Summary

Ubiquitin is small, globular protein present in all eukaryotes. The outstanding feature of ubiquitin is conservation of its protein sequence throughout evolution. Sequence variations occurred only at three positions from yeast to humans. The marked sequence conservation suggests crucial role of residues in maintaining structural and functional integrity of the protein (Gavilanes et al., 1982; Watson et al., 1978; Schlesinger et al., 1975; Schlesinger and Goldsteiner, 1975). Structurally, ubiquitin consists of α-helix, five strands of mixed β-sheet, nine reverse turns and two β-bulges (Vijay-Kumar et al., 1987). Extensive hydrogen bonding and compact hydrophobic core contribute to its remarkable stability. Ubiquitination involves covalent attachment of ubiquitin to substrate protein through three steps catalyzed by a cascade of three enzymes i.e., E1, E2 and E3. Ubiquitin serves as a tag to mark proteins for degradation by ubiquitin proteasome system (UPS) and to regulate many non-proteolytic functions. Ubiquitin is crucial player of the cell as it facilitates various functions to maintain cellular homeostasis.

Keeping the conserved nature of ubiquitin in mind, our laboratory aims to understand structural and functional correlation of conserved residues. Previously *in vitro* evolution was carried out in our laboratory using random mutagenesis approach in order to generate various ubiquitin mutations. Mutant forms of ubiquitin were selected by expressing the ubiquitin mutants in the UBI4 lacking, stress hypersensitive SUB60 strain of *Sachharomyces cerevisiae*,. One of the mutants UbEP42 displayed lethal phenotype even under permissive conditions for SUB60 strain. Sequencing of UbEP42 unravelled four novel point mutations *viz*. S20F, A46S, L50P and I61T. Site-directed mutagenesis approach was used to generate single mutant derivatives to understand their effects on cellular functions. UbEP42, UbS20F, UbA46S, UbL50P and UbI61T were cloned in *CUP1* inducible yeast expression vector. Expression of UbEP42, UbL50P and UbI61T displayed lethal phenotype whereas UbS20F and UbA46S did not show any adverse effects on cellular functions.

Functional studies with SUB60 and detailed characterization at molecular levels revealed several interesting facts about the mutations UbEP42, UbL50P and UbI61T. Results of cell cycle analysis and BrDU cell proliferation assay suggest that UbEP42, UbL50P and UbI61T expressing cells were arrested at G0/G1 phase and showed delayed entry into S phase. Cdc28 protein kinase levels were decreased. Cdc28 acts as molecular switch for cell cycle progression through G0/G1 to S phase in association with G1 cyclines. The MAPK, Fus3 showed opposite trend in the above cells. The mRNA levels of *CDC28*, *FUS3*,

molecular chaperone, *CDC37* and G1 cyclin, *CLN3* remained unaltered. This suggests that variation in the levels of these kinases occurred post-translationally. However, our results have ruled out the possibility of ubiquitin mediated degradation of Cdc28 protein kinase and Fus3 MAPK. Further, the ROS levels decreased significantly in lethal mutant expressing cells. UbEP42, UbL50P and UbI61T expression hampered lysosomal degradation of a membrane protein uracil permease and endosomal sorting of carboxypeptidase S (CPS). Western blot analysis of Lys48 and Lys63 linkages was also found to be defective. Moreover, lethal mutants displayed adverse effects over ubiquitin fusion degradation pathway with elevated half-life of substrate protein. UbEP42, UbL50P and UbI61T expressing yeast cells failed to withstand stress conditions like exposure to various antibiotics and elevated temperature. However, expression of UbS20F and UbA46S did not have any detrimental effects over SUB60 cells (Doshi et al., 2014; Doshi et al, 2017).

Structural changes occurred by replacement of amino acids were studies using circular dichroism (CD) and fluorescence spectroscopy techniques. UbF45W, a pseudo-wild type ubiquitin was mutated at desired positions by site-directed mutagenesis. Mutant ubiquitins were cloned and expressed in *Escherichia coli* and purified to homogeneity. Results of spectroscopic studies revealed drastic alteration in secondary structure of UbEP42, UbL50P and UbI61T with increased β-sheet content and decreased helical content. However, UbS20F and UbA46S did not show any significant changes (Doshi et al., 2017).

Candida albicans was chosen as model organism to study the effects of mutant forms of ubiquitin for two reasons: (i) its is an opportunistic pathogen of humans and (ii) it shares common ancestry with *S. cerevisiae*. The mutant forms of ubiquitin were introduced into CAI4 strain of *C. albicans* extra-chromosomally using *CUP1* inducible yeast expression vector. The genome of CAI4 strain has all sets of ubiquitin coding genes intact. We have observed similar effects as *S. cerevisiae* with *C. albicans*. Expression of UbEP42, UbL50P and UbI61T caused lethality. These mutations have negative effects over growth rate with increased doubling time, decreased Cdc28 protein kinase levels. The Lys48 and Lys63 polyubiquitination were also found to be defective. UbS20F and UbA46S had no significant effects over the above functions. Interestingly, all five mutations could not show yeast-to-hyphae morphogenesis, which is necessary for causing pathogenesis in host.

Ubiquitin sequence of humans is 97% identical to that of yeast. We hypothesized that if these mutations displayed similar adverse effect on cancer cells, then it would be useful to develop novel therapeutic approaches. To study the effects of mutant forms of ubiquitin in humans, we used MCF-7 cell line as model system. MCF-7cell line is estrogen responsive, breast cancer cell line. Human ubiquitin gene was used for the studies and amino acids were replaced at identical positions using site-directed mutagenesis and expressed under tetracycline inducible system. Overexpression of HUbEP42, HUbL50P and HUbI61T led to the lysis of MCF-7 cells. Also doubling time of MCF-7 cells was found to be increased with decreased cell proliferation. Moreover, results suggest significantly decreased clonogenic ability of cells expressing the three lethal mutants. HUbS20F and HUbA46S expressing cells remained unaltered. However, there was no alteration found in the localization pattern of the mutant forms of ubiquitin with respect to wild type.

Summarizing, the ubiquitin mutant UbEP42 was found in a random mutagenesis carried out with ubiquitin gene in order to understand the reasons behind evolutionary conservation of its sequence. It consists of four novel amino acid replacements. These are UbS20F, UbA46S, UbL50P and UbI61T. We have tested several cellular functions in *S. cerevisiae*, *C. albicans* and MCF-7 human breast cancer cell line to monitor effects of mutant forms of ubiquitin from lower to higher eukaryotes. Collectively, the results established that the expression of UbEP42, UbL50P and UbI61T had negative effects over cell functions in all three organisms. However, UbS20F and UbA46S did not show any significant effects on the biology of *S. cerevisiae* and MCF-7 cell line. However, in *C. albicans* morphogenesis was inhibited. Besides, Structural studies using yeast ubiquitin revealed drastic variation in secondary structural content of UbEP42, UbL50P and UbI61T, whereas secondary structural content of UbS20F and UbA46S was similar to that of ubiquitin wild type. The inhibitory effects of these mutations over cell proliferation have several implications for the control of infectious diseases and cancer.