Structural and Functional Characterization of Chimeric Ubiquitin Conjugating Enzyme (c-UBC1) in *S.cerevisiae*

Varsha Raimalani and C. Ratna Prabha*

Department of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara – 390002, INDIA

Introduction

In eukaryotes, post-translational modification of proteins by ubiquitin conjugation is catalysed by ubiquitin machinery. It comprises of three enzymes, ubiquitin activating enzyme (E1) that binds free ubiquitin and transfers it to ubiquitin conjugating enzyme (E2), which further transfers ubiquitin to substrate with the help of ubiquitin ligase (E3). In the present study, we have focused on yeast UBC1 (E2) which plays important role in degradation of short-lived and abnormal proteins[1]. It is also essential for cell growth and viability. The yeast UBC1 homolog in humans is E2-25K, which is one of the crucial components for regulation of key pathways involved in neurodegenerative disorders[2] .

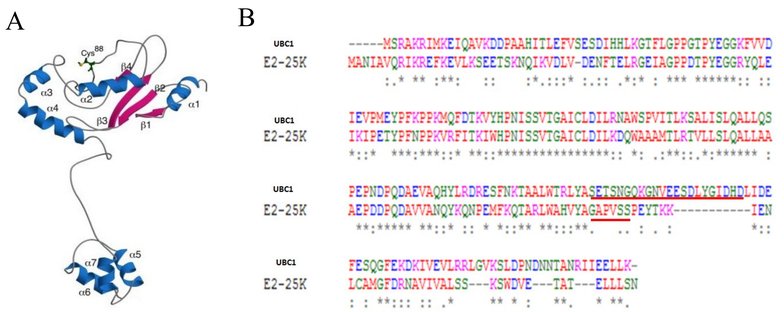
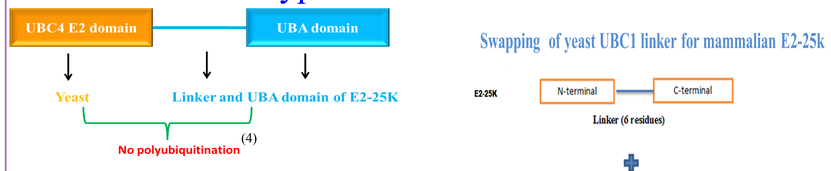
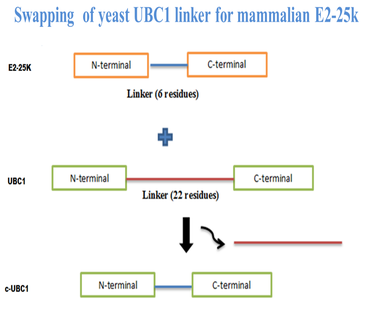


Figure1 A) Secondary structure of UBC1[3]. **B)** Sequence alignment of UBC1 and E2-25K using clustal omega.

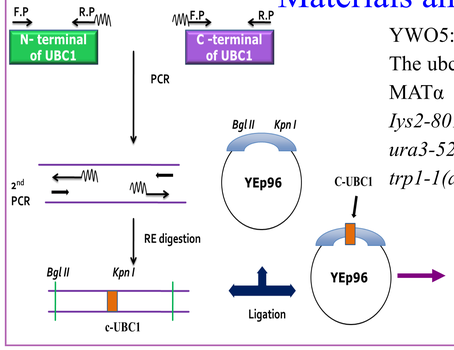
Hypothesis and Rationale





- E2-25K interacts with UBB^{+1} leading to amyloid –β neurotoxicity
- Able to synthesize Lys^{48} linked free polyubiquitin chains in the absence of an E3 ligase
- Both UBC1 and E2-25k belonging to the same class (I) of E2s can form polyubiquitin chains then why there is difference in the linker region?

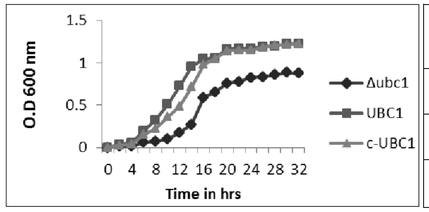
Materials and method



YWO5: ubc1::HlS3 was used for *in vivo* studies. The ubc mutant strain used in this study is haploid MATα derivative of strain DF5 *(MATα/MATα, lys2-801/lys2-801, leu2-3,2-112/leu2-3, 2-112, ura3-52/ura3-52, his3Δ200/his3Δ200, trp1-1(am)/ trp1-1(am)*; Finley et al., 1987).

Sub Cloning into Bacterial Expression Vector for purification and structural studies

Results



Sample	Doubling time in hours
Δubc1	2.5
UBC1	1.6
c-UBC1	1.8

Figure2 Growth profile of Δubc1 cells of *S. cerevisiae* transformed with plasmids expressing YEp96/UBC1 WT and mutant YEp96/c-UBC1

	Survival after 5 min at 52°C without induction	
	Without pre-treatment (%)	After pre-treatment (%)
Δubc1	21.5	25.5
Yep96/UBC1	23	30
YEp96/c-UBC1	34	36

Table1 Resistance to thermotolerance of YEp96/UBC1WT andYEp96/c-UBC1. For pre-treatment cultures were shifted to 37°C for 1 hr. Prior to or after incubation cultures were shifted to 52°C. Appropriate aliquots were spread on minimal agar plates containing the selection. UBC1 and Δubc1 were used as controls. Numbers give the fraction of colonies formed after heat treatment.

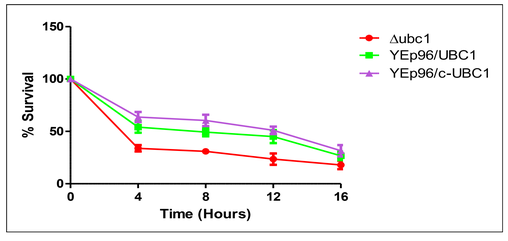


Figure3 Survival of YEp96/UBC1 and YEp96/c-UBC1 under heat stress (37°C) at various time intervals. UBC1 and Δubc1 were used as controls.

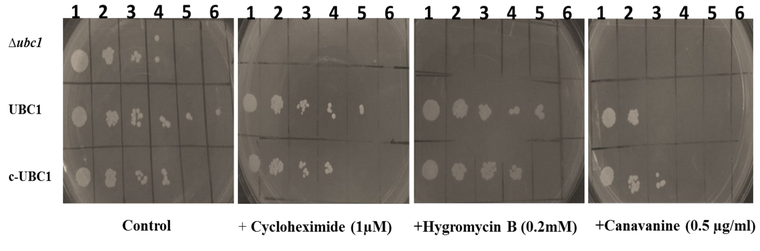


Figure4 Antibiotic stress complementation of *Saccharomyces cerevisiae* Δubc1 strain by variants of E2 (UBC1). The strain Δubc1 and Yep96/UBC1WT were used as controls. The transformants expressing Yep96/UBC1 and Yep96/c-UBC1 were tested for L-canavanine, hygromycin-B and cycloheximide resistance. Undiluted stock and fivefold dilutions (2), (3), (4),(5) and (6) were spotted on SDA plates consisting of antibiotics

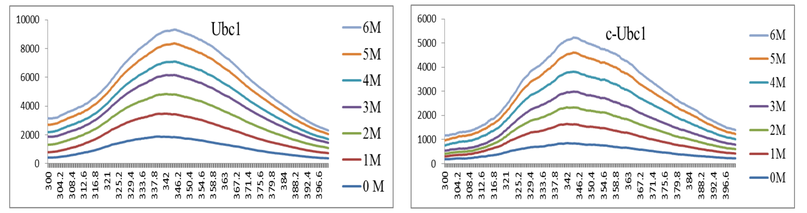


Figure 5 Gaunidinium chloride denaturation curves of Ubc1 and c-Ubc1. The denaturation of the proteins was followed by recording the intrinsic fluorescence spectra by exciting it at 280 nm. The wavelength of maximum emission was plotted against concentration of guanidinium chloride

Conclusion

- c-UBC1 was found to be functionally active and comparable with UBC1
- Compared to UBC1, c-UBC1 showed increased resistance under heat stress and similar resistance under thermotolerance
- c-UBC1 can not resist to canavanine as compared to other two translation inhibitors suggesting that it behaves as enzymatic component of the ubiquitin-mediated proteolysis involved in selective degradation of proteins
- With change in concentrations of GuHCL, change in fluorescence intensity was observed which indicates that the protein has a compact structure
- Thus, c-UBC1 with shorter linker behaved equally well in the yeast system as compared to longer linker suggesting it to be non-essential.

References

1. Seufert,W., Mcgrath, J. P., and Jentsch, S. (1990). The EMBO Journal Vol.9 no.13, pp.4535-4541
2. Sunggeon Ko, *et al.*,(2010). *JBC*, Vol. 285, no. 46, pp. 36070–36080
3. Nadine Merkley and Gary S. Shaw (2004). *JBC,* Vol. 279, no. 45, pp. 47139–47147, 2004
4. Haldeman, *et al.*,(1997). *JBC,* Vol*36,* no. 34, pp.0526-10537

Acknowledgement

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)

Contact Information

Prof. C. Ratna Prabha
chivukula_r@yahoo.com
Macromolecular Structure & Biology Laboratory
Department of Biochemistry, Faculty of Science,
The Maharaja Sayajirao University of Baroda
Vadodara – 390002,Gujarat, INDIA

Functional Characterization of Ubiquitin Conjugating Enzyme UBC4 with N82S Mutation in *Saccharomyces cerevisiae*

Varsha Raimalani and C. Ratna Prabha*

Department of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara – 390002, INDIA

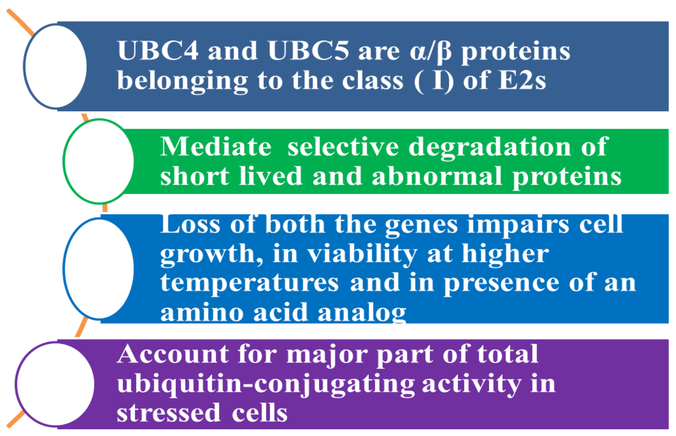
Abstract

In eukaryotes, post-translational modification of proteins by ubiquitin conjugation is catalyzed by a cascade of enzymes namely, ubiquitin activating enzyme which binds free ubiquitin and transfers it to ubiquitin conjugating enzyme, which further transfers ubiquitin to substrate with the help of ubiquitin ligase. In the present study we are focusing on ubiquitin conjugating enzyme (E2) UBC4 of *S. cerevisiae*. UBC4 and UBC5 are α/β proteins belonging to the same class of E2s with ~ 92% sequence identity, differing only at 11 amino acid residues. The two E2s functionally complement each other in mediating selective degradation of short lived and abnormal proteins[1]. Hence, they are essential during stress response. The reason behind maintenance of two identical proteins suggests specialized functions for these enzymes under certain selective conditions. To understand the reason for the residue differences between the two E2s, we have decided on creating single mutations in UBC4 by replacing the residue from UBC5 and studying the functional differences arising out of the substitutions. Here, the gene of UBC4 with N82S mutation was constructed and introduced in *Δubc4ubc5* strain of *S. cerevisiae* and functional studies were carried out. We found that this mutation is functionally active. Moreover, it displays better resistance to various translational inhibitors and greater thermotolerance compared to Ubc4 and even wild type (WT). However, it showed less resistance when tested for heat stress. N82 is present in β-turn of the protein. Using the set of potential given by Hutchinson and Thornton in 1994, the preferences of Asn and Ser were compared for (i+1) position in β-turn. Interestingly, Ser showed higher preference over Asn for adopting the structure. This supports our *in vivo* data and proves to be an important mutation for UBC4.

Introduction

In eukaryotes, Ubiquitin Conjugating enzymes ensure protein quality control via the Ubiquitin Proteasome system. The system is highly substrate specific and comprises of single E1, several E2s and hundreds of E3s. E2 family comprises of a conserved UBC domain providing a binding platform for E1, E3s and activated ubiquitin. E2s actively participate in ubiquitin chain assembly reaction in the UPS. In the present study we are focusing on ubiquitin conjugating enzyme UBC4 of *S. cerevisiae* system.

(A)



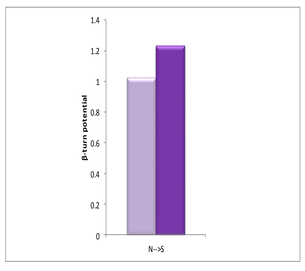
(B)

```
Ubc4   MSSSKRIAKELSDLERDPPTSCSAGPVGDDLYHWQASIMGPADSPYAGGVFFLSIHFPTD 60
Ubc5   MSSSKRIAKELSDLGRDPPASCSAGPVGDDLYHWQASIMGPSDSPYAGGVFFLSIHFPTD 60
       ************ ****:**********************:******************

Ubc4   YPFKPPKISFTTKIYHPNINANGNICLDILKDQWSPALTLSKVLLSICSLLTDANPDDPL 120
Ubc5   YPFKPPKVNFTTKIYHPNINSSGNICLDILKDQWSPALTLSKVLLSICSLLTDANPDDPL 120
       *******:.**********:.**************************************

Ubc4   VPEIAHIYKTDRPKYEATARENTKKYAV 148
Ubc5   VPEIAQIYKTDKAKYEATAKENTKKYAV 148
       *****:*****:.******:********
```

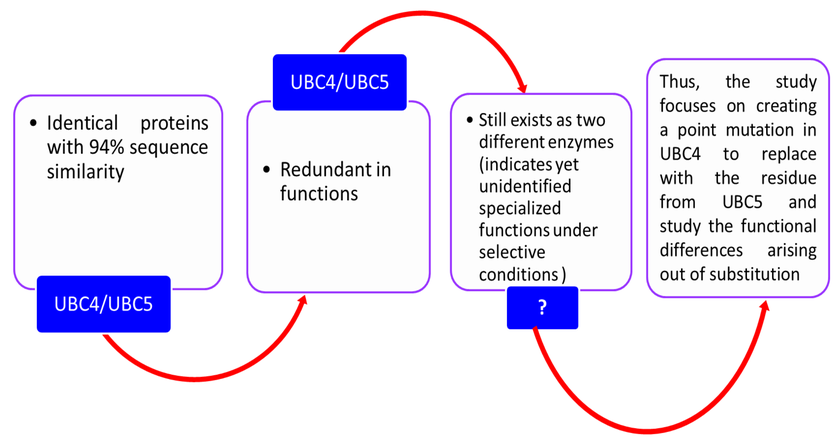
(C)



Position of the amino acid	Substitution	β-turn value	Residue
82			
Ubc4	N	1.02	(i+1)
Ubc5	S	1.23	of turn

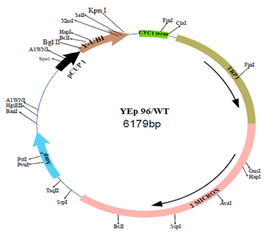
Figure 1. (A) Functions of UBC4 & UBC5 (B) Sequence alignment of two proteins. (C) Comparison of β-turn propensities of residue no. 82 in Ubc4 & Ubc5 according to Hutchinson *et al.*,1994

Hypothesis



Materials and Methods

The *S. cerevisiae* strains used in the study are Wild type cells (Mat α his3-Δ200 leu2-3,112 ura3-52, lys2-801, trp1-1, gal 2), Δubc4Δubc5 *(*Mat α his3-Δ200 leu2-3,112 ura3-52, lys2-801, trp1-1, ubc4-Δ1::HIS3 ubc5-Δ1::LEU2) is a double *knock out* strain lacking UBC4 and UBC5

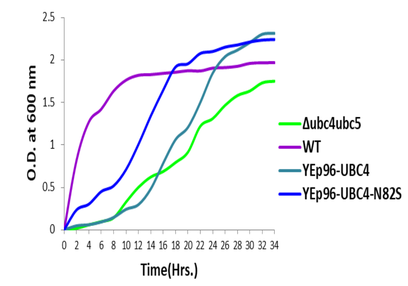


Construction of UBC4-N82S by Site directed mutagenesis in a shuttle vector (YEp96) which replaces Asn of UBC4 with Ser of UBC5

Transformation of the plasmid carrying YEp96-UBC4-N82S into Δubc4Δubc5. Transformation of yeast cells Δubc4Δubc5 with UBC4 plasmid was used as control

Functional evaluation of YEp96-UBC4-N82S under various stress conditions like heat, thermotolerance and post translational inhibitors. Wild-type & YEp96-UBC4 were used as controls

Results



Strain	Doubling time (Hrs.)
Δubc4ubc5	5
WT	1.1
YEp96-UBC4	2.7
YEp96-UBC4-N82S	2.3

Figure 2. Growth profile of Δubc4Δubc5 cells of *S. cerevisiae* transformed with plasmids expressing YEp96-UBC4 and mutant YEp96-UBC4-N82S along with WT and Δubc4Δubc5 as controls.

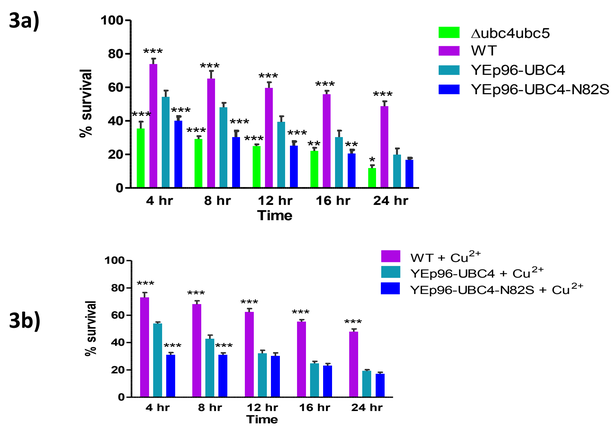


Figure 3. a) Survival of YEp96-UBC4-N82S under heat stress (37°C) at various time intervals in absence of an inducer (copper sulphate) and **b)** in presence of inducer.

% Survival is given as fraction of colonies formed after heat treatment.***p<0.001, **p<0.01,*p<0.05 in comparison to YEp96-UBC4

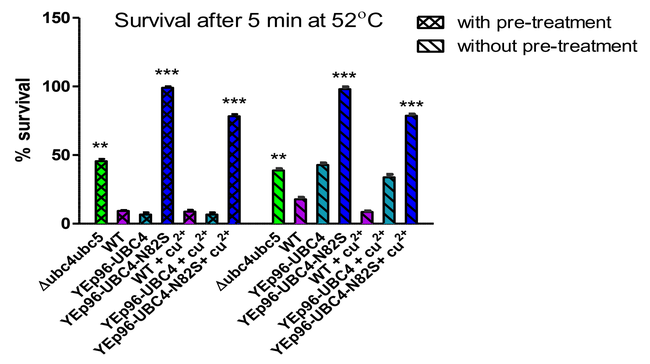


Figure 4. Resistance to thermotolerance of YEp96-UBC4-N82S in presence & absence on an inducer. For pre-treatment cultures were shifted to 37°C for 1 hr. Prior to or after incubation cultures were shifted to 52°C . Appropriate aliquots were spread on minimal agar plates containing the selection. %Survival was calculated.***p<0.001, **p<0.01 in comparison to YEp96-UBC4

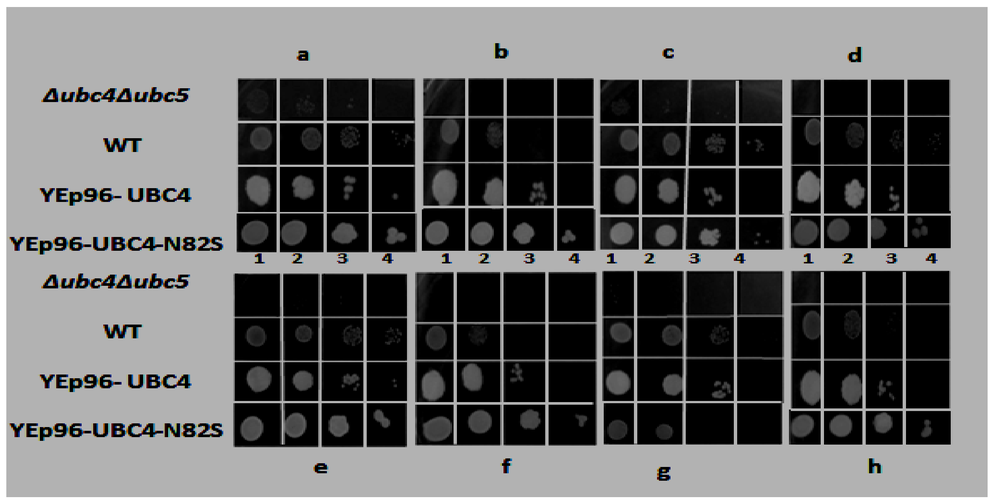


Figure 5. Resistance to translation inhibitors of *S. cerevisiae* cells Δubc4Δubc5 and Δubc4Δubc5 transformed with UBC4 and mutant YEp96-UBC4-N82S.
a) Control plate with no antibiotics (b) In presence of canavanine (0.5 µg/ml)
(c) In presence of hygromycin-B (0.2 mM) (d) In presence of cycloheximide (1µM)
(e) control plate with $CuSO_4$and no antibiotics (f) In presence of $CuSO_4$ plus canavanine
(g) In presence of $CuSO_4$ plus hygromycin-B (h) In presence of $CuSO_4$ plus cycloheximide

Conclusion and Significance

1.The point mutation N82S is functionally active.

2. In case of thermotolerance stress and antibiotic complementation N82S shows 100 % resistance compared to wild type (WT) and UBC4; while not under heat stress.

3. The experimental observations suggests that the mutation N82S provides greater stability to the protein structure .

References

1.Seufert W and Jentsch S. , *The EMBO Journal* vol.9 no.2,(1990), 543-550

2.Hutchinson *et al.,* Protein science, 1994

Acknowledgements

We would like to thank Prof. S. Jentsch (Department of Molecular Cell Biology, Max Planck Institute of Biochemistry, Germany) for providing UBC4 plasmid and Prof. Mark Hochstrasser (Department of Molecular Biophysics & Biochemistry, Yale University, Connecticut) for providing the wild type and Δubc4Δubc5 yeast strains.

Functional studies on Ubiquitin Conjugating Enzyme UBC4 with R133K mutation in *S.cerevisiae*

Varsha Raimalani and C. Ratna Prabha*

Department of Biochemistry, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara – 390002, INDIA

E-mail : varsharai1512@gmail.com, * Corresponding author: chivukula_r@yahoo.com

Introduction

In eukaryotes, Ubiquitin Conjugating enzymes assure the protein quality control via the Ubiquitin Proteasome system. The system is highly substrate specific in spite of presence of single E1, several E2s and thousands of E3s. E2 family comprises of a conserved UBC domain providing a binding platform for E1, E3s and activated ubiquitin. E2s have been classified based on the additional extensions present around this catalytic domain. In the present study we are focusing on ubiquitin conjugating enzyme UBC4 of *S. cerevisiae* system .

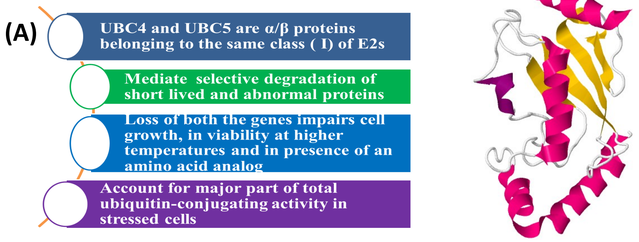
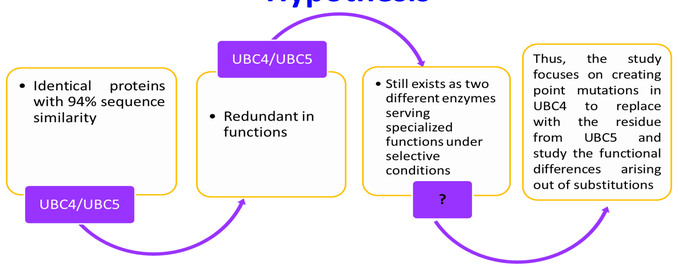


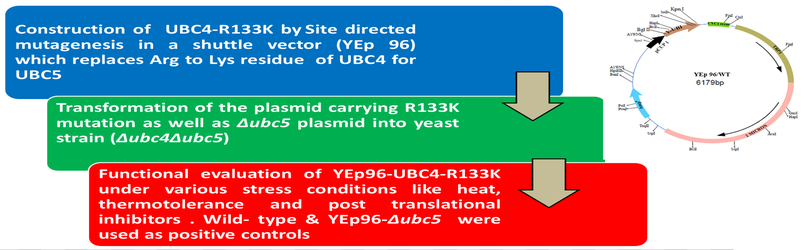
Figure 1. (A) Functions of UBC4 & UBC5.
(B) Secondary structure of UBC4.
(C) Sequence alignment of two proteins.

Hypothesis

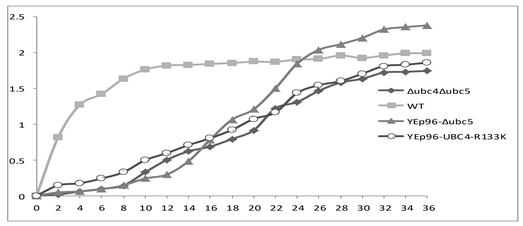


Materials and Methods

The S. *cerevisiae* strains used in the study are Wild type cells (Mat α his3-Δ200 leu2-3,112 ura3-52, lys2-801, trp1-1, gal 2) , *Δubc4Δubc5 (* Mat α his3-Δ200 leu2-3,112 ura3-52, lys2-801, trp1-1, ubc4-Δ1::HIS3 ubc5-Δ1::LEU2) is a double *knock out* strain lacking UBC4 and UBC5 .



Results



Strain	Doubling time (Hrs.)
Δubc4Δubc5	3.4
Wild type	3.2
YEp96-*Δubc5*	3.2
YEp96-UBC4-R133K	3.3

Figure 2. Growth profile of *Δubc4Δubc5* cells of S. *cerevisiae* transformed with plasmids expressing YEp96-*Δubc5* and mutant YEp96-UBC4-R133K.

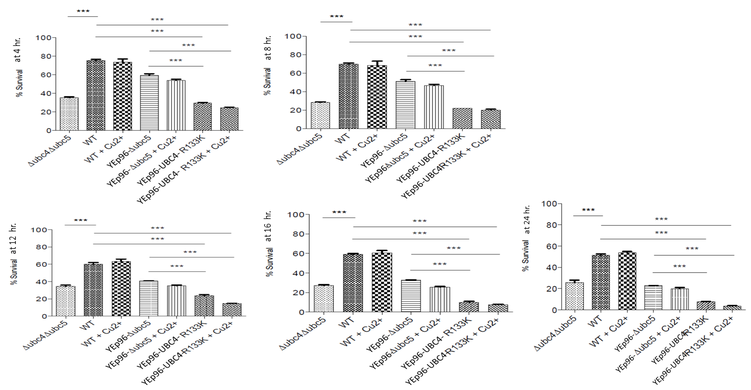


Figure 3. Survival of YEp96-UBC4-R133K under heat stress (37°C) at various time intervals in presence and absence of inducer (copper sulphate) . Numbers give the fraction of colonies formed after heat treatment.***p<0.001

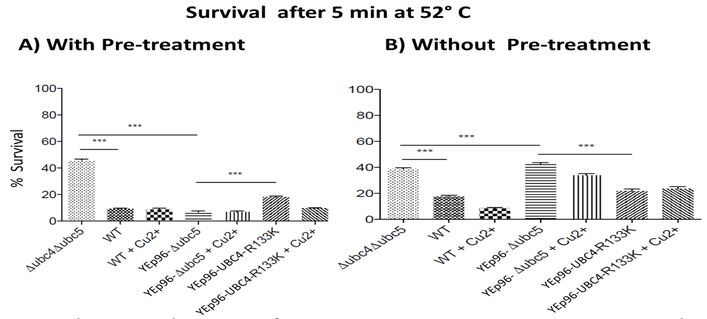


Figure 4. Thermotolerance of YEp96-UBC4-R133K in presence & absence on an inducer. For pre-treatment cultures were shifted to 37°C for 1 hr. Prior to or after incubation cultures were shifted to 52 °C . Appropriate aliquots were spread on minimal agar plates containing the selection .Numbers give the fraction of colonies formed after heat treatment.***p<0.001

Results

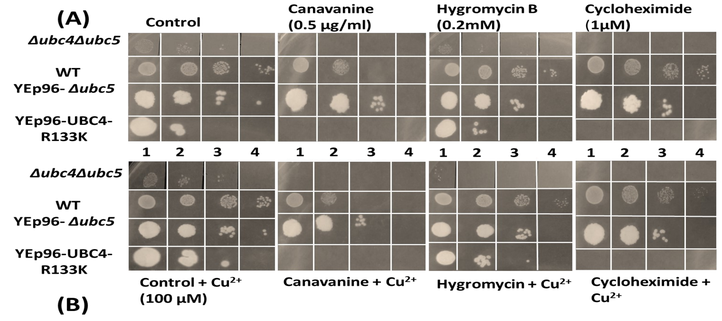


Figure 5. Antibiotic stress complementation of S. *cerevisiae* cells *Δubc4Δubc5* and *Δubc4Δubc5* transformed with UBC4 WT (YEp96-*Δubc5)* and mutant YEp96-UBC4-R133K. (A) without inducer
(B) with inducer (copper sulphate 100μM)

Conclusion

1.The point mutation R133K is functionally active.

2. In case of heat & thermotolerance stress R133k is less tolerant compared to wild type and *Δubc5.*

3.R133K also affects cell machinery as it is inefficient in degrading truncated proteins in presence of translational inhibitors.

4.Thus, this point mutation may be affecting the structure and hence altering the E2-E3 interaction pairing and so it is detrimental to the cell functioning.

Acknowledgements

We would like to thank Prof. Jentsch (Department of Molecular Cell Biology, Max Planck Institute of Biochemistry, Germany) for providing UBC4 plasmid and Prof. Mark Hochstrasser (Department of Molecular Biophysics & Biochemistry, Yale University, Connecticut) for providing the wild type and *Δubc4Δubc5* yeast strains.

Contact Information

Prof. C.Ratna Prabha
chivukula_r@yahoo.com
Macromolecular Structure & Biology Laboratory
Department of Biochemistry, Faculty of Science,
The Maharaja Sayajirao University of Baroda
Vadodara – 390002,Gujarat, INDIA