2.3.2.4. Antibiotic sensitivity test

Antibiotic complementation study of ubiquitin variants was carried out as per standard protocol (Hanna et al., 2003). SUB60 transformants were allowed to grow till they reached optical density values around 0.2 and cells were three fold serially diluted and spotted on SD media. Cells were induced with 25μM copper sulphate in the presence of 4μg/ml concentration of cycloheximide. Plates were incubated for ten days to assess the complementation of mutant ubiquitin by examining growth.

2.3.2.5. Western blot analysis

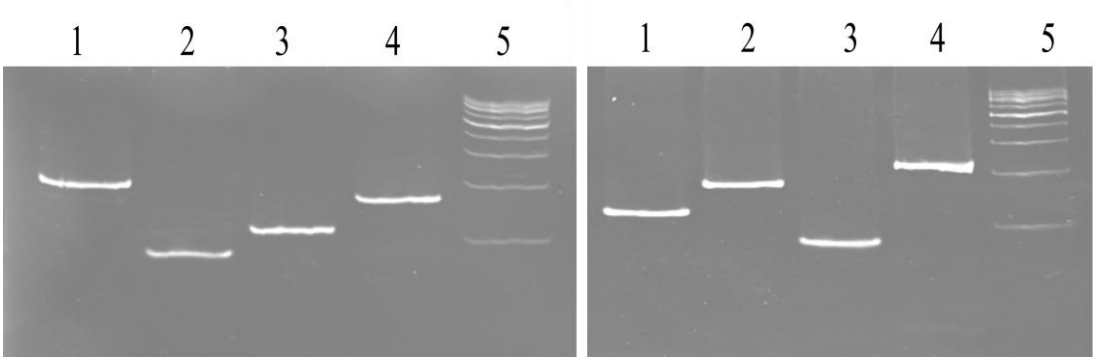
Yeast transformants were grown till mid-log phase to get optical density around 0.5 then the cells were induced with 25μM $CuSo_4$ for 4 hours and shifted to 40°C for 2 hours. Yeast cells were pelleted and washed twice with distilled water, suspended in normal saline and treated with gel loading buffer (Hochstrasser et al., 1991). Equal amount of proteins were loaded in each well. Discontinuous SDS-PAGE was performed. Wet transfer of proteins on Nitrocellulose membrane was performed for 1 hour at 4°C. Incubation of nitrocellulose membrane for four hours in 1% BSA in phosphate buffered saline (PBS), pH-7.8 was used for blocking. Anti-c-myc-horse radish peroxidase conjugated antibody procured from Roch was used at 1/1000 dilution. The blot was developed with enhanced luminol in presence of H_2O_2 and $NiCl_2$.

2.4. Results

2.4.1. Construction and functional evaluation of single mutant of UbEP42 derivative in *Saccharomyces cerevisiae*

2.4.1.1. Generation of single mutant forms of ubiquitin S20F, A46S, L50P and I61T by site directed mutagenesis using recombinant PCR

All four mutations namely UbS20F, UbA46S, UbL50P and UbI61T have been amplified from the combination of mutagenic and non-mutagenic primers (Primer sequences are given in Table 2.2 material and methods section). Resultant amplicons were used as a template in subsequent PCRs, further amplified to give rise full length mutated ubiquitin gene respectively. Amplified product is shown in Fig. 2.4. Restriction enzymes *Bgl*II and *Kpn*I were used to clone the fragment in yeast expression vector YEp96.



(A) **(B)**

Fig. 2.4. Gel picture showing amplified PCR products from vector YEp96 Wt ubiquitin by using sets of mutagenic and non-mutagenic primers in combination.

(A) Lane 1, 2 shows 203 and 77bp amplification by using 20FP, 175RP and 175 FP, 20RP respectively for constructing S20F mutation. Lane 3, 4 shows 106 and 162 bp amplification from 46FP, 175 RP and 175 FP, 46RP respectively for constructing A46S mutation. Lane 5 shows 100bp DNA ladder.

(B) Lane 1, 2 shows 101 and 162bp amplification from 50FP, 175RP and 175FP, 50RP respectively for constructing L50P mutation. Lane 3, 4 shows 61 and 208bp amplification from 61FP,175RP and 175 FP and 61RP respectively for constructing I61T mutation. Lane 5 shows 100bp DNA ladder.

2.4.1.1.1. Cloning of UbS20F, UbA46S, UbL50P and UbI61T mutant in yeast expression vector YEp96

For the construction of the single mutants namely UbS20F, UbA46S, UbL50P and UbI61T in yeast-bacterial shuttle vector YEp96 (Fig. 2.2), ubiquitin gene carrying above mentioned mutations were amplified and subjected to *Bgl*II and *Kpn*I double digestion. After restriction digestion the mutated gene is ligated into YEp96 vector. Vector carrying mutations in the ubiquitin gene were confirmed with restriction digestion pattern of both vector and its PCR amplicon by digesting it with *Xba*I, *Xho*I and *Sal*I restriction enzyme. Detail strategy used to make and confirm the mutations

are mentioned in the table 2.3. Further, mutated ubiquitin gene were sequenced to ensure the incorporation of respective mutations.

2.4.1.1.1.1. Cloning of UbS20F in yeast expression vector YEp96

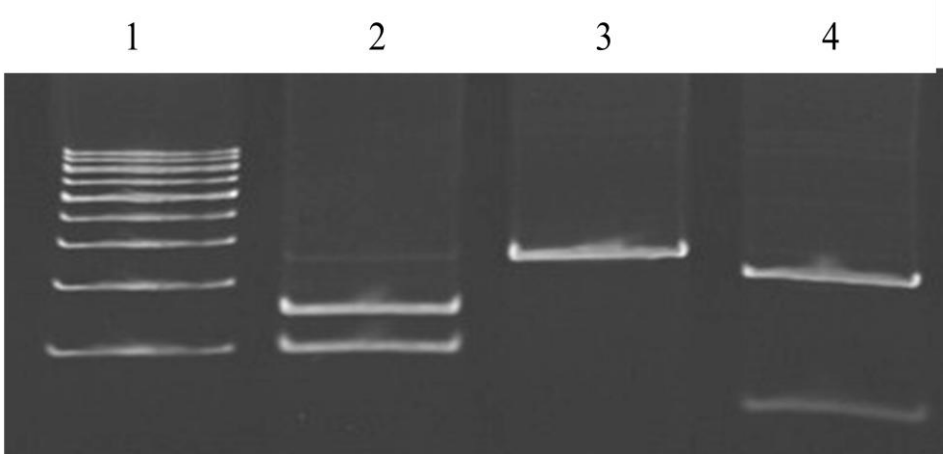
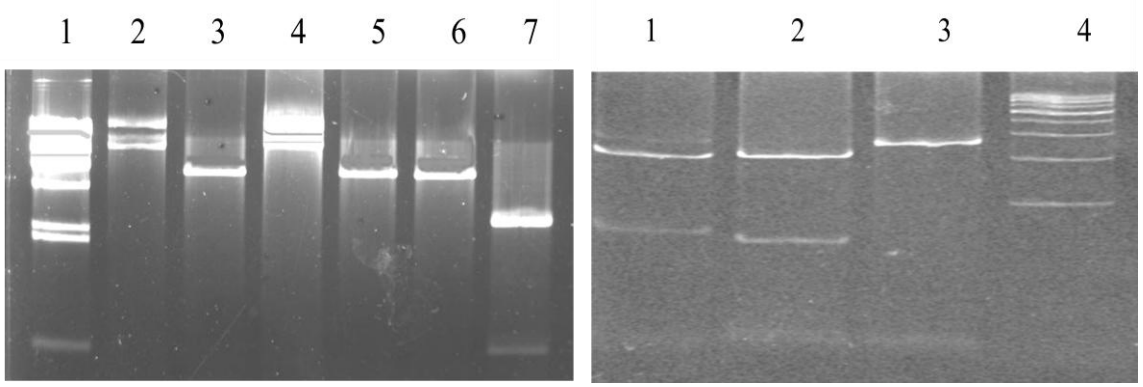


Fig. 2.5. Gel picture showing PCR product digestion pattern of UbS20F cloned in YEp96

Lane 1 presents 100bp DNA ladder. Lane 2 shows *Xho*I digestion of gene into two fragment of 150 and 100bp. Lane 3 shows failure of *Xba*I digestion suggests clone confirmation. Lane 4 shows *Sal*I digest with two fragments of 220 and 30bp.

2.4.1.1.1.2. Cloning of UbA46S mutant in yeast expression vector YEp96



(A) **(B)**

Fig. 2.6. Gel picture showing digestion pattern of YEp96 UbA46S plasmid and its PCR product

(A) Gel pictures showing digestion Pattern of YEp96 UbA46S plasmid and 250bp PCR Product digestion. Lane1 shows λ *Hind*III Digest Marker. Lane2 has undigested plasmid. Lanes 3,4,5,6 and 7 show digestion pattern of the plasmid by *Bgl*II, *Xho*I, *Sal*I, *Kpn*I and *Eco*RI. In Lane 4 *Xho*I does not digest the plasmid indicating incorporation of mutation

(B) Lane 1 and 2 show digestion of 250bp PCR amplicon with *Sal*I and *Xba*I respectively. Lane 3 shows failure of digestion of 250bp amplicon with *Xho*I indicates incorporation of desired mutation, Lane 4 shows 100bp DNA ladder.

2.4.1.1.1.3. Cloning of UbL50P mutant in yeast expression vector YEp96

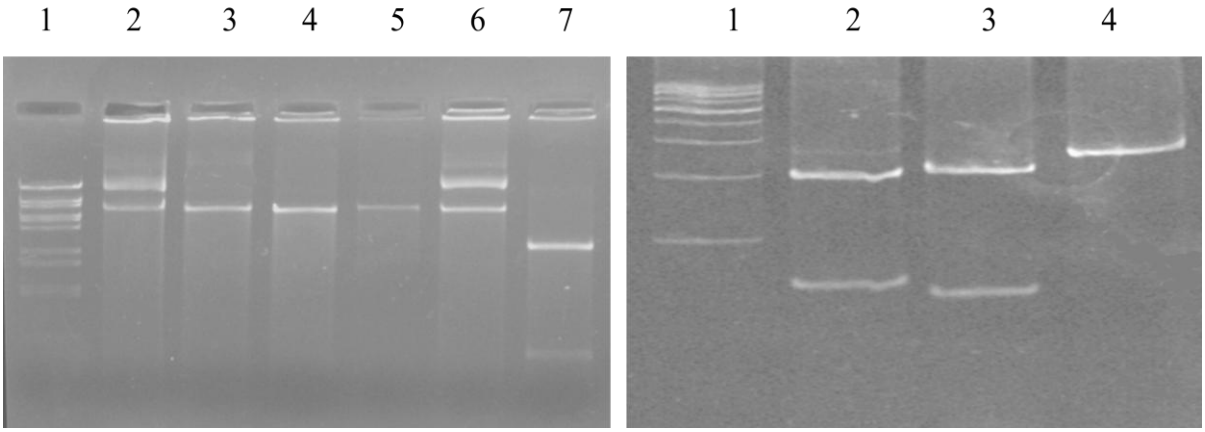
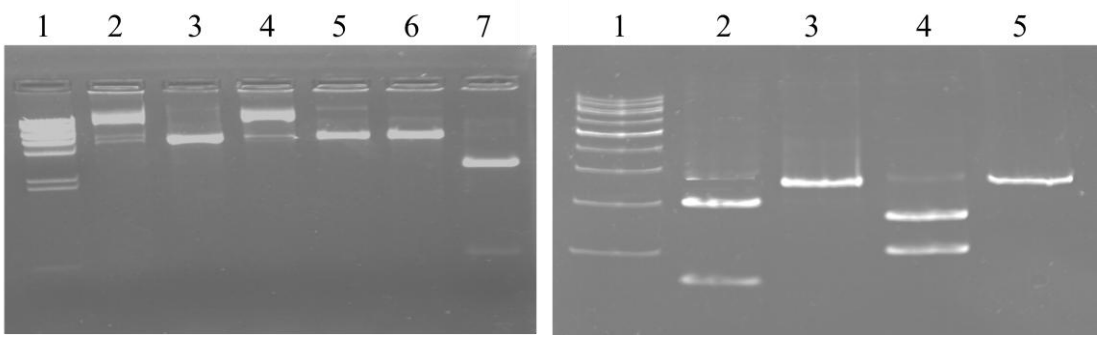


Fig. 2.7. Gel pictures showing digestion Pattern of YEp96 UbL50P plasmid and 250bp PCR Product digestion.

(A) Lane1 shows *Bst*I Digest Marker. Lane2 has undigested plasmid. Lanes 3,4,5,6 and 7 show digestion pattern of the plasmid by *Bgl*II, *Sal*I, *Kpn*I, *Xho*I, and *Eco*RI. In Lane 6 *Xho*I does not digest the plasmid indicating incorporation of mutation

(B) Lane 1 shows 100bp DNA ladder. Lane 2 and 3 show digestion of 250bp PCR amplicon with *Sal*I and *Xba*I respectively. Lane 4 shows failure of digestion of 250bp amplicon with *Xho*I indicates incorporation of desired mutation.

2.4.1.1.1.4. Cloning of UbI61T mutant in yeast expression vector YEp96



(A) **(B)**

Fig. 2.8. Gel picture showing digestion Pattern of YEp96 UbI61T plasmid and PCR product of UbI61T

(A) Gel pictures showing Lane1 shows λ *Hind*III Digest Marker. Lane2 has undigested plasmid. Lanes 3,4,5,6 and 7 show digestion pattern of the plasmid by *Bgl*II, *Sal*I, *Kpn*I, *Xho*I and *Eco*RI. In Lane 4 *Sal*I does not digest the plasmid indicating incorporation of mutation

(B) Lane 1 shows 100bp DNA ladder, lane 2, 3 and 4 show digestion of 250bp PCR amplicon with *Xba*I, *Sal*I and *Xho*I respectively. Lane 5 shows undigested PCR product of the mutant ubiquitin gene. Lane 3 shows failure of digestion of 250bp amplicon with *Sal*I indicates incorporation of desired mutation.

2.4.1.1.1.5. Cloning of UbL50P mutant in myc tagged yeast expression vector pUb221

UbL50P mutant ubiquitin gene has been subcloned in myc tagged yeast expression vector PUb221 (Fig. 2.3) by using *Bgl*II and *Kpn*I restriction site. Clone confirmation was done by analysing restriction digestion pattern of the PCR product (Fig. 2.9). *Xho*I restriction site was used for the screening of vector carrying desired mutation followed by sequencing of gene to ensure the incorporation of the mutation in ubiquitin.

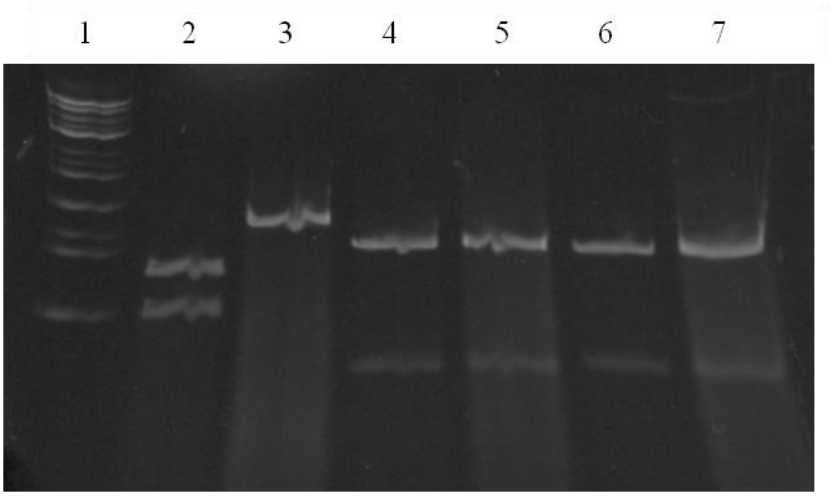


Fig. 2.9. Gel picture showing digestion Pattern of PCR Product of pUb221/UbL50P

Gel pictures showing digestion of pUb221/L50P. Lane1 shows 100bp Marker. Lane 2 and Lane 3 shows digestion of Pub221/Wt and UbL50P with *Xho*I Enzyme. Lanes 4 and 5 show digestion pattern of the PCR product of Pub221/wt and UbL50P with *Sal*I Restriction enzyme. Lane 6 and 7 show digestion pattern of the PCR product of 221/wt and UbL50P with *Xba*I restriction enzyme. In lane 3 PCR product remain undigested after digestion with *Xho*I Enzyme confirms incorporation of proline instead of leucine at 50^{th} position.

2.4.1.1.1.6. Cloning of UbI61T mutant in myc tagged yeast expression vector pUb221

UbI61T mutant ubiquitin gene has been subcloned in yeast expression vector pUb221 (Fig. 2.3) by using *Bgl*II and *Kpn*I restriction site. Clone confirmation was done by analysing restriction digestion pattern of the PCR product (Fig. 2.10). *Sal*I restriction site was used for the screening of vector carrying desired mutation followed by sequencing of vector to ensure incorporation of the mutation in ubiquitin.

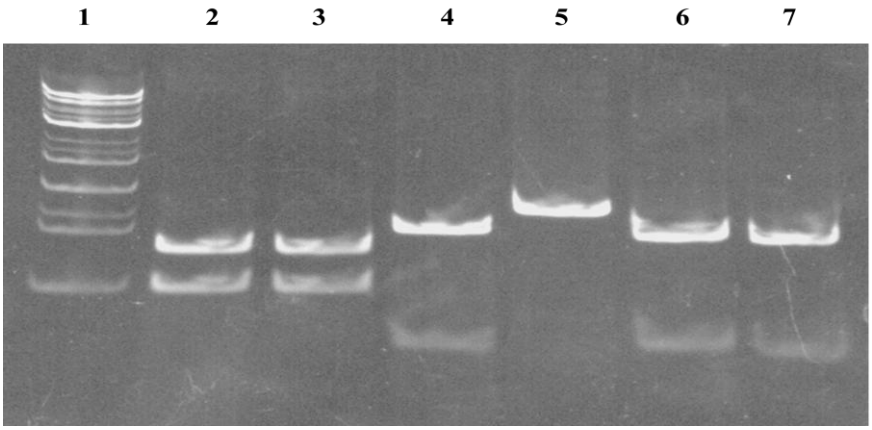


Fig. 2.10. Gel picture showing digestion Pattern of PCR Product of Pub221/c-myc UbI61T.

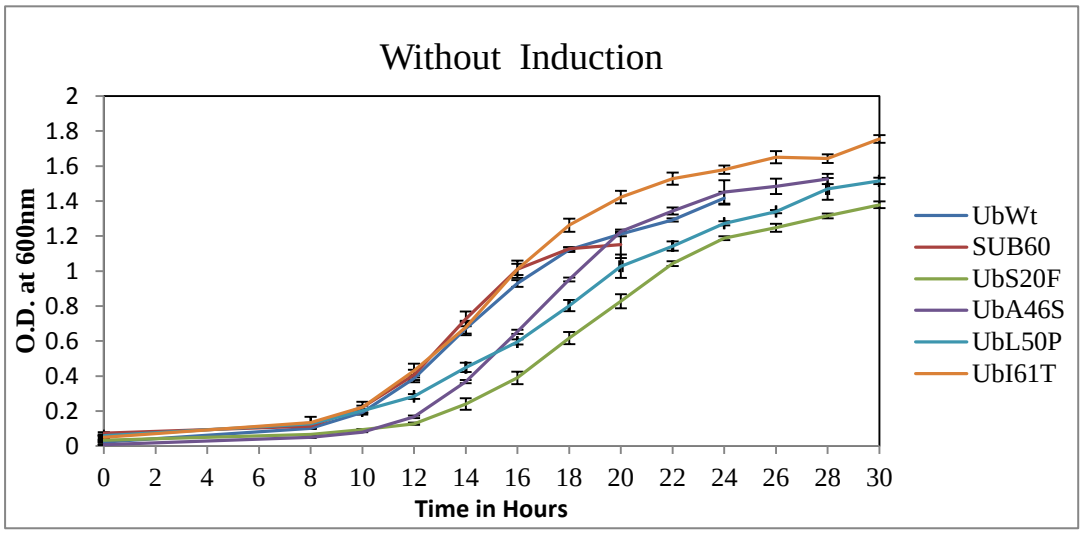
Gel pictures showing lane1 shows 100bp Marker. Lane 2 and lane 3 shows digestion of Pub221/Wt and UbI61T with *Xho*I Enzyme. Lanes 4 and 5 show digestion pattern of the PCR product of 221/wt and UbI61T with *Sal*I restriction enzyme. In Lane 6 and 7 show digestion pattern of the PCR product of 221/wt and UbI61T with *Xba*I restriction enzyme. In lane 5 PCR product remain undigested after digestion with *Sal*I enzyme confirms incorporation of thrionine instead of isoleucine at 61st position.

2.4.2. Functional evaluation of single mutants of UbEP42 derivative in *Saccharomyces cerevisiae*

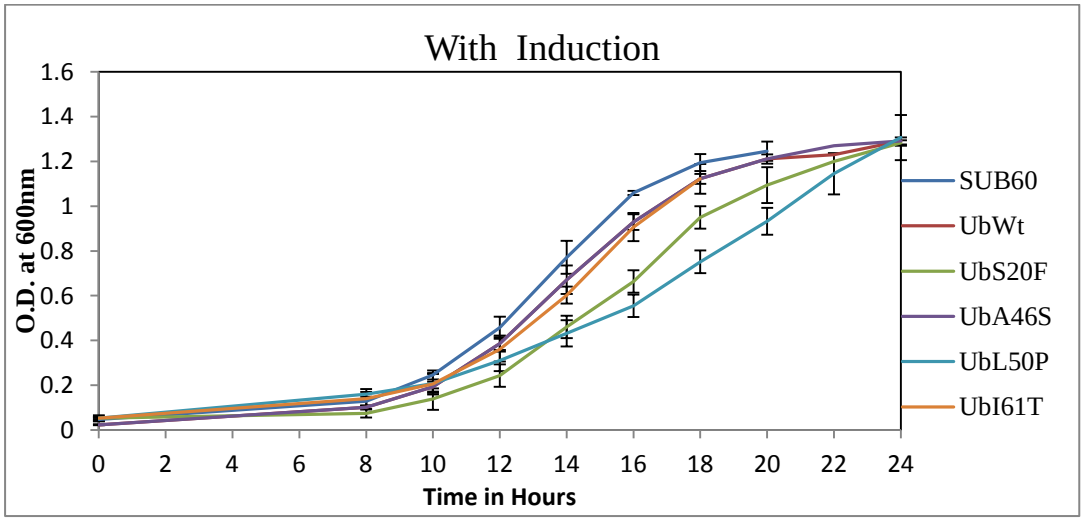
2.4.2.1. Effect of ubiquitin single mutant's gene expression on growth of *S. cerevisiae*

Ubiquitin has a significant role in the regulation of different physiological processes so the single mutations may have adverse effect on the growth rate. Single mutants i.e. UbS20F, UbA46S, UbL50P and UbI61T were transformed into *UBI*4 deleted SUB60 strain of *Saccharomyces cerevisiae* and the effect of mutations in ubiquitin gene on the growth of *S. cerevisiae* was analysed by following its growth curve under uninduced and induced conditions condition in the presence of 25µM of $CuSO_4$ (Fig. 2.11). Among the mutants, the two single mutants A46S and I61T do not

appear to have any influence on the growth rate, while the other two single mutants i.e. UbS20F and UbL50P showed comparatively low growth rates.



(A)



(B)

Fig. 2.11. Growth profile of SUB60 and SUB60 cells transformed with plasmid UbWt, UbS20F, UbA46S, UbL50P and I61T under uninduced and induced condition

2.4.2.2. Generation Time of Ubiquitin single mutants in SUB60 cells

Generation time of ubiquitin mutants i.e. UbS20F, UbA46S, UbL50P and UnI61T, SUB60 cells transformed with wild type ubiquitin and SUB60 were estimated by considering two consecutive values from the log phase stage (Fig. 2.12). Amongst these, mutants namely UbS20F and UbL50P showed significantly increased rate of generation time compared to UbWt whereas no change observed in other mutants.

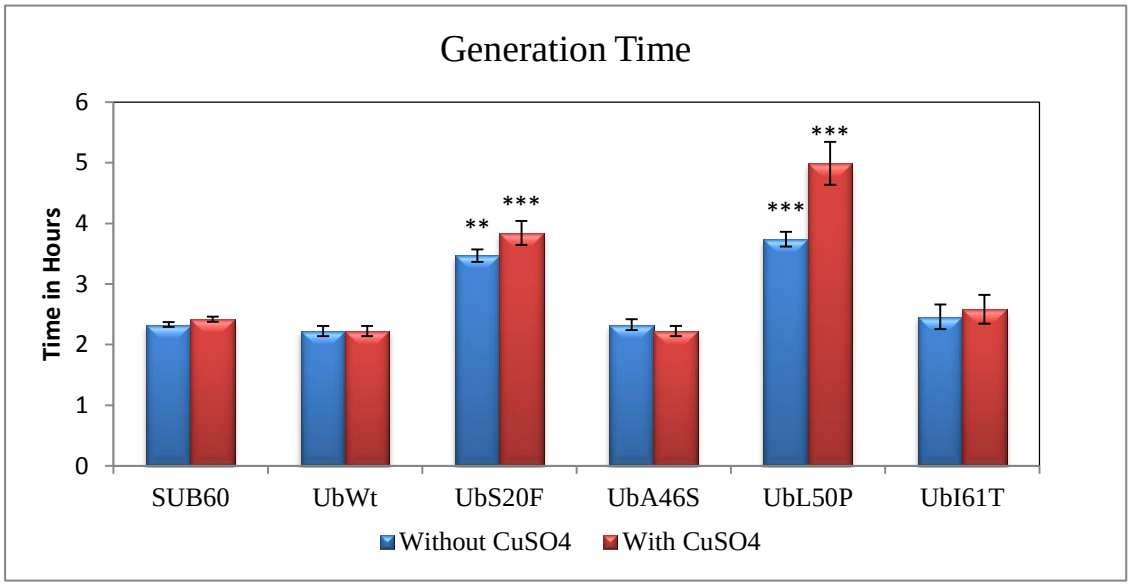


Fig. 2.12. Generation time of SUB60, and SUB60 cells transformed with plasmids UbWt, UbS20F, UbA46S, UbL50P and UbI61T in uninduced and induced condition in the presence of 25 µM of $CuSO_4$.

2.4.2.3. Effect of single mutant ubiquitin proteins on viability of cells

To check the effect of mutant ubiquitin proteins on viability of cells, single mutants namely UbS20F, UbA46S, UbL50P and UbI61T and wild type ubiquitin gene were transformed in *Saccharomyces cerevisiae* strain SUB60 lacking *UBI*4 gene. Viability was studied by taking log phase cultures (OD_{600} ~ 0.5 to 0.6), diluting them serially fourfold followed by plating on selective media. The cells were allowed

to grow at permissible temperature (Fig. 2.13). Result showed that mutants UbS20F and UbL50P with substantial loss of viability in comparison to wild type ubiquitin. Surprisingly, viability of cells expressing UbA46S was increased while viability of UbI61T got decreased in induced condition.

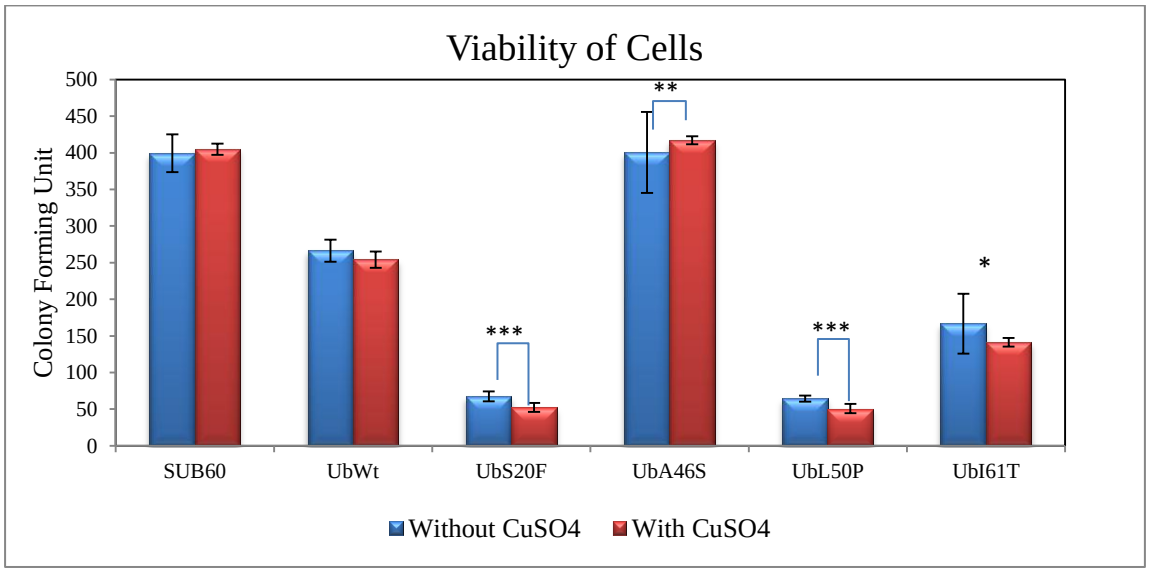


Fig. 2.13. Graph showing colony forming units of *Saccharomyces cerevisiae* strain SUB60 and SUB60 transformed with mutants UbWt, UbS20F, UbA46S, UbL50P and UbI61T. Mutant UbS20F and UbL50P reveal reduced viability in both uninduced and induced conditions (25µM of $CuSO_4$) at permissive temperature.

2.4.2.4. Complementation of Stress hypersensitive phenotype SUB60 cells

SUB60 strain is a UBI4 gene deletion mutant of *S. cerevisiae*, UBI4 gene expresses polyubiquitin under stress conditions. Lack of UBI4 gene in SUB60 makes it hypersensitive to various stress conditions, i.e. exposure to high temperature, antibiotic, amino acid analogue and nutrient depletion. However SUB60 strain transformed with wild type ubiquitin is proved to complement the hypersensitive phenotype. Hence, functionality of mutant ubiquitin gene can be checked by transforming them into SUB60 cells followed by complementation studies. SUB60 and SUB60 transformed with UbWt and single mutants namely UbS20F, UbA46S,

UbL50P and UbI61T were streaked on a plate containing selective media and exposed to permissive temperature at 30°C and non-permissive temperature at 40°C (Heat stress). Plates have analyzed after 2 days for their complementation (Fig. 2.14.). Results suggested that mutants UbL50P and I61T could not complement the heat sensitive phenotype while UbS20F could complement it partially and UbA46S complemented the phenotype completely.

In another set of experiments, the cells were exposed to restrictive temperature (40°C) for variable time intervals as well to assess the functional integrity of the mutant ubiquitin. To start with, SUB60 yeast transformants were grown to log phase and fourfold serially diluted cells were plated on SD selection media and then incubated at higher temperature 40°C for various time interval of 0, 4,8,12 and 16 hours and shifted back to 30°C to resume their growth, the colonies were counted and percentage survival was calculated (Fig. 2.15).

Result suggested that mutants UbL50P and UbI61T could not complement the heat sensitivity phenotype and percentage survival less than that of SUB60, it can be concluded that the substitutions impair some or all of the functions of ubiquitin and UbL50P and UbI61T might act as competitive inhibitors and display more severe effects than UBI4 deletion. There was no difference observed between un-induced and induced conditions. Ubiquitin mutant UbS20F showed percentage survival of ~20% after 16 hrs of heat stress under uninduced condition. While under induced condition in the presence of 25μM of $CuSO_4$ the mutant UbS20F could not survive after 16 hrs. While UbA46S could survive after temperature stress so could complement the heat sensitive phenotype.

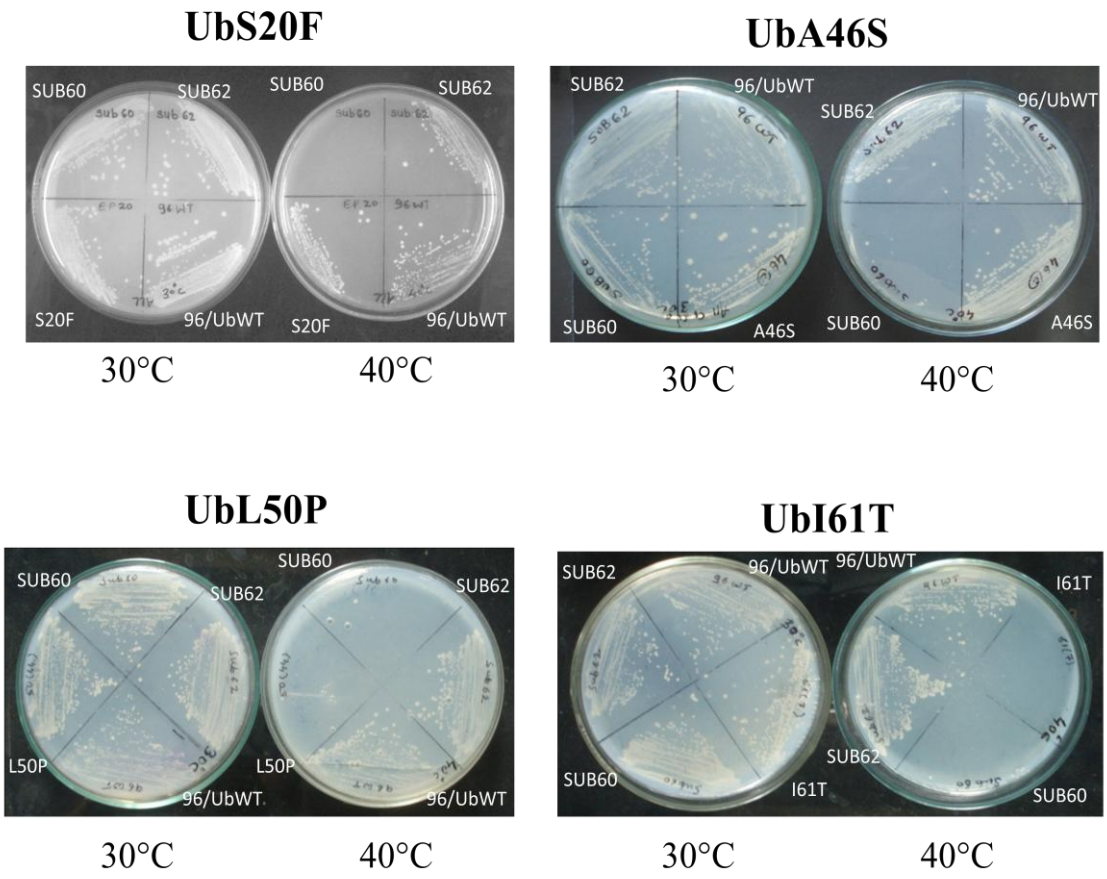
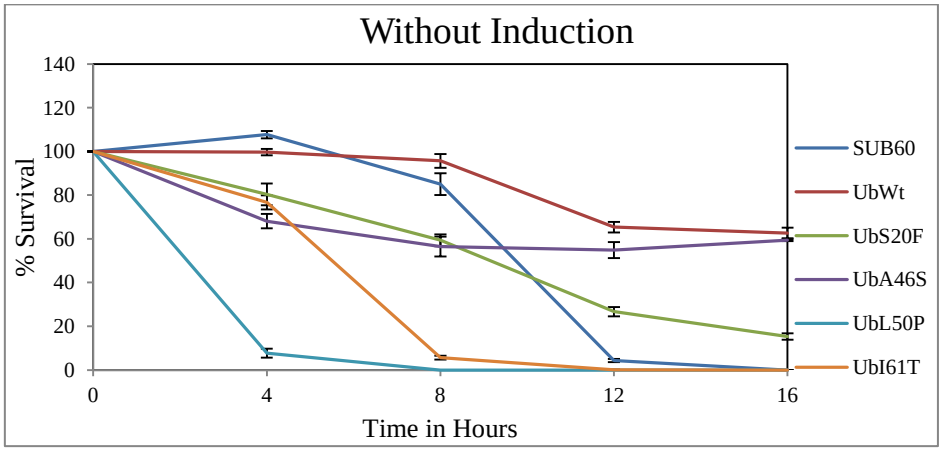
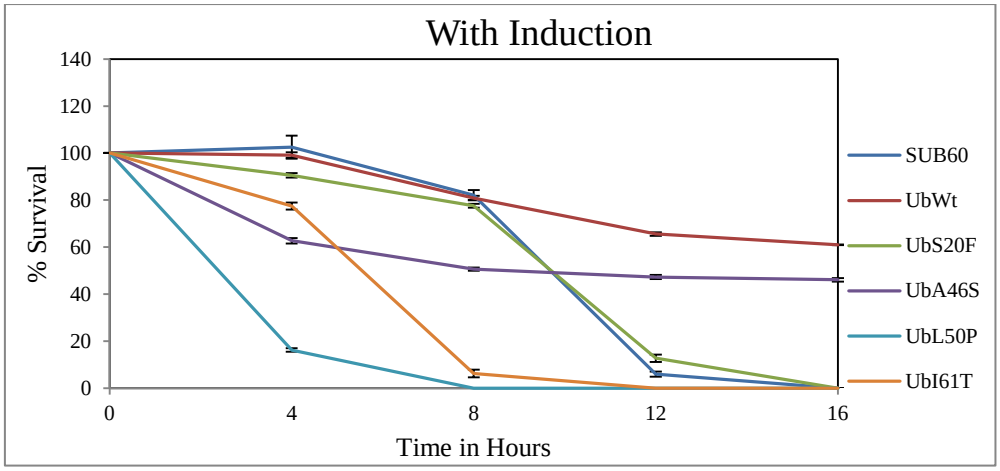


Fig. 2.14. Failure of complementation of stress hypersensitive phenotype by mutated ubiquitin gene in yeast cell transformants. It shows growth of control strains SUB60, SUB62, UbWt along with mutant S20F, A46S, L50P and I61T at permissive temperature 30ºC and at restrictive temperature 40ºC. Fig. shows non complementation of heat sensitive phenotype by hypersensitive strain SUB60, UbL50P and I61T. However rest of the control strain SUB62, UbWt and mutant S20F and A46S show complementation of the same.



(A)



(B)

Fig. 2.15. Graph showing heat stress complementation, heat stress given at 40ºC to *Saccharomyces cerevisiae* strain SUB60 cells and SUB60 cells transformed with mutants UbWt, UbS20F, UbA46S, UbL50P, UbI61T for various time period i.e. 0,4,8,12 and 16 hours in absence and presence of 25µM $CuSO_4$.

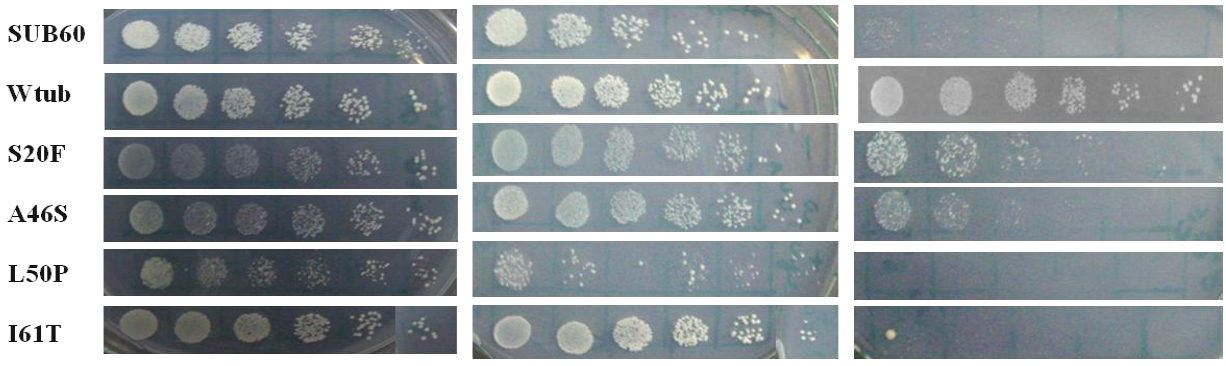
2.4.2.5. Study of the effects of antibiotic cycloheximide on SUB60 cells

In order to check effect of the mutations on ubiquitin's function at translational level, eukaryotic translational inhibitor cycloheximide was used.

Cycloheximide suppresses protein synthesis by binding on large ribosomal subunit. Ubiquitin plays two different roles in helping the cells overcome cycloheximide induced stress. Primarily, one of the proteins in large subunit of ribosome L-28 is linked to polyubiquitin chains with Lys63 linkage. Failure of this post-translational modification makes cells more sensitive to cycloheximide (Hanna et al., 2003). Secondarily, the translational inhibitor causes accumulation of prematurely terminated nascent polypeptide chains. UPS rescues the cells by degrading these unwanted proteins. Cells detect the presence of cycloheximide and stimulate stress sensing UBI4 gene, which in turn helps producing more ubiquitin to degrade nascent proteins and rescue cells from the deleterious effects exerted by cycloheximide.

Ubiquitin pool confers resistance while suppressed level renders sensitivity towards cycloheximide. Presence of non-functional ubiquitin in cytoplasmic pools can lead to breakdown of this machinery by non-productive competition with functional ubiquitin.

Plasmids expressing single mutants of ubiquitin were transformed into stress sensitive SUB60 strain of *S. cerevisiae*, which is a deletion mutant for UBI4 gene. Wild type ubiquitin expressed extra-chromosomally in SUB60 can make the cells stress resistant. *S. cerevisiae* cells transformed with plasmids carrying wild type ubiquitin were used as controls. All transformants were studied to check complementation phenotype towards cycloheximide in *S. cerevisiae* under uninduced and induced condition (Fig. 2.16). Results showed that mutant UbA46S could overcome the deleterious effect of cycloheximide and thus remained resistant towards it while other mutants namely UbS20F, UbL50P and UbI61T became hypersensitive in presence of cycloheximide and could not survive which proves their non-functionality due to mutation. In addition upon over-expression UbL50P showed retardation of growth in presence of 25µM $CuSO_4$, while the growth of other mutants remained unaltered.



(A) (B) (C)

Fig. 2.16. Antibiotic stress complementation of *Saccharomyces cerevisiae* SUB60 and SUB60 transformed with wild type ubiquitin gene and mutants UbS20F, UbA46S, UbL50P, UbI61T.

(A) SUB60, UbWt and ubiquitin single mutants with no $CuSO_4$ and no cycloheximide.

(B) SUB60, UbWt and ubiquitin single mutants with 25µM $CuSO_4$ and no cycloheximide.

(C) SUB60, UbWt and ubiquitin single mutants with 25µM $CuSO_4$ and 4µg of cycloheximide. SUB60 cells transformed with UbWt, UbS20F and UbA46S shows resistance towards antibiotic cycloheximide while stress hypersensitive strain SUB60 and SUB60 transformed with UbL50P, UbI61T fail to grow in presence of cycloheximide hence shows sensitivity towards cycloheximide. Three fold serial dilutions spotted on SD agar plates containing 4µg/ml of cycloheximide.

2.4.2.6. Over-expression of single error prone mutant of ubiquitin

Ubiquitin variant UbEP42 shows lethal phenotype upon over-expression and comprises four mutations i.e. UbS20F, A46S, UbL50P and UbI61T. In order to check which of the single mutations is responsible for such phenotype they were individually over-expressed by adding 100µM and 200µM of copper sulphate (Fig. 2.17). Over-expression results show that lethal phenotype of UBEP42 is contributed by UbL50P and UbI61T. UbL50P shows lethal phenotype at 200µM concentration of copper sulphate whereas UbI61T shows lethal effect at 100 and 200µM concentration of copper sulphate. However UbA46S and UbS20F did not show any lethal consequences.

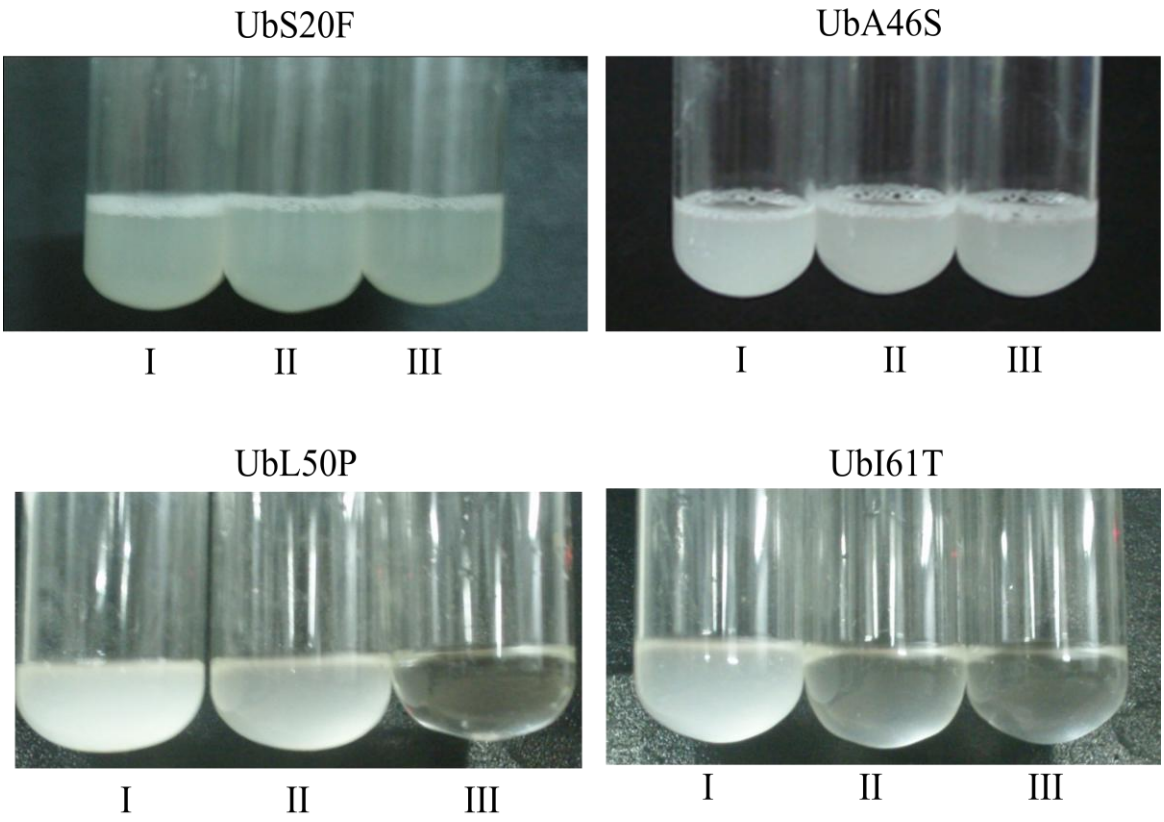


Fig. 2.17. Shows expression of mutants UbS20F, UbA46S, UbL50P, UbI61T and UbEP42 in presence of 0, 100 and 200 µM of copper sulphate.

2.4.2.7. Western blot analysis of ubiquitin mutant UbL50P and UbI61T in *S. cerevisiae*

From previous complementation analysis and over-expression studies it is clear that UbA46S complements the heat sensitive phenotype and UbS20F complements partially. While UbL50P and UbI61show non-complementation or functional failure of protein. To answer the questions, whether these mutant ubiquitins are unable to make polyubiquitin chains or despite forming chains they are unable to act as degradation signals on substrate proteins at the level of proteasomes, UbL50P and UbI61T have been tested for their ability to make polyubiquitin chains, UbL50P and UbI61T mutants have been cloned in myc tagged pUb221 yeast expression

vector. Polyubiquitination ability of the mutants has been checked by western blot analysis using anti-myc antibodies (Fig. 2.18).

Tagging with myc was necessary to distinguish the ubiquitin molecules carrying the mutation from basal levels of ubiquitin which is present in the background, as ubiquitin is expressed by more than one gene in the yeast cells. The myc-tagged UbL50P and UbI61T were utilized for polyubiquitination and were not discriminated by ubiquitination machinery. But increased intensity of signal in higher molecular weight protein range indicates their failure in being recognized as tags by proteasomal machinery while degrading substrate protein, whereas myc tagged wild type ubiquitin shows lesser intensity of higher molecular weight protein which causes timely degradation of substrate protein.

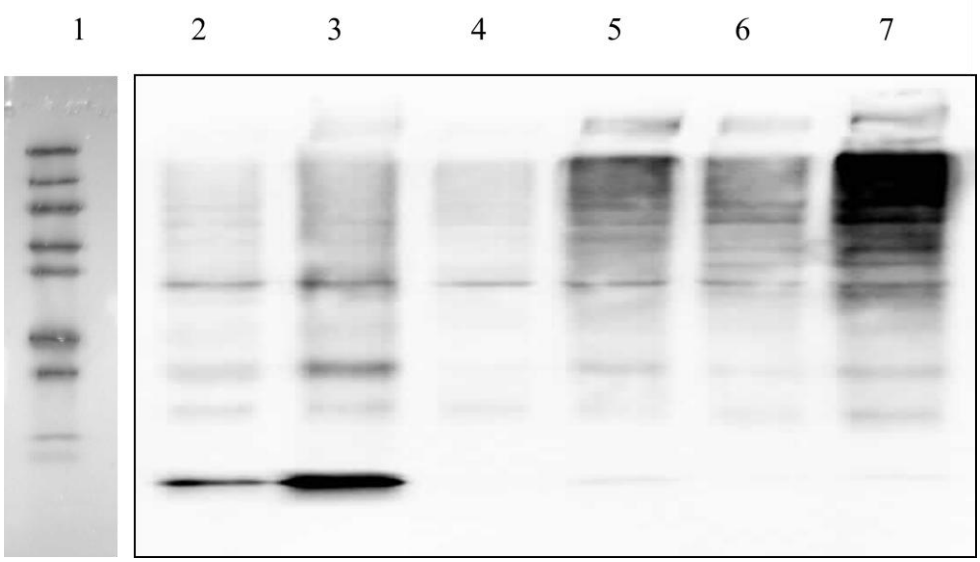


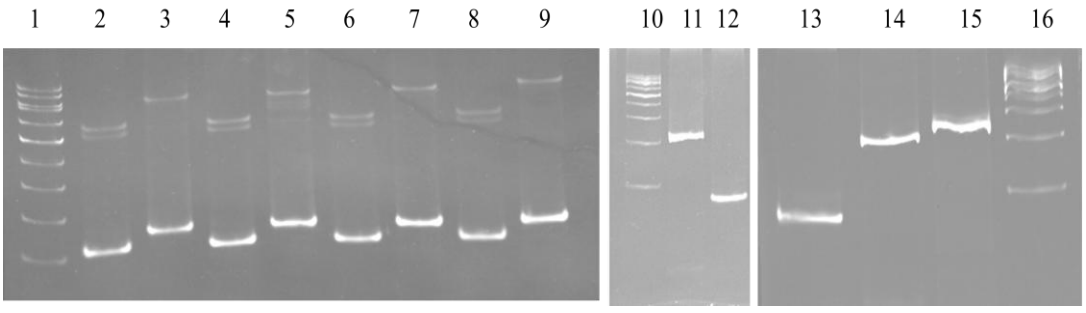
Fig. 2.18. Western blot showing polyubiquitination profile. Lane 1. Rain bow molecular weight marker. Lane 2. pUb221/Wt Lane 3. pUb221/Wt with 25 µM $CuSO_4$, Lane 4. pUb221/UbL50P Lane 5. pUb/UbL50P with 25 µM $CuSO_4$ Lane 6. pUb221/UbI61T with 25 µM $CuSO_4$

2.4.3. Construction and functional evaluation of double mutant of UbEP42 derivative in *Saccharomyces cerevisiae*

2.4.3.1. Generation of double mutant forms of ubiquitin UbS20F-A46S, UbS20F-L50P, UbS20F-I61T, UbA46S-UbL50P, UbA46S-I61T and UbL50P-I61T by site directed mutagenesis using recombinant PCR

Primer based recombinant PCR strategy has been used to construct all the above mentioned double mutants. The site directed mutants namely UbS20F-A46S, UbS20F-L50P, UbS20F-I61T were generated by taking UbS20F as a template for PCR and then amplified with the help of mutagenic primers for 46^{th}, 50^{th} and 61^{st} sites respectively. Mutants UbA46S-I61T and UbL50P-I61T were generated by taking UbI61T as a template and amplified with mutagenic primers for 46^{th} and 50^{th} sites respectively. Mutant UbA46S-L50P was generated with the help of primer designed to bear mutations at 46^{th} and 50^{th} positions. Amplified PCR produt by different combination of primers are shown in Fig. 2.19.

Incorporation of the mutation in amplified PCR product has been confirmed by digesting PCR product of mutant ubiquitin gene by *Xba*I for UbS20F, *Xho*I for UbA46S and UbL50P and *Sal*I for UbI61T mutations. PCR products of all the confirmed mutants were double digested with *Bgl*II and *Kpn*I restriction enzymes and cloned in yeast expression vector YEp96 for their functional evaluation in *S. cerevisiae*.



(A) **(B)** **(C)**

Fig. 2.19. Gel picture showing amplified PCR products from vector YEp96/ Wt ubiquitin by using sets of mutagenic and their non-mutagenic counterparts in combination.

(A) Lane 1,10 and 16 shows 100bp ladder, lane 2, 3 show 106 and 162bp amplification by using 46FP, 175RP and 175 FP, 46RP respectively for constructing UbS20F-A46S mutation Lane 4, 5 shows 106 and 162bp amplification from 50FP, 175 RP and 175 FP, 50RP respectively for constructing S20F-L50P mutation. Lane 6,7 shows 101 and 162bp from 61FP, 175 RP and 175 FP, 61RP for constructing UbS20F-I61T mutation respectively. Lane 8, 9 show 106 and 162bp from 46-50FP, 175 RP and 175 FP, 46-50RP respectively for constructing UbA46S-L50P mutation.

(B) Lane 11 and 12 shows 200 and 30bp amplification from 61FP, 175RP and 175FP, 61RP respectively for constructing UbA46S-I61T.

(C) Lane 13, 14 shows 106 and 162bp amplification from 50FP, 175 RP and 175 FP, 50RP respectively for constructing UbL50P-I61T mutation. Lane 15 shows recombined PCR product comprising full length mutant ubiquitin gene.

2.4.3.1.1. Cloning of UbS20F-A46S, UbS20F-L50P, UbA46S-L50P, UbS20F-I61T, UbA46S-I61T and UbL50P-I61T mutant in yeast expression vector YEp96

In order to construct above mentioned double mutations, single mutants UbS20F, UbI61T and UbWt have been taken as a template and amplified by mutagenic primer for requisite mutation sites. Incorporation of mutations in PCR product have checked by digesting it with *Xba*I , *Xho*I and *Sal*I restriction enzymes and cloned back in YEp96 vector by using *Bgl*II and *Kpn*I restriction sites. Detail strategy used to make and confirm the mutations are mentioned in the table 2.3 listed below. Further PCR products were sequenced to confirm the presence of mutations. Screening of the mutant was done by digesting plasmid with *Xho*I and *Xba*I.

2.4.3.1.1.1. Cloning of UbS20F-A46S mutant in yeast expression vector YEp96

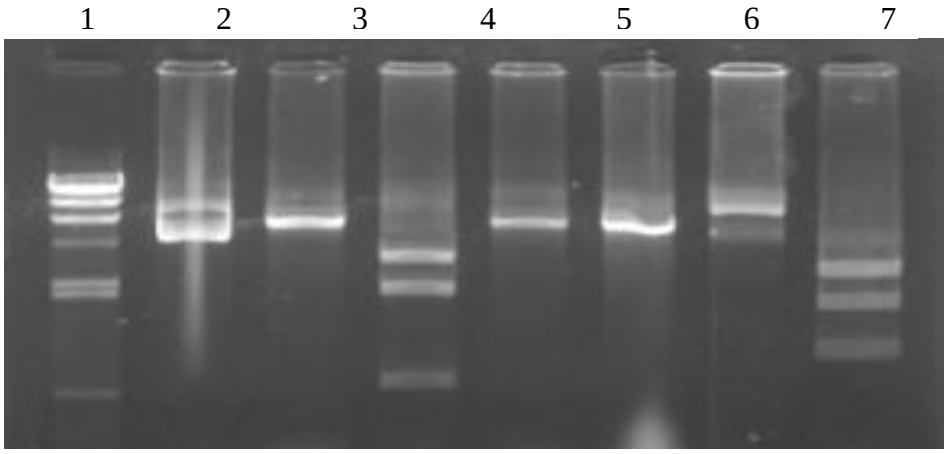
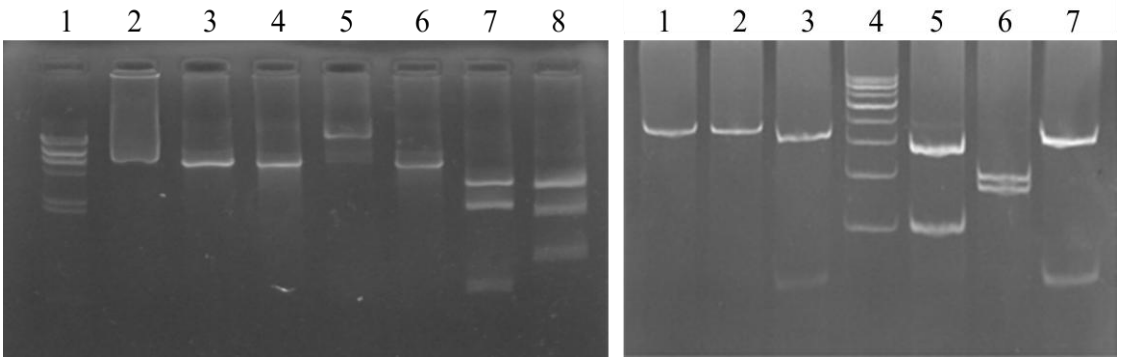


Fig.2.20. Gel picture showing digestion Pattern of UbS20F-A46S plasmid digestion

Lane1 shows λ *Hind*III Digest Marker. Lane2 has undigested plasmid. Lanes 3, 4,5,6,7 and 8 show digestion pattern of the plasmid by *Bgl*II, *Hind*III, *Kpn*I, *Sal*I, *Xho*I and *Xba*I respectively. In Lane 7 *Xho*I does not digest the plasmid and in lane 8 *Xba*I gives three bands instead of four indicating incorporation of mutations.

2.4.3.1.1.2. Cloning of UbS20F-L50P mutant in yeast expression vector YEp96



(A) **(B)**

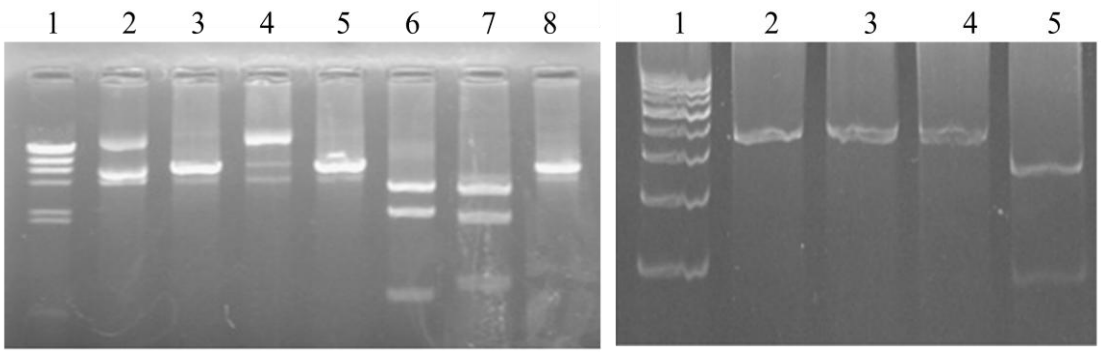
Fig. 2.21. Gel picture depicts digestion Pattern of UbS20F-L50P plasmid and PCR Product digestion.

Gel pictures showing (A) digestion Pattern of UbS20F-L50P plasmid and (B) 350bp PCR Product UbS20F-L50P digestion.

(A) Lane1 shows λ *Hind*III digest Marker. Lane2 has undigested plasmid. Lanes 3, 4,5,6,7 and 8 show digestion pattern of the plasmid by *Bgl*II, *Kpn*I, *Xho*I, *Sal*I, *Hind*III and *Xba*I. In Lane 5 *Xho*I does not digest the plasmid and in lane 6 *Xba*I gives 3 bands instead of 4 indicating incorporation of mutations.

(B) Lane 1 and 2 shows failure of digestion of 350bp amplicon with *Xho*I and *Xba*I, indicates incorporation of desired mutation, Lane 3 shows *Sal*I digestion, Lane 4 shows 100bp DNA marker, Lane 5, 6 and 7 shows UbWt digestion pattern with *Xho*I, *Xba*I and *Sal*I respectively.

2.4.3.1.1.3. Cloning of UbS20F-I61T mutant in yeast expression vector YEp96



(A) **(B)**

Fig. 2.22. Gel picture showing digestion Pattern of UbS20F-I61T plasmid and PCR Product.

(A) Gel pictures showing digestion Pattern of UbS20F-I61T plasmid and 350bp PCR Product UbS20F-I61T digestion. Lane1 shows λ *Hind*III Digest Marker. Lane2 has undigested plasmid. Lanes 3,4,5,6,7 and 8 show digestion pattern of the plasmid by *Bgl*II, *Sal*I *Kpn*I, *Hind*III, *Xba*I and *Xho*I. In Lane 4 Sal I does not digest the plasmid and in lane 7 *Xba*I gives 3 bands instead of 4 indicating incorporation of mutations.

(B) Lane 1shows 100bp DNA Ladder, lane 2 shows 350bp undigested PCR amplicon and lane 3, 4 shows failure of digestion of 350bp amplicon with *Xba*I and *Sal*I, indicates incorporation of desired mutation, Lane 5 digestion with *Xho*I.

2.4.3.1.1.4. Cloning of UbA46S-L50P mutant in yeast expression vector YEp96

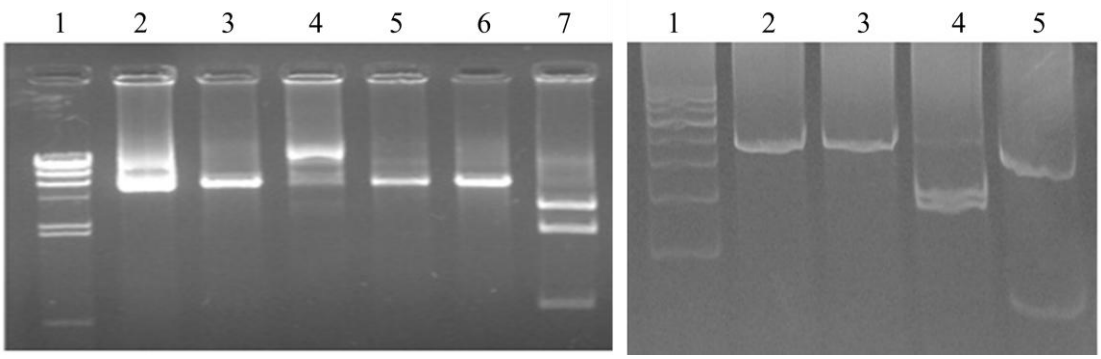


Fig. 2.23. Gel picture showing digestion Pattern of UbA46S-L50P plasmid and its PCR Product

(A) Gel pictures showing digestion Pattern of UbA46S-L50P plasmid and 350bp PCR Product UbA46S-L50P digestion. Lane1 shows λ *Hind*III Digest Marker. Lane2 has undigested plasmid. Lanes 3, 4, 5, 6, 7 and 8 show digestion pattern of the plasmid by *Bgl*II, *Xho*I, *Kpn*I, SalI, *Hind*III. In Lane 4 *Xho*I does not digest the plasmid.

(B) Lane 1 shows 100bp DNA ladder, Lane 2 shows undigested 350bp PCR amplicon, lane 3 shows digestion failure of 350bp amplicon with *Xho*I indicates incorporation of desired mutation. Lane 4 and 5 show digestion with *Xba*I and *Sal*I respectively.

2.4.3.1.1.5. Cloning of UbA46S-I61T mutant in yeast expression vector YEp96

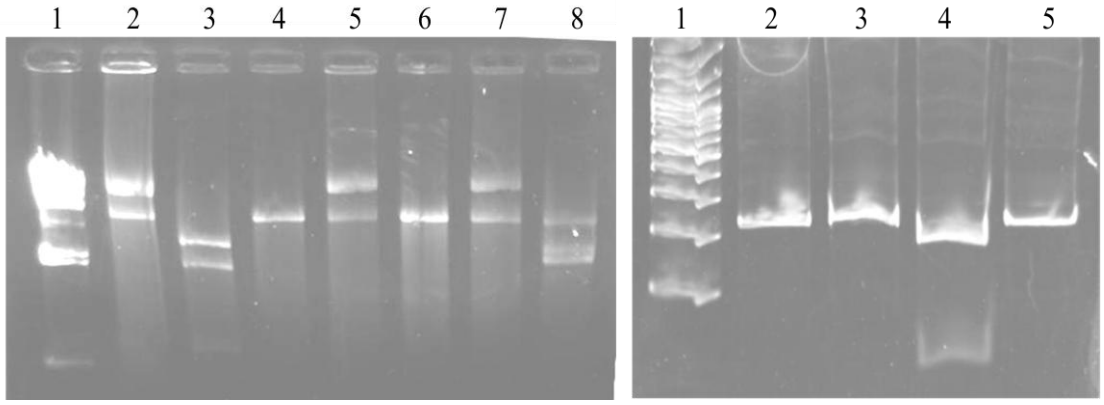
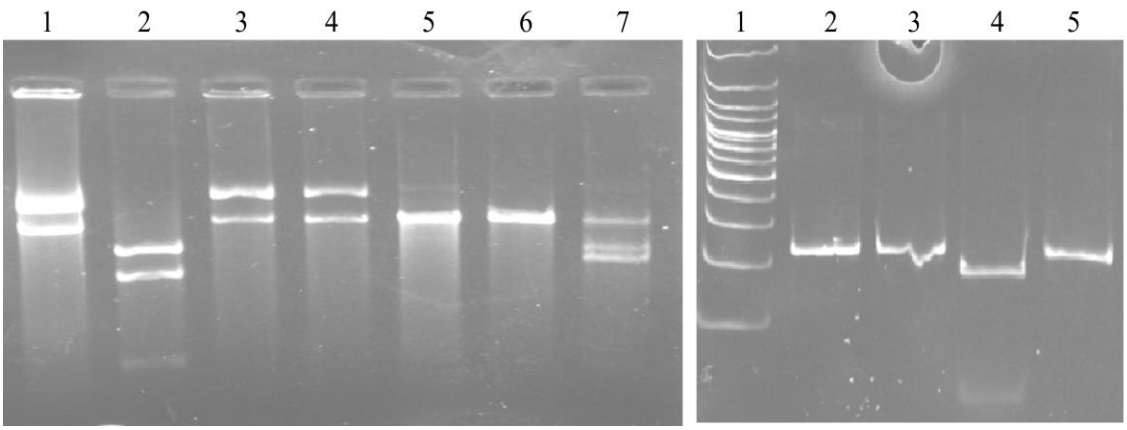


Fig. 2.24. Gel pictures showing (A) digestion Pattern of YEp96 UbA46S- I61T plasmid and (B) 250bp PCR Product of mutant UbA46S-I61T digestion.

(A) Lane1 shows λ *Hind*III Digest Marker. Lane2 has undigested plasmid. Lanes 3,4,5,6,7 and 8 show digestion pattern of the plasmid by *Hind*III, *Bgl*II *Xho*I, *Kpn*I, *Sal*I and *EcoR*I Lane 5 and 7 shows failure of digestion of mutant with restriction enzyme *Xho*I and *Sal*I hence confirm the incorporation of respective mutations.

(B) Lane 1 shows 100bp DNA ladder, Lane 2 shows undigested 250bp PCR amplicon, lane 3 and 5 shows digestion failure of 250bp amplicon with *XhoI* and *Sal*I respectively indicates incorporation of desired mutation. Lane 4 shows digestion of PCR amplicon with *Xba*I restriction enzyme.

2.4.3.1.1.6. Cloning of UbL50P-I61T mutant in yeast expression vector YEp96



(A) **(B)**

Fig. 2.25. Gel pictures showing digestion Pattern of YEp96 UbL50P-I61T plasmid and digestion pattern of 250bp PCR Product of UbA46S-L50P.

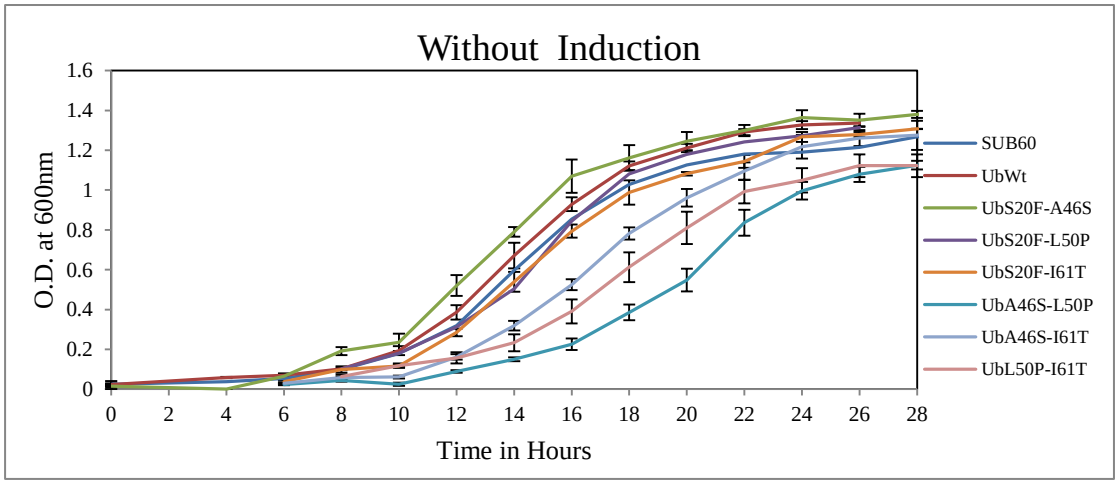
(A) Lane1 has undigested plasmid. Lanes 2, 3,4,5,6,7 and 8 show digestion pattern of the plasmid by *Hind*III, *Xho*I, *Sal*I, *Kpn*I, *Bgl*II and *EcoR*I Lane 3 and 4 shows failure of digestion of mutant with restriction enzyme *Xho*I and *Sal*I hence confirm the incorporation of respective mutations.

(B) Lane 1 shows 100bp DNA ladder, Lane 2 shows undigested 250bp PCR amplicon, lane 3, 4 and 5 shows digestion of PCR product with *Xho*I, *Xba*I and *Sal*I respectively, lane 3 and 5 shows digestion failure of 250bp amplicon with restriction enzyme *Xho*I and *Sal*I hence indicates incorporation of desired mutation.

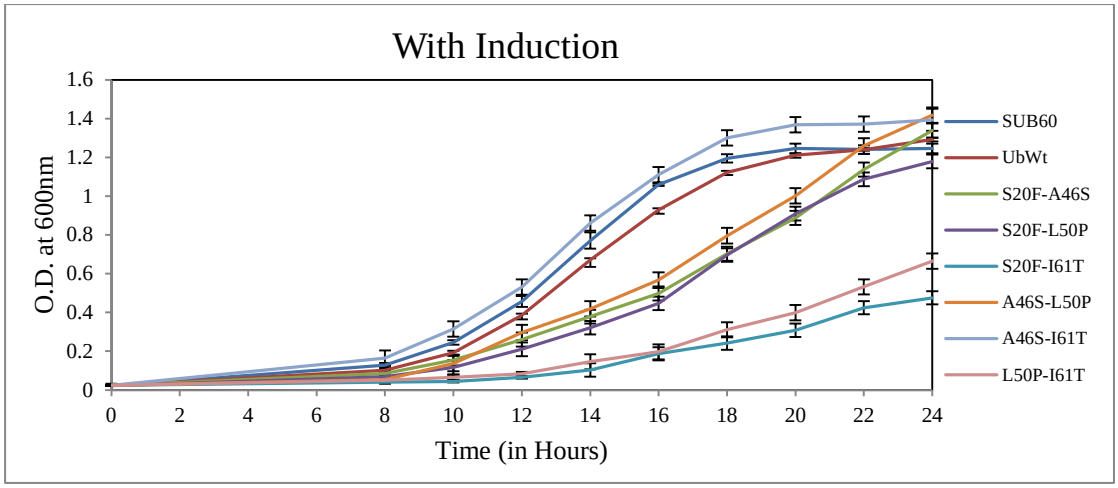
2.4.3.2. Functional evaluation of double mutants of UbEP42 in *Saccharomyces cerevisae*

2.4.3.2.1. Effect of ubiquitin double mutant's gene expression on growth of *S. cerevisiae.*

Freshly grown cultures were used as inoculum for the growth curve. Optical density was recorded at 600nm at regular intervals of two hours (Fig. 2.26). The cultures of SUB60 cells expressing wild type and mutant forms of ubiquitin were grown and their growth was compared in uninduced and induced conditions. Among all double mutants, UbA46S-L50P has slower growth in comparison to wild type ubiquitin under basal level expression while overexpression of mutant ubiquitins UbS20F-L50P, UbS20F-I61T, UbA46S-UbL50P, UbL50P-I61T with 25µm $CuSO_4$ have drastically reduced the growth of mutants. Result shows the interference of mutant ubiquitin with the normal growth supporting mechanism.



(A)



(B)

Fig. 2.26. Growth profile of SUB60 and SUB60 cells transformed with plasmids UbWt, UbS20F-A46S, UbS20F-L50P, UbS20F-I61T, UbA46S-UbL50P, UbA46S-I61T, UbL50P-I61T under un-induced and induced condition (25 µm $CuSO_4$)

2.4.3.2.2. Generation Time of double mutants of ubiquitin

The effect of mutations on the generation time was analyzed, (Fig.2.27) using the relation given in Materials and Methods. For the calculation of Generation time, two OD600 values from the mid log phase were considered. The generation time increased in all double mutants in induced state except UbA46S-I61T mutant. Generation time of UbA46S-L50P is 3.5 and 4.3 hrs respectively under uninduced and induced condition. While under induced conditions S20F-A46S, S20F-L50P, S20F-I61T and L50P-I61T show generation times of approximately 3.58, 3.56, 6.16 and 5.40 hrs respectively. On the other hand generation time of UbA46S-I61T remains near to UbWt.

Increased generation time can be attributed to combinations of mutations which can bring more structural deformities affecting their ability to bind other proteins. Consequently, the mutations influence the overall growth of cells.

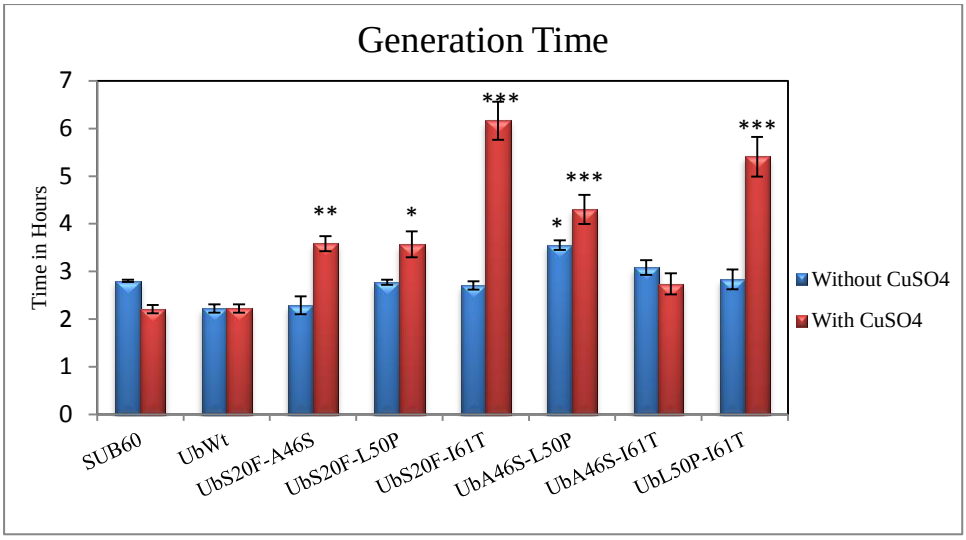


Fig. 2.27. Generation time of SUB60 and SUB60 cells transformed with plasmids UbWt, UbS20F-A46S, UbS20F-L50P, UbS20F-I61T, UbA46S-UbL50P, UbA46S-I61T and UbL50P-I61T in absence and presence of 25 µm $CuSO_4$.

2.4.3.2.3. Viability of double mutants of ubiquitin

SUB60 yeast transformants were grown to log phase with optical density of the cultures reaching a value between 0.5 and 0.6 and serially diluted fourfold and plated on SD selection media. Plates were incubated at 30°C and the colonies were counted.

Colony forming units decreased in case of all double mutants except UbA46S-I61T under uninduced and induced conditions. Number of colonies of ubiquitin mutants S20F-L50P, S20F-I61T, A46S-L50P and L50P-I61T were found to be decreased to 35%, 22%, 13% and 23% under uninduced condition while 33%, 17%, 10% and 14% under induced condition respectively. While colony count for S20F-A46S and A46S-I61T under uninduced and induced conditions remained close to positive control UbWt (Fig.2.28). The reduction in viabilty of S20F-L50P, S20F-I61T, A46S-L50P and L50P-I61T can be attributed to additive effects of mutations within ubiquitin.

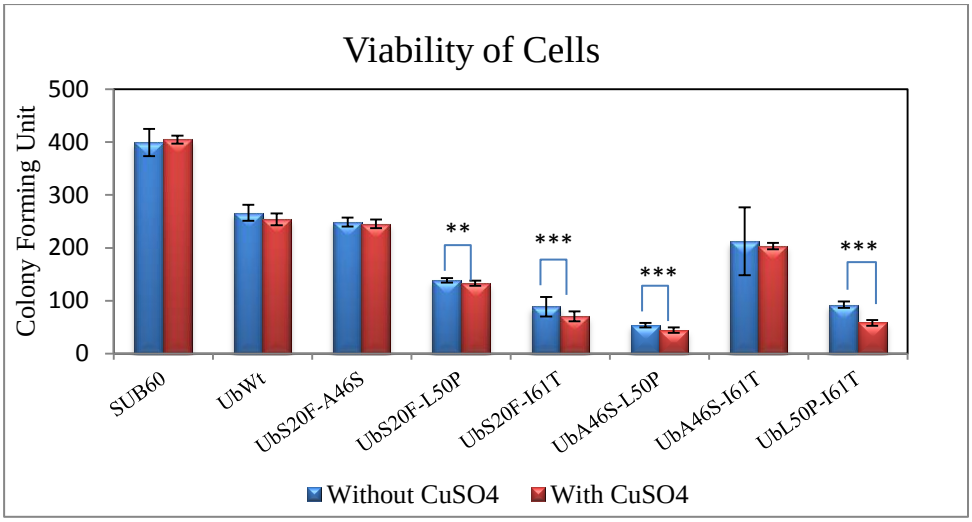
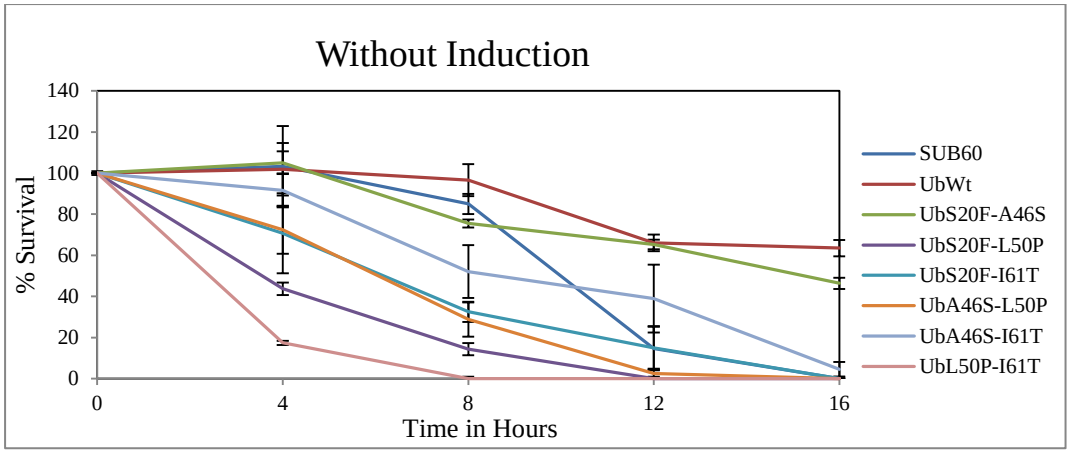


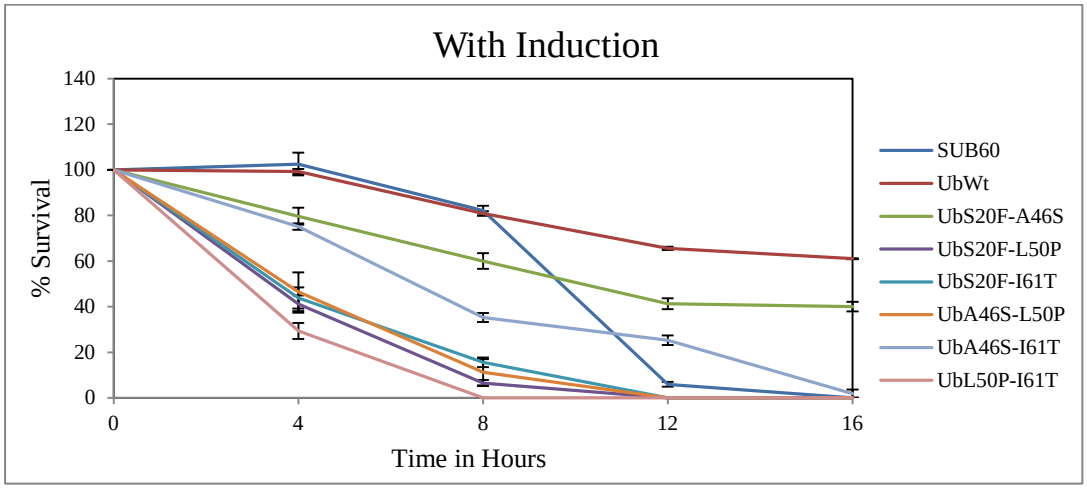
Fig. 2.28. Graph showing colony forming units of *Saccharomyces cerevisiae* strain SUB60, and SUB60 transformed with mutants UbWt, UbS20F-A46S, UbS20F-L50P, UbS20F-I61T, UbA46S-UbL50P and UbL50P-I61T in absence and presence of 25 µm $CuSO_4$.

2.4.3.2.4. Heat Stress complementation of double mutants of ubiquitin

Heat stress hypersensitivity of the mutants has been analyzed by applying chronic heat stress which shows that mutant ubiquitins UbA46S-I61T, UbS20F-I61T, UbS20F-L50P, UbA46S-L50P and UbL50P-I61T are hypersensitive to stress heat under uninduced and induced conditions (Fig. 2.29). Mutant ubiquitins A46S-I61T and S20F-L50P, S20F-I61T, A46S-L50P and L50P-I61T have shown decrease in percentage survival under both induced and uninduced conditions. The effect under induced conditions is more pronounced. The mutants UbS20F-L50P, UbA46S-L50P and UbL50P-I61T are more severe in terms of degree of sensitivity towards heat and reduction in time of exposure when compared to UBI4 deleted SUB60 strain. Non-complementation of mutant ubiquitin shows that mutant ubiquitin is unable to participate in the normal functioning of the disposal system and probably due to competitive inhibition exerted by mutant ubiquitin.



(A)



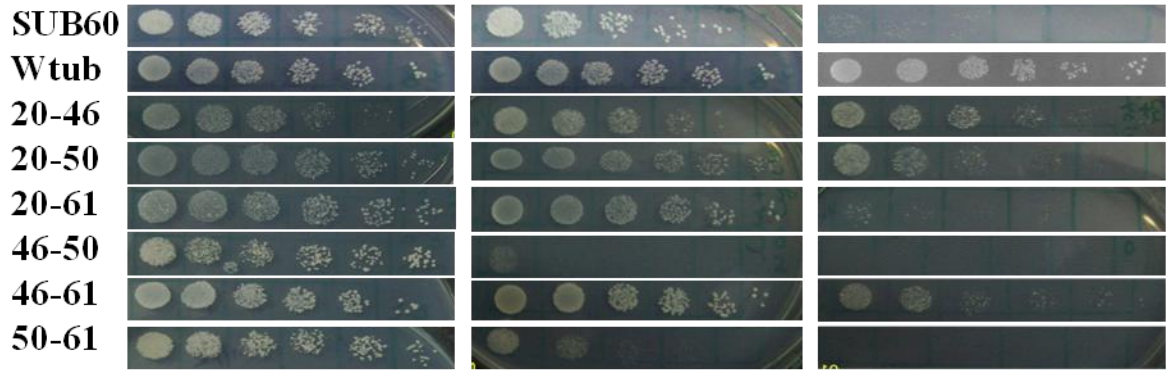
(B)

Fig. 2.29. Graph showing heat stress complementation, heat stress given to *Saccharomyces cerevisiae* strain SUB60 cells and SUB60 cells transformed with mutants UbA46S-I61T, UbS20F-I61T, UbS20FL50P, UbS20F-A46S, UbL50P-I61T and UbA46S-L50P for various time period i.e. 0,4,8,12 and 16 hours.

2.4.3.2.5. Cycloheximide Sensitivity Test

SUB60 cells transformed with UbWt and mutants UbS20F-A46S, UbS20F-L50P, UbS20F-I61T, UbA46S-L50P, UbA46S-I61T and UbL50P-I61T exposed to antibiotic stress by adding cycloheximide in the selection medium to test the functionality of mutant ubiquitin. Results showed that mutants UbS20F-L50P,

UbA46S-L50P and UbL50P-I61T became failed to show complementation in cycloheximide, while other mutants UbS20F-A46S, UbS20F-I61T, UbA46S-I61T showed resistance towards antibiotic, a sign of complementation (Fig. 2.30).



(A) (B) (C)

Fig. 2.30. Antibiotic stress complementation of *Saccharomyces cerevisiae* SUB60 strain and SUB60 strain transformed with wild type ubiquitin gene (UbWt) and ubiquitin double mutants UbS20F-A46S, UbS20F-L50P, UbS20F-I61T, UbA46S-L50P, UbA46S-I61T, UbL50P-I61T. SUB60, UbWt (A) with no $CuSO_4$ and no cycloheximide, (B) with 25µM $CuSO_4$ and no cycloheximide, (C) with 25µM $CuSO_4$ and 4µg of cycloheximide.

2.4.3.2.6. Over-expression of double mutants of dose dependent lethal mutant Ubiquitin

Earlier laboratory studies have shown that mutant ubiquitin UbEP42 caused lysis of host cells when over expressed by adding $CuSO_4$ in the selection medium. Over-expression produces more copies of mutant ubiquitin inside cell hence its effects are more drastic. Double mutant combination of UbEP42 were analysed by over-expression to know which of the combinations execute dose dependant lethality.

In order to check the effect of over-expression of mutant ubiquitin gene UBI4 deleted SUB60 Strain of *S. cerevisiae* was selected and plasmid YEp96 carrying the double mutants i.e. UbS20F-A46S, UbS20F-I61T, UbS20F-L50P and UbA46S-L50P,

UbA46S-I61T and UbL50P-I61T were transformed in YEp96, with UbWt was used as a positive control. All ubiquitin mutants were expressed by adding 0,100 and 200µM $CuSO_4$ in the medium. Mutants UbS20F-L50P, UbA46S-L50P and UbL50P-I61T showed lethality while grown in presence of inducer. However remaining mutants i.e. UbS20F-A46S, UbS20F-I61T and UbA46S-I61T could survive even at 200µM $CuSO_4$ concentration and thus do not interfere with normal functioning of cells (Fig. 2.31).

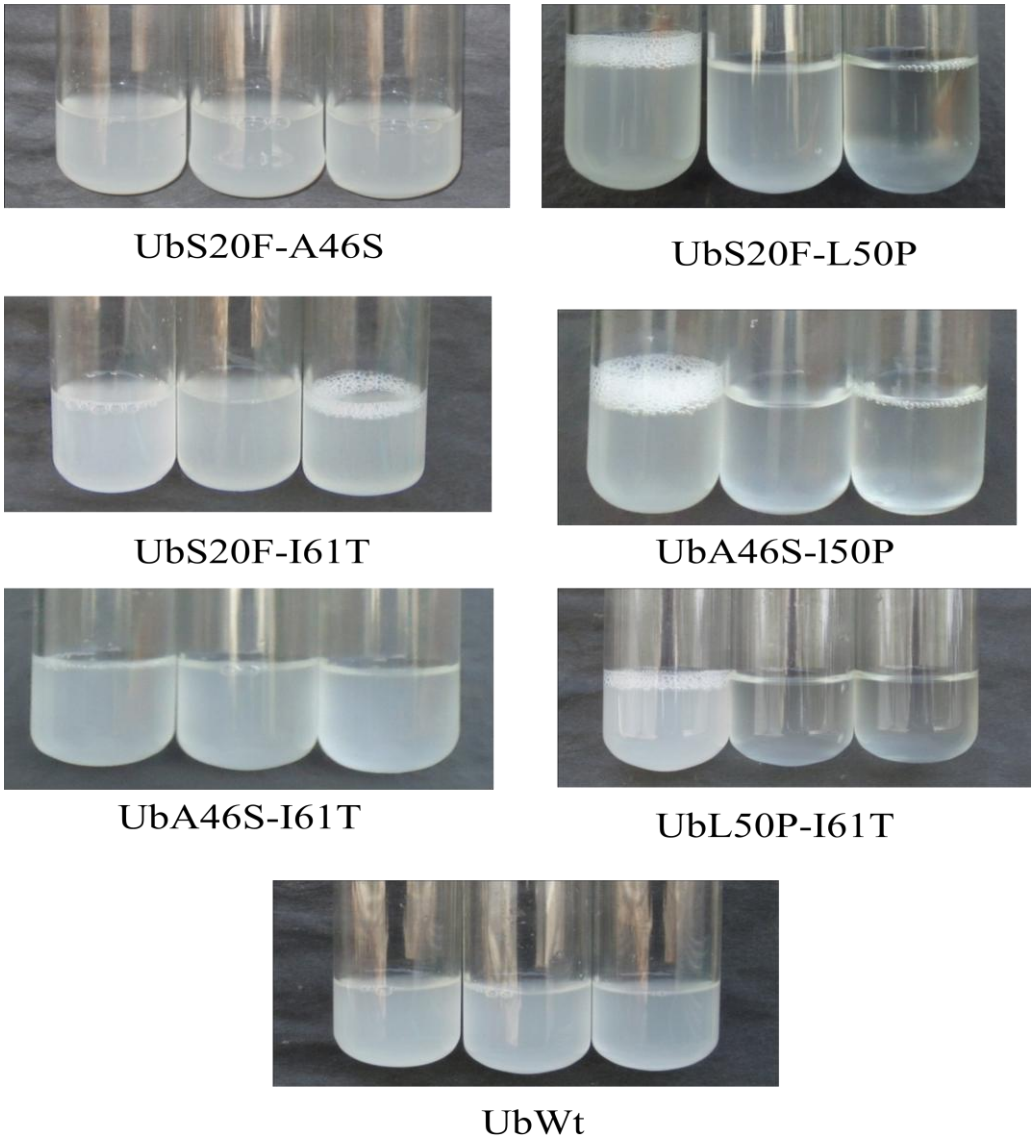


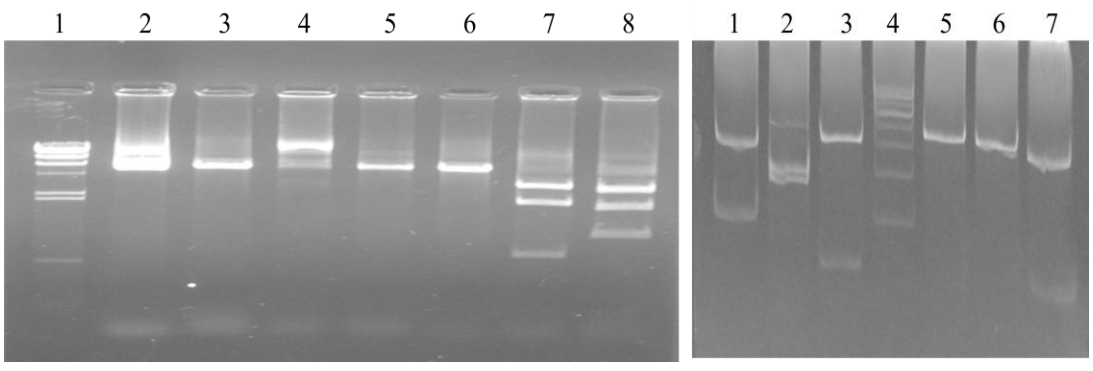
Fig. 2.31. Effect of expression of mutant ubiquitins UbS20F-A46S, UbA46S-L50P, UbL50P-I61T, UbA46S-I61T, UbS20F-L50P, UbS20F-I61T and UbEP42 in presence of 0, 100 and 200µM of copper sulphate.

2.4.4. Construction and functional evaluation of Triple mutant of UbEP42 derivative in *Saccharomyces cerevisiae*

2.4.4.1. Generation of Triple mutant forms of ubiquitin UbS20F-A46S--L50P, UbS20F-A46S-I61T, UbS20F-L50P-I61T and UbA46S-L50P-I61T by site directed mutagenesis

Double mutants UbS20F-A46S, UbA46S-L50P and UbS20F-L50P have been taken as starting templates for PCR to generate the Triple mutants bearing combinations of S20F, A46S, L50P and I61T mutations and amplified by primers bearing point mutations at 20^{th} , 46^{th} , 50^{th} and 61^{st} sites using recombinant PCR and then whole gene harboring combinations of three mutations was amplified by gene specific PCR primers. PCR products were double digested with *Bgl*II and *Kpn*I restriction enzymes and then cloned in yeast expression vector YEp96. Incorporation of mutation was confirmed by digestion pattern of mutant plasmids and their PCR amplicons by digesting them with *XbaI, XhoI and Sal*I restriction enzymes. Detailed strategy used to construct and confirm the mutations is given in the Table 2.3. Further the gene constructs were sequenced to confirm the presence of desired mutations.

2.4.4.1.1. Construction of UbS20F-A46S-L50P mutant in yeast expression vector YEp96



(A) (B)

Fig. 2.32. Gel picture shows digestion pattern of YEp96 UbS20F-A46S-L50P mutant.

(A) Gel pictures showing digestion Pattern of UbS20F-A46S-L50P plasmid and 350bp PCR Product UbS20F-A46S-L50P digestion. Lane1 shows λ *Hind*III digest as marker. Lane2 has undigested plasmid. Lanes 3, 4, 5, 6, 7 and 8 show digestion pattern of the plasmid by *Bgl*II, *Xho*I, *Sal*I, *Kpn*I, *Xba*I and *Hind*III. In Lane 4 *Xho*I failed to digest the plasmid. In lane 7 *Xba*I gives 3 bands instead of 4 indicating incorporation of mutation at 20th position.

(B) Lane 1, 2, 3 show digestion pattern of YEp96 Wt gene 350bp PCR product with *Xho*I, *Xba*I and *Sal* I. lane 4 shows 100bp DNA marker. Lane 5, 6, 7 show digestion with *Xba*I, *Xho*I and *Sal*I. In lane 5 and 6 PCR products remain undigested by *Xba*I and *Xho*I respectively, which confirms incorporation of respective mutations.

2.4.4.1.2. Construction of UbS20F-A46S-I61T mutant in yeast expression vector YEp96

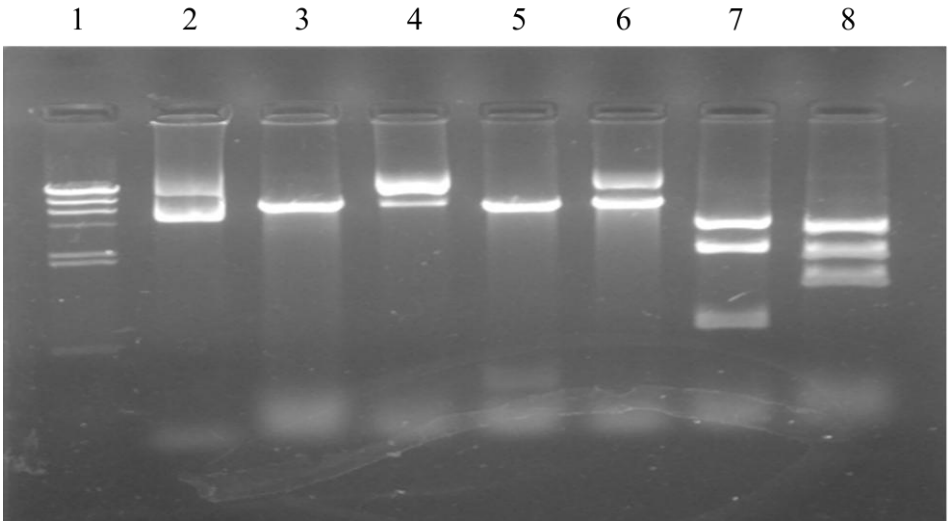


Fig. 2.33. Gel picture shows digestion Pattern of YEp96 UbS20F-A46S-L50P mutant.

Gel pictures showing - Lane1 shows λ *Hind*III digestion marker. Lane 2 has undigested plasmid. Lanes 3, 4, 5, 6, 7 and 8 show digestion pattern of the plasmid by *Bgl*II, *Xho*I, *Kpn*I, *Sal*I, , *Xba*I and *Hind*III, and. In Lanes 4 and 6 *Xho*I and *Sal*I does not digest the plasmid. In lane 7 *Xba*I gives 3 bands instead of 4 indicating incorporation of mutation at 20th, 46th and 61st positions.

2.4.4.1.3. Construction of UbA46S-L50P-I61T mutant in yeast expression vector YEp96

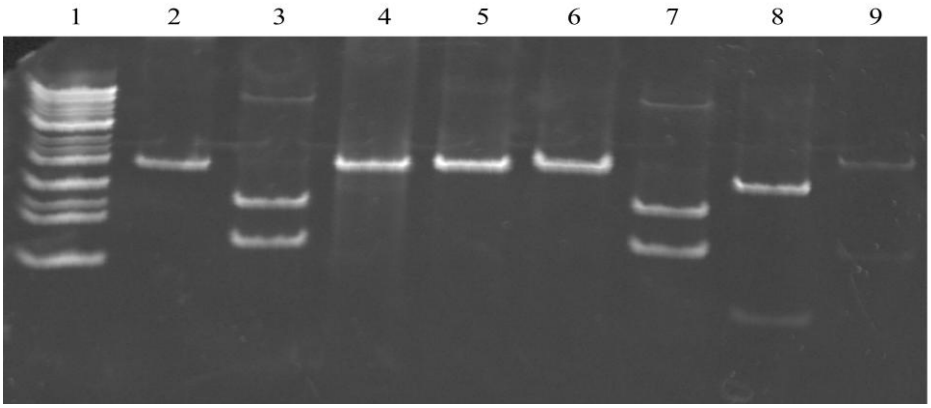


Fig. 2.34. Gel picture showing confirmation of the mutant A46S-L50P-I61T by plasmid digestion pattern by restriction enzymes.

Lane 1 show 100bp DNA ladder, Lane 2 show undigested PCR product, lane 3, 4, 5 and 6 show digestion patterns of mutant with restriction enzymes *Bgl*II, *Xho*I, *Sal*I and *Pdi*I respectively. Lane 7, 8 and 9 show digestion pattern of wildtype ubiquitin gene's 350bp PCR poduct with *Bgl*I, *Sal*I and *XhoI*. In lane 4 and 5 PCR product remain undigested by *XhoI* and *Sal*I respectively which confirms incorporation of respective mutations.

2.4.4.1.4. Construction of UbS20F-L50P-I61T mutant in yeast expression vector YEp96

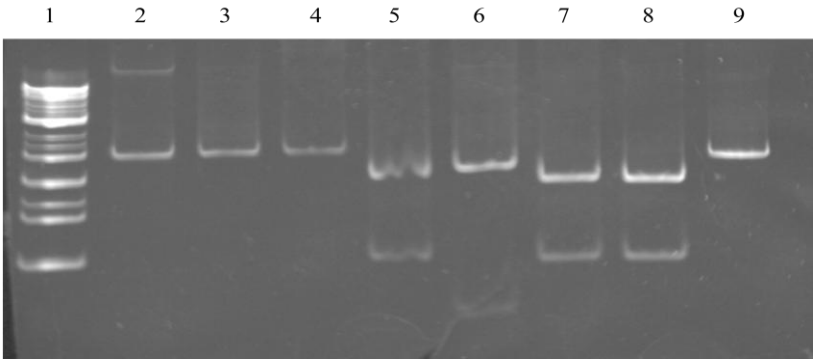


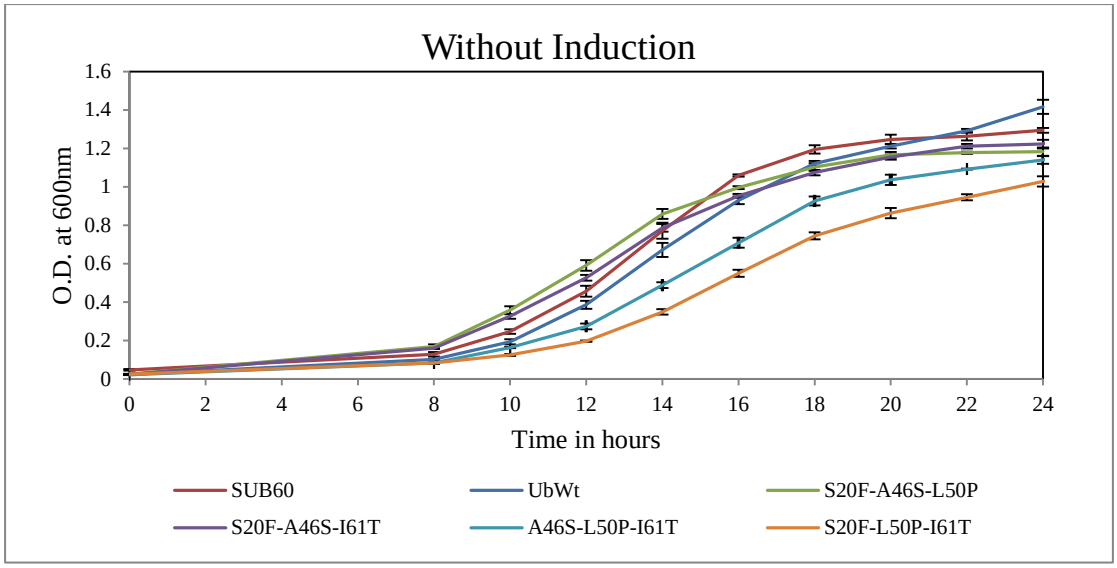
Fig. 2.35. Gel picture showing confirmation of the mutant UbA46S-L50P-I61T construction in YEp96 vector

Lane 1 show 100bp DNA ladder, Lane 2, 3 and 4 show digestion patterns of mutant with restriction enzymes *Xba*I, *Xho*I and *Sal*I respectively. Lane 5 and 6 show digestion pattern of mutant with 7, 8 and 9 show digestion pattern of wild type ubiquitin gene's 350bp PCR product with *Xho*I, *Sal*I and *Bgl*II. In lane 2, 3 and 4 PCR product remain undigested by *Xba*I, *Xho*I and *Sal*I respectively which confirms incorporation of respective mutations. Lane 9 shows undigested PCR product.

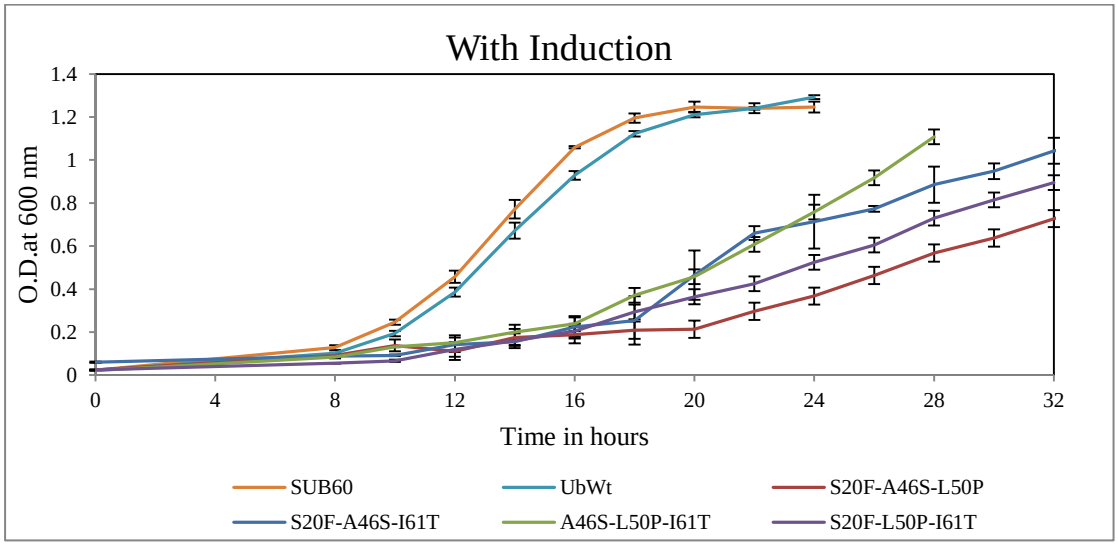
2.4.4.2. Functional evaluation of triple mutants of UbEP42 derivative in *Saccharomyces cerevisiae*

2.4.4.2.1. Effect of expression of mutant ubiquitin gene on growth of *S. cerevisiae*

The cultures of SUB60 cells expressing wild type and mutant forms of ubiquitin were grown and their growth was compared with the wild type ubiquitin gene transformed in SUB60 which lacks UBI4 (Finley et al., 1994). Freshly grown cultures were used as inoculum for the growth curve. Optical density was recorded at 600nm at regular intervals of two hours (Fig. 2.36). Results indicated that all of the four triple mutants showed decreased growth rate in presence of 25µM $CuSO_4$ and UbS20F-L50P-I61T showed decreased growth rate even in the absence of inducer.



(A)



(B)

Fig. 2.36. Growth profile of SUB60 cells transformed with plasmids UbS20F-A46S-L50P, UbS20F-A46S-I61T, UbA46S-L50P-I61T , UbS20F-L50P-I61T (A) Uninduced (B) Induced with 25µM of $CuSO_4$.

2.4.4.2.2. Effects of mutant ubiquitin on the generation time of *S.Cerevisiae*

To analyze the effect of mutations on the generation time, two values from the mid log phase have been taken for calculations (Fig.2.37). Generation time has

increased for the mutants UbS20F-A46S-L50P, UbS20F-A46S-I61T, UbS20F-L50P-I61T and UbA46S-L50P-I61T up to 7.5, 4.15, 8.1 and 5.8 hrs respectively in presence of inducer while UbS20F-L50P-I61T showed generation time of 4.56 hr in absence of inducer which shows severe additive effects of combinations of mutations.

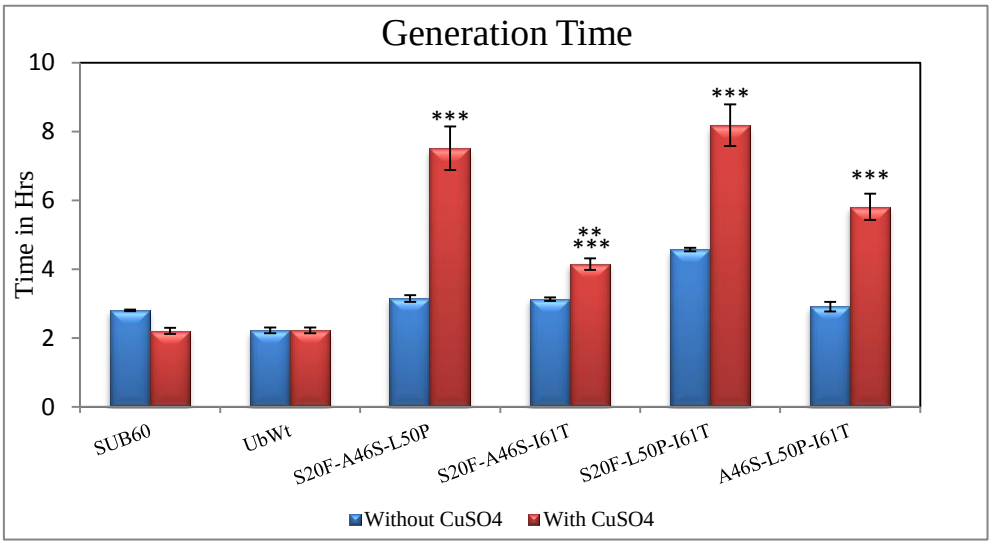
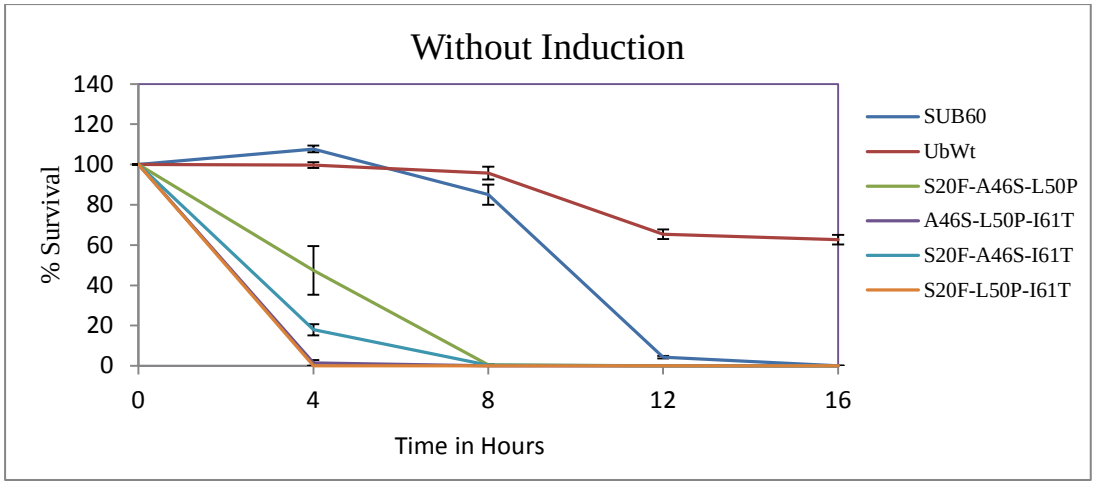


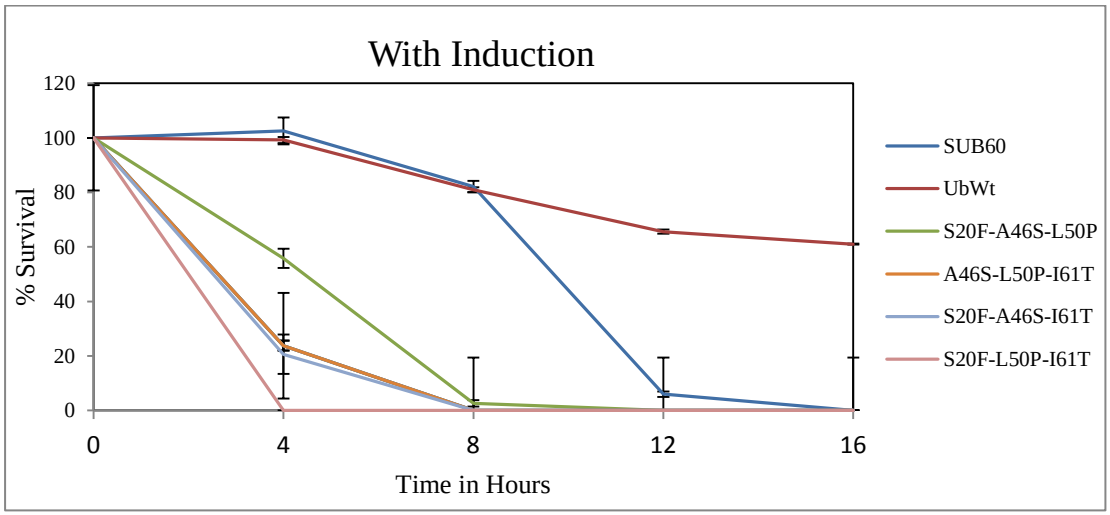
Fig. 2.37. Generation time of SUB60 and SUB60 cells transformed with plasmids UbWt, UbS20F-A46S--L50P, UbS20F-A46S-I61T, UbS20F-L50P-I61T and UbA46S-L50P-I61T in absence and presence of 25µM of $CuSO_4$.

2.4.4.2.3. Complementation of Stress hypersensitive phenotype in SUB60 cells

Heat stress hypersensitivity of the mutants has been analyzed by applying chronic heat stress, which shows that all the triple mutants of ubiquitin i.e. S20F-A46S-L50P, S20F-A46S-I61T, A46S-L50P-I61T and S20F-L50P-I61T are hypersensitive to chronic heat (Fig. 2.38). S20F-A46S-L50P and S20F-A46S-I61T show 0.4, 1.4, percent survival after 8 hrs incubation. A46S-L50P-I61T and S20F-L50P-I61T show 0.3 and 0.0 percent survival after 4 hrs incubation. Non-complementation of mutant ubiquitin shows the mutant ubiquitin lost its function under heat stress. Values for percent survival were close for uninduced and induced conditions, due to leaky expression of the promoter.



(A)



(B)

Fig. 2.38. Graph showing heat stress complementation, heat stress given to *Saccharomyces cerevisiae* strain SUB60 cells and SUB60 cells transformed with mutants UbS20F-A46S-I61T, UbS20F-L50P-I61T, UbA46S-L50P-I61T and UbS20F-A46S-L50P for various time period i.e. 0,4,8,12 and 16 hours in absence and presence of 25µM of $CuSO_4$.

2.4.4.2.4. Viability of ubiquitin mutants in *S. cerevisiae*

SUB60 yeast transformants were grown to log phase with optical density of the cultures reaching values of 0.5- 0.6 then the cultures were serially diluted fourfold

and plated on SD selection media. Plates were incubated at 30°C and the colonies were counted (Fig.2.39). S20F-A46S-L50P has shown 2.5 fold and 4.9 fold reduction under uninduced and induced condition. S20F-L50P-I61T has shown 4.7 and 6.19 fold under uninduced and induced condition. A46S-L50P-I61T has shown 12.9 and 21 fold under uninduced and induced condition. While S20F-A46S-I61T mutant has shown CFU count comparable to that of UbWt. Combination of mutations has exerted effects on the overall physiology of the cells.

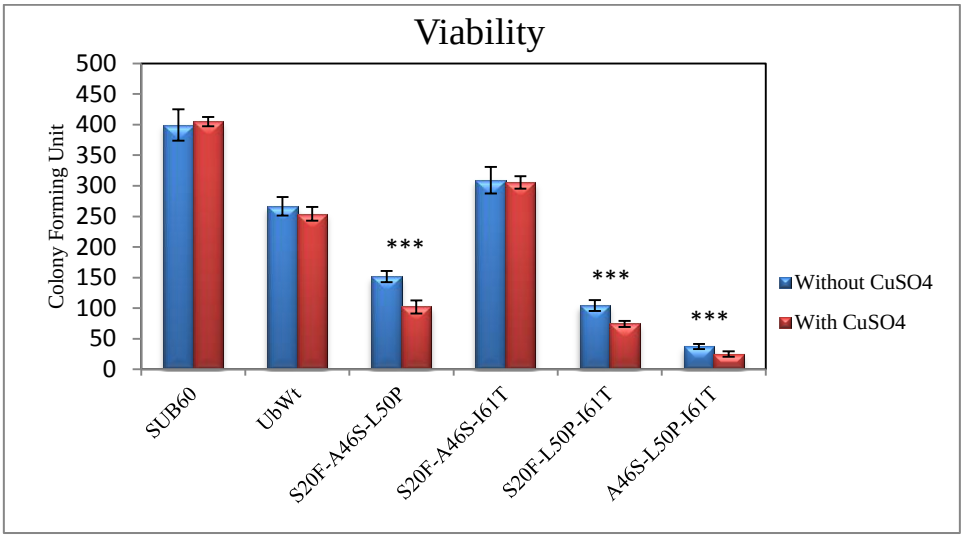


Fig.2.39 Graph showing colony forming units of *Saccharomyces cerevisiae* strain SUB60 and SUB60 transformed with UbWt, UbS20F-A46S-L50P, UbA46S-UbL50P-I61T, UbS20F-L50P-I61T, UbS20F-A46S-I61T in absence and presence of 25µM of $CuSO_4$.

2.4.4.2.5. Cycloheximide Sensitivity Test

SUB60,SUB60 cells transformed with UbWt and mutants UbS20F-A46S-L50P, UbA46S-L50P-I61T, UbS20F-L50P-I61T, UbS20F-A46S-I61T exposed to antibiotic stress by adding cycloheximide in the selective medium to test the functionality of mutant ubiquitin. Results showed that all of the above mentioned mutants are sensitive towards cycloheximide (Fig. 2.40).

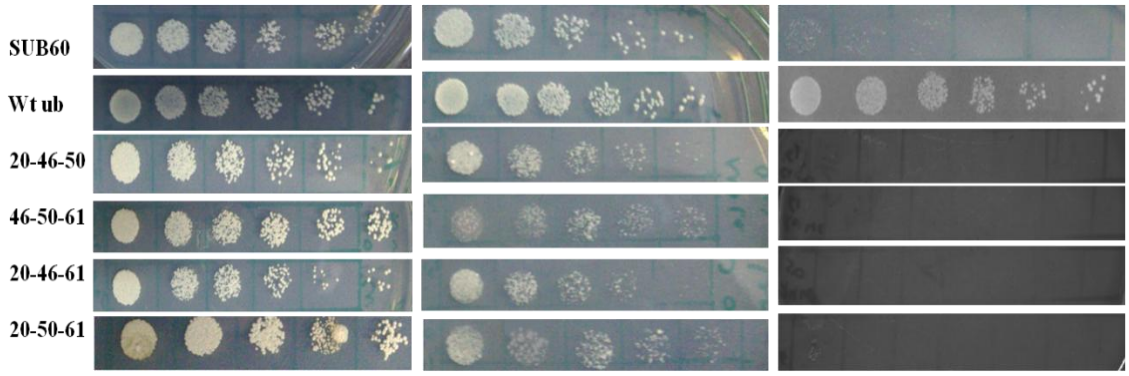


Fig.2.40. Antibiotic stress complementation of *Saccharomyces cerevisiae* SUB60 and SUB60 transformed with wild type ubiquitin gene (Ubwt) and mutants UbS20F-A46S-L50P, UbA46S-L50P-I61T, UbS20F-L50P-I61T, UbS20F-A46S-I61T.

(A) SUB60, UbWt and ubiquitin Triple mutants with no $CuSO_4$ and no cycloheximide.

(B) SUB60, UbWt and ubiquitin Triple mutants with 25µM $CuSO_4$ and no cycloheximide.

(C) SUB60, UbWt and ubiquitin Triple mutants with 25µM $CuSO_4$ and 4µg of cycloheximide.

2.4.4.2.6. Over-expression of Triple mutants of lethal Ubiquitin

In order to check the effect of over-expression of mutant ubiquitin gene in *Saccharomyces cerevisiae*, all triple mutants i.e. UbS20F-A46S-I61T, UbS20F-A46S-L50P, UbS20F-L50P-I61T and UbA46S-L50P-I61T were cloned in YEp96 vector and expressed by adding copper sulphate in the medium in varying concentration i.e. 0,100 and 200µM. Results showed that mutants UbS20F-L50P-I61T and UbA46S-L50P-I61T showed lethality upon induction at 200µM $CuSO_4$ however remaining mutants could grow in presence of 200µM $CuSO_4$ concentration (Fig. 2.41).

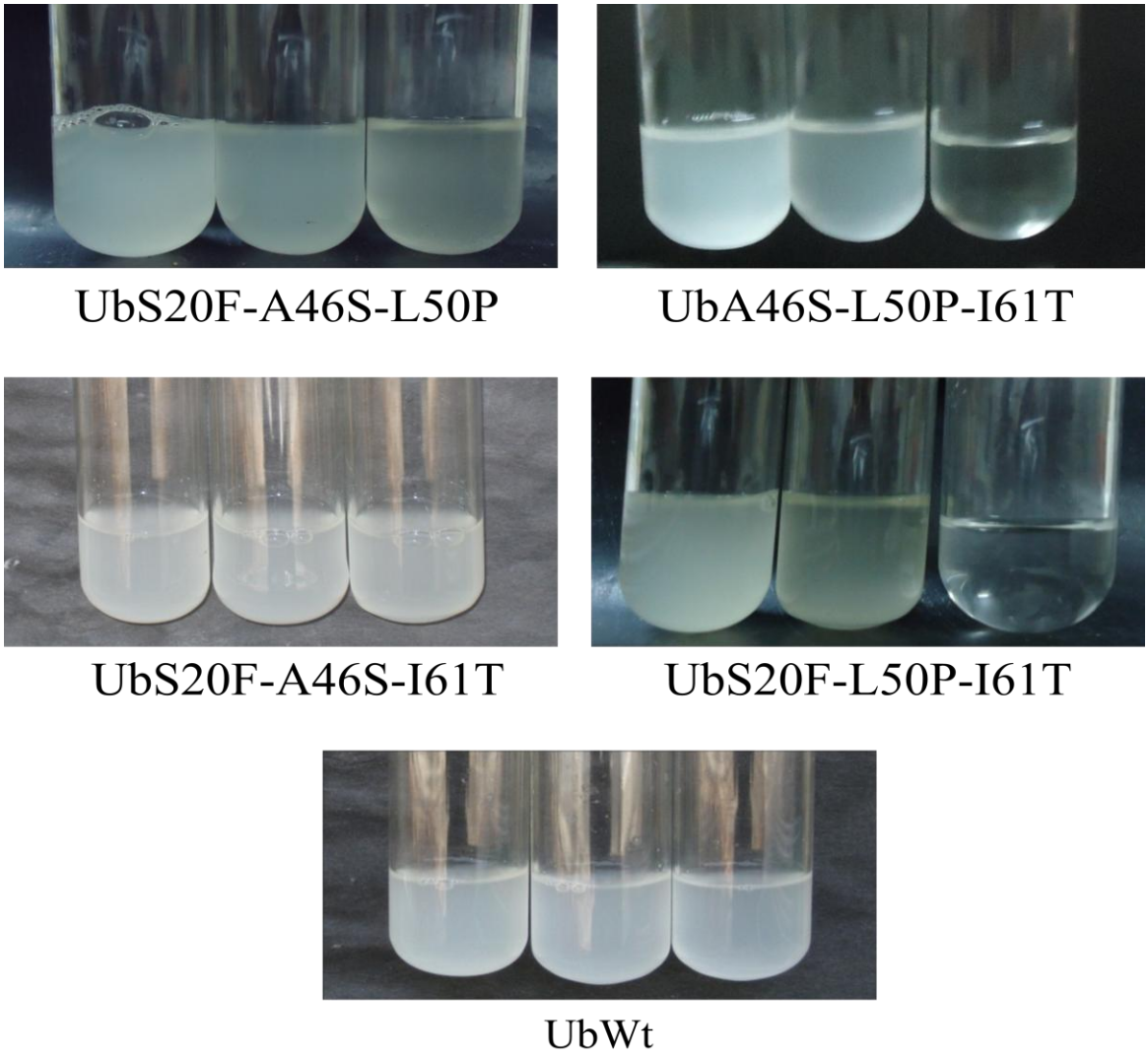


Fig. 2.41. Effect of expression of mutant ubiquitin UbS20F-A46S-L50P, UbA46S-L50P-I61T, UbS20F-L50P-I61T in presence of 0, 100 and 200 µM of copper sulphate after 36 hrs of incubation

2.5. Discussion

Temperature sensitive dose dependent lethal mutant of ubiquitin UbEP42 comprised four mutations at four novel sites i.e. 20, 46, 50 and 61. Study has been carried out in order to know which of them or their combinations are responsible for such phenotype. These all four mutations and their all possible double and triple mutation have been generated by site directed mutagenesis and cloned in yeast expression vector YEp96 and analyzed for function in *Saccharomyces cerevisiae* strain SUB60. Growth profile, heat stress complementation, viability and cycloheximide sensitivity test have been performed in order to evaluate the functionality of mutant ubiquitin. Wild type ubiquitin is known to complement the stress conditions in SUB60 cells, if extra-chromosomally expressed. Hence, complementation tests conducted with mutant ubiquitin can answer about the functional integrity of mutant ubiquitins. Among four single mutants in SUB60 cells two mutants namely UbL50P and UbI61T could not complement the heat stress phenotype and antibiotic assay, which shows that in stress condition these two mutants are functionally not active and apparently are not recognized as tags by proteasomes for the process of protein degradation by 26S proteasome. In addition they may be defective in the functions associated with tagging of ribosomal subunit L-28, because of which the organism is unable to withstand exposure to cycloheximide. However, our results show that these two forms of ubiquitin can be successfully incorporated into polyubiquitin chains. UbS20F shows moderate effect with ~20% survival and no growth in presence of cycloheximide whereas UbA46S complements all the phenotypic characters checked for complementation study. Proline in place of leucine at 50^{th} position shows drastic effect which can be attributed to the kink formed by proline which in fact can change the surface topology around it, ubiquitin is a protein with number of interacting partners like E1, E2, E3 enzymes of ubiquitination pathway, deubiquitinating enzymes and those proteins which recognize ubiquitin tag on substrate proteins. Hence any change in surface residue can bring a

loss of function of ubiquitin. Isoleucine at 61st position is a part of buried hydrophobic pocket made up of alanine at 46th and leucine at 61st position. Substitution of polar amino acid threonine in place of nonpolar isoleucine can destabilize the hydrophobic pocket, leading to alteration in the structure of ubiquitin resulting in failure of complementation under stress conditions and a lethal phenotype upon over-expression. This result implies that isoleucine is important for maintaining the functional role of ubiquitin. While surprisingly there is no effect of replacement of alanine by serine at 46th position, implying that the mutation is permissible with respect to the complementation analysis carried out in the present study.

Further all combinations of double mutants have been generated to analyse the effect of mutations on each other. Growth curve and generation time revealed that except S20F-A46S and S20F-I61T, all other mutant show increased generation time and reduced growth rate under induced condition, viability decreased in case of all the mutants except in S20F-A46S and A46S-I61T.

Heat stress complementation study revealed that except S20F-A46S all mutants show 0% survival after 16 hrs. Induction of mutant ubiquitins with copper sulphate, resulted in reducing the incubation times further for causing 0% survival under stress conditions. Cycloheximide sensitivity test revealed that S20F-A46S and A46S-I61T are resistant towards the antibiotic and S20F-I61T, A46S-L50P, L50P-I61T are sensitive while S20F-L50P showed moderate effects. Over-expression analysis has revealed that there is no lethality upon over-expression of S20F-A46S, S20F-I61T, A46S-I61T, while mutants S20F-L50P, A46S-L50P and L50P-I61T show lethality upon over-expression.

Most prominent effect has been seen in the case of double mutant L50P-I61T in terms of dose dependent lethality, growth retarding effect and non-complementation of stress hypersensitivity, another mutant A46S-L50P also exert severe effects on cell

survival which can be attributed to non-functionality of mutant ubiquitin due to close proximity of the two mutations at the positions 46^{th} and 50^{th}. Proline introducing a bend in the protein backbone introduces deviation in the structure of the protein, which can hamper the function of ubiquitin. Hence it can be concluded that all the combinations, which possess L50P as one of the mutations exert deleterious effect on *S.cerevisiae* under both uninduced and induced conditions. Hence it can be concluded that combination of mutations which bear L50P or I61T mutation as one of the mutants exerts the intermediate phenotype and double mutants which do not posses L50P or I61T complements the phenotype very well whereas double mutant which bear both L50P and I61T mutations are non-complementing and these show lethal effects. Thus, mutants do show an additive effect on each other.

Triple mutants have been generated to analyse the effect of mutations on each other. Growth curve and generation time revealed that all triple mutants show increased generation time and reduced growth rate in induced condition whereas S20F-L50P-I61T shows decreased growth rate and increased generation time in uninduced condition as well. Heat stress complementation study revealed that all triple mutants show 0% survival after 16 hrs whereas induction with copper sulphate reduces survival rate in stress condition. All combination of triple mutation shows non-complementation due to presence of either L50P or I61T whereas A46S-L50P-I61T and S20F-L50P-I61T show lethality upon over-expression which can be attributed to presence of both mutation L50P or I61T together.

Functional analysis of double and triple mutants show that the effects of mutations are additive as UbL50P-I61T becomes more lethal than the single mutations and exert non-complementation of the phenotypes studied. However, combinations of S20F–L50P, S20F–I61T, A46S-I61T remain moderately affected. The severe effects which are seen with A46S-L50P could be due to the proximity of two mutations causing greater distortion in the structure of the protein.

Cycloheximide sensitivity test revealed that all triple mutants show sensitivity towards cycloheximide. Over expression analysis has revealed that there is no lethality upon over-expression in S20F-A46S-L50P, S20F-A46S-I61T whereas A46S-L50P-I61T and mutant S20F-L50P-I61T shows lethality upon over-expression. Interestingly, the mutations S20F and A46S seem to have compensatory effects on the more lethal L50P and I61T. However, when the two mutations L50P and I61T are together or alone, either S20F or A46S are not sufficient to counteract the lethality produced. Originally when UbEP42 was generated, it was also observed that the two mutations S20F and A46S could not be palliative to the lethal mutations L50P and I61T.

CHAPTER 3

Functional assessment of site directed mutants of Parallel G1 β-bulge of ubiquitin in *Saccharomyces cerevisiae*

3.1. Introduction

In eukaryotes large fractions of proteins get degraded by being tagged to ubiquitin. Ubiquitin's C-terminal glycine makes an isopeptide bond with substrate protein's internal lysine residue. More molecules of ubiquitin are added, to substrate attached ubiquitin through its lys 6,11, 27, 33, 48 and 63 positions, forming polyubiquitin chains (Arnason and Ellison, 1994; Haas et al., 1991; Spence et al., 1995).

The relation between in vivo half life of a protein and the identity of its N-terminal residue is referred to as the N-end rule (Varshavsky, 1992). N-terminal residues are divided into two types; stabilizing and destabilizing. Ubiquitin fused with reporter protein can be assayed in vivo to find the half life of the test protein and its dependence on N-terminal residue, for example Ub-X-β-gal in which X represents any amino acid at N-end of β-galactosidase. All amino acid are cleaved by deubiquitinating enzymes except where junction residue is proline (Bachmair et al., 1986). Other 19 Ub-X- β -gal are either long lived or metabolically unstable depending on their N-end residue.

Deubiquitination is completely inhibited if C-terminal glycine is converted to any other amino acid i.e. alanine or valine (Johnson, 1999; Khorasanizadeh et al., 1993). In the yeast Saccharomyces cerevisiae a ubiquitin fusion such as Ub-P-gal, UbV76V-β-gal is rapidly degraded t1/2`~ 4 min by ubiquitin dependent system termed the UFD pathway (ubiquitin fusion degradation). The targeting of a ubiquitin fusion by the UFD pathway results in multiubiquitination of the fusions on nonremovable ubiquitin moiety. There are 5 genes in UFD pathway, they are UFD1,UFD2, UFD3, UFD4 and UFD5. UFD 1 and UFD5 function at post-ubiquitination step while UFD3 plays a role in controllng the concentration of ubiquitin in cell. UFD2 and UFD4 appear to influence the formation and topology of

a multiubiquitin chain linked to the fusion's ubiquitin moeity. Physiological substrates of UFD pathway are Rpn4, the regulatory protein for proteasome subunit (Wang et al., 2004), and integrases of lentiviruses including that of HIV (Mulder and Meusing, 2000).

3.1.1. Physiological functions and stress response

Ubiquitin gene family comprised of the four gene locations of the ubiquitin gene in which *UBI1, UBI2* and *UBI3* expresses single ubiquitin gene fused with the ribosomal subunit protein while *UBI4* expresses polymer of ubiquitin having five repeats of ubiquitin in head to tail manner. Naturally *UBI4* is regulated by heat shock box present upstream of it, which in turn can sense various stresses (Ozkaynak et al., 1987). SUB60 strain of *Saccharomyces cerevisiae* is an engineered strain which lacks the UBI4 gene thus becomes hypersensitive towards various stresses, albeit functions normally in the absence of any kind of stress (Finley et al., 1987). SUB60 cells can be taken as a tool to study by complementation analysis, i.e. heat, amino acid analogue, antibiotic, starvation and UV exposure. Under the stress condition various proteins are denatured, which have to be removed early and normal ubiquitin pool inside the cell helps to combat against stresses.

Earlier work from our laboratory has addressed the biological role of the residues Glu64, Ser65 and Gln2 in the second β-bulge of ubiquitin by substituting them with Gly64, Asp65 and Asn2 (Mishra et al., 2011; Mishra et al., 2009). Structural characterization of the single mutants namely UbE64G, UbS65D and UbQ2N have revealed only minor changes where structure and stability of the protein were not compromised (Mishra et al., 2009; Mishra et al., 2011). Results were in agreement to the hypothesis as residues naturally present inside the bulge were replaced with more preferred residues. Further the mutant forms of ubiquitin complement and rescue UBI4 deleted SUB60 cells. It was observed that ubiquitin

mutants UbE64G, UbS65D and UbQ2N have extended the half life of unstable ubiquitin fusion of substrate proteins with N-terminal Pro residue (Ub-Pro-β-galactosidase). It was also noticed that the sensitivity of ubiquitin single mutants towards cycloheximide has been increased compared to the wild type ubiquitin control. However they complemented the heat sensitive phenotype (Mishra et al., 2009; Mishra et al., 2011).

Present chapter describes the biological effect of combination of the mutations where the whole β-bulge has been replaced serially with more preferred amino acids i.e. Q2N-E64G, UbQ2N-S65D, UbE64G-S65D and UbQ2N-E64G-S65D. The functional integrity of mutant ubiquitins was assessed by expressing them from a plasmid vector and carrying out complementation studies in *UBI4* deleted SUB60 strain of *S. cerevisiae* by looking at heat stress tolerance, antibiotic sensitivity and half-life of β-galactosidase in Ub-Pro-β gal fusions.

3.2. Materials and methods

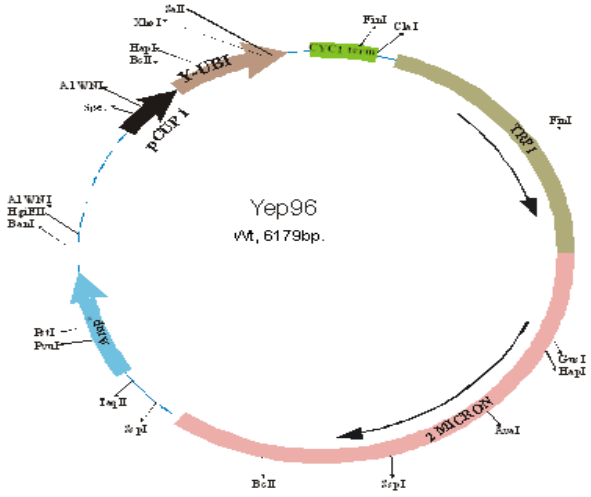
3.2.1. Strains, media, and plasmids

The *S. cerevisiae* strains used in the study are as follows- SUB62 (MATa lys2-801 leu2-3,112 ura3-52 his3-Δ200 tr1-1) is a wild type strain for ubiquitin genes and SUB60 (MATa ubi4-Δ2:: LEU2 lys2-801 leu2-3,112 ura3-52 his3-Δ200 trp1-1) is a deletion mutant lacking *UBI4* polyubiquitin gene (Finley et al., 1987; Spence et al., 1995). (Discussed in detail in materials and methods section of **Chapter 2**).

Escherichia coli DH5α culture was taken for all plasmid manipulations and grown at 37ºC at 200 rpm in nutrient rich Luria broth from Hi-media. Strain was maintained under a selection pressure of 50µg/ml of ampicillin.

3.2.2. Yeast expression vector used in the study

Ubiquitin gene mutations were carried out in plasmid YEp96 which express a synthetic yeast ubiquitin gene under the *CUP1* promoter and are a shuttle vector between *E.coli* and *S.cerevisiae* (Fig. 3.1) (Finley et al., 1994). It has a tryptophan and ampicillin as a selection marker in yeast and bacteria respectively. Ubiquitin mutant gene amplicons generated by PCR were cloned into the *Bgl*II and *Kpn*I sites of YEp96. Screening of mutations for UbE64G and UbS65D were done with loss of *Sal*I site whereas for UbQ2N was done with loss of *Bgl*II site.



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

*Eco*RI Met *Bgl*II Gly Gly *KpnI*

Fig. 3.1. The DNA sequence of the *Eco*RI-*Kpn*I insert in YEp96-UbWt (Finley et al., 1987)

pUb23 vector is a 2 micron based galactose inducible shuttle vector which expresses the ubiquitin fused with β-galactosidase (Ub-X-β gal) where X denotes different amino acids depending upon their primary, secondary or tertiary destabilizing categorization. It has a uracil and ampicillin as a selection marker for yeast and bacteria respectively. pUb23 vector containing proline at the X position was used to assess the activity of test protein fused with the ubiquitin (Fig. 3.2), yeast cells

containing pUb23 vector were double transformed with ubiquitin mutants cloned in Yep96 yeast expression vector.

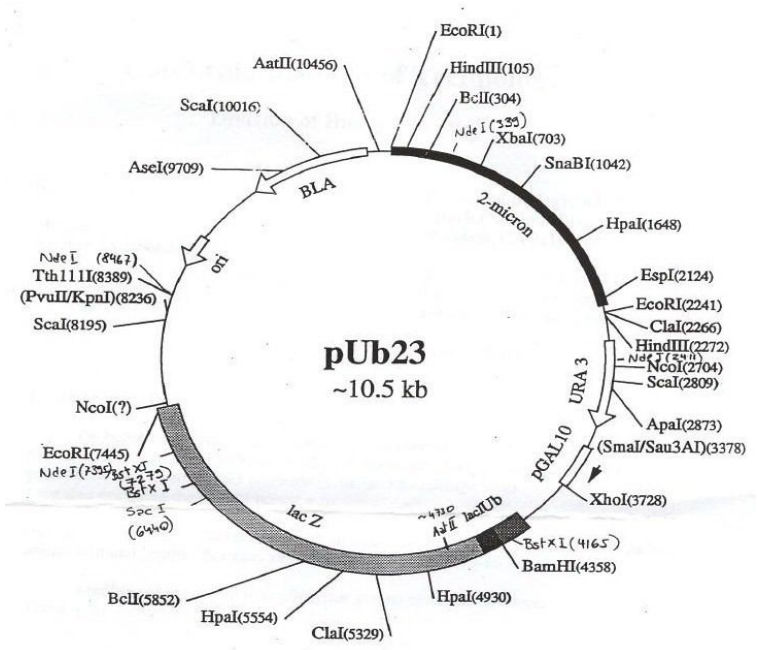


Fig. 3.2. Map of pUb23 yeast expression vector (Spence et al., 1995)

3.2.3. Sequence Analysis

The plasmid with mutant gene was sequenced using dideoxy sequencing method for the detection of the mutation incorporation in the DNA. Mutants ubiquitin genes were sequenced in a single forward reaction using primer sequence 5' GCGCCGACATCATAACGGTTCTGGC 3'.

3.2.4. Phenotype analysis

3.2.4.1. Growth Effects

The cultures of SUB60 cells expressing wild type and mutant forms of ubiquitin were grown and their growth was compared with wild type strain for *UBI*4, SUB62 (Finley et al., 1994). Freshly grown stationary phase cells were used as

inoculums for the growth curve. Optical density was recorded at intervals of two hours at 600nm. Generation time has been calculated from mid log phase of cultures.

3.2.4.2. Viability assay

SUB60 yeast transformants were grown to log phase with optical density of the cultures reaching a value in between 0.5 to 0.6 and serially diluted fourfold and plated on SD selection media. Plates were incubated at 30°C and the colonies were counted.

3.2.4.3. Heat Stress Complementation

Stress hypersensitive UBI4 mutant, the SUB60 strain of yeast was transformed with YEp96 plasmid carrying wild type or mutated ubiquitin gene under *CUP*1 promoter and was tested for complementation under stress (discussed in detail in material and method section of chapter 2).

3.2.4.4. Antibiotic sensitivity test

Complementation potential of the ubiquitin variants was tested using antibiotic sensitivity test (Hanna et al., 2003). SUB60 transformants were grown to log phase with their optical density values around 0.2 and serially diluted to three fold and spotted on YPD media with induction by 25µM copper sulphate and cycloheximide (4µg/ml). Plates were incubated for ten days for assessment of growth revival.

3.2.4.5. N-end rule as degradation signals

Yeast double transformants of YEp96 and pUB23 were grown to exponential log phase on SD-galactose selection media. Cells were pelleted and washed twice

with distilled water and resuspended in saline and the optical density was adjusted to 0.5 to 0.6. Protein estimation was done by modified Lowry method (Lowry et al., 1951) and β-galactosidase assay was done (Baker and Board, 1991).

3.3. Plan of work

The site directed mutants generated for the ubiquitin (UbQ2N-E64G, UbQ2N-S65D, UbE64G-S65D and UbQ2N-E64G-S65D) were cloned in the shuttle vector for yeast and bacteria**.** These cloned genes were introduced into the UBI4 polyubiquitin gene mutant of *Saccharomyces cerevisiae* (SUB60) and examined for complementation of phenotype (Fig. 3.3).

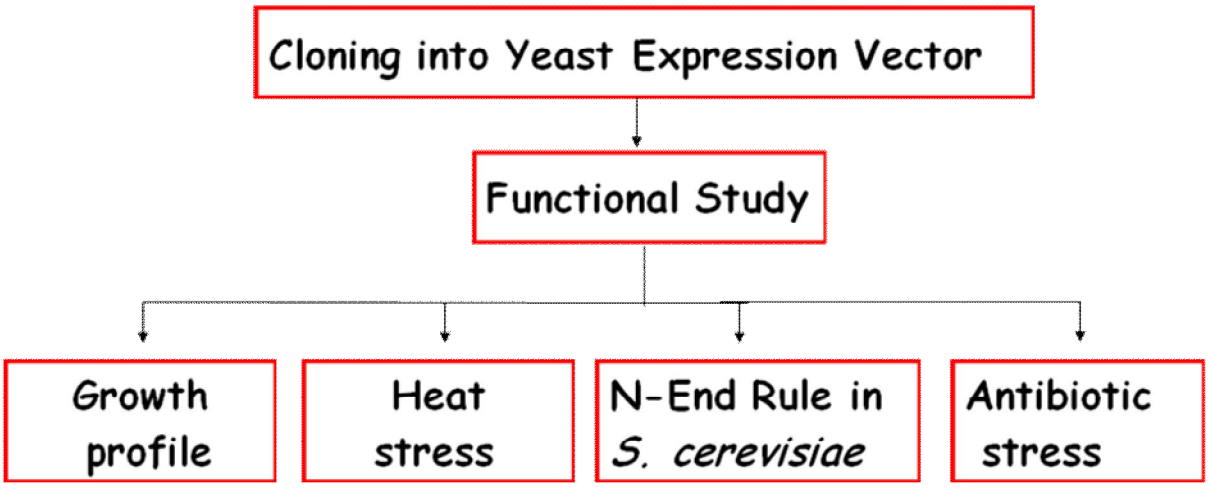


Fig. 3. 3. Schematic representation of functional complementation studies

3.4. Results

3.4.1. Construction of mutations of β-bulge in ubiquitin

3.4.1.1. Construction of UbQ2N-S65D in yeast expression vector YEp96

In order to construct double mutation UbQ2N-S65D in yeast-bacterial shuttle vector YEp96, S65D mutant ubiquitin was amplified by PCR and subjected to *Xho*I and *Kpn*I double digestion. Further this fragment was sub-cloned in YEpUbQ2N

mutant. Incorporation of both the mutations has been confirmed by digestion pattern of mutant plasmid and its PCR amplicon, further sequence confirmation has been done to assure the incorporation of both of the mutations. Screening of the mutant has been done by digesting plasmid with *Sal*I restriction enzyme (Fig. 3.4).

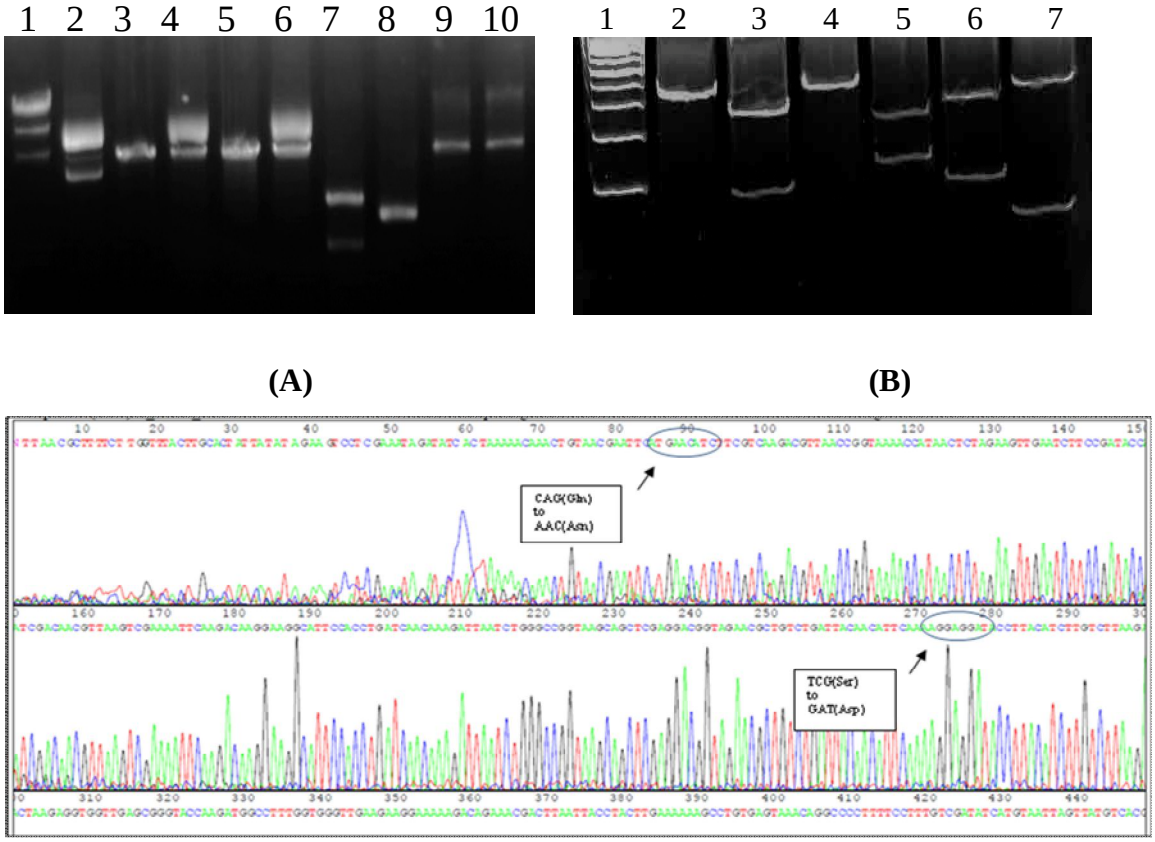


Fig. 3.4. Gel pictures showing digestion Pattern of (A) YEp96 UbQ2N-S65D plasmid and (B) 350bp PCR Product UbQ2N-S65D digestion (C) Electrophoretogram of UbQ2N-S65D gene in YEp96 vector.

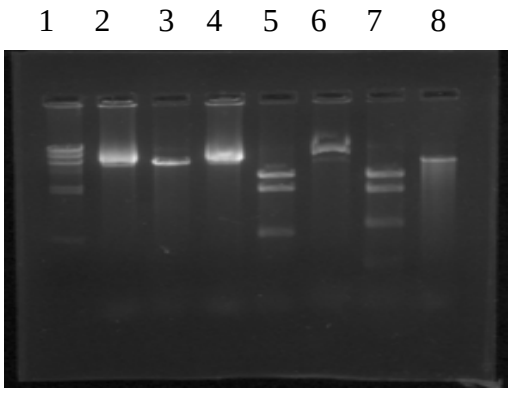
(A) Lane1 shows λ *Hind*III Digest Marker. Lane2 has undigested plasmid. Lanes 3,4,5,6,7,8 and 9,10 shows digestion pattern of the plasmid by *Xho*I, *Bgl*II *Kpn*I *Sal*I *Hind*III, *EcoR*II restriction digestion of mutant *UbQ2N-S65D* and BglII, *SalI* shows restriction digestion of UbWt respectively. Lane 4 and 6 shows failure of digestion of mutant with restriction enzyme *Bgl*II and *Sal*I hence confirm the incorporation of respective mutations.

(B) Lane 1 shows 100bp DNA ladder, Lane 2, 3 and 4 shows digestion with *Bgl*II, *Xho*I *and Sal*I respectively. lane 2 and 4 shows failure of digestion 250 with *Bgl*II and *Sal*I respectively indicates incorporation of desired mutation. Lane 5,6 and 7 shows digestion of PCR amplicon with *Bgl*II, *Xho*I and *Sal*I of YEp 96Wt.

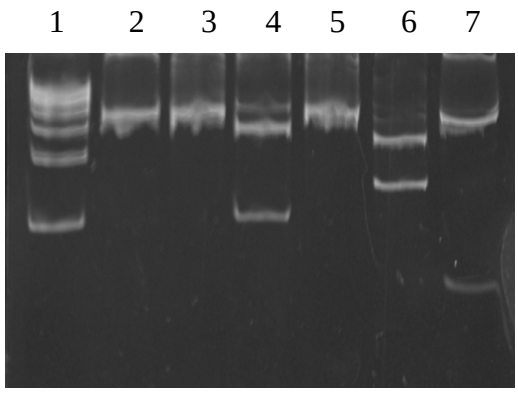
(C) The marked region confirms the incorporation of UbQ2N-S65D substitution.

3.4.1.2. Construction of UbQ2N-E64G in yeast expression vector YEp96

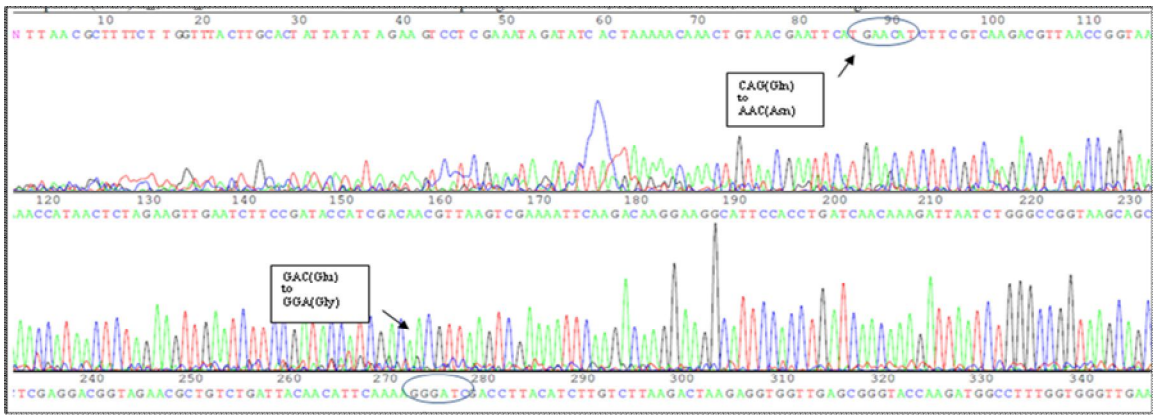
UbQ2N-E64G mutation has been introduced in yeast-bacterial shuttle vector YEp96 by PCR amplifying mutant ubiquitin UbE64G and double digested by *Xho*I and *Kpn*I restriction enzymes. This fragment bearing E64G mutation was subcloned in YEpUbQ2N vector. Screening of the mutant has been done by digesting plasmid with *Sal*I (Fig. 3.5). Incorporation of both the mutations has been confirmed by digestion pattern of mutant plasmid and its PCR amplicon, followed by sequence confirmation.



(A)



(B)



(C)

Fig. 3.5. Gel pictures showing digestion Pattern of (A) YEp96 UbQ2N-E64G plasmid and (B) 350bp PCR Product UbQ2N-E64G digestion (C) Electrophoretogram of UbQ2N-E64G gene in YEp96 vector.

(A) Lane1 shows λ *Hind*III Digest Marker. Lane2 has undigested plasmid. Lanes 3,4,5,6,7,8 and 9,10 shows digestion pattern of the plasmid by *Xho*I, *Bgl*II, *Hind*III, *Sal*I, *Xba*I and *Kpn*I restriction digestion of mutant UbQ2N-E64G and *Bgl*II, *Sal*I shows restriction digestion of UbWt respectively. Lane 4 and 6 shows failure of digestion of mutant with restriction enzyme *Bgl*II and *Sal*I hence confirm the incorporation of respective mutations.

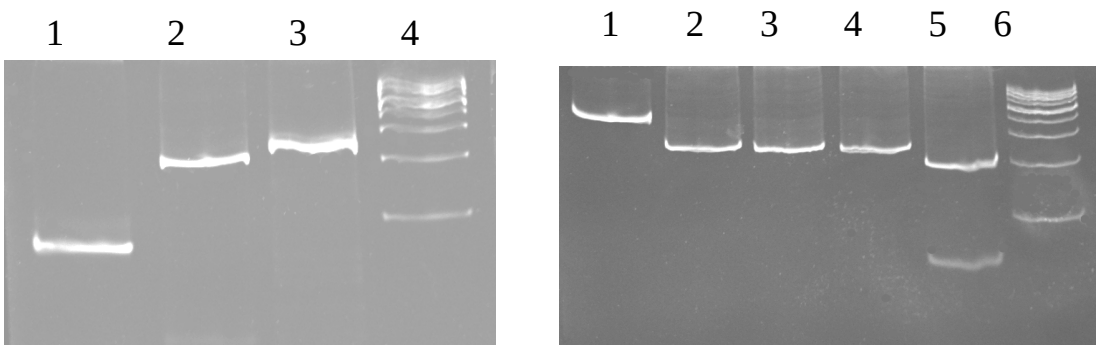
(B) Lane 1 shows 100 bp DNA ladder, Lane 2, 3 and 4 shows digestion with *Bgl*II, *Xho*I and *Sal*I respectively. lane 2 and 4 shows failure of digestion 250 with *Bgl*II and *Sal*I respectively indicates incorporation of desired mutation. Lane 5,6 and 7 shows digestion of PCR amplicon with *Bgl*II, *Xho*I and *Sal*I of YEp 96Wt.

(C) The marked region confirms the incorporation of UbQ2N-E64G substitution.

3.4.1.3. Construction of UbE64G-S65D mutation in yeast expression vector YEp96

3.4.1.3.1. Recombinant PCR and clone confirmation in cloning vector pTZ57R/T

Primer based recombinant PCR strategy has been used to construct the mutant UbE64G-S65D. Two mutagenic complementary primers were designed to incorporate the mutations in ubiquitin gene. Combination of both mutagenic and its counterpart have been used to amplify ubiquitin gene in two halves, further these two fragments had recombined by full length PCR with the help of non-mutagenic gene specific primers. Incorporation of the mutation in amplified PCR product has been confirmed by digesting it with *Sal*I restriction enzyme. PCR fragment bearing mutation E64G-S65D has been cloned in the cloning vector pTZ57R/T (Fig. 3.6).



(A) (B)

Fig. 3.6. (A) Showing generation of mutant by recombinant PCR. (B) showing restriction digestion pattern of pTZ57 R/T-UbE64G-S65D.

(A) Lane 1 and 2 shows 62 and 217bp amplicon generated by the combination of UBFP, E64G-S65D RP and E64G-S65DFP and UBRP, lane 3 shows recombinant PCR, lane 4 shows 100 bp ladder.

(B) Lane1 shows 391 bp extension of amplicon by using universal M-13 primers. Lane 2 and 3. Show 252 extension pattern of mutant and Wild type gene using primers UbFP and UbRP, Lane 4. Shows 252 bp extension which could not digest by *Sal*I enzyme indicates incorporation of mutation Lane 5. Show *Xba*I site digestion pattern of PCR product which cuts gene into two fragments 52 and 200 respectively.

3.4.1.3.2. Clone confirmation of UbE64G-S65D

Double mutant E64G-S65D of ubiquitin has been sub-cloned from cloning vector pTZ57R/T to yeast expression vector YEp96 using *Bgl*II and *Kpn*I restriction sites. Incorporation of double mutation has been confirmed by digestion pattern of plasmid and PCR product, further sequence analysis has done to confirm the same. Screening of the positive clones has been done by digesting them with *Sal*I enzyme (Fig. 3.7).

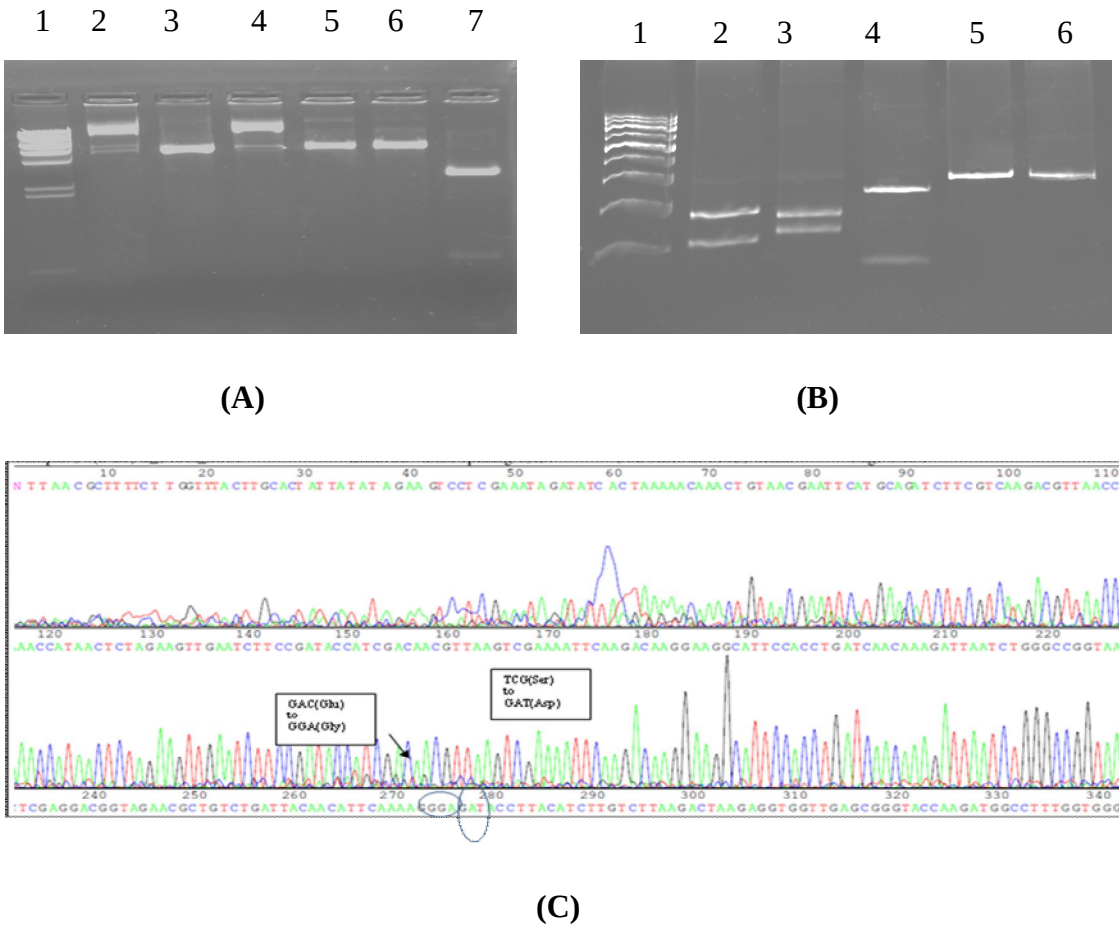


Fig. 3.7. Gel pictures showing digestion Pattern of (A) YEp96 UbE64G-S65D plasmid and (B) 250bp PCR Product UbE64G-S65D digestion (C) Electrophoretogram of UbE64G-S65D gene in YEp96 vector

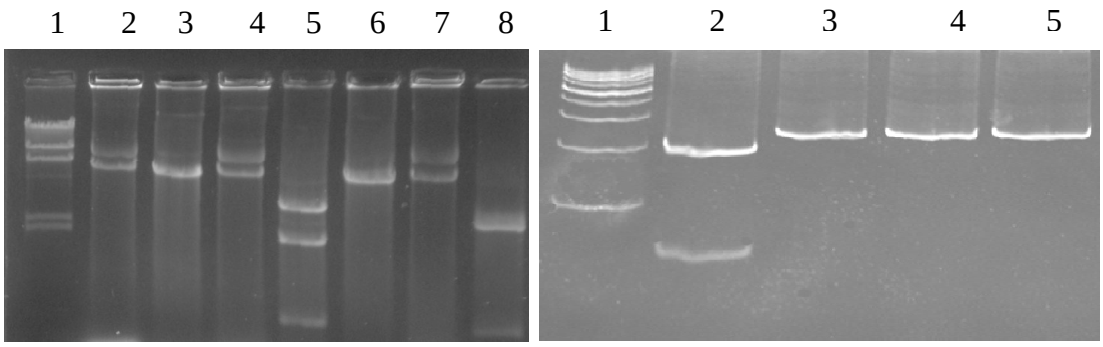
(A) Lane1 shows λ *Hind*III Digest Marker. Lane2 has undigested plasmid. Lanes 3,4,5,6,7,8 and 9,10 shows digestion pattern of the plasmid by *Bgl*II, *Sal*I, *Xho*I, *Kpn*I and *EcoR*I respectively. Lane 4 shows failure of digestion of mutant plasmid with restriction enzyme *Sal*I hence confirm the incorporation of desired mutation.

(B) Lane 1 and 6 shows 100 bp DNA ladder and Undigested PCR product. Lane 2, 3, 4 and 5 shows digestion with *Xho*I, *Sau*96, *Xba*I and *Sal*I respectively.

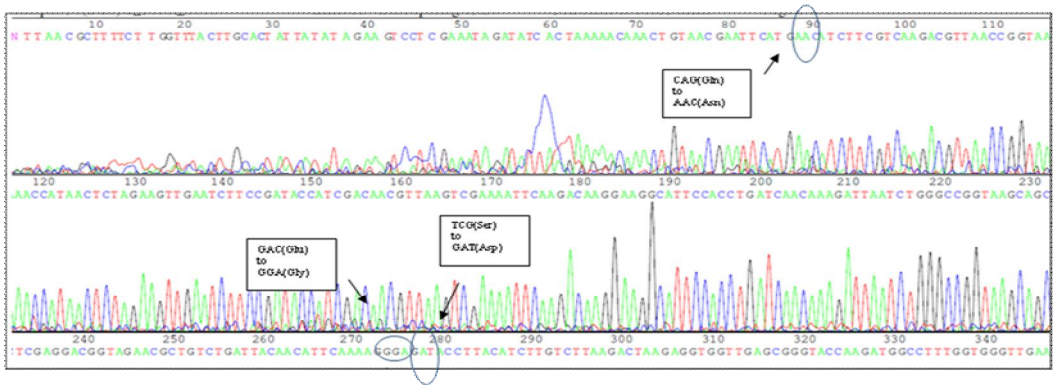
(C) The marked region confirms the incorporation of UbE64G-S65D substitution.

3.4.1.4. Construction of UbQ2N-E64G-S65D in yeast expression vector YEp96

The whole β-bulge replacement has been made by inserting all three mutations Q2N, E64G and S65D in yeast expression vector YEp96. E64G-S65D mutant generated by recombinant PCR has been digested by *XhoI* and *Kpn*I restriction enzyme and cloned in YEpUbQ2N vector backbone. Incorporation of all three mutants in ubiquitin gene has been confirmed restriction digestion pattern analysis of its plasmid and PCR product, further the same was confirmed by sequence analysis of the mutant gene. Screening of the positive clones has been made by digesting the vector with *Bgl*II and *Kpn*I restriction enzymes (Fig. 3.8 A and B).



(A) **(B)**



(C)

Fig. 3.8. Gel pictures showing digestion Pattern of (A) YEp96 UbQ2N-E64G-S65D plasmid and (B) 250bp PCR Product UbQ2N-E64G-S65D digestion (C) Electrophoretogram of UbQ2N-E64G-S65D gene in YEp96 vector.

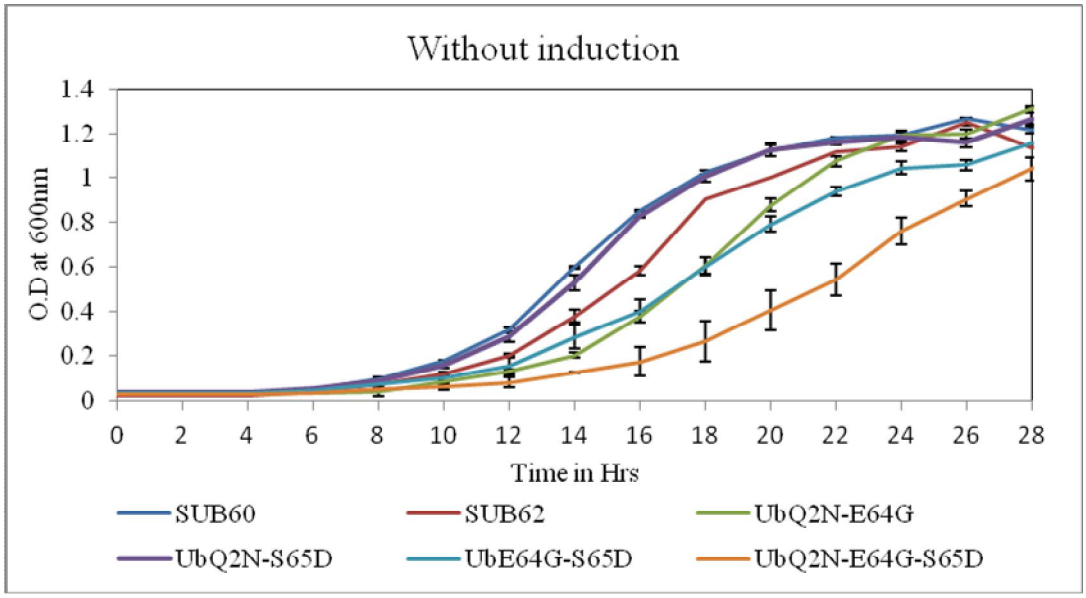
(A) Lane1 shows λ *Hind*III Digest Marker. Lane2 has undigested plasmid. Lanes 3,4,5,6,7 and 8 shows digestion pattern of the plasmid by *Kpn*I, *Bgl*II, *Hind*III, *Xho*I, *Sal*I and *EcoR*I respectively. Lane 4 and 7 shows failure of digestion of mutant plasmid with restriction enzyme *Bgl*II and *Sal*I respectively hence confirm the incorporation of desired mutation.

(B) Lane 1 and 5 shows 100 bp DNA ladder and undigested PCR product. Lane 2, 3, 4 and 5 shows digestion with *Xba*I, *Bgl*II and *Sal*I respectively. Lane 3 and 4 remain undigested after digestion due to site loss confirms incorporation of desired mutation.

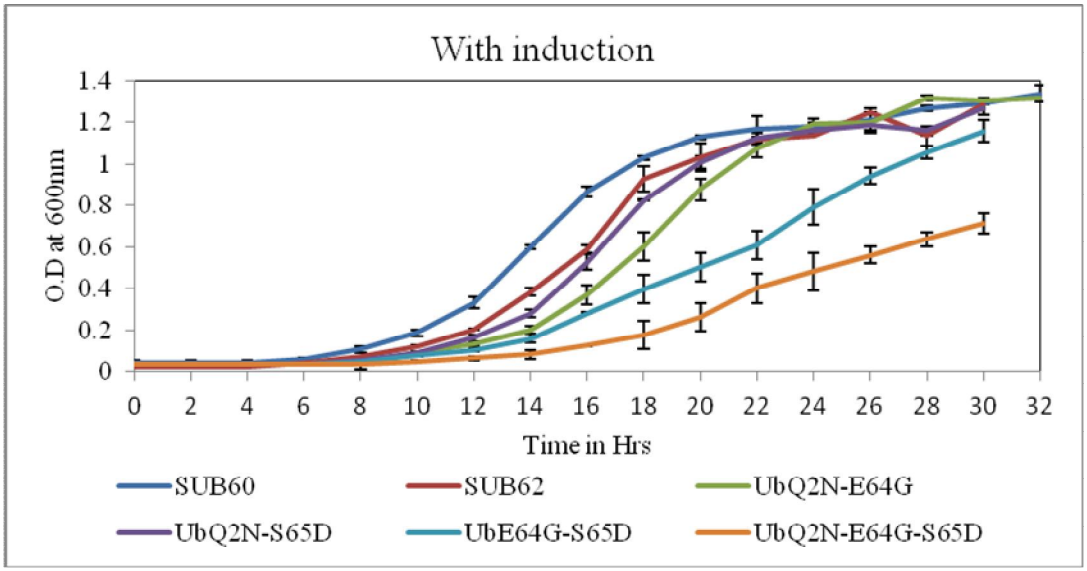
(C) The marked region confirms the incorporation of UbQ2N-E64G-S65D substitution.

3.4.2. Effects of mutants gene expression on growth of *S. cerevisiae*

In order to check the effect of mutations made in ubiquitin gene on the growth of cells, mutant ubiquitin Q2N-E64G, Q2N-S65D, E64G-S65D and Q2N-E64G-65 have been transformed in UBI4 deleted engineered strain SUB60 of *Saccharomyces cerevisiae* (Fig. 3.9). As ubiquitin plays a central role in the variety of physiological processes adverse effect on growth is expected due to mutations. Mutant ubiquitin was over-expressed under the influence of *CUP*1 promoter. Among four mutants UbE64G-S65D and UbQ2N-E64G-S65D have shown less growth rate and increased lag phase under uninduced and induced conditions. Growth further diminished in the presence of inducer while effect of UbQ2N-E64G and UbQ2N-S65D on the growth of cells remains comparable to wild type yeast cells SUB60 and SUB60 cells transformed with wild type ubiquitin gene.



(A)



(B)

Fig. 3.9. Growth profile of SUB 60 and SUB 60 cells transformed with plasmids UbWt, UbQ2N-E64G, UbQ2N-S65D, UbE64G-S65D and UbQ2N-E64G-S65D under uninduced and induced condition (induced with 50µM of $CuSO_4$).

3.4.2.1. Generation Time of Ubiquitin mutants in SUB60 cells

Generation time of ubiquitin mutant and wild type cells SUB62 and SUB60 has been measured by taking two consecutive values from the log phase stage (Fig. 3.10). Generation time has been inceased in the case of mutants UbE64G-S65D and UbQ2N- E64G-S65D and increased considerably under induced condition. While, generation time of Q2N-S65D and Q2N-E64G remained unaltered under both uninduced and induced condition.

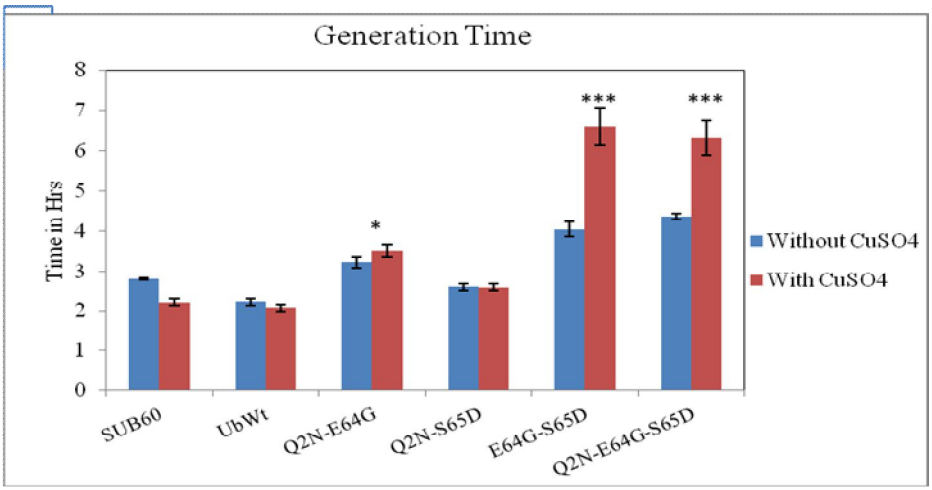


Fig. 3.10. Generation time of SUB 60, SUB 62 and SUB 60 cells transformed with plasmids Ub96Wt, UbQ2N-E64G, UbQ2N-S65D, UbE64G-S65D, and UbQ2N-E64G-S65D in uninduced and induced condition in the presence of 50 µM of $CuSO_4$.

3.4.2.2. Effect of mutant ubiquitin proteins on viability of cells

Viability of the transformant bearing all four mutations UbQ2N-E64G, UbQ2N-S65D, UbE64G-S65D, UbQ2N-E64G-S65D and UbWt cells have been analysed by plating them on selective media after diluting them four fold. Cells having mutations UbE64G-S65D and UbQ2N-E64G-S65D have shown 6 fold less viability as compared with wild type ubiquitin. Whereas viability of mutant ubiquitin namely UbQ2N-E64G, UbQ2N-S65D were found to be comparable to SUB60 and

SUB62 cells. Result indicates the severe effect on growth by variant of ubiquitin gene UbE64G-S65D, and UbQ2N-E64G-S65D (Fig. 3.11).

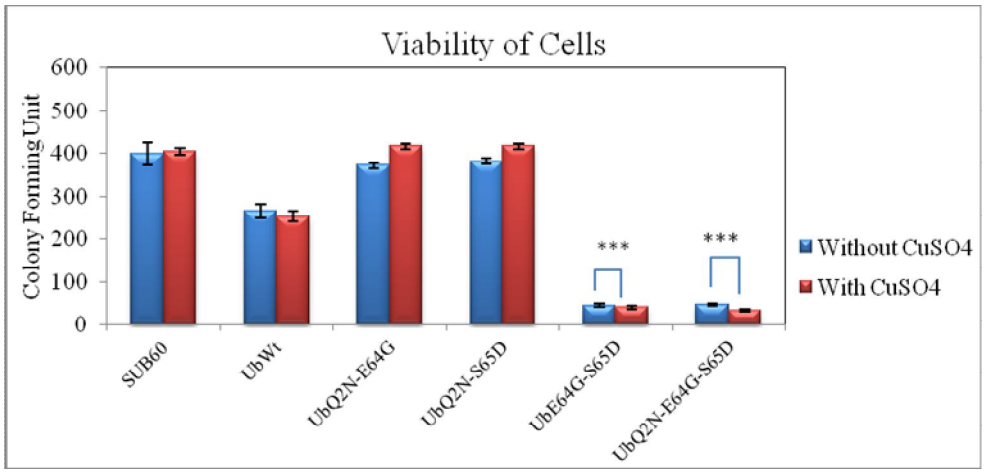


Fig. 3.11. Graph showing colony forming units of *Saccharomyces cerevisiae* strain SUB60 and SUB60 transformed with mutants UbQ2N-E64G, UbQ2N-S65D, UbE64G-S65D and UbQ2N-E64G-S65D under uninduced and induced condition (50µM of $CuSO_4$) at permissive temperature.

3.4.2.3. Complementation studies

3.4.2.3.1. Heat stress complementation

Sacchromyces cerevisiae strain SUB60 becomes hypersensitive to various stresses since it lacks UBI4 gene which is responsible to generate more ubiquitin during stress condition. Wild type ubiquitin gene transformed in SUB60 can complement the heat sensitive phenotype. All mutants namely UbQ2N-E64G, UbQ2N-S65D, UbE64G-S65D and UbQ2N-E64G-S65D have been transformed in SUB60 and exposed to heat stress for variable time periods to confirm the functional integrity of the mutant ubiquitin (Fig. 3.12).

Heat stress hypersensitivity of the mutants has been analysed after applying chronic heat stress which shows that mutant ubiquitin UbE64G-S65D and UbQ2N-E64G-S65D are hypersensitive to chronic heat and shows ~10% and ~ 5% survival at

12 and 8 hours of heat stress respectively under uninduced condition and the effect under induced conditions is more pronounced however UbQ2N-E64G, UbQ2N-S65D show moderate sensitivity towards heat i.e. ~ 25% and 20 % survival at 16 hours and 10% and no survival under induced condition (Fig. 3.13). Non-complementation of mutant ubiquitin shows the functional loss of mutant ubiquitin as this ubiquitin is unable to participate in the normal functioning of the disposal system.

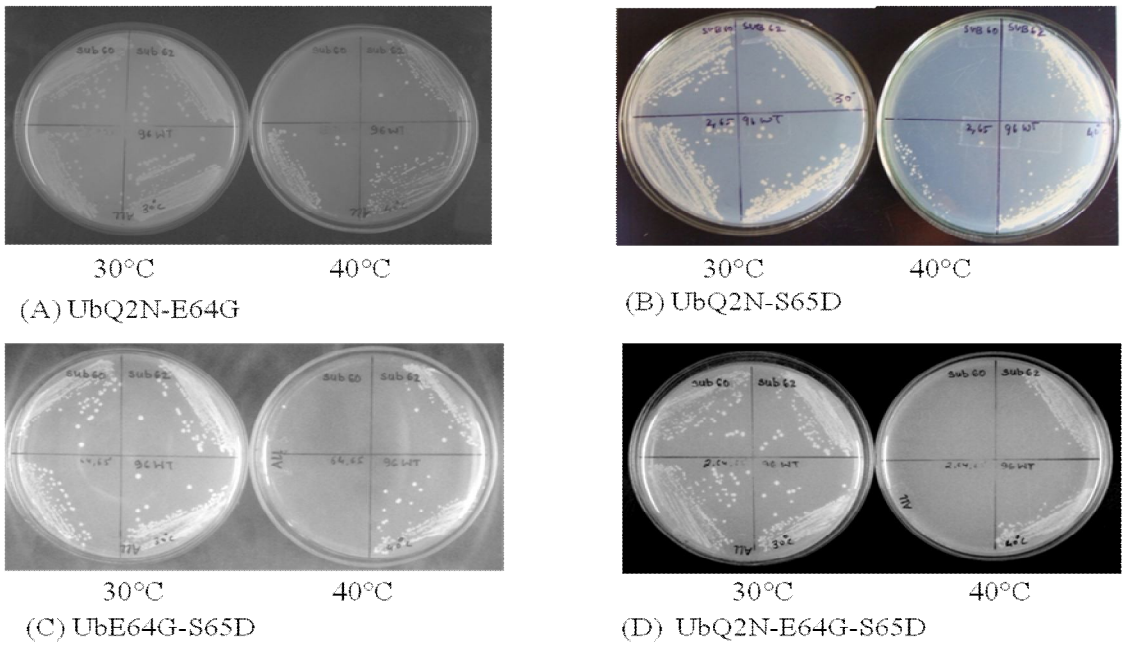
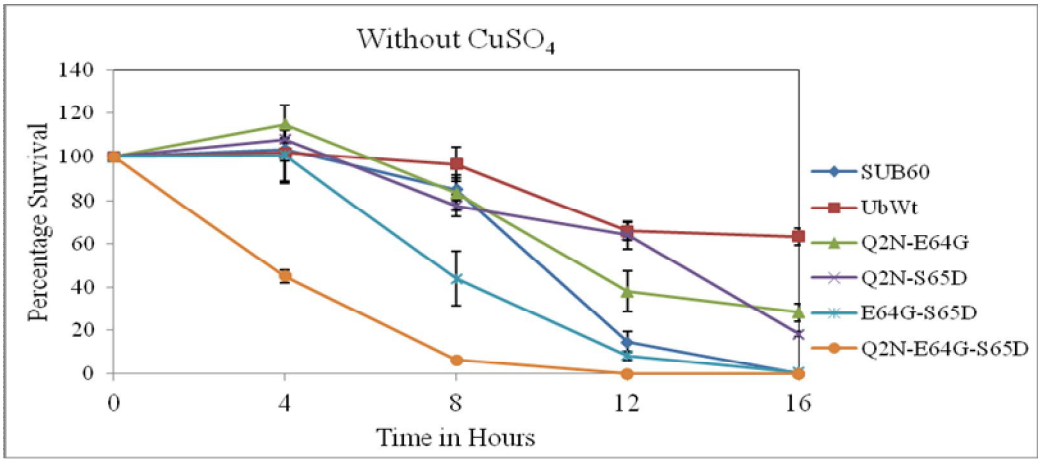
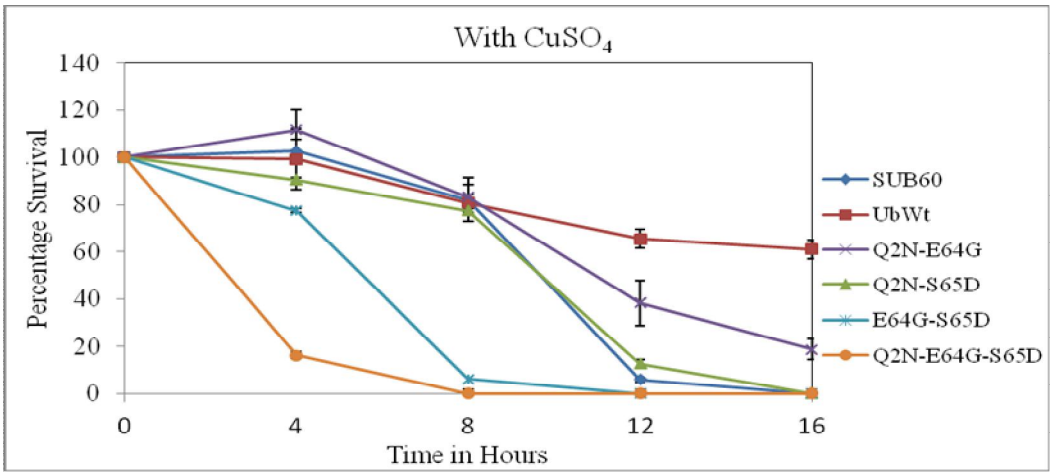


Fig. 3.12. Heat stress complementation test of SUB60 and SUB60 transformed with wild type ubiquitin gene and SUB60 transformed with mutant ubiquitin gene.



(A)



(B)

Fig. 3.13. Shows graph of percentage survival of cells SUB60 and SUB60 transformed with wild type gene, UbQ2N-E64G, UbQ2N-S65D, UbE64G-S65D and UbQ2N-E64G-S65D under heat stress (A) Un-induced condition (B) Induced with 50 µm $CuSO_4$

3.4.2.3.2. Cycloheximide sensitivity assay

Cycloheximide is a eukaryote translational inhibitor which inhibits the protein synthesis by binding at large subunit of the ribosomes. UPS plays significant role to combat the effect of antibiotic by removing the consequent nascent and wrongly folded peptide. Over-expression of ubiquitin confers resistance while reduced level

confers sensitivity towards cycloheximide. L28 protein of large subunit of ribosome undergoes polyubiquitination through lysine 61 linked polyubiquitin chains. Failure of polyubiquitination on L28 makes ribosomes more sensitive to cycloheximide. Cycloheximide also induces the stress sensing UBI4 gene which upon over expression synthesizes more ubiquitin. In order to check the functionality of mutant ubiquitin they were transformed in SUB60 cells which is cycloheximide sensitive due to lack of UBI4 gene, wild type ubiquitin gene transformed in SUB60 can revert sensitivity to resistance towards cycloheximide. Thus all mutants have been checked whether they confer resistance or sensitivity towards cycloheximide in *Saccharomyces cerevisiae* (Fig. 3.14). Results indicated that all mutants show sensitivity towards cycloheximide, sensitivity was found to be more in case of UbE64G-S65D and UbQ2N-E64G-S65D.

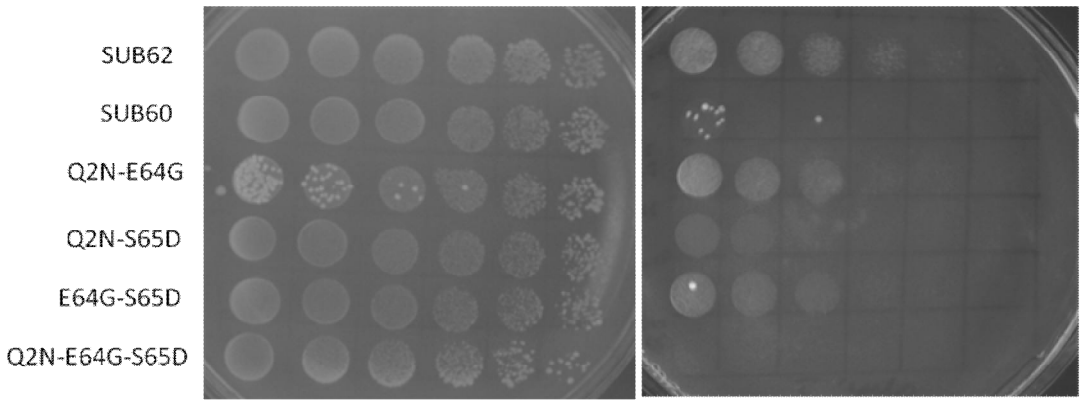


Fig. 3.14. Antibiotic stress complementation of *Saccharomyces cerevisiae* SUB60 and its transformants. SUB62 and SUB60 show cycloheximide resistance and sensitivity respectively. The SUB60 cells transformed with UbQ2N-E64G, UbQ2N-S65D, UbE64G-S65D shows sensitivity while UbQ2N-E64G-S65D shows hypersensitivity towards cycloheximide. Three fold serial dilutions (2), (3) and (4) spotted on SD agar plate in the presence and absence of 4µg/ml of cycloheximide.

3.4.2.3.3. N-end rule – β galactosidase assay

N-end rule says that the stability of protein is dictated by the identity of its N terminal residue. Several proteins change their N-end identity post-translationally, which decides their rate of degradation by UPS system. β-galactosidase enzyme has been chosen as a reporter protein. Methionine belongs to the class stabilizing residue while proline belongs to the most destablizing residue. So when methionine is at N-end of β-gal in Ub-Met-β gal, deubiquitinating enzymes recognize them and remove ubiquitin and the half life of β-gal increases. While in the case of Ub-Pro-β gal, proline at N-end it is not recognized by deubiquitinating enzymes. Hence polyubiquitin chain forms on the existing ubiquitin and β-gal becomes vulnerable for degradation by UPS. Efficient degradation of substrate protein depends on the sufficiency of healthy ubiquitin pool in the cells. SUB60 is the engineered strain, which does not have UBI4 gene hence ubiquitin pool is less in this strain. While SUB62 which is wild type for UBI4 and SUB60 transformed with plasmid carrying wild type ubiquitin gene, have normal ubiquitin pools as compared to SUB60. Hence, mutants of ubiquitin have been transformed in SUB60 cells and checked whether the mutant ubiquitin can paticipate in the normal functioning of the UPS.

Our results show β-galactosidase activity remained low for SUB62 and UbWt, for Ub-Pro-β-gal, where Pro was present as the N-end residue. However, the β-galactosidase activity increased for Ub-Pro-β-gal in the mutants UbQ2N-E64G, UbQ2N-S65D and UbE64G-S65D and UbQ2N-E64G-S65D (Fig. 3.15) which indicates the non-functionality of the mutant ubiquitin.

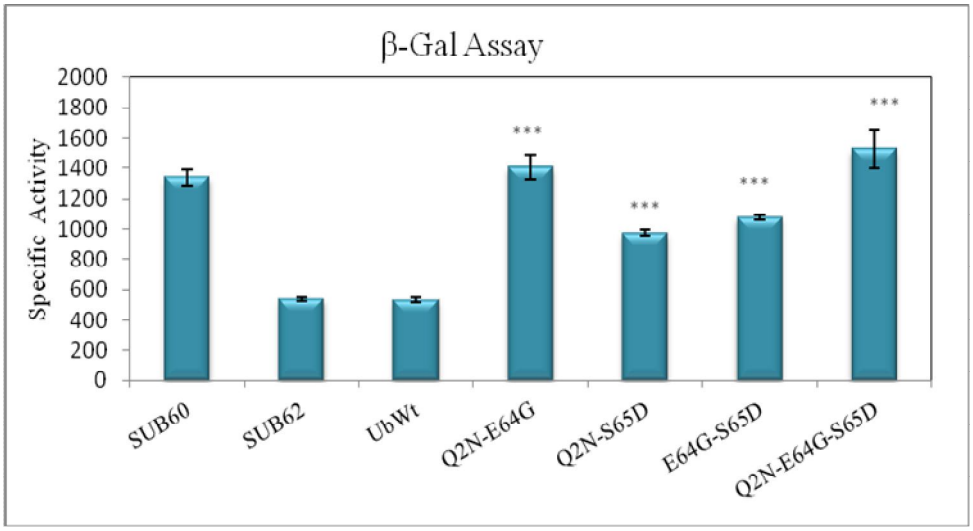


Fig. 3.15. Effect of UbQ2N-E64G, UbQ2N-S65D, UbE64G-S65D and UbQ2N-E64G-S65D mutations on the half-life of β-gal in UBI4 background estimated by measuring β-gal activity.

S. Crevisiae strain SUB60, SUB62, SUB60 transformed with wild type ubiquitin, UbWt (60/Wt) and mutants UbQ2N-E64G, UbQ2N-UbS65D, UbE64G-S65D and UbQ2N-E64G-S65D. These transformant were also co-transformed with pUb23 expressing Ub-β-galactosidase fusion with Pro (P) as the N-terminal residues. SUB60 and SUB62 cells were transformed by plasmid pUb23 expressing Ub-β-galactosidase fusion with Pro (P) as the N-terminal residues.

3.5. Discussion

In order to analyze functional consequences of the residues of the second β-bulge of ubiquitin, residues Gln at 2nd position, Glu at 64th position and Ser at 65th position were replaced by more preferred residues i.e. Asn, Gly and Asp at 2nd, 64th and 65th positions respectively. Hence four site directed mutants having combinations of the single mutations namely UbQ2N-E64G, UbQ2N-S65D, UbE64G-S65D and UbQ2N-E64G-S65D were constructed in yeast expression vector.

Growth profile of the mutants UbQ2N-E64G and UbQ2N-S65D have shown no effect on the overall growth of the organism. While due to UbE64G-S65D and UbQ2N-E64G-S65D mutations, growth of the organism has reduced significantly and it became slow growing which confirms the importance of the residues to ubiquitin structure and in the balance of cellular homeostasis.

Complementation analysis of above mentioned mutants has revealed that mutants UbQ2N-E64G and UbQ2N-S65D could complement the heat sensitive phenotype partially while UbE64G-S65D and UbQ2N-E64G-S65D showed non-complementation of the phenotype. As tertiary structure of these two mutants remained unaltered and imparted more stability to the protein (results presented in **Chapter 5**), confirms that the loss of phenotype can be attributed to the potential role of the residues of the β-bulge in proteasomal degradation of the substrate protein. Possible explanation for the results observed could be that the mutants may act as potential antagonists and interfere with degradation by proteasomes, when incorporated in the polyubiquitin chains in place of wild type ubiquitin.

Cycloheximide is the potential eukaryotic translational inhibitor which acts on the large subunit of ribosome and disrupts the normal functioning of them in protein

synthesis which results in the formation of truncated, partially folded proteins. UBI4 deleted SUB60 strain is sensitive towards the antibiotic due to lack of sufficient ubiquitin pool to remove all truncated proteins. L28 protein of large subunit of ribosome undergoes polyubiquitination through lysine 63 linked polyubiquitin chains. Failure of polyubiquitination on L28 makes ribosomes more sensitive to cycloheximide. Mutations addressed in the present study occur adjacent to lysine 63 and are likely to interfere with the ubiquitination of L28. All of the above mentioned mutants transformed in SUB60 have become sensitive towards the antibiotic. Among all mutants, UbQ2N-E64G-S65D has showed more sensitivity which proves non-functionality of the same.

Half life of the test protein, β-gal has been assayed to know the functionality of the ubiquitin mutants. Mutant ubiquitin were co-expressed with Ub-Pro- β-gal. All of the mutants showed extended half life of the test protein with N terminal proline, which could not be removed efficiently by the ptroteasome due to non-functionality of the mutant ubiquitin.

It may be concluded that the residues which are present naturally in the β-bulge of ubiquitin are extremely important and they work in combination to maintain the various cellular event. As the residues of the β-bulge of ubiquitin were replaced by more preferred amino acids, to keep the structure of the protein unaffected by the mutations. Hence, functional failure of the ubiquitin may be attributed specifically to the residues which were mutated.

CHAPTER 4

Construction, expression and structural analysis of dose dependent lethal mutation of ubiquitin

4.1. Introduction

Nature has kept the sequence of ubiquitin conserved all the way through the evolution process, in order to preserve its structure. Highly conserved structure of ubiquitin plays central role in the normal homeostasis and regulation of cell. Ubiquitin interacts with a variety of proteins and becomes an active participant in the regulation process. Hence, mutations are not allowed naturally in this system. Amino acid replacements in ubiquitin reveal significant information on its structure and the functions associated with them. Earlier work from our laboratory has generated mutant forms of ubiquitin by successive rounds of PCR in error prone conditions, which resulted in the isolation of a novel dose dependant lethal mutant of ubiquitin. Sequencing of the same revealed changes in seven bases, out of them three gave rise to silent mutations, while the other four brought changes in the codon leading to replacement of amino acid residues. Position of mutations and the replacements are S20F, A46S, L50P and I61T (Ratna Prabha et al., 2010). The mutations Ser20 to Phe and Ala46 to Ser have occurred in type I and type III turns respectively (Vijay-Kumar et al., 1985). These two are surface residues and hence the substitution of a hydrophilic residue Ser by a hydrophobic residue Phe may cause changes in the interaction behaviour of ubiquitin with other proteins, however A46S did not show any change in functionality of mutant ubiquitin. Formation of a hydrogen bond between the N of ε-amino group of Lys48 and Ala46 was reported in wild type ubiquitin (Trempe et al., 2010). The third mutation Leu50 to Pro occurring in the β-sheet seems to be significant as well since the side chain of Leu is buried in the interior of the protein. Furthermore, Pro being restricted in geometry due to its torsion angle φ, introduces kink in the protein backbone hence can direct the polypeptide chain in a new direction drastically changing the pattern of surface residues, which are known to participate in the interaction with many proteins inside the cell. Substitution of Ile61 by Thr is change of hydrophobic residue to hydrophilic residue. Ile61 is one of the first residues to be protected from H-D exchange during refolding of ubiquitin

(Briggs and Roder, 1992). Hence, it may be considered as an important residue from folding viewpoint. Present study deals with structural characterization of the mutant UbEP42, to understand the impact of mutations on the structure of mutated residues which functions of this multi-tasking protein are affected due to the mutations accrued, giving rise to a dosage dependent lethal phenotype.

4.2. Material and methods

4.2.1. Strain and reagents used in the study

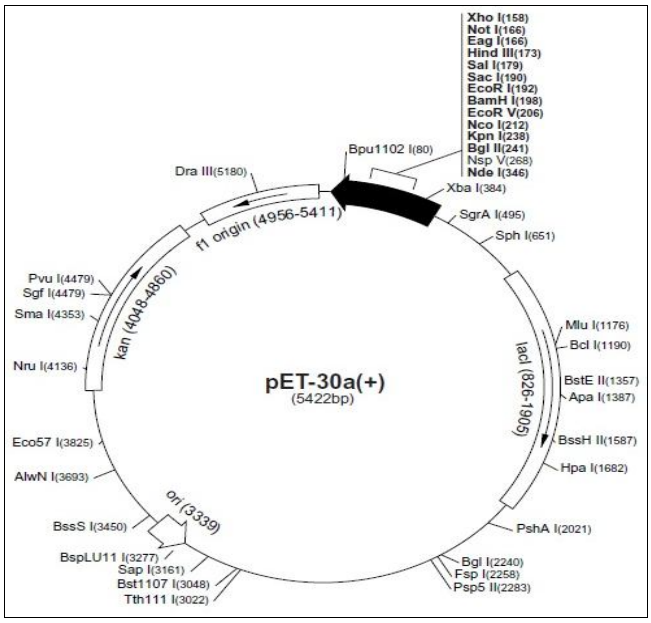
E.coli DH5α (F^-, 80dlacZ M15, endA1, recA1, hsdR17 (rk^-,mk^+), supE44, thi-1, gyrA96, relA1, (lacZYA-argF)U169) strain was used for recombinant DNA manipulation and storage.

BL21(DE3) pLys S F^-ompT $hsdS_B(r_B^- m_B^-)$ gal dcm (DE3)pLysS(Cam^R), high stringent expression strain was used to express recombinant DNA with chloramphenicol (30µg/ml) selection pressure. Cultures were grown at 37°C at 200 rpm in Luria broth (Hi-media) and selected on ampicillin (50µg/ml) containing medium. Bacterial transformation was done by $CaCl_2$ method (Dagert and Ehrlich, 1979). Plasmid isolation was done by standard alkaline lysis protocol (Sambook et al., 2001).

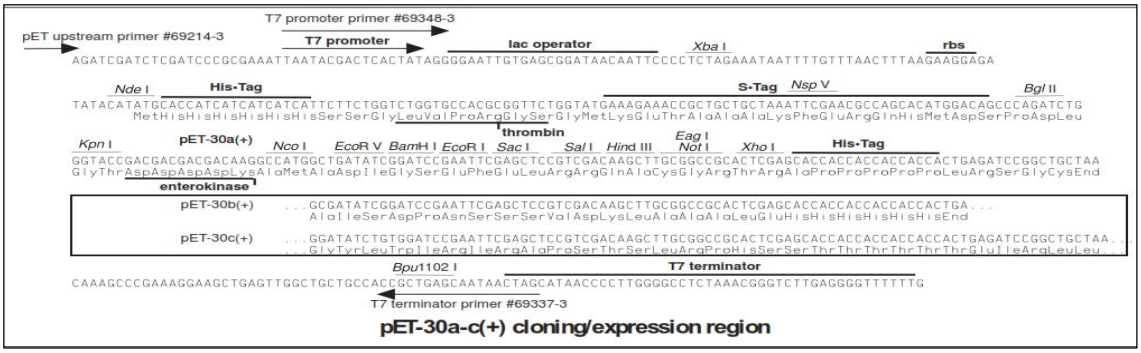
4.2.2. Plasmid construction in expression vector pET-30a

The pET vector system was used in the study for the cloning and expression of all ubiquitin variant in *E. coli*. Mutant ubiquitin genes were cloned in pET-30a plasmid under the control of strong bacteriophage T7 promoter (Fig. 4.1). Target genes were initially cloned using hosts that do not contain the T7 RNA polymerase gene i.e. DH5α, thus eliminating the possible plasmid instability. Once established in a non-expression host, target protein expression was initiated by transferring the plasmid into host cell BL21(DE3) containing a chromosomal copy of the T7 RNA polymerase gene under lacUV5 control. T7 RNA polymerase is very selective and

active. It utilises cellular resources to express target gene when fully induced. Expression was induced by the addition of IPTG (isopropyl-β-D-1-thiogalactoside) at a final concentration of 0.1mM-2mM to the medium.



(A)



(B)

Fig.4.1. Vector map of bacterial expression vector pET-30a. The plasmid carries multiple cloning site under T7 promoter. (A) vector map showing promoter, antibiotic resistance, origin of replication and restriction sites. (B) shows sequence wise detail of cloning/ expression region (Frankel and Leinwand., 1996)

4.2.3. Sequence Analysis

Plasmid pET-30a carrying the mutant ubiquitin gene was sequenced in a single forward reaction using T7 forward primer 5'TAATACGACTCACTATAGG 3'.

4.2.4. Protein purification by size exclusion chromatography

A pKK223-3 plasmid construct containing the mutant ubiquitin gene UbEP42 was expressed in *Escherichia coli* strain BL21(DE3) under the control of IPTG inducible p*tac* promoter. Cells were grown at 37°C in Luria broth (LB) containing ampicillin (100µg/ml) to mid-log stage and harvested after 4hours of induction (1mM IPTG). Cells were resuspended in lysis buffer containing 50mM Tris, 2mM EDTA, 5% glycerol, pH7.8. Cells were treated with 100µg of lysozyme for 30 minutes at 37°C. Lysed cells were sonicated (50 duty cycles for 15 sec/3-4 times in an interval of 5 sec) and centrifuged at 8,000g for 10 min at 4°C. The cell lysate was treated with protease inhibitor cocktail and 1mM PMSF (Phenyl methyl sulfonyl fluoride). Following centrifugation the supernatant containing the remaining soluble cellular proteins was collected. The collected supernatant was treated with 0.2% polyethylene imine (v/v) (PEI) to precipitates the DNA, removed by centrifugation at 16,000g for 30 minutes. Mutant ubiquitin UbEP42 was further precipitated out of the supernatant at 20% (w/v) ammonium sulphate. After dialysis ubiquitin containing fraction was purified on Sephadex G-50 column. The protein was concentrated by precipitation and dialysis.

Protein concentration for UbEP42 was determined using its extinction coefficient 1280 M^{-1} cm^{-1} at 280nm.

4.2.5. Protein purification by affinity chromatography

Mutant ubiquitin protein UbF45W, UbEP42 and UbI61T were cloned in pET-30a expression system, downstream to the affinity tag consisting polyhistidine residues and purified by affinity chromatographic purification using immobilized metal affinity chromatography (IMAC) method (Porath et al., 1975). IMAC is based on the interaction between a transition metal ion (Co^{2+}, Ni^{2+},Cu^{2+}, Zn^{2+}) immobilized on a matrix. Among all, histidine exhibits the strongest interaction with immobilized metal ion matrices, as electron donor groups on the histidine imidazole ring readily form coordination bonds with the immobilized transition metal. Peptides containing sequences of consecutive histidine residues are efficiently retained on IMAC (Hochuli et al., 1987). Poly-histidine affinity tags are normally placed on either the N- or the C-terminus of target proteins depending on their characteristics. Purification using poly-histidine tags has been carried out effectively using a number of expression systems including bacteria (Chen and Hai, 1994; Rank et al., 2001), yeast (Kaslow and Shiloach, 1994), mammalian cells (Janknecht et al., 1991; Janknecht and Nordheim, 1992) and baculovirus-infected insect cells (Kuusinen et al., 1995; Schmidt et al., 1998). Affinity purification is the method of choice when purification based on proteins characteristics become difficult either due to its nature or induced structural changes because affinity purification is based on the tag attached with protein rather than characteristics of protein itself. Tagged protein can be eluted from free imidazole as it has high affinity towards Ni^{+2}, concentration of imidazole lies in the range of 20-250mM imidazole (Hefti et al., 2001; Janknecht et al., 1991) or else from low pH buffer which can also replace the histidine tagged protein.

Mutant ubiquitin protein UbEP42, UbI61 and UbF45W control were expressed from pET-30a vector. Lysis of the cells has been carried out by incubating cells with lysozyme followed by sonication in lysis buffer containing 50mM

NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0. Initial cell lysis protocol and lysate preparation of induced culture is discussed in section 2.4.2.

Ni-NTA resins (Qiagen) were supplied in 20% ethanol to prevent it from degradation by bacterial growth. Ethanol content in the resin was removed by washing with deionized water and subsequently washed with equilibration buffer containing 50mM NaH_2PO_4, 300mM NaCl, pH 8.0. After several washes resins were incubated with lysate for an hour to allow binding of His tagged protein to the resin, after incubation cell lysate was removed and resin was washed with buffer containing 50mM NaH_2PO_4, 300mM NaCl with low concentration of imidazole (20mM), pH 8.0 to remove unbound protein and to reduce nonspecific binding of host proteins with histidines. After washing steps, target protein was eluted from the buffer containing 50mM NaH_2PO_4, 300mM NaCl, 250mM imidazole, pH 8.0. Alternatively elution with low pH buffer, pH < 4.5 may also be used, here histidine is replaced by proton instead of imidazole. Percent purity of the target proteins have been checked with 15% SDS-PAGE, further imidazole, NaCl were removed by dialysis with buffer containing 10mM Tris, pH 7.4, purified proteins were concentrated by vacuum evaporation, and salting out with ammonium sulphate precipitation.

4.2.6. Circular dichroism and fluorescence spectroscopy

CD spectra were recorded using a Jasco J-815 spectropolarimeter. For far UV CD spectra (200 to 250nm) cells of 1mm path length were used. Spectra were recorded at 2nm resolution. The data pitch was 0.2nm. Scan speed was 50nm/sec. three spectra were accumulated to reduce the noise. Protein solutions were prepared in 10mM Tris HCl, pH7.4 and concentration of protein was 15μM. Proper blanks were prepared for all samples and the spectra of the samples were blank corrected.

Fluorescence spectra were recorded using Hitachi F7000-FL fluorescence spectrophotometer. Slit width was 5nm. Protein solutions were prepared in 10mM

Tris HCl, pH7.4 and concentration of protein was 5μM. Samples were excited at 280nm to record the intrinsic fluorescence due to tryptophan (Trp). Emission was recorded between 320-440nm.

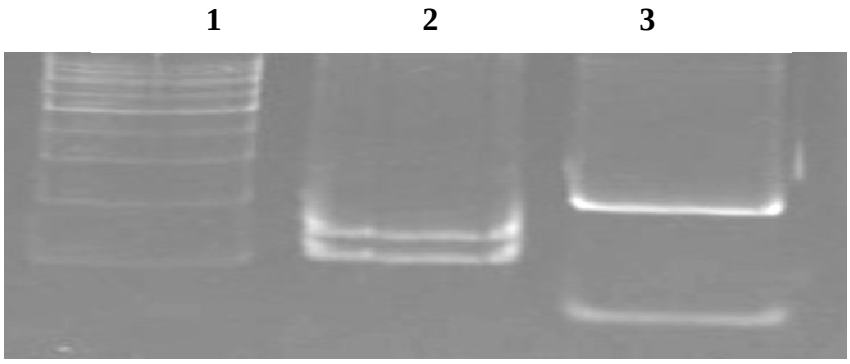
For denaturation studies, ubiquitin mutants were denatured by Guanidine hydrochloride (Sigma) in the range of 0M to 6M concentration, proper blanks were prepared and the spectra were blank corrected.

4.3. Results

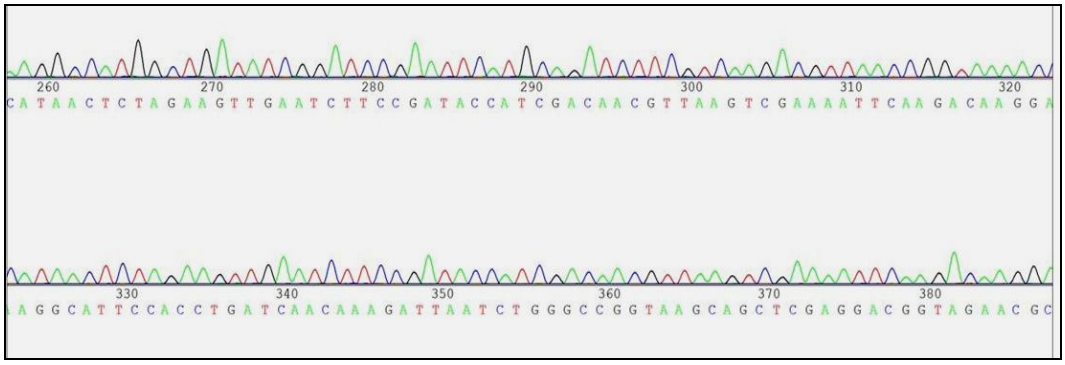
4.3.1. Construction of UbF45W, UbEP42 and UbI61T in bacterial Expression vector pET-30a

4.3.1.1. Construction of UbF45W in bacterial expression vector pET-30a

For the construction of UbF45W in bacterial expression vector pET-30a, the gene for UbF45W was amplified from pKK223-3 vector and subjected to *Eco*RI and *Hind*III double digestion. After restriction digestion the mutated gene was ligated in the pET-30a vector by using the same restriction enzyme sites. Vector carrying F45W mutation of ubiquitin gene in pET-30a vector was confirmed with restriction digestion pattern of its PCR amplicon by digesting it with *Sau*96 enzyme to confirm the mutation incorporated into the vector (Fig. 4.2). Further, gene was sequenced to ensure the incorporation of mutant gene.



(A)



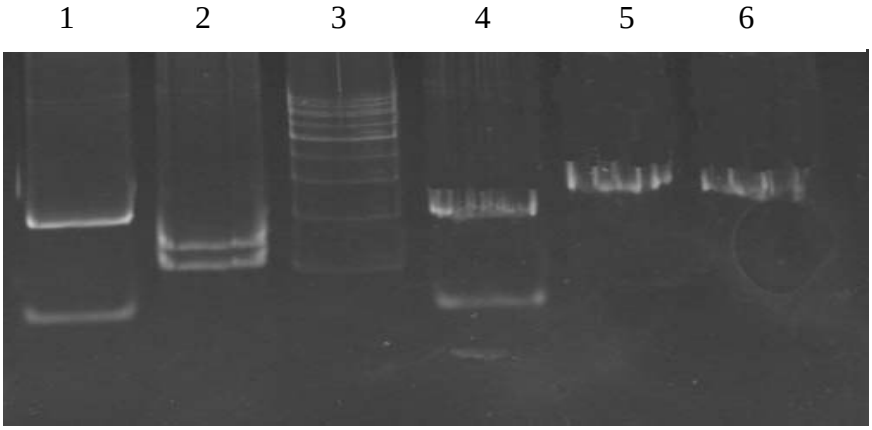
(B)

Fig. 4.2. Restriction digestion and sequence confirmation of UbF45W

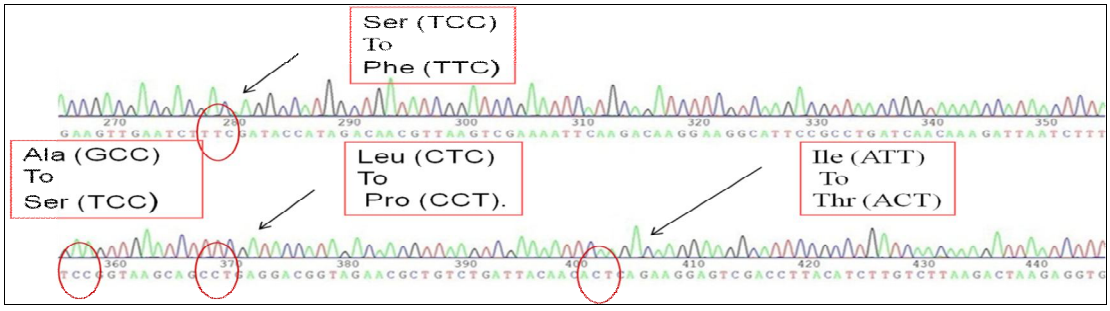
(A) Lane 1 shows 100 bp ladder, Lane 2 shows digestion of pET-F45W with *Sau*96 restriction enzyme and Lane 3 shows digestion of pET-F45W with *Sal*I restriction enzyme (B) Electrophoretogram of UbF45W gene in pET-30a vector.

4.3.1.2. Construction of UbEP42 in bacterial expression vector pET-30a

For the construction of the single mutant ubiquitin UbEP42 in bacterial expression vector pET-30a, wild type ubiquitin gene (UbWt) carrying mutations at four places i.e. S20F, A46S, L50P and I61T mutations was amplified and subjected to *Eco*RI and *Hind*III double digestion. After restriction digestion the mutated gene was ligated into the pET-30a vector by using the same restriction enzyme sites. Vector carrying all above mentioned mutations of ubiquitin gene in pET-30a vector was confirmed with restriction digestion pattern of its PCR amplicon by digesting it with *Xba*I, *Xho*I and *Sal*I restriction enzymes (Fig. 4.3). Further, gene was sequenced to ensure the incorporation of mutant gene.



(A)



(B)

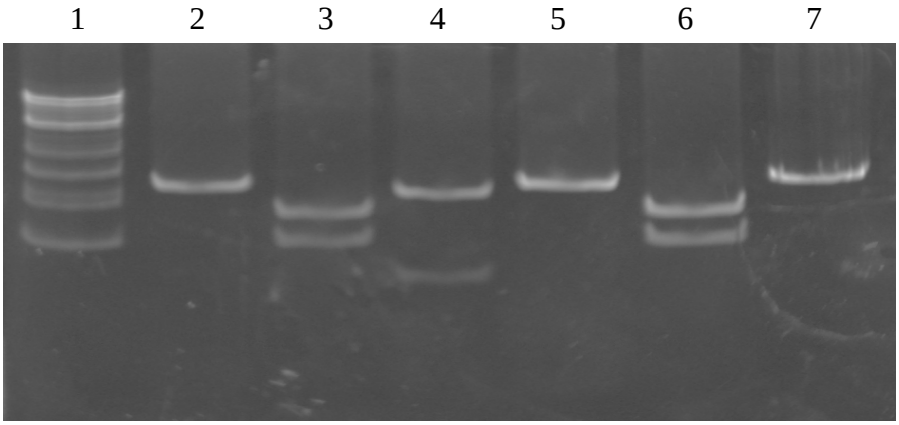
Fig.4.3. Gel picture showing restriction digestion Pattern of PCR product pET-30a UbEP42 and UbF45W (wild type control) and sequence confirmation

(A) Lane1 and lane 2 show digestion of PCR amplicon of UbF45W with *Sal*I and *Xho*I respectively. Lane 3 shows 100 bp DNA ladder, lane 4 and 5 show PCR product digestion of UbI61T with *Sal*I and *Xho*I respectively. Lanes 6 shows undigested PCR amplicon as a control. Lane 5 shows failure of digestion of mutant with restriction enzyme *Xho*I hence confirms the presence of desired mutation.

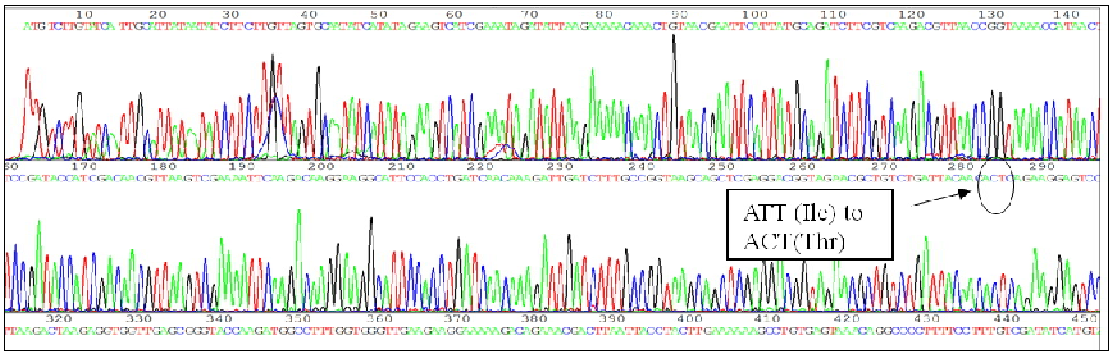
(B) Electrophoretogram of UbEP42 gene in pET-30a vector. The marked region confirms the presence of S20F, A46S, L50P and I61T substitution.

4.3.1.3. Construction of UbI61T in bacterial expression vector pET-30a

For the construction of the single mutant ubiquitin UbI61T in bacterial expression vector pET-30a, wild type ubiquitin gene carrying mutations at I61T mutations was amplified and subjected to *EcoR*I and *Hind*III double digestion. After restriction digestion the mutated gene was ligated into the pET-30a vector by using the same restriction enzyme sites. Vector carrying all above mentioned mutations of ubiquitin gene in pET-30a vector was confirmed with restriction digestion pattern of its PCR amplicon. *Sal*I restriction enzyme was used to confirm the incorporated mutation into the vector (Fig. 4.4). Further, gene was sequenced to ensure the incorporation of mutant gene.



(A)



(B)

Fig.4.4. Gel picture showing digestion Pattern of PCR product pET-30a UbI61T and UbF45W (control) and sequence confirmation of Ub61T

(A) Lane1 shows 100bp DNA ladder. Lane 2 and lane 5 show undigested PCR product of UbWt and pET UbI61T respectively, lane 3 and 4 show digestion of PCR product for wild type ubiquitin gene with *Xho*I and *Sal*I restriction enzymes respectively. Lanes 6 and 7 show digestion pattern of the PCR product UbI61T by *Xho*I and *Sal*I respectively. Lane 7 shows failure of digestion of mutant with restriction enzyme *Sal*I hence confirm the incorporation of desired mutation.

(B) Electrophoretogram of UbI61T gene in pET-30a vector. The marked region confirms the I61T substitution.

4.3.2. Expression and Purification of UbEP42 cloned in pKK223-3 expression vector by size exclusion chromatography

A pKK223-3 plasmid construct containing the mutant ubiquitin gene UbEP42 was expressed in *E. coli* strain BL21 (DE3) by inducing it with 1mM IPTG at 37°C under selection pressure of 50µg/ml of ampicillin (Fig. 4.5).

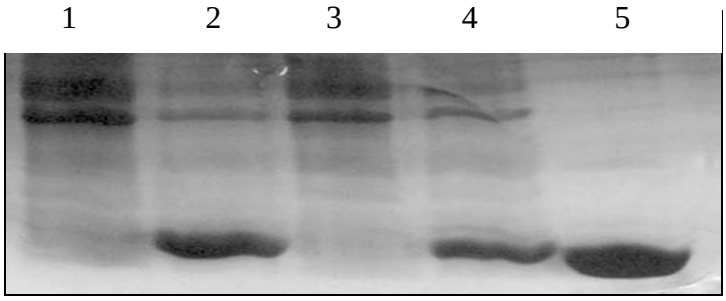
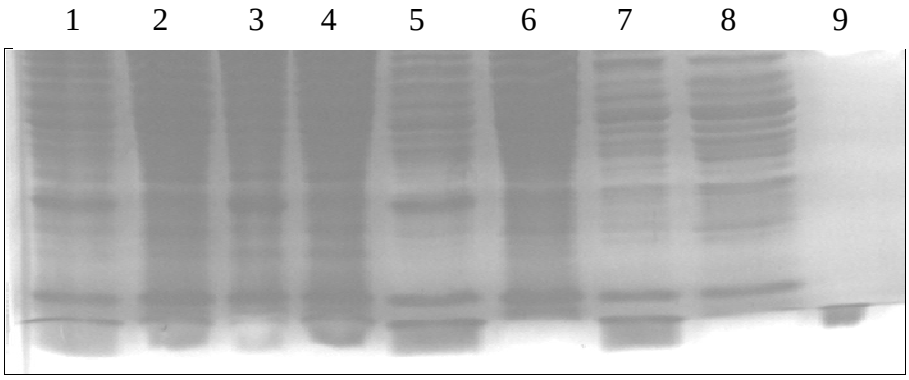


Fig.4.5. Ubiquitin expression gel profile

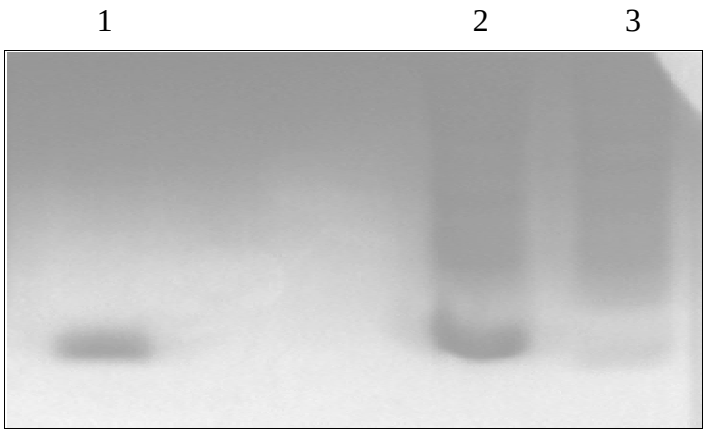
Lane 1. Shows protein profile of un-induced pET-F45W. Lane 2. Shows protein profile of pET-F45W with 1mM of IPTG. Lane 3. Shows un-induced pET-EP42 Lane 4. Shows induced pET-EP42 Lane 5. Shows the standard ubiquitin.

Wild type ubiquitin purification involves two crucial steps to get rid of the rest of the proteins in cell; one is to remove those cellular proteins which are heat sensitive by giving heat treatment to the lysate as ubiquitin is the most stable protein at wide range of pH and temperature. Hence ubiquitin remains stable and active even after

heat treatment. Another step of screening involves salting out method in which ubiquitin gests precipitated at high concentration of ammonium sulphate i.e. 85%. Thus, 60% ammonium sulphate cut can remove many undesired proteins. However due to incorporation of four residues at different places in mutant ubiquitin UbEP42 has undergone a change in its structure in such a way that it cannot be purified by the method used for wild type ubiquitin purification. Two major changes in its behaviour are temperature sensitivity and hydrophobicity. In order to get purified protein, heat treatment step has been omitted out from the standard protocol. It has been also noted out that instead of precipitation at 85% saturation of ammonium sulphate, it could precipitated out at even 20% of ammonium sulphate concentration (Fig. 4.6).



(A)



(B)

Fig. 4.6. Purification strategy of UbEP42 involving salting out method by ammonium sulphate salt Precipitation

(A) Lane 1 and 3 show protein profile of UbF45W cell, taken as a control and whole cell protein profile of UbEP42, Lane 2 and Lane 5 show pellet from 85% Ammonium sulphate precipitation of F45W and F45W 60 % supernatant then 85 %, Lane 4 and Lane 6 show 85% pellet from UbEP42 and 60% supernatant then 85% cut from EP42, Lane 7 shows F45W 45 % supernatant then 85% cut, Lane 8 shows EP42 45% supernatant then 85% cut, Lane 9 shows ubiquitin standard.

(B) Lane 1 shows standard ubiquitin used as a marker, lane 2 shows 45% ammonium sulphate cut of UbEP42 protein and Lane 3 shows 45% ammonium sulphate cut of UbF45W (used as a control).

Result showed that UbEP42 has become more hydrophobic due to mutations as compared to wild type strain and less amount of ammonium sulphate is sufficient enough to allow mutatnt ubiquitin to get precipitated. Hydrophobicity also caused insolubility of protein in solution, most of the mutant protein resided in the pellet rather than in soluble form hence it was difficult to purify the protein in standard conditions. To overcome this problem UbEP42 was purified by using 3M urea in the buffer. In presence of 3M urea it was possible to go further for the purification of protein by size exclusion chromatography. After chromatographic separation of UbEP42, it was also noticed that it has become prone to the rapid degradation even in the presence of protease inhibitor and storage at low temperature. From the above experiments it may concluded that UbEP42 displays the lack of stability, temperature sensitivity and increased hydrophobicity due to above mentioned mutations. Therefore to improve the mutant protein yield and stability it was decided to clone UbEP42 in bacterial expression vector pET-30a to exploit the benefits of affinity chromatography, as the purification procedure depends on vector based histidine tag rather than the characteristics of protein per se. Hence His-tagged purification of mutant ubiquitin was adopted. Detail purification procedure of the same is mentioned in Materials and Methods section. Due to difficulties encountered in cloning and solubility problems faced in purification of mutant forms of ubiquitin, structural studies had to be restricted to UbEP42 and one of its derivatives UbI61T.

4.3.3. Expression and Purification of UbF45W, UbEP42 and UbI61T cloned in pET-30a expression vector

The protein UbEP42 cloned in pET-30a vector was expressed in BL21(DE3) under the control of IPTG inducible T7 promoter. Cells were induced by IPTG in mid-log phase and checked on 15% SDS PAGE (Fig. 4.7).

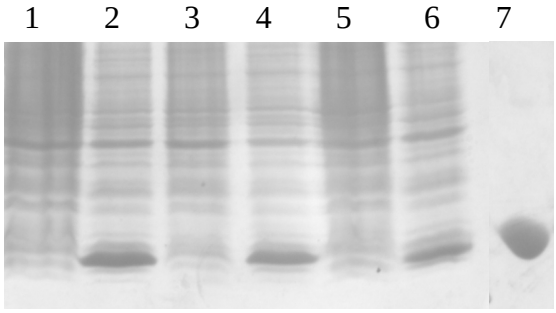


Fig. 4.7. Ubiquitin expression gel profile

Lane 1, Lane 3 and Lane 5 Show protein profile of un-induced cultures of pET-UbF45W, pET-UbI61T and pET-UbEP42. Lane 2, Lane 4 and Lane 6 Show protein profile of pET-F45W, pET-UbI61T and pET-UbEP42 cultures with 1mM of IPTG induction. Lane 7 Shows Lysozyme, used as a marker.

After checking expression mutant protein, purification was carried out by suspending cells in lysis buffer and lysed by lysozyme and sonication methods (Details of the purification methods is discussed in detail in material and method section). The cell lysate was treated with protease inhibitor cocktail and PMSF. After removing DNA, supernatant was incubated with pretreated Ni-NTA resin and washed with buffer containing 20mM imidazole to remove non-specific proteins which were bound to resin, His tagged ubiquitin mutant proteins have been eluted by 250mM imidazole. Eluted purified proteins were checked by 15% SDS PAGE (Fig. 4.8) Eluted purified proteins were dialysed against stabilizing buffer containing Tris 10mM, pH-8.0, 5% glycerol and 5mM EDTA. Dialysed proteins were checked on

SDS-PAGE. Proteins were concentrated by precipitation method and dialysed again for spectral studies.

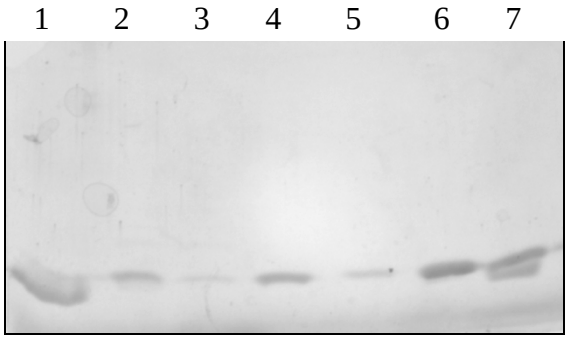


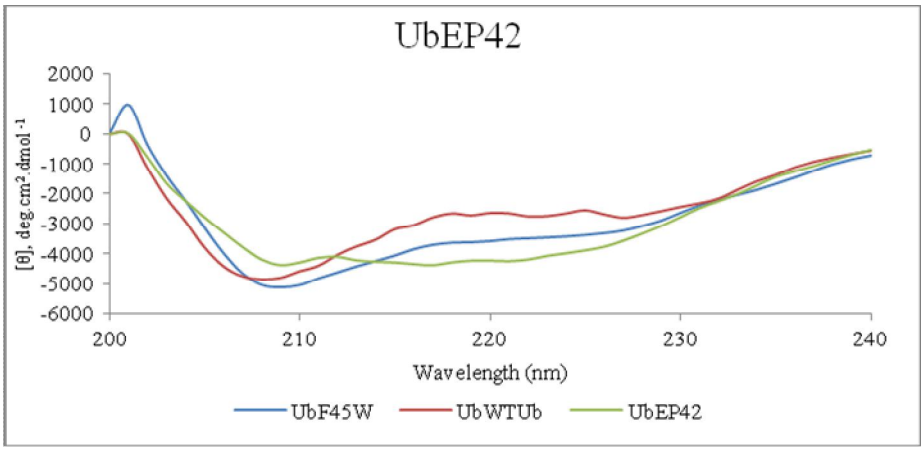
Fig. 4.8. Shows purified protein of UbF45W, UbI61T and UbEP42

Lane 1 shows lysozyme used as a marker, Lane 2 and Lane 3 show Elution 1 and 2 of mutant UbF45W, Lane 4 and 5 show elution 1 and 2 of UbI61T, Lane 6 and 7 show Elution 1 and 2 of UbEP42.

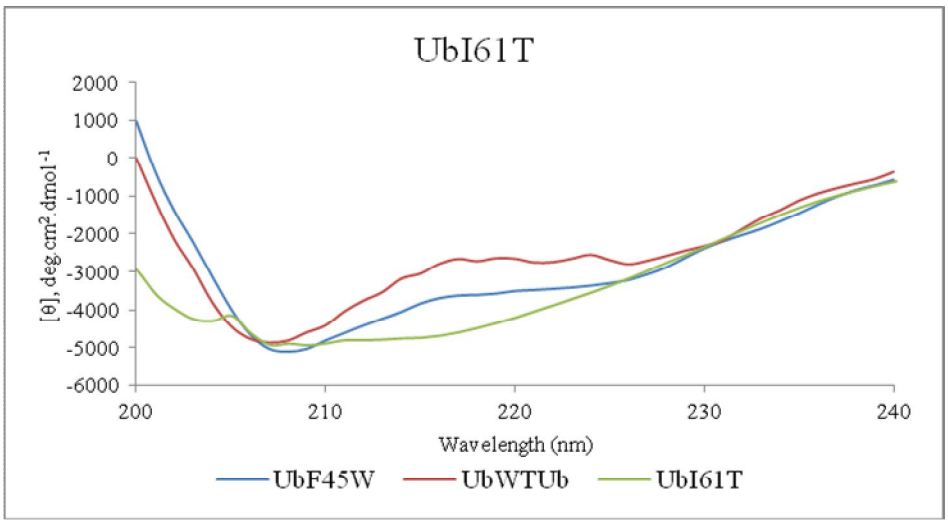
4.3.4. Far UV CD spectra of pETUbF45W, pETUbEP42 and pETUbI61T

Far UV CD spectra were recorded to study the changes in secondary structure due to mutations in the sequence of ubiquitin. UbF45W is used as a control protein which resembles the structure of wild type ubiquitin and has advantage of having Trytophan for fluorescence analysis. It was used as a reference in far UV CD spectra. UbEP42 shows a structural change in secondary structure with respect to UbF45W. The CD spectrum of UbEP42 shows a change in secondary structure and indicates that it has acquired more of a β-sheet structure when compared to native protein (Fig. 4.9). UbI61T also resembles the structure to UbEP42 and displays more β-sheet content in the protein (Table 4.1). Structural changes of UbEP42 was expected as there are four substitution in the ubiquitin gene which are important to maintain the structure, one of the change is L50P, proline is known to introduce kink in the structure and it can change the direction of polypeptide chain. However, UbEP42 and UbI61T did not show proper secondary structure, which indicates that they may remain in the cell as improperly folded structures or the conditions used in vitro do

not allow stabilization of secondary structure of marginally stable UbEP42. Functional non-complementation and western blot analysis of UbEP42 (Pradeep Mishra and C. Ratna Prabha, unpublished observations) and UbI61T (results presented in **Chapter 2**) also support the second proposition as this mutant ubiquitin may resemble wild type ubiquitin and hence may be incorporated in polyubiquitin chain but can not be recognized by ubiquitin proteasome system.



(A)



(B)

Fig.4.9. Far UV CD spectra of three forms of ubiquitin UbF45W, UbEP42 and UbI61T.

Type of secondary structure	UbWt (X-ray)	UbWt	UbF45W	UbEP42	UbI61T
α-helix	16	17.3	18.3	7.9	10.4
β-sheet	32	32.2	32.1	41.3	55.6
Turns and random coil	52	50.5	49.6	50.8	34

Table 4.1. Secondary structural analysis of far-UV CD spectra of UbF45W, UbEP42, UbI61T using CD Pro software

4.3.5. Fluorescence spectra of the UbF45W, UbEP42 and UbI61T

Fluorescence spectra of the UbF45W, UbEP42 and UbI61T variants of ubiquitin were recorded by exciting the protein at 280nm (Fig. 4.10). UbWt does not give fluorescence as it lacks Trp, the only potential probe for fluorescence analysis in wild type ubiquitin is tyrosine at 59th position which is exposed to the solvent and displays only small changes in fluorescence upon folding (Jenson et al., 1980). While on the other hand engineered variant of ubiquitin UbF45W, which is otherwise comparable to wild type ubiquitin, contains tryptophan at 45th position in place of phenylalanine indicates the environment around aromatic amino acid residue (Khorasanizadeh et al., 1993). UbEP42 and UbI61T give fluorescence spectra upon excitation at 280nm despite lacking tryptophan. UbEP42 contains Ser to phe mutation at 20th position and tyr at 59th position which may contribute to fluorescence to some extent.

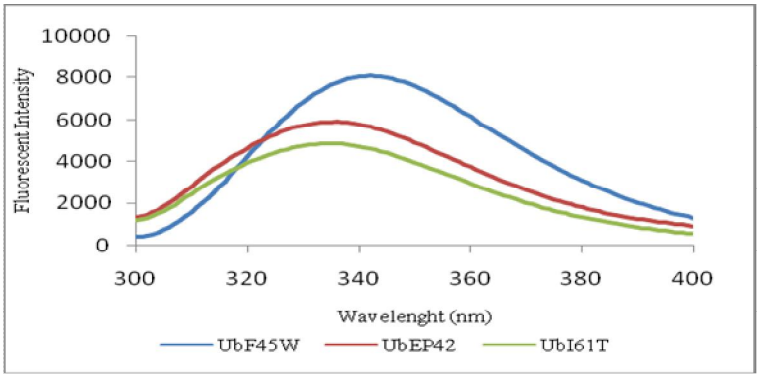
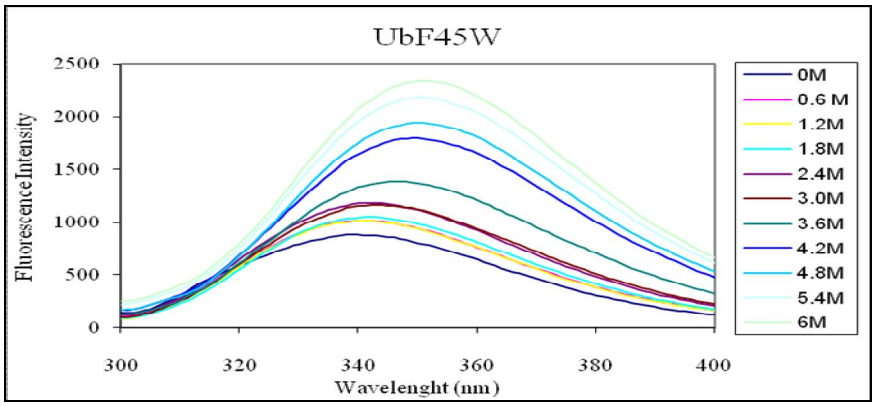


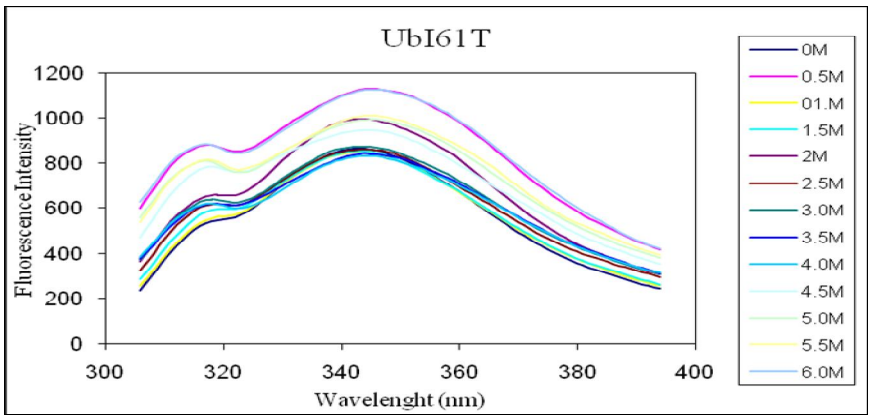
Fig.4.10. Fluorescence emission spectra of UbF45W, UbEP42 and UbI61T recorded after exciting the protein at 280nm

4.3.5.1. Guanidine hydrochloride denaturation of UbF45W, UbEP42 and UbI61T

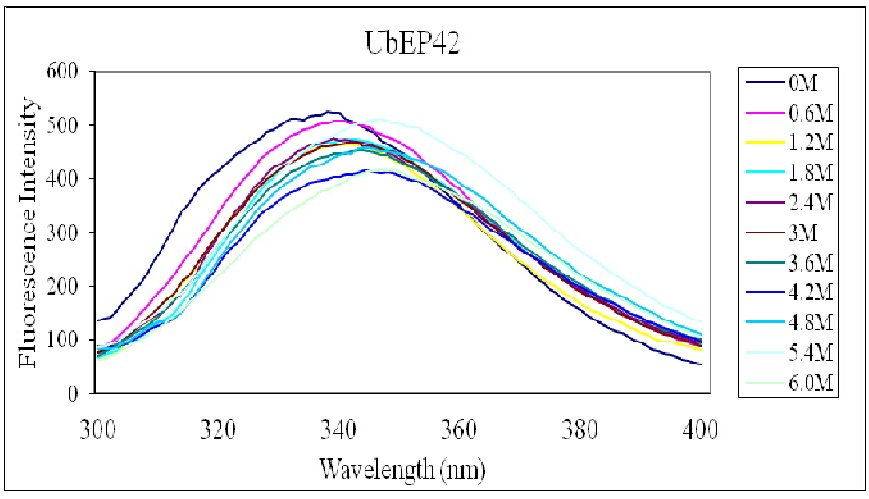
Guanidine hydrochloride denaturation of UbF45W, UbEP42 and UbI61T were studied by fluorescence. Increasing concentration of guanidine hydrochloride causes loss of protein secondary and tertiary structures and subsequent increase in the fluorescence intensities, along with a red shift in λ_{max} of emission. The stability can be evaluated by measuring the intensity and the shift in spectra as the protein which is more stable tends to loss its structure at higher concentration of GdHCl rather than the unstable protein. UbF45W showed increased intensity of fluorescence and red shift upon denaturation while UbEP42 and UbI61T did not show any change with respect to the two parameters confirming the absence of tertiary structure (Fig. 4.11).


UbF45W
Fluorescence Intensity
Wavelenght (nm)
0M
0.6 M
1.2M
1.8M
2.4M
3.0M
3.6M
4.2M
4.8M
5.4M
6M

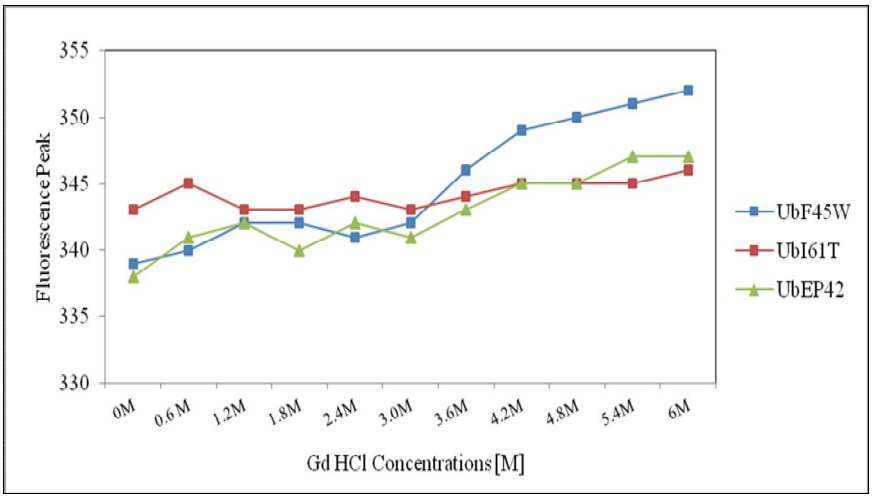
(A)


UbI61T
Fluorescence Intensity
Wavelenght (nm)
0M
0.5M
01.M
1.5M
2M
2.5M
3.0M
3.5M
4.0M
4.5M
5.0M
5.5M
6.0M

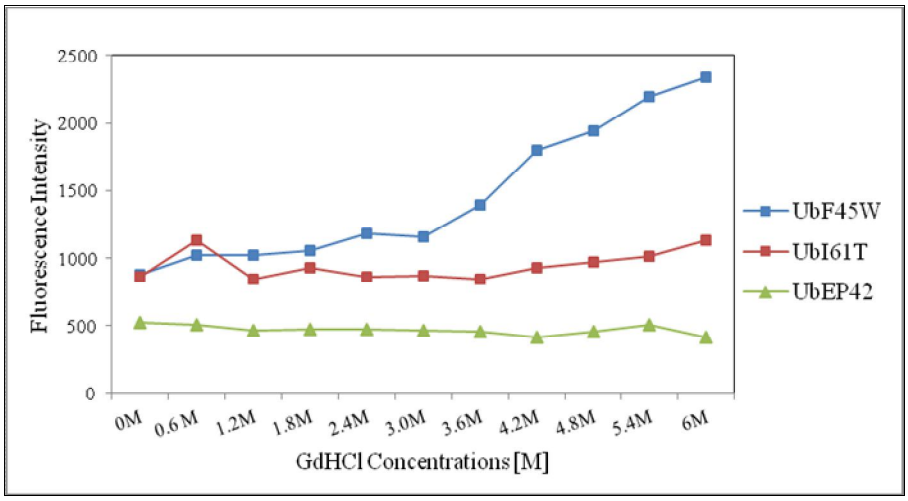
(B)


UbEP42
Fluorescence Intensity
Wavelength (nm)
0M
0.6M
1.2M
1.8M
2.4M
3M
3.6M
4.2M
4.8M
5.4M
6.0M

(C)



(D)



(E)

Fig.4.11. Intrinsic fluorescence emission spectra for guanidine hydrochloride denaturation

(A) UbF45W (B) UbI61T (C) UbEP42 (D) fluorescent peak of UbF45W, UbI61T and UbEP42 and (E) fluorescence intensity of UbF45W, UbI61T and UbEP42.

4.4. Discussion

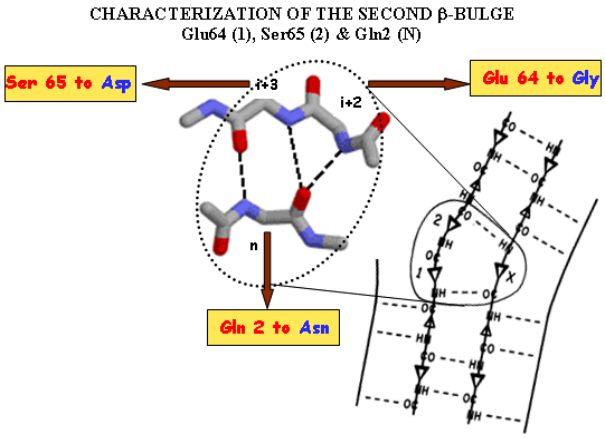
From the results it can be concluded that substitutions in the ubiquitin variant, UbEP42 comprising four mutations at different places in ubiquitin gene i.e. S20F, A46S, L50P and I61T affect the structure of protein drastically. Due to mutations properties of UbEP42 have been changed adversely, as it became heat sensitive and hydrophobic in nature. Structural studies of UbEP42 demonstrated by circular dichrometry and fluorescence spectra showed that it could retain only its secondary structure and didn't show any sign of presence of tertiary structure. Structural characterization of UbI61T showed that it follows the structure of UbEP42. UbF45W taken as a control has displayed the secondary, tertiary structure and unfolding of the structure in presence of GdHCl. In conclusion, replacing the Ile at position 61 with Thr leads to changes in structure as indicated by decrease in helicity and increased β-sheet structure. Guanidine hydrochloride denaturation curve showed the lack of structure for UbI61T and UbEP42 compared to UbF45W control.

CHAPTER 5

Structural analysis of mutations of Parallel G1 β-bulge of ubiquitin

5.1. Introduction

Ubiquitin is the most conserved protein in eukaryotes, the reasons for total conservation can be attributed to the importance of ubiquitin in a variety of cellular event that regulates the cell and to preserve structural integrity of the protein. Generation of ubiquitin mutants may therefore help to understand the importance of the residues in maintaining the structure and or functions associated with it. There are two β bulges in this protein, which partially overlap with β-turns. The first turn and the bulge, present on the N terminal have been studied in detail and found to be crucial in early folding of ubiquitin (Cox et al., 1993). The second G1 β-bulge is present at the C-terminal end in a parallel β-sheet and associated with a type II turn which is rarely found in proteins (Vijay-Kumar et al., 1987). Residues involved in the second β-bulge are Glutamine, Glutamate and Serine at 2nd, 64th and 65th positions respectively.



Glu64 is the first residue in β-bulge and third residue in a type II turn (Vijay-Kumar et al., 1987). Glu64 displays unusual Φ and Ψ angles in Ramachandran plot (Ramachandran et al., 1963). Furthermore these angles are observed with Gly. Percentage frequency of Glu in a bulge is 0.23 which is much lower than that of Gly which is 11.3. It suggests that Gly prefers the first position in a β bulge rather than the Glu as shown in Fig. 5.1. In addition Gly is also observed at the 1st position in the

structural homologs of ubiquitin RUB1 and NEDD8 (Jentsch and Pyrowolakis, 2000).

Amino acid	I	F	V	L	W	M	A	G	C	Y	P	T	S	H	E	N	Q	D	K	R
Faai/bulge	0.00	0.17	0.00	0.00	1.13	0.00	0.00	11.30	0.39	0.00	0.00	0.00	0.00	0.00	0.23	3.95	0.34	0.71	0.34	0.25
Naai	0	1	0	0	2	0	0	84	1	0	0	0	0	0	1	13	1	3	2	1

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

Fig.5.1. Percentage frequencies of amino acids in the first position of β-bulge.(Chan et al., 1993)

Glu64 to Gly mutation has been generated in our lab and it was found that replacement of the conserved glutamate at 64th position with glycine leads to slight changes in structure as indicated by 4-5% decrease in helicity and increased stability towards guanidine hydrochloride denaturation (Mishra et al., 2009).

Coming to the next residue of the second β- bulge of the ubiquitin, Ser65 which is at the second position in the parallel β-bulge is the last residue of a type II turn accompanying β-bulge (Vijay-Kumar et al., 1987). Percentage frequency of occurrence of amino acids in β-bulge indicates that Ser65 has less preference having a value of 1.5, whereas Asp shows percentage frequency of 2.4 (Chan et al., 1993) (Fig. 5.2). In addition Asp is also observed at the 2nd position in the structural homologs of ubiquitin SMT3, SUMO-1, SUMO-2 and SUMO-3 and NEDD8 (Jentsch and Pyrowolakis, 2000).

Amino acid	I	F	V	L	W	M	A	G	C	Y	P	T	S	H	E	N	Q	D	K	R
Faai/bulge	0.00	0.17	0.00	0.00	1.13	0.00	0.00	11.30	0.39	0.00	0.00	0.00	0.00	0.00	0.23	3.95	0.34	0.71	0.34	0.25
Naai	0	1	0	0	2	0	0	84	1	0	0	0	0	0	1	13	1	3	2	1

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

Fig. 5.2. Percentage frequencies of amino acids in the second position of β-bulge. (Chan et al., 1993)

Ser at the 65th position has been replaced by more preferred Asp in our laboratory. This replacement indicates subtle changes in structure, such as change in the exposed hydrophobic regions of the protein whereas stability of the protein remains unchanged hence the mutation is well accommodated in ubiquitin structure (Mishra et al., 2011).

Third position of the β-bulge (X-position) holds Glutamine, present at the N-terminal end in β-sheet, forms hydrogen bonds with the Glu64 and Ser65 which are bulged out. Gln2 shows total conservation in all ubiquitins irrespective of species. Percentage frequency of occurrence of Gln at X position of β-bulge is 0.34, whereas Asn shows frequency 5.4 which is much higher than Gln (Fig. 5.3). Asn is also found at the X-position in the structural homologs of ubiquitin, namely SMT3, SUMO-2 and SUMO-3.

Amino acid	I	F	V	L	W	M	A	G	C	Y	P	T	S	H	E	N	Q	D	K	R
Faai/bulge	0.53	0.17	0.76	0.8	0.57	0.00	0.81	1.0	2.3	0.20	0.00	0.9	1.6	2.6	0.68	5.4	0.34	3.5	0.17	0.25
Naai	5	1	10	8	1	0	6	8	6	1	0	7	11	6	3	18	1	15	1	1

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

Fig. 5.3. Percentage frequencies of amino acids in the X-position of β-bulge. (Chan et al., 1993)

Gln to Asn mutation at the 2nd position of ubiquitin shows that the mutation has been accommodated well locally and does not affect the secondary and tertiary structures of the protein. However it decreases the stability of the protein (Mishra et al., 2011).

5.2. Material and methods

5.2.1. Strains and vector used in the study

E.coli DH5α (F^-, 80dlacZ M15, endA1, recA1, hsdR17 (rk^-,mk^+), supE44, thi-1, gyrA96, relA1, (lacZYA-argF)U169) strain was used for recombinant DNA manipulation and storage.

E. coli BL21(DE3) pLys S F^-ompT $hsdS_B(r_B^- m_B^-)$ gal dcm (DE3)pLysS(Cam^R), strain was used to express recombinant DNA. Cultures were grown at 37°C at 200 rpm in Luria broth (Hi-media) and selected on ampicillin (50µg/ml) containing medium. Bacterial transformation was done by $CaCl_2$ method. Plasmid isolation was done by standard alkaline lysis protocol.

Mutant ubiquitin genes were cloned in pET30-a plasmid under the control of strong T7 promoter. Expression was induced by the addition of IPTG (isopropyl-β-D-1-thiogalactoside) at a final concentration of 1mM. (Strain and vector information is described in detail in Materials and Methods section of **Chapter 4**).

5.2.2. Sequence Analysis

Plasmid pET30-a carrying the mutant ubiquitin gene was sequenced in a single forward reaction using T7 forward primer 5'TAATACGACTCACTATAGG 3'.

5.2.3. Purification of mutant ubiquitin by affinity chromatography

Mutant ubiquitin protein UbF45W, UbQ2N-E64G, UbQ2N- S65D, UbE64G-S65D and UbQ2N-E64G-S65D were cloned in pET30-a expression system, downstream to the affinity tag consisting polyhistidine residues and purified by affinity chromatographic purification using immobilized metal affinity chromatography (IMAC) method.

Lysis of the cells has been carried out by incubating cells with lysozyme followed by sonication in lysis buffer containing 50mM NaH_2PO_4, 300 mM NaCl, 10mM imidazole, pH 8.0. Initial cell lysis protocol and lysate preparation of induced culture is discussed in detail in materials and methods section of chapter 4.

Ni-NTA resins were washed with equilibration buffer containing 50mM NaH_2PO_4, 300mM NaCl, pH 8.0 followed by incubation with lysate for an hour to allow binding of His tagged protein to the resin, after incubation cell lysate was removed and resin was washed with buffer containing 50mM NaH_2PO_4, 300mM NaCl with low concentration of imidazole (20mM) to remove unbound protein and to reduce nonspecific binding of host proteins with histidines. After washing steps, target proteins were eluted from the buffer containing 50mM NaH_2PO_4, 300mM NaCl, 250mM imidazole, pH 8.0. Percent purity of the target proteins have been checked with 15% SDS-PAGE. Imidazole and NaCl were removed by dialysis with buffer containing 10mM Tris, pH 7.4, purified proteins were concentrated by vacuum evaporation and salting out with ammonium sulphate precipitation.

5.2.4. Circular dichroism and fluorescence spectroscopy

CD spectra were recorded using a Jasco J-815 spectropolarimeter. For far uv CD spectra (200 to 250nm) cells of 1mm path length were used. Spectra were recorded at 2nm resolution. The data pitch was 0.2nm. Scan speed was 50nm/sec.

Three spectra were accumulated to reduce the noise. Protein solutions were prepared in 10mM Tris HCl, pH7.4 and concentration of protein was 15μM. Proper blanks were prepared for all samples and the spectra of the samples were blank corrected. CD spectra were deconvulated by CONTIN/LL method with SMP56 reference set [6 (αR, αD, βR, βD, T, U)] (Johnson, 1999; Keiderling, 1991; Sreerama et al., 1999; Yang et al., 1986) of CDPro software (Sreerama et al., 1999). Where α and β denotes α helix and β strand, R and D denotes regular and distorted structures respectively, T and U denotes turns and unordered structure respectively.

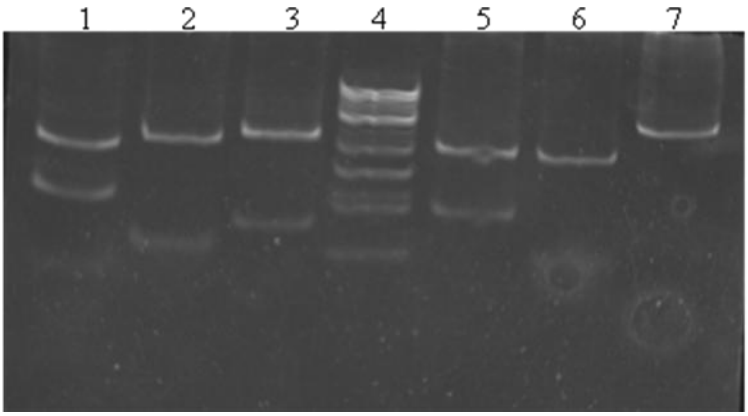
Fluorescence spectra were recorded using Hitachi F7000-FL fluorescence spectrophotometer. Slit width was 5nm. Protein solutions were prepared in 10mM Tris HCl, pH7.4 and concentration of protein was 5μM. Samples were excited at 280nm to record intrinsic fluorescence due to tryptophan (Trp). Emission was recorded between 300-400nm.

For denaturation studies, ubiquitin mutants were denatured by Guanidine hydrochloride (Sigma) in the range of 0M to 6M concentration, proper blanks were prepared and the spectra were blank corrected.

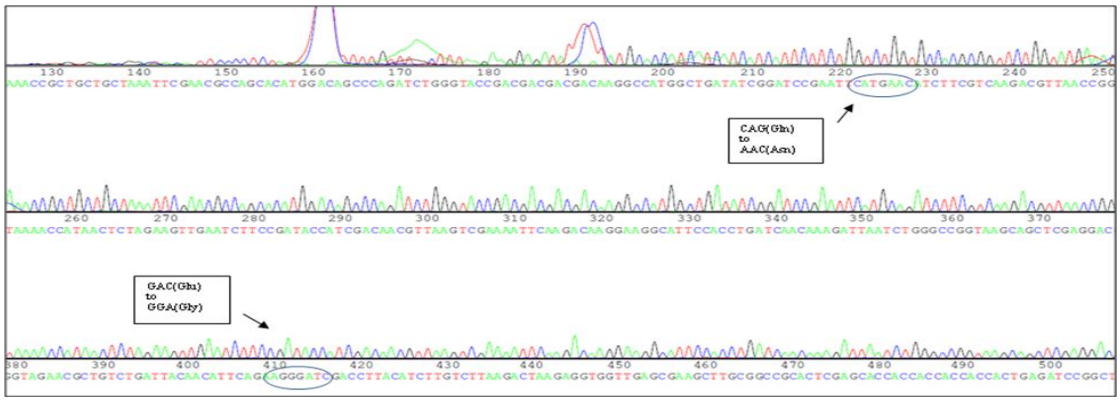
5.3. Result and discussion

5.3.1. Construction of UbQ2N-E64G in pET30-a bacterial expression vector

For the construction of UbQ2N-E64G in bacterial expression vector pET-30a, ubiquitin gene carrying double mutation, Q2N and E64G mutations was amplified from YEp-UbQ2N-E64G vector. Amplified gene product and pET-30a vector were double digested with *EcoR*I and *Hind*III restriction enzymes, subsequently the two DNA fragments were ligated. Incorporation of desired mutation has been confirmed by digesting the mutant gene with *Bgl*II and *Sal*I restriction enzyme as these two sites are damaged due to mutations at 2^{nd} and 64^{th} position (Fig. 5.4). Further, gene was sequenced to ensure the incorporation of mutations.



(A)



(B)

Fig. 5.4. Gel picture showing restriction digestion and sequence confirmation of pET-UbQ2N-E64G

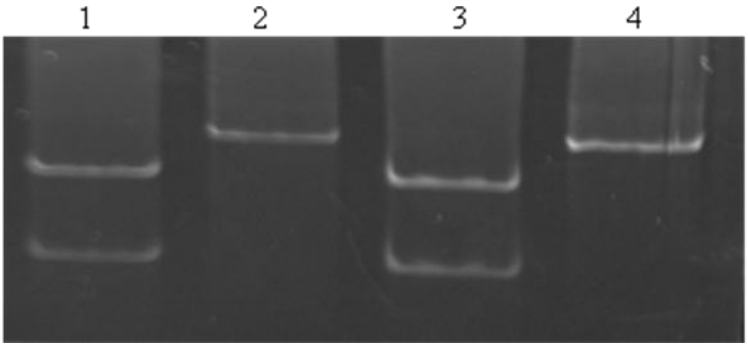
(A) Lane1, Lane 2 and Lane 3 show digestion pattern of pET-UbF45W after digestion with *Xho*I, *Bgl*II and *Sal*I respectively. Lane 4 shows 100 bp DNA ladder whereas Lane 5, Lane 6 and Lane 7 show digestion pattern of pET-UbQ2N-E64G after digestion with *Xho*I, *Bgl*II and *Sal*I respectively. Failure of digestion by *Bgl*II and *Sal*I in Lane 6 and 7 confirms the incorporation of desired mutations.

(B) The marked region confirms the incorporation of UbQ2N-E64G substitution.

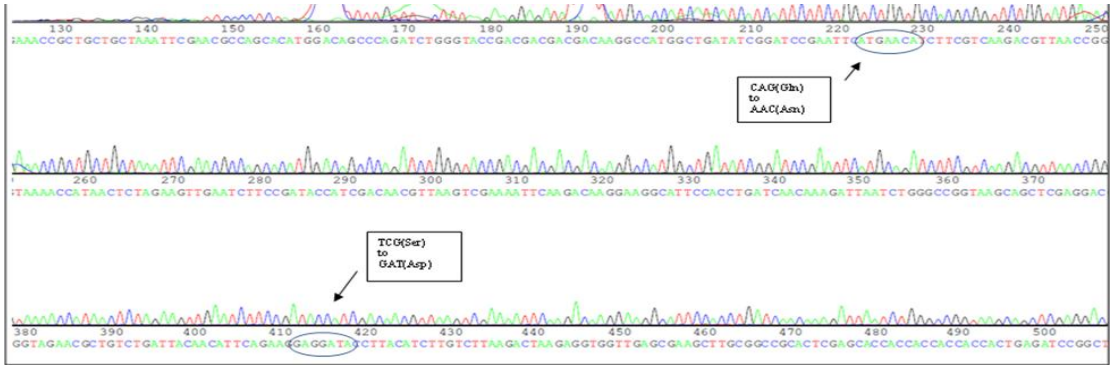
5.3.2. Construction of Q2N-S65D in pET30-a bacterial expression vector

For the construction of UbQ2N-S65D in bacterial expression vector pET30-a, ubiquitin gene carrying double mutation, Q2N and S65D mutations was amplified

from YEp-UbQ2N-S65D vector. Amplified gene product and pET30-a vector were double digested with *EcoR*I and *Hind*III restriction enzymes and subsequently cloned. Incorporation of desired mutation has been confirmed by digesting the mutant gene with *Bgl*II and *Sal*I restriction enzyme as these two sites are damaged due to mutations at 2nd and 65th positions (Fig. 5.5). Further, gene was sequenced to confirm the incorporation of mutations.



(A)



(B)

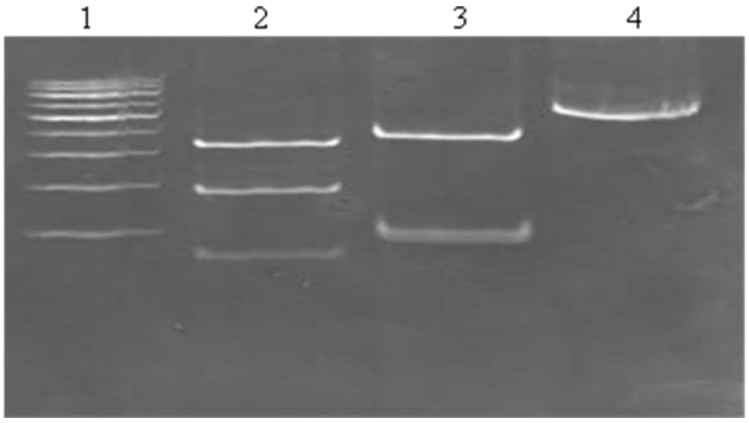
Fig. 5.5. Gel picture showing restriction digestion and sequence confirmation of pET-UbQ2N-S65D

(A) Lane1, Lane 2 and Lane 3 show digestion pattern of pET-UbQ2N-S65D by digesting it with *Bgl*II, *Sal*I and *Xho*I respectively. Lane 4 shows undigested PCR product. Lane 1 shows failure of digestion by *Bgl*II resulting in two bands instead of three and lane 2 show failure of digestion by *Sal*I restriction enzyme.

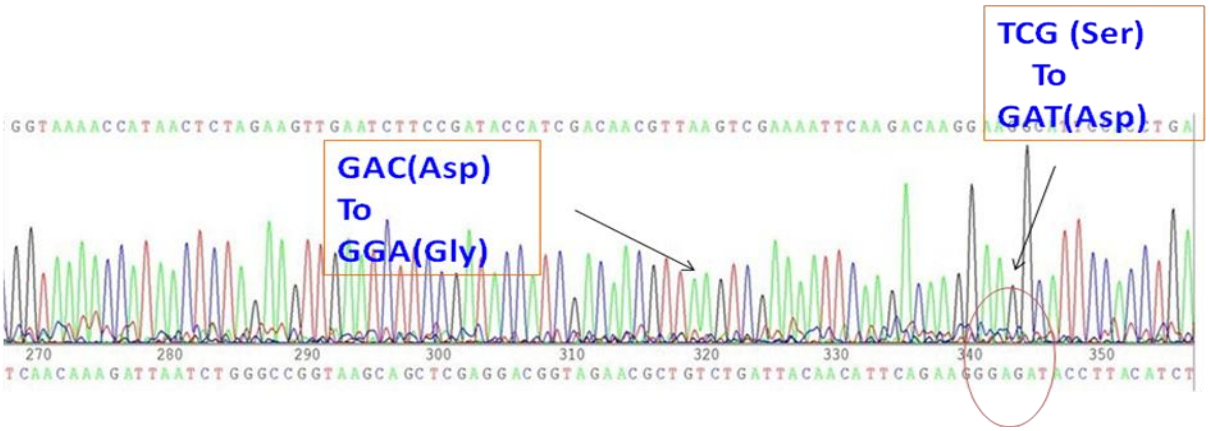
(B) The marked region confirms the incorporation of UbQ2N-S65D substitution.

5.3.3. Construction of E64G-S65D in pET30-a bacterial expression vector

For the construction of UbE64G-S65D in bacterial expression vector pET30-a, ubiquitin gene carrying double mutation, E64G and S65D mutations was amplified from YEp-UbE64G-S65D vector. Amplified gene product and pET30-a vector were double digested with *Eco*RI and *Hind*III restriction enzymes and subsequently cloned. Incorporation of desired mutations have been confirmed by digesting the mutant gene with *Sal*I restriction enzyme as this site is damaged due to mutations at 64th and 65th positions (Fig. 5.6A). Further, gene was sequenced to confirm the incorporation of mutations.



(A)



(B)

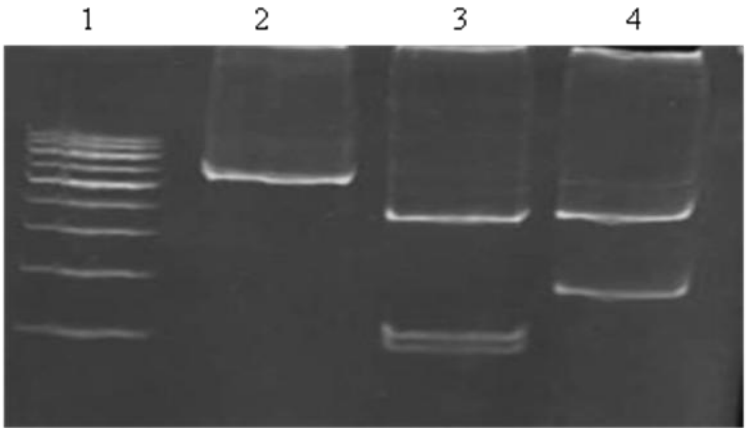
Fig. 5.6. Showing restriction digestion and sequence confirmation of pET-E64G-S65D

(A) Lane 1 shows 100 bp DNA ladder. Lane 2.shows *Bgl*II digestion cuts gene into three fragments of 52,194,and 340 bp. Lane 3 shows *Xho*I digestion gives three fragments of 90,103 and 393 bp. Lane 4.shows failure of *Sal* I digestion gives desired clone confirmation.

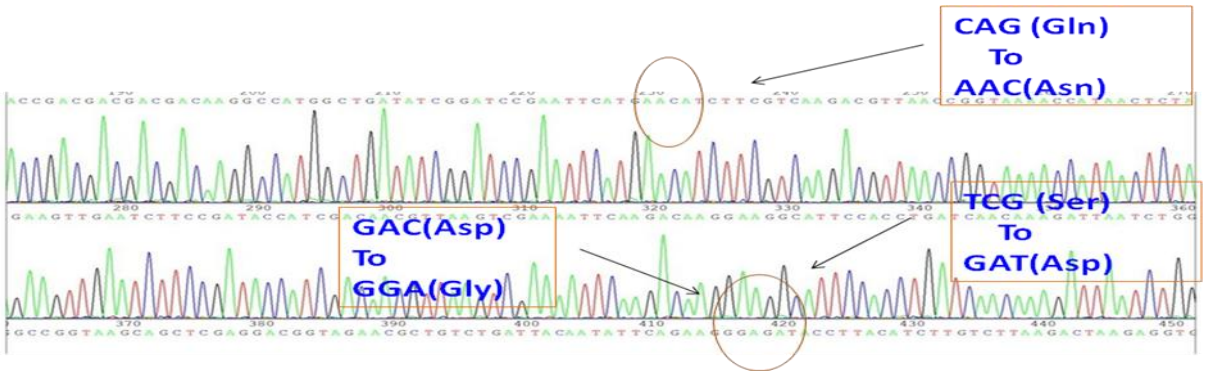
(B)The marked region confirms the incorporation of UbE64G-S65D substitution.

5.3.4. Construction of Q2N-E64G-S65D in pET30-a bacterial expression vector

For the construction of Q2N-E64G-S65D in bacterial expression vector pET30-a, ubiquitin gene carrying Triple mutation, Q2N-E64G-S65D mutations were amplified from YEpUb-Q2N-E64G-S65D vector and subjected to *Eco*RI and *Hind*III double digestion. After restriction digestion the mutated gene was ligated in the pET30-a vector by using the same restriction enzyme sites. Vector carrying Q2N, E64G and S65D mutations of ubiquitin gene in pET30-a vector was confirmed with restriction digestion pattern of its PCR amplicon by digesting it with *Bgl*II and *Sal*I restriction enzyme to confirm the mutation incorporated into the vector (Fig. 5.7A). Further, gene was sequenced to confirm the incorporation of mutant gene.



(A)



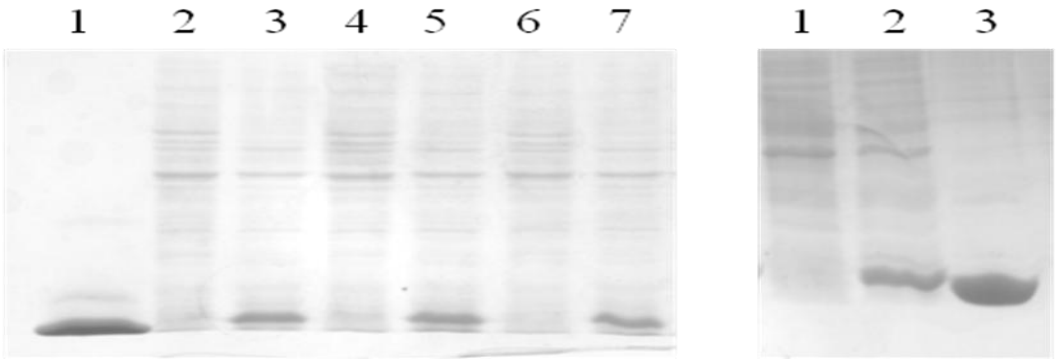
(B)

Fig. 5.7. Gel picture showing restriction digestion and sequence confirmation of pET30-UbQ2N-E64G-S65D

(A) Lane 1 shows 100 bp DNA ladder Lane 2 shows digestion with *Sal*I, which remains undigested hence confirms the incorporation of mutation, Lane 3 shows digestion with *Xho*I which gives three fragments of 90,103 and 393 bp, Lane 4 shows digestion with *Bgl*II which gives three fragments of 194 and 392 bp.

(B) The marked region confirms the incorporation of Q2N, E64G and S65D substitution.

5.3.5. Expression of ubiquitin variant Q2N-E64G, Q2N-S65D, E64G-S65D and Q2N-E64G-S65D cloned in pET-30a expression vector



(A) **(B)**

Fig. 5.8. Expression profile of UbQ2N-E64G, UbQ2N-S65D, UbE64G-S65D and UbQ2N-E64G-S65D

(A) Lane 1 shows Lysozyme as a marker. Lane 2, Lane 4 and Lane 6 show protein profile of un-induced pET-UbQ2N-E64G, pET-UbQ2N-S65D and pET-UbE64G-

S65D respectively. Lane 3, Lane 5 and Lane 7 show pET-UbQ2N-E64G, pET-UbQ2N-S65D and pET-UbE64G-S65D induced with 1mM IPTG.

(B) Lane 1 and Lane 2 show Mutant ubiquitin proteins UbQ2N-E64G-S65D under the conditions of un-induced and induced with 1mM IPTG concentration respectively. Lane 3 shows lysozyme as molecular weight marker.

5.3.6. Purification of UbF45W, UbQ2N-E64G, UbQ2N-S65D, UbE64G-S65D and UbQ2N-E64G-S65D cloned in pET30-a expression vector by Affinity chromatography

After confirming the expression of mutant protein, purification was carried out by suspending cells in lysis buffer and the cells were lysed by lysozyme and sonication methods. The cell lysate was incubated with Ni-NTA resin and washed with buffer containing 20mM imidazole to remove non specific proteins, His tagged ubiquitin mutant proteins have been eluted by 250mM imidazole (Details of the purification methods is discussed in Materials and Methods section of **Chapter 4**). Eluted purified proteins were checked for their integrity by 15% SDS PAGE (Fig. 5.9). Eluted purified proteins were dialyzed against buffer containing Tris 10mM, 5% glycerol, 5mM EDTA, pH 8. Dialyzed proteins were checked on SDS-PAGE. Proteins were concentrated by precipitation method and dialyzed again for spectral studies.

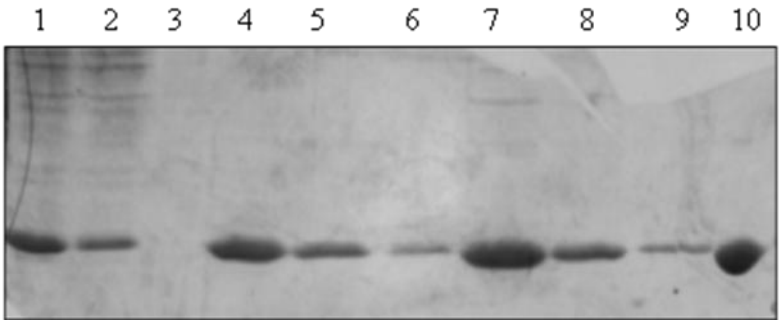


Fig. 5.9. Purification of of UbF45W, UbQ2N-E64G, UbQ2N-S65D, UbE64G-S65D and UbQ2N-E64G-S65D

Lane 1 and 2 show induced culture of ubiquitin variant control UbF45W, Lane 4, Lane 5, Lane 7 and Lane 8 show elution from 250mM imidazole of ubiquitin mutants UbQ2N-E64G, UbQ2N-S65D, UbE64G-S65D and UbQ2N-E64G-S65D respectively, Lane 6 and Lane 9 show second elution of UbQ2N-S65D and UbQ2N-E64G-S65D respectively, Lane 10 shows lysozyme used as a marker.

5.3.7. Structural characterization of ubiquitin variant UbQ2N-E64G

5.3.7.1. Far UV CD spectra of UbQ2N-E64G

Far uv CD spectra were recorded to study the changes in secondary structure due to mutations in the sequence of ubiquitin. UbWt and UbF45W were used as references in far uv CD spectra. UbWt and UbF45W showed negative band at 208nm and small negative shoulder at 222nm indicates presence of helical structure (Fig. 5.10). Further wild type and mutant spectra have been analyzed by CONTIN/LL method in CD pro software. Reference set containing 56 proteins (SMP56) was chosen for deconvulating the spectra. The values of wild type ubiquitin spectra were in agreement with the values of X-ray diffractions of ubiquitin. Mutant ubiquitin UbQ2N-E64G showed less helical structure with 8.8% of α helix content, β-sheet content and turns were increased up to 40.6 and 55.6 % respectively (Table 5.1).

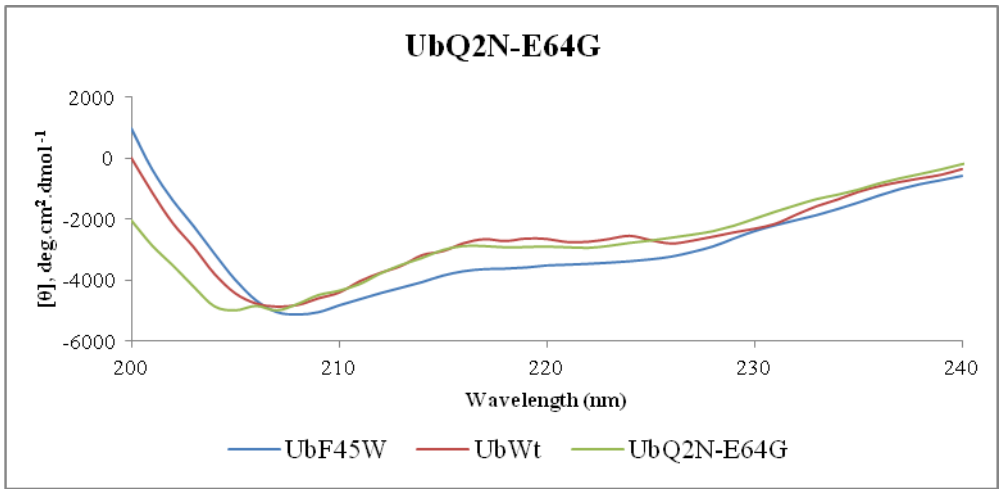


Fig. 5.10. Far UV CD spectra of UbQ2N-E64G with UbWt

Type of secondary structure	UbWt (X-ray)	UbWt	UbF45W	UbQ2N-E64G
α-helix	16	17.3	18.3	8.8
β-sheet	32	32.2	32.1	40.6
Turns and random coil	52	50.5	49.6	55.6

Table 5.1. Secondary structural analysis of far-uv CD spectra of UbQ2N-E64G using CD Pro software

5.3.7.2. Fluorescence spectra of UbQ2N-E64G

Fluorescence spectra of the ubiquitin variants UbQ2N-E64G was recorded by exciting the protein at 280nm and compared with control protein UbF45W. UbE64G and UbF45W show comparable intensities of fluorescence, indicating that the environment around aromatic amino acid residue is not changed much (Fig. 5.11).

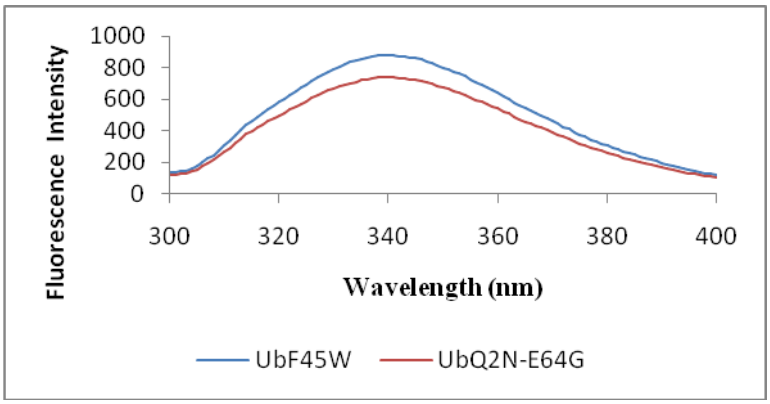
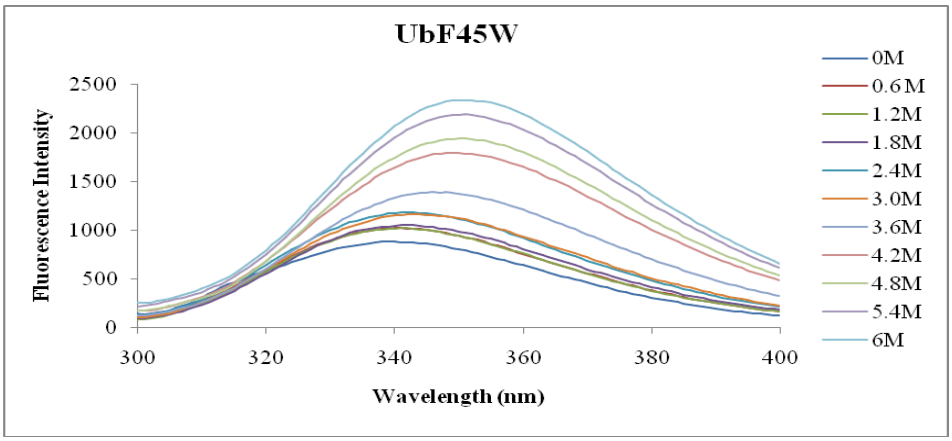


Fig. 5.11. Fluorescence emission spectra of UbF45W and UbQ2N-E64G recorded after exciting the protein at 280nm

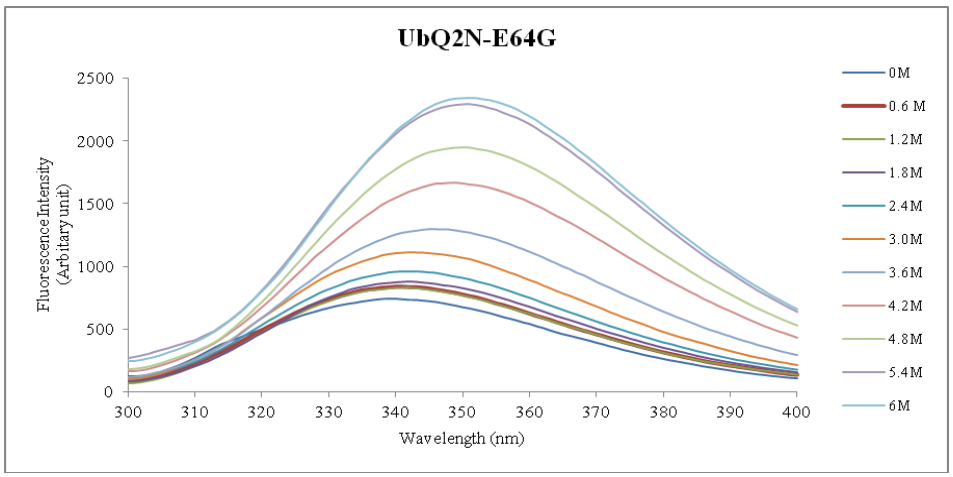
5.3.7.3. Guanidine hydrochloride denaturation of UbQ2N-E64G

Guanidine hydrochloride denaturation of UbF45W and UbQ2N-E64G were studied by fluorescence. As naturally ubiquitin does not contain any strong intrinsic

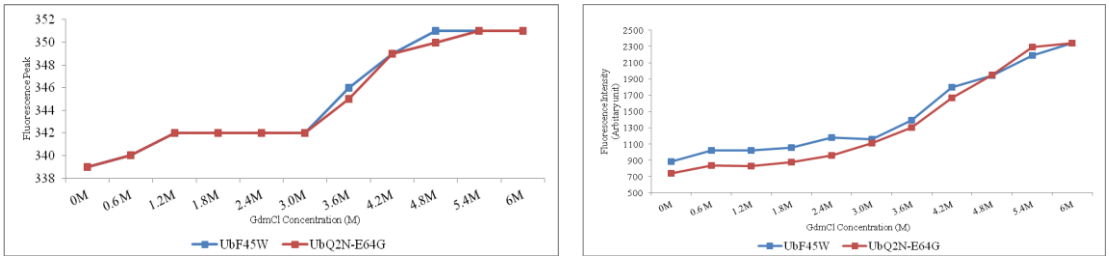
fluorophore, tryptophan has been introduced in its structure to facilitate the folding studies of ubiquitin (Khorasanizadeh et al., 1993). All mutations have been constructed in UbF45W background to facilitate folding studies. Guanidine hydrochloride is a denaturing agent which is known to cause loss of protein secondary and tertiary structures and subsequent increase in the fluorescence intensities, along with a red shift in λ_{max} of emission due to exposure of tryptophan in more soluble environment. The mutant protein UbQ2N-E64G was found to be less stable than UbF45W. Earlier results from the laboratory have indicated that the UbQ2N mutant is less stable and UbE64G is more stable by denaturation studies (Mishra et al., 2009). Combination of the above two mutants in UbQ2N-E64G does not indicate any change in overall stability compared to UbF45W, as observed from GdHCl denaturation curve (Fig. 5.12).



(A)



(B)



(C) **(D)**

Fig. 5.12. Intrinsic fluorescence emission spectra of UbQ2N-E64G with increasing concentration of GdHCl

(A) Guanidine hydrochloride denaturation curve of UbF45W (B) Guanidine hydrochloride denaturation curve of UbQ2N-E64G (C) Plot between guanidine hydrochloride concentration and fluorescent peak of UbF45W and UbQ2N-E64G (D) Plot between guanidine hydrochloride concentration and fluorescent intensity of UbF45W and UbQ2N-E64G.

5.3.8. Structural characterization of ubiquitin variant UbQ2N-S65D

5.3.8.1. Far UV CD spectra of UbQ2N-S65D

Far uv CD spectra were recorded to study the changes in secondary structure due to mutations in the sequence of ubiquitin. UbWt and UbF45W were used as references in far uv CD spectra. UbWt and UbF45W showed negative band at 208nm and small negative shoulder at 222nm indicates presence of helical structure. Further wild type and mutant spectra have been analysed by CONTIN/LL method in CD pro software, reference set containing 56 proteins (SMP56) was chosen for deconvulating the spectra. The values of wild type ubiquitin spectra were in agreement to the values of X-ray diffraction structure of ubiquitin. Mutant ubiquitin UbQ2N-S65D showed increased helical structure and β-sheet content with 23.7% of α helix content and 50.4% of β-sheet content and turns were decreased to 25.6 % (Table 5.2).

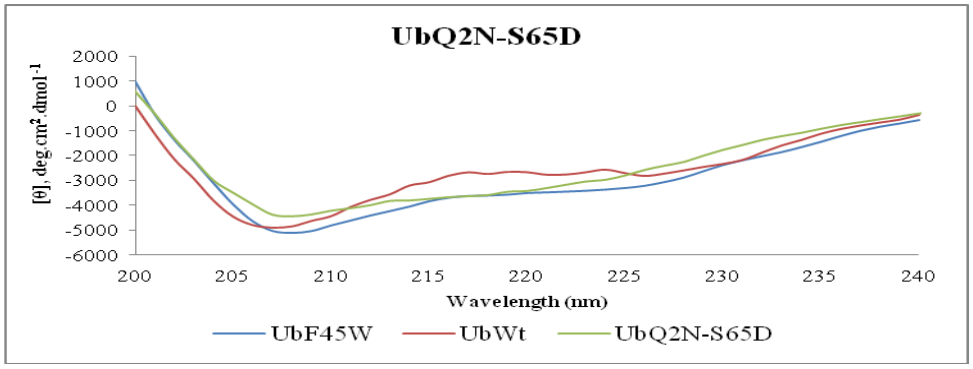


Fig. 5.13. Far UV CD spectra of UbQ2N-S65D

Type of secondary structure	UbWt (X-ray)	UbWt	UbF45W	UbQ2N-S65D
α-helix	16	17.3	18.3	23.7
β-sheet	32	32.2	32.1	50.4
Turns and random coil	52	50.5	49.6	25.9

Table 5.2. Secondary structural analysis of far-uv CD spectra of UbQ2N-S65D using CD Pro software

5.3.8.2. Fluorescence spectra of UbQ2N-S65D

Fluorescence spectra of the ubiquitin variants UbQ2N-S65D was recorded by exciting the protein at 280nm and compared with control protein UbF45W. UbQ2N-S65D shows lesser intensity upon excitation than UbF45W which indicates changes in the surrounding of tryptophan.

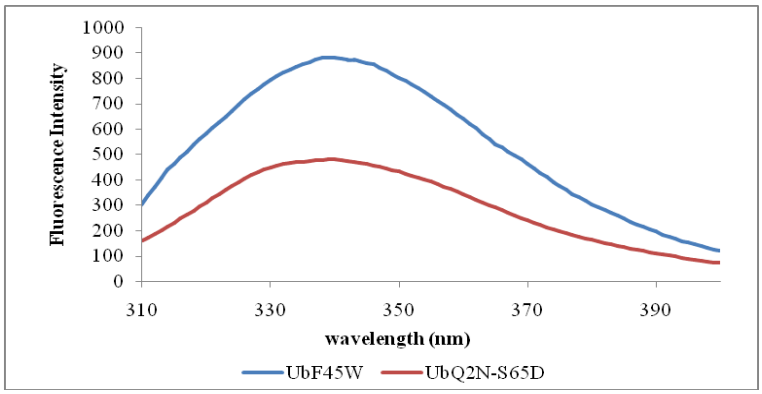
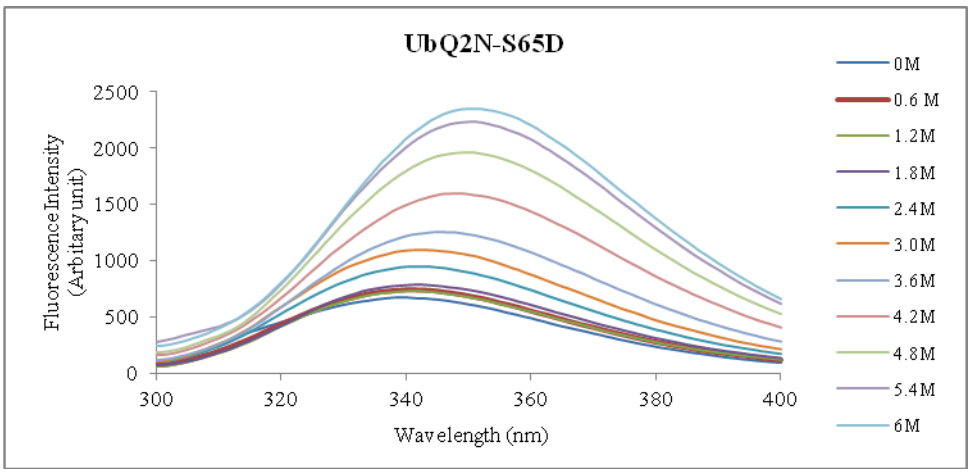


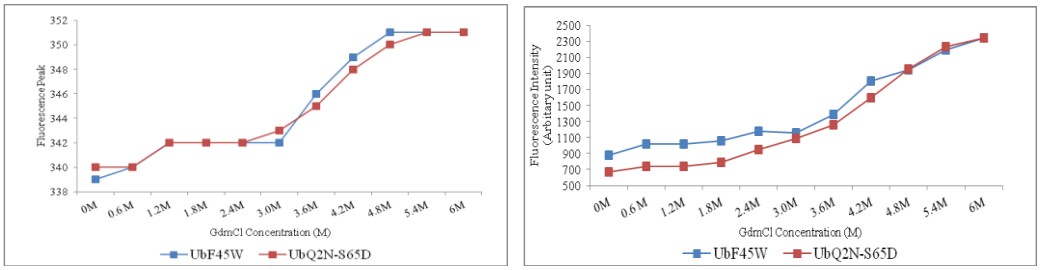
Fig. 5.14. Fluorescence emission spectra of UbF45W and UbQ2N-S65D recorded after exciting the protein at 280nm

5.3.8.3. Guanidine hydrochloride denaturation of UbQ2N-S65D

Guanidine hydrochloride denaturation of UbQ2N-S65D was studied by exciting the protein at 280nm and compared with the UbF45W. Denaturation of ubiquitin by Guanidine hydrochloride cause loss of protein secondary and tertiary structures and subsequent increase in the fluorescence intensities, along with a red shift in λ_{max} of emission due to exposure of tryptophan in more soluble environment. The mutant protein UbQ2N-S65D was found to be less stable than UbF45W. Earlier results from the laboratory have indicated that the UbQ2N mutant shows less stability and UbS65D exhibits no change in stability upon denaturation (Mishra et al., 2011). Combination of the above two mutations increased the stability of the protein marginally, which may be attributed to the changes in the secondary structure leading to changes in tertiary structure (Fig. 5.15).



(A)



(B) **(C)**

Fig. Error! No text of specified style in document.**.85. Intrinsic fluorescence emission spectra of UbQ2N-S65D in GdHCl**

(A) Guanidine hydrochloride denaturation curve of UbQ2N-S65D (B) Plot between guanidine hydrochloride concentration and fluorescent peak of UbF45W and UbQ2N-S65D (C) Plot between guanidine hydrochloride concentration and fluorescent intensity of UbF45W and UbQ2N-S65D.

5.3.9. Structural characterization of ubiquitin variant UbE64G-S65D

5.3.9.1. Far UV CD spectra of the ubiquitin variant UbE64G-S65D

Far uv CD spectra were recorded to study the changes in secondary structure due to mutations in the sequence of ubiquitin. UbWt and UbF45W were used as references in far uv CD spectra. UbWt and UbF45W showed negative band at 208nm and small negative shoulder at 222nm indicates presence of helical structure. Further wild type and mutant spectra have been analysed by CONTIN/LL method in CD pro software, reference set containing 56 proteins (SMP56) was chosen for deconvulating the spectra. The values of wild type ubiquitin spectra were in agreement to the values of X-ray diffractions structure of ubiquitin. Mutant ubiquitin UbE64G-S65D showed decreased helical structure with 7% helical content while β-sheet content remained almost same and turns have an increment of 5% (Table 5.3).

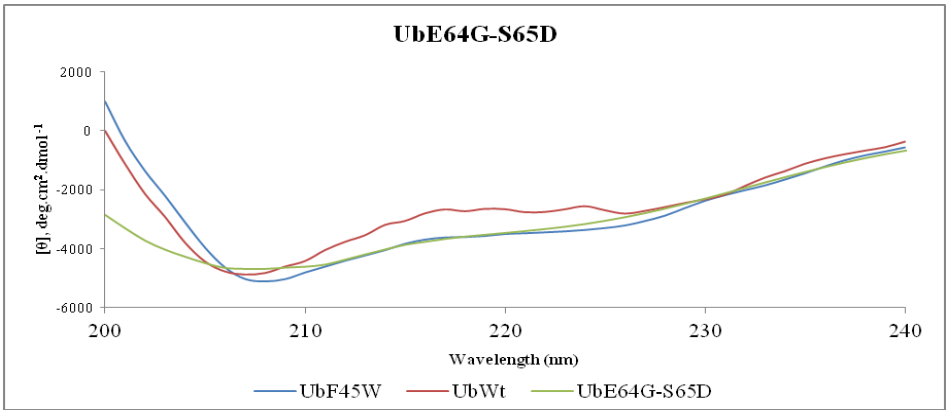


Fig. 5.16. Far UV CD spectra of UbE64G-S65D

Type of secondary structure	UbWt (X-ray)	UbWt	UbF45W	UbE64G-S65D
α-helix	16	17.3	18.3	7
β-sheet	32	32.2	32.1	34.2
Turns and random coil	52	50.5	49.6	58

Table 5.3. Secondary structural analysis of far-uv CD spectra of UbE64G-S65D using CD Pro software

5.3.9.2. Fluorescence spectra of UbE64G-S65D

Fluorescence spectrum of the ubiquitin variant UbE64G-S65D was recorded by exciting the protein at 280nm and compared with that of control protein UbF45W. UbE64G- S65D shows lesser intensity upon excitation than UbF45W which indicates minor changes in the surrounding of tryptophan.

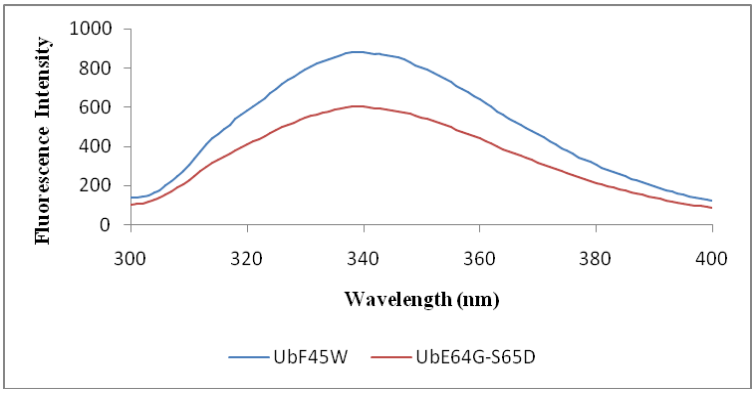
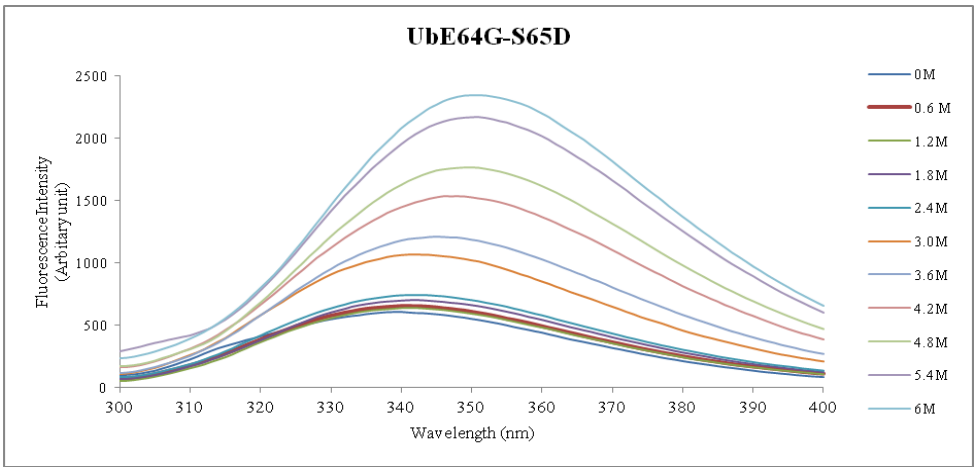


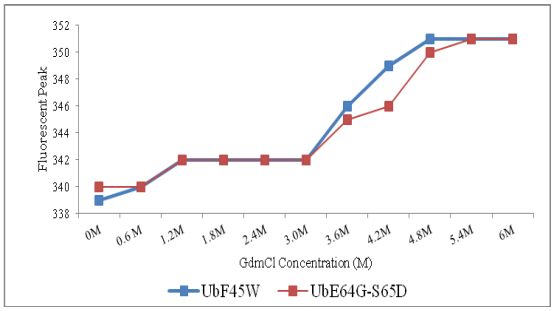
Fig. 5.17. Fluorescence emission spectra of UbF45W and UbE64G-S65D recorded after exciting the protein at 280nm

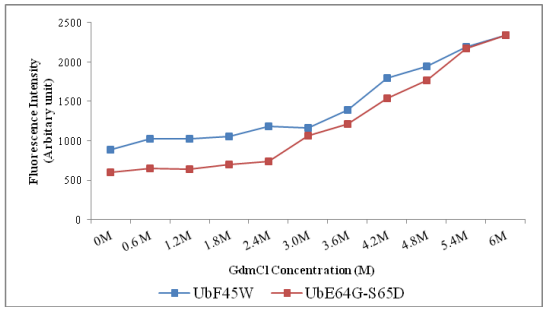
5.3.9.3. Guanidine hydrochloride denaturation of UbE64G-S65D

Guanidine hydrochloride denaturation of UbE64G-S65D was studied by exciting the protein at 280nm and compared with UbF45W. Denaturation of ubiquitin by guanidine hydrochloride causes loss of protein secondary and tertiary structures and subsequent increase in the fluorescence intensities, along with a red shift in λ_{max} of emission due to exposure of tryptophan in more soluble environment. The mutant protein UbE64G-S65D was found to be more stable than UbF45W (Fig. 5.18). Earlier results from the laboratory have indicated that the UbE64G mutant shows more stability and UbS65D exhibits no change in stability upon denaturation.



(A)





(B) **(C)**

Fig. 5.18. Intrinsic fluorescence emission spectra UbE64G-S65D in GdHCl

(A) Guanidine hydrochloride denaturation curve of UbE64G-S65D (B) Plot between guanidine hydrochloride concentration and fluorescent peak of UbF45W and UbE64G-S65D (C) Plot between guanidine hydrochloride concentration and fluorescent intensity of UbF45W and UbE64G-S65D.

5.3.10. Structural characterization of ubiquitin variant UbQ2N-E64G-S65D

5.3.10.1. Far UV CD spectra of the ubiquitin variant UbQ2N-E64G-S65D

Far uv CD spectra were recorded to study the changes in secondary structure due to mutations in the sequence of ubiquitin. UbWt and UbF45W were used as a reference in far uv CD spectra. UbWt and UbF45W showed negative band at 208nm and small negative shoulder at 222nm indicating presence of helical structure. Further wild type and mutant spectra have been analysed by CONTIN/LL method in CD pro software, Reference set containing 56 proteins (SMP56) was chosen for deconvulating the spectra. The values of wild type ubiquitin spectra were in agreement to the values of X-ray diffraction structure of ubiquitin. Mutant ubiquitin UbQ2N-E64G-S65D showed decreased helical structure with 6.8 % helical content while β-sheet content increased up to 38% turns and random coil have increment of ~6 % (Table 5.4).

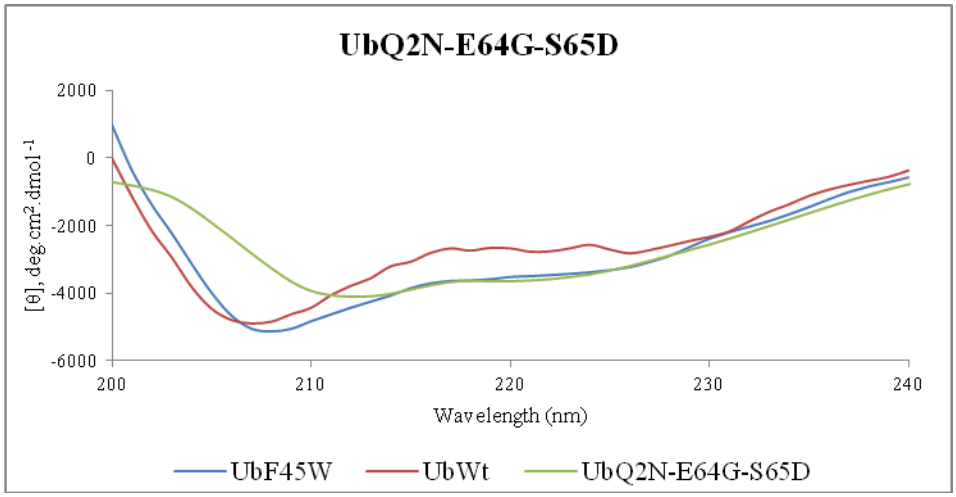


Fig. 5.19. Far UV CD spectra of UbQ2N-E64G-S65D

Type of secondary structure	UbWt (X-ray)	UbWt	UbF45W	UbQ2NE64G-S65D
α-helix	16	17.3	18.3	6.8
β-sheet	32	32.2	32.1	38
Turns and random coil	52	50.5	49.6	55.3

Table 5.4. Secondary structural analysis of far-uv CD spectra of UbQ2N-E64G-S65D using CD Pro software

5.3.10.2. Fluorescence spectra of UbQ2N-E64G-S65D

Fluorescence spectra of the ubiquitin variants UbQ2N-E64G-S65D was recorded by exciting the protein at 280nm and compared with the structure of control protein UbF45W. UbQ2N-E64G- S65D shows almost same intensity as UbF45W than UbF45W which indicates that there is no change in the surrounding of tryptophan due to mutations.

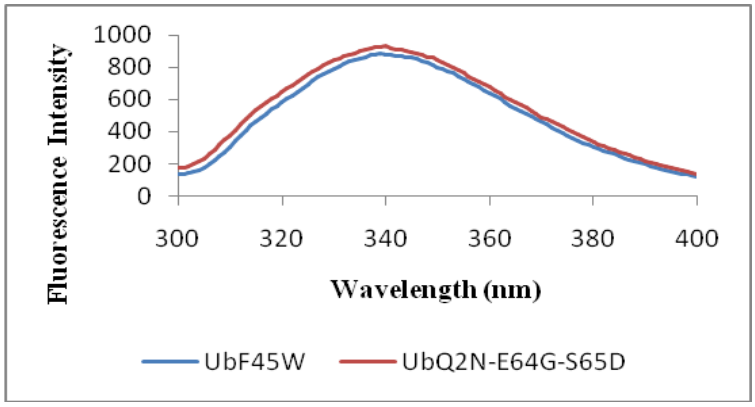
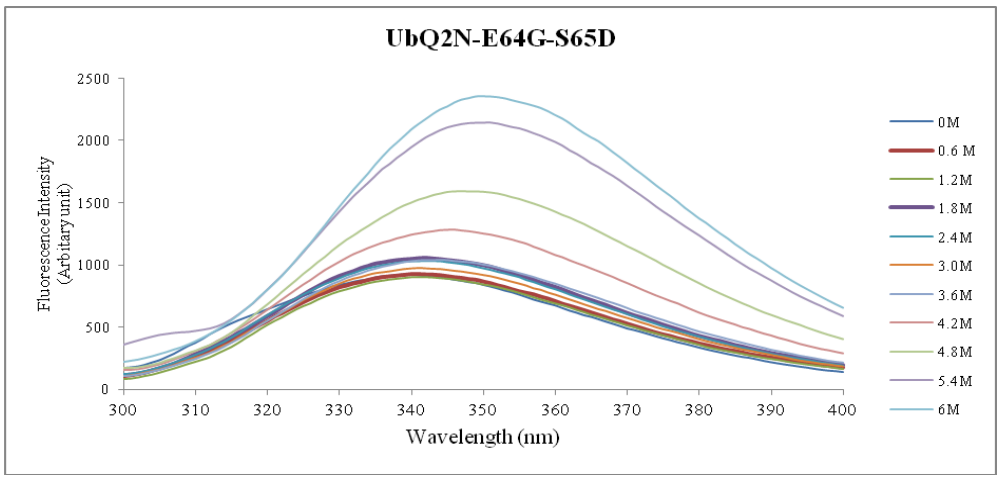


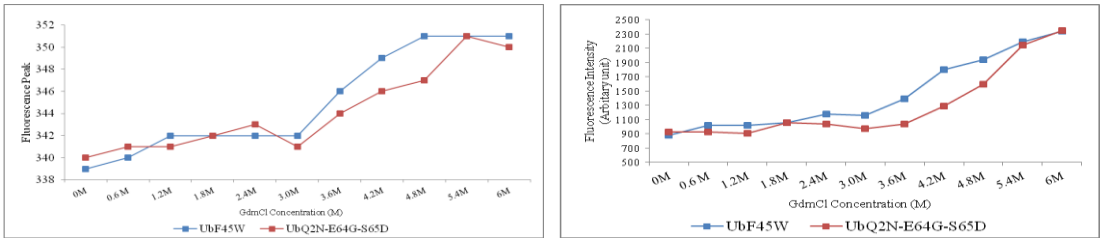
Fig. 5.20. Fluorescence emission spectra of UbF45W and UbQ2N-E64G-S65D recorded after exciting the protein at 280nm

5.3.10.3. Guanidine hydrochloride denaturation of UbQ2N-E64G-S65D

Guanidine hydrochloride denaturation of UbQ2N-E64G-S65D was studied by exciting the protein at 280nm and compared with UbF45W. Denaturation of ubiquitin by guanidine hydrochloride causes loss of protein secondary and tertiary structures and subsequent increase in the fluorescence intensities, along with a red shift in λ_{max} of emission due to exposure of tryptophan in more soluble environment. The mutant protein UbQ2N-E64G-S65D was found to be more stable than UbF45W (Fig. 5.21). Earlier results from the laboratory have indicated that the UbE64G mutant shows more stability whereas UbQ2N mutant is less stable and UbS65D exhibits no change in stability upon gdHCl denaturation.



(A)



(B) **(C)**

Fig. 5.21. Intrinsic fluorescence emission spectra UbQ2N-E64G-S65D

(A) Guanidine hydrochloride denaturation curve of UbQ2N-E64G-S65D (B) Plot between guanidine hydrochloride concentration and fluorescent peak of UbF45W and UbQ2N-E64G-S65D (C) Plot between guanidine hydrochloride concentration and fluorescent intensity of UbF45W and UbQ2N-E64G-S65D.

5.4. Discussion

From the above study it is clear that the replacement targeted to unusual residues of β- bulge by more preferred residues has affected secondary structure considerably while showing minor effects on tertiary structure.

Q2, E64 and S65 amino acid residues are at X, 1 and 2 position of β- bulge, which act together to hold the structure and in turn impart stability to protein. Secondary and tertiary structure of ubiquitin mutant UbQ2N-E64G, UbQ2N-S65D, UbE64G-S65D and UbQ2N-E64G-S65D along with wild type control have been analysed by CD spectroscopy and fluorescence spectroscopy respectively.

Far uv CD spectra of mutant ubiquitins UbQ2N-E64G, UbQ2N-S65D, UbE64G-S65D and UbQ2N-E64G-S65D showed change in secondary structure. This signifies the role of Q, E and S at 2^{nd}, 64^{th} and 65^{th} position respectively. Replacement of these residues in pairs or all of them together with more preferred residues was not well accommodated in the secondary structure. Tertiary structure of UbQ2N-E64G and UbQ2N-E64G-S65D as gauged from intrinsic fluorescence, do not show much change in the environment around aromatic residues. However, UbQ2N-

S65D and UbE64G-S65D show slight difference in intrinsic fluorescence compared to the control UbF45W. Stability of the molecules studied by guanidine hydrochloride denaturation showed there is no change in the stability of UbQ2N-E64G and UbQ2N-S65D with respect to UbF45W. On the other hand, UbE64G-S65D and UbQ2N-E64G-S65D are more stable than control UbF45W as revealed by GdHCl denaturation. Functional complementation of the mutants UbQ2N-E64G and UbQ2N-S65D in vivo supports moderate effects on structure due to these mutations. Functional complementation of the mutants UbE64G-S65D and UbQ2N-E64G-S65D has revealed drastic effects in vivo as the mutants could not complement various stress conditions. Replacement of whole β-bulge by preferred residues makes protein more stable however mutant was not found functionally active.

SUMMARY

Ubiquitin is a small, highly conserved protein present in all eukaryotes. Its sequence is totally conserved from insects to higher eukaryotes such as human. Sequence variations are seen only in three positions in organisms as distantly related as yeast and plant. The X- ray crystallographic structure of ubiquitin reveals tightly-packed globular structure with five strands of mixed parallel and anti-parallel β-sheet packed against α-helix to form the hydrophobic core and a flexible tail formed by four protruding residues. Due to extensive hydrogen bonding and hydrophobic core, ubiquitin forms extremely stable structure. Ubiquitin is stable in wide ranges of pH and temperature. The compact structure of ubiquitin shows nine reverse turns and two β bulges (Vijay-Kumar et al., 1987). The first β bulge, which is situated at N terminal region in the type I turn of the β hairpin has been extensively studied and shown to involve in directing the formation of the hairpin, stabilizing the transition state and act as a nucleation centre (Cox et al., 1993).

The second β-bulge which is located at the C- terminal region in continuation of the type II turn, is formed by three residues Glu64 (1), Ser65 (2), Gln2 (X). Based on sequence analysis of 182 proteins and a total of 362 bulges it is observed that the % frequency of occurrence of these amino acids in such β-bulges is very low (Chan et al, 1993). This structure has been conserved in ubiquitin throughout the evolution, leaving no scope for interspecies comparison to understand the role of individual residues. Whether these residues are important for folding and structural stability or for function of ubiquitin is an interesting question in ubiquitin structural biology. Earlier work from our laboratory chose to answer the above question by constructing and characterizing three site directed mutants, namely UbQ2N, UbE64G and UbS65D (Mishra et al., 2011; Mishra et al., 2009). The residues for replacement Gly64 (1), Asp65 (2) and Asn2(X) in site directed mutants were chosen on the basis of their preference for β-bulges. These residues are also found in the β-bulges of ubiquitin homologues. Our results established that *Sacchromyces cervisiae* expressing the mutated proteins UbE64G, UbS65D and UbQ2N, do not complement *UBI4* ubiquitin deletion mutants and fail to confer resistance to the antibiotic cycloheximide. They show altered rate of degradation with Ub-Pro-β galactosidase, a substrate of UFD pathway. Even though, they

can be employed in polyubiquitin chain formation, proteins with destabilizing N-terminal residues show longer half-lives, an indication that the chain is not recognized by the degradation machinery of the proteasome. However, heat sensitivity of the organism and thermal stability of the protein remained unaltered.

In order to understand if the combinations of double mutants and the triple mutant have more detrimental effects, or being closer to ideal β-bulge have some compensatory effects we have constructed and expressed the double mutant forms of ubiquitin UbQ2N-E64G, UbQ2N-S65D, UbE64G-S65D and the triple mutation UbQ2N-E64G-S65D in *UBI*4 deletion mutant strain of SUB60 of *S. cerevsiae*. Cells carrying UbE64G-S65D and UbQ2N-E64G-S65D show compromised growth and viability (Sharma M. and Prabha., 2011) (Mrinal Sharma, AnkitaDoshi and C. RatnaPrabha, Manuscript under preparation). The mutations do not complement SUB60 cells under stress conditions like exposure to cycloheximide and heat. UbQ2N-E64G and UbQ2N-S65D have milder effects on growth and viability (Sharma M. and Prabha., 2011). UbQ2N-E64G and UbQ2N-S65D are found to complement the SUB60 cells better compared to UbE64G-S65D and UbQ2N-E64G-S65D under heat and cycloheximide stresses. However, all these mutations have more severe effects compared to any of the single mutations. Hence, it can be concluded that the residues inside the β-bulge are crucial for interacting with the other proteins to maintain ubiquitin's functions.

The double mutant forms of ubiquitin UbQ2N-E64G, UbE64G-S65D, UbQ2N-S65D and the triple mutant UbQ2N-E64G-S65D were constructed in pET30-a vector and expressed in *Escherichia coli* BL21strain. The proteins were purified and spectroscopic characterization was carried out using circular dichroism and fluorescence spectroscopic studies. Far-UV CD spectra analysis suggests the alteration in the secondary structure of ubiquitin implicating the role of residues to maintain the proteins secondary structure. Tertiary structure and the stability of the proteins have been evaluated by fluorescence spectroscopy which suggests that protein could maintain their tertiary structure despite mutations. UbE64G-S65D and Q2N-E64G-S65D mutant have shown more stability than that of wild type ubiquitin while

other two mutants namely Q2N-E64G and Q2N-S65D have shown that the tertiary structure comparable to that of wild type ubiquitin.

A second approach of error prone PCR was adopted to generate random mutations in ubiquitin with functional effects. A dosage dependent lethal mutation of ubiquitin UbEP42 was isolated. UbEP42 was found to have no effect on SUB62 strain of *S.cerevisiae* which is wild type for UBI4 and can withstand heat stress and cycloheximide stress. On the other hand expression of UbEP42 in SUB60 cells, which carry *UBI*4 deletion, could not survive the expression of UbEP42 both under stressed and normal conditions. Sequencing of gene for UbEP42 showed 4 mutations namely S20F, A46S, L50P and I61T.

Earlier studies from the laboratory showed UbEP42 is thermo-labile. It does not complement SUB60 cells under stress conditions like heat treatment and exposure to antibiotics. Though UbEP42 can form polyubiquitin chains, substrate protein degradation may not be effective with this tag. UbEP42 altered the half-life of substrate proteins with destabilizing N-terminal residue. This mutant is likely to produce lethality in a dosage dependent manner in higher eukaryotes as well, leaving a possibility for selective targeting of diseased cells by expressing under native tissue specific regulatable promoters with an immediate application in cancer therapy. Considering the importance of this mutation, we decided to look into the effect of individual mutations found in UbEP42. Single mutant derivatives of UbEP42, namely UbS20F, UbA46S, UbL50P and UbI61T were constructed and expressed in SUB60 strain of *S.cerevisiae*. Further double and triple mutations were made in combination to dissect out the compensatory role of the mutations in *S. cerevisiae*. Complementation analysis has been done with all mutants to study the impact of mutations on the cell. Functional analysis of double and triple mutants show that the effects of mutations are additive as UbL50P-I61T becomes more lethal than the single mutations and exert non-complementation of the phenotypes studied, however combinations of S20F and A46S with L50P and I61T remain moderately affected.

UbEP42 and its derivative UbI61T has been cloned in pET30-a bacterial expression vector to study the structure and stability of the protein. Purification of the same protein by size exclusion chromatography has revealed the major changes in the purification procedure as the protein has lost its solubility and became hydrophobic in nature. Urea has been used to solubilise and to purify the protein. Because of difficulties in the purification procedure the mutant ubiquitin was cloned in pET30-a expression vector to take the advantage of affinity based purification. Secondary structure of the protein was analysed by circular dichroism and it was observed that the β-sheet content of the protein was increased significantly. GdHCl denaturation curve has shown that the protein could not maintain the tertiary structure, which explains the dose dependent lethality of the protein as it is observed using in vivo analysis that the protein acts as an antagonist hence it may be concluded that the recognition of the protein by proteasome is compromised.

In summary, the studies demonstrate that the combinations of double mutations UbQ2N-E64G, UbE64G-S65D, UbQ2N-S65Dand the triple mutation UbQ2N-E64G-S65Daffect the functions of ubiquitin more severely than individual mutations. Interestingly none of the mutations has a palliative effect on the other mutations. Hence, despite their low % frequency of occurrence the residues in parallel G1β-bulge show complete conservation through evolution because of their importance in the functional roles of ubiquitin. This studyestablishes relationship between the primary structure of the parallel G1β-bulgeand the functions of ubiquitin unequivocally. Besides, the mutant UbEP42 isolated earlier in the laboratory by error prone PCR has been found to confer several interesting phenotypic characters to the host cell, the most important feature among them is dosage dependent lethality.Sequence of UbEP42 revealed four substitutions, namely S20F, A46S, L50P and I61T. In order to dissect out individual effects of these mutations, single mutants of ubiquitin with the above mutations were constructed. Further characterization of these mutants revealed that the mutations L50P and I61T are mainly responsible for lethality. Mutation S20F has moderate effects and A46S does not show any detrimental effect in case of the functions studied. Considering the variable impacts of these mutations on the multiple

biological roles played by ubiquitin, further studies in higher systems may have find biomedical relevance.

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Publications,

Oral and Poster presentations

Publication :

Sharma M., and Prabha., C. R. (2011). **Construction and functional characterization of double and triple mutants of parallel β-bulge of ubiquitin.** Indian Journal of Experimental Biology *49*, 919-924.

Sharma M., and Prabha., C. R. **Compensatory effects of S20F and A46S over L50P and I61T mutations in ubiquitin** (Manuscript under preparation).

Sharma M., Doshi A., Prabha., C. R. **Ubiquitin with Q2N-E64G substitution in parallel β- bulge of ubiquitin in *Saccharomyces cerevisiae* displays functional alterations** (Manuscript under preparation).

Oral/ Poster presentation

Oral presentation entitled:

Mrinal Sharma and C. Ratna Prabha " **Functional importance of mutated residues in dosage dependent lethal mutation of ubiquitin "** at XXVI Gujarat Science Congress organized by The M.S. University of Baroda, Vadodara on 26th February 2012.

Mrinal Sharma, Ankita Doshi and C. Ratna Prabha **" Functional Importance of the mutant ubiquitin Q2N-E64G of parallel β-bulge of ubiquitin in *S. cerevisiae*"** Regional Science Congress on Science for Shaping the Future of India, organized by The M.S. University of Baroda, Vadodara on 15th-16th September 2012.

Poster Presentation entitled:

Mrinal Sharma and C. Ratna Prabha. **Functional importance of the residues of parallel β- bulge of ubiquitin.** International conference on molecular medicine, organized by CHARUSAT- 2010, Changa, Anand, Gujarat, India.

Functional importance of the residues of parallel β-bulge of Ubiquitin

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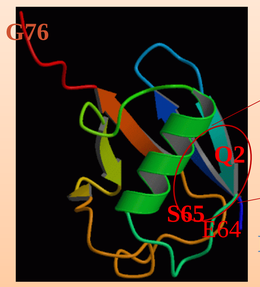
ABSTRACT

Ubiquitin is a small, low molecular weight globular protein bearing 76 amino acid residues. The characteristic function of this highly conserved polypeptide is to target proteins through proteasome mediated degradation in eukaryotic cells through a consecutive process of ubiquitin activation and substrate recognition. The substrate proteins for Ubiquitin-proteosome system (UPS) consist cell cyclines, antitumour proteins and transcription factors. As ubiquitin-proteasome system plays central role in cellular homeostasis and cell cycle regulation, it can be a potential target for drugs with anticancer potential. Ubiquitin features two β bulges. The second β-bulge which is located at the C- terminal region in the type II turn, holds three residues Glu64 (1), Ser65 (2), Gln2 (X). The % frequency of occurrence of such a β-bulge is very low. However, this structure has been conserved in ubiquitin throughout the evolution. Earlier work from our laboratory addressed the importance of this remarkable feature of Ubiquitin, through functional evaluation of three site directed mutants namely Gln2, Glu64 and Ser65. Present study involves the replacement of residues in this unusual β bulge of ubiquitin by introducing mutations in combination through site directed mutagenesis. Mutant ubiquitin genes have been cloned in yeast expression vector YEp 96 and the presence of mutations confirmed through restriction digestion patterns of vector and sequence analysis of the gene. Growth profile and viability assay have revealed significant decrease in growth rate and loss of viability in case of double mutants E64G-S65D however Q2N-S65D remained same. Heat stress complementation study has been done in *Saccharomyces cerevisiae* UBI 4 deleted SUB 60 strain. Though, Q2N-S65D complemented heat sensitive phenotype partially with 25% survival, E64G-S65D failed to complement the heat sensitive phenotype altogether. Our results establish that replacement of residues in β bulge of ubiquitin exerts severe effects on growth and viability in *Saccharomyces cerevisiae* due to functional failure of the mutant ubiquitin.

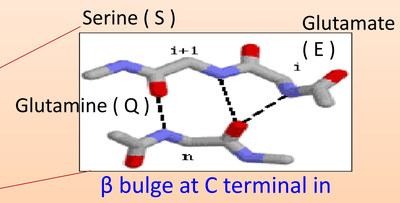
INTRODUCTION

Ubiquitin as the name indicates, is present ubiquitously in eukaryotic cells. It is a small, highly conserved, highly stable, 76 amino acid residues long tightly packed globular protein. It has two β-bulges(3). The first β-bulge present at N-terminal region and the second one is located at C terminal part of protein.

The three amino acids residues Gln2, Glu64 and Ser65 have low propensity for their positions in β-bulge. Here we have addressed the question of importance of these invariable residues in ubiquitin biology. We have chosen to answer the question by replacing these amino acids with those found in structural homologues of ubiquitin, which have higher propensity for β-bulge to generate the structural variants E64G-S65D and Q2N-S65D and by characterizing their function.



Structure of Ubiquitin

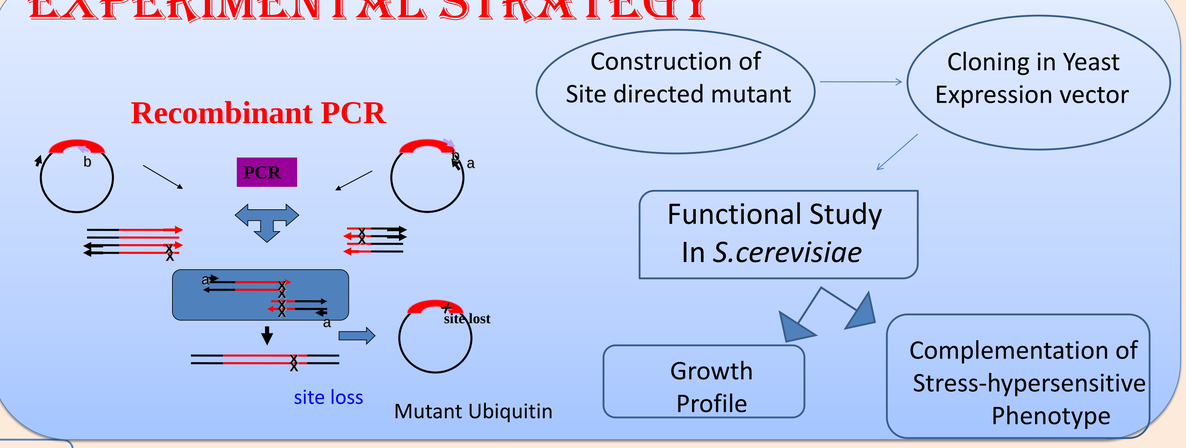


β bulge at C terminal in Ubiquitin

Class	1	2	X
G1	**Gly(G),** Asn	Arg, Lys, **Asp (D)** Glu, Gln	**Asn (N),** Asp, His Cys, Ser

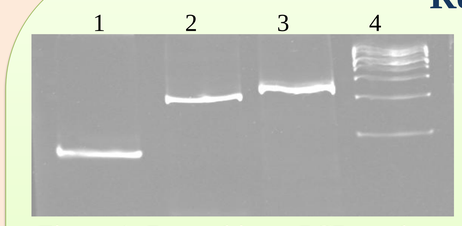
Table-1 Shows Preferred amino acid residues in G1 β-bulge after analyzing with several bulges. (Chan et.al. 1993)

EXPERIMENTAL STRATEGY



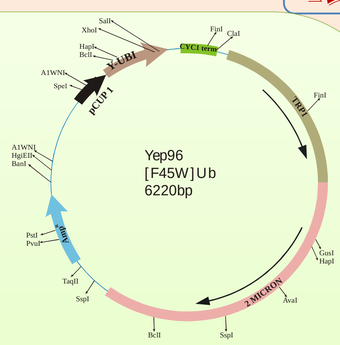
RESULTS

Recombinant PCR Product



Lane 1- 62 bp Amplicon
Lane 2- 217 bp Amplicon
Lane 3- 252 bp Amplicon
Lane 4- 100 bp Ladder

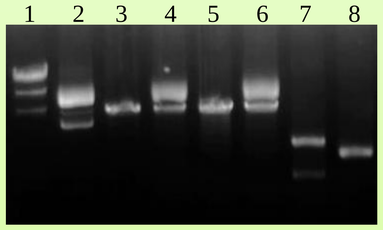
Figure-1: Recombinant PCR product of E64G-S65D mutant



Vector used in the study

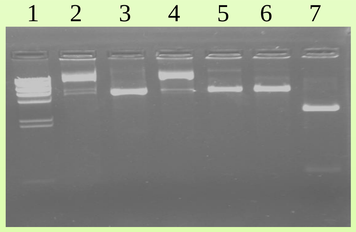
Clone confirmation by Restriction enzyme digestion

Recombinant PCR products bearing the two mutations were cloned in yeast expression vector Yep96 by using *Bgl*II and *Kpn*I *restriction enzyme. Below gel images [Figure-2(a-* and b)] show confirmation of mutants by plasmid digestion pattern.



Lane1 λ *Hin*dIII Marker
Lane2 Undigested plasmid.
Lane3 *Xho*I
Lane4 *Bgl*II
Lane5 *Kpn* I
Lane6 *Sal* I
Lane7 *Hin*dIII
Lane8 *EcoRI*

Figure-2(a)- Yep96Q2N-S65D



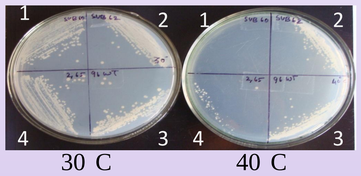
Lane1 λ HindIII Marker.
Lane2 Undigested plasmid.
Lane3 *Bgl*II
Lane4 *Sal* I
Lane5 *Xho* I
Lane6 *Kpn*I
Lane7 *Eco*RI

Figure-2(b)Yep96E64G-S65D

Heat Stress Complementation

Stress-hypersensitive phenotype

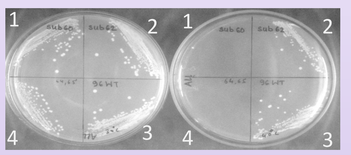
SUB60 strain of *S. cerevisiae* lacks gene for polyubiquitin *UBI4*, which makes the cells sensitive to stresses such as heat, UV and cycloheximide. Expression of wild type ubiquitin gene from a plasmid can complement the SUB60 cells under stress conditions and help them in overcoming the stress. In order to check the functionality of ubiquitin variants Q2N-S65D and E64G-S65D were expressed in SUB60 background under stress conditions.



1. SUB60
2. SUB62
3. YEp96 WT
4.UbQ2N-S65D

30 C 40 C

YEp96 [Q2N-S65D]



1. SUB60
2. SUB62
3. YEp96 WT
4.UbE64G-S65D

30 C 40 C

YEp96 [E64G-S65D]

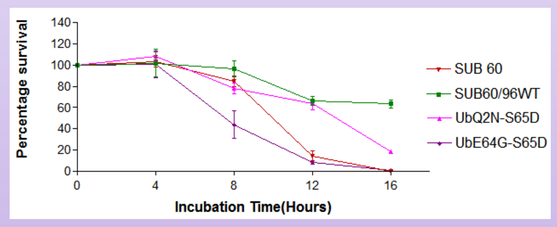


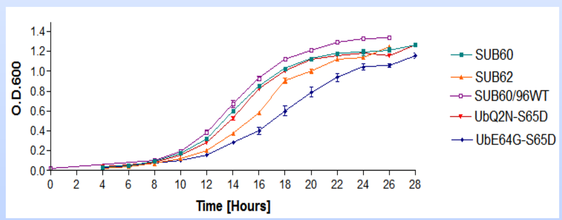
Figure3-: Effect of heat stress in mutants at different time points.

Strain used in the study

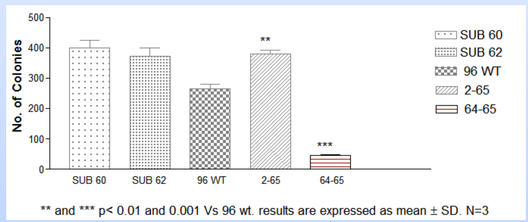
- **SUB 62 [Control Strain]**
  MATα lys2-801 leu2-3, 112 ura3-52 his3-Δ200 trp1-1
- **SUB 60 [Ubi4 Mutant Strain]**
  MATα ubi4-Δ2::LEU2 lys2-801 leu2-3,112 ura3-52 his3-Δ200 trp1-1

Functional Characterization

Growth Profile



Viability Assay



** and *** p< 0.01 and 0.001 Vs 96 wt. results are expressed as mean ± SD. N=3

CONDITIONS :

- 30°C , 200 rpm
- 1% inoculums of 24 hours grown culture.
- Culture Medium-
  1. Yeast nitrogen base [Without amino acid]
  2. Glucose 2%
  3. Amino acids-Leu,Trp,Lys,His & Ura.

✓Mutants transformed in UBI4 deleted SUB60 yeast strain were grown to log phase with O.D. of the cultures reaching a value of 0.6.

✓Serial diluted fourfold and plated on SD selection media. Plates were incubated at 30°C and the colonies were counted.

CONCLUSIONS

Our results establish that replacement of residues in β bulge of ubiquitin affect growth and viability in *Saccharomyces cerevisiae* due to functional failure of the mutant ubiquitin. The results observed in *Saccharomyces cerevisiae* can be extrapolated to higher eukaryotic systems, suggesting a possibility for biomedical applications especially under conditions of cancer and certain neurodegenerative disorders.

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