Synopsis of the thesis on

Structural Alterations of Mutant Forms of Ubiquitin and their Relevance to its Multitudinous Functions

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The Maharaja Sayajirao University of Baroda

For the degree of

Doctor of Philosophy in Biochemistry

By

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Under the
Supervision of

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To
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The M. S. University of Baroda,
Vadodara - 390 002.

28.07.2016

Subject: Submission of synopsis of the Ph. D. work entitled – "Structural Alterations of Mutant Forms of Ubiquitin and heir Relevance to its Multitudinous Functions "

Respected Sir/Madam,

Kindly accept the synopsis of my Ph. D work entitled – **"Structural Alterations of Mutant Forms of Ubiquitin and their Relevance to its Multitudinous Functions"**. My date of registration is 02/05/2011 and registration no. is 615.

Thanking you,

Yours sincerely,

(Doshi Ankita Arvindkumar)

(Prof. C. Ratna Prabha)
Guide

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INTRODUCTION

Proteins have a finite life span and continually synthesized and degraded in a constant state of flux. Intracellular Proteolysis involves both nonprocessive (single) cleavages of polypeptide chains of cellular proteins and the processive (complete or nearly complete) degradation of specific proteins to short peptides and/or individual amino acids. Many Intracellular proteins are either conditionally or constitutively short-lived, with in vivo half-lives that can be as brief as a few minutes to few hours. One Major role of proteolytic pathways is the selective destruction of regulatory proteins whose concentrations must vary with time and alterations in the state of a cell. The other functions of intracellular proteolysis includes the elimination of misfolded or otherwise abnormal proteins, the maintenance of amino acid pools in cells affected by stresses such as starvation, and the generation of protein fragments that act as hormones, antigens, or other effectors. [1] Short in vivo half-life of a protein provides a way to generate its spatial gradient and to rapidly adjust its concentration through changes in the rate of its degradation.[2] In conjunction with molecular chaperones, autophagy, and lysosomal proteolysis, selective degradation of proteins largely occurs through the ubiquitin proteasome system (UPS). [1, 3]

Ubiquitin is present ubiquitously in eukaryotic cells playing an indispensable role in selective protein degradation by marking off the target protein. It is a small, highly conserved protein bearing 76 amino acid residues. It has tightly-packed globular structure with mixed parallel and anti-parallel β-sheet, which is packed against α-helix to form the hydrophobic core and a flexible tail formed by protruding residues.[4] Among eukaryotes, ubiquitin is highly conserved and only a difference of three amino acids is found in the sequence from yeast to mammalian system. This high degree of conservation can be attributed to its central role in the cellular metabolism.[5, 6, 7]

In *Saccharomyces cerevisiae*, ubiquitin is expressed from four different genes namely UBI1, UBI2, UBI3 and UBI4, as a natural translational fusion to either ubiquitin or other proteins. Ubiquitin encoded by the genes *UBI1*, *UBI2* and *UBI3* is C-terminally fused to ribosomal subunit L40 and S31 respectively.[8] UBI4 is expressed as polymeric head to tail fusion containing five ubiquitin repeats (polyubiquitin). Ubiquitin C-terminal hydrolase cleaves the fusion proteins at the C-terminal end of ubiquitin, liberating free

ribosomal proteins and individual ubiquitin monomers. Polyubiquitin gene, *UBI4* is an important component of the stress response system and it helps the cell to cope up with stress. The adverse conditions are sensed by the element present upstream to the coding region, the 'heat shock box'.[9, 10]

Ubiquitination is carried out by seven lysine residues of ubiquitin i.e. K6, K11, K27, K29, K33, K48 and K63.[11, 12] Through these lysine residues variety of Ub chains forms, which function as different molecular signals in the cell. Ub chains that form through K48 serve as tag for degradation of substrate protein.[13] K63 linked ubiquitin chain is known to participate in protein trafficking, signalling and DNA damage response, inflammatory response and protein synthesis. [14, 15, 16] Lys11 linked chains can signal proteasome degradation in vitro.[17] Lys6 linked chains show conjugation to tumour suppressor protein BRCA1 implicated in the pathogenesis of breast and ovarian cancer.[18] Lys29 and Lys33 linked ubiquitination serves as tag for kinase modification.[19]

Ubiquitin mediates proteolysis through the enzymatic conjugation of Ub to proteins that contain degradation signals, called degron.[20] Ub protein conjugation marks proteins for their recognition and degradation by the 26S proteasome, a processive, ATP dependent protease. The conjugation of Ub to other proteins involves a preliminary ATP Dependent step in which the last residue of Ub (Gly76) is joined, via a thioester bond, to a Cys residue of the E1, Ub activating enzyme. The ''activated'' Ub moiety is transferred to a Cys residue in one of several Ub conjugating (E2) enzymes. In the third step catalyzed by a ubiquitin protein ligase or E3 enzyme, ubiquitin is linked by its C-terminus in an amide isopeptide linkage to an "-amino group of the substrate protein's Lys residues.

Recent studies regarding the role of Ubiquitin proteasome pathway in the regulation of human diseases revealed that mutations in ubiquitin and some of the ubiquitin ligase enzymes lead to many diseases like Alzheimer's disease, Parkinson's disease, Fanconi anaemia, various types of cancers, etc. So mutations in the lysines and other residues in the ubiquitin gene result in undesirable effects in the structural configuration as well as on function. Therefore, it is not surprising that aberrations of ubiquitination have been linked to a wide range of pathologic states.

Our laboratory has explored in vitro evolution of ubiquitin gene using error prone PCR to study the effect of residue variations on organism. Several mutations of ubiquitin have been successfully generated. The mutations were screened in *UBI4* deleted *Saccharomyces cerevisiae* strain. One of the mutations isolated, namely EP42 happened to be dosage dependent lethal mutation. Sequence of the gene has revealed seven codon replacements, of which three variations were coding the same amino acid and four variations were coding novel residue changes. The replacements are S20F, A46S, L50P and I61T.[21] Our previous results show that UbEP42, UbL50P and UbI61T are imparting dosage dependent lethality to the *UBI4* knocked out hypersensitive strain of *S. cerevisiae*, SUB60. These variants show increased generation time and less viability. In addition to this phenotype, these mutants are hypersensitive to antibiotic stress as well as heat stress, whereas other two mutants, UbS20F and UbA46S are phenotypically and functionally as good as wild type strain. [22]

Present work aims to extend the previous study from our laboratory, the effect of dosage dependent lethal mutant of yeast ubiquitin, UbEP42 and its single mutant derivatives, UbS20F, UbA46S, UbL50P and UbI61T using *S. cerevisiae* as model organism. Study focuses on the effects of above mentioned mutants on cell cycle regulation, UFD pathway, lysosomal degradation, polyubiquitation, effect of translational antibiotics on the organism in the presence of mutant ubiquitins. Changes caused in the structure of ubiquitin due to replacement of residues were studied using circular dichroism (CD) and fluorescence spectroscopy. Further, the studies on the effects of mutant forms of ubiquitin were extended to pathogenic organism *Candida albicans* and human cancer cell line MCF7.

Specific objectives: Major objectives of the present study are –

1. Functional characterization of dosage dependent effects of yeast ubiquitin mutations in *Saccharomyces cerevisiae*
2. Structural characterization of mutant forms of ubiquitin.
3. To study the effects of ubiquitin mutants on growth and propagation of *Candida albicans*
4. To determine the lethal effects of altered residues of ubiquitin on breast cancer cell line MCF7.

Objective 1: Functional characterization of dosage dependent effects of yeast ubiquitin mutations in *Saccharomyces cerevisiae*

Results of the growth curve, generation time and % viability of the mutant expressing cells show that there was increase in generation time and % viability decreased significantly in dosage dependent lethal mutants UbEP42, UbL50P and UbI61T whereas UbS20F and UbA46S phenotype behaved similar to UbWt. Lethal mutants were also unable to complement heat stress as well as antibiotic stress.

Our laboratory had already generated variants of yeast ubiquitin by using site directed mutagenesis and cloned yeast ubiquitin mutants, UbEP42, UbS20F, UbA46S, UbL50P and UbI61T in yeast expression vector, yEP96. These constructs were used for the functional characterization. YEP96 contains TRP and ampicillin as selection marker for yeast and bacteria respectively. Expression of ubiquitin is driven by *CUP1* promoter.

(i) Effects of mutants on cell cycle regulation.

S. cerevisiae UBI4 knockout cells, SUB60 were transformed with yEP96 vector which contains gene for mutant ubiquitin. These transformants were selected on TRP negative plates. Screened transformants were grown in the presence of $CuSO_4$ so as to overexpress mutant ubiquitin. Cells were stained with PI and FACS analysis was carried out. Results show that overexpression of UbEP42, UbL50P and UbI61T causes cell cycle arrest at G1 phase of the cell cycle whereas UbS20F and UbA46S were as good as UbWt.

(ii) Levels of Cdc28 and Fus3 in the presence of mutant ubiquitin

Cdc28 is the major regulator of cell cycle. Fus3 a MAPK, belongs to mating–pheromone response pathway. Cells respond to Fus3 expression by arresting cell cycle in G1 phase of cell cycle.

SUB60 cells transformed with mutant ubiquitin were overexpressed. At log phase cells were lysed and western blot analysis was carried out. Proteins were probed with Anti-Cdc28 antibody and Anti-Erk1/2 antibody for Cdc28 and Fus3 blots respectively. Result shows that levels of Cdc28 were decreased in presence of UbEP42, UbL50P and UbI61T whereas with UbS20F and UbA46S the levels were equal as UbWt. Pattern of Fus3 expression was found to be reciprocal to Cdc28. i.e. with UbEP42, UbL50P and UbI61T levels were increased whereas with UbS20F and UbA46S levels were low as compared to lethal mutant protein and at the same time equal to UbWt. RNA levels of

the Cdc28 and Fus3 remain constant in all mutants whereas after MG132 treatment protein Cdc28 and Fus3 levels remains same as without MG132 treatment which suggests that at transcriptional level concentration of kinases remains same in all the transformants and they may be changing somewhere downstream.

(iii) Consequence of ubiquitin mutants on lysosomal degradation of uracil permease

The yEP96 vectors containing ubiquitin variants were co-transformed with uracil permease coding vector. Expression of uracil permease protein is under endogenous promoter, *FUR4*. Cells were grown till log phase and simultaneously mutant ubiquitins were over expressed by using inducer. After giving cycloheximide treatment, the cells were harvested at regular intervals and lysates were prepared. Proteins were probed with ~~A~~anti-Fur4 antibody and western blot analysis was carried out.

For further confirmation, fluorescence spectroscopy was carried out. For this experiment, yEP96 constructs carrying the gene for mutant ubiquitin were co-transformed with GFP-uracil permease containing vector. The chimeric construct for GFP fused to uracil permease was expressed from GAL10 promoter. Protein synthesis was blocked by treating cells with protein translational inhibitor, cycloheximide. After giving cycloheximide treatment, the cells were harvested at regular intervals. The cells were excited at 488 nm and emission spectra were scanned in the range of 505 to 550 nm.

Western blot result indicates that till 90 minutes after cycloheximide treatment, there is no degradation of uracil permease protein when it is expressed in cells containing UbEP42, UbL50P and UbI61T. The same results were observed in fluorescence spectroscopy. There was more GFP expression in cells containing lethal mutants. UbWt, UbS20F and UbA46S expressing cells showed degradation of uracil permease in due course of time. It clearly suggests that there is accumulation of membrane permease and it is not degraded.

(iv) Degradation of substrate ubiquitin fusions by N-end rule and UFD pathways

For this study, we used pUB23 vector, which expresses Ub-X-β-Galactosidase under GAL10 promoter and has *URA3* as selection marker. The two forms of Ub-X-β-Galactosidase used here i.e. Ub-Met-β-Galactosidase and Ub-Pro-β-Galactosidase, in which X was Met and Pro respectively. Ub-Met-β-Galactosidase is processed ubiquitin

dependent proteases to release Met-β-Galactosidase. Met at the N-terminal of a protein stabilizes the protein and confers extended half-life according to N-end rule pathway for degradation of proteins. On the other hand, Ub of Ub-Pro-β-Galactosidase is not cleaved off by ubiquitin dependent proteases and polyubquitin chain is built on it, marking the protein for degradation by UFD pathway. These plasmid were co-transformed with yEP96 vector expressing ubiquitin variants. Mutants were overexpressed in the background of Ub-X- β-Galactosidase. To check the degradation of fusion substrate, western blot analysis was carried out using anti-β-Galactosidase antibody. To further confirm β-Galactosidase enzyme assay was carried out using ONPG as substrate.

Result shows that UbEP42, UbL50P and UbI61T are not able support the degradation of Ub-Pro-β-Galactosidase and hence there is a accumulation of Ub-Pro-β-Galactosidase. UbS20F and UbA46S along with UbWt are capable of assisting the degradation of Ub-Pro-β-Galactosidase and hence the level of Ub-Pro-β-Galactosidase is decreased. The results indicate that UbEP42, UbL50P and UbI61T hamper the degradation of chimeric fusions of ubiquitin degraded by UFD pathway.

(v) Influence of variants of ubiquitin on resistance to antibiotic stress caused by various antibiotics

Mutant ubiquitin gene carrying plasmids were transformed in SUB60 cells and the cultures were grown till log phase. ODs of the transformants were equalized and serially diluted. Dilutions were spotted on the plate containing different translational inhibitors, Hygromycin, Gentamicin, Geneticin, ER tress inducer i.e. Tunicamycin and Arginine homologue i.e. Canavanine.

(vi) Cloning of mutants in yeast expression vector and study of polyubiquitin chain formation

To carry out this experiment, we have used pUB221 vector which contains 6X HIS - myc tag, URA as selection marker. Ubiquitin variants namely UbEP42 construct is already present in our lab. UbS20F, UbA46S, UbL50P and UbI61T were PCR amplified and cloned in pUB221 using *BglII* and *KpnI* RE sites. Constructs were transformed in SUB60 strain. Transformants were selected on Uracil lacking plates. Transformants were grown in presence of inducer. At log phase cells were harvested and western blot analysis was carried out probing it with HRP tagged Anti-C myc antibody. We found

that all the mutants regardless of its lethality are capable in taking part in polyubiquitin chain formation.

Objective 2: Structural characterization of mutant forms of ubiquitin

Construction of plasmids, expression and purification of protein

We have used synthetic yeast ubiquitin with F45W mutation for this study. Reports show that Yeast F45W is structurally and functionally similar to UbWt. Site directed mutagenesis was carried out using sets of mutagenic and non-mutagenic primers. The mutants were confirmed by sequencing. These mutants were PCR amplified using gene specific primers and were cloned in pkk-223 vector using *EcoRI* and *HindIII* sites. Restriction digestions and ligations were done using standard methods (Sambrook, 1989). The constructs were transformed in *E. coli* BL21 (DE3) cells. Freshly transformed cells were grown in Luria−Bertani medium (Himedia) containing 50 µg/ml ampicillin at 37°C up to log phase, at this point protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were further allowed to grow overnight. Cells were harvested and lysed using sonication. Depending on the heat stability of protein, lysates were given heat shock for 15 minutes to remove other proteins. Salting out method was used to further nullify other unwanted protein. Final precipitates of proteins were solubilized in 10 mM Tris-Cl buffer pH 8.0 and dialysed. The lysate was loaded in pre-equilibrated column which is filled with Sephadex G-50 as stationary phase. Ubiquitin variants were purified by using size exclusion chromatography. The fractions were collected and checked for the presence of protein by recording OD at 280nm. Further the purity of sample was checked by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE).

a) Circular dichroism and fluorescence spectroscopy

To study the secondary structure content of the peptides far UV-CD spectra were recorded in the range of 180-260 nm (Jasco J-815). Purified 30 µM yeast ubiquitin mutants in 10 mM Tris buffer, pH 7.4 were used for recording CD. Spectral study shows destabilization and distortion in structure of UbEP42, UbL50P and UbI61T. From CDPro analysis, we found that there is a significant decrease in percentage of helix in all 3 lethal mutants. UbL50P is showing remarkably more beta-sheet like structure. CD spectra of UbS20F and UbA46S remain unaltered as of UbF45W. So the structure of UbEP42,

UbL50P and UbI61T are getting distorted due to residue replacements and hence due to this phenotype of cells and stability is compromised.

b) Fluorescence spectroscopic studies to characterize the structure of the ubiquitin mutants:

Fluorescence studies were carried out to characterize tertiary structural features of the mutant forms of ubiquitin using intrinsic and extrinsic fluorophores. Fluorescence spectra were recorded using Hitachi Fluorescence Spectrophotometer FL-7000.

Intrinsic fluorescence spectra were recorded to know the tertiary structure of the variants. Mutant proteins were excited at 280 nm and emission spectra were recorded in the range of 300-400 nm wavelength. Guanidine hydrochloride denaturation study was also performed. Increasing concentrations of guanidine hydrochloride (0 to 6M) were used for the study. Result shows that the mutant proteins contain tertiary structure. UbEP42, UbS20F and UbL50P showed decrease in intensity upon increasing concentration of guanidine hydrochloride and UbWt, UbA46S and UbI61T Extrinsic fluorophore 1-anilino 8-naphthalene sulphonic acid (ANS) was used in fluorescence resonance energy transfer (FRET) studies. All the mutants showed fluorescence resonance energy transfer from intrinsic (aromatic amino acids) to extrinsic (ANS) fuorophore in FRET study but the intensity was less in UbEP42 and UbA46S as compared to other mutants which suggest that changes in surface hydrophobic residues.

Objective 3: To study the effects of ubiquitin mutants on growth and propagation of *Candida albicans*

Candida albicans being near relative of *S.cerevisiae* and also a facultative pathogenic organism provides a good model organism to study the effects of ubiquitin mutations. Mutant ubiquitin cloned in pUB221 vector which have *URA* marker and expression is driven under *CUP1* promoter was used for the study. Mutants were transformed in CAI4 strain of *C. albicans*. Mutants were overexpressed and following experiments were carried out.

(i) Effect of overexpression of mutant ubiquitins on phenotype

(ii) Effect of mutants on growth profile

(iii) Heat stress complementation test

(iv) Thermotolerence capacity of transformants in presence of mutants

(v) Polyubiquitation chain formation capability of mutants

Results of the above studies show that cells were sensitive to overexpression UbEP42, UbL50P and UbI61T and caused lethality to the cells. Cells transformed with these mutants had slow growth profile and hence longer generation time. They showed compromised heat stress complementation and their thermotolerance capacities were lowered significantly. The functions of UbS20F and UbA46S were remain unaltered and cells expressing them behaved as good as those expressing UbWt.

Objective 4: To determine the lethal effects of altered residues of ubiquitin on breast cancer cell line MCF7.

To extend the study in higher eukaryotes, we have chosen breast cancer cell line, MCF7. For this study mammalian ubiquitin was used with same residue replacements as in UbEP42, UbL50P and UbI61T studied with yeast system. Mutants were generated by site directed mutagenesis. For this objective, pTRE-IRES-EGFP vector was used to clone the mutants. This vector is tetracycline inducible with G418 selection marker. It has IRES site which is fuse with EGFP. So the cloned fragment expresses in tetracycline inducible fashion individually and EGFP expression is driven independently by IRES site. The mutants were cloned in pEGFP vector which expressed EGFP fused chimeric protein. Transfected MCF7 cells were subjected to inducer and the concentration, dosage and incubation period were optimized i.e. 1µg inducer, incubation for a maximum period of 48 hours and GFP expression was monitored. The conditions were maintained in the following experiments:

(i) Effect of overexpression of Human ubiquitin variants on MCF7 cells

(ii) Viability of cells by MTT assay

(iii) Colony forming capacity of variants

Overexpression of mutants HUbEP42, HUbL50P and HUbI61T showed decreased growth rate and the mutants were found to be cytotoxic to the cells. Viability and colony forming capacity were significantly decreased in cells expressing above mutants. HUbS20F, HUbA46S were showing normal growth rate, as well as colony forming capacity and viability remains unaffected.

Conclusion:

Functional studies of yeast ubiquitin reveal that change in residues of conserved ubiquitin sequence leads to the erratic changes in physiology of the organism. The dosage dependent lethal mutants of the ubiquitin namely UbEP42, UbL50P and UbI61T are responsible for the increased generation time with less viability and lethality of the hypersensitive SUB60 *S.cerevisiae* cells. Levels of Cdc28 lower and due to which cells are not getting START signal for replication in the cells expressing these lethal mutants hence in cell cycle analysis experiment we found cells are stuck in G1 phase of cell cycle. Our results shows that RNA levels of Cdc28 and Fus3, the Mitogen-activated protein kinase remain constant and degradation is not carried out by UPS pathway so they may be regulated differently probably at the level of translation. These lethal mutants also obstructed lysosomal degradation of membrane proteins and also altered the half-life of substrate proteins with Pro residue. However, they are able to take part in polyubiquitin chain formation. These mutants are unable to complement heat stress as well as antibiotic stress. UbS20F and UbA46S remain unaltered and able to perform all the functions normally as UbWt. CD and fluorescence spectroscopic studies showed structural changes occurring due to the mutations of UbEP42, UbL50P and UbI61T. So the structural defects might be responsible for the changes observed in functioning of the organism. Similar results were observed in the facultative phathogen *C. albicans* as well as human cancer cell line MCF7. Considering the of the ubiquitin protein sequence and various but critical roles played by ubiquitin, it can be concluded that alteration in the residues L50 and I61 of ubiquitin protein hamper its functions and lead cells to lethality.

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Functional characterization of dosage dependent lethal mutation of ubiquitin in *Saccharomyces cerevisiae.*
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FEMS Yeast research, 2014

Oral/Poster presented & conference/ workshop attended in Ph.D. duration:

1. Presented poster entitled "The Effects of Ubiquitin Mutations on Ubiquitin Biology of *Saccharomyces cerevisiae"* at 'Cell Biology of Yeasts' Meeting, 5th - 9th November 2013, Cold Spring Harbor Laboratory, New York, USA.

2. Presented poster entitled "Effects of Ubiquitin Mutation on *Candida albicans*" at Ramanbhai Patel College of Pharmacy International Conference (RAPCOPINC – 2014) "Current Perspectives in Drug Discovery, Development and Therapy", 27th & 28th February, 2014 at Ramanbhai Patel College of Pharmacy, CHARUSAT, Changa

3. Attended Three day National Symposium on "Emerging Trends In Biochemical Sciences" at department of Biochemistry, Faculty of sci., The M.s.University of Baroda, Vadodara , organised during 29th to 31st December, 2014.

4. Attended Two Day National Seminar on "Molecular Basis of diseases" at department of Biochemistry, Faculty of sci., The M.s.University of Baroda, Vadodara , organised during 1st & 2nd August, 2014.

5. Presented a research paper entitled "Functional importance of the mutant ubiquitin Q2N-E64G of parallel β-bulge of ubiquitin in *S.cerevisiae"* at Regional Science Congress organized by The M.S.University of Baroda, Vadodara, held on 15-16 september, 2012.

6. Presented poster entitled "Structural and Functional Characterization of Dosage Dependent Lethal Mutation of Ubiquitin in *Saccharomyces cerevisiae"* held at DBT-MSUB-ILSPARE programme at Faculty of Science, The M.S.University of baroda, at 30th September, 2014.

7. Attended one week National workshop on "Frontiers of NMR Spectroscopy: "Nucleus to Nucleotides", held at Centre of Excellence, NFDD Complex, Department of Chemistry, Saurashtra University during 15 - 21, January, 2016

8. Attended DBT-MSUB-ILSPARE sponsored Five Day Workshop on "Basics of Cell Culture and Stem Cell Techniques" at The M.S.University of Baroda, Vadodara during 28th February to 3rd March, 2012.

Date: **Signature of the candidate**

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