Chapter 3

Construction and Characterization of UBC4 Mutants with Single Residues Swapped from UBC5

3.1. Introduction

It is known that, E3s are responsible for selection of substrate proteins for ubiquitination. However, E2s are the ones which determine the topology and length of the ubiquitin chains to be conjugated to the substrate (Ye and Rape, 2009). Targeting the UPS system with small molecules has been of substantial importance for treatment of diseases such as neurodegenerative disorders and cancer (Bedford et al., 2011). Even though, the targets for E1 enzymes and proteasome are in clinical trials, they lack the strategic advantage that can be realized with developing inhibitors to E2 and E3 enzymes, since they are responsible for substrate selectivity and for the formation of type of ubiquitin linkage (Harper and King, 2011). Regulating cell cycle using a recently identified allosteric inhibitor against the E2 enzyme Cdc34 (Ceccarelli et al., 2011), emphasizes the importance of understanding E2 enzymes, as there is a strong possibility for targeting them with small-molecule therapy in the treatment of neurodegenerative disorders and cancer (Bedford et al., 2011; Skaar and Pagano, 2009).

The E2 enzymes UBC4 and UBC5 of *S. cerevisiae*, possessing only the UBC domain belong to Class I ubiquitin-conjugating enzymes (Cook et al., 1993). They were first isolated and characterized by Seufert and Jentsch (Seufert and Jentsch, 1990). UBC4 is an α/β protein consisting of UBC domain, which is highly conserved with respect to its secondary structural makeup involving four helices and a β-sheet consisting of 4 strands (Cook et al., 1993). It shares 92% sequence identity with UBC5 in the yeast system, differing by only 11 residues (Seufert and Jentsch, 1990). Interestingly, these residues are scattered along the sequence located in different secondary structural features of the protein (**Fig. 3.1**).

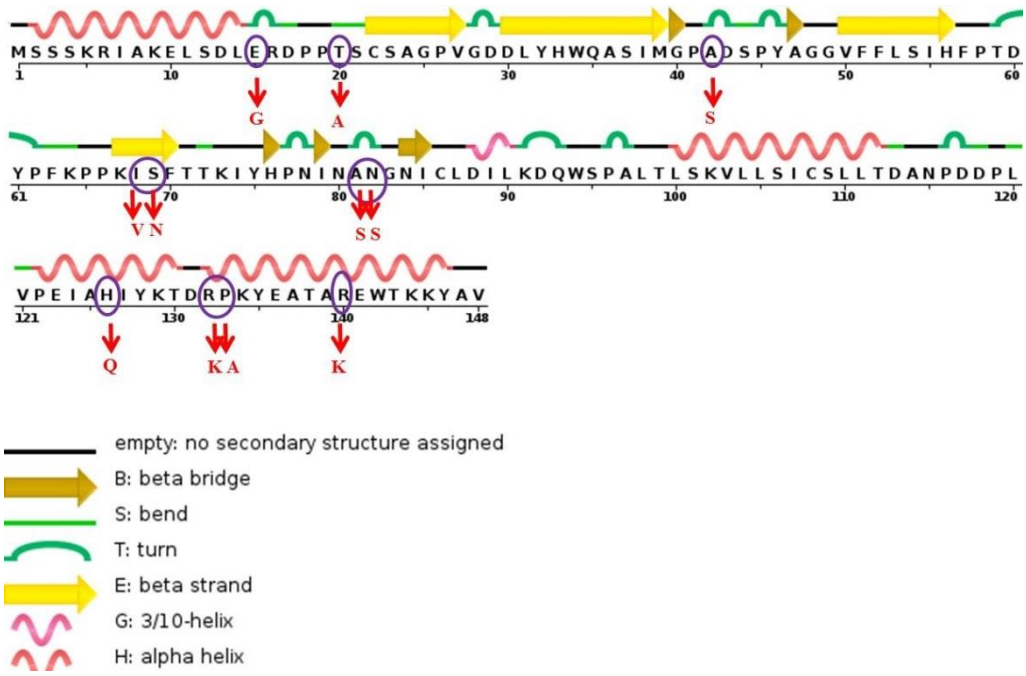

Fig. 3. 1. Sites of amino acid replacement in UBC4 (PDB ID: 1QCQ). The substituting residues from UBC5 are highlighted below the UBC4 sequence.

UBC4 is expressed in exponentially growing cells, while UBC5 is present in stationary phase (Cook et al., 1993). UBC4 and UBC5 are believed to be functionally redundant, as the cells lacking either *UBC4* or *UBC5* can live normally as long as the other gene is functional. Yeast cell's dependence on UBC4 is almost indispensable to overcome cellular responses to protein misfolding caused by amino acid analogues, heat shock and oxidative damage (Chuang and Madura, 2005). Yeast cells lacking both *UBC4* and *UBC5* are incompetent to overcome stress conditions, thus, establishing UBC4 and/or UBC5 mediated ubiquitination is indispensable, when there is a requirement for major proteolytic turnover in yeast cells (Seufert et al., 1990). Since, UBC4 and UBC5 are key enzymes of the ubiquitination cascade they interact with many ubiquitin ligases as well as other proteins. The information available in literature on the interacting proteins of UBC4 and UBC5 was collected and analysed here. The proteins have been classified on the basis of their activity and location to gain insight into their functional uniqueness and redundancy.

Even though the enzymes are considered to be functionally redundant for the properties tested, UBC4 and UBC5 exist as two separate enzymes in nature. Their independent identities and conservation through evolution, indicate yet unidentified specialized functions for each one under certain conditions. Mutagenesis studies are often used to understand the structural and functional significances of individual residues in proteins (Mishra et al., 2011; Mishra et al., 2009; Sharma and Prabha, 2011, 2015). In this study, we tried to understand the importance of the residues that differ between UBC4 and UBC5, by swapping individual residues of UBC4 with those of UBC5. The single mutants of UBC4 generated were tested for their functional efficacy by expressing them in *S. cerevisiae* strain lacking both *UBC4* and *UBC5* genes. Growth under normal conditions, survival under heat stress and resistance to antibiotics were monitored to assess the effects of mutations on UBC4 functions.

Moreover, available information in literature on the contribution of individual residues to the structure of these two E2s is limited. Theoretical studies were carried out with the mutant forms of UBC4 generated by swapping single residues from UBC5, to see if residues of UBC5 confer greater structural stability to UBC4. Results generated from these three lines of study were analysed collectively to understand the reason for the existence of two separate enzymes with considerable functional overlap.

3.2. Materials and Methods

3.2.1. In silico analysis of the binding partners of UBC4 and UBC5

The information available in open source database Bio-GRID[3.5] (https://thebiogrid.org) on binding partners of UBC4 and UBC5, had been analysed to identify interacting proteins specific to either of them and common to both (Oughtred et al., 2016; Oughtred et al., 2019).

3.2.2. Theoretical analysis for the variants of UBC4

In silico protein stability was performed by using the online bioinformatics tool, mutation Cutoff Scanning Matrix (mCSM) available on http://biosig.unimelb.edu.au/mcsm/stability. Specific mutation (e.g. E15G, T20A) was used as input and wild-type protein sequence was used for UBC4 protein. This tool models distance patterns between atoms to represent protein residue environments and delivers output in terms of change in Gibbs free energy (ΔΔG) (Pires et al., 2014).

3.2.3. Strains used for in vivo studies

The S. cerevisiae strains used in the study MHY501 (MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1) and MHY508 (MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801trp1-1 ubc4-Δ1::HIS3 ubc5-Δ1::LEU2) (Chen et al., 1993) were provided generously by Prof. Mark Hochstrasser (Yale University, New Haven, CT). Wild-type cells (MHY501) and the mutant MHY508 with Δubc4Δubc5 deletions are haploid Matα strains congenic to DF5 (Finley et al., 1987). Yeast episomal plasmid YEp96 with TRP1 as the selection marker, is a shuttle vector between S. cerevisiae and E. coli (Finley et al., 1994). YEp96 carrying ubiquitin gene under CUP1 promoter was used for the construction of UBC4 WT, by replacing gene for ubiquitin with that of wild-type UBC4 and mutated forms of UBC4 gene by swapping the codons of UBC4 with those of UBC5. **Table 3.1** gives details of mutated proteins. The UBC4 gene and its derivatives were expressed from the CuSO4 inducible CUP1 promoter, by using 100μM copper sulphate. S. cerevisiae cultures were grown in Yeast Extract, Peptone, Dextrose (YPD) medium containing 2% glucose, 1% peptone, 1% yeast extract and synthetic dextrose (SD) medium containing 2% glucose as carbon source, 0.67% yeast nitrogen base. The SD medium did not contain any amino acids. Solid media contained 2% agar additionally. Histidine (20mg L^{-1}), lysine (30mg L^{-1}), uracil (20mg L^{-1}), leucine (100 mg L^{-1}) or tryptophan (20mgL^{-1}) were added for selection, depending on the experimental requirement (Finley et al., 1994). The cultures were grown at

30°C and 200 rpm. Cells were transformed by lithium acetate method (Chen et al., 1992). Glucose and all amino acid stocks were obtained from SRL, India. Peptone, agar, yeast extract, yeast nitrogen base were of Himedia, India. All chemicals used in the study were of highest grade.

Table 3.1 UBC4 variants constructed, codons originally present in UBC4 and codons substituted from UBC5 for required amino acid substitutions are listed in this table.

UBC4 protein variants after amino acid substitution	**Abbreviation used for indicating the substitution**	**Position**	**UBC4**		**UBC5**	
			Original codon	**Amino acid encoded**	**Codon swapped from UBC5**	**Amino acid encoded**
UBC4-E15G	E15G	15	GAA	Glu	G**GG**	Gly
UBC4-T20A	T20A	20	ACT	Thr	**G**CT	Ala
UBC4-A42S	A42S	42	GCC	Ala	**T**C**A**	Ser
UBC4-I68V	I68V	68	ATC	Ile	**G**T**A**	Val
UBC4-S69N	S69N	69	TCC	Ser	**AA**C	Asn
UBC4-A81S	A81S	81	GCC	Ala	**TC**G	Ser
UBC4-N82S	N82S	82	AAT	Asn	A**G**T	Ser
UBC4-H126Q	H126Q	126	CAT	His	CA**A**	Gln
UBC4-R132K	R132K	132	AGA	Arg	A**AG**	Lys
UBC4-P133A	P133A	133	CCC	Pro	**G**C**T**	Ala
UBC4-R140K	R140K	140	AGA	Arg	A**AG**	Lys
UBC4	UBC4 WT	-	-	-	-	-

3.2.4. Plasmids and plasmid construction/Construction of yeast vectors with variants of UBC4 gene generated by swapping with codons from UBC5

The plasmid pQE9 carrying UBC4 gene was a kind gift from Prof. Stefan Jentsch (Department of Molecular Cell Biology, Max Planck Institute of Biochemistry, Germany). Mutagenic and non-mutagenic primers were designed to generate the amplicons UBC4 and its mutants carrying the mutation essential to get the desired amino acid substitution (Table 3.2). YEp96 was double digested by *Bgl*II and *Kpn*I restriction enzymes to remove ubiquitin gene. Amplicons of UBC4 and its variants were cloned into the plasmid backbone of YEp96 using compatible end cloning strategy, using T4 DNA ligase to generate UBC4 WT series of plasmids (**Table 3.1**). The plasmids were transformed into DH5α strain of E. coli. The transformant cells were grown in LB medium supplemented with the antibiotic ampicillin (100μg/mL) to maintain the plasmids. The genes were placed under CUP1 promoter. The respective plasmids were confirmed by DNA sequencing using non-mutagenic primer as listed in **Table 3.2**.

Table 3.2 List of primers used for the construction of variants of UBC4 gene. The highlighted sequence in FR (forward primer) of UBC4 WT represents restriction site for *BamH*I and *Kpn*I in RE (reverse primer).

Substitution	Primer sequence
UBC4 WT (Non-mutagenic primers)	FR:5' ATCTCGGATCCATGTCTTCTTCTAAACGTATTGC 3' RE: 5' AATTGGTACCTTATACAGCGTATTTCTTTGTCC 3'
E15G	FR: 5' GAACTAAGTGATCTAGGGAGAGATCCACC 3' RE: 5' GGTGGATCTCTCCCTAGATCACTTAGTTC 3'
T20A	FR: 5' AGAGATCCGCCGGCTTCATGTTCAGCC 3' RE: 5' GGCTGAACATGAAGCAGGTGGATCTCT 3'
A42S	FR: 5' GGACCTTCAGATTCCCCATAT 3' RE: 5' ATATGGGGAATCTGAAGGTCC 3'
I68V	FR: 5’ AAG CCA CCA AAG GTA TCC TTC ACA ACC 3’ RE: 5’GGT TGT GAA GGA TAC CTT TGG TGG CTT 3’
S69N	FR: 5' CCAAAGATCAACTTCACA 3' RE: 5' TGTGAAGTTGATCTTTGG 3'

A81S	FR: 5’ AAT ATC AAT TCG AAT GGT AAC AT 3’ RE: 5’ATG TTA CCA TTC GAA TTG ATA TT 3’
N82S	FR: 5' AATATCAATGCCAGTGGTAACATCTGT 3' RE: 5' ACAGATGTTACCACTGGCATTGATATT 3'
H126Q	FR: 5' GTACCAGAAATCGCTCAAATCTACAAGACTGAC 3'RE:5' GTCAGTCTTGTAGATTTGAGCGATTTCTGGTAC 3'
R132K	FR: 5'ACTGACAAGCCCAAGTAC 3' RE: 5' GTACTTGGGCTTGTCAGT 3'
P133A	FR: 5' AAGACTGACAGAGCTAAGTACGAAGC 3' RE: 5' GCTTCGTACTTAGCTCTGTCAGTCTT 3'
R140K	FR: 5’AA GCT ACA GCC AAG GAA TGG ACA AAG 3’ RE: 5’CTT TGT CCA TTC CTT GGC TGT AGC TT 3’

3.2.5. Testing the expression levels and stabilities of UBC4 WT and its variants

The transformant cultures were grown in the presence and absence of an inducer (copper sulphate, 100μM) at 30 °C in YPD medium for various time intervals (4, 8, 12, 16, 24 h). MHY508 and UBC4 WT were used as controls. The cells were harvested by centrifugation at room temperature and 10,000 rpm for 1 min, and were washed and re-suspended in normal saline. Cell lysis was carried out by sonication (80% amplitude, 0.2 s pulse on/off) until a clear solution was obtained. Sonicated samples were centrifuged at room temperature and 10,000 rpm for 1 min. The supernatants were mixed with laemmli sample buffer and boiled for 15 min and were analysed on 12% SDS-PAGE. The gels were stained with coomassie Blue R-250.

3.2.6. Effect of mutations on growth of cells

The vectors carrying the wild-type UBC4 and variants of UBC4 were transformed into yeast Δubc4Δubc5 strain. Later, the cultures were grown at 30 °C at 200 rpm, except where indicated in synthetic dextrose medium. One hundred micromolars of copper sulphate was used as an inducer to check the effects of expression of mutant UBC4 genes on *S. cerevisiae* cells. The growth rate was monitored by measuring OD at 600nm every 2h. Growth of the cultures expressing UBC4 mutations was compared with control cells

expressing wild-type UBC4 extrachromosomally from UBC4 WT. Wild-type strain (MHY501), as well as Δubc4Δubc5 (MHY508), were used as another set of controls.

3.2.7. Heat stress test

Heat sensitivity test was performed to confirm the functional integrity of the mutant ubiquitin-conjugating enzyme (Seufert and Jentsch, 1990). The transformant cultures were grown at 30°C till they reached an absorbance of 1.0. Fourfold serial dilution was made and spread on SDA plates in the presence or absence of an inducer (100 μM). The plates were then incubated at 37°C for various time intervals (0, 4, 8, 12, 16 and 24 h) and then shifted back to 30°C to assess colony formation.

3.2.8. Antibiotic sensitivity test

Various translational inhibitors were tested to find if these mutants can function as efficiently as wild-type UBC4, when abnormal and truncated proteins are generated. MHY508 cells transformed by plasmid UBC4 WT and its variants were grown to log phase, and were diluted till their optical density values reached around 0.1. The cultures were further diluted three-fold serially and all the dilutions were spotted on SDA plates with and without the antibiotics cycloheximide (0.001M), L-canavanine (0.14mM) and hygromycin-B (0.2mM) in the presence of the inducer (100μM copper sulphate). The plates were incubated for 7–10 days at 30°C to assess survival and growth.

3.3. Results

3.3.1. In silico analysis of binding partners of UBC4 and UBC5

Binding partners of UBC4 and UBC5 were collected from the publicly available open source database for protein–protein interactions, BioGRID3.5 (Oughtred et al., 2016; Oughtred et al., 2019). UBC4 interacts with >400 interactors, while UBC5 has only 71 interactors. Out of them chemical and genetic interactions were filtered out and we focussed on physical interactions. We looked for partners to find which are common between both UBC4 and UBC5 (**Fig. 3.2 (a–c**)). Our results show that out of 74 interactive partners of UBC4 and 28 of UBC5, only 14 are common (**Fig. 3.3**). The common interacting partners of UBC4 and UBC5 indicate the complementary roles the two ubiquitin conjugating enzymes play with respect ubiquitin proteasome system, whereas individually both of them can participate in the regulation of various biological processes through their partners exclusive to either one (**Table 3.3 (a-c**)). Hence, both UBC4 and UBC5 also play crucial but distinct roles in regulating biological processes.

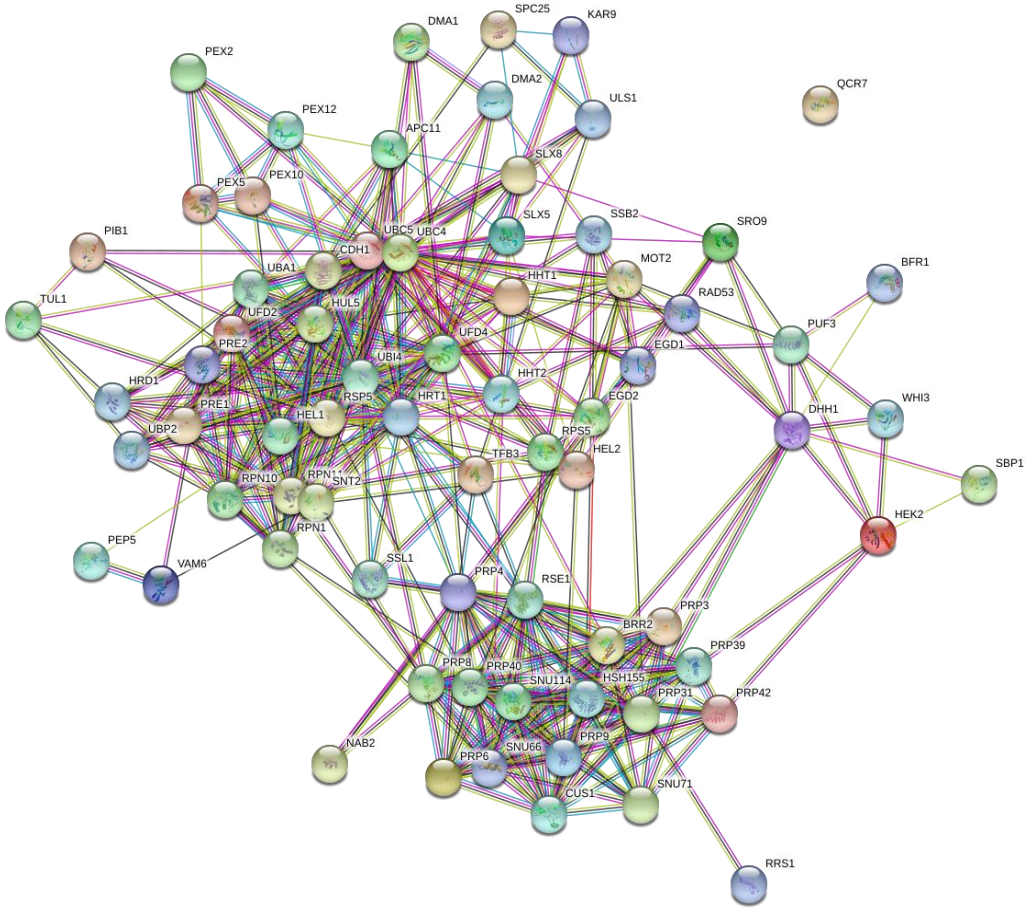

Fig. 3.2 (a) Interactors of UBC4. The thickness of the lines indicates the strength of interaction between the proteins. Interactions of UBC4 and UBC5 plotted on STRING (Szklarczyk et al., 2017) (data collection source: The Biological General Repository for Interaction Datasets (BioGRID)[3.5]) (Oughtred et al., 2016; Oughtred et al., 2019).

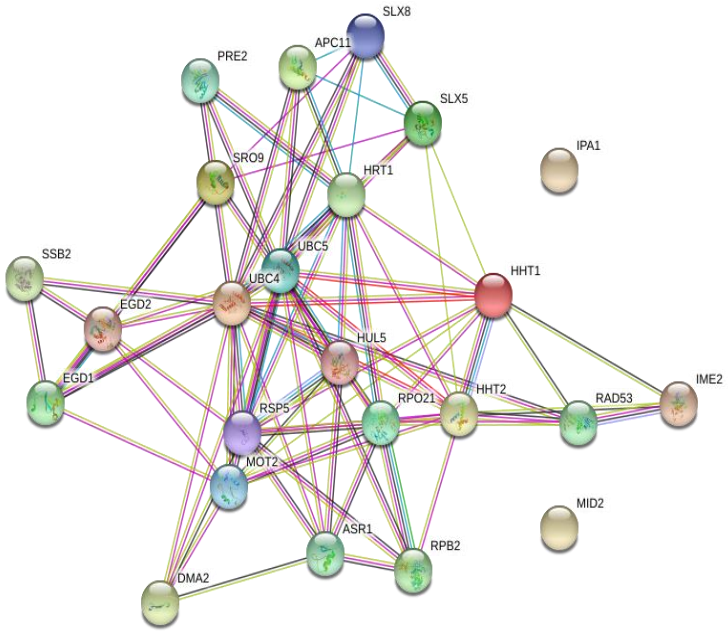

Fig. 3.2 (b) Interactors of UBC5. The thickness of the lines indicates the strength of interaction between the proteins. Interactions of UBC4 and UBC5 plotted on STRING (Szklarczyk et al., 2017) (data collection source: The Biological General Repository for Interaction Datasets (BioGRID)[3.5]) (Oughtred et al., 2016; Oughtred et al., 2019).

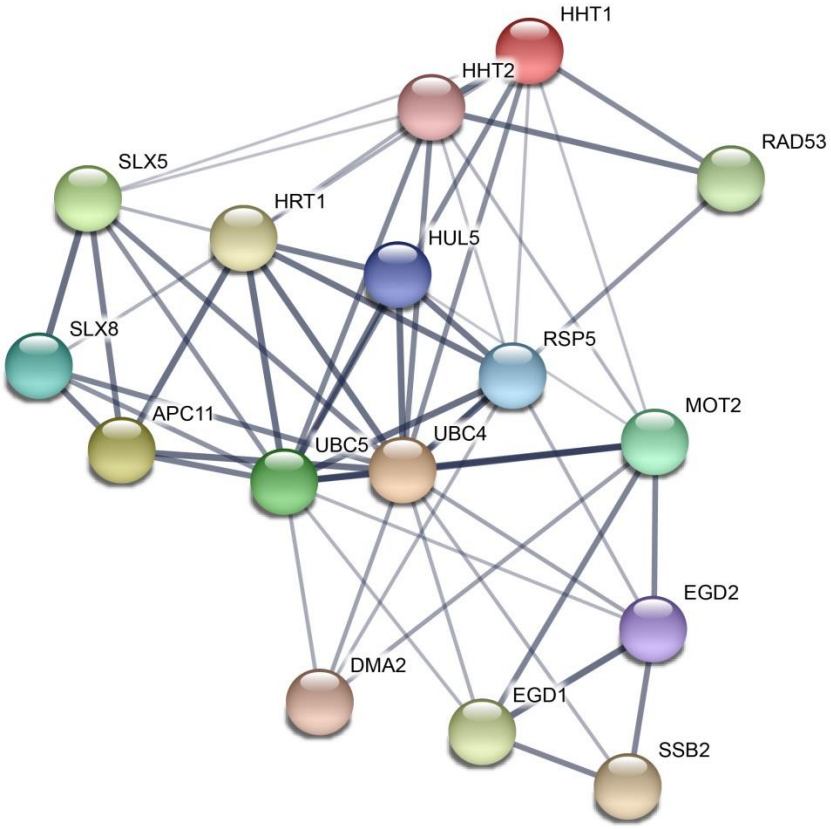

Fig. 3.2 (c) Interactors of UBC4 and UBC5. The thickness of the lines indicates the strength of interaction between the proteins. Interactions of UBC4 and UBC5 plotted on STRING (Szklarczyk et al., 2017) (data collection source: The Biological General Repository for Interaction Datasets (BioGRID)[3.5]) (Oughtred et al., 2016; Oughtred et al., 2019).

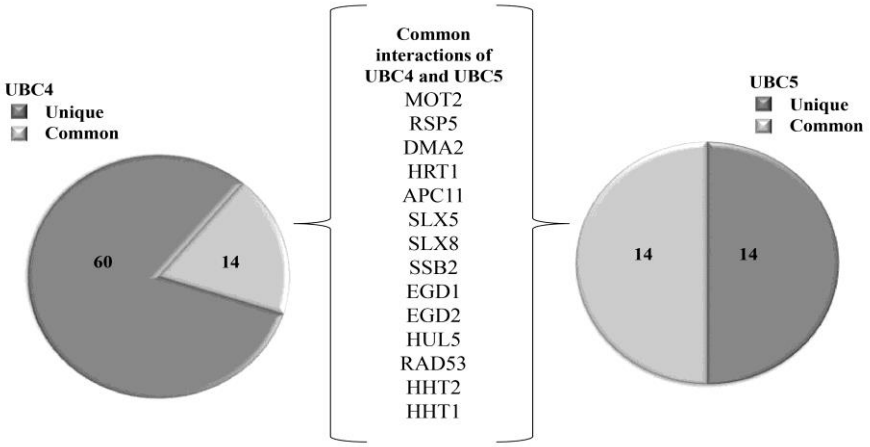

Fig. 3.3 Diagrammatic representation for physical interactions of UBC4 and UBC5. The values represent the number of interactions for individual proteins and common interactors shared between two genes.

Table 3.3 (a) Interactions of UBC4 with their cellular localisation and functions (source: The Biological General Repository for Interaction Datasets (BioGRID) [3.5]).

Interactions of UBC4	Cellular component	Molecular Function/ Biological process
TUL1	• Golgi apparatus	• Ubiquitin-Protein Transferase Activity
PRE2	• Endoplasmic reticulum • Nucleus • proteasome core complex • proteasome storage granule	• Endopeptidase Activity
PRE1	• Endoplasmic reticulum nucleus • proteasome core complex • proteasome storage granule	• Endopeptidase Activator Activity

SPC25	• Ndc80 Complex • Condensed Nuclear Chromosome Kinetochore • Condensed Nuclear Chromosome, Centromeric Region	• Structural Constituent Of Cytoskeleton
HEK2	• Cytoplasm • Cytoplasmic mRNA Processing Body • Nuclear Chromosome, Telomeric Region	• mRNA Binding
WHI3	• Cytoplasm • Cytoplasmic mRNA Processing Body • Cytoplasmic Stress Granule	• mRNA Binding
NAB2	• Cytoplasm • Nucleus	• mRNA Binding • Poly(A) Binding
RPN11	• Cytosol • Mitochondrion • Nucleus • Proteasome Regulatory Particle, Lid Subcomplex • Proteasome Storage Granule	• Metallopeptidase Activity • Ubiquitin-Specific Protease Activity
PEX2	• Peroxisomal Importomer Complex • Peroxisomal Membrane	• Protein Binding • Ubiquitin-Protein Transferase Activity
KAR9	• Cell Cortex • Mating Projection Tip • Spindle Pole Body	• Establishment Of Spindle Localization • Mitotic Spindle Orientation Checkpoint • Nuclear Migration Along Microtubule
SRO9	• Cytoplasmic Stress Granule • Polysome	• RNA Binding • MRNA Binding
RSP5	• Golgi Apparatus • Cellular Bud Tip • Cytoplasm • Endosome Membrane • Extrinsic Component Of Cytoplasmic Side Of	• Phosphatidylinositol Binding • Ubiquitin Binding • Ubiquitin-Protein Transferase Activity

	Plasma Membrane • Mitochondrion • Nucleus • Plasma Membrane • Ubiquitin Ligase Complex	
MOT2	• CCR4-NOT Core Complex • Cytoplasm	• Sequence-Specific DNA Binding • Ubiquitin-Protein Transferase Activity
HRD1	• Hrd1p Ubiquitin Ligase ERAD-L Complex • Hrd1p Ubiquitin Ligase ERAD-M Complex • Endoplasmic Reticulum Membrane • Integral Component Of Membrane	• Ubiquitin-Protein Transferase Activity
RPS5	• 90S Preribosome • Cytosolic Small Ribosomal Subunit	• Structural Constituent Of Ribosome
UFD4	• Cytoplasm • Mitochondrion • Nucleus	• Ubiquitin-Protein Transferase Activity
TRIM21	• Cytoplasm • Nucleus • Autophagosome	• DNA binding • identical protein binding • RNA binding • ubiquitin-protein transferase activity • zinc ion binding
APC11	• Anaphase-Promoting Complex	• Ubiquitin-Protein Transferase Activity
SLX5	• SUMO-Targeted Ubiquitin Ligase Complex • Chromosome, Centromeric Region • Nucleus	• SUMO Binding • Ubiquitin-Protein Transferase Activity
SLX8	• SUMO-Targeted Ubiquitin Ligase Complex • Cytoplasm Nucleus •	• Ubiquitin-Protein Transferase Activity
PEP5	• CORVET Complex • HOPS Complex	• Phosphatidylinositol Binding

	• Fungal-Type Vacuole Membrane	• Ubiquitin-Protein Transferase Activity
SNT2	• Snt2C Complex • Cytoplasm • Nucleus	• DNA Binding • Ubiquitin-Protein Transferase Activity
HEL1	• Cytoplasm	• metal ion binding • ubiquitin conjugating enzyme binding • ubiquitin protein ligase activity • ubiquitin-protein transferase activity
HEL2	• Cytoplasm • Ribosome	• Ubiquitin-Protein Transferase Activity
SBP1	• Cytoplasm • Cytoplasmic mRNA Processing Body • Cytoplasmic Stress Granule • Nucleolus	• RNA Binding • Eukaryotic Initiation Factor 4G Binding • mRNA Binding
CDH1	• Cytoplasm • Nucleus	• anaphase-promoting complex binding • cyclin binding • ubiquitin ligase activator activity
UBA1	• Cytoplasm • Nucleus	• Ubiquitin Activating Enzyme Activity
PUF3	• Cytoplasm • Cytoplasmic Side Of Mitochondrial Outer Membrane	• mRNA Binding
UBP2	• Cytoplasm	• Ubiquitin-Specific Protease Activity
PEX5	• Cytosol • Peroxisomal Importomer Complex • Peroxisomal Membrane • Peroxisome	• Peroxisome Matrix Targeting Signal-1 Binding • Protein Binding, Bridging
PIB1	• Fungal-Type Vacuole Membrane • Late Endosome	• Phosphatidylinositol-3-Phosphate Binding • Ubiquitin-Protein Transferase Activity

DMA1	• Cytoplasm	• Ubiquitin-Protein Transferase Activity
DMA2	• Cellular Bud Neck Split Septin Rings • Cellular Bud Tip • Cytoplasm	• Ubiquitin-Protein Transferase Activity
PEX12	• Integral Component Of Peroxisomal Membrane • Peroxisomal Importomer Complex •	• Protein Binding • Ubiquitin-Protein Transferase Activity
PEX10	• Peroxisomal Importomer Complex • Peroxisomal Membrane	• Protein Binding • Ubiquitin-Protein Transferase Activity
HRT1	• Cul3-RING Ubiquitin Ligase Complex • Cul8-RING Ubiquitin Ligase Complex • SCF Ubiquitin Ligase Complex • Cytoplasm • Nucleus	• Protein Binding, Bridging • Ubiquitin-Protein Transferase Activity
UFD2	• Cytoplasm • Nucleus •	• Ubiquitin-Ubiquitin Ligase Activity
SSB2	• Cytoplasm • Plasma Membrane • Polysome	• ATPase Activity • Unfolded Protein Binding
anapc11	• Cytoplasm • Nucleus	• cullin family protein binding • metal ion binding • ubiquitin protein ligase activity • ubiquitin-ubiquitin ligase activity
fzr1	• Cytoplasm • Nucleus	• anaphase-promoting complex binding • ubiquitin-protein transferase activator activity
UBC5	• Proteasome Complex	• ubiquitin-protein transferase Activity
VAM6	• HOPS Complex • Fungal-Type Vacuole • Fungal-Type Vacuole	• Rab GTPase Binding • Phosphatidylinositol Binding

	Membrane	
RPN1	• Cytoplasm • Endoplasmic Reticulum • Nucleus • Proteasome Regulatory Particle, Base Subcomplex • Proteasome Storage Granule	• Endopeptidase Activity • Protein Binding, Bridging
RPN10	• Proteasome Complex • Proteasome Regulatory Particle, Base Subcomplex	• Polyubiquitin Binding • Structural Molecule Activity •
DHH1	• Cytoplasm • Cytoplasmic MRNA Processing Body • Cytoplasmic Stress Granule	• ATP-Dependent RNA Helicase Activity • Chromatin Binding • mRNA Binding • Protein Binding • Translation Regulator Activity, Nucleic Acid Binding •
BFR1	• Cytoplasm • Endoplasmic Reticulum • Nuclear Outer Membrane-Endoplasmic Reticulum Membrane Network • Polysome • Ribonucleoprotein Complex	• RNA BINDING • mRNA Binding
QCR7	• Integral Component Of Membrane • Mitochondrial Respiratory Chain Complex III • Mitochondrion	• Ubiquinol-Cytochrome-C Reductase Activity
RRS1	• Nucleus	• 5S rRNA binding • RNA binding
EGD1	• Nascent Polypeptide-Associated Complex	• Unfolded Protein Binding
EGD2	• Nascent Polypeptide-Associated Complex	• Phosphatidic Acid Binding • Phosphatidylinositol-3,5-Bisphosphate Binding • Phosphatidylinositol-3-Phosphate Binding

		• Phosphatidylinositol-4-Phosphate Binding • Unfolded Protein Binding
ULS1	• Cytoplasm • Mitochondrion • Nucleolus • Nucleus	• DNA-Dependent ATPase Activity • SUMO Binding
RAD53	• Cytosol • Nucleus	• DNA Replication Origin Binding • Protein Kinase Activity • Protein Serine/Threonine/Tyrosine Kinase Activity
HHT2	• CENP-A Containing Nucleosome • Nuclear Nucleosome	• DNA Binding
TFB3	• TFIIK Complex • Holo TFIIH Complex	• Core RNA Polymerase Binding Transcription Factor Activity
SSL1	• Core TFIIH Complex • Holo TFIIH Complex • Nucleotide-Excision Repair Factor 3 Complex	• Core RNA Polymerase Binding Transcription Factor Activity
HUL5	• Cytoplasm • Nucleus • Proteasome Complex	• Ubiquitin-Ubiquitin Ligase Activity
UBI4	• Cytoplasm • Peroxisomal Importomer Complex	• ATP-Dependent Protein Binding • Protein Tag
HHT1	• CENP-A Containing Nucleosome • Nuclear Nucleosome • Nucleus • Replication Fork Protection Complex	• DNA Binding
PRP9	• U2-Type Prespliceosome	• RNA Binding
PRP42	• U1 SnRNP • U2-Type Prespliceosome	• RNA Binding
PRP3	• U4/U6 X U5 Tri-SnRNP Complex	• mRNA Splicing, Via Spliceosome
PRP6	• U4/U6 X U5 Tri-SnRNP Complex	• mRNA Splicing, Via Spliceosome

SNU71	• U1 SnRNP • U2-Type Prespliceosome	• RNA Binding
PRP31	• U4/U6 X U5 Tri-SnRNP Complex • Mitochondrion	• mRNA Splicing, Via Spliceosome • Spliceosomal SnRNP Assembly
SNU114	• U4/U6 X U5 Tri-SnRNP Complex • U5 SnRNP	• GTPase Activity • U5 SnRNA Binding
PRP40	• U1 SnRNP • U2-Type Prespliceosome • Nucleus	• RNA Binding
SNU66	• U4/U6 X U5 Tri-SnRNP Complex	• mRNA Splicing, Via Spliceosome • Maturation Of 5S RRNA
RSE1	• U2 SnRNP • U2-Type Prespliceosome	• U2 SnRNA Binding
PRP39	• U1 SnRNP • U2-Type Prespliceosome • Commitment Complex	• Pre-mRNA 5'-Splice Site Binding
CUS1	• U2 SnRNP • U2-Type Prespliceosome	• mRNA Splicing, Via Spliceosome • Spliceosomal Complex Assembly
HSH155	• U2 SnRNP • U2-Type Prespliceosome	• mRNA Binding
PRP4	• U4/U6 X U5 Tri-SnRNP Complex	• mRNA Splicing, Via Spliceosome • SnoRNA Splicing
PRP8	• U4/U6 X U5 Tri-SnRNP Complex • U5 SnRNP • Nucleus	• U1 SnRNA Binding • U2 SnRNA Binding • U5 SnRNA Binding • U6 SnRNA Binding • mRNA Binding • Pre-mRNA Intronic Binding • Second Spliceosomal

		Transesterification Activity
BRR2	• U4/U6 X U5 Tri-SnRNP Complex • U5 SnRNP • Nucleus	• ATP-Dependent RNA Helicase Activity

Table 3.3 (b) Interactions of UBC5 with their cellular localisation and functions (source: The Biological General Repository for Interaction Datasets (BioGRID)[3.5]).

Interact-ions of UBC5	**Cellular component**	**Molecular Function/ Biological process**
PRE2	• Endoplasmic reticulum • Nucleus • proteasome core complex • proteasome storage granule	• Endopeptidase Activity
UBC4	• Proteasome Complex	• Protein Binding, Bridging • Ubiquitin Binding • Ubiquitin-Protein Transferase Activity
IME2	• Nucleus	• Protein Kinase Activity
SRO9	• Cytoplasmic Stress Granule • Polysome	• RNA Binding • mRNA Binding
MOT2	• CCR4-NOT Core Complex • Cytoplasm	• Sequence-Specific DNA Binding • Ubiquitin-Protein Transferase Activity
CNOT4	• Nucleus • Cytoplasm	• metal ion binding • RNA binding • ubiquitin-protein transferase activity
RNF5	• Endoplasmic reticulum • Mitochondrion	• identical protein binding • ubiquitin-like protein conjugating enzyme binding

		• ubiquitin protein ligase activity • ubiquitin-protein transferase activity • zinc ion binding
RSP5	• Golgi Apparatus • Cellular Bud Tip • Cytoplasm • Endosome Membrane • Extrinsic Component Of Cytoplasmic Side Of Plasma Membrane • Mitochondrion • Nucleus • Plasma Membrane • Ubiquitin Ligase Complex	• Phosphatidylinositol Binding • Ubiquitin Binding • Ubiquitin-Protein Transferase Activity
Rbx1	• Cul3-RING Ubiquitin Ligase Complex • Cul8-RING Ubiquitin Ligase Complex • SCF Ubiquitin Ligase Complex • Cytoplasm • Nucleus	• Protein Binding, Bridging • Ubiquitin-Protein Transferase Activity
DMA2	• Cellular Bud Neck Split Septin Rings • Cellular Bud Tip • Cytoplasm	• Ubiquitin-Protein Transferase Activity
RPO21	• DNA-Directed RNA Polymerase II, Core Complex • Mitochondrion • Nucleus	• RNA Polymerase II Activity • RNA-Directed RNA Polymerase Activity
RPB2	• DNA-Directed RNA Polymerase II, Core Complex • Mitochondrion • Nucleus	• RNA Polymerase II Activity • RNA-Directed RNA Polymerase Activity • mRNA Binding
HRT1	• Cul3-RING Ubiquitin Ligase Complex • Cul8-RING Ubiquitin Ligase Complex • SCF Ubiquitin Ligase Complex • Cytoplasm • Nucleus	• Protein Binding, Bridging • Ubiquitin-Protein Transferase Activity
APC11	• Anaphase-Promoting	• Ubiquitin-Protein Transferase

	Complex	Activity
SLX5	• SUMO-Targeted Ubiquitin Ligase Complex • Chromosome, Centromeric Region • Nucleus	• SUMO Binding • Ubiquitin-Protein Transferase Activity
SLX8	• SUMO-Targeted Ubiquitin Ligase Complex • Cytoplasm Nucleus	• Ubiquitin-Protein Transferase Activity
SSB2	• Cytoplasm • Plasma Membrane • Polysome	• ATPase Activity • Unfolded Protein Binding
YJR141W	• Cytosol • Nucleus •	• ubiquitin conjugating enzyme binding
EGD1	• Nascent Polypeptide-Associated Complex	• Unfolded Protein Binding
EGD2	• Nascent Polypeptide-Associated Complex	• Phosphatidic Acid Binding • Phosphatidylinositol-3,5-Bisphosphate Binding • Phosphatidylinositol-3-Phosphate Binding • Phosphatidylinositol-4-Phosphate Binding • Unfolded Protein Binding
ASR1	• Cytoplasm • Nucleus	• Ubiquitin-Protein Transferase Activity
HUL5	• Cytoplasm • Nucleus • Proteasome complex	• ubiquitin conjugating enzyme activity • ubiquitin-ubiquitin ligase activity
RAD53	• Cytosol • Nucleus	• DNA Replication Origin Binding • Protein Kinase Activity • ProteinSerine/Threonine/Tyrosine Kinase Activity
HHT2	• CENP-A Containing Nucleosome • Nuclear Nucleosome	• DNA Binding
HHT1	• CENP-A Containing Nucleosome • Nuclear Nucleosome • Nucleus • Replication Fork Protection Complex	• DNA Binding

TRAF6	• Nucleus • Cytoplasm • Cell cortex • Lipid droplet	• histone deacetylase binding • mitogen-activated protein kinase kinase kinase binding • protein kinase B binding • protein N-terminus binding • thioesterase binding • tumor necrosis factor receptor binding • ubiquitin conjugating enzyme binding • ubiquitin protein ligase activity • ubiquitin protein ligase binding • ubiquitin-protein transferase activity • zinc ion binding
TRIM39	• Cytosol • Mitochondrion	• identical protein binding • transferase activity • zinc ion binding
MID2	• Integral Component Of Plasma Membrane • Mating Projection Tip	• Protein Binding • Transmembrane Signaling Receptor Activity

Table 3.3 (c) Common interactions of UBC4/5 with their cellular localisation and functions (source: The Biological General Repository for Interaction Datasets (BioGRID) [3.5]).

Interactions of UBC4/UBC5	Cellular component	Molecular Function/ Biological process
MOT2	• CCR4-NOT Core Complex • Cytoplasm	• Sequence-Specific DNA Binding • Ubiquitin-Protein Transferase Activity
RSP5	• Golgi Apparatus • Cellular Bud Tip • Cytoplasm • Endosome Membrane • Extrinsic Component Of Cytoplasmic Side Of Plasma Membrane • Mitochondrion • Nucleus • Plasma Membrane • Ubiquitin Ligase Complex	• Phosphatidylinositol Binding • Ubiquitin Binding • Ubiquitin-Protein Transferase Activity
DMA2	• Cellular Bud Neck Split Septin Rings • Cellular Bud Tip • Cytoplasm	• Ubiquitin-Protein Transferase Activity
HRT1	• Cul3-RING Ubiquitin Ligase Complex • Cul8-RING Ubiquitin Ligase Complex • SCF Ubiquitin Ligase Complex • Cytoplasm • Nucleus	• Protein Binding, Bridging • Ubiquitin-Protein Transferase Activity
APC11	• Anaphase-Promoting Complex	• Ubiquitin-Protein Transferase Activity
SLX5	• SUMO-Targeted Ubiquitin Ligase Complex • Chromosome, Centromeric Region • Nucleus	• SUMO Binding • Ubiquitin-Protein Transferase Activity
SLX8	• SUMO-Targeted Ubiquitin Ligase Complex	• Ubiquitin-Protein Transferase Activity

	• Cytoplasm Nucleus	
SSB2	• Cytoplasm • Plasma Membrane • Polysome	• ATPase Activity • Unfolded Protein Binding
EGD1	• Nascent Polypeptide-Associated Complex	• Unfolded Protein Binding
EGD2	• Nascent Polypeptide-Associated Complex	• Phosphatidic Acid Binding • Phosphatidylinositol-3,5-Bisphosphate Binding • Phosphatidylinositol-3-Phosphate Binding • Phosphatidylinositol-4-Phosphate Binding • Unfolded Protein Binding
HUL5	• Cytoplasm • Nucleus • Proteasome Complex •	• Ubiquitin-Ubiquitin Ligase Activity
RAD53	• Cytosol • Nucleus	• DNA Replication Origin Binding • Protein Kinase Activity • Protein Serine/Threonine/Tyrosine Kinase Activity
HHT2	• CENP-A Containing Nucleosome • Nuclear Nucleosome	• DNA Binding
HHT1	• CENP-A Containing Nucleosome • Nuclear Nucleosome • Nucleus • Replication Fork Protection Complex	• DNA Binding

3.3.2. In silico analysis of protein stability of UBC4 variants in comparison to UBC4 wild type

Online bioinformatics tool mCSM was used to investigate the effect of individual mutations on the in vitro or inherent protein stability, as the stability of a protein influences its function directly. Alteration in stability of protein can be measured by the difference of change in Gibbs free energy between wild type and mutant forms of UBC4, the ΔΔG. Our results show that out of

11 mutations, 2 mutations (N82S, H126Q) were stabilizing and the remaining nine mutations were found to be destabilizing based on ΔΔG values (**Table 3.4**). With these theoretical results in hand, single mutations for all the 11 residues were generated in the lab and studied in vivo.

Table 3.4 Theoretical prediction of change in protein stability of UBC4 after incorporating the substitution from UBC5 calculated using mCSM (mutation Cutoff Scanning Matrix).

MUTATION	PROTEIN STABILITY(ΔΔG)
E15G	-0.937 Kcal/mol (Destabilizing)
T20A	-0.186 Kcal/mol (*Destabilizing*)
A42S	-0.333 Kcal/mol (*Destabilizing*)
I68V	-1.463 Kcal/mol (*Destabilizing*)
S69N	-0.782 Kcal/mol (*Destabilizing*)
A81S	-0.281 Kcal/mol (*Destabilizing*)
N82S	0.076 Kcal/mol (*Stabilizing*)
H126Q	0.518 Kcal/mol (*Stabilizing*)
R132K	-0.856 Kcal/mol (*Destabilizing*)
P133A	-0.872 Kcal/mol (*Destabilizing*)
R140K	-1.33 Kcal/mol (*Destabilizing*)

3.3.3. Construction of UBC4 variants

For generating the variants of UBC4, as listed in table 3.2 (materials and methods section 3.2.4) a combination of mutagenic and non-mutagenic primers were used against pQE9/UBC4 WT plasmid which gave us the desired amplicons for the respective mutation. Following the compatible end strategy, the amplicons and backbone YEp96 were simultaneously double-digested with restriction enzymes, ligated and transformed. The transformants were screened by PCR (**Fig**.**3.4 (a-c)**) and confirmed by DNA sequencing as shown in **Fig 3.5 (a-l).**

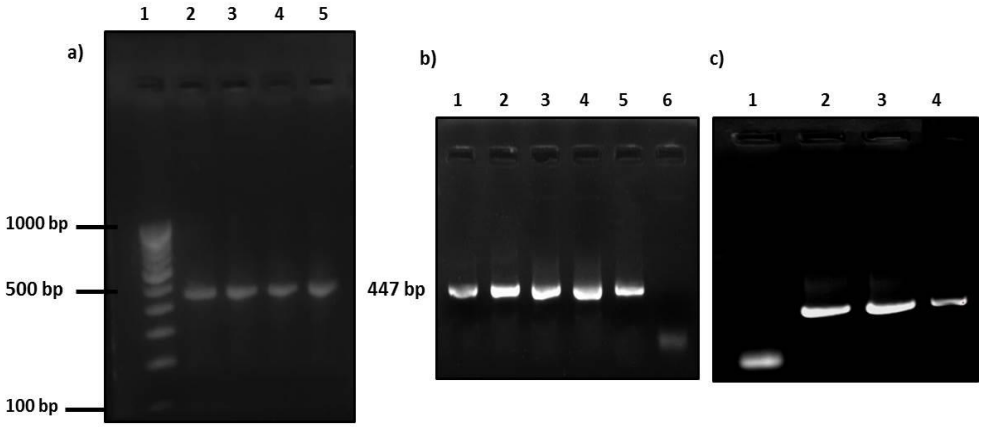

Fig 3.4. Agarose gel showing screening for variants of UBC4. The gel shows 447 bp amplicon of variants of UBC4**. a)** Lane 1 shows 100 bp DNA marker, lane 2 shows E15G, lane 3 shows T20A, lane 4 shows A42S and lane 5 shows I68V. **b)** Lane 1 shows S69N, lane 2 shows A81S, lane 3 shows N82S, lane 4 shows H126Q, lane 5 shows R132K and lane 6 shows negative control. **c)** Lane 1 shows negative control, lane 2 shows P133A, lane 3 shows R140K and lane 4 shows UBC4 WT.

```
>1st_BASE_2195454_wt_ubc_4_fr
AGCATCCAAAAAAACATGTCTTCTTCTAAACGTATTGCTAAAGAACTAAGTGATCTAGAAAGGTATGTCTAAAGTTATGGCCACGTTTCAAATGCGTG
CTTTTTTTTTAAAACTTATGCTCTTATTTACTAACAAAATCAACATGCTATTGAACTAAGATCCACCTACTTCATGTTCAGCCGGTCCAGTCGGCGAT
GATCTATATCACTGGCAAGCATCCATCATGGGACCTGCCGATTCCCCATATGCCGGCGGTGTTTTCTTCTTGTCTATCCATTTCCCAACCGACTACCC
ATTCAAGCCACCAAAGATCTCCTTCACAACCAAGATATATCATCCAAATATCAATGCCAATGGTAACATCTGTCTGGACATCCTAAAGGATCAATGGT
CTCCAGCTCTAACTCTATCGAAGGTCCTATTATCCATCTGTTCTTTGTTAACAGACGCTAATCCTGACGATCCTTTAGTACCAGAAATCGCTCATATC
TACAAGACTGACAGACCCAAGTACGAAGCTACAGCCAGAGAATGGACAAAGAAATACGCTGTATAAACAGA
```

Fig. 3.5 (a) DNA sequencing showing confirmation of UBC4 WT in YEp96. The red highlighted sequences indicate start and stop codons respectively.

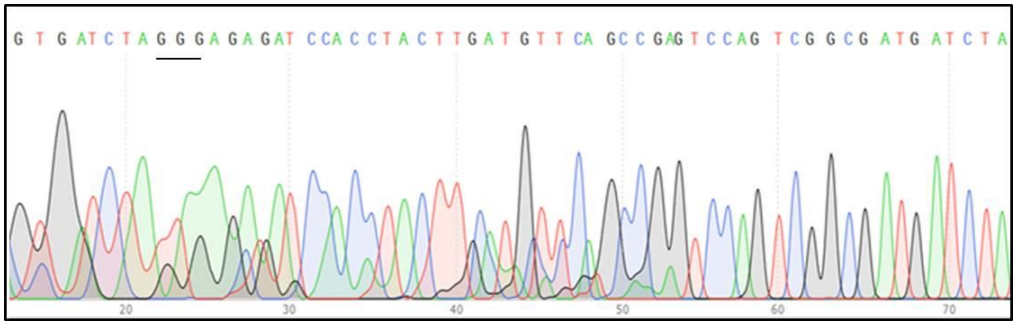

Fig. 3.5 (b) DNA sequencing showing confirmation of E15G substitution in UBC4 in YEp96 plasmid. The underlined sequence indicates substitution from GAA (Glu) to GGG (Gly).

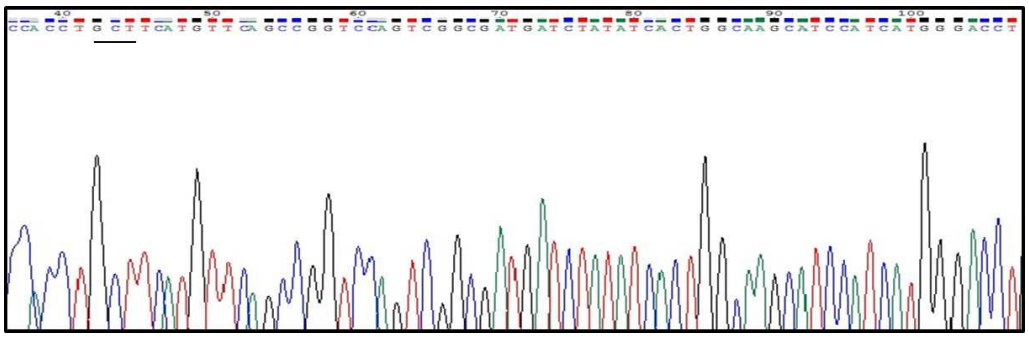

Fig. 3.5 (c) DNA sequencing showing confirmation of T20A substitution in UBC4 in YEp96 plasmid. The underlined sequence indicates substitution from ACT (Thr) to GCT (Ala).

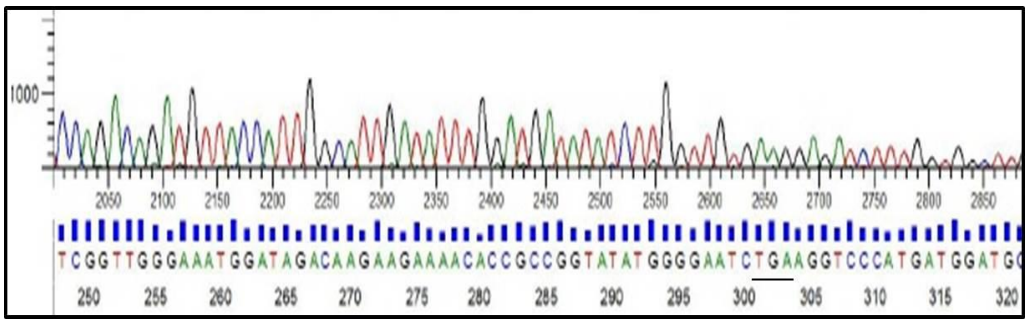

Fig. 3.5 (d) DNA sequencing showing confirmation of A42S substitution in UBC4 in YEp96 plasmid. The underlined sequence indicates substitution from GCC (Ala) to TCA (Ser). This sequencing was done using non-UBC4 reverse primer.

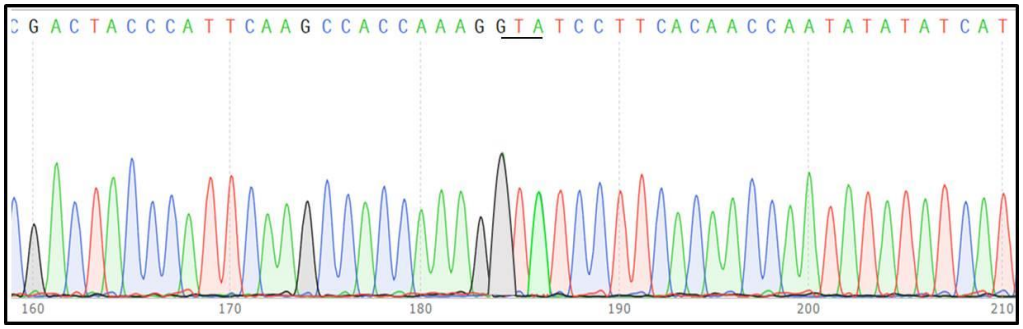

Fig. 3.5 (e) DNA sequencing showing confirmation of I68V substitution in UBC4 in YEp96 plasmid. The underlined sequence indicates substitution from ATC (Ile) to GTA (Val).

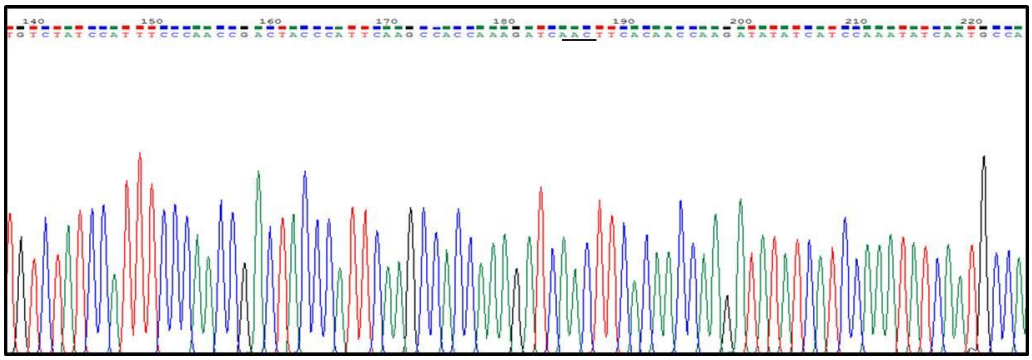

Fig. 3.5 (f) DNA sequencing showing confirmation of S69N substitution in UBC4 in YEp96 plasmid. The underlined sequence indicates substitution from TCC (Ser) to AAC

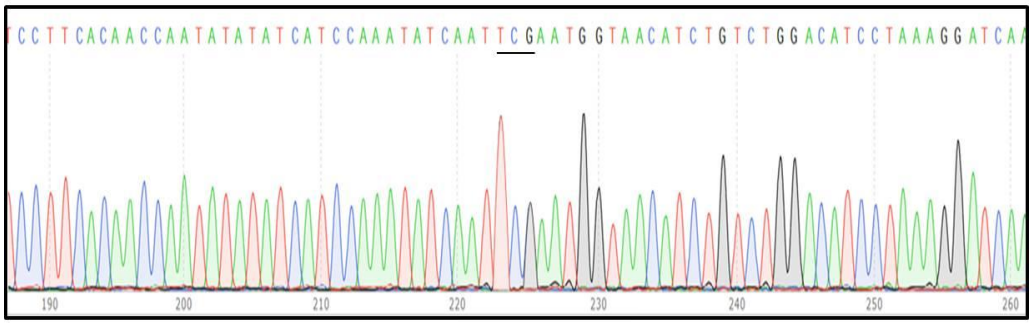

Fig. 3.5 (g) DNA sequencing showing confirmation of A81S substitution in UBC4 in YEp96 plasmid. The underlined sequence indicates substitution from GCC (Ala) to TCG (Ser).

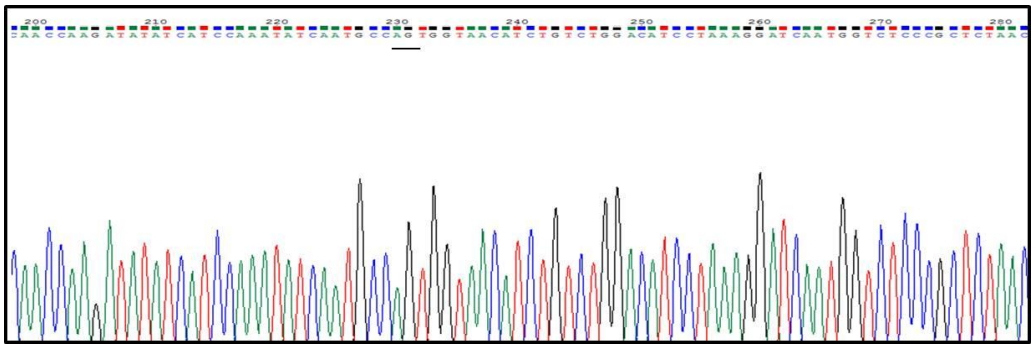

Fig. 3.5 (h) DNA sequencing showing confirmation of N82S substitution in UBC4 in YEp96 plasmid. The underlined sequence indicates substitution from AAT (Ceccarelli et al.) to AGT (Ser).

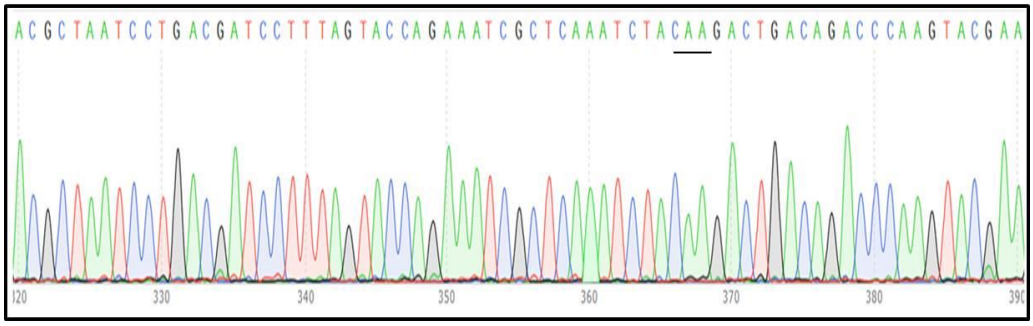

Fig. 3.5 (i) DNA sequencing showing confirmation of H126Q substitution in UBC4 in YEp96 plasmid. The underlined sequence indicates substitution from CAT (His) to CAA (Gln).

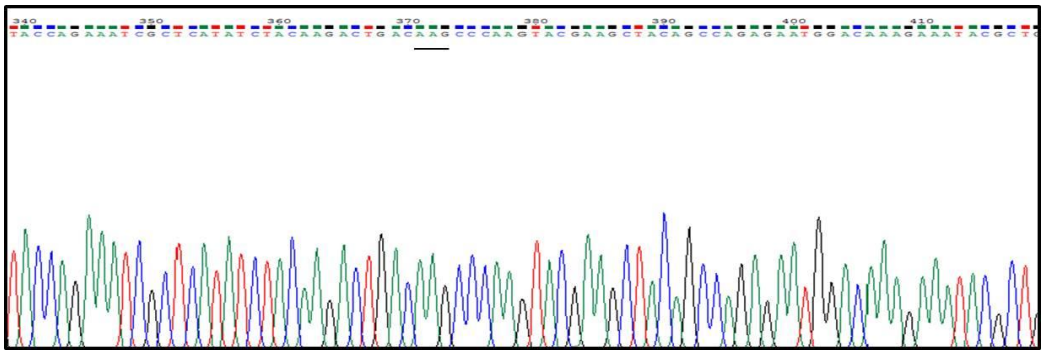

Fig. 3.5 (j) DNA sequencing showing confirmation of R132K substitution in UBC4 in YEp96 plasmid. The underlined sequence indicates substitution from AGA (Arg) to AAG (Lys).

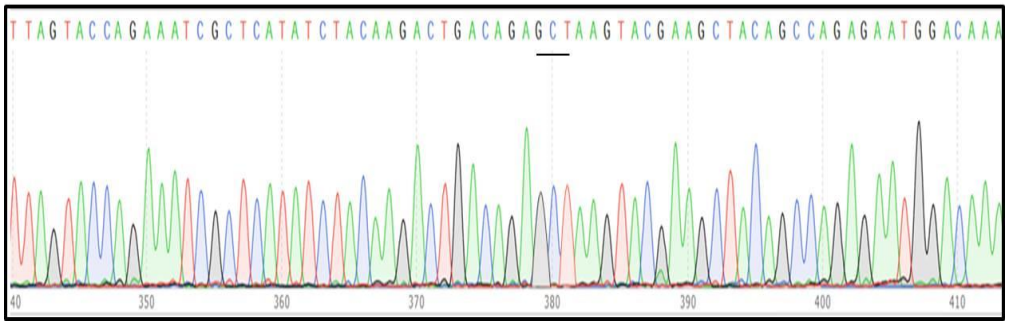

Fig. 3.5 (k) DNA sequencing showing confirmation of P133A substitution in UBC4 in YEp96 plasmid. The underlined sequence indicates substitution from CCC (Pro) to GCT (Ala).

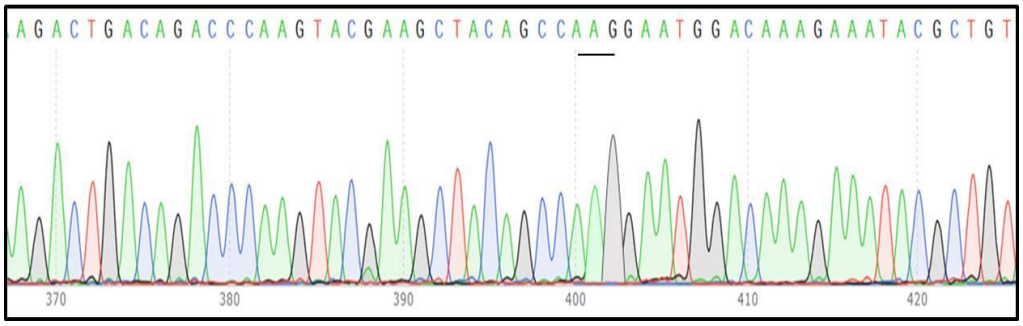

Fig. 3.5 (l) DNA sequencing showing confirmation of R140K substitution in UBC4 in YEp96 plasmid. The underlined sequence indicates substitution from AGA (Arg) to AAG (Lys).

3.3.4 Analysis of expression levels and stabilities of UBC4 WT and its variants at various time points in the time period of 24 h

The transformant cultures expressing UBC4WT and its variants were analysed for determining whether there is any difference in the protein levels either due to difference in their expression levels or stabilities at different time intervals (4, 8, 12, 16 and 24 h). Culture expressing UBC4WT was used as positive control, while uninduced cultures of respective variants were used as negative controls. The intensity of bands corresponding to UBC4 and its variants were compared. The band intensities showed no significant differences indicating that the protein levels are unaffected by the mutations introduced in them (**Fig. 3.6**).

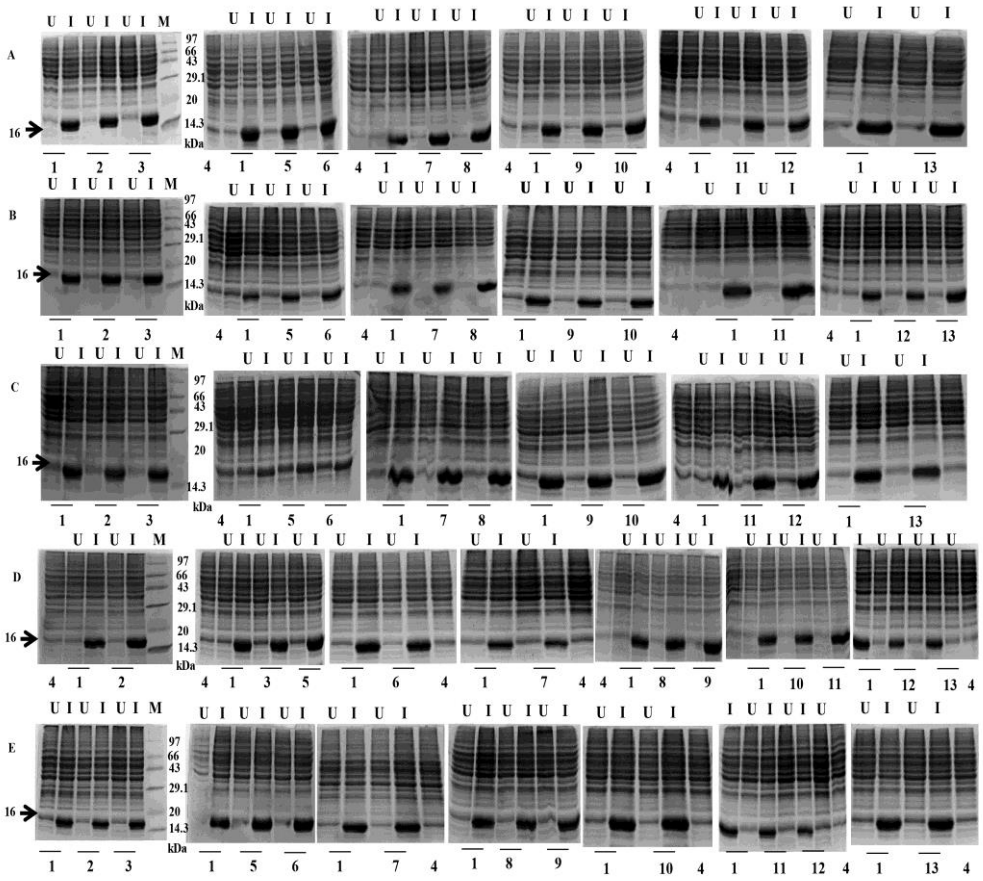

Fig. 3.6 Analysis of expression levels and stabilities of variants of UBC4 at various time points (a, b, c, d, e represent 4, 8, 12, 16, 24 h, respectively). MHY508 and UBC4 WT were used as controls. 100 μM copper sulphate was used as an inducer. U indicates Uninduced, I indicates induced samples and M indicates protein marker. The sequences of samples are as follows: (1) UBC4WT, (2) E15G, (3) T20A, (4) MHY508, (5) A42S, (6) I68V, (7) S69N, (8) A81S, (9) N82S, (10) H126Q, (11) R132K, (12) P133A and (13) R140K.

3.3.5. Effect of expression of UBC4 variants on the growth of *S. cerevisiae*

Since, UBC4 and UBC5 are conserved and maintained as two separate enzymes in S. cerevisiae, the 11 residues which are different must serve some structural and/or functional role. Substitution with the residues of UBC5 in these 11 positions in the sequence of UBC4 may have effect on its function by interfering with its binding to either E1 or E3 enzymes and arresting the degradation of substrate test this hypothesis, the UBC4 gene with mutations

corresponding to single residue substitutions from UBC5 was cloned under CUP1 promoter generating a series of plasmids (**Table 3.1**). The plasmids carrying wild-type UBC4 gene and its variants were introduced into MHY508 and the growth of transformants was monitored. S. cerevisiae strains MHY501, MHY508 transformed by UBC4 WT and MHY508 were used as positive and negative controls in the study. The cultures of all the mutants showed growth. Thus, UBC4 variants are not lethal to S. cerevisiae and are functional (**Fig. 3.7 and Table 3.5**). However, significant difference in the growth of the transformants of MHY508 expressing the mutations was observed when compared with transformants of UBC4 WT. The cells carrying H126Q and P133A grew at a rate much slower than all other mutations.

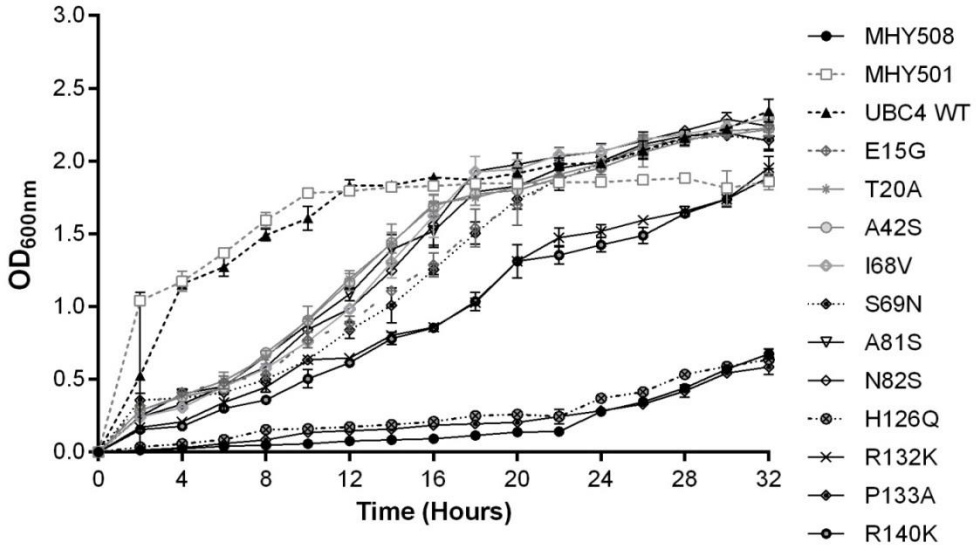

Fig. 3.7 Growth curves of UBC4 mutants of MHY508 cells of *S. cerevisiae* transformed by plasmids expressing variants of UBC4 gene namely, WT, E15G, T20A, A42S, I68V, S69N, A81S, N82S, H126Q, R132K, P133A, and R140K expressing UBC4-WT, UBC4-E15G,UBC4-T20A, UBC4-A42S, UBC4-I68V, UBC4-S69N, UBC4-A81S,UBC4-N82S, UBC4-H126Q, UBC4-R132K, UBC4-P133A and UBC4-R140K, respectively. MHY501, which is a wild-type strain and MHY508, which is a double deletion strain for UBC4 and UBC5 were used as positive and negative controls, respectively. The experiment was carried out three times independently. This is a representative graph from a single experiment, which was performed with two replicates.

Table 3.5 Determination of doubling time of UBC4 variants. Cultures were grown in YPD liquid medium at 30 °C. OD600 was monitored at 2 h intervals to calculate the doubling time.

Strain/ UBC4 variant	Doubling time (hrs.)	Strain/ UBC4 variant	Doubling time (hrs.)
MHY508	3.4	S69N	2.4
MHY501	1.1	A81S	2.4
UBC4 WT	1.1	N82S	2.4
E15G	2.4	H126Q	3.4
T20A	2.4	R132K	2.4
A42S	2.4	P133A	3.4
I68V	2.4	R140K	2.4

3.3.6 Complementation of MHY508 Cells of *S. cerevisiae* by UBC4 variants under heat stress

Protein denaturation increases under heat stress, leading to elevated demand on ubiquitin proteasome system for denatured protein degradation. Since UBC4 plays a crucial role in the removal of denatured proteins, the mutations of UBC4 were tested by submitting the transformants to heat stress. The transformant cells were plated on SDA medium containing 100μM inducer and subjected to heat stress at 37 °C for varying periods from 0 to 24 h, with 4 h intervals. The plates were shifted to 30 °C after heat stress. MHY501, transformants of MHY508 with UBC4 WT and MHY508, were used as positive and negative controls. Colonies were counted on plates after 24 h of growth. The transformants of S69N showed near 100% survival like

the two positive controls, namely MHY501 and MHY508 transformant with UBC4 WT wild type. The transformants of I68V and A81S showed 50% survival, while E15G, T20A, A42S and N82S showed 35% survival. Transformants of H126Q and P133A displayed 20% survival. In contrast, the transformants R132K and R140K behaved similar to the negative control MHY508 with 10% survival (**Fig. 3.8 and Table 3.6**).

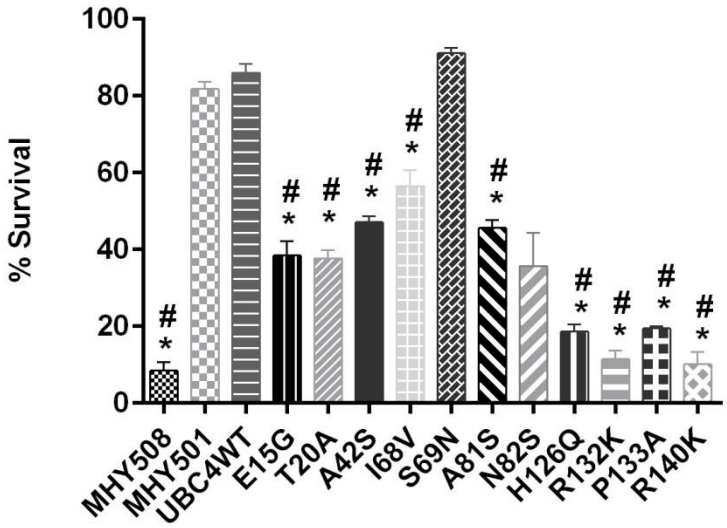

Fig. 3.8. Functional complementation under heat stress at 24 h. by the variants of UBC4, UBC4-E15G, UBC4-T20A, UBC4-A42S, UBC4- I68V, UBC4-S69N, UBC4-A81S, UBC4-N82S, UBC4-H126Q, UBC4-R132K, UBC4-P133A and UBC4-R140K in MHY508, the double deletion strain of UBC4 and UBC5 of S. cerevisiae. MHY508 cells were transformed by plasmids UBC4 WT, E15G, T20A, A42S, I68V, S69N, A81S, N82S, H126Q, R132K, P133A and R140K. MHY501 and MHY508 were used as positive and negative controls, respectively. In comparison to MHY501 and MHY508 all the variants of UBC4 except S69N and N82S showed significant reduction in % survival. Statistical significance was calculated and represented as *$p < 0.001$ and #$p < 0.001$ represents comparison of UBC4WT and MHY501 with all other strains, respectively. This experiment was independently performed three times in triplicates.

Table 3.6. Survival of *S. cerevisiae* MHY508 cells, expressing UBC4 variants from UBC4 WT series of plasmids exposed to heat stress (37°C) for various time intervals. MHY501 and MHY508 were used as controls. Statistical significance was determined by two-way ANOVA using Tukey's multiple comparisons test.*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001(n=3 independent experiment).

Strain/ UBC4 variant	% Survival after 0 hr of heat stress at 37°C	% Survival after 4 hr of heat stress at 37°C	% Survival after 8 hr of heat stress at 37°C	% Survival after 12 hr of heat stress at 37°C	% Survival after 16 hr of heat stress at 37°C	% Survival after 24 hr of heat stress at 37°C
MHY508	100	76.1****	55****	40.3****	26****	8.4****
MHY501	100	97	91.4	92	85.1	82
UBC4 WT	100	99	94.4	93	92	86
E15G	100	68.1 ****	56****	46****	47.3****	38.4****
T20A	100	80.2***	75****	58****	41.1****	38****
A42S	100	53****	53****	53****	50****	47.1****
I68V	100	82**	79**	71.3****	64.4****	57****
S69N	100	100	100	91.7	95	91.1
A81S	100	50****	50****	48****	47****	46****
N82S	100	81.3****	70****	63****	49.1	36
H126Q	100	46.1****	41.4****	33.3****	25****	19****
R132K	100	53****	39****	31****	17****	11.4****
P133A	100	50****	43****	34.4****	27****	19.4****
R140K	100	50****	33.2****	24.1****	17****	10.1****

3.3.7. Complementation by UBC4 variants under protein translation inhibitors

Translational inhibitors lead to premature termination of translation and produce truncated peptides. To determine if any of these transformants expressing mutant forms of UBC4 have negative effect over protein degradation, we spotted them on culture plates containing translational inhibitors (cycloheximide, L-canavanine and hygromycin- B), along with the controls. Our results revealed, out of 11 variants, H126Q, R132K, P133A and R140K could not support the MHY508 cells in the presence of translational inhibitors, suggesting that under antibiotic stress, these variants cannot

complement the cells as they may be functionally less efficient than the wild-type protein (**Fig. 3.9**).

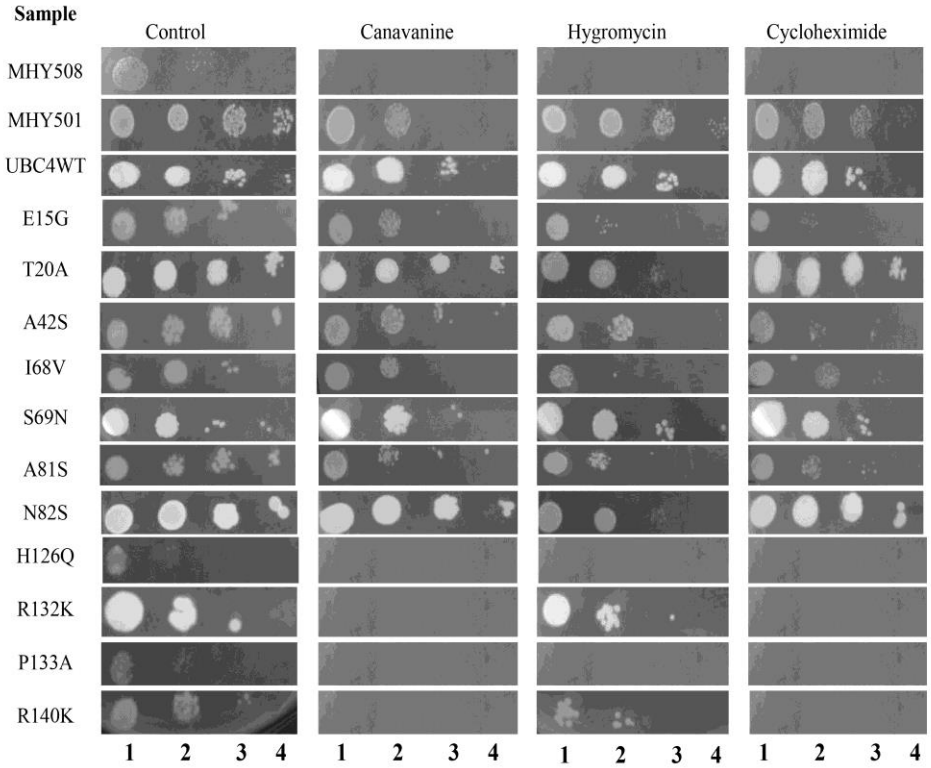

Fig. 3.9 Complementation by UBC4 variants under protein translation inhibitors in MHY508 strain of *S. cerevisiae*. The strain MHY501 and MHY508 were used as positive and negative controls respectively. MHY508 cells transformed by plasmids UBC4 WT, E15G, T20A, A42S, I68V, S69N, A81S, N82S, H126Q, R132K, P133A and R140K expressing UBC4WT, UBC4E15G, UBC4T20A, UBC4A42S, UBC4I68V, UBC4S69N, UBC4A81S, UBC4N82S, UBC4H126Q, UBC4R132K, UBC4P133A and UBC4R140K respectively were tested for L-canavanine, hygromycin-B and cycloheximide resistance. Undiluted stock and three fold dilutions (2), (3) and (4) were spotted on SDA plates in two sets. First set was control plates which contained no antibiotic and second set of plates contained antibiotics. 100µM copper sulphate was used as an inducer.

3.4. Discussion

E2s play central role in the ubiquitin proteasome pathway [12]. In *S. cerevisiae* the E2s, UBC4 and UBC5 bear 92% sequence identity, differing only in 11 positions in the entire amino acid sequence. They have been reported to serve redundant functions. The conserved presence of these two enzymes indicates some specialized roles assigned to them by nature under specific conditions, which have not been identified so far.

In humans two E2 enzymes, UbcH6 (UBE2E1) and UbcH8 (UBE2E2) are highly homologous. Though, N-terminals of the two enzymes have low identity, their E3 binding UBC domains are almost identical with only three residues being different. The three positions in UbcH6 are D58, T103 and E105, while in UbcH8 they are E66, S111 and D113. However, they show remarkably different E3 interaction patterns. UbcH6 interacts with ~24 RING E3s, while UbcH8 binds to only two RING E3s (van Wijk et al., 2012). Previously, it was reported that E3 interaction pattern of UbcH8 could be made to resemble that of UbcH6 by introducing any one of the two single mutations E66D and D113E (van Wijk et al., 2012). This observation raises a question regarding the importance of individual residue differences in UBC4 and UBC5 with respect to their structure, stability and function. In order to answer these questions we decided to generate and characterize single residue variants of UBC4 by swapping with residues from UBC5.

In this study we investigated the functional role of UBC4 by creating eleven variants. The variants of UBC4 with E15G, T20A, A42S, I68V, S69N, A81S, N82S, H126Q, R132K, P133A and R140K were engineered by substituting single residues of UBC4 with the respective residues from UBC5.

This study reveals that the transformants, which received H126Q and P133A grow at a slower rate in comparison to UBC4 WT. Moreover, the

plasmids with H126Q, R132K, P133A and R140K were the only variants which conferred sensitive phenotype towards all three translational inhibitors. In contrast, in P133A, proline in the 133 position with its unique structure cannot be substituted with any other amino acid. These findings were further supported by our observations on survival under heat stress. The only substitution that showed a survival similar to MHY501 wild type was S69N, where in serine (Ser) was replaced by aspargine. Both these amino acids have similar propensities for adopting β-sheet structure (Chou and Fasman, 1974) and show similar hydrophilicity profiles (Hopp and Woods, 1981). In summary, the other enzymes of ubiquitination cascade show only mild changes in their affinity for the variant E2 and wild-type and hence polyubiquitination was not severely affected.

The mutations R132K and R140K involve replacement of arginine (Arg) by lysine (Lys), which make the cells resemble the double deletion mutant of UBC4 and UBC5, suggesting that the two mutations lead to drastic decrease in functionality of the E2 enzyme. Overexpressing of the protein was not sufficient to rescue the phenotype. These observations show that minimal differences in side chain characteristics can have huge impact on the E3 binding profiles of strictly conserved E2 enzymes. This reinforces the fact that a point mutation from arginine to lysine is not always neutral. In certain structural or functional contexts, such a mutation can be devastating to function (Betts and Russell). The results of the present study with ubiquitin-conjugating enzyme UBC4 are in line with the studies on UbcH6 and UbcH8 and shows how single amino acid substitution can significantly impact the functionality of a protein (van Wijk et al., 2012).

The paralogs UBC4 and UBC5 arose from an ancestral gene during whole genome duplication. The proteins differ by 11 residues distributed over the entire sequence. Interestingly enough the DNA sequences maintain high degree of identity at the level of 46.73%. Analysis of secondary structure preferences of single residue replacements in UBC4 showed the incoming residues selected from UBC5 are preferred over the residues present in the

wild-type UBC4 (Chou and Fasman, 1974; Hutchinson and Thornton, 1994). However, results generated with mutation cut off scanning matrix (mCSM), meant to predict the effect of mutations on protein, suggest that the residues present in UBC4 confer more stability on protein and can have more interacting partners. Unlike the first method which takes single residues for evaluation and is purely statistical, the mCSM method considers interatomic distances for its evaluation. Hence, the results are more reliable and explain the experimental results better.

In conclusion, UBC4 and UBC5 are the products of a gene duplication event and have some common interacting partners (which are mostly E3 enzymes) to serve the important purpose of degrading misfolded proteins, even when one of them is absent. Gene duplication and subsequent diversification in sequence of the two proteins led to partial separation of their interactomes, equipping the cell with higher degree of regulation. UBC4 being an important E2 during exponential growth interacts with far larger number of proteins, while UBC5 interacts with a smaller set as it is the enzyme expressed during stationary phase and under stress conditions. The two proteins diverged in their sequence are conserved and maintained by the yeast cell to act selectively and exclusively on two different sets of proteins.