Chapter 2

Construction, Expression and Purification of UBC1 and c-UBC1 followed by Their Structural-Functional Analysis

2.1. Introduction

The ubiquitin-conjugating enzyme UBC1 of *S. cerevisiae* is a typical Class II E2 enzyme possessing two domains: (i) the N-terminal domain of 150 residues, also known as catalytic or ubiquitin conjugating (UBC) domain and (ii) a C-terminal domain or ubiquitin associated domain. The two distinct domains of UBC1 are conjoined by a linker of 22 residues (Merkley et al., 2005). The characteristic α/β fold found in the catalytic domain of UBC1 is similar to that observed in the crystal structures of class I E2 proteins including UBC2 (Worthylake et al., 1998), UBC4 (Cook et al., 1993), UBC7(W. J. Cook et al. 1997), and UBC2b (Miura et al., 2002). Unlike the catalytic domain, the UBA domain is able to interact with mono or polyubiquitin chains in a non-covalent manner (Merkley et al., 2005). Binding of Ub to UBA domain does not affect the thioester formation in the catalytic domain. UBC1 normally functions in conferring viability and stress resistance to yeast, sharing functional similarity with UBC4 and UBC5 (Seufert and Jentsch, 1990; Seufert et al., 1990). *UBC1* mutants display slow growing phenotype with impaired growth following germination (Seufert et al., 1990). Thus, it has a unique role in the early stages of spore germination.

Ubiquitin proteasome system (UPS) is implicated to play important role in AD pathogenesis since ubiquitin and ubiquitin conjugates are found in the AD senile deposits (Alves-Rodrigues et al., 1998). Human homolog of UBC1, E2-25k is up-regulated in the neurons and in the brain of patients suffering from Alzheimer's disease (AD), as a consequence of exposure to Aβ peptide. A frameshift mutant of ubiquitin UBB^{+1}, interacts with E2-25k leading to inhibition of the enzyme, which in turn contributes to the neurotoxic disorder (Ko et al., 2010b). Previously a chimeric protein was constructed in which the UBA domain and linker of E2-25K was fused to the E2 domain of yeast UBC4. The resultant chimeric protein failed to show polyubiquitination activity, suggesting that catalytic activity of E2-25K is dependent on the relative conformations of UBC and UBA domains and their specific interactions (Haldeman et al., 1997).

Yeast UBC1 is a homolog of the mammalian enzyme E2-25K. Sequence alignment of UBC1 and E2-25K protein sequences by Clustal Omega tool, shows sequence conservation in N and C-terminal domains (**Fig. 2.1**). However, the linker regions present in both the proteins does not show any resemblance in their sequences. The UBC1 linker is of 22 amino acids; while E2-25k has 6 amino acids. In the present study, to understand the importance of difference in the length and sequence of the linkers, a chimeric protein (c-UBC1) by swapping E2-25k linker with that of yeast UBC1 was constructed and characterized. The main objective of this study was to appreciate the functional or structural differences arising out of swapping, in the wake of the role of this protein.

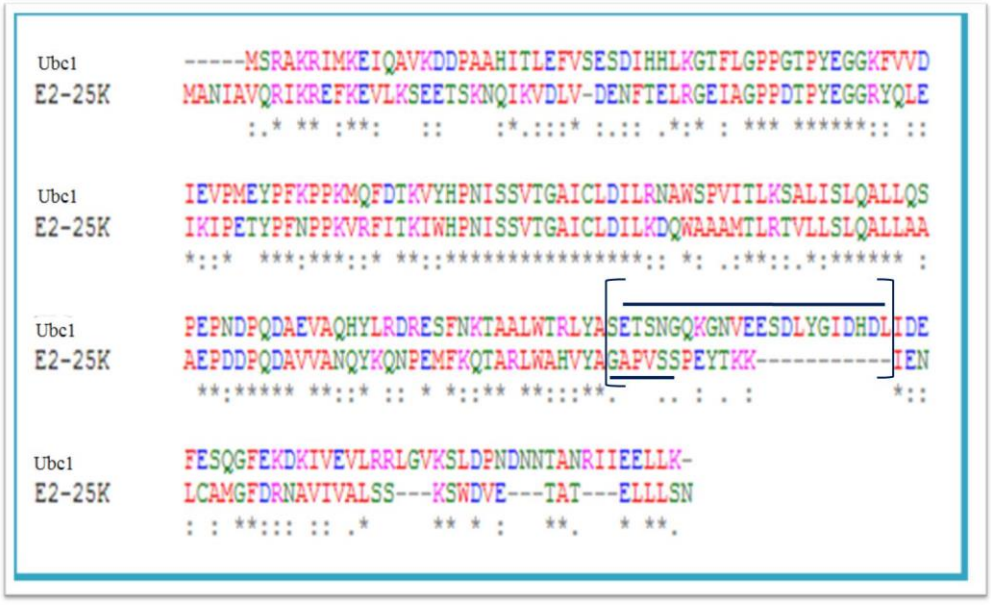

Fig. 2.1. **Sequence comparison of two proteins UBC1 (*S. cerevisiae*) and E2-25K *(Homo sapien)* using Clustal omega (htpp://www.ebi.ac.uk/Tools/msa/clustalo).** The linker sequence is underlined and the highlighted box shows the major difference between two proteins.

However, on comparing sequences of E2-25k and c-UBC1 as shown in **Fig. 2.2**, it was again noticed that they have a lot of similarity in sequence. Such similarities in sequence of c-UBC1 to E2-25K had led us to perform functional and structural studies using the chimera.

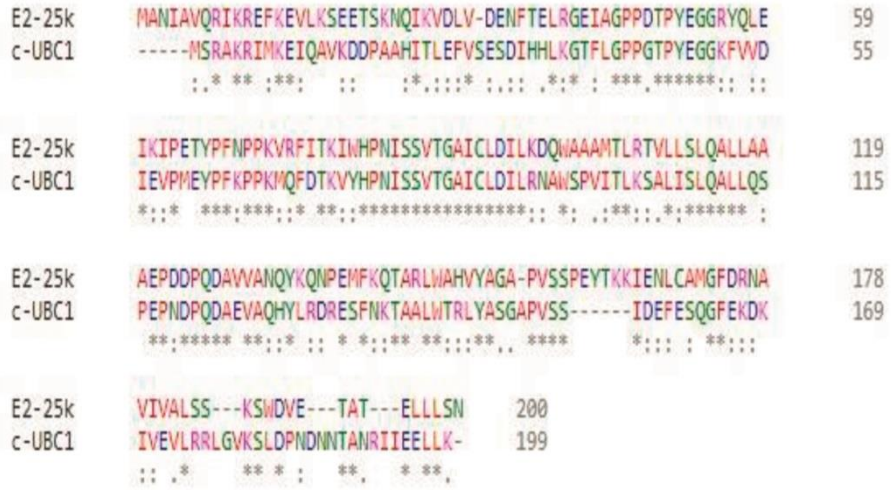

Fig. 2.2. Sequence alignment of E2-25K and c-UBC1.

2.2. Materials and methods

2.2.1. Comparison of linker sequence of UBC1 (*Saccharomyces cerevisiae)* with other organisms.

To study the linker conservation among various phyla, sequences of homologs of UBC1 enzyme of various organisms were searched and collected from National Center for Biotechnology Information (NCBI). Later, using the alignment tool, Clustal Omega (Sievers et al., 2011) the sequences were subjected to multiple sequence alignment and compared to *S. cerevisiae.*

2.2.2. Strain used for in vivo studies

The *S. cerevisiae* strain used in the study was a generous gift from Prof. Stefan Jentsch (Department of Molecular Cell Biology, Max Planck Institute of Biochemistry, Germany). The *ubc* mutant strain used in this study was haploid MATα derivative of strain DF5 *(MATα/MATα, Iys2-801/lys2-801, leu2-3,2-112/leu2-3, 2-112, ura3-52/ura3-52, his3Δ200/his3Δ200, trp1-1(am)/ trp1-1(Winn et al.)*; Finley et al., 1987). YWO5: UBC1::HlS3 was used for in vivo studies.

2.2.2.1. Bacterial strains and media

For transforming recombinant DNA, *E.coli* DH5α (F-, 80dlacZ M15, *endA*1, *recA*1, *hsdR*17 (*rk-,mk+), supE44, thi-1, gyrA96, relA1,(lacZYA-argF)U169*) strain was used. Cultures were grown at 37°C at 200 rpm in Luria broth (Hi-media). 50µg/ml of ampicillin concentration was used for selection of plasmid. Bacterial transformation was done by $CaCl_2$ method (Dagert and Ehrlich, 1979). Plasmid isolation was done by standard alkaline lysis protocol (Sambrook and Russell, 2001).

For purification purpose, BL21 (DE3), high stringent expression strain was used to express recombinant protein. Cultures were grown at 37°C at 200 rpm in Luria broth (Hi-media) and selected on kanamycin (50µg/ml) containing medium. Bacterial transformation was done by $CaCl_2$ method (Dagert and Ehrlich, 1979).

2.2.1.2. Construction of yeast vectors carrying genes for wild type UBC1 and c-UBC1 by site directed mutagenesis

The pET23d plasmid carrying *UBC1* gene was a generous gift from Prof. David O. Morgan (Yale University, San Francisco, CA). In this study *UBC1* was used as control and c-*UBC1* was generated from *UBC1* by swapping the linker sequence with linker sequence of human *E2-25K* gene. In order to achieve the above objective of swapping the linker, a combination of mutagenic and non-mutagenic primers were used to generate c-UBC1. Two non-mutagenic primers were designed for the 5' and 3' ends of *UBC1* gene, while two mutagenic primers were designed complimentary to the 3' end of the sequence encoding N-terminal domain and 5' end of C-terminal domain, with 18 bp long complimentary sequences for linker of *E2-25K* as the flanking regions (**Table 2.1** and **Figure 2.3**). From the first PCR reaction we got two products encoding the N and C terminal domains flanked by E2-25K linker. In the next set of PCR reaction, amplicons from the first reaction were used as templates and a reaction was run using the non-mutagenic primers to get the product c-UBC1. So, the final chimeric construct (c-*UBC1*) had DNA sequence encoding N and C-terminal domains of yeast UBC1 and linker of

mammalian E2-25K. For generating wild type UBC1 only non-mutagenic primers were used. The backbone of YEp96, a shuttle vector between yeast and bacteria was used for cloning *UBC1* and *c-UBC1* genes (**Fig. 2.4**). The genes were cloned into YEp96 under *CUP1* promoter, using *Bgl*II and *Kpn*I restriction sites and replacing the ubiquitin gene *Y-UBI*. Expression of the genes could be induced by 10-100µM copper sulphate. To ease screening procedure, restriction site (*Sau96*) was created in the sequence carrying the linker region. While doing this manipulation in the sequence care was taken to avoid amino acid changes. Final confirmation was done by DNA sequencing.

Table 2.1. Primers used for the study to generate c-UBC1 in YEp96. The flanking regions highlighted in the sequence carry the linker region of *E2-25K*.

Name	Primer sequence
UBC1 5' FR (Non-mutagenic)	***Bgl* II** **5'**GGCGCTC**AGATCT**ATGTCTAGGGCTAAGAGAATTATGAAAGAA ATCCAGC **3'**
UBC1 5' RE (mutagenic)	**5'ACTAGAAACTGGGGCCCC**ACTGGCGTATAACCTCGTCC **3'** Flanking
UBC1 3' FR (Non-mutagenic)	**5'GGGGCCCCAGTTTCTAGT**ATTGACGAGTTTGAATCTCAAG **3'** Flanking
UBC1 3' RE (mutagenic)	5' CCTCTGG**GGTACC**TCACTTCAACAATTCC 3' ***Kpn* I**

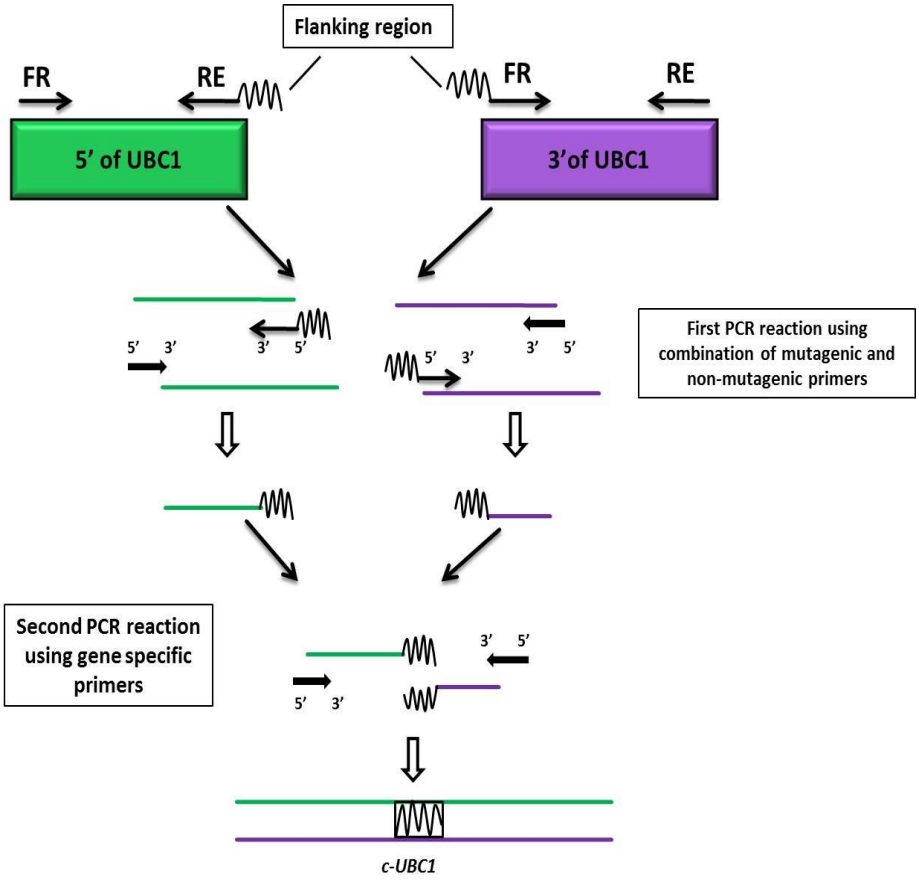

Fig. 2.3. Graphical representation of strategy used to generate *c-UBC1*.

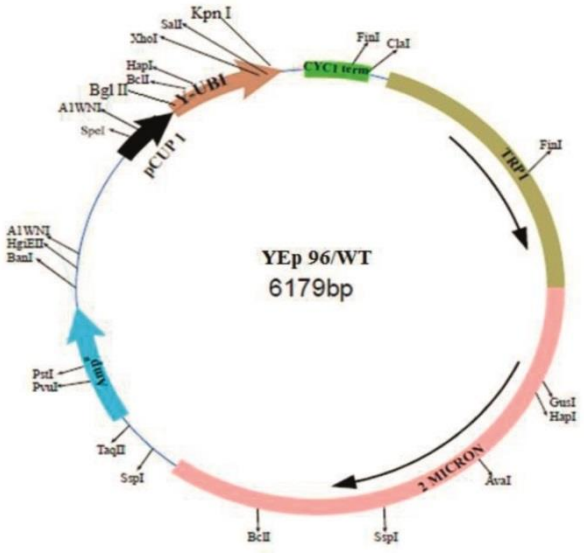

Fig. 2.4. Yeast expression vector YEp96/UbWT, expresses synthetic ubiquitin under CUP1 promoter (Finley et al., 1987).

2.2.3. Phenotype analysis

2.2.3.1. Effect of c-UBC1 on Growth of Yeast Cells

The *ubc1* deletion mutant YWO5 cells were transformed by plasmid YEp96/UBC1 and YEp96/c-UBC1 were grown at 30°C at 200 rpm, except where indicated in synthetic dextrose medium (SD) consisting of 0.67% Hi-media yeast nitrogen base supplemented without amino acids and 2% glucose as carbon source, as and when required.100µM copper sulphate was used as an inducer to check the effect of the expression of *c-UBC1* gene on *S. cerevisiae* cells. Optical density at 600nm was measured every 2h. Their growth was compared with the positive control YWO5 cells transformed by plasmid YEp96/UBC1. YWO5 cells with Δubc1 genotype were used as negative control.

2.2.3.2. Heat stress test

Heat sensitivity test was performed to confirm the functional integrity of the chimeric ubiquitin-conjugating enzyme c-UBC1. Cells were grown in YPD broth at 30°C till the OD at 600 nm reached 1.0. Four-fold serial dilutions were made and spread on SDA plates supplemented with amino acids in the presence or absence of 100µM copper sulphate inducer. The plates were then incubated at 37°C for various time intervals (0, 4, 8, 12, 16 and 24 hr) and then shifted back to 30°C to assess colony formation. This experiment was done three times in triplicates.

2.2.3.3. Antibiotic sensitivity test

Resistance to various post-translational inhibitors was tested to check if the chimeric construct, c-UBC1 can function like UBC1 or not. Transformants of YWO5 carrying the plasmids YEp96/UBC1 and YEp96/c-UBC1 were grown to log phase till their optical density values reached around 0.1 and were serially diluted five-fold and spotted on SDA plates in the

presence and absence of cycloheximide (0.001M), L-canavanine (0.14mM) and hygromycin-B (0.2mM), with and without the inducer (100µM copper sulphate). The plates were incubated for 7-10 days to assess revival and growth.

2.2.3.4. Thermotolerance test

This assay was performed as described previously (Seufert and Jentsch, 1990). Cells were grown in YPD liquid medium at 30°C. The cultures were shifted to 37°C for 1 hr. for pre-treatment. Then the cultures were subjected to heat shock at 52°C for 5 min. Prior to and after heat shock, 100 µl aliquots were spread on SDA plates and incubated at 30°C to assess colony formation. Colonies were counted and data is presented as percentage of colonies formed after heat shock.

2.2.4. In vitro studies

2.2.4.1. Construction of plasmids carrying *UBC1* and *c-UBC1* genes in bacterial expression vector pET-28 a

Plasmid pET-28a was used for expression and purification of UBC1 and c-UBC1 proteins. Both the genes *UBC1* and *c-UBC1* were cloned under bacteriophage T7 promoter in *Nco*I and *Xho*I sites of pET-28a. The plasmid carries a sequence for C-terminal 6X His-tag for affinity purification (**Fig. 2.5**). Primers were designed as listed in **Table 2.2**.Target genes were initially cloned into the non-expression host DH5α, as it does not contain the T7 RNA polymerase gene and hence eliminates the possible plasmid instability. Once established in non-expression host, the plasmid was transferred into host cell BL21 (DE3) containing a chromosomal copy of the T7 RNA polymerase gene as it is very selective and active. T7 RNA polymerase utilises cellular resources to express the target gene when fully induced. T7 RNA polymerase expression was induced by the addition of IPTG (isopropyl-β-D-1-thiogalactoside) at a final concentration of 0.1mM-2mM to the medium.

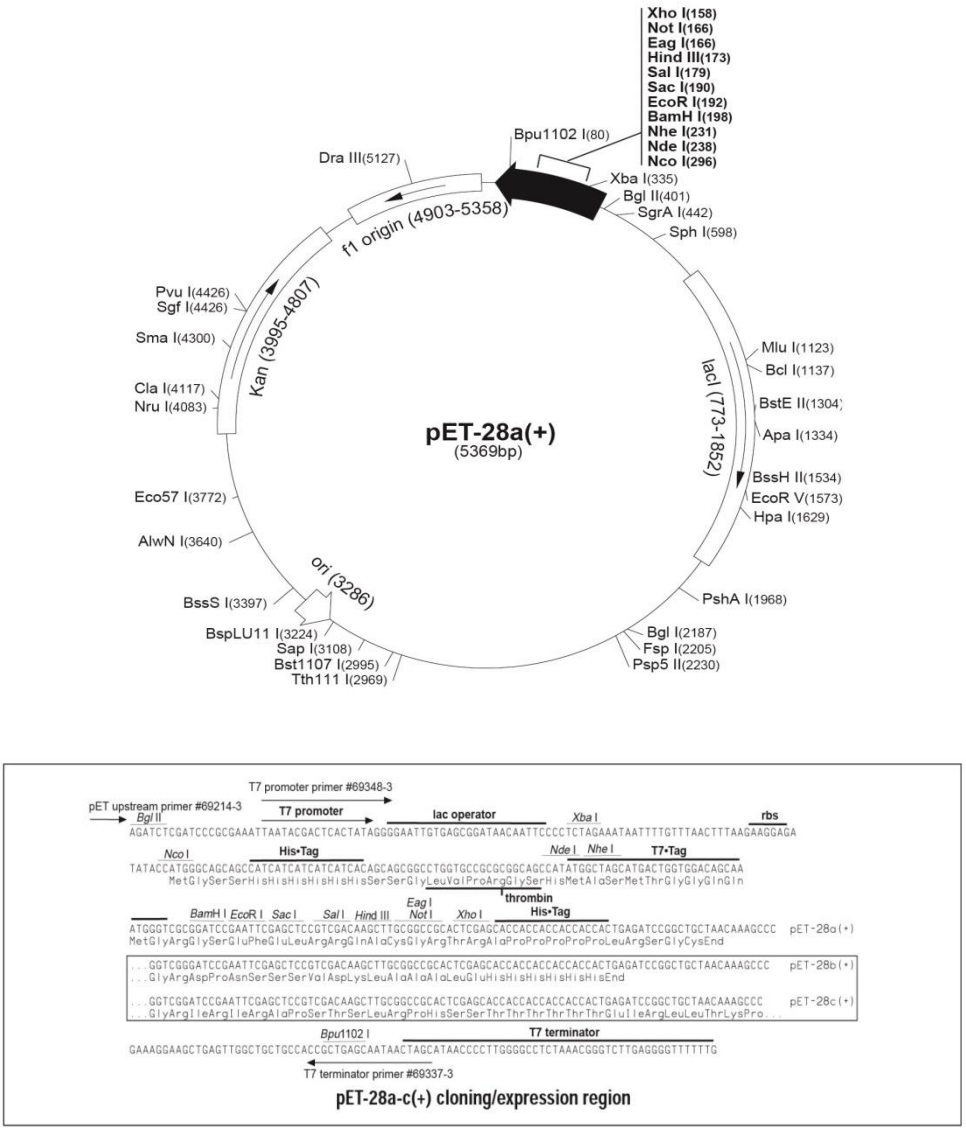

Fig. 2.5. Vector map of pET-28a. The vector carries sequence for an N-terminal His•Tag/thrombin/T7•Tag configuration plus sequence for an optional C-terminal His•Tag sequence. Unique sites are shown on the circle map.

Table 2.2 Primers used for the study.

Name	Primer sequence
pET UBC1 FR	***Nco I*** **5'**CCGGCCATGGATGTCTAGGGCTAAGAGAAT **3'**
pET UBC1 RE	**5'** CGCCGGCTCGAGCTT CAACAA TTCCTC **3'** ***Xho I***

2.2.4.2. Purification of UBC1 and c-UBC1

The vectors carrying the plasmids UBC1WT and c-UBC1 were transformed in E.*coli* BL21 (DE3) cells. The cultures were allowed to grow till they reached log phase. Later on, they were induced by the addition of 1mM IPTG and incubated on shaker for overnight at RT. After checking expression of proteins, purification was carried out by suspending cells in lysis buffer pH7.8 containing 5% glycerol, 50mM Tris, pH 8.0 and 2mM EDTA. Cells were sonicated at 30% amplitude and pulse 2 sec on/off for 4 min. The lysates were centrifuged at 10,000 rpm for 10 min. The cell lysates were treated with protease inhibitor cocktail and phenylmethylsulphonyl fluoride (PMSF) (1mM final concentration). Following centrifugation the supernatants containing the remaining soluble cellular proteins were collected. The collected supernatants were treated with 0.2% polyethyleneimine (PEI) and the precipitates were removed by centrifugation at 12,000 rpm for 30 minutes at 4°C. After removing DNA, the lysates were purified by standard Ni-NTA superflow technique under native conditions (IMAC method) (Porath et al., 1975). The supernatants were incubated with pre-treated Ni-NTA resin and washed with buffer containing 20mM imidazole to remove non-specific proteins which were bound to the resin. His tagged E2 proteins were eluted with 250mM imidazole. Eluted purified proteins were checked on 15% SDS-PAGE. Eluted purified proteins were dialysed against stabilizing buffer containing phosphate buffered saline (PBS). Dialysed proteins were checked for purity on SDS-PAGE.

2.2.4.3. Circular dichroism and fluorescence spectroscopy of UBC1 and c-UBC1

CD spectra of UBC1 and c-UBC1 were recorded using a Jasco J-815 CD spectrometer. Far-UV-CD spectra were recorded using 1-mm path length cells in the wavelength range of 190–250 nm with a scan speed of 50 nm/min. The spectra were accumulated five times to improve the signal-to-noise ratio. Protein solutions were prepared in 50 mM PBS buffer, pH 8.0 and concentration of the protein was 0.2mg/ml.

Fluorescence spectra of the samples were recorded using Hitachi F-7000 fluorescence spectrophotometer with excitation and emission band-passes of 5 nm. Protein solutions were prepared in 50 mM PBS buffer, pH 8.0 with protein concentration of (0.2mg/ml). Samples were excited at 280 nm, and the intrinsic fluorescence was recorded in the range of 300–400 nm.

For denaturation studies, UBC1 and c-UBC1 were denatured by guanidine hydrochloride (Sigma) in the range of 0M to 6M concentration, proper blanks were prepared and the spectra were blank corrected.

1-Anilino 8-naphthalene sulphonic acid (ANS) was used as an extrinsic fluorophore at a concentration of 50 μM. ANS was excited at 390nm and emission was recorded between 450-550nm. Slit width was 5nm. Protein concentration was 0.2mg/ml (23.4μM). Protein solutions were prepared in PBS buffer pH8.0.

2.2.4.4. In vitro analysis to check polyubiquitination by c-UBC1

UBC1 is known to auto-ubiquitinate and form polyubiquitin chains (Hodgins et al., 1996). To detect whether c-UBC1 can also have the same activity as UBC1 or not, ubiquitination activity was studied. Ubiquitination assay kit from abcam (ab139467) was used and the reaction was set up according to the protocol provided by the company as described below (**Fig.2.6**).

Assay Protocol

Note: recommended total reaction volume = 50µL

Component	E1-Ub	E1-Ub –ve Control	E2-Ub	E2-Ub –ve Control
	volume / µL			
dH_2O	26.5	24	21.5	19
10X Ubiquitinylation Buffer	5	5	5	5
IPP (100 U/mL)	10	10	10	10
DTT (50 mM)	1	1	1	1
Mg-ATP (0.1 M)	2.5	-	2.5	-
EDTA (50 mM)	-	5	-	5
20X E1 (2 µM)	2.5	2.5	2.5	2.5
10X E2 (25 µM)	-	-	5	5
20X Ub (50 µM)	2.5	2.5	2.5	2.5

Fig. 2.6. Various components used to set up polyubiquitination assay for UBC1 and c-UBC1.

The reactions were set up by mixing reagents as mentioned above and incubated for 1-4 hours at 37°C. After incubation, the reactions were terminated by adding 2X non-reducing gel loading buffer. The samples were further analysed by western blot. 20 µL of each quenched assay solution were applied to 12% SDS-PAGE, along with molecular weight marker and electrophoresed. After electrophoresis, transfer of proteins to a PVDF membrane was done as described under:

Transferring

1) In a shallow tray filled with transfer buffer, place two fiber pads and four pre-cut whatman papers for a few minutes.

2) Disassemble gel apparatus and carefully pry plates apart. Then cut off stacking gel with a clean razor blade and soak gel in transfer buffer for a few minutes.

3) Prepare the transfer sandwich in the tray filled with transfer buffer.

i. One fiber pad

ii. Two whatman papers

iii. SDS gel

iv. PVDF membrane

v. Two whatman papers

vi. One fiber pad

4) Cover the sandwich, fasten with the latch, and insert the gel cassette into the electrode module.

5) Insert the bio-ice cooling unit into the buffer chamber, and fill the buffer chamber with transfer buffer.

Transfer for 1-2 hours at 4 ºC, stirring at a constant current of 100V.

Blocking and Incubating

1) Incubate the membrane with BSA/PBS-T blocking buffer on a shaker for 1-2 hours at 37°C or overnight at 4 ºC.

2) Dilute primary antibody with primary antibody dilution buffer and incubate the membrane with the diluted primary antibody on a shaker for 1 hour at 37 °C or overnight at 4 ºC.

3) Wash the membrane 3 x 10mins with washing buffer on the shaker for 5-10 minutes each time.

4) Dilute anti-mouse IgG secondary antibody with blocking buffer and incubate the membrane with the diluted secondary antibody on a shaker for 1 hour at 37 °C or overnight at 4 ºC.

5) Wash the membrane 6 x 10 mins with washing buffer on the shaker for 5-10 minutes each time.

Detection

Detect protein with ECL kit. In a separate tube, mix two ECL solutions in 1:1 ratio and incubate the membrane with it and visualise the blot on chemidoc imaging system.

2.3. Results

2.3.1. Theoretical analysis of homologs of UBC1 sequences of various phyla

From the information collected from NCBI and multiple sequence alignment done by Clustal Omega it was observed that there are differences in the sequence length and composition of the residues of linker region among various organisms across the phyla as shown in **Table 2.3**. Also, differences were observed in percent identity of the protein sequences.

Table 2.3. Comparison of linker sequence of UBC1 homologs of *Saccharomyces cerevisiae* with organisms of different phyla.

Linker sequence of *S. cerevisiae* (ETSNGQKGNVEESDLYGIDHDL)		
Organisms	**Linker sequence**	**% conservation between protein sequences**
Caenorhabditis elegans	TAVYWTSYFANSKK	38.22
Danio rerio (zebra fish)	GAPVSS	44.33
Nothobranchius kuhntae (Bony fish)	SHVYGGAAVSSPDY	43.30
Callorhinchus milii (Elephant shark)	HVYAGAPVPSPEY	41.54
Xenopus laevis (Frog)	HVYAGAPVTSPEY	42.27
Anolis carolinensis (Green lizard)	VYAGAPVSSPE	43.81

Gallus gallus (chicken)	GAPVSS	40.62
Columba livia (pigeon)	GAPVSS	40.62
Homo sapien	GAPVSS	43.81

2.3.2. Construction of plasmids carrying genes encoding the proteins UBC1 and c-UBC1 in *Saccharomyces cerevisiae*

For generating c-*UBC1*, initially the 5' and 3' regions of gene *UBC1* were amplified using combination of mutagenic and non-mutagenic primers as listed in Table 2.1 (Materials and Method section). The non-mutagenic primers were specifically designed to replace the linker region *UBC1* with that of linker of *E2-25K* gene. A second set of PCR reaction using the amplicons from first reaction as templates against non-mutagenic primers gave us the final product *c-UBC1* (**Fig. 2.7**). Non-mutagenic primers were used to amplify wild type *UBC1*. Restriction enzymes *Bgl*II and *Kpn*I were used to clone the fragments in yeast expression vector YEp96, in place of ubiquitin gene originally present in it. The primary screening of the constructs was done by restriction digestion using *Sau*96 (**Fig. 2.8**). This site is present in 5'-region of *UBC1* as well as in the linker region of *c-UBC1*. Insert release by restriction digestion was used as a test for successful cloning of *c-UBC1* as shown in **Fig. 2.9**. Final confirmation of the construct *c-UBC1* was done by DNA sequencing (**Fig. 2.10**).

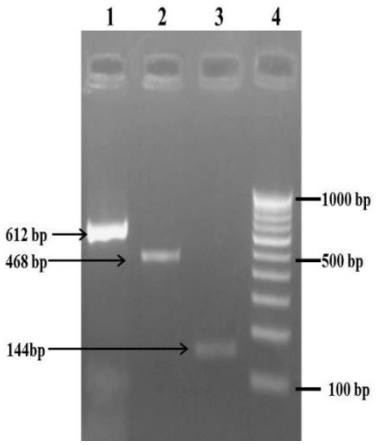

Fig. 2.7. Gel picture showing amplicons of c-UBC1. Lane 1 shows full fragment of *c-UBC1*, lane 2 shows 5'-end, lane 3 shows 3'-endand lane 4 shows DNA marker.

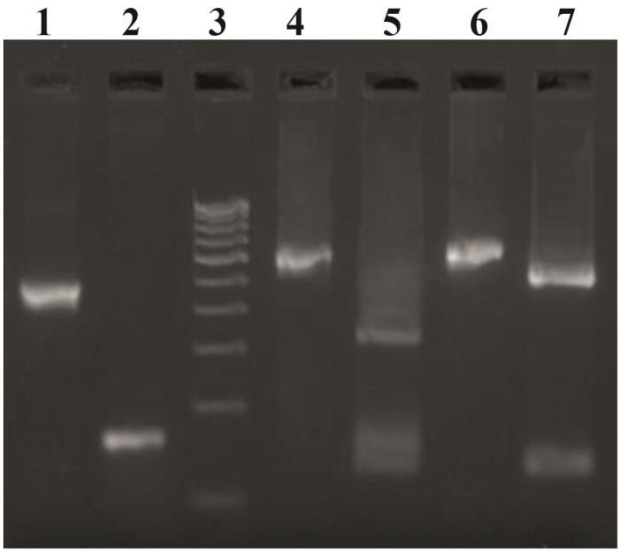

Fig. 2.8. **Gel picture showing screening for c-UBC1 and UBC1 in YEp96.** Lane 1 shows N-terminus amplicon of c-UBC1 (468bp), lane 2 shows C-terminus of c-UBC1 (144bp), lane 3 shows 100 bp ladder, lane 4 shows amplicon for c-UBC1 (612bp), lane 5 shows R.E digestion of c-UBC1 by *Sau96* (fragments 123bp, 143bp and 328bp), lane 6 shows amplicon of UBC1 (645bp) and lane 7 shows R.E digestion of UBC1 by *Sau96* (fragments 121bp and 524bp).

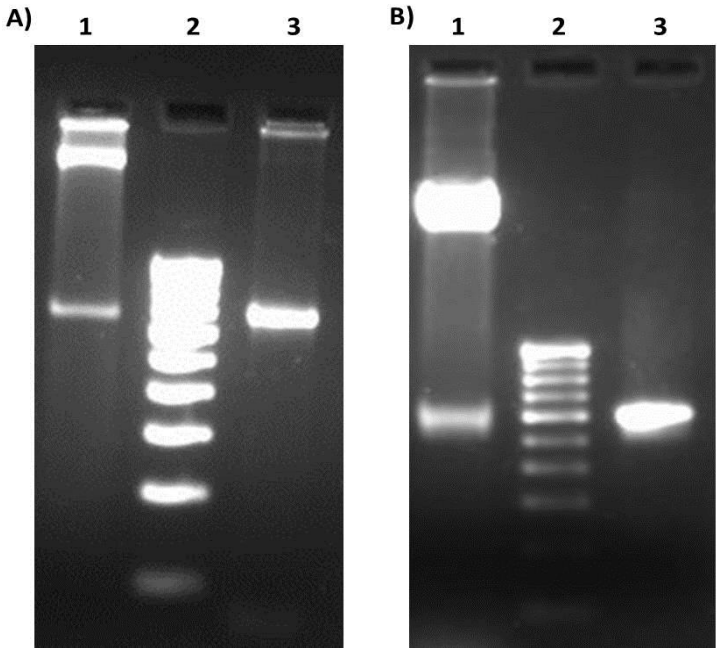

Fig. 2.9. Gel picture showing insert release of UBC1 and c-UBC1 fragments from YEp96 plasmid. A) Lane 1 consists of *UBC1* plasmid

double digested by *Bgl* II and *Kpn I* restriction enzymes. A fragment release of 645bp can been seen which can be compared to 100 bp DNA marker as seen in lane 2 as well as with amplicons of UBC1 in lane 3. B) Lane 1 consists of *c-UBC1* plasmid double digested by *Bgl* II and *Kpn* I restriction enzymes. A fragment release of 612bp can been seen which can be compared to 100 bp DNA marker as seen in lane 2 as well as with amplicons of *c-UBC1* in lane 3.

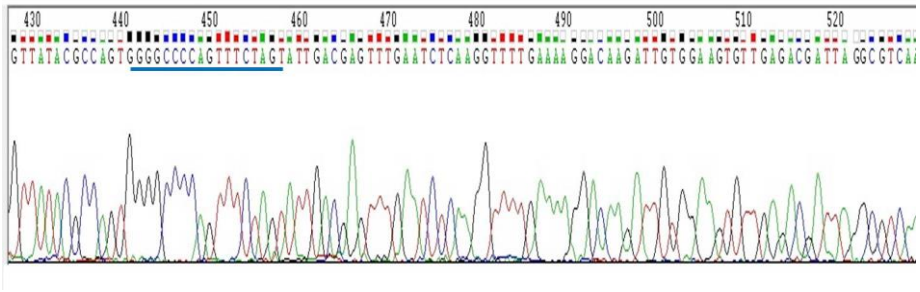

Fig. 2. 10 Confirmation of c-UBC1 in YEp96 by DNA sequencing.

2.3.3. Functional evaluation of chimeric protein c-UBC1 in *S. cerevisiae*

2.3.3.1. Effect of c-UBC1 expression on the growth of *S. cerevisiae*

UBC1 is essentially required in the early stages of growth of the organism during G_0-G_1 transition phase following spore germination (Seufert et al., 1990). Hence, if c-UBC1 loses its structure and becomes non-functional as a result of linker swapping, it may not bind to E1 or E3. Thus, possible functional defect in c-UBC1 may lead to a drop in the rate of protein degradation, leading to retention of substrate proteins, which may eventually affect the growth and survival of the organism. To test this possibility, the chimeric gene under the *CUP1* promoter (copper inducible) was expressed in yeast cells. Along with the cells expressing chimeric protein, cells expressing wild type UBC1 and the untransformed YWO5 with Δubc1deletion mutation were used as positive and negative controls for growth conditions. However, expression of c-UBC1 is not lethal to *S.cerevisiae*, however growth was slowed down when compared to cells expressing UBC1 (**Fig. 2.11** and **Table 2.4**).

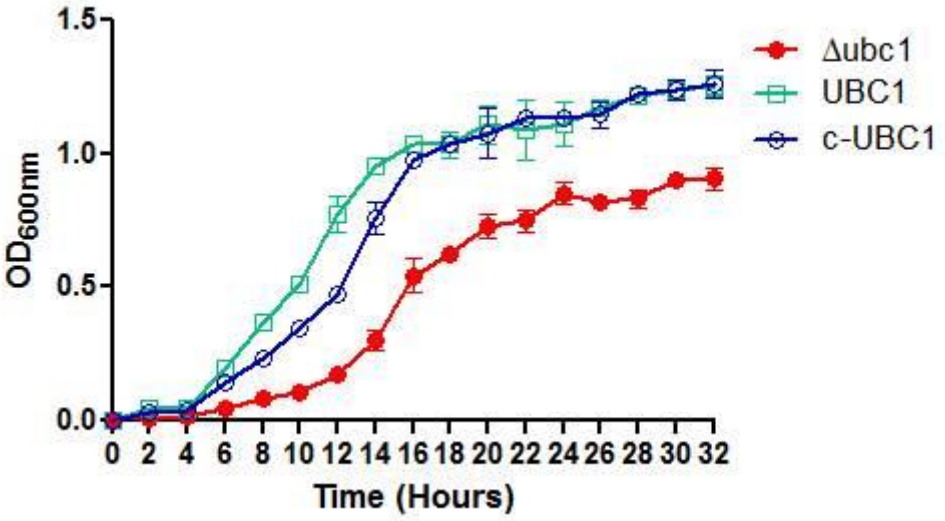

Fig. 2.11. Representative graph showcasing growth profile of YWO5 cells with Δubc1 mutation transformed with plasmids expressing UBC1 and c-UBC1. This experiment had been performed independently three times.

Table 2.4. Doubling times of YWO5 cells with Δubc1 mutation transformed with plasmids expressing UBC1 and c-UBC1. Cultures were grown in YPD liquid medium at 30°C. OD_{600} was followed to calculate the doubling time. This is representative of single experiment which had been carried out independently three times.

Strain	Doubling time in hrs.
Δubc1	2.5
UBC1	1.6
c-UBC1	1.8

2.3.3.2. Complementation of c-UBC1 under heat stress

UBC1 is involved in the turnover of misfolded and denatured proteins. UBC1 plays important role under stress conditions as the proteins tend to undergo denaturation. Cells lacking UBC1 show poor survival under heat stress. Swapping of the linker leads to a chimeric protein decreases length of

the linker. Cells expressing c-UBC1 were subjected to heat stress to test whether the two domains of the protein fold properly and form a functional chimeric protein. The ability to show survival similar to that of UBC1 expressing cells is indication of a functional c-UBC1. Comparison of % survival of cells expressing UBC1 and c-UBC1 established that c-UBC1 was functional and interestingly, c-UBC1 displayed increased resistance under heat stress compared to UBC1 (**Fig. 2.12**).

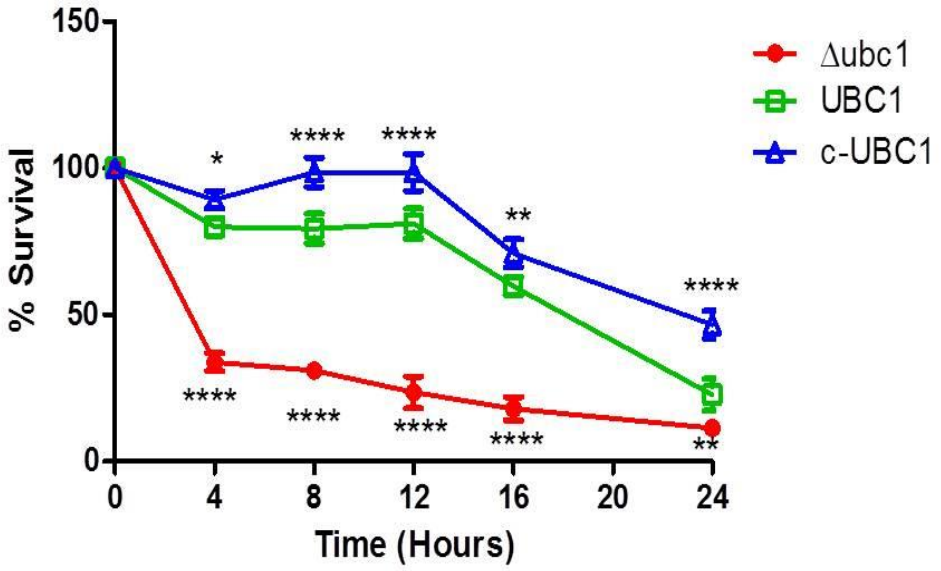

Fig. 2.12. Survival of *Saccharomyces cerevisiae* strain YWO5 transformed with plasmids expressing UBC1 and c-UBC1 under heat stress (37°C) at various time intervals. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (n=3 independent experiments repeated in triplicate).

2.3.3.3. Complementation of c-UBC1 under thermotolerance

Yeast cells can withstand higher temperatures if they are subjected to increased temperatures in two steps, a pretreatment step and a heat shock step. During pretreatment step they develop thermotolerance by expressing chaperones for refolding the proteins which have undergone denaturation at higher temperatures. When the chaperones cannot fold the denatured proteins, ubiquitin proteasome system comes to rescue by degrading them. When the

cells are given heat shock after pretreatment they show better survival than those cells which are subjected to heat shock directly. Thermotolerance levels of cells expressing UBC1 and c-UBc1 were compared. YWO5 cells carrying Δubc1 mutation were used as negative control. The results of thermotolerance established that cells expressing c-UBC1 showed better thermotolerance than those expressing UBC1 (**Fig. 2.13**).

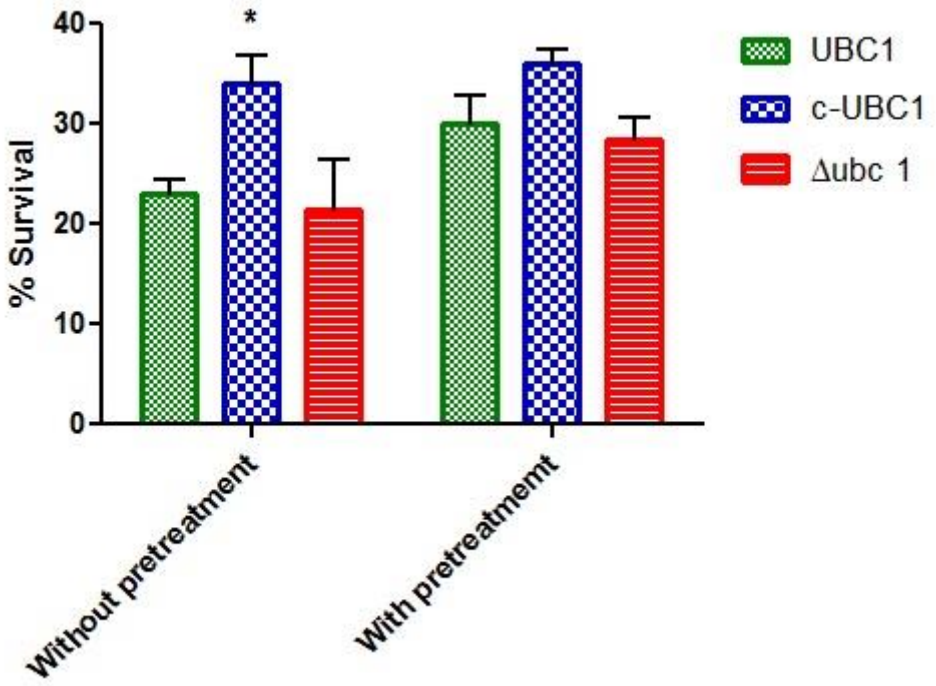

Fig. 2.13. Graph showing thermotolerance profiles of *Saccharomyces cerevisiae* strain YWO5 with Δubc1 mutation transformed with plasmids carrying genes for UBC1 and c-UBC1. p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 (n=3 independent experiments repeated in triplicate).

2.3.3.4. Complementation of c-UBC1 under protein translation inhibitors

Translational inhibitors lead to accumulation of truncated proteins. UPS degrades truncated proteins. To evaluate the involvement of c-UBC1 in ubiquitin- mediated turnover, we examined the sensitivity of it towards various protein translation inhibitors and found that, c-UBC1 exhibited almost similar resistance as UBC1 suggesting that c-UBC1 also behaves as an

enzymatic component of the ubiquitin-mediated proteolysis involved in selective degradation of proteins (**Fig. 2.14**).

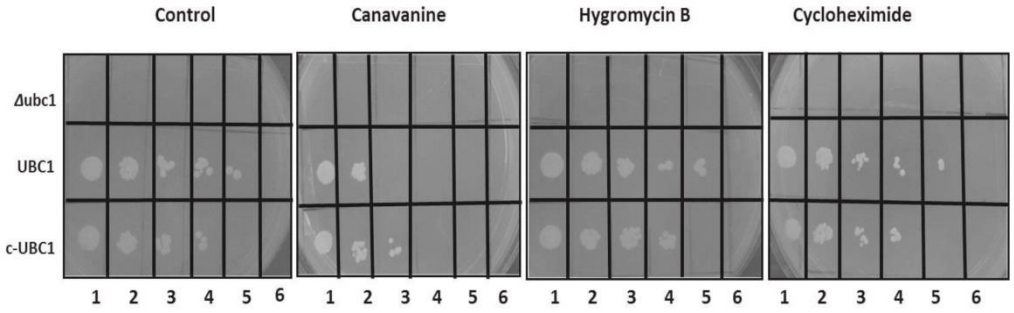

Fig. 2.14. Antibiotic stress complementation of *Saccharomyces cerevisiae* strain YWO5 by variants of E2 namely, UBC1 and c-UBC1. The strain Δubc1 and UBC1 were used as negative and positive controls respectively. The transformants expressing UBC1 and c-UBC1 were tested for resistance to L-canavanine, hygromycin-B and cycloheximide antibiotics. Undiluted stock and fivefold serial dilutions (2), (3), (4), (5) and (6) were spotted on SDA plates in the presence and absence of antibiotics. 100μM copper sulphate was used as an inducer.

2.4. Construction of *UBC1* and *c-UBC1* in bacterial expression vector for structural studies

For the construction of *UBC1* and *c-UBC1* in bacterial expression vector pET 28a, the genes were amplified from YEp96 vector and subjected to *Nco*I and *Xho*I double digestion. After restriction digestion the genes was ligated in the pET-28a vector by using the same restriction enzyme sites. The genes were confirmed by insert release and PCR amplification (**Fig. 2.15**).

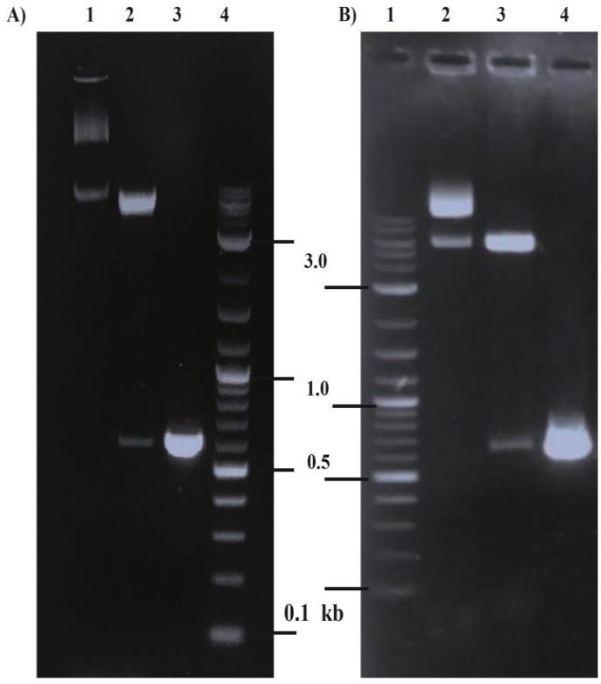

Fig. 2.15. **Gel picture showing confirmation of cloning of *UBC1* and c-*UBC1* in pET 28 by insert release.** A) Lane 1 contains plasmid *UBC1*, lane 2 shows insert release (645bp) of *UBC1* gene from plasmid after double digestion by *Nco I* and *Xho I* restriction enzymes, which matches with the PCR product as shown in lane 3 and lane 4 contains DNA ladder. B) Lane 1 contains plasmid c-UBC1, lane 2 shows insert release (612bp) of *c-UBC1* from plasmid after double digestion by *Nco I* and *Xho I* restriction enzymes, which matches with the PCR product as shown in lane 3 and lane 4 contains DNA ladder.

2.4.1. Expression, purification and dialysis of UBC1 and c-UBC1

The proteins UBC1 and c-UBC1 cloned in pET-28a vector were expressed in BL21 (DE3) under the control of IPTG inducible T7 promoter. Cells were induced by IPTG in mid-log phase and observed on 15% SDS-PAGE to confirm over-expression of UBC1 and c-UBC1 proteins. Both UBC1 and c-UBC1 were purified by methods described in detail under Materials and Methods section 2.2.3.2 (**Fig. 2.16 and Fig. 2.17**).

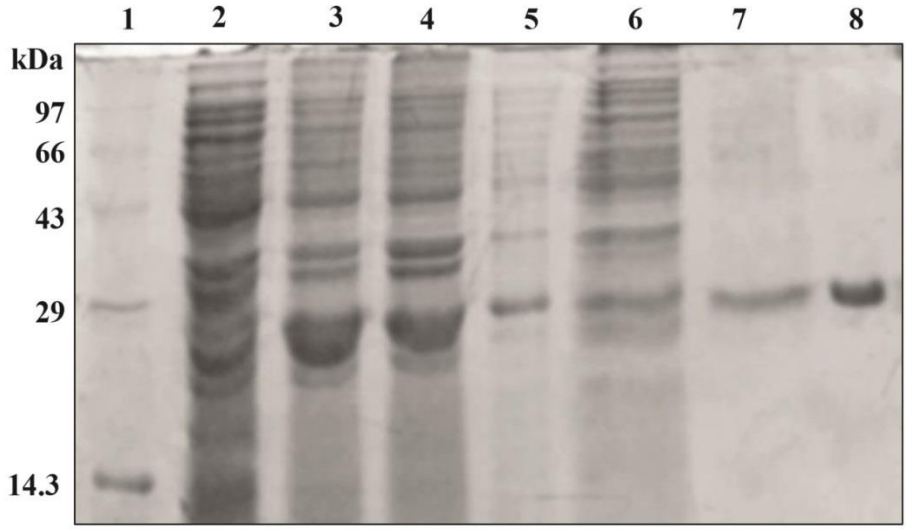

Fig. 2. 16. Gel profile showing over expression and purification of UBC1. Lane 1 displays protein marker, lane 2 contains uninduced UBC1, lane 3 shows induced UBC1, lane 4 contains pellet after sonication, lane 5 contains supernatant after sonication, and lane 6 contains flow through sample collected during purification; while lane 7 and lane 8 contain eluted fractions.

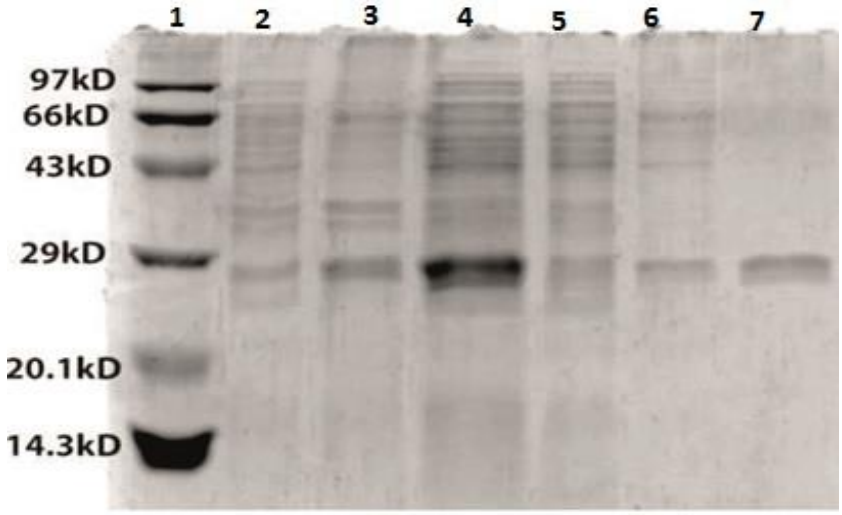

Fig. 2. 17. Gel profile showing over expression and purification of c-UBC1. Lane 1 displays protein marker, lane 2 contains uninduced c-UBC1, lane 3 shows induced c-UBC1, lane 4 contains supernatant after sonication, and lane 5 contains flow through sample collected during purification; while lane 6 and lane 7 contain eluted fractions.

After purification the purified proteins were dialysed against stabilizing buffer containing PBS. Dialysed proteins were observed on SDS-PAGE for homogeneity (**Fig. 2.18**).

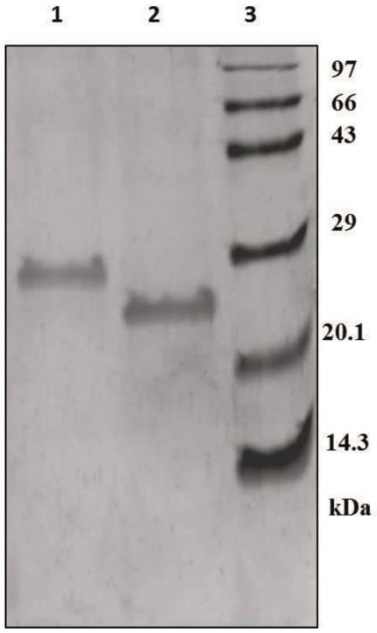

Fig. 2.18. Gel picture displaying purified proteins UBC1 and c-UBC1. Lane 1 shows UBC1 (24.2kDa), lane 2 shows c-UBC1 (21.8kDa) and lane 3 shows protein marker.

2.4.1.1. Circular dichorism and fluorescence spectra

Far-UV CD spectra of UBC1 and c-UBC1 were recorded to study the changes in secondary structure arising due to linker swapping in the sequence of ubiquitin conjugating enzyme, UBC1. The spectra of the wild type UBC1 and its chimeric construct c-UBC1 shown in **Fig. 2.19,** indicate that there are a few changes in secondary structural features of the proein c-UBC1 compared with control UBC1. The far-UV CD spectra were analyzed using two software's namely, Phyre^2 and BeStSel (Kelley et al., 2015; Micsonai et al., 2018). The results presented in **Table 2.5** are compared with the values reported in Protein Data Bank after NMR studies of UBC1 (1TTE) (Merkley and Shaw, 2004).

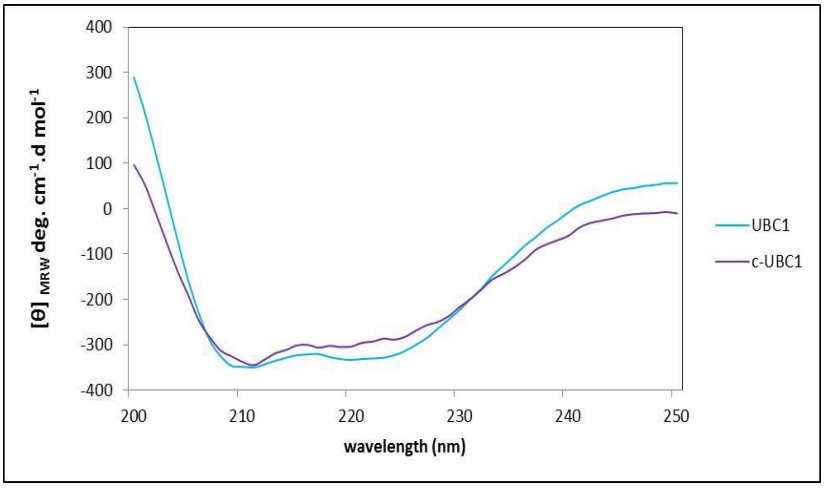

Fig. 2. 19. Far-UV CD spectra of UBC1 and c-UBC1.

Table 2.5. Values of various secondary structures obtained from CD spectral analysis of UBC1 and c-UBC1 done by BeStSel and Phyre softwares (Kelley et al., 2015; Micsonai et al., 2018) The values of UBC1 were compared to Protein Data Bank values obtained from its NMR structure (Merkley and Shaw, 2004).

Secondary structure		**UBC1**	**c-UBC1**
% α-helix	Values obtained from CD spectra	33.1	25.5
	Values reported by Phyre[2]	37.2	38.6
	PDB Value	35	-
% β-sheet	Values obtained from CD spectra	9.3	14.6
	Values reported by Phyre[2]	13.4	12.5
	PDB Value	11	-
Turns/Coils	Values obtained from CD spectra	57.6	59.9
	Values reported by Phyre[2]	49.4	48.9
	PDB Value	54	-

Observation of changes in secondary structure prompted investigations on protein stability, which were carried out using fluorescence spectroscopy. Guanidine hydrochloride (GdnHCl) denaturation of UBC1 and c-UBC1 were studied by fluorescence spectroscopy. Increase in concentration of guanidine hydrochloride causes denaturation of secondary and tertiary structures of a protein leading to increase in the fluorescence intensities, along with a red shift in λ_{max} of emission. Hence, stability of a protein can be assessed by measuring the fluorescence intensities and the shift in λ_{max} of intrinsic fluorescence spectra, as the protein which is thermodynamically stable tends to lose its structure at higher concentration of GdnHCl. Both UBC1 and c-UBC1 showed increase in fluorescence intensity with increase in GdnHCl concentration. At 0M GdnHCl the λ_{max} values of UBC1 and c-UBC1 are 339 nm and 342.2 nm respectively (**Fig. 2.20 and Fig. 2.21**). Concentration of GdnHCl required to bring about half denaturation is referred to as $C_{1/2}$. The $C_{1/2}$ value of c-UBC1 is less than that of UBC1. λ_{max} values of UBC1 and c-UBC1 have been plotted (**Fig. 2.22)**.

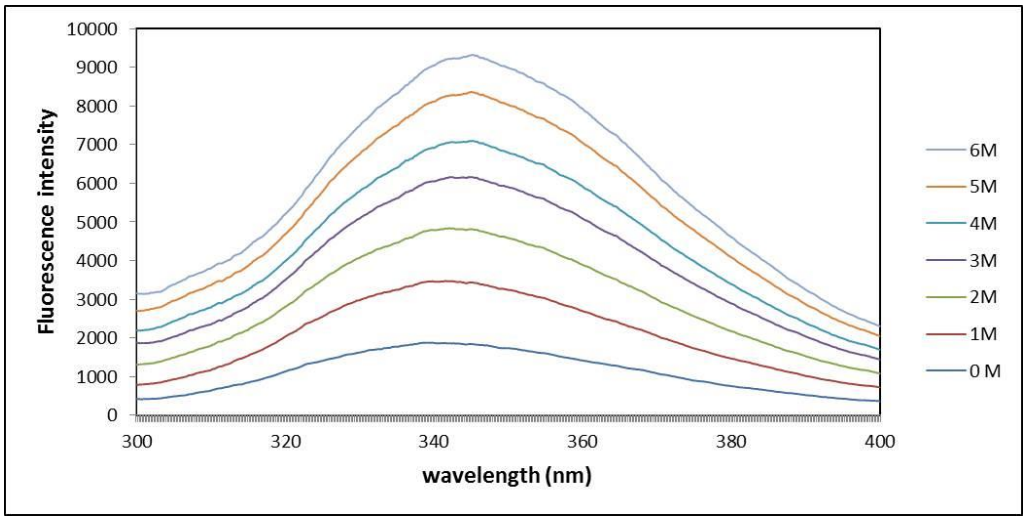

Fig. 2.20. Intrinsic fluorescence emission spectra of guanidine hydrochloride denaturation of UBC1.

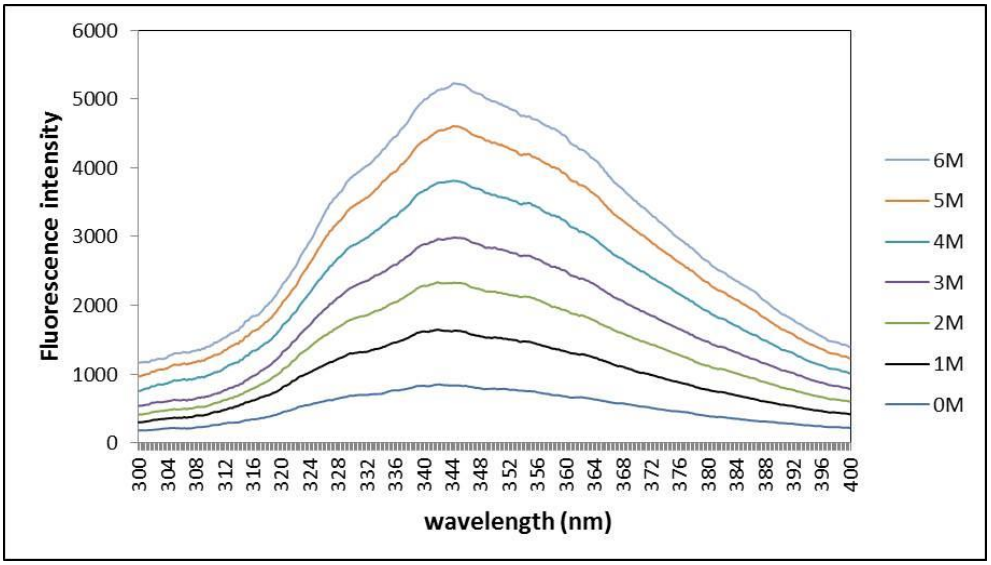

Fig. 2.21. Intrinsic fluorescence emission spectra of guanidine hydrochloride denaturation of c-UBC1.

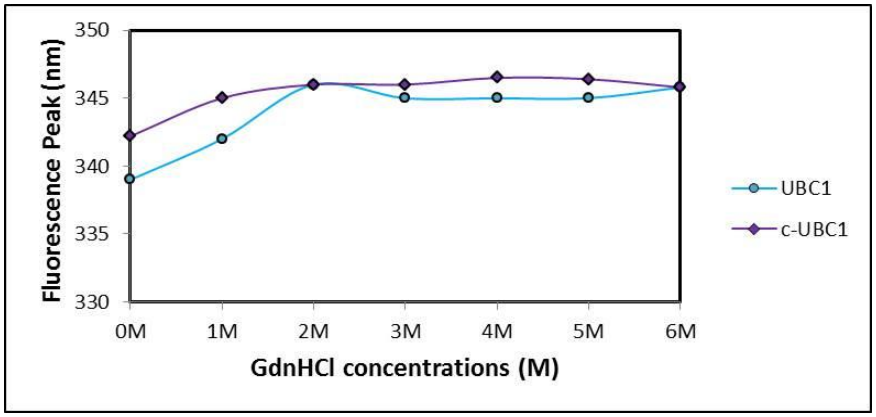

Fig. 2.22. Change in λ_{max} values of guanidine hydrochloride denaturation curves of UBC1 and c-UBC1.

1-anilinonaphthalene-8-sulphonate (ANS), an extrinsic fluorophore was used to check surface hydrophobicity of proteins. ANS binds to exposed hydrophobic residues on the surface of a protein and emits fluorescence (Hawe et al., 2008). To examine changes in the protein structure, ANS was added to the proteins, in the presence of various concentrations of guanidine hydrochloride. With increasing concentrations of guanidine hydrochloride, both UBC1 and c-UBC1 showed increase in fluorescence intensity, revealing that hydrophobic residues which were initially buried in the core, became

increasingly accessible for binding to ANS with denaturation. Our results show that there is not much difference in the exposure of hydrophobic residues in both proteins (**Figs. 2.23 and 2.24**).

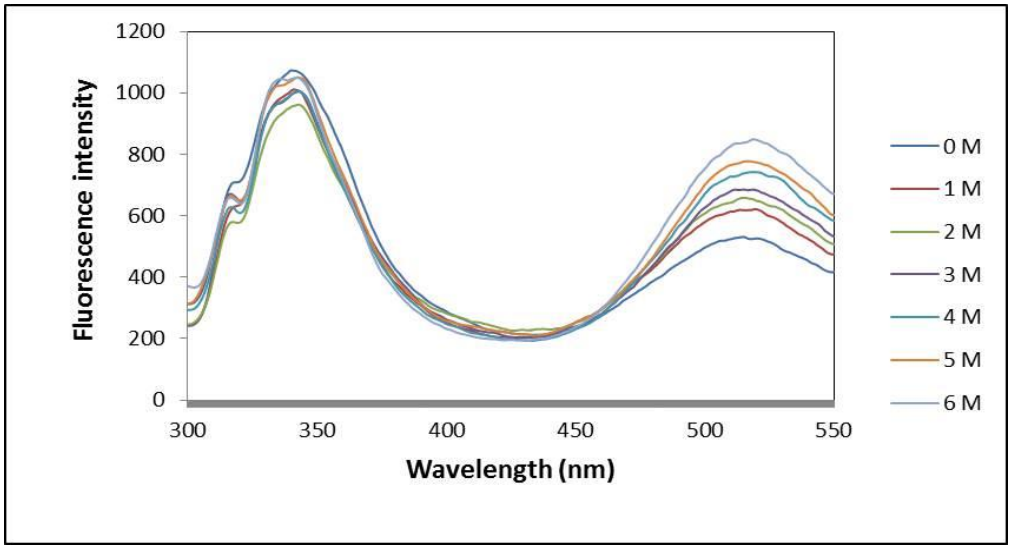

Fig. 2.23. Fluorescence resonance energy transfer spectra showing the fluorescence of extrinsic fluorophore ANS bound to UBC1

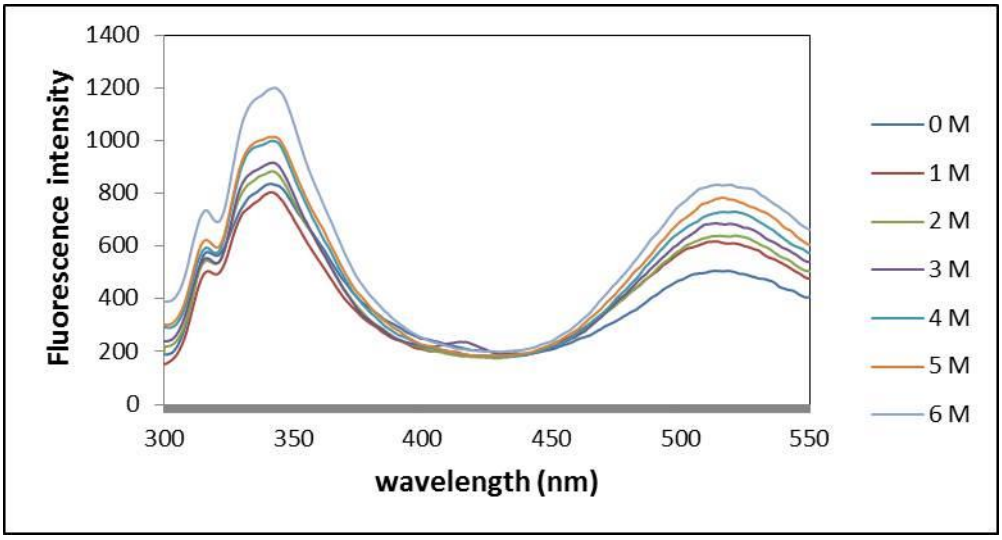

Fig. 2.24. Fluorescence resonance energy transfer spectra showing the fluorescence of extrinsic fluorophore ANS bound to c-UBC1.

2.4.1.3. Polyubiquitination assay

Ubiquitination is a result of three enzymes E1, E2 and E3 acting in a cascade. In the presence of Mg^{2+} cofactor, ubiquitin is activated by the ubiquitin activating enzyme (E1) which catalyzes the formation of a reactive thioester bond with ubiquitin, in an ATP-dependent process. This is followed by its subsequent transfer to the active site cysteine of a ubiquitin conjugating enzyme (E2). Generally, subsequent association of the E2-ubiquitin with a substrate specific ubiquitin-protein ligase (E3) leads to ubiquitin ligation between ubiquitin and its target protein. However, in this case ubiquitin itself reacts as a substrate and E3 ligase is not required as UBC1 is known to show poly-ubiquitination activity in vitro (Hodgins et al., 1996). Our results show that, c-UBC1 shows same poly-ubiquitination activity as UBC1 (**Fig. 2.25**) and hence shorter linker did not have any negative influence over the structure of and relative orientations of the N-terminal or C-terminal domains or their overall function.

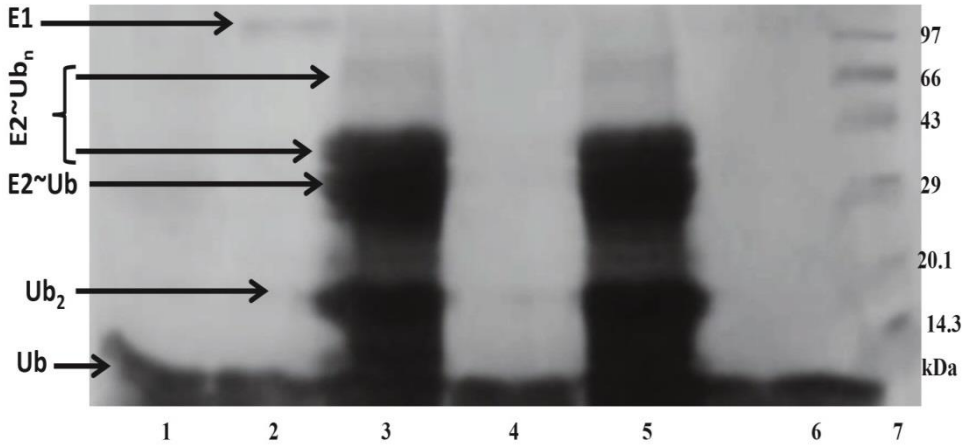

Fig. 2.25 Western blot of ubiquitin thioester assay of the E2 conjugating enzymes UBC1 and c-UBC1. Ubiquitin-enzyme conjugates were detected by western blotting using the ubiquitin antibody. The blot displays E1-Ub (Lanes 1 and 2), UBC1-Ub (Lanes 3 and 4), and c-UBC1-Ub (Lanes 5 and 6). The enzyme reactions were carried out in the presence of Mg-ATP co-factor (in lanes 2, 3 and 5) and in the absence of Mg-ATP co-factor (in lanes 1, 4 and 6).

Lane 7 is of marker. Results demonstrate the formation of polyubiquitin by c-UBC1 similar to UBC1.

2.5. Discussion

It is well known that E2s are key mediators of the ubiquitination pathway. So far, the best examples explored among E2s are UBC1 and E2-25k. E2-25K shows high degree of sequence and structural similarity with the cores of UBC1, UBC4, and UBC5 proteins of yeast and their homolog in higher organisms (Haldeman et al., 1997). Moreover, UBC1 and E2-25k are class II E2s consist of UBA domain C-terminal to UBC domain. The UBA domain in both UBC1 and E2-25k has been found to play crucial role in the assembly of long poly-Ub chains (Hodgins et al., 1996). Besides, UBC1 and E2-25k also play role in polyglutamine protein aggregation (Howard et al., 2007). E2-25K has been shown to synthesize polyubiquitin chains independent of any E3 ligase. However, UBC1 has been shown to catalyse polyubiquitin synthesis in vitro, there are no reports on polyubiquitin synthesis by UBC1 in vivo.

E2-25K and UBC1 show a major difference in the length of their linkers. Studies have revealed that domain linkers can play an essential role in maintaining cooperative inter-domain interactions (Gokhale and Khosla, 2000). In general, linkers are defined as flexible regions connecting two adjacent domains within modular proteins. Although linkers are highly divergent in their lengths and sequences, some show a bias toward certain amino acids, such as proline, glycine, serine, and threonine (Sammond et al., 2012). Some studies have also shown that linker length plays a fundamental role as is the case involving activity of processive cellulases, because shortening or deleting the linker reduces enzymatic activity on crystalline cellulose (Srisodsuk et al., 1993). Also, linker length appears to play a role in the thermal adaptation of enzymes (Batista et al., 2011; Sonan et al., 2007).

Here, we swapped the mammalian E2-25K linker for yeast UBC1 to study the effect of length of linker on protein folding and in vivo characterization. Subsequently, this may either result in the inhibition of the degradation process or may follow the normal route via ubiquitin conjugation.

We modeled the secondary structure of c-UBC1 using online Bioinformatics tool SWISS-MODEL (Waterhouse et al., 2018) and compared its structure with UBC1 as shown in **Fig. 2.26 and Fig. 2.27.** However, the subtle changes observed in far-UV CD spectra are not visible here, as modeling is based on information already existing in literature and does not successfully reflect global changes in when short stretches of sequence are replaced in proteins.

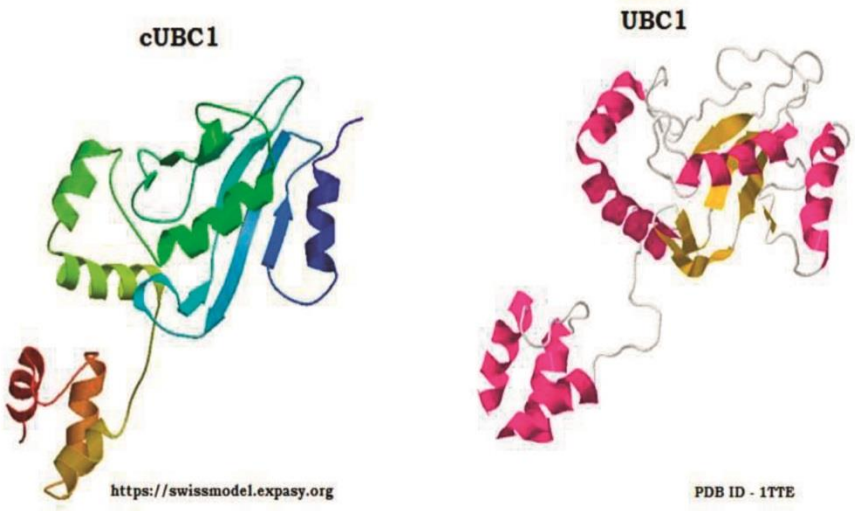

Fig. 2.26. Secondary structure prediction of c-UBC1 using SWISS-MODEL and its comparison to UBC1.

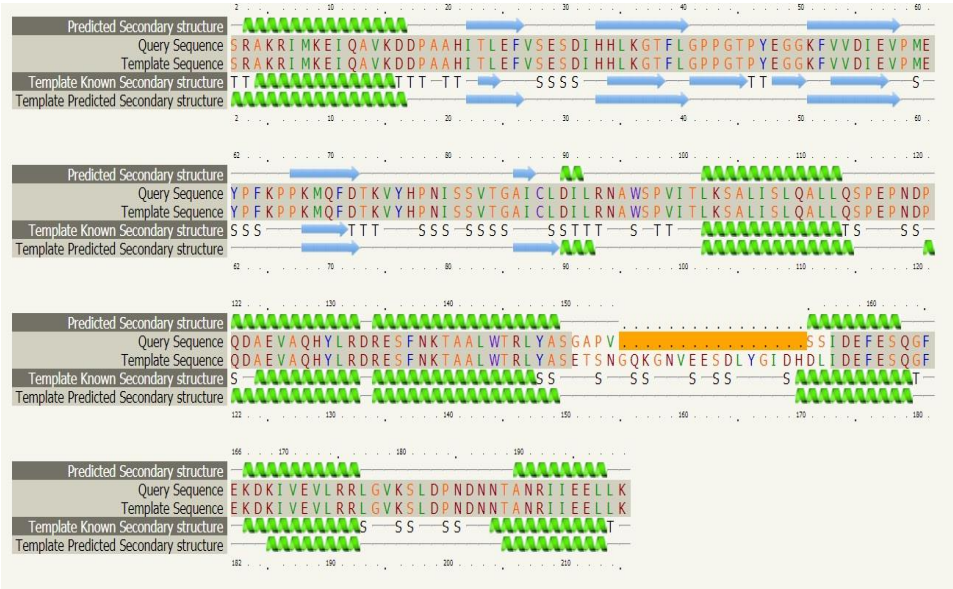

Fig. 2.27. Secondary structure prediction of c-UBC1 using Phyre[2] (Kelley et al., 2015) and its comparison to UBC1 (chosen as template by the database).

The fluorescence spectra studies revealed that c-UBC1 is a stable protein and has a compact structure which unfolds in presence of increasing guanidine hydrochloride concentrations like UBC1. The CD spectra displayed subtle changes with respect to c-UBC1's secondary structure. The increased proximity between the domains possibly reduced the α-helical content, probably in the regions adjacent to linker region. The results of fluorescence studies supported the observations made in CD spectra. There are several intrinsic fluorophores present in and adjacent to linker region of UBC1. While Trp144, Tyr148 present very close to linker, were probably affected by changes in the environment in c-UBC1, while residue Y166 was completely lost in the process of swapping, which may explain the changes observed in intrinsic fluorescence with c-UBC1 with respect to UBC1.

c-UBC1 displayed polyUb activity similar to that of UBC1. UBC1 has a flexible 22 residues linker which allow N and C terminal domain of the enzyme to work with greater spatial independence. Whereas, E2-25k has 6 residues linker and the resulting proximity forces extensive interactions between the two domains resulting in a single globular topology (Ko et al.,

2010a). Hence, it was hypothesized that because of the structural differences, the polyubiquitin synthesis activity of c-UBC1 would be affected. However, polyubiquitin activity of c-UBC1 remained unaffected by swapping of the linker.

The protein sequence analysis of UBC1 homologs of various organisms revealed that, a) there is difference in their % conservation and b) linker region is not consistent but variable with respect to length as well as residue composition. As we go towards higher phylum it was observed that linker region has reduced in length.

In vivo experiments conclude that overall c-UBC1 functions similar to UBC1 under normal as well as stress conditions except heat stress. In conclusion, c-UBC1 construct having the E2-25k linker gave a functionally active enzyme. Secondary structure of the enzyme showed some changes due to decreased length of linker as the two domains were brought into proximity. Enzyme c-UBC1 performed equally well in the yeast system as compared to UBC1 with original yeast linker suggesting that linker length does not have any significance in relation to the functions tested and hence it could be concluded that during the course of evolution, the linker must have incorporated changes which would not interfere with the protein's function.