Chapter 3

Cloning and Functional Characterization of Four Domains of Ubiquitin Activating Enzyme E1

3.1. Introduction

Cells respond to stress in different ways, like activating pathways to start programmed cell death that eliminates damaged cells, necrosis or other modes of cellular death. Depending on the severity of cellular stress, the cell's response varies. Cells try surviving, if the stress does not go beyond a certain threshold, and allow cells to cope up by having an appropriate protective cellular response. If the stress is too strong which leads to the failure to activate a protective response, it results in cell death pathways (Weston and Davis, 2007).

One of the survival activities of cells is response to heat shock, where cells are exposed to mild heat stress. One of the major cellular outcomes of this stress is protein damage leading to the aggregation of misfolded proteins. In order to overcome this stress, the cell starts degrading aggregated misfolded proteins to ensure survival.

In **Chapter 2**, it was already discussed; E1 is modular protein and has a multidomain architecture. The observations based on structural characterization of four domains of ubiquitin activating enzyme indicated the domains are attaining secondary structure.

As it is already known, a protein domain does its function by interacting with various molecules inside the cell. In the current study, the domains were expressed in cells, to answer the question of whether they by virtue of possessing structure have the potential to interact with any binding partners and interfere or slow down cellular machinery by blocking the binding site. To check that, domains were overexpressed in cells and cells were exposed to stress conditions. To respond to protein aggregates formed due to stress, cells undergo an increased rate of ubiquitination. If the domains interfere at any point under this condition, the cell's survival will be affected.

3.2. Plan of Work

Ubiquitin activating enzyme E1's four domains namely FCCH, 4HB, SCCH and UFD were isolated by standard PCR techniques and inserted into yeast shuttle vector Yep96. All four domains were overexpressed using $CuSo_4$ *in vivo* and exposed to various cellular stress (**Fig. 3.1**).

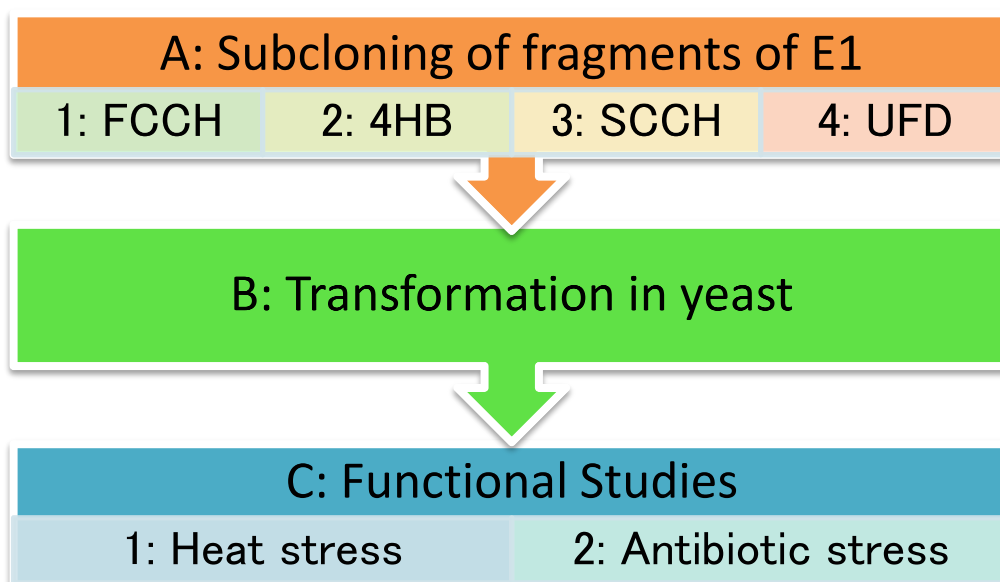


Fig. 3. 1. Schematic diagram of the work plan. A) subcloning of all four DNA fragments encoding FCCH, 4HB, SCCH and UFD into yeast vector, B) Transformation of recombinant plasmids into yeast C) Functional studies of all four peptides by heat stress and antibiotic stress.

3.3. Materials and Methods

3.3.1. Strains and Media

Bacterial strain used for cloning purpose was *E. coli* DH5α (F^-, 80dlacZ M15, *endA*1, *recA*1, *hsdR*17 (*rk*$^-$,*mk*$^+$*), supE44, thi-1, gyrA96, relA1, (lacZYA-argF)U169*). *E. coli* was grown on Luria broth and Luria agar (Hi-media). The selection marker for plasmid was ampicillin used at the concentration of 100µg/ml.

For yeast cultures, YEPD medium (1% yeast extract, 2% peptone, 2% glucose/dextrose) and Synthetic Dextrose (SD) medium (0.67% Hi-media yeast nitrogen base (without amino acids) and 2% glucose as carbon source) were used. 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}) was added for selection, 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).

3.3.2. Vector Selection

The vector used for functional studies for all four domains of yeast E1 was Yep96. It is a shuttle vector between *S. cerevisiae* and *E. coli* with *TRP1* as a selection marker (**Fig. 3.2**). Genes are regulated under the *CUP1* promoter, the expression of which can be induced by adding 10–100 µM $CuSO_4$.

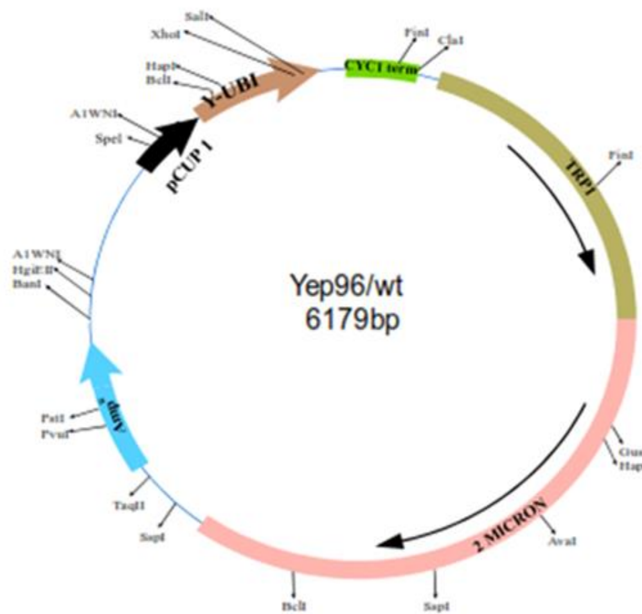


Fig. 3. 2. **Vector map of Yep96 carring wild type-yeast ubiquitin gene.**

3.3.3. Cloning Strategy

DNA fragments encoding the domains FCCH, 4HB, SCCH and UFD were amplified from the gene for Uba1 using standard PCR techniques. Primers used to amplify all four domains are listed in **Table 3.1**. Primers were designed to amplify residues 175-265 to make FCCH construct, residues 269-356 generated 4HB construct, residues 594-860 amplified to make SCCH construct and residues 1121-1220 amplified to generate UFD construct. An additional stop codon was added at the C-terminus of all the fragments. These fragments were cloned in the Yep96 vector between *Bgl*II and *Sal*I sites to remove the ubiquitin gene which is already present in the vector, was named YRPB1, YRPB2, YRPB3 and YRPB4 respectively. All four clones were sequenced and confirmed.

Table 3.1. **Primer sequences used to clone domains of E1 in Yep96 vector**

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’

3.3.4. Transformation of Recombinant Plasmids after Subcloning in Yeast cells

After all four clones YRPB1, YRPB2, YRPB3 and YRPB4 were confirmed, they were transformed into yeast strain MHY501 cells separately. Trp1 was used as a selection marker to screen positive transformants. Later positive transformants were picked and grown in YEPD medium for further studies.

3.3.5. 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 100µM $CuSO_4$ as an inducer. To check the effect of overexpression of the peptides on cell viability and growth, OD_{600} was measured every two hours, and the doubling time was calculated.

Formula to calculate doubling time:
Doubling Time = t/g
$g = [\log_{10} (N_t/N_0)]/ 0.3$

N_t = Number of cells or OD at start
N_0 = Number of cells or OD at end
t = time cultured (in Hours)

3.3.6. 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 dilution of the cultures was plated on SD agar with and without inducer (100µM $CuSO_4$). Plates were incubated at 37˚C for different time intervals i.e., 0h, 4h, 8h and 12h. 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.

3.3.7. Antibiotic Sensitivity Test

Three antibiotics cycloheximide, L-canavanine and hygromycin B were used to check sensitivity. Hygromycin B inhibits translation in eukaryotes by binding to ribosomes (Eustice and Wilhelm, 1984), whereas cycloheximide blocks translation elongation by binding to 60S ribosomal unit (Schneider-poetsch et al., 2010). L-canavanine, the structural analog of L-arginine, leads to form altered protein structures causing cell death (Rosenthal, 1977). 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. The cultures were serially diluted fivefold and spotted on SD plates containing one of 0.001M cycloheximide, 0.14 mM L-canavanine, 0.2mM Hygromycin B with and without inducer (100µM $CuSO_4$). Plates were incubated at 30˚C for 7 days to check survival in the presence of antibiotics.

3.4. Results

3.4.1. Construction of YRPB1, YRPB2, YRPB3 and YRPB4 Plasmids

Fragments of FCCH, 4HB, SCCH and UFD were amplified from pETE1 to construct YRPB1, YRPB2, YRPB3 and YRPB4 plasmids, respectively, using site-specific primers. The vector used for cloning was Yep96 and restriction sites used were *Bgl*II and *Sal*I. Each of them was sequenced to confirm the insertion of fragments in vector.

3.4.1.1. Cloning of YRPB1 Plasmid Construct

Clone YRPB1 was constructed by inserting the FCCH fragment in the Yep96 vector (**Fig. 3.3**). Samples were run on 1% agarose gel.

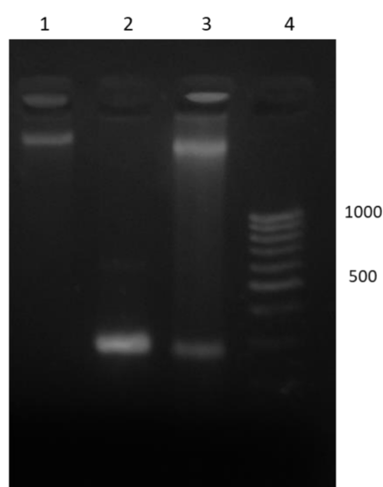


Fig. 3.3. **Gel picture showing YRPB1 clone in Yep96 backbone.** Lane 1 is YRPB1 plasmid; Lane 2 is double digestion of YRPB1 plasmid by *Bgl*II and *Sal*I restriction enzyme releasing the DNA fragment of 270bp from vector Yep96 (6.1kbp), Lane 3 is PCR amplification of FCCH fragment (270bp) and lane 4 is 100bp molecular weight marker.

3.4.1.2. Cloning of YRPB2 Plasmid Construct

Clone YRPB2 was constructed by inserting the 4HB fragment in the Yep96 vector (**Fig. 3.4**). Samples were run on 1% agarose gel.

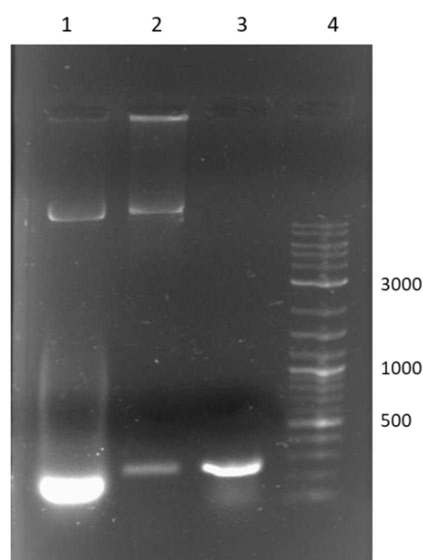


Fig. 3.4. **Gel picture showing YRPB2 clone in Yep96 backbbone.** Lane 1 is YRPB2 plasmid; Lane 2 is double digestion of YRPB2 plasmid by *Bgl*II and *Sal*I restriction enzyme releasing the DNA fragment of 237bp from vector Yep96 (6.1kbp), Lane 3 is PCR amplification of 4HB fragment (237bp) and lane 4 is 1kbp molecular weight marker.

3.4.1.3. Cloning of YRPB3 Plasmid Construct

Clone YRPB3 was constructed by inserting the SCCH fragment in the Yep96 vector (**Fig. 3.5**). Samples were run on 1% agarose gel.

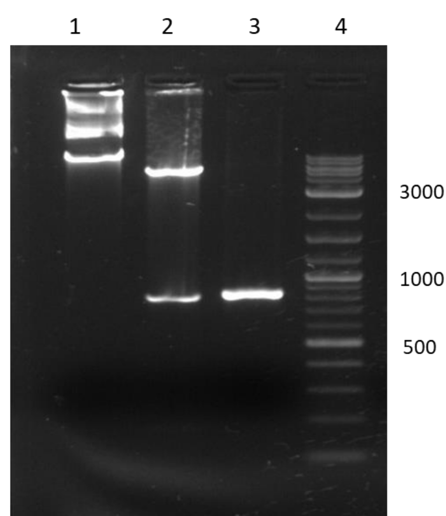


Fig. 3.5. Gel picture showing YRPB3 clone in Yep96 backbone. Lane 1 is YRPB3 plasmid; Lane 2 is double digestion of YRPB3 plasmid by *Bgl*II and *Sal*I restriction enzyme releasing the DNA fragment of 786bp from vector Yep96 (6.1kbp), Lane 3 is PCR amplification of SCCH fragment (786bp) and lane 4 is 1Kbp molecular weight marker.

3.4.1.4. Cloning of YRPB4 Plasmid Construct

Clone YRPB4 was constructed by inserting the UFD fragment in the Yep96 vector (**Fig. 3.6**). Samples were run on 1% agarose gel.

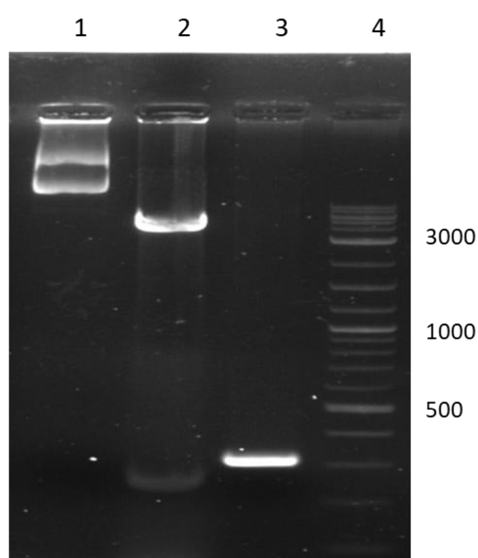


Fig. 3. 6. **Gel picture showing YRPB4 clone in Yep96 backbone.** Lane 1 is YRPB4 plasmid; Lane 2 is double digestion of YRPB4 plasmid by *Bgl*II and *Sal*I restriction enzyme releasing the DNA fragment of 300bp from vector Yep96 (6.1kbp), Lane 3 is PCR amplification of UFD fragment (300bp) and lane 4 is 1Kbp molecular weight marker.

3.4.2. 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 the 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 to wild type MHY501 cells, which were used as a positive control in the experiment. (**Fig. 3.7 and Table 3.2**)

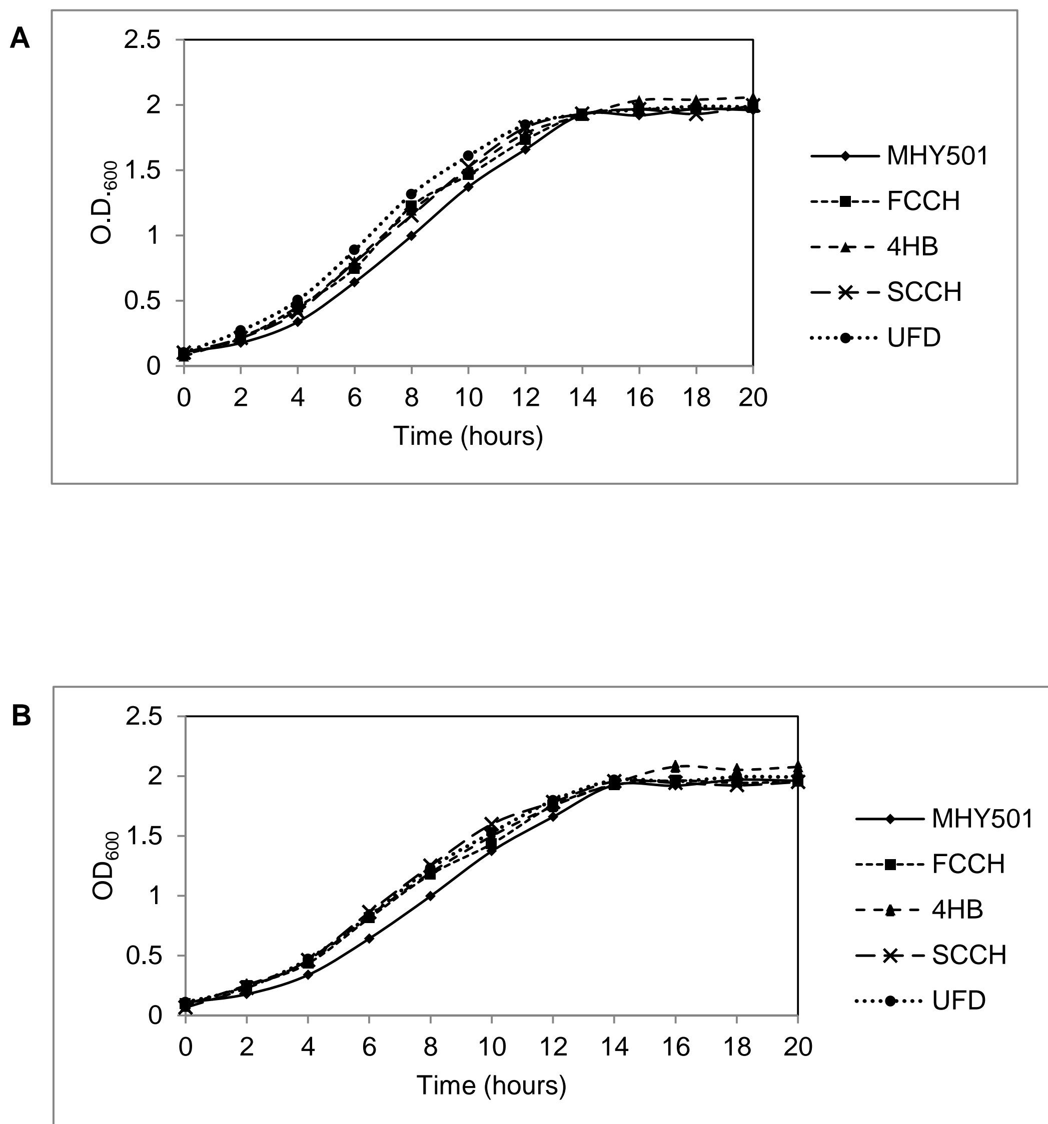


Fig. 3.7. **Growth curves of MHY501 cells expressing domains FCCH, 4HB, SCCH, UFD of ubiquitin activating enzyme E1**, A) without inducer B) with $CuSO_4$ inducer. Untransformed MHY501 cells were used as negative control.

Table 3.2. Determination of doubling time of MHY501 cells transformed with domains 4HB, FCCH, SCCH, 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

3.4.3. Effects of Expression of the Domains 4HB, FCCH, SCCH and UFD on Cell Growth under Heat Stress

Ubiquitination machinery works more rigorously in cells under stress to remove the denatured proteins than 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. 3.8**).

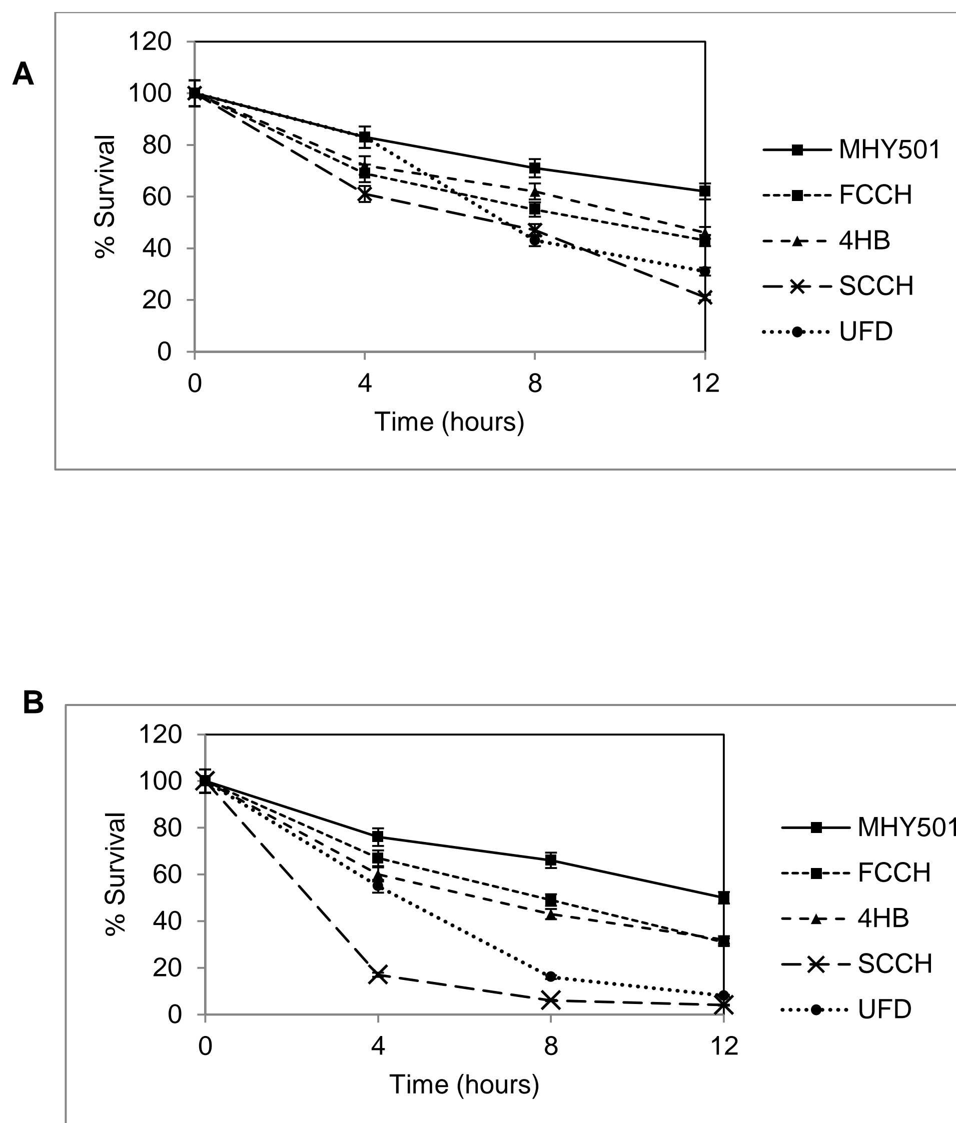
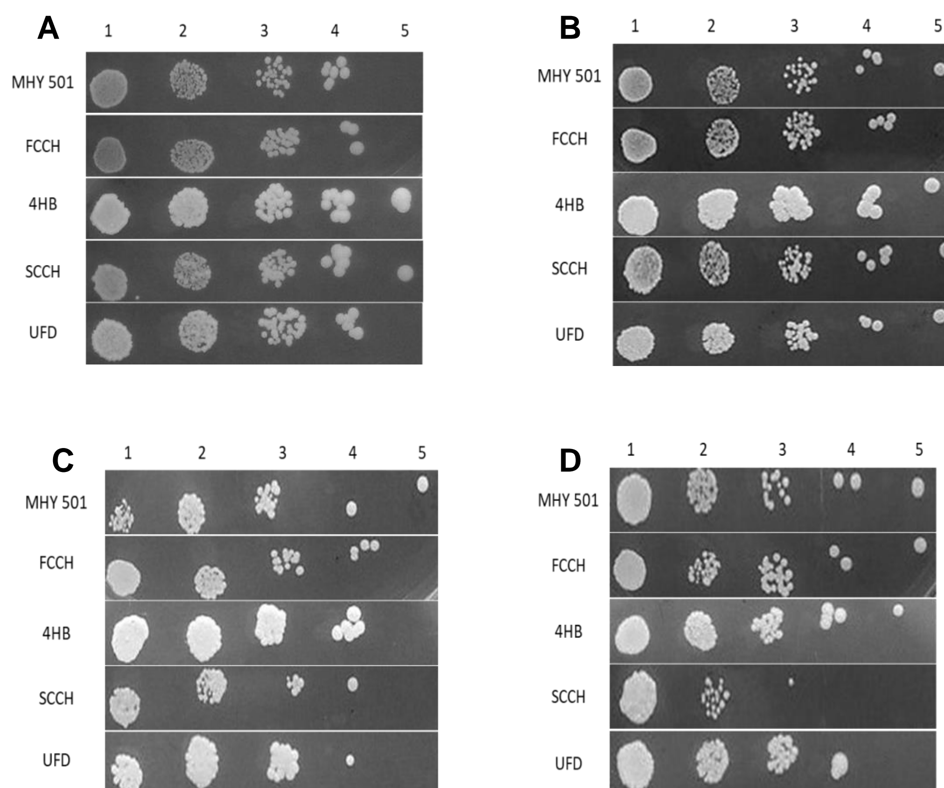


Fig. 3. 8. **Survival of MHY501 strain expressing the domains of ubiquitin activating enzyme E1 under heat stress:** A) without inducer B) with $CuSO_4$ inducer. Cells expressing SCCH and UFD showed decreased survival under heat stress.

3.4.4. Effects of 4HB, FCCH, SCCH and UFD Expression on Cell Growth in the Presence of Antibiotics

The presence of Hygromycin B decreased cell growth in the presence of SCCH peptide. No change in cell growth observed in presence of L-canavanine, whereas in the presence of cycloheximide cells with UFD showed a decrease in growth. SCCH and UFD 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 and 4HB were able to complement the antibiotic stress as effectively as MHY501 cells (**Fig. 3.9**).



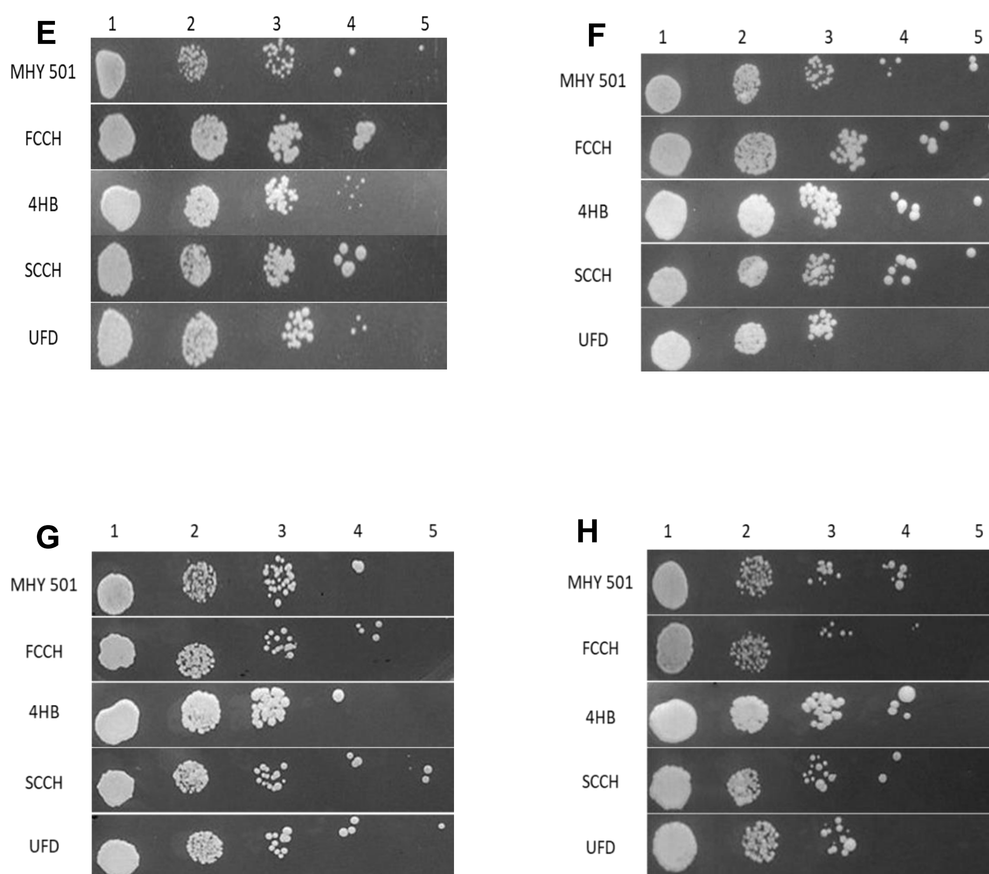


Fig. 3.9. **Effect of overexpression of domains on cell growth under antibiotic stress.** Petri dishes contain undiluted cultures (1), and fourfold serial dilutions (2–5) have combinations of antibiotic and inducer as follows: A) control petri dishes without inducer and without antibiotic, B) petri dishes with inducer and without antibiotic, C) petri dishes without inducer and with Hygromycin B, D) petri dishes with inducer and with Hygromycin B, E) petri dishes without inducer and with Cycloheximide, F) petri dishes with inducer and with Cycloheximide, G) petri dishes without inducer and with L-canavanine, H) petri dishes with inducer and with L-canavanine. Inducer used is 100 µM $CuSO_4$.

3.5. Discussion

The domains were expressed in cells, to answer the question whether they by virtue of possessing structure 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 (Finley et al., 1994). 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 at some level in the cellular processes under heat stress and in the presence of the antibiotic. 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 leads to the 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 native like structure and can bind to antizyme (Joshi et al., 2015). 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.

In summary, the SCCH domain could act as a potential inhibitor of ubiquitin proteasome system.