



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

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## Abstract

Ubiquitination is tightly regulated to control degradation, localization and function of various proteins. Ubiquitination is catalysed by three enzymes, namely E1, E2 and E3. The specificity shown by E2s for E3s holds key to regulation of ubiquitination. Here we focussed on the E2 enzymes, UBC4 and UBC5 of *Saccharomyces cerevisiae*, which are almost identical differing only by 11 residues. They show functional complementation in protein degradation, especially during stress response. Existence of two almost identical proteins suggests specialized requirement of one of them under selective conditions. To understand the reasons for the residue differences between them, mutations were introduced in the *UBC4* gene to generate single residue variants by swapping with codons from *UBC5*. Though the variants are found to be functionally active in  $\Delta ubc4\Delta ubc5$  strain of yeast, they cause reduced growth under normal conditions, altered survival under heat and antibiotic stresses, when compared with UBC4. The variants indicated decrease in protein stability theoretically. Hence, the residues of UBC5 individually do not confer any structural advantage to UBC4. Interactive proteins of UBC4 are nearly three times more than those of UBC5. UBC5, therefore, is a functionally minimized version, evolved as another means of regulation to meet cell stage specific needs.

**Keywords** UBC4 · UBC5 · Heat stress · Antibiotic stress · Growth ·  $\Delta\Delta G$

## Introduction

Functional diversity of the proteome is increased by post-translational modifications (PTMs) such as ubiquitination, influencing both normal cell biology and pathogenesis. The post-translational modification of ubiquitination serves three main purposes in eukaryotic cells. First, it regulates the function of several proteins by modulating their activities [1–5]. Second, ubiquitination serves as a tag for transporting proteins to the site of their activity [6, 7]. Third and most important function is to catalyse the selective degradation of proteins either misfolded or those that have served their function, by ubiquitin proteasome system (UPS) [8, 9].

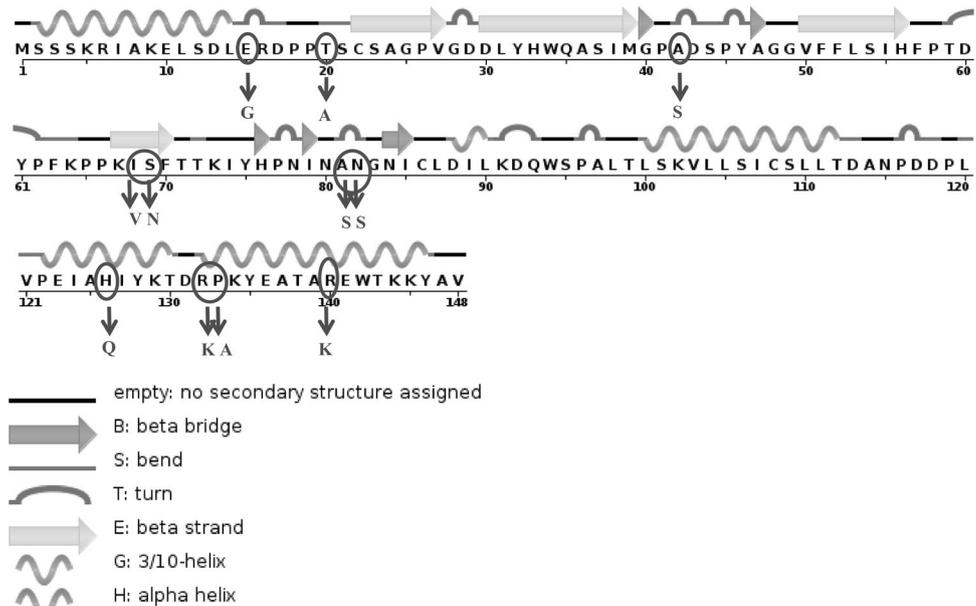
The ubiquitination system comprises of ubiquitin (Ub), a small protein modifier and a set of three enzymes, ubiquitin activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3) [10]. Ub is activated by E1 in an ATP dependent reaction and mobilized to E2 and from E2 activated ubiquitin is transferred to a substrate with the help of E3 [11]. E3s are responsible for selective presentation of substrate proteins for ubiquitination. However, E2s determine the topology and length of the ubiquitin chains to be conjugated to the substrate [12]. Targeting the UPS system with small molecules is seen as a potential strategy for treatment of diseases such as neurodegenerative disorders and cancer [13]. 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 [14]. Regulating cell cycle using a recently identified allosteric inhibitor against the E2 enzyme Cdc34 [15], 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 [13, 16].

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



There are thirteen E2 enzymes known in *S. cerevisiae* and around forty E2 enzymes present in humans [12]. All E2s have a conserved catalytic domain termed as, “ubiquitin-conjugating core domain” or UBC domain [17]. Depending on the presence and absence of N-terminal or C-terminal extensions to UBC domain they have been grouped into four classes as Class I, II, III and IV, respectively [17].

The E2 enzymes UBC4 and UBC5 of *S. cerevisiae*, possessing only the UBC domain belong to Class I ubiquitin-conjugating enzymes [17]. They were first isolated and characterized by Seufert and Jentsch [18]. UBC4 is an  $\alpha/\beta$  protein consisting of UBC domain, which is highly conserved with respect to its secondary structural makeup involving four helices and a  $\beta$ -sheet consisting of 4 strands [17]. It shares 92% sequence identity with UBC5 in the yeast system, differing by only 11 residues [18]. Interestingly, these residues are scattered along the sequence located in different secondary structural features of the protein (Fig. 1).

UBC4 is expressed in exponentially growing cells, while UBC5 is present in stationary phase [17]. UBC4 and UBC5 have functional overlap and 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 [19]. 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 [20]. 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 [21–27]. 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.

**Table 1** UBC4 variants constructed, codons originally present in *UBC4* and codons substituted from *UBC5* for required amino acid substitutions are given below

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
UBC4WT	UBC4 WT	–	–	–	–	–
UBC4-E15G	E15G	15	GAA	Glu	GGG	Gly
UBC4-T20A	T20A	20	ACT	Thr	GCT	Ala
UBC4-A42S	A42S	42	GCC	Ala	TCA	Ser
UBC4-I68V	I68V	68	ATC	Ile	GTA	Val
UBC4-S69N	S69N	69	TCC	Ser	AAC	Asn
UBC4-A81S	A81S	81	GCC	Ala	TCG	Ser
UBC4-N82S	N82S	82	AAT	Asn	AGT	Ser
UBC4-H126Q	H126Q	126	CAT	His	CAA	Gln
UBC4-R132K	R132K	132	AGA	Arg	AAG	Lys
UBC4-P133A	P133A	133	CCC	Pro	GCT	Ala
UBC4-R140K	R140K	140	AGA	Arg	AAG	Lys

## Materials and Methods

### In Silico Analysis of the Binding Partners of UBC4 and UBC5

The information available in open source database BioGRID<sup>3,5</sup> (<https://thebiogrid.org>) on binding partners of UBC4 and UBC5, has been analysed to identify interacting proteins specific to either of them and common to both [28, 29].

### 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 ( $\Delta\Delta G$ ) [30].

### Strains Used for In Vivo Studies

The *S. cerevisiae* strains used in the study MHY501 (MAT $\alpha$  his3- $\Delta$ 200 leu2-3,112 ura3-52 lys2-801 trp1-1) and MHY508 (MAT $\alpha$  his3- $\Delta$ 200 leu2-3,112 ura3-52 lys2-801 trp1-1 *ubc4- $\Delta$ 1::HIS3 ubc5- $\Delta$ 1::LEU2*) [31] were provided generously by Prof. Mark Hochstrasser (Yale University, New Haven, CT). Wild-type cells (MHY501) and the mutant MHY508 with  $\Delta$ *ubc4* $\Delta$ *ubc5* deletions are haploid MAT $\alpha$  strains congenic to DF5 [32]. Yeast episomal plasmid YEp96 with *TRP1* as the selection marker, is a shuttle vector between *S. cerevisiae* and *E. coli* [33]. 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 1 gives details of mutated proteins. The *UBC4* gene and its derivatives were expressed from the CuSO<sub>4</sub> inducible *CUP1* promoter, by using 100  $\mu$ M copper sulfate.

*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 (20 mg L<sup>-1</sup>), lysine (30 mg L<sup>-1</sup>), uracil (20 mg L<sup>-1</sup>), leucine (100 mg L<sup>-1</sup>) or tryptophan (20 mg L<sup>-1</sup>) were added for selection, depending on the experimental requirement [33]. The cultures were grown at 30 °C and 200 rpm. Cells were transformed by lithium acetate method [34]. 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.

### 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 2). YEp96 was double digested by *Bgl*III and *Kpn*II restriction enzymes to remove ubiquitin gene. Amplicons of

**Table 2** List of primers used for the construction of variants of *UBC4* gene

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' GGACCTTCAGATTCCCATAT 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' CCAAAGATCAACTCACA 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' GTAAGTGGGCTTGTCAGT 3'
P133A	FR: 5' AAGACTGACAGAGCTAAGTACGAAGC 3' RE: 5' GCTTCGTAAGTCTAGCTCTGTCAGTCTT 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'

The highlighted sequence in FR (forward primer) of UBC4 WT represents restriction site for *Bam*HI and in RE (reverse primer) *Kpn*I

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 1). The plasmids were transformed into DH5 $\alpha$  strain of *E. coli*. The transformant cells were grown in LB medium supplemented with the antibiotic ampicillin (100  $\mu$ g/mL) to maintain the plasmids. The genes were placed under *CUP1* promoter. The respective plasmids were confirmed by sequencing.

### 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 sulfate, 100  $\mu$ M) at 30  $^{\circ}$ 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 added to Laemmli sample buffer and boiled for 15 min and were analysed on 12% SDS-PAGE. The gels were stained with Coomassie Blue G-250.

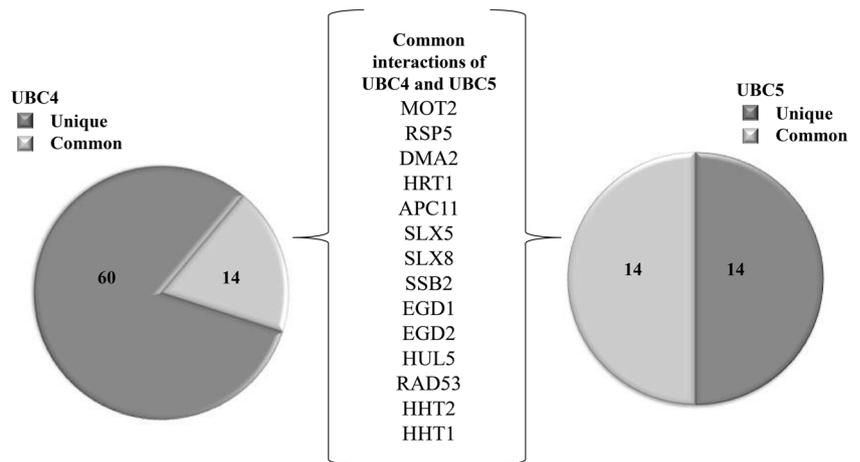
### Effect of Mutations on Growth of Cells

The vectors carrying the wild-type *UBC4* and variants of *UBC4* were transformed into yeast  $\Delta$ ubc4 $\Delta$ ubc5 strain. Later, the cultures were grown at 30  $^{\circ}$ C at 200 rpm, except where indicated in synthetic dextrose medium. One hundred micromolars of copper sulfate 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 600 nm every 2 h. 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  $\Delta$ ubc4 $\Delta$ ubc5 (MHY508), were used as another set of controls.

### Heat Stress Test

Heat sensitivity test was performed to confirm the functional integrity of the mutant ubiquitin-conjugating enzyme

**Fig. 2** 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



[18]. 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  $\mu$ M). 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.

### 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 threefold serially and all the dilutions were spotted on SDA plates with and without the antibiotics cycloheximide (0.001 M), L-canavanine (0.14 mM) and hygromycin-B (0.2 mM) in the presence of the inducer (100  $\mu$ M copper sulfate). The plates were incubated for 7–10 days at 30 °C to assess survival and growth.

## Results

### 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, BioGRID<sup>3,5</sup> [28, 29]. 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. Our results show that out of 74

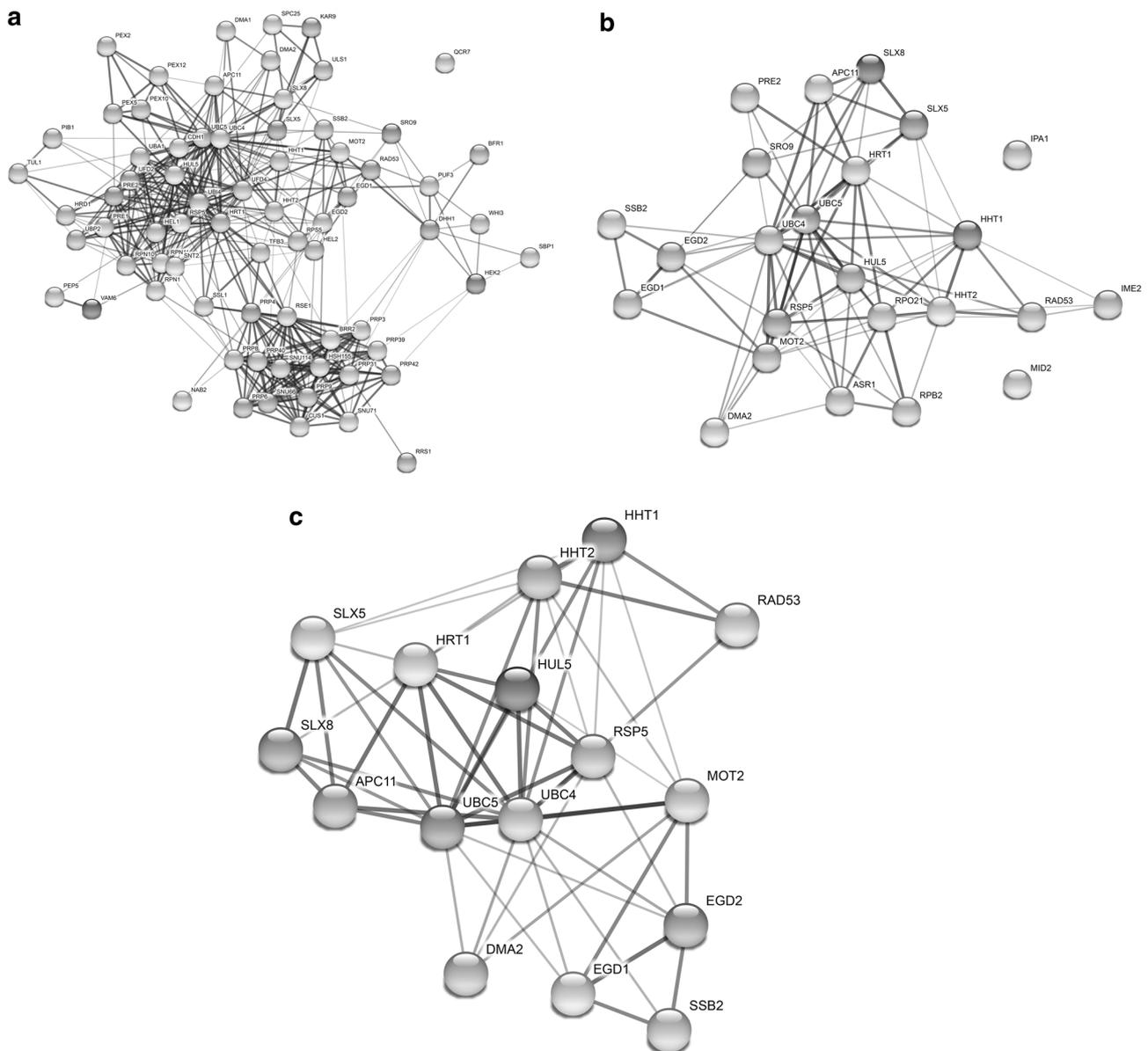
interactive partners of UBC4 and 28 of UBC5, only 14 are common (Results presented in Supplementary information in Table 6). The common interacting partners of UBC4 and UBC5 indicate the complementary roles the two ubiquitin-conjugating enzymes play with respect ubiquitin proteasome system, whereas individually both of them can participate in the regulation of various biological processes through their partners exclusive to either one (Fig. 2). Hence, both UBC4 and UBC5 also play crucial but distinct roles in regulating biological processes (Fig. 3a–c).

### 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  $\Delta\Delta G$ . 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  $\Delta\Delta G$  values (Table 3). With these theoretical results in hand, single mutations for all the 11 residues were generated in the lab and studied in vivo.

### 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



**Fig. 3** **a**, **b** and **c** represent interactors of UBC4, UBC5, UBC4 and UBC5, respectively. The thickness of the lines indicates the strength of interaction between the proteins. Interactions of UBC4 and UBC5

plotted on STRING [40] (data collection source: The Biological General Repository for Interaction Datasets (BioGRID)<sup>3,5</sup>) [28, 29]

proteins, which may affect the growth of the organism. To 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 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. 4 and Table 4). However, significant difference in the growth of the transformants of MHY508

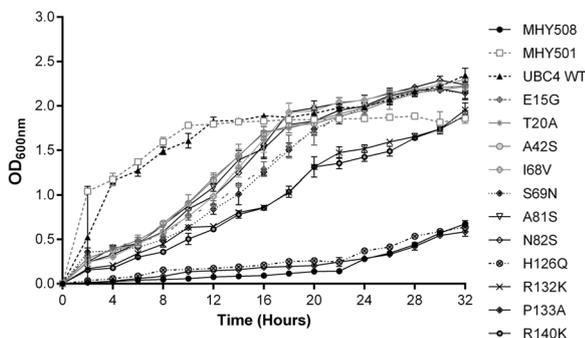
expressing the mutations was observed when compared with transformants of UBC4 WT. The cells carrying H126Q and P133A grew at a rate much slower than all other mutations.

### **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

**Table 3** Theoretical prediction of change in protein stability of UBC4 after incorporating the substitution from UBC5 calculated using mCISM (mutation Cutoff Scanning Matrix) [30]

Substitution in UBC4 protein	Protein stability ( $\Delta\Delta G$ )
E15G	-0.937 kcal/mol ( <i>Destabilizing</i> )
T20A	-0.186 kcal/mol ( <i>Destabilizing</i> )
A42S	-0.333 kcal/mol ( <i>Destabilizing</i> )
I68V	-1.463 kcal/mol ( <i>Destabilizing</i> )
S69N	-0.782 kcal/mol ( <i>Destabilizing</i> )
A81S	-0.281 kcal/mol ( <i>Destabilizing</i> )
N82S	0.076 kcal/mol ( <i>Stabilizing</i> )
H126Q	0.518 kcal/mol ( <i>Stabilizing</i> )
R132K	-0.856 kcal/mol ( <i>Destabilizing</i> )
P133A	-0.872 kcal/mol ( <i>Destabilizing</i> )
R140K	-1.33 kcal/mol ( <i>Destabilizing</i> )

**Fig. 4** 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 4** Determination of doubling time of UBC4 variants

Strain/UBC4 variant	Doubling time (h)	Strain/UBC4 variant	Doubling time (h)
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

Cultures were grown in YPD liquid medium at 30 °C. OD<sub>600</sub> was monitored at 2 h intervals to calculate the doubling time. The experiment was carried out independently three times

intervals (4, 8, 12, 16, 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. 5).

### Complementation of MHY508 Cells of *S. cerevisiae* by UBC4 Variants under Heat Stress

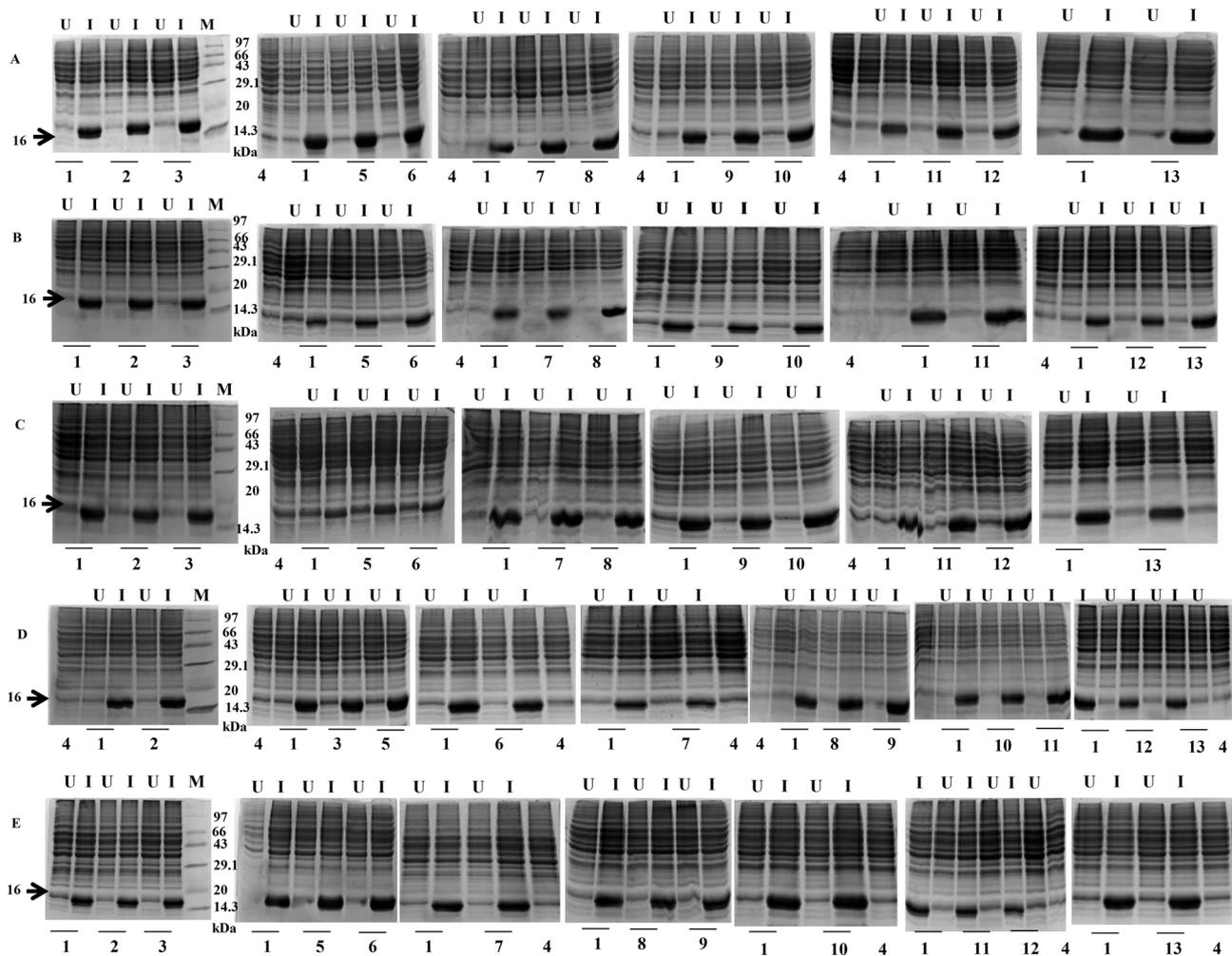
Protein denaturation increases under heat stress, leading to elevated demand on ubiquitin proteasome system for denatured protein degradation. Since UBC4 plays a crucial role in the removal of denatured proteins, the mutations of UBC4 were tested by submitting the transformants to heat stress. The transformant cells were plated on SDA medium containing 100  $\mu$ M 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. 6 and Table 5).

### 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. 7).

## Discussion

E2s play indispensable role in the ubiquitin proteasome pathway [12]. In *S. cerevisiae* the E2s, UBC4 and UBC5



**Fig. 5** 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  $\mu$ M copper sulfate 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, (13) R140K

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  $\sim$ 24 RING E3s, while UbcH8 binds to only two RING E3s [35]. 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 [35]. 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.

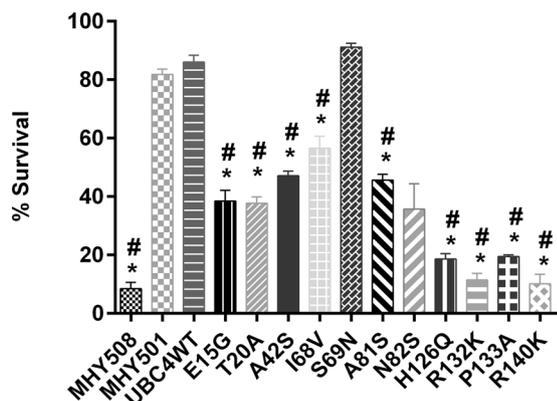
This study reveals that the transformants, which received H126Q and P133A grow at a slower rate in comparison to UBC4 WT. Moreover, the plasmids with H126Q, R132K, P133A and R140K were the only variants which conferred sensitive phenotype towards all three translational inhibitors. In contrast, in P133A, 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 asparagine (Asn). Both these amino acids have similar

propensities for adopting  $\beta$ -sheet structure [36] and show similar hydrophilicity profiles [37]. 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 [38]. 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 [35].

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 [36, 39]. However, results generated with mutation cut off scanning matrix (mCSM), meant to predict the effect

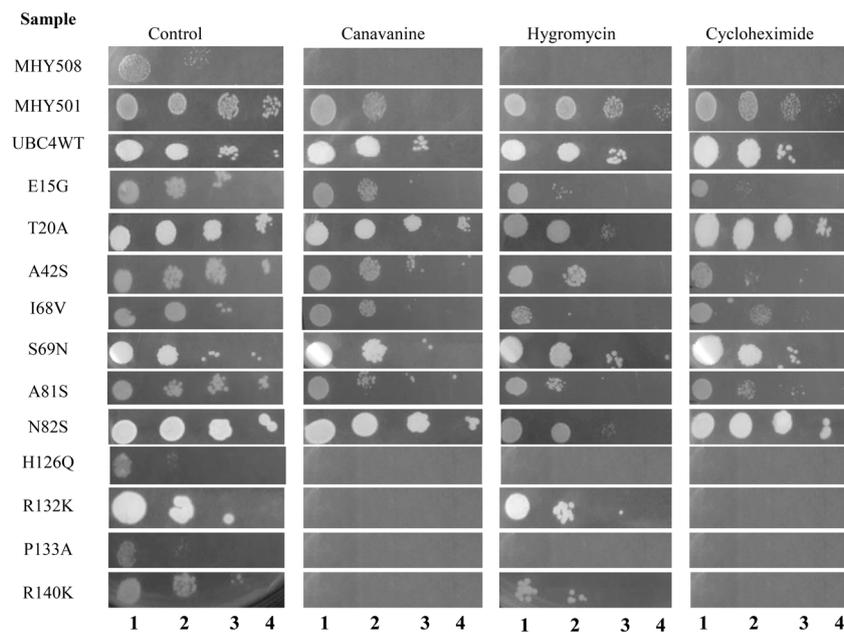


**Fig. 6** Functional complementation under heat stress at 24 h. by the variants of UBC4, UBC4-E15G, UBC4-T20A, UBC4-A42S, UBC4-I68V, UBC4-S69N, UBC4-A81S, UBC4-N82S, UBC4-H126Q, UBC4-R132K, UBC4-P133A and UBC4-R140K in MHY508, the double deletion strain of *UBC4* and *UBC5* of *S. cerevisiae*. MHY508 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 5** 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

Strain/UBC4 WT and its variants	% Survival after 0 h of heat stress at 37 °C	% Survival after 4 h of heat stress at 37 °C	% Survival after 8 h of heat stress at 37 °C	% Survival after 12 h of heat stress at 37 °C	% Survival after 16 h of heat stress at 37 °C	% Survival after 24 h 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****

MHY501 and MHY508 were used as controls. Statistical significance is indicated as \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  ( $n = 3$  independent experiments)



**Fig. 7** Complementation by UBC4 variants in the presence of protein translational 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 encoding the variants of UBC4 namely, UBC4WT, UBC4E15G, UBC4T20A, UBC4A42S, UBC4I68V, UBC4S69N, UBC4A81S, UBC4N82S, UBC4H126Q, UBC4R132K, UBC4P133A and UBC4R140K,

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.

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respectively, were tested for resistance to L-canavanine, hygromycin-B and cycloheximide. Undiluted stock and three fold serial 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. One hundred micromolars of copper sulfate was used as an inducer

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## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

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