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ORIGINAL PAPER

Structural and Functional Characterisation of the Domains of Ubiquitin-Activating Enzyme (E1) of *Saccharomyces cerevisiae*

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Abstract

Ubiquitin-activating enzyme (E1) is the first enzyme of the ubiquitination pathway and is required to activate ubiquitin. E1 of *Saccharomyces cerevisiae* is a large multidomain monomeric protein. There are no studies available on the domains of yeast E1 as independent entities. Four domains of E1 namely, first catalytic cysteine half-domain (FCCH), four-helix bundle (4HB), second catalytic cysteine half-domain (SCCH) and ubiquitin fold domain (UFD) were characterised to understand their structural and functional independence vis-a-vis full length E1. Spectroscopic characterisation using circular dichroism and fluorescence suggested that these domains can act as independent folding units and attain native-like secondary structure. The structural features obtained with the peptides SCCH and FCCH of *S. cerevisiae* bear a high degree of structural similarity to the corresponding fragments of mouse from literature. Nearly 50% of the residues of the 4HB domain of the *S. cerevisiae* sample showed helical conformation. They displayed a high degree of conservation when compared with 4HB of mouse with respect to their identity and arrangement. The fragment UFD of yeast formed an α/β domain as in the whole protein and exhibited 45% homology with that of mouse, showing a similar arrangement of α and β elements in its secondary structure. Overexpression of the domains in vivo indicated that the SCCH domain and to some extent UFD apparently interfere with cellular functions such as survival under various stresses.

Keywords Ubiquitin-activating enzyme · E1 · Yeast · Domain · Structure · Function

Introduction

Most of the proteins are subjected to post-translational modifications in order to either regulate their functions or to maintain their concentrations in a cell [1–3]. Ubiquitination is a major modification occurring in the eukaryotic cell wherein a small protein ubiquitin (Ub) is tagged to substrate proteins to regulate their activity or to earmark them for degradation through Ub-mediated pathways of degradation [4, 5].

A cascade of three enzymes namely, Ub-activating enzyme (E1), Ub-conjugating enzyme (E2) and Ub protein ligase (E3), catalyse ubiquitination in sequential reactions [6–8]. The yeast Ub system comprises of single E1 enzyme, while numerous E2s and E3s are instrumental in imparting substrate specificity to ubiquitination [9]. In the first step of ubiquitination pathway E1 activates Ub, by an ATP-dependent mechanism and subsequently transfers it to E2 enzymes [10]. Initially E1 forms Ub adenylate and forges a thioester linkage between its active site cysteine and C-terminus of Ub. Later, it transfers activated Ub present in the E1~Ub complex to the active site of E2 [11]. Subsequently, E2 transfers Ub either directly to the target protein bound on E3 or to E3 ligase, which then transfers the Ub to target protein [4]. Target proteins undergo either monoubiquitination or polyubiquitination [12, 13].

In *S. cerevisiae* E1 is encoded by *UBA1* gene, which translates into ~110 kDa (1024 amino acids) monomeric protein (Uba1/E1). Deletion of the *UBA1* gene is lethal to the organism [14]. E1 along with other enzymes plays a role in the regulation of cell cycle proteins like histone H2A and p53, which are essential for cell cycling [15, 16]. Therefore defect in *UBA1* gene results in detrimental effects on cell cycle progression. The crystal structure of yeast E1 with a Ub molecule bound to its adenylation site has been reported

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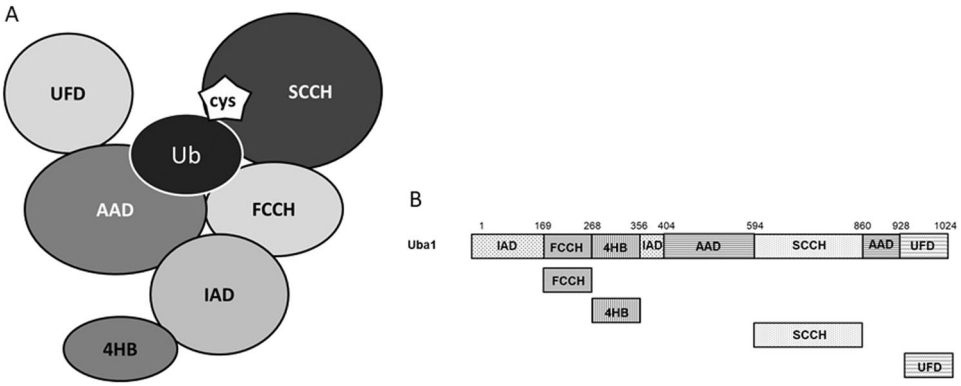

Fig. 1 **a** Diagrammatic representation of the domains of yeast ubiquitin-activating enzyme E1 along with ubiquitin; **b** linear representation of yeast ubiquitin-activating enzyme E1 and its domains namely, FCCH, 4HB, SCCH and UFD

earlier [17]. The structure of E1 is organised into six domains namely, inactive adenylation domain (IAD), first catalytic cysteine half-domain (FCCH), four-helix bundles (4HB), active adenylation domain (AAD), second catalytic cysteine half-domain (SCCH) and Ub fold domain (UFD) [17] (Fig. 1). E1 displays complex architecture due to the discontinuous and interspersed organisation of its six domains. IAD is present interspersed in the N-terminal half of E1, while AAD is present in the C-terminal half of the enzyme. ATP and Ub are bound noncovalently to AAD as the domain is catalytically active, and serves as the site for adenylation reaction [18, 19]. Adenylation domains of eukaryotic E1 enzyme are homologous to MoeB and ThiF proteins of bacteria [20]. MoeB and ThiF catalyse the C-terminal adenylation of MoaD and ThiS proteins, which are structural homologues of Ub [18, 21, 22].

The domains carrying the catalytic cysteine exist as two parts namely, FCCH and SCCH, which are found inserted into each of the adenylation domains [23]. SCCH domain consisting of catalytic cysteine forms a thioester bond with Ub. On the other hand FCCH, which associates with IAD is non-functional. 4HB domain present immediately after the FCCH, represents the second insertion in the IAD. UFD present in the C-terminus of E1 has a role in the recruitment of specific E2s to the ubiquitination pathway [24, 25].

Arrangement of the domains UFD, FCCH and SCCH is crucial for the overall tertiary structure of E1. They are connected to their respective adjacent domains by flexible linkers [17]. These structural features suggest that E1 might undergo large-scale conformational changes during the ubiquitination process. Moreover, the detailed catalytic mechanism of E1 is still not well understood.

It would be crucial to study the domains of E1 as independent units to understand if isolated domains can retain native structure and to what extent they enjoy functional independence. Hence, the main focus of this work is the characterisation of structural and functional aspects of the four domains of E1, i.e., FCCH, SCCH, 4HB and UFD as independent units. Structural characterisation of the four domains was carried out by circular dichroism (CD) and fluorescence spectroscopy. Growth and survival of *S. cerevisiae* transformants expressing the domains were monitored under normal conditions and under heat and antibiotic stresses in order to understand if the domains produce any negative effects on the functioning of cellular machinery by interfering with ubiquitination.

Materials and Methods

Yeast and Bacterial Strains and Culture Media

YEPD medium containing 1% yeast extract (Hi-media), 2% peptone (Hi-media), 2% glucose/dextrose (SRL) and Synthetic Dextrose (SD) medium 0.67% yeast nitrogen base without amino acids (Hi-media) and 2% glucose as carbon source (SRL) were used to grow yeast cultures. All amino acids and uracil were supplied by SRL. Histidine (20 mg L^{-1}), lysine (30 mg L^{-1}), leucine (100 mg L^{-1}) and uracil (20 mg L^{-1}) were added to SD medium and tryptophan (20 mg L^{-1}) was added, depending on the experimental requirements. *S. cerevisiae* strain used for the functional study was MHY501 (MATα his3-Δ200 leu2–3,112 ura3–52 lys2–801 trp1–1). *Escherichia coli* strains used for experiments were DH5α (F^- *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20* φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17($r_K^- m_K^+$), λ^-) and BL21 DE3 (F^- *ompT gal dcm lon hsdS*$_B$($r_B^- m_B^-$) λ(DE3 [*lacI lacUV5-T7p07 ind1 sam7 nin5*]) [*malB*$^+$]$_{K\text{-}12}$(λ^S)).

Engineering of the Constructs Encoding the Domains for Structural Characterisation

The *UBA1* gene (Swiss protein databank entry P22515) inserted in the bacterial expression vector pET28a, which has 6x His-tag on both N and C terminals [17]. DNA fragments encoding the domains FCCH, 4HB, SCCH and UFD, corresponding to residues 175–265, residues 269–356, residues 594–860 and residues 1121–1220 of E1

respectively, were amplified from the *UBA1* gene, using PCR technique employing PR polymerase [26]. Primers were designed to amplify DNA fragments of peptides individually (Table 1). To use only N-terminal His-tag stop codon was added at the 3′ end of all the fragments. The fragments were cloned in pET28a vector between *Nhe*I and *Xho*I sites. All four clones were sequenced by outsourcing to 1st BASE. Sequencing was carried out using the BigDye Terminator v3.1 Terminator sequencing kit. The sequences were confirmed by comparing the sequencing file with the sequence of the domains obtained from Swiss protein databank entry P22515.

Expression and Purification of Peptides Corresponding to the Domains

The plasmids were transformed into *E. coli* BL21 (DE3) cells and the cultures were grown at 37 °C to an A_{600} of 0.6–1.0. 1 mM isopropyl β-D-thiogalactopyranoside was added to the cultures and incubated overnight. Cell lysates were added to Ni^{2+}-NTA agarose (Qiagen) and subjected to native affinity chromatography for the purification of FCCH, 4HB and SCCH. UFD was purified by affinity chromatography under denaturing conditions to avoid aggregate formation. The buffer used for UFD purification contained 6 M Urea. All four peptides were dialysed against 20 mM Tris-Cl (pH 8.0) and 100 mM NaCl buffer. Finally, the samples were concentrated by ultrafiltration.

CD and Fluorescence Spectroscopy

Peptide solutions were prepared in 20 mM Tris-Cl (pH 8.0) and 100 mM NaCl buffer and the concentration of peptides used in all the experiments was 23.4 μM.

Jasco J-715 CD spectrophotometer was used to record the Far-UV CD spectra of all four peptides. The range for Far-UV-CD spectra was 200–250 nm. The path length was 1 mm. All the spectra were blank corrected. The spectra were accumulated for five times to improve the signal-to-noise ratio. Scan speed was 50 nm/s. The spectra were then analysed by BeStSel software [27].

Separately, the secondary structure content of the peptides was predicted by PSIPRED protein secondary structure prediction tool [28]. PSIPRED predicts the structure based on PSI-BLAST and neural network machine learning algorithm [29].

Fluorescence spectra were recorded using Hitachi FL-7000 fluorescence spectrophotometer. For intrinsic fluorescence spectroscopy, the buffer used was 20 mM Tris-Cl (pH 8.0) and 100 mM NaCl buffer. The excitation wavelength was 280 nm and emission spectra were recorded in the range of 300–400 nm. Band-pass was 5 nm.

An extrinsic fluorophore, 1-Anilino 8-naphthalene sulphonic acid (ANS) was used at the concentration of 50 mM. FRET assay was performed using an excitation wavelength of 300 nm. ANS was excited at 390 nm, and emission spectra were recorded between 450 and 550 nm. The slit width was kept at 5 nm. Fluorescence experiments were repeated three times in independent sets.

Construct Preparation for Functional Studies

Yep96 is a shuttle vector between *S. cerevisiae* and *E. coli* with *TRP1* as a selection marker. DNA fragments encoding FCCH, 4HB, SCCH and UFD were inserted into *Bgl*II and *Sal*I sites of Yep96 plasmid replacing the Ub gene. Primers used to construct the following clones are listed in Table 2. The plasmid constructs YRPB1, YRPB2, YRPB3 and YRPB4 generated were transformed into wild-type strain MHY501 of *S. cerevisiae*. Notably, these transformed strains contained wild-type E1 as a part of normal cellular machinery. The inserts are under the regulation of the *CUP1* promoter, the expression of which can be induced by adding 10–100 μM $CuSO_4$.

Cell Growth and Viability

Cultures of wild-type strain MHY501 of *S. cerevisiae* and its transformants with the plasmids YRPB1, YRPB2, YRPB3 and YRPB4 were grown in SD medium. Another set was prepared with 100 μM $CuSO_4$ as an inducer. To check the effect of overexpression of the peptides on cell

Table 1 Primer sequences used to amplify domains of E1 to transform in vector pET28a

Primer name	Primer sequence
FCCH FR	5′ GATAAGCTAGCGACCCAACGGGTGAAG 3′
FCCH RE	5′ GCGCACTCGAGTCATGAGATTTTACG 3′
4HB FR	5′ GCACAGGCTAGCTTGAAACAACAACTGTCC 3′
4HB RE	5′ GATAACTCGAGTCATCTTGCCTGATAGGAAAGC 3′
SCCH FR	5′ GATAAGCTAGCAAGTCTATCCCATTG 3′
SCCH RE	5′ GATACTCGAGTCAGCCAGCTATGAATTTG 3′
UFD FR	5′ GCACAGGCTAGCATGATTTGGGATAGATTTG 3′
UFD RE	5′ GTTAGCAGCCGGATCCTCGAGTCATAGATGAATGG 3′

Table 2 Primer sequences used to amplify domains of E1 to transform in vector Yep96

Primer name	Primer sequence
YepFCCH FR	5′ ATGCAAGATCTGACCCAACGGGTGAAG 3′
YepFCCH RE	5′ GCCGAGTCGACTCATGAGATTTTACG 3′
Yep4HB FR	5′ GCACAGAGATCTTTGAAACAACAACTGTCC 3′
Yep4HB RE	5′ ATAAGTCGACTCATCTTGCCTGATAGGAAAGC 3′
YepSCCH FR	5′ GCGGAAGATCTAAGTCTATCCCATTG 3′
YepSCCH RE	5′ GAGAGTCGACTCAGCCAGCTATGAAT 3′
YepUFD FR	5′ GCACGGAGATCTATGATTTGGGATAGA 3′
YepUFD RE	5′ GGATCGTCGACTCATAGATGAATGG 3′

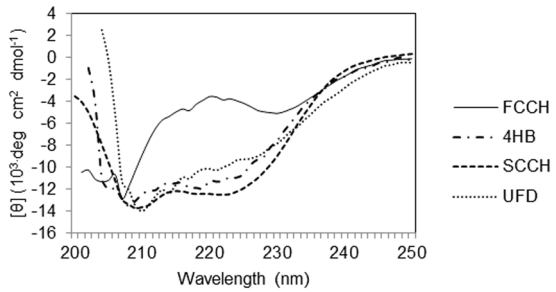

Fig. 2 Far-UV CD spectra of domains of ubiquitin-activating enzyme E1 namely, FCCH, 4HB, SCCH and UFD

viability and growth, OD_{600} was measured every 2 h, and the doubling time was calculated.

Heat Sensitivity Test

Cultures MHY501 strain of *S. cerevisiae* and its transformants with the plasmids YRPB1, YRPB2, YRPB3 and YRPB4 were grown in YPD medium till OD_{600} reached 1. Fourfold serial dilutions of the cultures were plated on SD agar with and without inducer (100 μM $CuSO_4$). Plates were incubated at 37 °C for different time intervals i.e., 0, 4, 8 and 12 h. After the exposure the cells were shifted to 30 °C. The colonies were allowed to grow and were counted. The experiment was repeated three times in independent sets and the mean values have been presented with error bars.

Antibiotic Sensitivity Test

Hygromycin B inhibits translation in eukaryotes by binding to ribosomes [30]. Cultures MHY501 strain of *S. cerevisiae* and its transformants with the plasmids YRPB1, YRPB2, YRPB3 and YRPB4 were grown in YPD medium till OD_{600} reached 1.0. The cultures were serially diluted fivefold and spotted on SD plates containing 0.2 mM Hygromycin B with and without inducer (100 μM $CuSO_4$). Plates were incubated at 30 °C for 7 days to check survival in the presence of antibiotics.

Results

CD Spectra of 4HB, FCCH, SCCH and UFD domains

To assess the folding of various domains, far-UV CD spectra were recorded for 4HB, FCCH, SCCH and UFD (Fig. 2). The spectra were analysed by BeStSel software. Secondary structural patterns of the peptide fragments were modelled using PSIPRED (Fig. 3). The results obtained by analysing CD spectra using BeStSel were compared with values obtained by secondary structure prediction tool PSIPRED (Table 3). Far-UV CD spectrum of FCCH shows ~33% β-sheet structure with very little helicity amounting to ~5%, the spectra of 4HB and SCCH with ~53% and 49% helicity, respectively, are predominantly helical, while the structure of UFD domain has mixed α/β structure containing 19% of α helices and nearly 30% of β-sheets, which is in agreement with the secondary structure predicted by PSIPRED. The PSIPRED predicted FCCH structure to be made up of nearly 43% β-sheet. Domains 4HB and SCCH were predicted to be helical with ~60% and 50% helices, respectively. UFD showed presence of nearly 34% helices and 27% β-sheets. Even though CD spectra of SCCH and 4HB displayed features corresponding to low amounts of β-sheet structure, the software fails to predict the presence of any β-sheet content. However, there are differences in the results obtained with PSIPRED and CD spectra with respect to the content of helices and turns. Isolated domains were characterised in CD spectra in the absence of rest of the sequences of E1, while PSIPRED considers the potential of the sequences based on the information from homologous sequences in full length proteins. Further, many times it is difficult to differentiate between helices and a series of turns (Table 3).

Denaturation of 4HB, FCCH, SCCH and UFD Domains of E1

Intrinsic fluorescence emission spectra of the domains were recorded to see if they have any structure. All four domains were individually exposed to denaturing conditions by

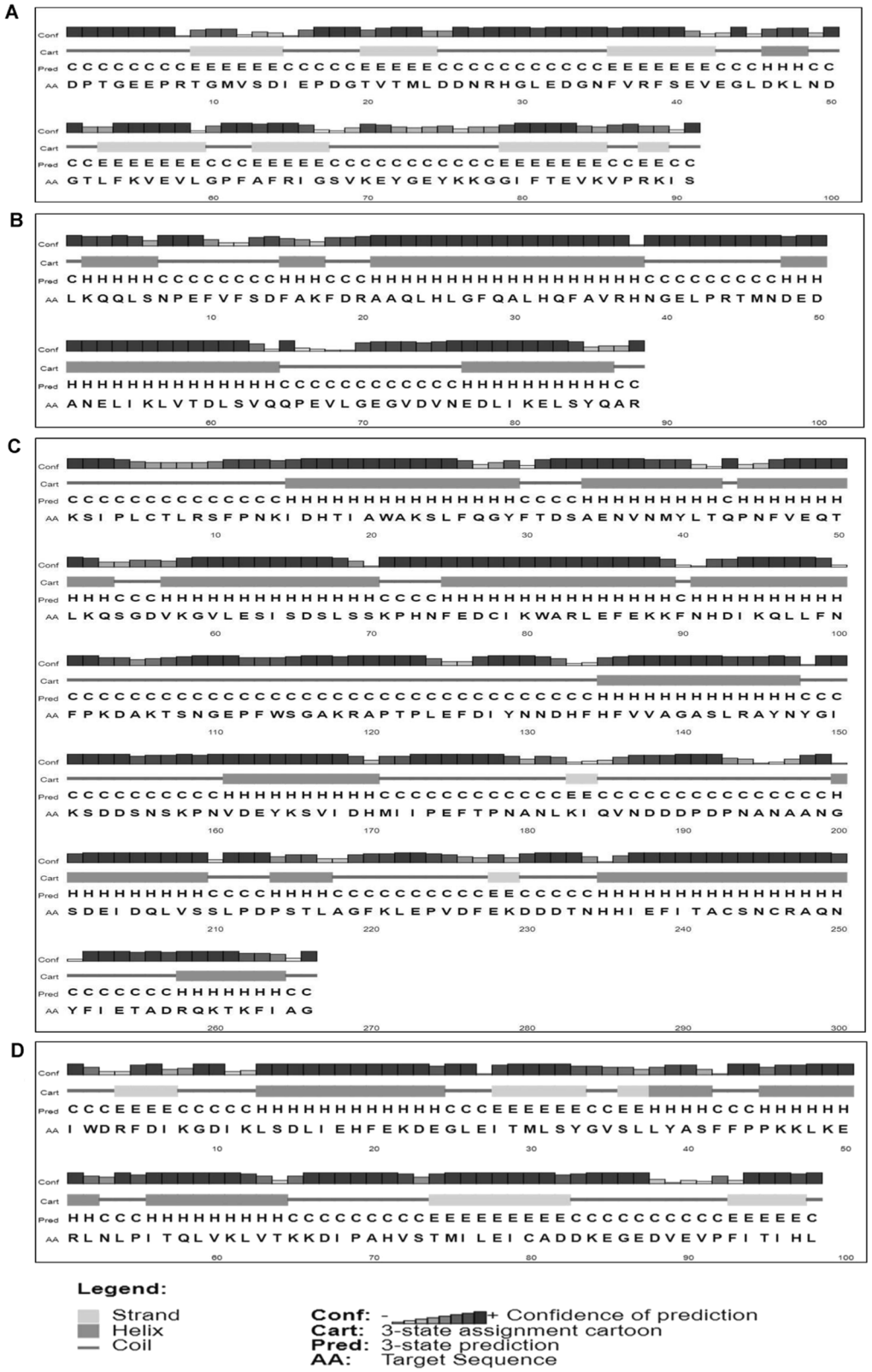

Fig. 3 Predicted secondary structures of the domains **a** FCCH, **b** 4HB, **c** SCCH and **d** UFD domains using PSIPRED software

Table 3 Values of secondary structure obtained from CD spectra analysis done by Bestsel and secondary structure prediction software PSIPRED shown in percentage

Peptide	α-helix		β-sheet		Turns/coils	
	Values obtained from PSIPRED	Values obtained from CD spectra	Values obtained from PSIPRED	Values obtained from CD spectra	Values obtained from PSIPRED	Values obtained from CD spectra
FCCH	3.3	4.9	42.85	33	53.84	62.2
4HB	60.22	52.9	0	17	39.77	30.2
SCCH	50	48.4	1.5	26	48.14	25.7
UFD	33.67	18.5	26.59	29.5	39.79	52

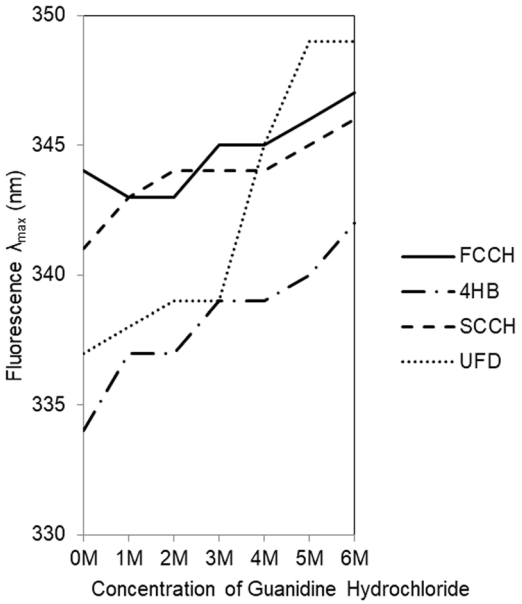

Fig. 4 Guanidine hydrochloride denaturation curves of domains of E1 namely; FCCH, 4HB, SCCH and UFD

adding guanidine hydrochloride. Fluorescence spectra of the domains showed a red shift upon exposure to denaturing conditions, indicating exposure of aromatic amino acids to polar environment as a consequence of a change in the overall structure of the domains. The results are presented in a graph showing a change in λ_{max} with an increase in guanidine hydrochloride concentration (Fig. 4).

Further, an extrinsic fluorophore 1-anilinonaphthalene-8-sulphonate (ANS) was used to check surface hydrophobicity of the isolated domains. ANS binds hydrophobic residues exposed on the surface of a protein and shows fluorescence [31]. Here, ANS was added to the domains, in the presence of various concentrations of guanidine hydrochloride to examine changes in the domain structure. 4HB, FCCH and SCCH showed an increase in fluorescence intensity with increasing concentrations of guanidine hydrochloride, indicating that hydrophobic residues were initially buried in the core and became increasingly accessible for binding to ANS with denaturation. With UFD, results indicated the presence of hydrophobic residues on the surface even in the absence of guanidine hydrochloride

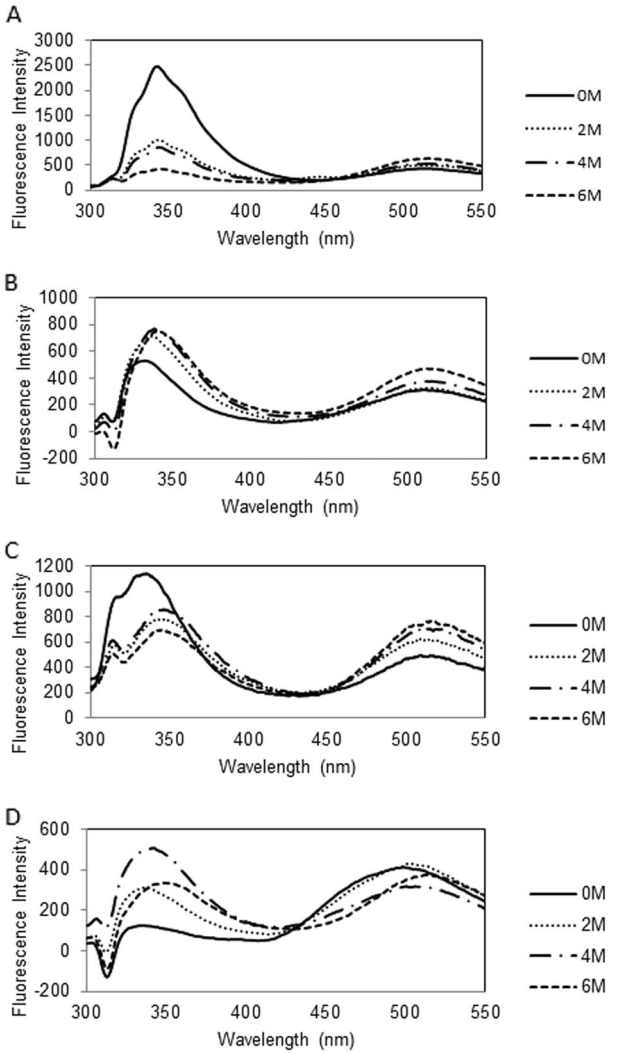

Fig. 5 Fluorescence emission spectra of extrinsic fluorophore ANS bound to the domains of E1 namely; **a** FCCH, **b** 4HB, **c** SCCH, **d** UFD domains in the presence of different concentrations of the denaturant guanidine hydrochloride

(Fig. 5). The spectra exhibited a small peak in some cases in the blue region, due to the presence of Raman peak that is generally observed within 30–50 nm of excitation. Further, the difference in the position of λ_{max} of emission could be due to the difference in the number and nature of aromatic residues and the chemical environment inherently present in

Table 4 The numbers and types of aromatic amino acid residues present in different domains are as listed below

Amino acid		Tryptophan	Tyrosine	Phenylalanine
Domain	Total number amino acid residues			
FCCH	91	0	2	6
4HB	88	0	1	6
SCCH	266	3	7	19
UFD	98	1	2	5

different peptides. The aromatic amino acids present in the four peptides are listed in Table 4.

Effects of Expression of Domains on Cell Survival and Growth

Ubiquitination is known to play a regulatory role in multiple pathways and various processes of the cell. The results obtained with structural studies raised questions on the possible effects produced by these domains in vivo. To test if the expression of the domains in vivo could have a negative influence over viability of yeast cells, DNA fragments encoding the four domains were cloned under copper-inducible *CUP1* promoter and transformed into yeast cells. $CuSO_4$-induced expression of the domains did not have any effect over cell growth and survival compared with wild-type MHY501 cells, which were used as a positive control in the experiment (Fig. 6 and Table 5).

Effects of Expression of the Domains on Cell Growth under Heat Stress

Ubiquitination machinery works more rigorously in cells under stress to remove the denatured proteins when they are under normal conditions. Here, the transformants expressing the domains were subjected to heat stress to check if the domains hinder the ubiquitination process and in turn affect survival. Expression of SCCH and UFD domains affected cell growth under heat stress and decreased cell survival (Fig. 7).

Effects of 4HB, FCCH, SCCH and UFD Expression on Cell Growth in the Presence of Hygromycin B

The presence of Hygromycin B decreased cell growth in the presence of SCCH peptide. SCCH might be interfering with the ubiquitination process, which hampered cell growth under stress. There was no negative effect observed with other peptides. Cells containing FCCH, 4HB and UFD were able to complement the antibiotic stress as effectively as MHY501 cells (Fig. 8).

Discussion

Domains are structural, functional and evolutionary units of proteins [32]. During evolution the core of a protein domain is largely stable relying on secondary and tertiary structural interactions and as it must be functionally conserved. Protein evolution quite possibly took place at the periphery of the relatively constant core [33]. During the course of evolution many a time domains are inserted or duplicated, despite conserving the function of a protein.

E1 is a modular protein, containing six domains. The domains are independent and have been evolved to carry out specific functions [17], with linkers present in between the domains conferring greater flexibility. Though the overall structure of yeast E1 has already been resolved using x-ray crystallography, there are no studies on the structure and function of individual domains of yeast. The work presented here deals with structural and functional characterisation of four of the six domains of E1 as independent units.

CD spectra of the domains of yeast E1 showed that they have the capacity to fold independently and have the potential to adopt a native-like structure. CD spectrum of FCCH of yeast confirmed the presence of β-sheets as a major structural feature. Interestingly, the CD spectrum of the Mouse FCCH showed a presence of around ~40% β-sheet [23], whereas the CD spectrum of yeast FCCH showed ~33% helicity. Mouse and yeast FCCH share ~47% sequence identity, besides both show prominent β-structure with the presence of very less or no helical content.

CD spectrum of SCCH showed that it is predominantly helical with ~50% helicity, which is in agreement with the structure predicted by PSIPRED. CD spectrum of mouse SCCH showed helical structure [23], which is strikingly similar to that of yeast SCCH at 48% helicity. Both the organisms share ~47% sequence identity in this domain. The CD spectrum of the 4HB domain displays helicity as the prominent secondary structural feature. Since there is no information available on the mouse 4HB domain in literature, it was predicted with the help of sequence homology. 4HB domains of both the organisms share ~48% sequence identity and predominant helicity as predicted by PSIPRED. UFD domain displays α/β structure, which matched the structure predicted by PSIPRED. E1 enzyme of yeast shares around 52% overall identity with that of mouse E1. Further, the domains show native-like characteristics even when they are expressed as individual units.

The domains were expressed in cells, to answer the question, whether they by virtue of possessing structure

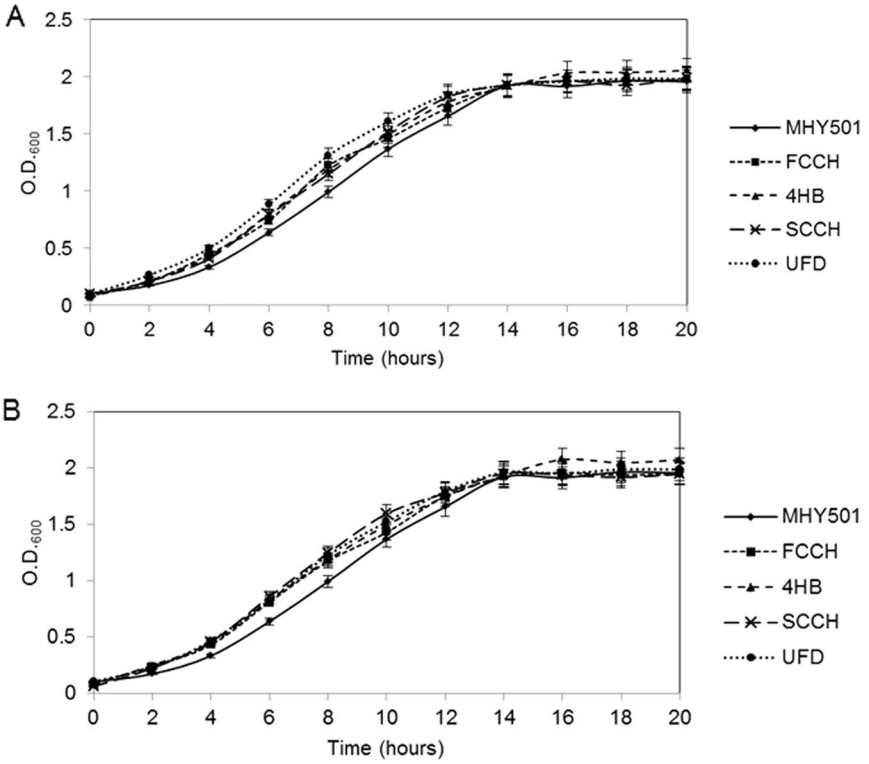

Fig. 6 Growth curves of cells MHY501-expressing domains FCCH, 4HB, SCCH and UFD of ubiquitin-activating enzyme E1, **a** without inducer **b** with $CuSO_4$ inducer. Untransformed MHY501 cells were used as control

Table 5 Determination of doubling time of MHY501 cells transformed with domains 4HB, FCCH, SCCH and UFD in absence and presence of inducer $CuSO_4$

Cells	Doubling time
MHY501	2.17
FCCH	2.26
FCCH $CuSO_4$	2.23
4HB	2.32
4HB $CuSO_4$	2.30
SCCH	2.20
SCCH $CuSO_4$	2.41
UFD	2.19
UFD $CuSO_4$	2.20

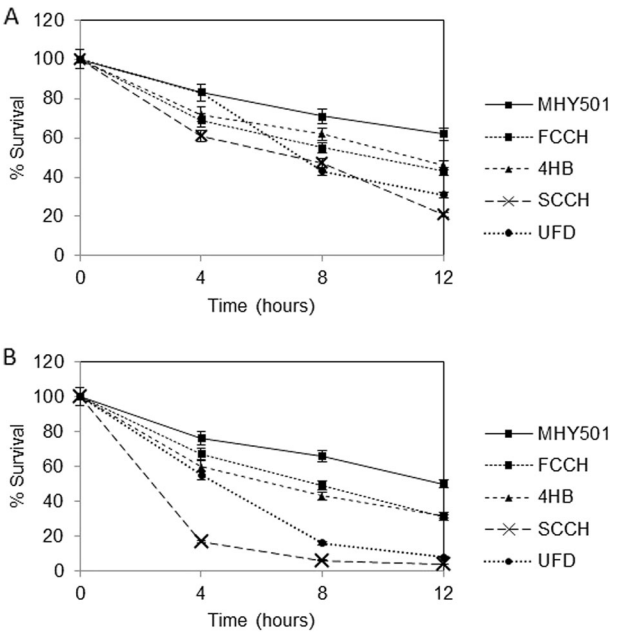

Fig. 7 Survival of MHY501 strain expressing the domains of ubiquitin-activating enzyme E1 under heat stress: **a** without inducer **b** with $CuSO_4$ inducer. Cells expressing SCCH and UFD decreased survival under heat stress

have the potential to interact with any binding partners and interfere or slow down cellular machinery by blocking the binding site. However, no change in growth was observed under normal conditions. Stress conditions were chosen to test the same, as the cells tend to increase the rate of protein degradation by ubiquitination, to remove the misfolded proteins [34]. The working hypothesis for this study was if the domains can compete with E1 for binding to other proteins, then their expression can affect cell survival and more so under stress conditions. Serving as proof for this hypothesis, the SCCH domain affected cell survival under heat stress and antibiotic stress. Even the presence of UFD caused interference in the cellular processes under heat stress. While the presence of the rest of the two domains FCCH and 4HB caused no effect on cell growth.

Ornithine decarboxylase (ODC) catalyzes the first step of polyamine biosynthesis. An increase in polyamine levels

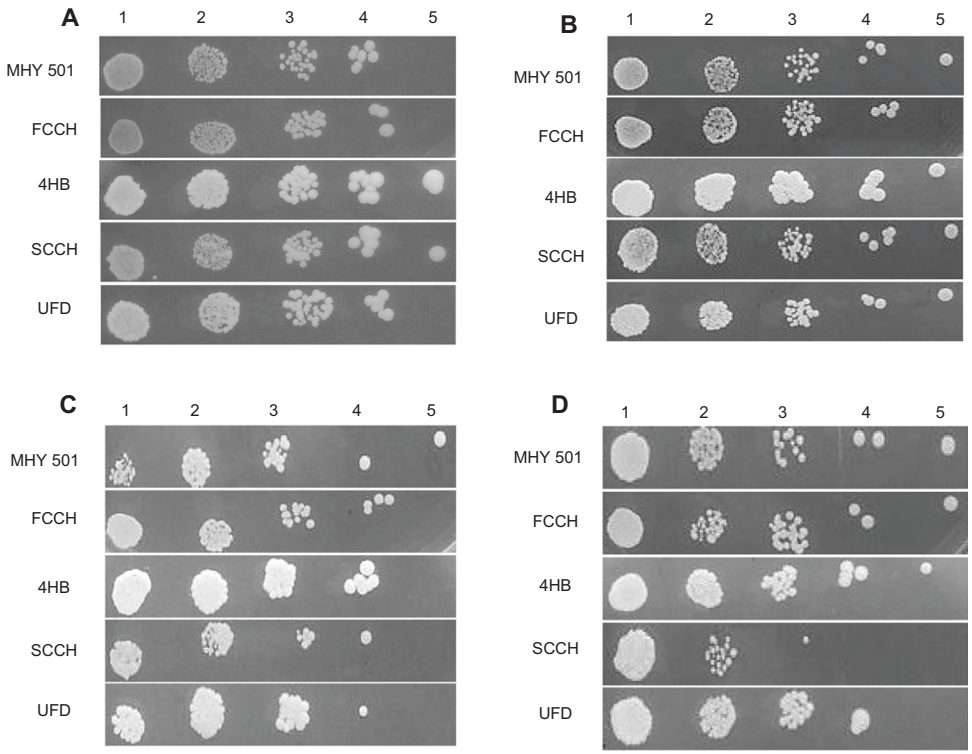

Fig. 8 Effect of overexpression of domains on cell growth under antibiotic stress. MHY501 cells were transformed with plasmids encoding the domains FCCH, 4HB, SCCH and UFD individually and were grown on plates containing 0.2 mM Hygromycin B. MHY501 cells were used as controls. MHY501-expressing SCCH showed reduced growth. Petri dishes contain undiluted cultures (1), and fourfold serial dilutions (2–5) have combinations of antibiotic and inducer as follows: **a** control petri dishes without inducer and without antibiotic, **b** petri dishes with inducer and without antibiotic, **c** petri dishes without inducer and with antibiotic, **d** petri dishes with inducer and with antibiotic. Inducer used is 100 μM $CuSO_4$

lead to expression of the protein Antizyme, which binds to ODC and delivers it to the proteasome for degradation. Domain Nα/β, isolated from ODC, can adopt the native-like structure and can bind to antizyme [35]. Thus, the ability of Nα/β to bind antizyme and be regulated by it suggested the possibility of competition for binding partners between isolated domains and whole proteins, which served as a motivation for this work. Further, mutant subunits of LacI protein with missense mutations could bind to wild-type subunits, leading to a reduction in functionally active assemblies [36]. Another example of alteration of protein structure to understand protein structure–function relationships comes from a lipid transporter protein apolipophorin III. The protein from insects possesses five helix bundle structure. The N-terminal helix and C-terminal helix have been deleted to convert it into a standard 4HB. The voltage sensor domain (VSD), constituting four helices, of the potassium ion channel protein from *Xenopus laevis* has been incorporated into lipid bilayer vesicles displayed structure similar to that of the native protein [37]. It was observed that both N- and C-terminal helix deletion variants were functionally effective. However, they possessed reduced secondary and tertiary structure content and decreased protein stability [38].

In summary, yeast Ub-activating enzyme E1 is a modular protein, evolved from small prokaryotic proteins, which had specific functions and in E1 enzyme they form domains that work together as part of a big enzyme. The main objectives of this study were to probe if the isolated domains could fold as independent units and exhibit functions associated with them. To answer the questions four domains of E1 enzyme have been chosen. They are FCCH, 4HB, SCCH and UFD. DNA fragments encoding the domains have been cloned and expressed in *E. coli*. CD and fluorescence studies of the peptides established that they adopt a native-like structure. To see if these domains can cause any functional aberrations, functional studies were carried out in vivo. Under normal conditions of growth, expression of the domains did not pose any problem to the growth and survival of yeast cells. However, under antibiotic and heat stresses expression of the domains SCCH and UFD proved to be detrimental to cell survival. Hence, it can be envisaged that individual domains like SCCH show the potential to interfere with cellular processes by acting as competitive inhibitors in protein–protein interactions involving complete proteins.

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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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ORIGINAL PAPER

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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 Δubc4Δubc5 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 · ΔΔG

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].

Supplementary information The online version of this article (https://doi.org/10.1007/s12013-019-00894-1) contains supplementary material, which is available to authorized users.

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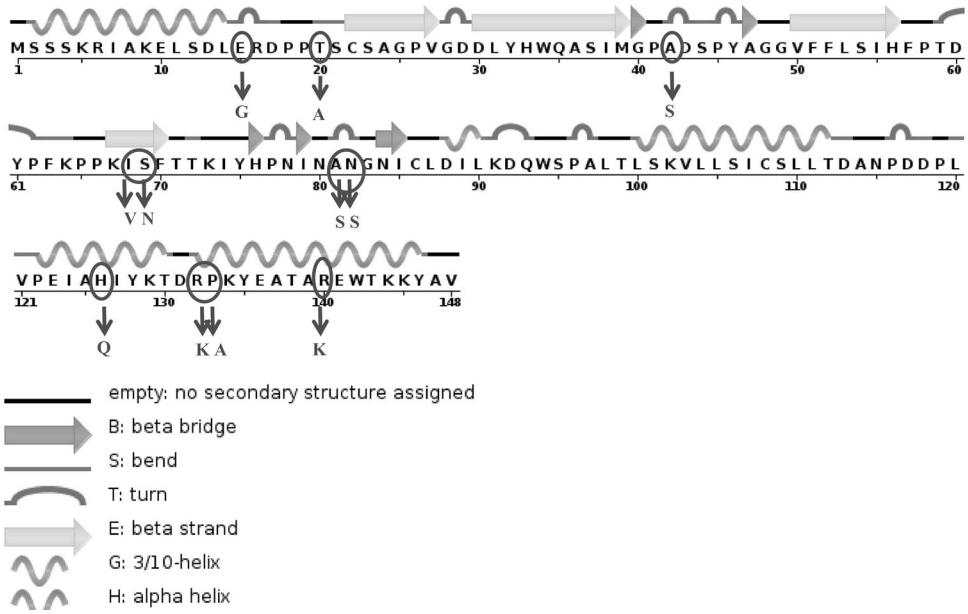

Fig. 1 Sites of amino acid replacement in UBC4 (PDB ID: 1QCQ). The substituting residues from UBC5 are highlighted below

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 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 [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	**G**CT	Ala
UBC4-A42S	A42S	42	GCC	Ala	**TCA**	Ser
UBC4-I68V	I68V	68	ATC	Ile	**GTA**	Val
UBC4-S69N	S69N	69	TCC	Ser	**AA**C	Asn
UBC4-A81S	A81S	81	GCC	Ala	**TCG**	Ser
UBC4-N82S	N82S	82	AAT	Asn	A**GT**	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	**GCT**	Ala
UBC4-R140K	R140K	140	AGA	Arg	A**AG**	Lys

Materials and Methods

In Silico Analysis of the Binding Partners of UBC4 and UBC5

The information available in open source database BioGRID[3.5] (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 (ΔΔG) [30].

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 lys2-801 trp1-1 ubc4-Δ1::HIS3 ubc5-Δ1::LEU2) [31] 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 [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 $CuSO_4$ inducible *CUP1* promoter, by using 100 μM 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^{-1}), lysine (30 mg L^{-1}), uracil (20 mg L^{-1}), leucine (100 mg L^{-1}) or tryptophan (20 mg L^{-1}) 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 *BglII* and *KpnI* 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′ 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′

The highlighted sequence in FR (forward primer) of UBC4 WT represents restriction site for *BamHI* and in RE (reverse primer) *KpnI*

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 DH5α strain of *E. coli*. The transformant cells were grown in LB medium supplemented with the antibiotic ampicillin (100 µg/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 µM) 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 added to Laemmmli 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 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 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 Δubc4Δubc5 (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

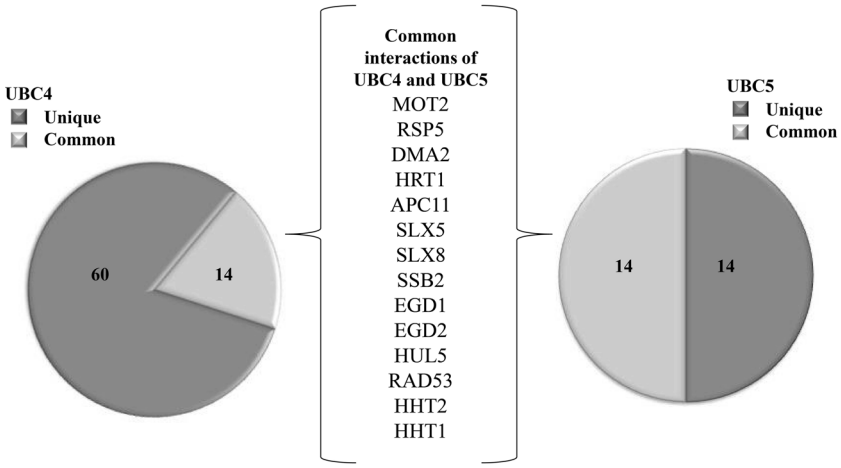

Fig. 2 Diagrammatic representation for physical interactions of UBC4 and UBC5. The values represent the number of interactions for individual proteins and common interactors shared between two genes

[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 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.

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 µM 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[3.5] [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 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). 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

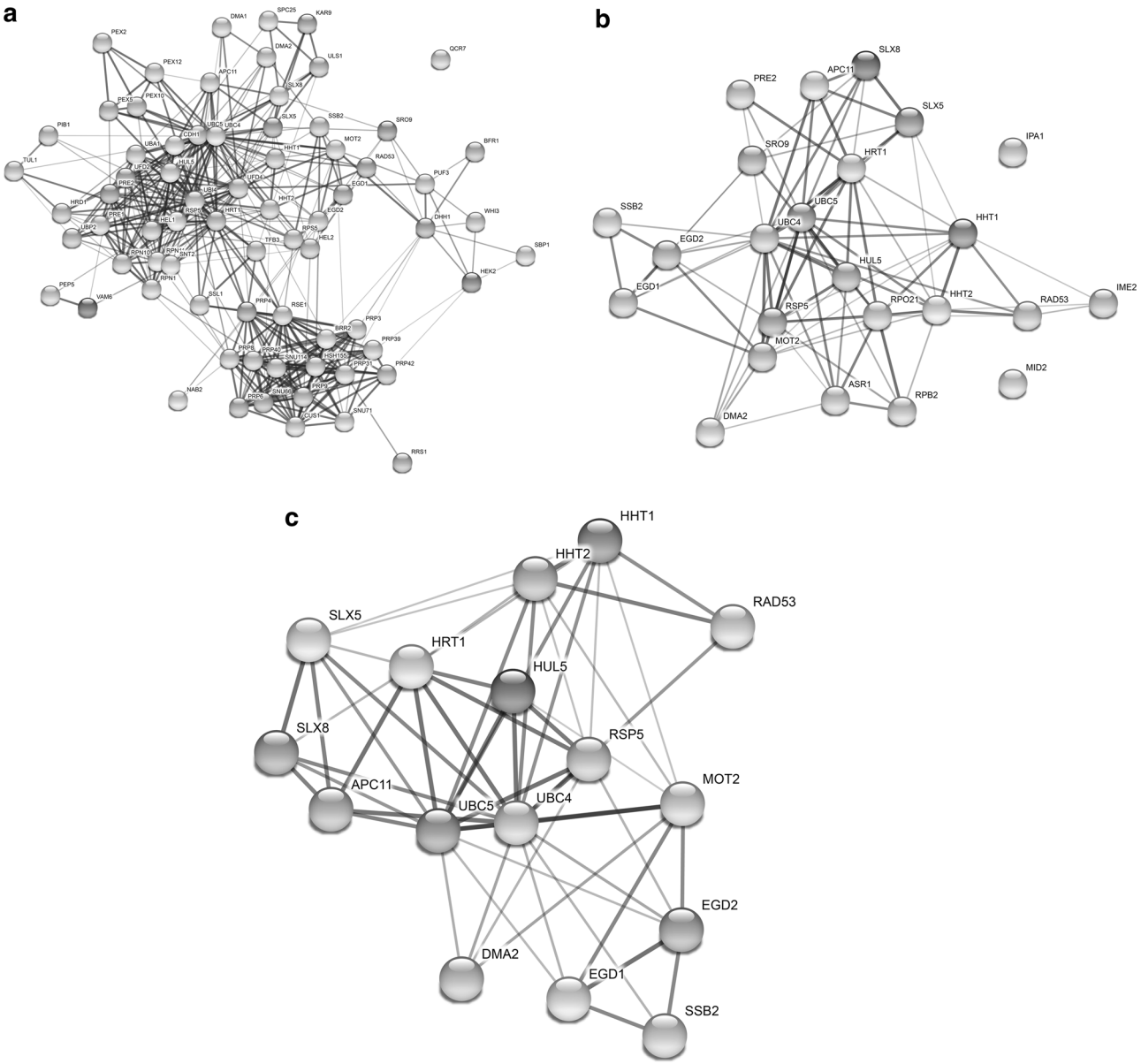

Fig. 3 **a**, **b** and **c** represent interactors of UBC4, UBC5, UBC4 and UBC5, respectively. The thickness of the lines indicates the strength of interaction between the proteins. Interactions of UBC4 and UBC5 plotted on STRING [40] (data collection source: The Biological General Repository for Interaction Datasets (BioGRID)[3.5]) [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 mCSM (mutation Cutoff Scanning Matrix) [30]

Substitution in UBC4 protein	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*)

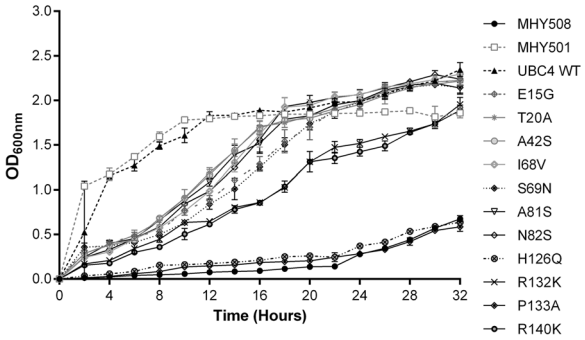

Fig. 4 Growth curves of UBC4 mutants of MHY508 cells of *S. cerevisiae* transformed by plasmids expressing variants of *UBC4* gene namely, WT, E15G, T20A, A42S, I68V, S69N, A81S, N82S, H126Q, R132K, P133A, and R140K expressing UBC4-WT, UBC4-E15G, UBC4-T20A, UBC4-A42S, UBC4-I68V, UBC4-S69N, UBC4-A81S, 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_{600} 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 µM 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

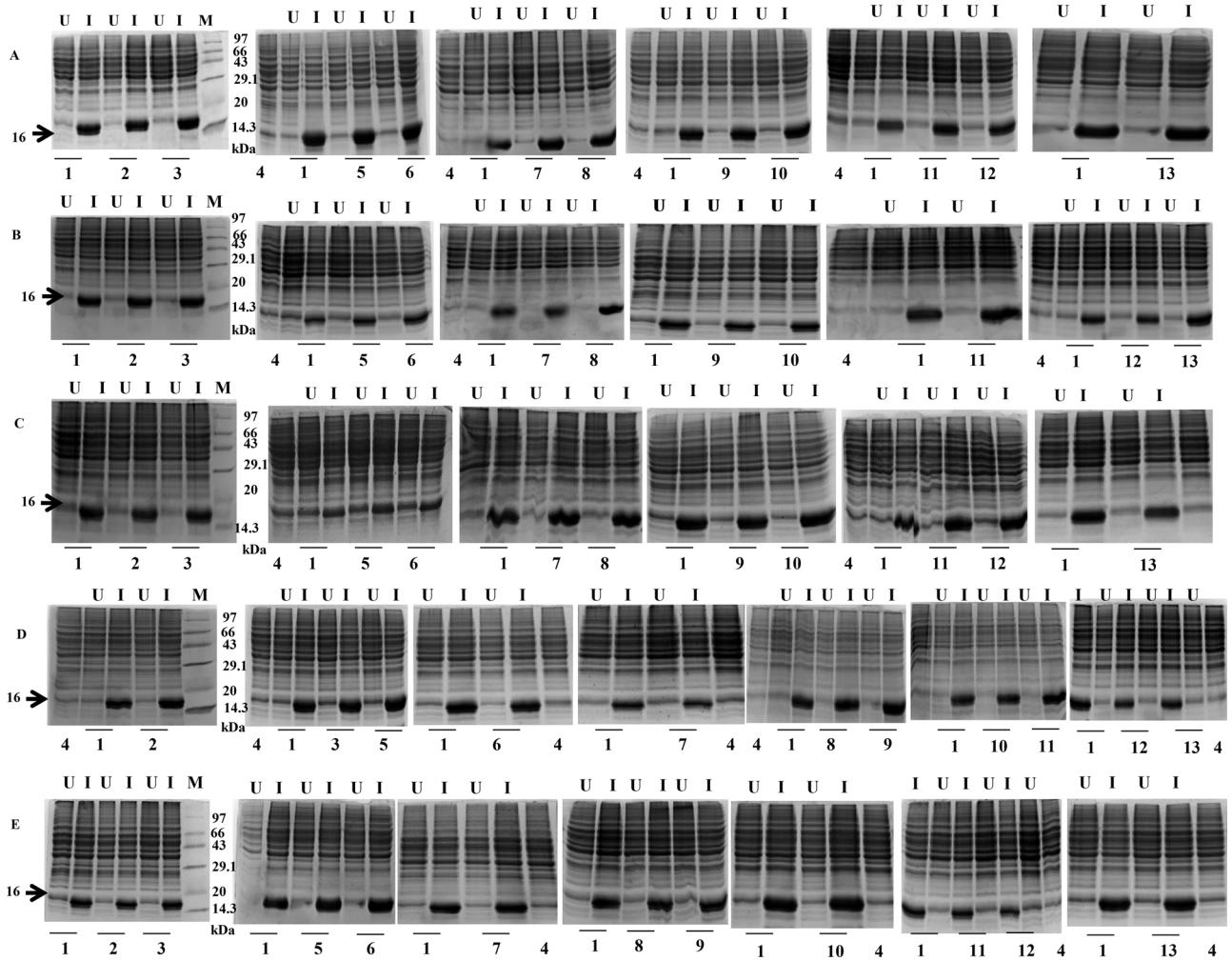

Fig. 5 Analysis of expression levels and stabilities of variants of UBC4 at various time points (**a**, **b**, **c**, **d**, **e** represent 4, 8, 12, 16, 24 h, respectively). MHY508 and UBC4 WT were used as controls. 100 µM copper sulfate was used as an inducer. U indicates Uninduced, I indicates induced 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 ~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 aspargine (Asn). Both these amino acids have similar propensities for adopting β-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.

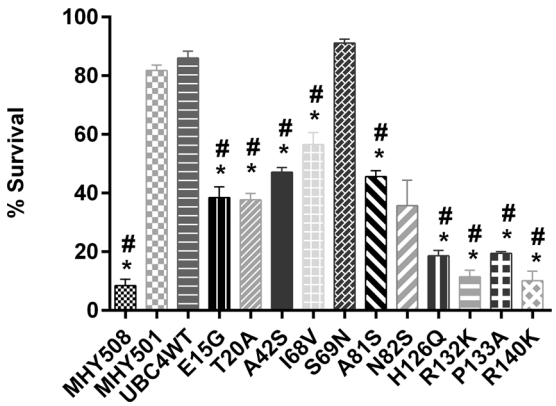

Fig. 6 Functional complementation under heat stress at 24 h. by the variants of UBC4, UBC4-E15G, UBC4-T20A, UBC4-A42S, UBC4-I68V, UBC4-S69N, UBC4-A81S, UBC4-N82S, UBC4-H126Q, UBC4-R132K, UBC4-P133A and UBC4-R140K in MHY508, the double deletion strain of *UBC4* and *UBC5* of *S. cerevisiae*. MHY508 cells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

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

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)

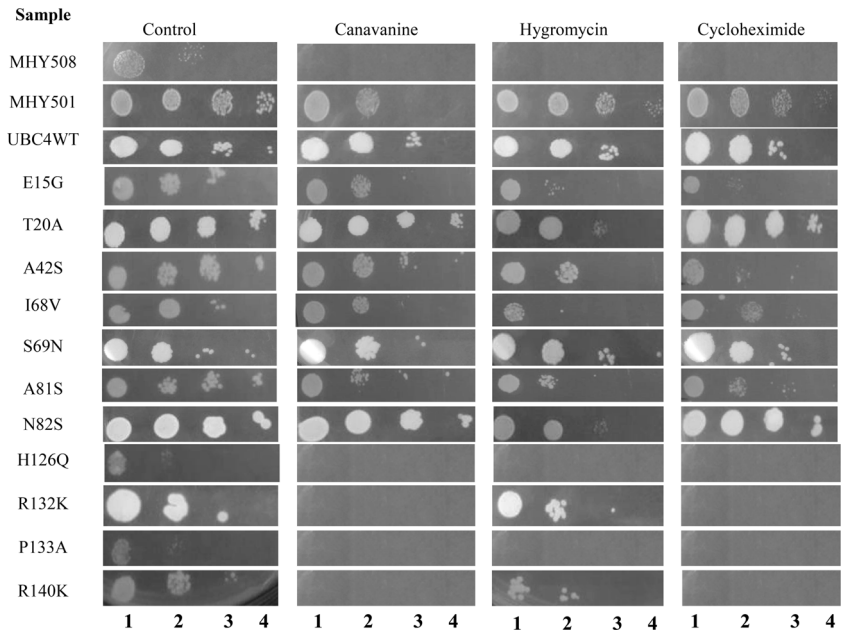

Fig. 7 Complementation by UBC4 variants in the presence of protein translational inhibitors in MHY508 strain of *S. cerevisiae*. The strain MHY501 and MHY508 were used as positive and negative controls, respectively. MHY508 cells transformed by plasmids encoding the variants of UBC4 namely, UBC4WT, UBC4E15G, UBC4T20A, UBC4A42S, UBC4I68V, UBC4S69N, UBC4A81S, UBC4N82S, UBC4H126Q, UBC4R132K, UBC4P133A and UBC4R140K, 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

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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Compliance with Ethical Standards

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

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