Studies on the lethal effects of mutated forms of ubiquitin on breast cancer cell line MCF-7

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5.1 INTRODUCTION

MCF-7 is the well-studied human breast cancer cell line. It was generated from the pleural effusion from a patient with metastatic breast cancer 45 years ago (Soule et al., 1973). MCF-7 expresses estrogen receptor and hence it shows proliferative response to estrogen. However, these cells do not show expression of HER2 oncogene. Breast cancer research utilize these features as MCF-7 expresses considerable levels of estrogen that mimics majority of estrogen expressing invasive human breast cancers (Brooks et al., 1973). According to global report, breast cancer is a serious, second most common cancer amongst women (Ferlay et al., 2010).

Accumulating evidence suggests that certain genetic variations, environmental factors and exposure to exogenous and endogenous agents cause mutations in DNA leading to malignanat transformation of cells. Malignant transformations in cells give rise to cancers (Thomson et al., 2005; Kotsopoulos and Narod, 2005; Clarke and Fuller, 2006; Ponti et al., 2006; Eyler and Rich, 2008; Kai et al., 2010). To remove damaged proteins, defective organelles and to maintain proteostasis, nature has evolved ubiquitin proteasome system (UPS) and autophagy driven lysosomal proteolysis (Nakatogawa et.al., 2009; Kirkin et.al., 2009; He et.al., 2009; Akerfelt et.al., 2010; Voisine et.al., 2010; Stolz and Wolf, 2010; Fredrickson, 2012).

As mentioned in the introduction in Chapter 1 the ubiquitin-proteasome system (UPS) is responsible for the proteolysis of regulatory proteins responsible for development, proliferation, differentiation, cell cycling, apoptosis, gene transcription, signal transduction, senescence, antigen presentation, protein trafficking, DNA repair, inflammation, and stress response, thereby governing basic cellular processes (Naujokat and Hoffmann, 2002; Rock et.al., 2002; Goldberg, 2003; Kruger et.al., 2004). Malfunctioning of UPS is responsible for various pathological conditions in humans like Angelman syndrome, Parkinson's disease,

Liddle syndrome, Fanconi's anemia, Type 2 diabetes, breast cancer, ovarian cancer and uterine cervical carcinoma (Kayed et al., 2003; Ross and Poirier, 2004; Chiti and Dobson, 2006; Ben-Zvi et al., 2009; Hartl et al., 2011).

Evolutionarily conserved protein ubiquitin has been changed only in three positions during evolution from yeasts to humans. These are serine to proline at 14th position, glutamic acid to aspartic acid at 19th position and serine to alanine at 24th position (Schlesinger and Goldsteiner, 1975; Gavilanes et al., 1982; Wilkinson et al., 1986). Mammalian genome consists of four ubiquitin coding genes. Uba52 (Ub monomer fused to L40 ribosomal protein) and Uba80 (Ub monomer fused to S27 ribosomal protein) are ubiquitin fusion proteins and Ubb and Ubc encode polyubiquitin genes and express four and nine tandem repeats of ubiquitin respectively. Ubiquitin hydrolase cleaves the fusion proteins at the C-terminal end of ubiquitin, liberating individual ubiquitin monomers and free ribosomal proteins. Reports suggest that expression of polyubiquitin genes is crucial. Disruption of Ubc in mice resulted in lethality of embryo. Ubb disrupted mice were found to be infertile due to failure of meiosis in germ cells (Redman and Rechsteiner, 1989; Ryu et al., 2007; Ryu et al., 2008(a) & 2008(b)).

As described in earlier chapters, the main focus of this work is to find the significance of conserved residues of ubiquitin. Results described in **Chapter 2** and **Chapter 4** suggest that mutant forms of ubiquitin are capable of disturbing the cellular homeostasis and thereby cause lethality to cells in laboratory conditions. Due to the marked sequence conservation, we hypothesised that, mutant forms of ubiquitin generated and tested in our laboratory for multiple biological functions using model organisms *S. cerevisiae* (Prabha et al., 2010; Doshi et al., 2014; Doshi et al., 2017) and *C. albicans* may also alter biology of human cells. If these mutant forms of ubiquitin display similar effect in cancer cells, then the results will pave way for developing novel therapeutic approaches. To extend the studies to human cancer cell lines, human equivalents of the mutant forms were generated using human ubiquitin gene employing site directed mutagenesis. Mutant forms of human uqbiquitin gene were cloned in mammalian expression vector pTRE-Tight-IRES-EGFP under tetracycline inducible promoter. The human cell line chosen in the study MCF-7 is estrogen responsive (ER+) human epithelial breast cancer cell line. As ubiquitin mutation, UbEP42, UbL50P and

UbI61T had displayed adverse effects on yeast cells, it was hypothesized that HUbEP42, HUbL50P and HUbI61T may have the potential to lyse human cancer cells by altering their biological functions. Stable cell lines expressing mutant forms of ubiquitin were generated and various functions were tested.

5.2 MATERIALS AND METHODS

5.2.1 Cell line and culture conditions

The experiments were carried out using MCF-7 as a model. It is an estrogen responsive (ER+) human epithelial breast cancer cell line. Cells were cultured in Dulbecco's modified Eagle medium (DMEM) from HIMEDIA supplemented with 10% (v/v) fetal bovine serum (FBS) from Gibco, 2 mM L-glutamine, 50 μg/ml streptomycin and 50 U/ml penicillin from Sigma-Aldrich. MCF-7 cell line was maintained at 37°C in a humidified atmosphere with 5% CO_2. Cells were routinely subcultured and were in exponential growth phase when used for experiments. Each experiment was independently performed at least in triplicate.

5.2.2 Mutagenesis

Mutant forms of ubiquitin *viz*. HUbEP42, HUbS20F, HUbA46S, HUbL50P and HUbI61T were generated using PCR based site-directed mutagenesis approach. Synthetic HUbWt (Figure 5.1) was used as template which contains several restriction sites. Mutagenic primers were designed in such a way that with each desired point mutation, one of the nearest restriction sites was disrupted. This strategy was used to confirm successful mutagenesis during primary screening. With the help of mutagenic and non-mutagenic primers listed in Table 5.1 mutant forms of ubiquitin were generated. Incorporation of point mutations was finally confirmed by DNA sequencing.

Primer	Oligonucleotide Sequence
UbWt-FR	5' ACA GAA TTC ATG CAG ATC TTC GTC AAG 3’
UbWt-RE	5' GCC ATC GGA TCC CGC TCA ACC ACC TCT TAG TCT 3’
UbS20F-FR	5’ CC ATA ACT CTG GAA GTT GAA CCA TTC GAT ACC ATC GAA 3’
UbS20F-RE	5’ TTC GAT GGT ATC GAA TGG TTC AAC TTC CAG AGT TAT GG 3’
UbA46S-FR	5’ GA TTG ATC TGG TCC GGT AAG CAG CTC GAA GAC GGT AGA AC 3’

UbA46S-RE	5’ GT TCT ACC GTC TTC GAG CTG CTT ACC GGA CCA GAT CAA TC 3’
UbL50P-FR	5’ G ATC TGG GCC GGC AAG CAG CCT GAG GAC GGT AG 3’
UbL50P-RE	5’ CT ACC GTC CTC AGG CTG CTT GCC GGC CCA GAT C 3’
UbI61T-FR	5’ CT GAT TAC AAC ACT CAG AAG GAG TCC ACC TTA CAT CTT G 3’
UbI61T-RE	5’ C AAG ATG TAA GGT GGA CTC CTT CTG AGT GTT GTA ATC AG 3’
EGFP-FR	5’ ACAGGTACCATGGTGAGCAAG 3’

Table 5.1: List of forward (FR) and reverse (RE) oligonucleotide used to generate single mutant derivatives of mammalian ubiquitin by site directed mutagenesis.

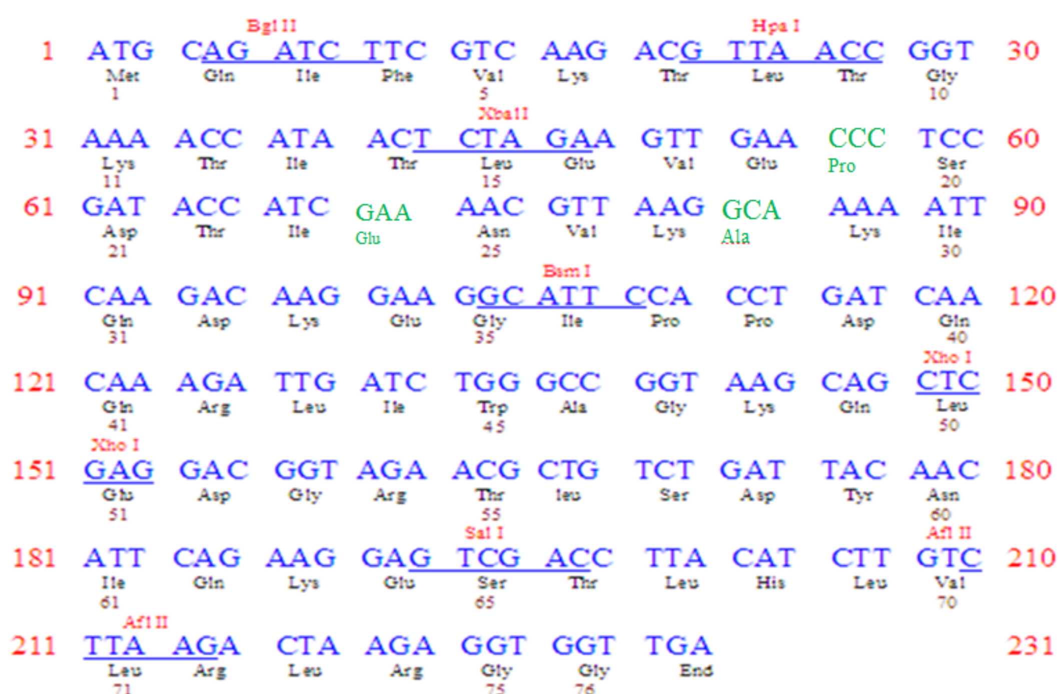


Figure 5.1: Synthetic human ubiquitin gene. It contains seven restriction sites which are underlined and codons marked with green colour represent difference in ubiquitin protein sequence from yeast to mammalian ubiquitin.

5.2.3 Plasmid construction

Plasmid construction was carried out using the strategy shown in Figure 5.2. The oligonucleotides used for cloning are listed in Table 5.1 and vector maps are shown in Figure 5.3. Briefly, mutant forms of ubiquitin *viz* HUbEP42, HUbS20F, HUbA46S, HUbL50P, HUbI61T along with HUbWt were PCR amplified and cloned in pTRE-Tight-IRES-EGFP

vector (kind gift from Prof. Han-Woong Lee, Yonsei University, Seoul, Korea) using *EcoR*I and *BamH*I restriction sites. These vectors express wild type or mutant forms of ubiquitin without any tag. Cloning of all mutant forms of ubiquitin were confirmed by RE mapping and DNA sequencing.

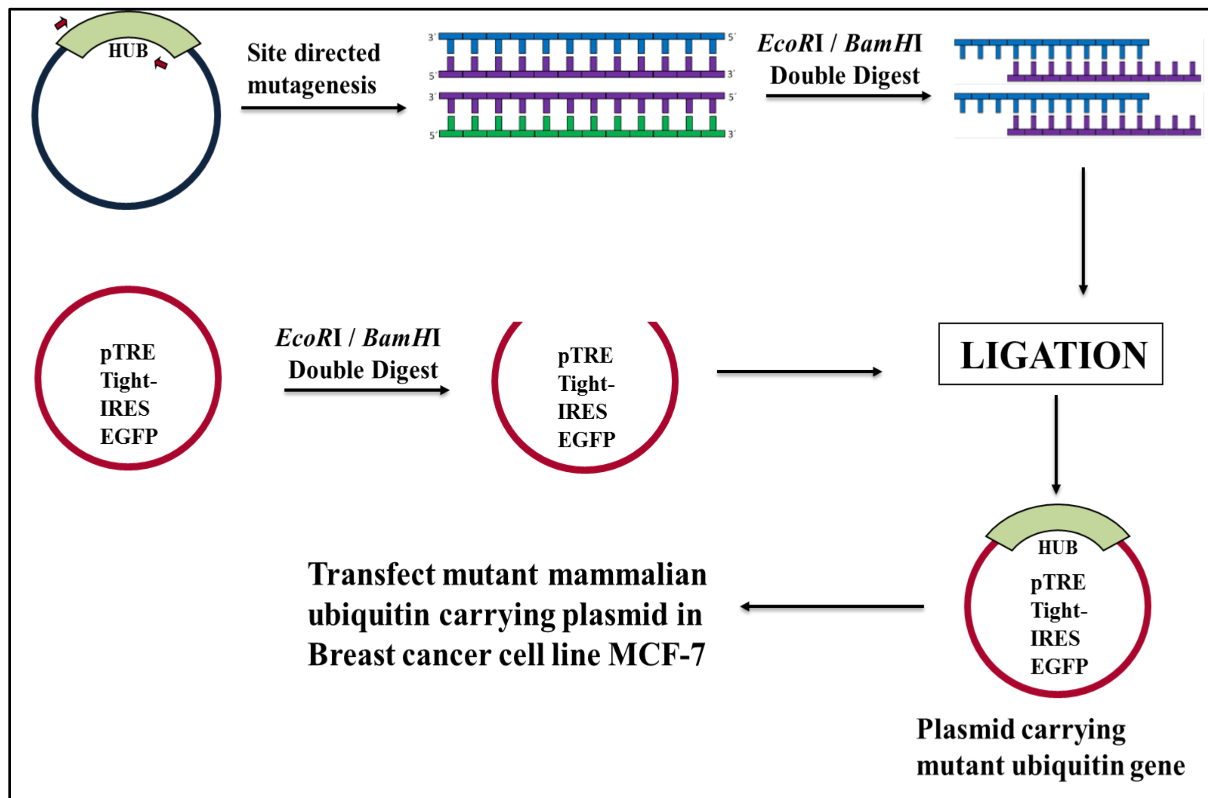


Figure 5.2: Strategy of cloning mutant forms and wild type ubiquitin encoding gene in mammalian expression vector.

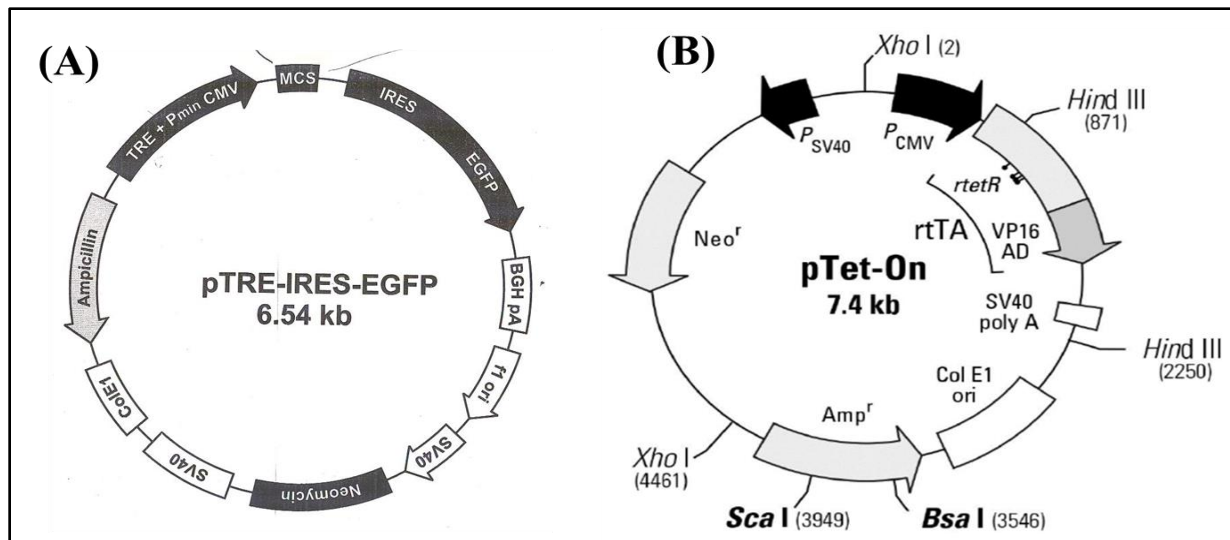


Figure 5.3: Vector maps of pTRE-Tight-IRES EGFP and pTet-On.

5.2.4 Transfection and Generation of stable cell line

5.2.4.1 Vector information:

pTRE-Tight and pTet-On vectors were used for the study. Both are shuttle vector between bacteria and mammalian cells. pTRE-Tight is a response plasmid that can be used to express a gene of interest with the help of Tet-On gene expression system. pTRE-Tight is a tetracycline-regulated responsive system (Gossen & Bujard, 1992; Gossen et al., 1995). Tet-On gene expression system expresses regulatory protein rtTA, the expression of which is regulated by tetracycline. The vector pTRE-Tight has MCS downstream to TRE promoter. The protein rtTA binds to P_{tight} promoter and induces its exression. The gene of interest inserted downstream to the Tet-responsive P_{tight} promoter is expressed in response to and rtTA regulatory proteins in Tet-On systems. P_{tight} contains a modified Tet response element (TRE_{mod}), which consists of seven direct repeats of a 36-bp sequence that contains the 19-bp tet operator sequence (*tetO*). The TRE_{mod} is just upstream of the minimal CMV promoter ($P_{minCMV\Delta}$). $P_{minCMV\Delta}$ lacks the enhancer that is part of the complete CMV promoter. Consequently, P_{tight} is silent in the absence of binding of rTetR. pTRe-Tight-IRES-EGFP vector consists of internal ribosome entry sight (IRES) which expresses EGFP present downstream. pTRe-Tight-IRES-EGFP contains neomycin antibiotic resistance gene for selection in mammalian cells. Along with that linear hygromycin marker was used to co-

transfect the cells. Vector maps are given in Figure 5.4.

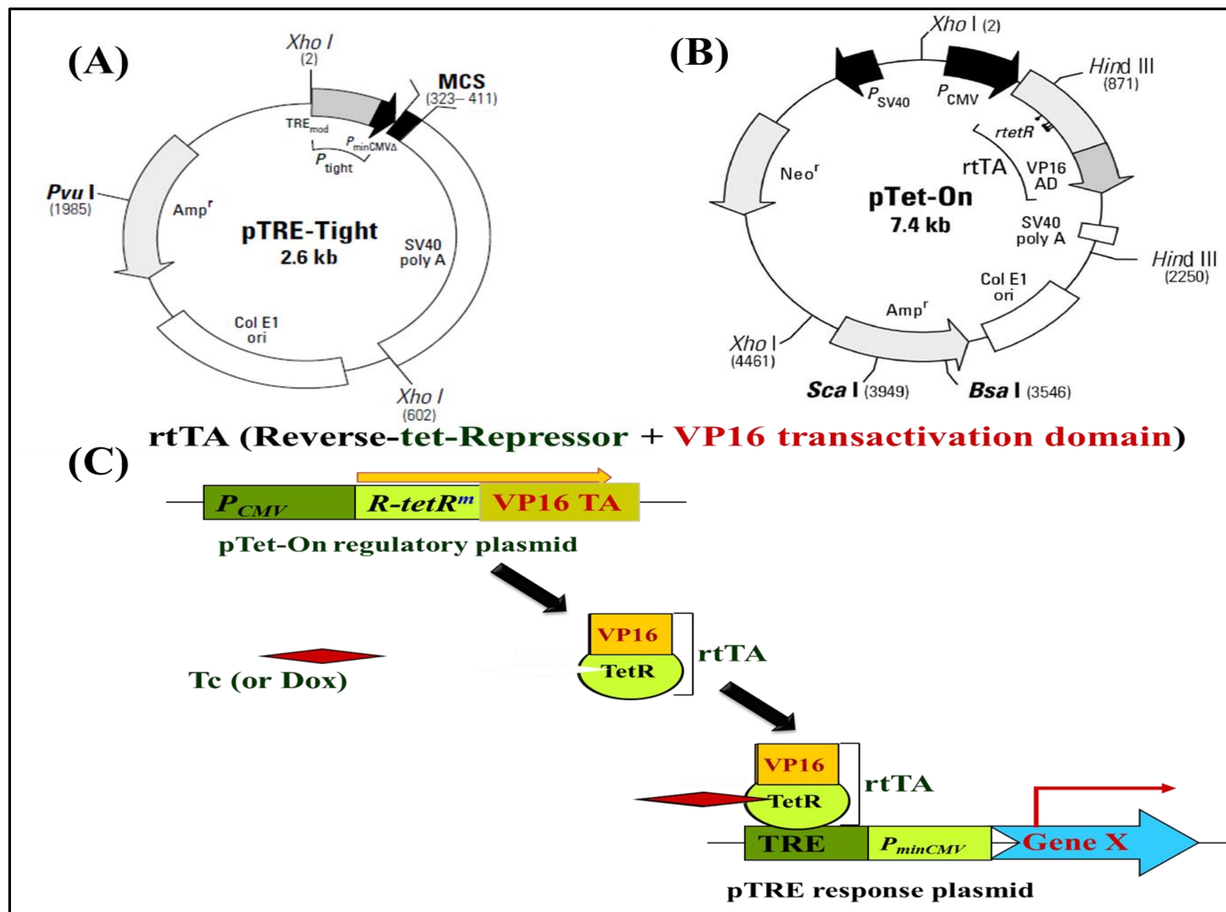


Figure 5.4: (A) and (B) shows vector maps of pTRE-Tight and pTet-On respectively which are used for inducing mutant ubiquitin protein in MCF-7 cells. The modulatory mechanism of Tet-On system in presence of doxycycline (Dox) is shown in (C).

5.2.4.2 Transfection

The MCF-7 cells were seeded in 12-well plate at 1x10^5 cells/ml density in DMEM media containing 10% FBS 24 h prior to transfection and were cultured in a humidified incubator at 37˚C under 5% CO_2 until 70% to 80 % confluency was reached. Lipofectamine 2000 from Invitrogen was used to transfect MCF-7 cells as per manufacturer's instruction. pTet-On and pTRE-Tight containing HUbWt, HUbEP42, HUbS20F, HUbA46S, HUbL50P, HUbI61T to final concentration of 1µg/1µl were diluted in Opti-MEM medium (Gibco Life Technologies, Carlsbad, CA, USA) and mixed with lipofectamine reagent followed by agitating lightly. DNA-lipid mixture was incubated for 15-20 minutes, then added to cells and

incubated for 4h. Later the medium was replaced by fresh media containing 10% serum. After 24 h cells were observed for the expression of enhanced green fluorescent protein to confirm transfection.

5.2.4.3 Generation of stable cell line

The stable cell lines expressing HUbWt, HUbEP42, HUbS20F, HUbA46S, HUbL50P, and HUbI61T were generated to study the effects of ubiquitin mutations on MCF-7 biology. For this firstly, MCF-7 cells were transfected with pTet-On plasmid, which contains rtTA element in order to control inducible expression of ubiquitin mutants using above described transfection protocol. The cells were supplemented with 500µg/ml G418 after 48h of transfection. Stable cells were harvested from 12-well plate, serially diluted and transferred to 96 well plate to get single clone. Stably transfected cells were maintained at higher scale in DMEM media supplemented with 50µg/ml of G418. In the second round, the MCF-7/pTet-On cells were used to co-transfect the pTRE-Tight expressing HUbWt, HUbEP42, HUbS20F, HUbA46S, HUbL50P, and HUbI61T along with linear hygromycin marker to ensure co-transfection. Stable clones were isolated using serial dilution procedure as above and cells were maintained in DMEM media supplemented with 50µg/ml G418 and 50µg/ml hygromycin. Stable cell lines expressing mutant proteins were subcultured regularly and were in exponential growth phase when used for experiments

5.2.5 Standardization of the Tet-On System for inducible expression of mutant ubiquitin protein

The conditions for expression of mutant forms of ubiquitin were standardized initially by taking exponentially growing MCF-7 cells harbouring pTRE-Tight/ EGFP, PTet-On vectors with no insert. Cells were seeded in 12-well plate at $1.5x10^5$ cells/ml density in DMEM media containing 10% FBS and required antibiotics in humidified incubator at 37˚C under 5% CO_2. Cells were allowed to attain 60-70% confluency. Following day when the cells adhered to the plate properly, were treated with 0 µg, 0.5 µg, 1 µg and 2 µg of doxycycline. Doxycycline is an inducer used to express the gene downstream to tetracycline inducible promoter. Cells were observed after 24h for EGFP expression by fluorescence microscope (EVOS, lifeTechnologies). Images were captured using green filter and 20X

objective.

5.2.6 Effect of overexpression of mutant forms of ubiquitin on MCF-7 cells

HUbWt, HUbEP42, HUbS20F, HUbA46S, HUbL50P, HUbI61T expressing MCF-7 cells were seeded in 6-well plate at $0.5x10^6$ cells/ml density in DMEM media containing 10% FBS and required antibiotics and incubated in humidified incubator at 37°C under 5% CO_2. Approximately at 60-70% confluency cells were treated with 1 µg/ml doxycycline for 48h. Sublethal concentration of the inducer and incubation period at which it was maximally effective was decided based upon optimization carried out with vector transfectant. Images were captured by fluorescent microscope (20X objective, green filter). The experiments were repeated thrice in three independent sets.

5.2.7 MTT assay to detect cell proliferation efficiency

To follow growth kinetics, cell lines expressing wild type and mutant forms of ubiquitin were seeded in triplicate in 96 well plate at the density of 1000 cells/well from exponentially growing cell culture. Culture conditions, media, supplements were used as described in above experiments. Next day cells were treated with 0.5 µg/ml of doxycycline for required period of time. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay was performed at specified time intervals. The cells were incubated with MTT (Sigma-Aldrich) at final concentration of 0.1 mg/ml at 37° C for 4h prior to estimating cells using ELISA plate reader. The supernatants were removed and visible blue crystals were solubilized in dimethylsulfoxide to terminate reaction. The absorbance was measured at 570nm using microplate reader. The OD value was measured for 5 days, and the growth curves were plotted. As the experiments were conducted for prolonged periods, the lower dose of inducer was used.

To test cell proliferation potential of the cell line after transfecting with mutant forms of ubiquitin, same procedure was followed as described above except that the final concentration of an inducer used was 1 µg of doxycycline and incubation period was 48h. Experiment was repeated three times independently.

5.2.8 Cell survival via clonogenic assay

To study consequences of mutant ubiquitin protein on cell survival, clonogenic assay was carried out as described by Franken's group (Franken et.al., 2006). Briefly, Mutants and wild type ubiquitin expressing cells were trypsinized and were seeded at density of 3000 cells in 35mm culture dish. They were allowed to adhere for one day and were treated with 1 μg/ml of doxycycline. Cells were incubated in a humidified incubator at 37° C under 5% CO_2 for 12-15 days, until the cell colonies were visible by eye. PBS washes were given followed by fixing cells with methanol. Later, the cells were stained with 0.5% crystal violet (Sigma-Aldrich) for 5 min, prior to washing and drying. Positive colonies, containing >50 cells, were observed under a microscope and images were captured. Effect of mutants was assessed by counting colonies. The experiment was repeated in three independent sets and Mean ± SEM values are presented in the form of graph.

5.2.9 Assay for monitoring antibiotic resistance

Mutants and wild type ubiquitin expressing MCF-7 cells were seeded at 1000 cells/ well in 96 well plate and incubated in humidified chamber at 37° C under 5% CO_2. Once the cells showed adherence, they were treated with 10 μg/ml tunicamycin (Tsirigotis et.al., 2001) and 20mM canavanine (Zhang et.al., 2014). 1 μg/ml of doxycycline was added to each well so as to induce expression of mutant as well as wild type ubiquitins, which were cloned under tetracycline inducible promoter. After incubation of 48 hours, MTT assay was carried out in order to check the effect of mutant ubiquitins towards antibiotic stress. Procedure of MTT assay was followed as described above. The experiment was repeated in three independent sets and Mean ± SEM values of percentage survival are presented in the form of graph.

5.2.10 Fluorimetric detection of ubiquitin mutant protein localization

5.2.10.1 Plasmid construction

Plasmid construction was carried out using the strategy shown in Figure 5.4, oligonucleotides used for cloning are listed in Table 5.1 and vector maps are shown in Figure 5.5. Briefly, DNA fragments encoding mutant forms of ubiquitin *viz* HUbEP42, HUbS20F, HUbA46S, HUbL50P, HUbI61T and HUbWt were PCR amplified and cloned in *EcoR*I and

*BamH*I restriction sites of pEGFP-C1 vector, to generate gene fusion of EGFP-ubiquitin and EGFP-mutant forms of ubiquitin. In the second round, the DNA fragments representing the gene fusions were subcloned into pTRE-Tight (Kind gift from Dr. Manoj K. Bhat, NCCS, Pune, India) between *Kpn*I and *BamH*I restriction sites. Cloning and subcloning of all mutant forms of ubiquitin were confirmed by RE mapping and DNA sequencing.

5.2.10.2 Microscopy

MCF-7 cells were transfected with vectors carrying genes for HUbEP42, HUbS20F, HUbA46S, HUbL50P, HUbI61T and HUbWt and stable cell lines were generated as described above in the same section. MCF-7 cells expressing EGFP tagged mutants and wild type ubiquitin were prepared for confocal microscopy by culturing on 35 mm non-coated cover slips. Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) in PBS at room temperature for 15 min and PBS wash was given. Confocal images were captured by Carl Zeiss LSM (laser scanning microscope) 710 by 63X oil immersion objective at a magnification of 630X. Fluorescence emission was monitored at 500-560 nm to detect the expression of EGFP. Experiments were carried out in triplicate.

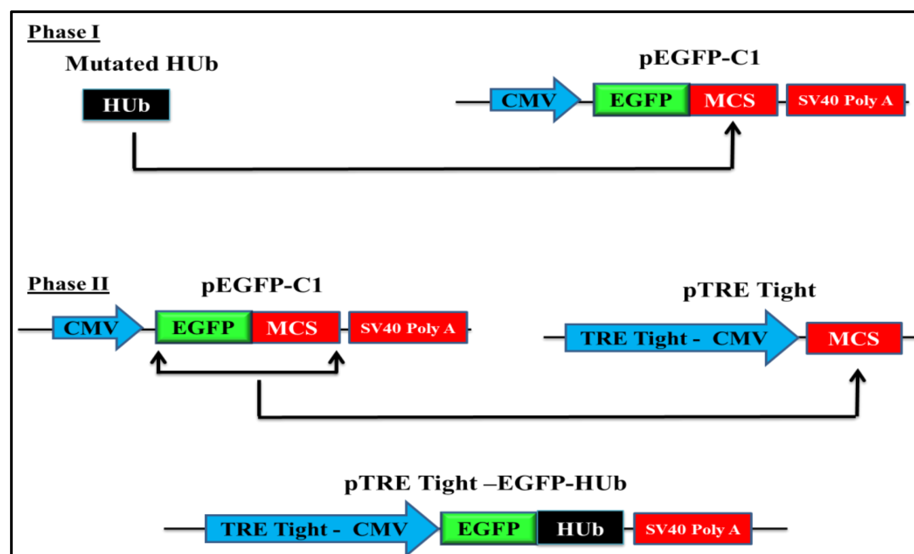
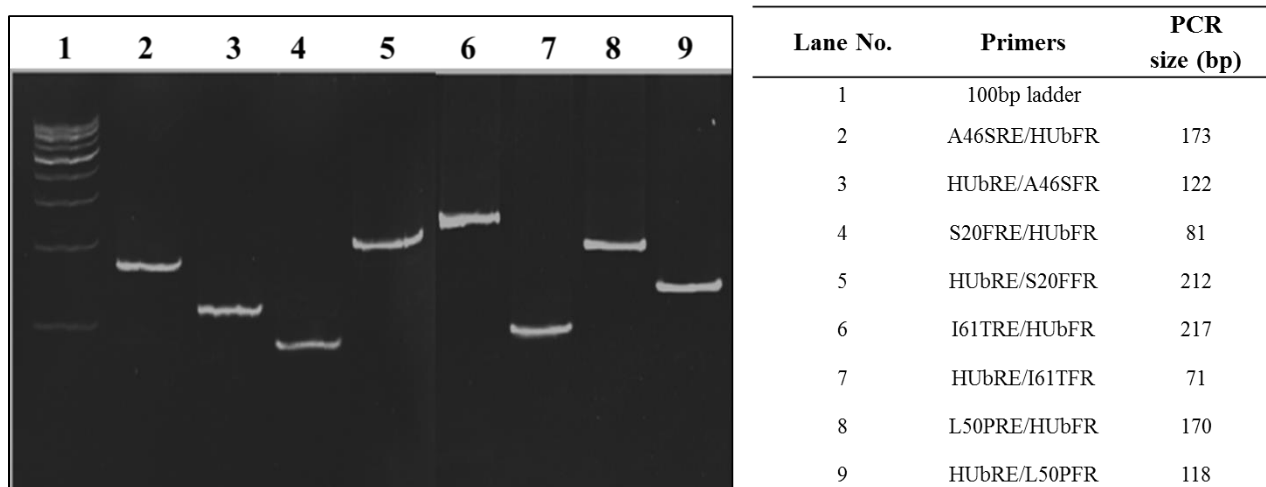


Figure 5.5: Strategy for generating chimeric EGFP tagged mutant ubiquitin.

5.3 RESULTS

5.3.1 Generation of the mutant forms of ubiquitin *HUbEP42, HUbS20F, HUbA46S, HUbL50P* and *HUbI61T* using recombinant PCR

Mutant forms of ubiquitin *HUbEP42, HUbS20F, HUbA46S, HUbL50P, HUbI61T* were generated using site directed mutagenesis approach. In the first round of PCR, sets of mutagenic and non-mutagenic primers listed in Table 5.1 were used to generate amplicons (Figure 5.6). These amplicons were further utilized in second round of PCR as templates to generate full length mutated ubiquitin genes (Figure 5.7). The full length mutated ubiquitin genes were used further for cloning purpose.



Lane No.	Primers	PCR size (bp)
1	100bp ladder	
2	A46SRE/HUbFR	173
3	HUbRE/A46SFR	122
4	S20FRE/HUbFR	81
5	HUbRE/S20FFR	212
6	I61TRE/HUbFR	217
7	HUbRE/I61TFR	71
8	L50PRE/HUbFR	170
9	HUbRE/L50PFR	118

Figure 5.6: Gel picture showing the amplified fragments of ubiquitin using mutagenic and non-mutagenic primers to generate mutant ubiquitin genes. Amplicon size and lane labelling are given in the Table next to gel image.

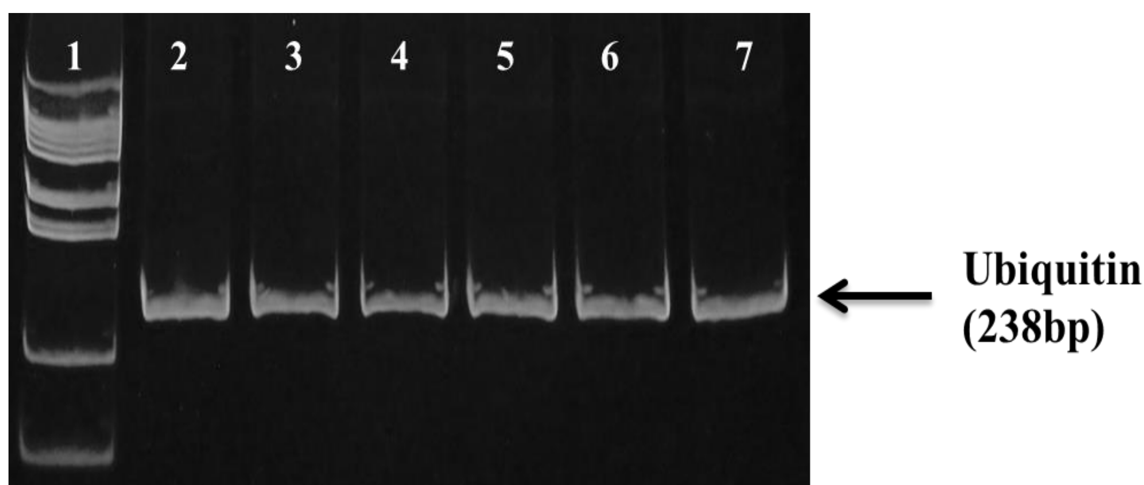


Figure 5.7: DNA-PAGE (15%) showing PCR amplicon of ubiquitin gene. Lane 1 shows 100bp DNA ladder and lanes 2-7 show full length ubiquitin gene of HUbWt, HUbEP42, HUbS20F, HUbA46S, HUbL50P, and HUbI61T respectively.

5.3.2 Construction of mammalian expression vectors for expressing HUbEP42 and its single mutant derivatives

All the mutant ubiquitin genes as well as wild type ubiquitin gene were cloned in mammalian expression vector pTRE-Tight-IRES-EGFP between *EcoR*I and *BamH*I restriction sites, so that the expression of mutant ubiquitin genes would be driven by tetracycline regulatory promoter. The restriction sites available in synthetic ubiquitin gene were utilized for primary screening to confirm the incorporation of mutation. HUbWt contains all seven restriction sites, which are functional. So upon digestion, PCR amplicon was cleaved by digesting with respective restriction enzyme (RE). While constructing and cloning HUbEP42, the *Xba*I and *Xho*I restriction sites were disrupted. Similarly, in the constructs of HUbS20F, HUbA46S, HUbL50P and HUbI61T the RE sites *Xba*I, *Xho*I, *Xho*I and *Sal*I were disrupted respectively (Figure 5.8). Failure of digestion by the enzymes whose sites were altered, was used as a primary means to confirm incorporation of mutations. Further, final confirmation was done by gene sequencing.

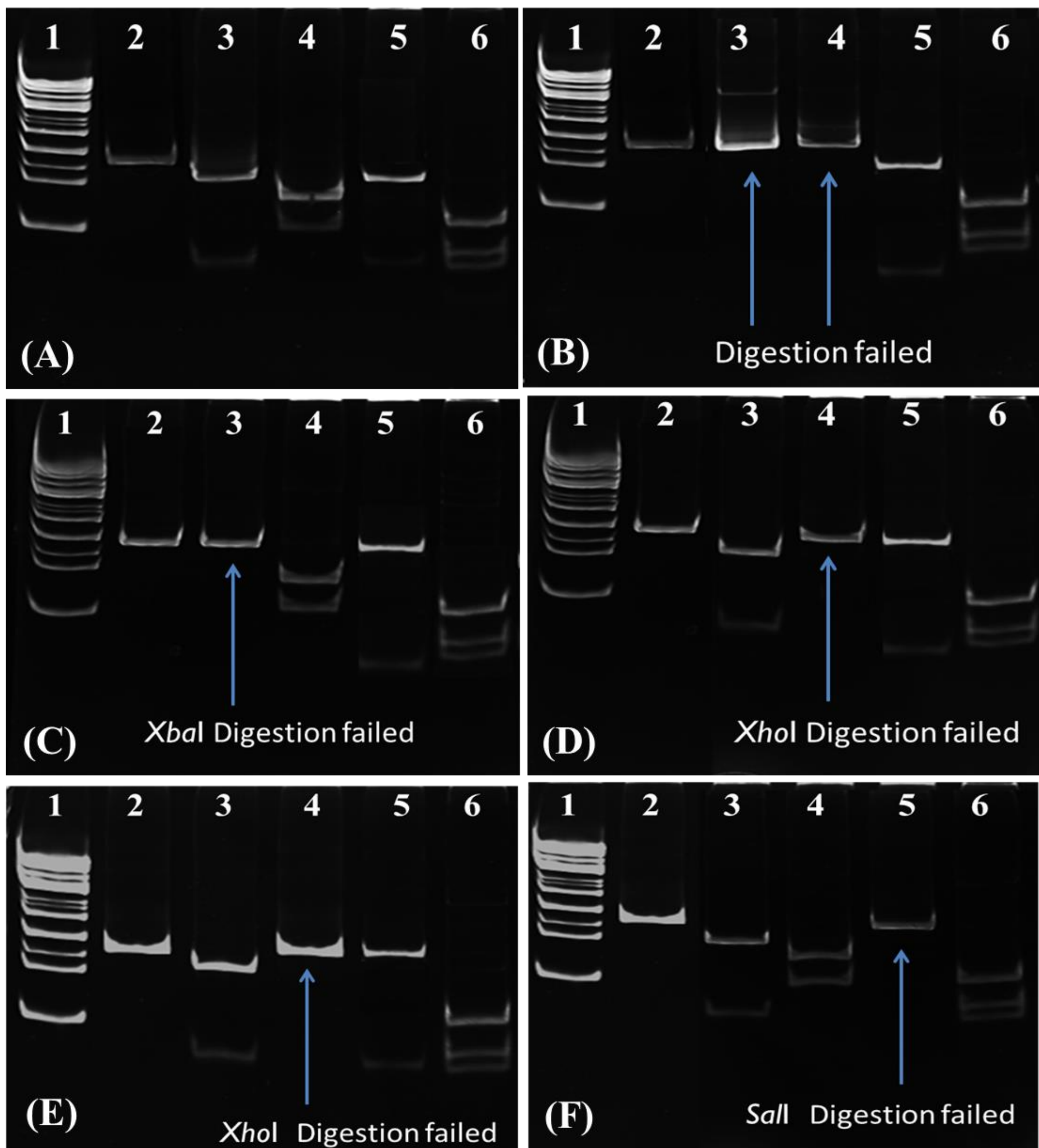


Figure 5.8: Agarose gel showing RE confirmation of ubiquitin mutant genes. A to F depicts HUbWt, HUbEP42, HUbS20F, HUbA46S, HUbL50P, and HUbI61T respectively. HUbWt was digested with all RE as it harbours all seven RE sites and HUbEP42 remains undigested with *Xba*I and *Xho*I, HUbS20F, HUbA46S, HUbL50P and HUbI61T remain undigested with *Xba*I, *Xho*I, *Xho*I and *Sal*I as their restriction sites were disrupted during mutagenesis. The gel pattern confirms the incorporation of mutations.

5.3.3 Optimization of Tet-On system for Inducible Expression of mutant ubiquitin proteins

The conditions suitable for overexpression of target gene were standardized using pTRE-Tight-IRES-EGFP vector by monitoring the levels of EGFP. Stably transfected MCF-7 cells were incubated with varying concentrations of the inducer i.e. doxycycline (tetracycline homologue) for different time points. The result showed that EGFP expression was maximum at 24h, when healthily growing vector transfected cell cultures were treated with 1 µg doxycycline (Figure 5.9). The vector transfected cells could not withstand treatment with 2 µg doxycycline and showed lysis. So for further experiments sublethal concentration of the inducer was used i.e. 1 µg doxycycline for 24 hours in order to overexpress mutant forms of ubiquitin protein in MCF-7 cells.

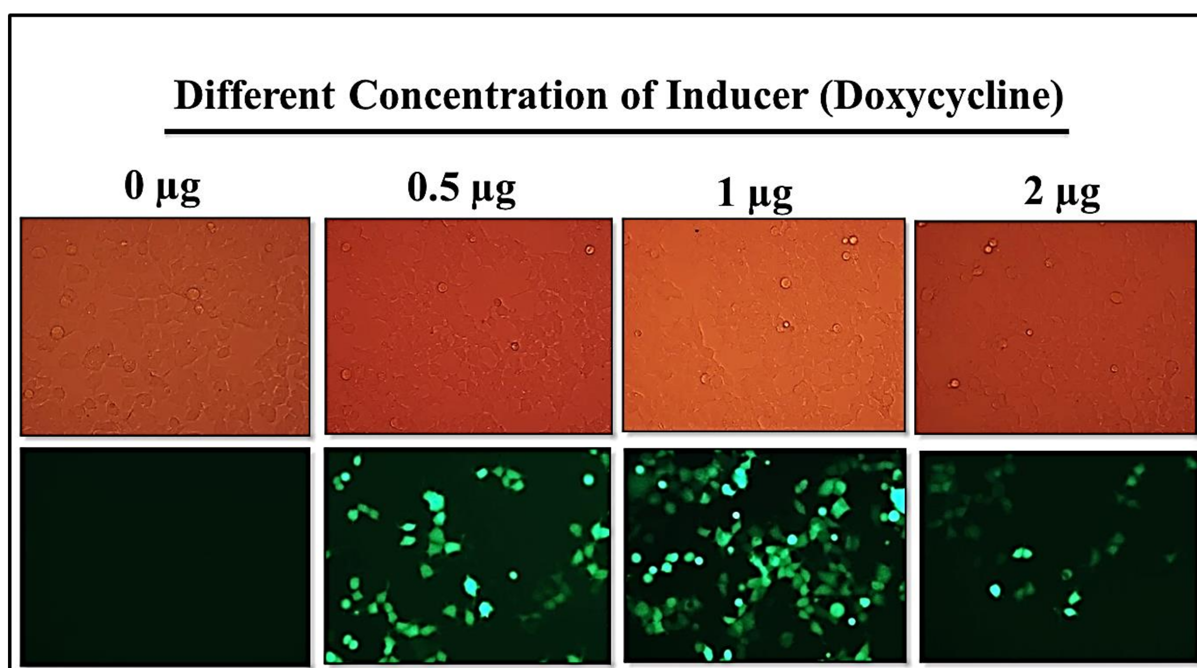


Figure 5.9: Overexpression of EGFP under Tet promoter. MCF-7 cells stably transfected with EGFP were subjected to varying concentrations of inducer doxycycline for different time points. Maximum expression of EGFP was observed at 24 hours with 1 µg/ml doxycycline.

5.3.4 HUbEP42, HUbL50P and HUbI61T cause lethality in MCF-7 cells, while HUbS20F and HUbA46S do not affect the cells

When incubated with 1 µg doxycycline for 48 hours MCF-7 cells transfected with *HUbEP42*, *HUbL50P* and *HUbI61T* mutants, showed lethal phenotype. HUbS20F and HUbA46S expressing MCF-7 cells remain unaffected and behaved as HUbWt transfected cells. The vector transfected and untransfected MCF-7 cells were used as controls (Figure 5.10).

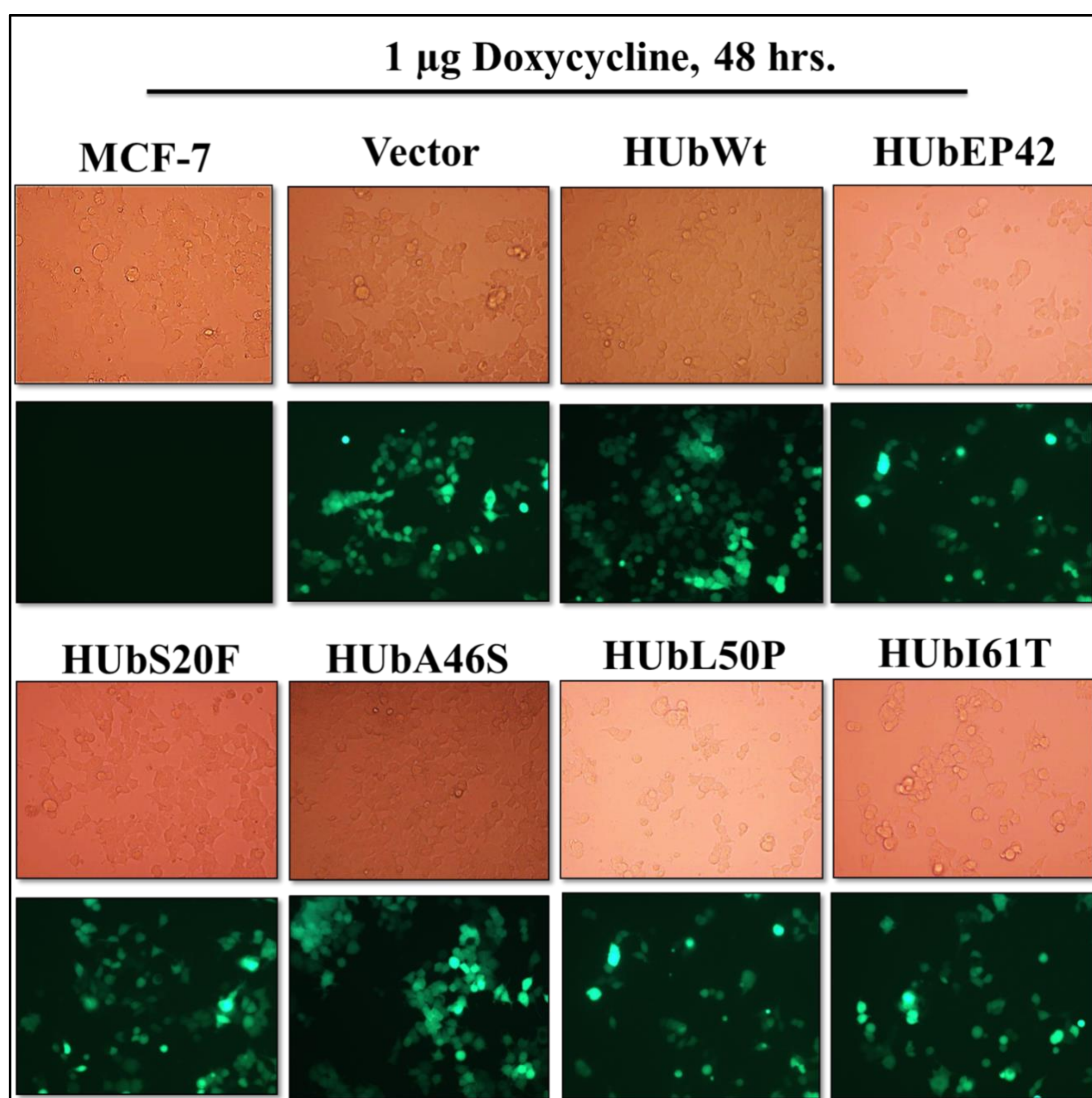


Figure 5.10: Consequences of mutant as well as wild type ubiquitin proteins on MCF-7 cells. Stably expressing MCF-7 cells were subjected to 1 µg/ml of doxycycline for 48

hours. Results show that HUbEP42, HUbL50P and HUbI61T transfected MCF-7 cells produced lethal phenotype. On the contrary, HUbS20F and HUbA46S expressing cells remained unaffected similar to HUbWt expressing cells. MCF-7 transfected with vector was considered as positive control whereas untransfected MCF-7 cells were taken as negative control.

5.3.5 Effect of expression of mutant forms of ubiquitin on the growth of MCF-7 cells monitored using MTT assay

It was clearly evident from the above experiment and earlier results of our laboratory regarding expressing identical mutants in *S. cerevisiae* and *C. albicans* that the growth kinetics of MCF-7 cells may get hamper upon high expression of these mutants. To study the same, the growth kinetics was observed of cells which stably express mutant protein as well as wild type ubiquitin. Ubiquitin was overexpress with help of inducer for required period of time. Results confer that the lethal mutants HUbEP42, HUbL50P and HUbI61T displayed slow growth pattern (Figure 5.11(A)). The cell proliferation ability of cells expressing lethal mutants were significantly decreased (Figure 5.11(B)). HUbS20F and HUbA46S could grow well as of HUbWt expressing cells and MCF-7 cells.

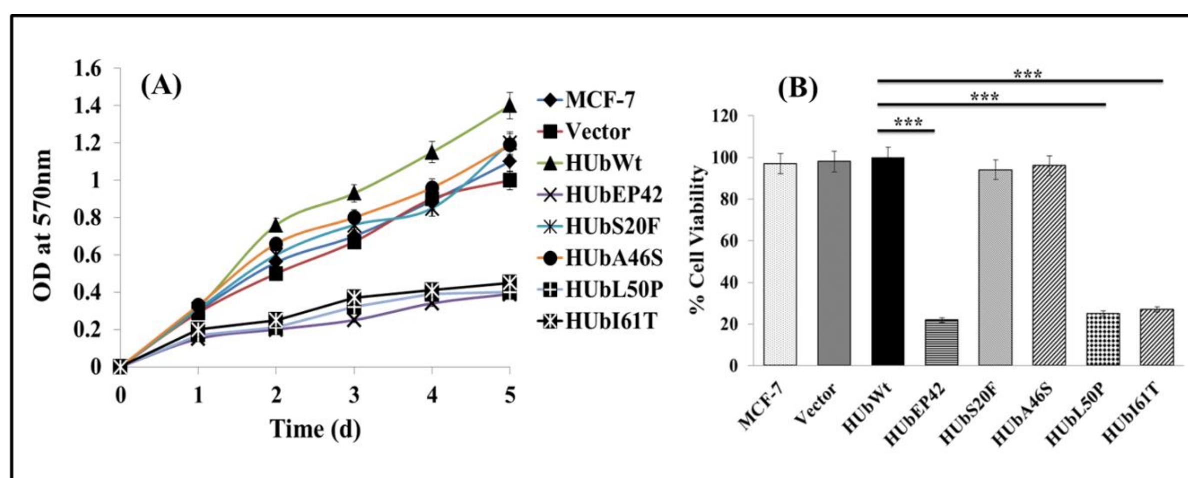


Figure 5.11: Influence of high level expression of HUbWt and mutant forms of ubiquitin on the growth rate of MCF-7 cells. (A) Growth curve of MCF-7 cells expressing wild type and mutant forms of ubiquitin. Results show that HUbEP42, HUbL50P and

HUbI61T grow at much slower rate as compared to other mutants and wild type ubiquitin protein expressing cells. (B) Cell proliferation ability of above mentioned lethal mutants were hampered significantly. However, HUbS20F and HUbA46S mutant forms of ubiquitin grew well and displayed unaltered cell proliferation ability. Results represented in Mean±SE. (n = 3, P* = <0.001, NS = non-significant).**

5.3.6 Influence of parent ubiquitin mutant HUbEP42 and its single mutant derivatives on clonogenic ability of MCF-7 cells.

Viability of MCF-7 cells expressing ubiquitin mutants and wild type protein were studied by clonigenic assay, wherein colony forming ability of these cells was observed. HUbWt and mutant transfected stable cells were added with an inducer till colonies were visible and colony counting was carried out. Results demonstrated that colony formation rate was markedly decreased in MCF-7 cells expressing HUbEP42, HubL50P and HUbI61T mutant forms of ubiquitin compared with HUbS20F, HUbA46S and HUbWt expressing cells. MCF-7 cells and vector transfected cells could form colonies at rates identical to HUbWt. This result clearly demonstrated that lethal mutants namely, HUbEP42, HUbL50P and HUbI61T reduced MCF-7 cell viability and cell proliferation (Figure 5.12).

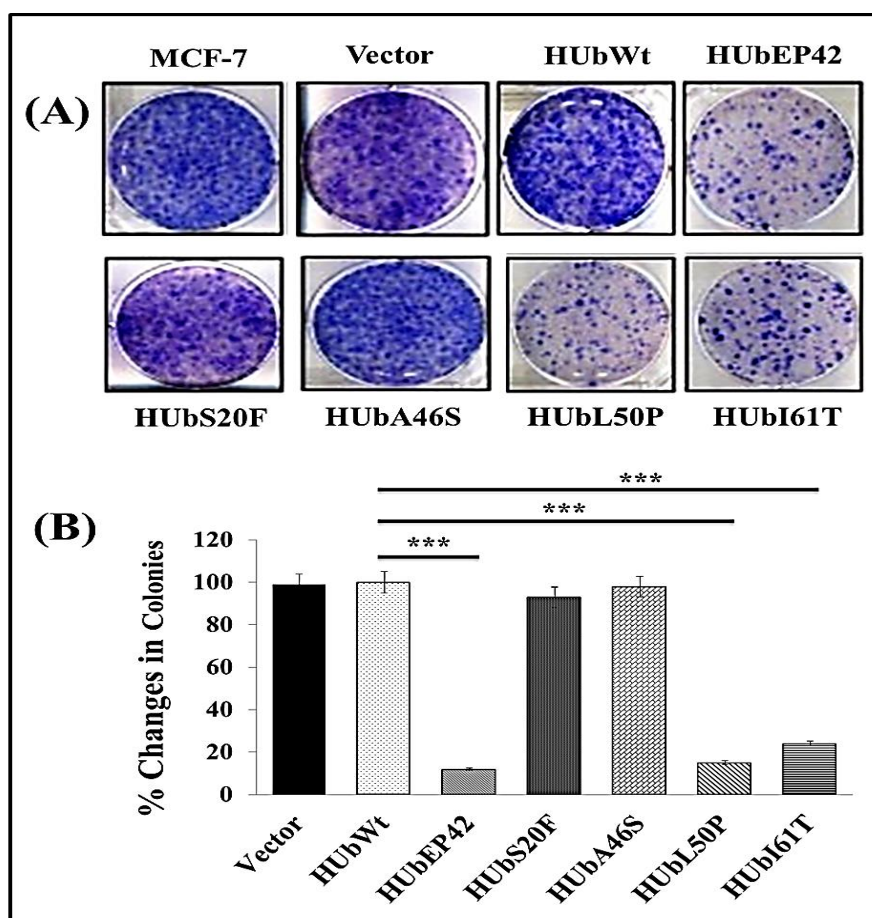


Figure 5.12: Implications of variants of ubiquitin HUbEP42 and its single mutant derivatives on clonigenic ability of MCF-7 cells. (A) Image showing clonogenic efficacy of mutant forms of ubiquitin accompanied by wild type expressing MCF-7 cells. Result shows that high expression of HUbEP42, HUbL50P and HUbI61T causes significant decrease in colony forming efficacy of MCF-7 cells. Other two mutants and HUbWt expressing cells grew well. Here, HUbWt transfected cells and MCF-7 cells were considered as positive controls. (B) Colonies were counted and data was shown as percentage changes in colonies of variants and wild type ubiquitin protein expressing cells along with MCF-7 cells. Results represented in Mean±SE. (n = 3, P* = <0.001, NS = non-significant).**

5.3.7 Effect of mutant ubiquitins over resistance to antibiotic stress.

Parent mutant HUbEP42 and its single mutant derivatives were seeded at equal

density in presence of doxycycline in addition to canavanine and tunicamycin antibiotics to study the effect of mutant ubiquitin over resistance of the cells to withstand antibiotic. These antibiotics cause increased generation of misfolded, truncated and non-functional proteins. The non-functional proteins are tagged by ubiquitin for removal by 26S proteasome. The transfectants were allowed to grow under identical conditions for 48h and MTT assay was performed. Results demonstrated that lethal mutants namely, HUbEP42, HUbL50P and HUbI61T could not withstand with the antibiotic stress. In contrary, HUbS20F and HUbA46S were capable of overcoming antibiotic stress similar to HUbWt expressing cells (Figure 5.13).

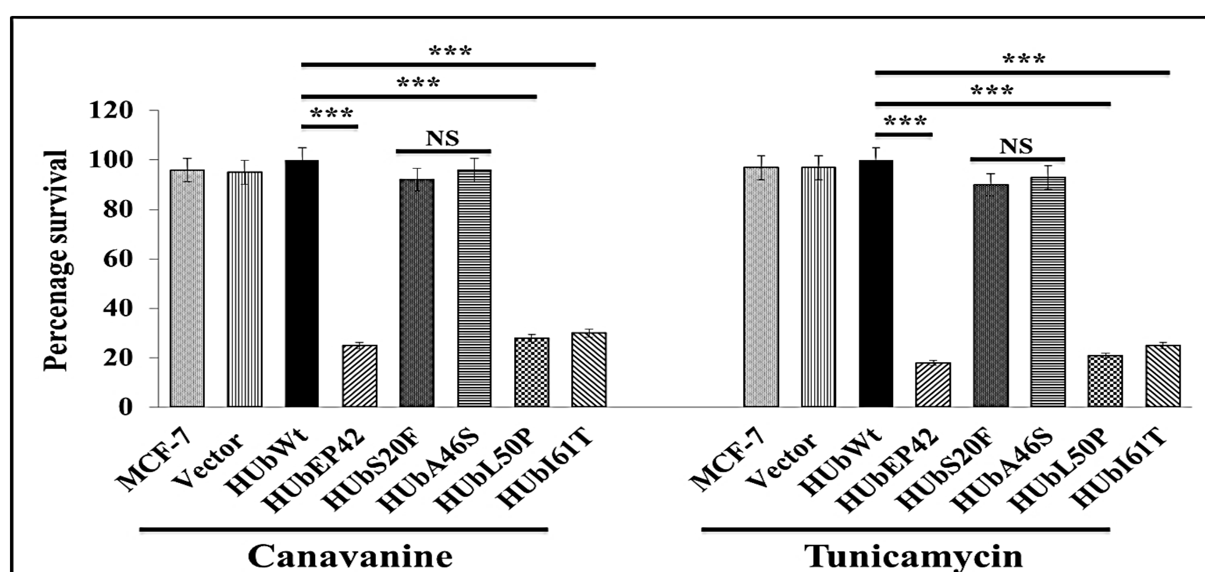


Figure 5.13: Stress response of HUbEP42 and its single mutant expressing MCF-7 cells upon antibiotics exposure. Result depicts that HUBEP42, HUbL50P and HUbI61T were unable to complement antibiotic stress as compared to HUbS20F and HUbA46S expressing cells. Results represented in Mean±SE. (n = 3, P* = <0.001, NS = non-significant).**

5.3.8 Fluorimetric detection of ubiquitin mutant protein localization

5.3.8.1 Construction of chimeric, EGFP tagged ubiquitin protein

As the earlier experiments shows that lethal variants of ubiquitin is disturbing

homeostasis of MCF-7 cells and unable to carry out biological functions, we hypothesised that ubiquitin is ubiquitous in the cell but due to point mutation the localization of mutant protein may limit to certain compartment of cells, leaving cells partially or non-functional. To elucidate this question, we generated EGFP tagged ubiquitin chimera which is cloned under tetracycline regulatory promoter in order to modulate protein expression. Ubiquitin was cloned in at C-terminus of EGFP, keeping C-terminus of ubiquitin free. Later this chimera was cloned in pTRE-Tight vector in which expression of EGFP-Ubiquitin was driven by Tet promoter with help of an inducer. Cloning was confirm by RE mapping of vector (Figure 5.14) followed by sequencing for further confirmation.

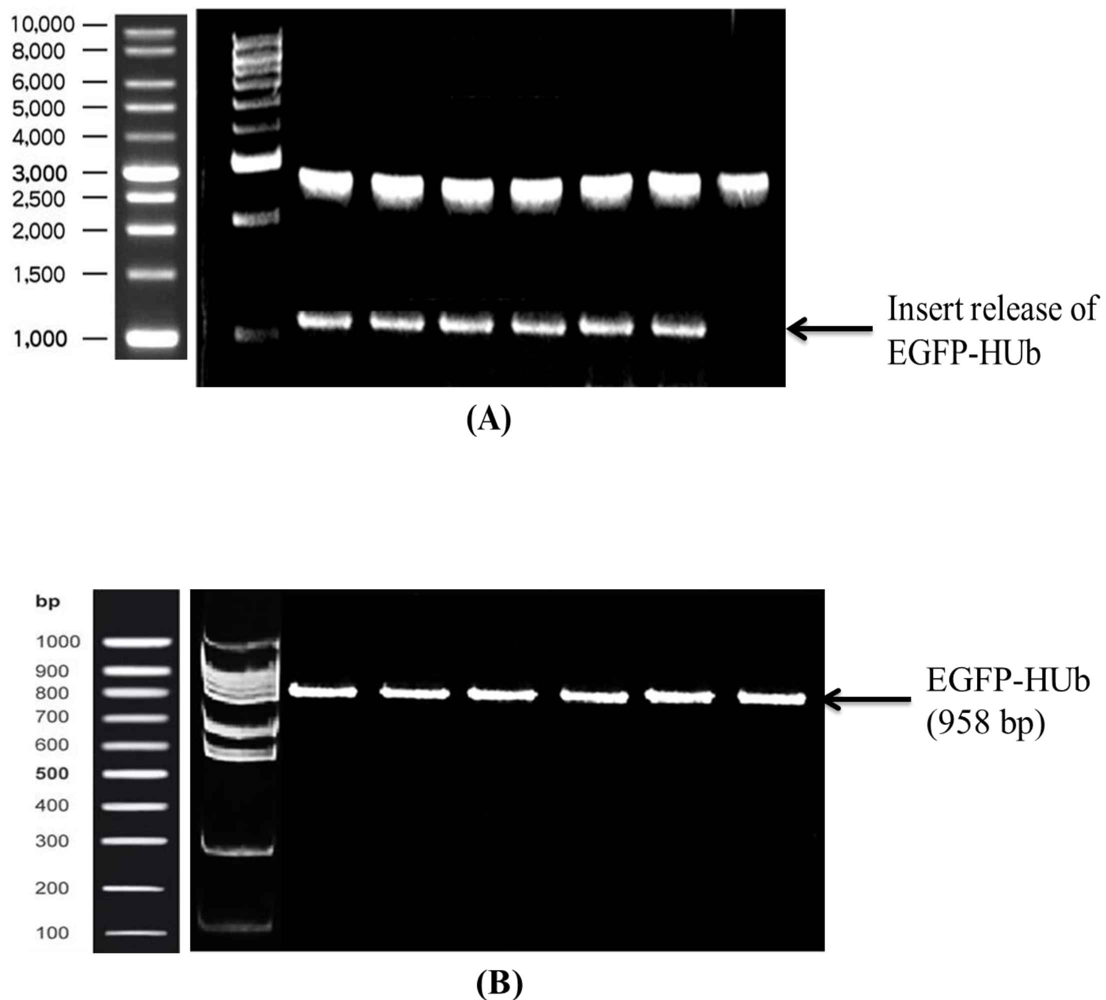


Figure 5.14: Agarose gel image showing RE mapping of pTRE-Tight vector harbouring

HUbWt and other ubiquitin variants. (A) Insert release of EGFP-Ubiquitin chimera upon digestion with *Kpn*I and *BamH*I. Lanes 1 to 6 show insert release of HUbWt, HUbEP42, HUbS20F, HUbA46S, HUbL50P and HUbI61T respectively. Lane 7 shows double digestion of pTRE-Tight vector which does not contain any insert and act as negative control. (B) PCR amplicon of EGFP-Ubiquitin chimera which amplified using EGFP forward primer and ubiquitin reverse primer (958bp). Lanes 1 to 6 show amplicon of HUbWt, HUbEP42, HUbS20F, HUbA46S, HUbL50P and HUbI61T respectively.

5.3.8.2 Localization of mutant ubiquitin protein in MCF-7 cells.

To study cellular distribution of mutant forms of ubiquitin, EGFP-ubquitin and EGFP-mutant forms of ubiquitin were employed. The engineering and expression of the chimeric constructs were described in Materials and Methods section of this chapter. Stably transfected MCF-7 cell line expressing HUbWt, HUbEP42 and its single mutant derivatives was subjected to inducer doxycycline at 1µg/ ml for 48h to induce expression of mutant ubiquitin protein. As ubiquitin was tagged to EGFP, green fluorescence of EGFP was monitored to find mutant protein localization in cells using confocal microscopy. In spite of the functional defects associated with HUbEP42, HUbL50P and HUbI61T, the localization pattern was very much similar to HUbS20F, HUbA46S and HUbWt. Results show no significant change in the distribution pattern of mutant proteins as compared to wild type protein (Figure 5.15).

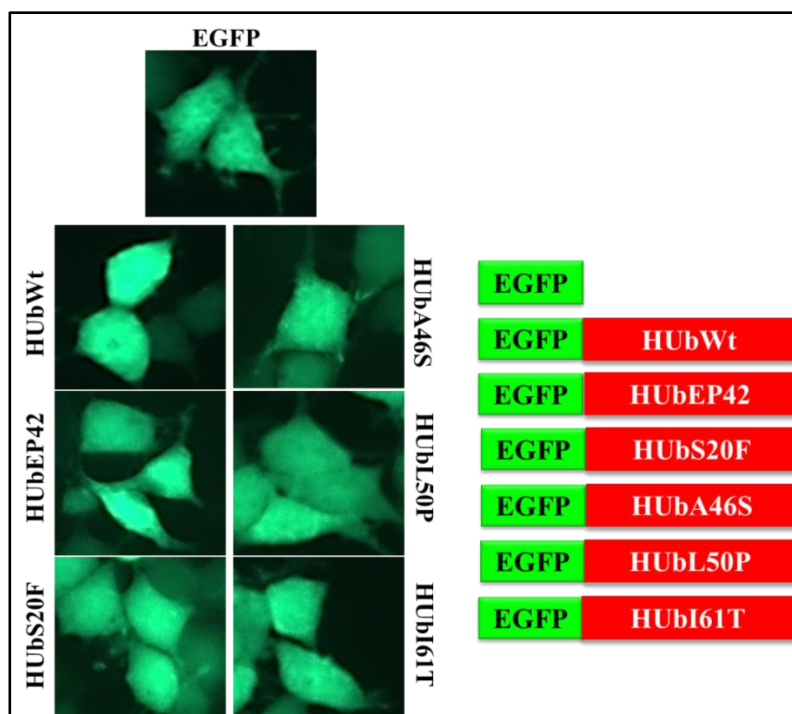


Figure 5.15: Microscopic evidence of mutant ubiquitin protein localization in MCF-7 cells. Wild type and mutant protein tagged with EGFP were overexpressed in MCF-7 cells in order to study mutant protein's localization by confocal microscopy. All the ubiquitin mutant proteins along with wild type protein were found to be distributed equally throughout the cell and no significant changes were observed. EGFP transfected pTRE-Tight vector was also subjected to identical conditions and it was considered as positive control for this experiment.

5.4 DISCUSSION

In the present study it was shown that variation of certain amino acid residues in ubiquitin led to alteration in the functions of ubiquitin. Ubiquitin was primarily known for its role in quality control of proteins. With advances in research, it is now evident that ubiquitin actively participate in carrying out myriad biological functions. Ubiquitin is highly stable and conserved throughout eukaryotes. Amino acids residues which are conserved in the sequence of ubiquitin are all essential, since natural selection must have removed any mutations that had interfered with either its structure or function. Mutational approach is a potent tool to study role of particular amino acids in maintaining structural and functional

integrity of the protein.

Ubiquitin mutations were generated in our laboratory using *in vitro* evolution and obtained ubiquitin mutant UbEP42, which has shown dosage dependent lethality in *S. cerevisiae* and *C. albicans*. The protein sequence of UbEP42 consists of four point mutations *viz.* S20F, A46S, L50P and I61T. These mutations were incorporated separately by site directed mutagenesis. Functional evaluation of the mutations demonstrated that UbEP42, UbL50P and UbI61T slow down growth and delayed S phase entry during cell cycle progression. The level of Cdc28 protein kinase was decreased and Fus3 MAPK was increased in the cells expressing lethal mutants (Doshi et al., 2014; Doshi et al., 2017). This could be reason for delayed entry in S phase as Cln3 activated Cdc28 complex plays important role in START of cell cycle and G0/G1 phase to S phase transition in yeasts (Cvrcková and Nasmyth, 1993). Similar results were observed in mammalian system. Mutants HUbEP42, HUbL50P and HUbI61T in MCF-7 cells displayed lethal phenotype and caused lysis of cells. Moreover, these mutant expressing MCF-7 cells showed slow growth phenotype and decreased cell proliferation. This could be due to hampered cell cycle progression at replication starting point as seen in yeast system with equivalent mutations. In mammalian cells the E2 enzyme UbcH7, is known to regulate entrance into and progression through S phase. Its overexpression delays entry of cells in S phase. Besides, level of UbcH7 is regulated by UPS (Whitcomb et al., 2009). Swi5 is transcriptional activator of Sic1 and inhibitor of S-phase CDKs. Swi5 is ubiquitinated by SCF^{Cdc4} and degraded by proteasomes. For efficient entry of cell in to S phase requires termination of Sic1 transcription at early G1 phase which in turn dependent of Swi5 (Kishi et al., 2008). Additionally, the anaphase-promoting complex/cyclosome (APC/C) is an E3 ubiquitin ligase that targets specific substrates for degradation by the 26S proteasome, which remain active during G1 phase through association with Cdh1. Entry to S phase is initiated by inactivation of APC^{Cdh1} complex, which is regulated by UPS (Nakayama et al., 2006). UPS is involved in activation, regulation and degradation of cyclin inhibitors during cell cycle (Chang et. al., 1990; Cross, 1990; Basco et al., 1995). The probable reasons for failure of mutants in G0/G1 to S phase transition could be structural changes in ubiquitin as a result of amino acids replacement in the lethal mutants HUbEP42, HUbL50P and HUbI61T.

Mammalian cells expressing HUbEP42, HUbL50P and HUbI61T were also shown to affect cell viability and colony forming ability of breast cancer cell line MCF-7. Whereas other two mutants, HUbS20F and HUbA46S did not show any adverse effect on the above cell functions and behaved similar to MCF-7 cell line and MCF-7 transfected with wild type ubiquitin gene. Previous studies on *S.cerevisiae* and *C.albicans* cells and with equivalent mutants established that these lethal mutants had lowered percentage survival, diminished levels of K48 and K63 linked polyubiquitination as compared to wild type ubiquitin, increased half-life of the substrates of ubiquitin fusion degradation (UFD) pathway, failure of sorting of carboxypeptidase S (CPS) protein to MVB, hampered uracil permease (membrane protein) degradation by lysosome and inability to withstand stress conditions like heat and antibiotics (Doshi et al., 2014; Doshi et al., 2017).

All Seven lysine residues of ubiquitin participate to the assembly of polyUb chain by forming isopeptide bond between C-terminus of Ub (G76) and the α-amino group of a lysine residue within the target protein, thereby generating variety of polyUb with diverse length and linkages to drive various functions (Peng et al., 2003b; Pickart and Fushman, 2004). Canonical K48 linked substrates are mediator of proteasomal degradation, whereas K63 linked substrates are believed to be directed for a range of processes including protein trafficking, DNA repair, lysosomal degradation of membrane proteins, inflammation etc. BRCA1/BARD1 E3 ubiquitination catalysed by K6 linkage might regulate DNA repair (Nishikawa et al., 2004). K11 linkages are known to support proteasomal degradation of certain protein targets as well as Ubc6 (ubiquitin conjugating enzyme) mediated functions in ERAD pathway (Baboshina and Haas, 1996; Kirkpatrick et al., 2006; Kim et al., 2007; Jin et al., 2008; Xu et al., 2009). During stress response U-box-type E3 ligases are known to form K27 and K33 linked polyUb whereas K29 linked chain may participate in UFD pathway (Hatakeyama et al., 2001; Johnson et al., 1995). Residues surrounding lysine and polyUb chain topologies determine substrate specificity, binding partners and docking site for ubiquitin mediated functions. The lethal mutants HUbEP42, HUbL50P and HUbI61T harbour point mutations proximal to K48 and K63 lysine residues of ubiquitin, which results in dysfunction of these mutants. Consequently undegraded substrate proteins accumulate in the cells, finally leading to death.

HUbEP42, HUbL50P and HUbI61T mutant forms of ubiquitin render MCF-7 cell more sensitive towards antibiotics Canavanine and tunicamycin. On contrary, HUbS20F and HUbA46S expressing MCF-7 cells remain unaffected. Owing to translational inhibitors, truncated and misfolded proteins are generated, which are eliminated by cells through UPS pathway (Hanna et al., 2003). The human ortholog of L28, a component if large ribosomal subunit undergoes K63 linked polyubiquitination and if the modification is absent ribosome shows increased sensitivity to ribosomal inhibitor antibiotics such as cycloheximide, tunicamycin and canavanine (Spence et al., 2000). Moreover, ribosomal proteins such as rpS3 and rpS6 (Kim et al., 2005) and pre-rRNA processing factors undergo UPS mediated degradation (Stavreva et al., 2006). Our results suggest that dysfunctional mutants HUbEP42, HUbL50P and HUbI61T potentially disturb ribosome biogenesis contributing to lysis MCF-7 cell line.

Wild type ubiquitin is present ubiquitously in the cell. Experiments carried out to see if mutations in conserved residues of protein had affected to the distribution, revealed no differences in their localization. Therefore, the damage caused due to functional failure of HUbEP42, HUbL50P and HUbI61T was not limited to any particular organelle and due to their distribution throughout the cell they could hamper myriad biological functions.

The topology of ubiquitin is a result of its compact structure provided by hydrophobic core and tightly packed secondary structural elements. Interacting partners of ubiquitin sample its surface features for diverse functions. As observed earlier, the mutations in UbEP42, UbL50P and UbI61T distort the structure of ubiquitin severely (Doshi et al., 2014; Doshi et al., 2017). Protein sequence identity 97% shared by human and yeast ubiquitin molecules implies that structural changes observed with yeast ubiquitin mutants must be very similar to human ubiquitin mutants. Hence, it can be concluded that the mutations produced identical results in both systems.

The ubiquitin proteasome system (UPS) is an elaborate and essential constituent of a eukaryotic cell that tightly modulates protein concentrations with specificity and precision to optimize cellular functions. Defects in UPS cause many disorders including cancer and neuronal diseases (Mani and Gelmann, 2005; Schmidt and Finley, 2014). The proximal activity of ubiquitin, the ubiquitination machinery comprising of E1, E2 and E3,

deubiquitinating enzymes (DUBs) and proteasome regulates expression of most proteins (Liu et al., 2015; Bielskiene et al., 2015; Johnson, 2015). Inhibition of the proteasome or a specific E3 ubiquitin ligase owing to its substrate has proven very effective in the treatment of multiple myeloma and neuronal disorders (Gong et al., 2016). However, the drugs introduced in the market like Bortezomyb have severe side effects and used only as final choice. Moreover, methods based on targeted protein degradation are being explored for inventing new strategies (Prabha et al., 2012; Joshi et al., 2015; Joshi and Prabha, 2016). The knowledge generated from mutational study of ubiquitin in the present study as well as advances in understanding roles of UPS system can identify attractive, novel and exciting targets for potential, future therapeutic interventions which can prove to be promising to regulate cellular dynamics. The results described here add to the information necessary for inventing drugs targeting components of UPS with greater potential and less side effects.