Chapter I

Introduction

1 INTRODUCTION

Proteins are the most important biological macromolecules of the cell taking part in synthesis and degradation of innumerable biomolecules and regulation of many biological pathways and processes, accounting for about half of the total dry mass (Ron Milo, 2013). Broadly they function as molecular instrument through which the genetic information of the cell is expressed. Proteins occur in great variety with their sizes ranging from relatively small peptides to huge polymers with molecular weights in the millions. Moreover, proteins exhibit enormous diversity of biological functions ranging from cellular functions including regulation of transcription, translation, cell replication, cell signalling, chaperoning, transport, defence, mediation of cell motility and many more. It has been estimated that there are ~ 800 macromolecular complexes involved in the functioning of a yeast cell, of which 500 have already been described and ~3000 molecular machines are estimated in humans (Gavin et al., 2006). Dynamic network of protein-protein interactions leads to the synchronization and regulation of different processes and cell responses through various signalling cascades which are indeed very critical. However, proteins are functional only when they are folded properly in their unique three-dimensional native structure, which is attainable due to the stereochemical code present in amino acid sequence. Remarkably, proteins are generated by joining twenty amino acids in different combinations and sequences using strikingly different properties and activities which leads to the production of various enzymes, hormones, antibodies, transporters, muscle fibers, the lens protein of the eye, antibiotics and myriad other substances having distinct biological activities. Proteins are continually synthesized and degraded. With the help of intracellular processive and nonprocessive proteolysis, constant state of protein degradation is maintained, which eventually regulates cell homeostasis and protein halflife. Major role of proteolytic pathways is the selective destruction of regulatory proteins, 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 (Weissmann, 1997). This highly complex and tightly regulated process that plays major roles in a variety of basic cellular pathways during both cell life and cell death is carried out by Ubiquitin Proteasome System (UPS) (Hershko and Ciechanover, 1998). "Ubiquitination" (or ubiquitylation), is known to mark proteins for degradation by the 26S proteasome and can also target proteins to

lysosomes by the endocytosis route or mediate other regulatory functions. (Jentsch and Pyrowolakis, 2000). This process involves transfer of ubiquitin in three sequential enzymatic reactions which are carried out by ubiquitin activating enzyme E1, ubiquitin conjugating enzyme E2, ubiquitin ligase E3, in which ubiquitin is added to the substrate protein targeted and directed to the multi-subunit protease complex proteasome for degradation (Varshavsky, 1997; Weissmann, 1997). Random mutagenesis can extend our understanding of some of the fundamental unsolved problems in protein evolution, especially with proteins whose sequence is largely conserved (Doolittle 1995a & 1995b). For example, ubiquitin protein displays 97% sequence conservation throughout evolution. Studies on sequence homology and phylogeny reveal that there are only three amino acid replacements that occurred during evolving from lower to higher eukaryotes (Schlesinger and Goldstein, 1975; Gavilanes et al., 1982; Dworkin-Rastl et al., 1984; Ozkaynak et al., 1984). The present research work aims to investigate indispensible role of conserved residues of ubiquitin protein in maintaining its structure in native state and its functions throughout evolutionary lineage.

1.1 UBIQUITIN

1.1.1 UBIQUITIN EVOLUTION:

During evolution, protein quality control has been the chief issue, with the need for maintaining steady state levels of proteins by rapid and selective removal of damaged proteins coupled to synthesis of required proteins. In 1978, Poole et al. documented that lysosome is actually not the crucial player for degradation of intracellular proteins, while investigating the mode of action of anti-malarial drugs. Instead, proteins in a cell are also degraded by a non-lysosomal mechanism now known as the ubiquitin proteasome system (UPS), so named by Hershko et al. in the 1970s (Ciechanover 2006). For many years, UPS was known to be the innovation of eukaryotes and found to be present only in eukaryota and absent from species of the other two super kingdoms, the Eubacteria and the Archaea. Subsequently, it has been shown that primitive versions of proteasomes and ubiquitin-like systems exist in certain bacteria and archaea and they have the ability to generate protein conjugates and target them for degradation (Hochstrasser, 2009).

In *Saccharomyces cerevisiae* Urm1 (ubiquitin-related modifier-1), acts as protein modifier and sulphur mobilizer. It carries sulfur in the thiolation of eukaryotic tRNA via a mechanism that requires the formation of a thiocarboxylated Urm1 like prokaryotic sulfur

carriers such as MoaD and ThiS. Urmylation also requires a thioester intermediate and forms isopeptide bonds between Urm1 and its substrates similar to ubiquitination and the enzyme Uba4 catalyzes the reaction similar to E1. This establishes an evolutionary link between ATP-dependent protein conjugation in eukaryotes and ATP-dependent cofactor sulfuration. Phylogenetic and structural analysis of Urm1 showed conserved sequence and structural features of the common ancestor of the entire ubiquitin superfamily and so it also known as ‘‘molecular fossil’’ (Xu et.al., 2006; Wang et.al., 2011; Eichler et.al., 2013).

In prokaryotes, the preparatory phase of thiamine and molybdenum cofactor biosynthesis involves the persulfide generation and a later step reveals similarity to the ubiquitin mediated protein degradation. In *E. coli*, the two step process of generating ThiS thiocarboxylate with help of ThiF is analogous to coupling of ubiquitin to ubiquitin conjugating enzyme (E1). ThiS is first adenylated by ThiF/ATP and then the activated sulfur is introduced by the action of IscS (Taylor et al., 1998, Xi et al., 2001). Likewise MoaD (molybdopterin synthase small subunit) gets adenylated by MoeB during synthesis of molybdenum cofactor (Lake et al., 2001). The sulphur-carrier proteins of *E. coli*, ThiS, which is of 7.3 KDa and MoaD, a protein of the size of 8.8 kDa share β-grasp fold and C-terminal Gly-Gly sequence with the ubiquitin and ThiF sequence is highly homologous to E1 (Taylor et al., 1998). Other examples of prokaryotic proteins homologous to ubiquitin and E1 are listed below:

Pathway	Ubiquitin homolog	E1 homolog
Protein degradation	Ubiquitin	E1/UBA1
Thiamin biosynthesis	ThiS	ThiF
Molybdoprotein biosynthesis	MoaD	MoeB
Quinolobactin biosynthesis	QbsE	QbsC
Cysteine biosynthesis	CysO	MoeZ

Table 1.1: Prokaryotic protein homologues of ubiquitin and ubiquitin activating enzyme E1.

Two Small Archaeal Modifier Proteins (SAMPs) of the Achaean *Haloferax volcanii* showed conjugation to protein targets, thus providing an evolutionary link in ubiquitin

protein conjugation systems. Along with β-grasp fold SAMP1 and SAMP2 genes are encoded as small, single domain proteins (of 87 and 66 amino acids, respectively) with the diglycine motif at their C-terminal end (Iyer, 2006; Burroughs, 2007; Burroughs,2008). The α-carboxyl of SAMPs forms an isopeptide bond to the ε-amino group of lysine residues on the target proteins by a mechanism that requires UbaA, an ubiquitin activating enzyme (E1) homolog of Archaea (Humbard et al., 2010; Miranda et al., 2011). However, the conjugation of SAMPs to substrate does not require E2 and E3 enzymes. Reduced proteasome activity in mutant forms *H. volcanii* led to accumulation of SAMP conjugates, indicating that SAMPs play a role in proteasome-dependent degradation (Humbard, 2010). Insight into the genome analysis of *Ca. Caldiarchaeum subterraneum*, a member of the phylum Aigarchaeota sheds light on novel view on the origin of ubiquitin and the ubiquitination system. Its genome consists of operon-like cluster representing complete set of ubiquitin system which includes single copy of ubiquitin, one ubiquitin activating enzyme (E1), one ubiquitin conjugating enzyme (E2), one RING-type ubiquitin-protein ligase (E3), and one deubiquitinating enzyme associated with the proteasome subunit Rpn11 (Nunoura et al., 2011). Various bacteria from phylum Actinobacteria, Planctomycetes and Acidobacteria contain related operons (Burroughs et.al., 2011). It also shows high sequence similarity to eukaryotic counterparts which place this archean species in the midst of eukaryotes (Koonin et al., 2014).

These studies establish that prototype of eukaryotic protein modifier system is present in the archaea. Hence, ancestral ubiquitin signalling genes might have undergone evolution through series of recombination and duplication events, which apparently resulted in modern day ubiquitin system.

1.1.2 UBIQUITIN CONSERVATION:

Ubiquitin is considered as one of the extremely conserved of all eukaryotic proteins (*Sharp & Li, 1987). Before 1984, known protein sequences of ubiquitin include diverse species ranging from mammals to insect but surprisingly they turn out to be identical. However, sequences from yeast (Oizkaynak, 1984), mammals, plant (Causing and Barkardottir, 1986) and slime mould (Westphal, 1986) revealed few variations but still sequence conservation is around 94.7% to 97%. Nevertheless, it is evident that the amino acid sequence of ubiquitin has not changed greatly across the different taxa between the ancestral "Proto-ubiquitin" and modern-day ubiquitin molecules. In past few years sequence-

comparison methods have become more sophisticated, revealing unexpected similarities between ubiquitin, components of ubiquitin-pathway and several bacterial proteins (Iyer, 2006) (Figure1.1).

Figure 1.1: Multiple protein sequence alignment of ubiquitin protein sequences. Proteins are grouped by eukaryotic kingdoms. The N-terminal methionine, seven lysine residues and the glycine-glycine C-terminal motifs involved in ubiquitin conjugation are shown in bold in first aligned sequence, from H. sapiens. Positions that show no variation are shown in black. Amino acid variations are highlighted in colours (Zuin et al., 2014).

In an attempt to derive the ancestral ubiquitin or proto-ubiquitin, Deocaris and group applied Bayesian statistical theory to estimate posterior probabilities of protein sequences from an evolution tree consisting 30 extant species. Their results suggest that ancestral sequence was homologous with the ubiquitin of *Brugia malayi*, a parasitic nematode with only nine residues were modified but there is absence of any major structural and functional changes (Deocaris, 2011). Studies on sequence homology of ciliates show 1-5 amino acid substitutions when it is compared with human ubiquitin. *Tetrahymena pyriformis*, a ciliate showed 4 amino acid substitutions at positions 16, 19, 24 and 28 (Neves et al. 1991; Guerreiro and Rodrigues-Pousada 1996; Xihan et. al, 2013). While investigating proportion *(pS)* of synonymous nucleotide differences amongst members of the ubiquitin gene family from 28 species of fungi, plants, and animals Masatoshi Nei et. al. observed that *pS* is very high and is often close to the saturation level even though the protein sequence is virtually identical (Masatoshi et al.,2000). Despite the ubiquitin gene redundancy, the genetic drift is observed. Upon comparing various sequences within various groups, no marked divergence was noticed. Therefore, it is believed that ubiquitin did not acquire major novel features

during eukaryote radiation and kept buffered to the date.

1.1.3 UBIQUITIN GENE FAMILY:

The covalent attachment of ubiquitin to its acceptor protein plays major role in down regulating protein degradation as well as various cellular processes in eukaryotes. Ub is a highly conserved protein known to bear 96% sequence homology among animals, plants and fungi (Graham et al., 1989). The gene family encoding ubiquitin is of multigene type, encoding natural fusion proteins (Ozkaynak et al., 1987). Ubiquitin is expressed as three different precursors. The polymeric gene, polyubiquitin (poly-u) is a polymer of ubiquitin genes arranged in head-to-tail fusions and by contrast in other three precursors, three monomeric genes are fused to the ribosomal proteins ([@]Sharp and Li, 1987 ; Baker and Board, 1991). These precursor proteins are cleaved by specific endopeptidases to release ubiquitin molecules, which are homologous (Tan et. al.. 1993; Jones and Candido, 1993). There is a marked variation in the number of ubiquitin units in a poly-u locus and the number of monomeric genes per genome among different eukaryotic species. However all ubiquitin genes encode identical amino acid sequences in a given species. Until recently it was believed that the present day ubiquitin family is a result of concerted evolution, meaning that there was a homogenization of the member genes of a family in due course of time (Mita et al., 1991; Nenoi et al., 1998; [#]Sharp & Li, 1987; Tan et al., 1993; Vrana & Wheeler, 1996). But later, the statistical studies of all available data depicted that it is a purification selection as in most species, non-synonymous nucleotide changes between the member genes was 0, whereas the synonymous differences were virtually saturated and the small nucleotide differences in some member genes were due to recent gene duplication (Nei et al., 2000).

The genome of *Saccharomyces cerevisiae* comprises of four ubiquitin encoding genes namely, *UBI1*, *UBI2*, *UBI3* and *UBI4* (Figure 1.2). The sequences of *UBI1* and *UBI2* are interrupted by introns at the same position in the sequence. However, the sequences of these introns are not identical. *UBI1*, *UBI2* and *UBI3* code precursor proteins, where ubiquitin is fused to some other protein sequences. *UBI1* and *UBI2* encode protein sequences of 52-residues, which are identical and *UBI3* encodes 76-residues long protein (Ozkaynak, et al., 1987). These tail protein sequences are highly conserved amongst yeast and mammals, with putative metal-binding, nucleic acid-binding domain of the form Cys-X_{2-4}-Cys-X_{2-15}-Cys- X_{2-4}-Cys (Miller et al., 1985; Berg, 1986; Harrison, 1986; Ozkaynak et al., 1987). Studies

revealed that the strains in which only ubiquitin was expressed were not viable without tail protein. Moreover, mutant strains lacking *UBI1*, *UBI2* and *UBI3* genes had sub-stoichiometric ratio of 18S to 25S rRNA. Later the proteins released after cleavage of the ubiquitin hybrid proteins were identified as ribosomal proteins and are important components of ribosomes. Thus it is evident that ubiquitin functions as a "chaperone" in the fusion facilitating the assembly of ribosome (Finley et al., 1989). The *UBI4* gene contains five consecutive repeats of ubiquitin gene in head to tail spacer-less arrangement, which encode 5 copies of ubiquitin protein as a translational fusion. The sequences in the upstream region of *UBI4* gene are strongly homologous to the 'heat shock box'. The *ubi4*/*UBI4* diploid cells are capable of forming four spores which are initially viable but two ubi4 spores in the ascus eventually lose viability while the *ubi4*/*ubi4* diploid cells are sporulation defective. When *ubi4* mutant cells are subjected to stress conditions like heat stress, starvation, amino acid analogues, they show hypersensitive phenotype. Hence, UBI4 is crucial player of the stress response system (Finley et al., 1987).

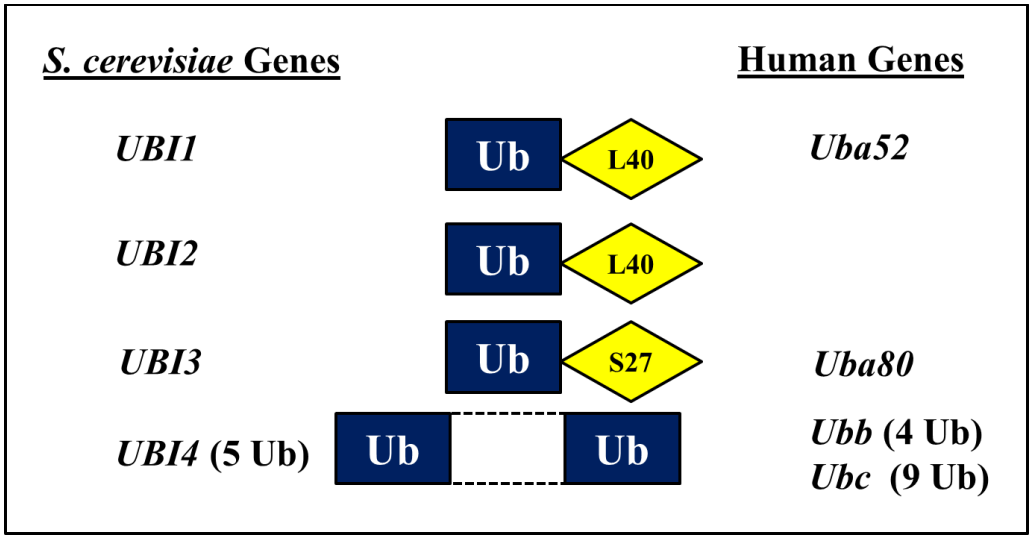

Figure 1.2: Ubiquitin encoding genes in *S. cerevisiae* and mouse.

Mammalian ubiquitin is also encoded by a multigene family comprising four genes namely, Uba (UBA52, Uba80), Ubb and Ubc (Figure 1.2). Of two Uba genes, Uba80 and Uba52 code for ubiquitin fused to ribosomal protein S27a (RPS27a) and L40 (RPL40), respectively (Redman and Rechsteiner, 1989; Webb, 1994; Kenmochi, 1998), where ubiquitin acts as a chaperone and assists in the formation of the holoribosome (Finley et al, 1989; Redman and Rechsteiner, 1989; Spence et al, 2000). Proteolytic process involving

specific enzymes is found to be responsible for generating monomer of ubiquitin from ubiquitin fusion precursor (Monia et al., 1989). Later in 1989, deubiquitinating enzymes (DUBs) were discovered as the enzymes with the ability to hydrolyze esters or ubiquitin fusion proteins (Miller, et al. 1989; Tobias and Varshavsky, 1991; Wilkinson, et al., 1989). Uba52 (*UBA52*) codes for ubiquitin fused to carboxyl extension protein (CEP) of 50-52 residues and Uba80 (RPS27a) codes for CEP of 76-80 residues. CEPs are known to have features typical of nucleic acid binding proteins, including many positively charged residues as well as hydrophobic domains (Redman and Rechsteiner, 1988). Ubb encodes 4 head-to-tail repeats of ubiquitin. Ubc gene contains 63 bp untranslated exon primarily, 812 bp intron followed by 9 tandemly repeated ubiquitin coding sequences (Wiborg et al., 1985). It is known to be a component of stress responsive system during UV irradiation (Nenoi, 1992; Arnason and Ellison, 1994), oxidative stress (Arnason and Ellison, 1994), exposure of cells to translational inhibitors like cycloheximide and amino acid analogue canavanine (Hanna et.al.,2003; Fernandes et al., 2006). The expression of UbC gene found to be unregulated in some pathological conditions. UbC carries out all these functions by maintaining steady state level of total Ub reservoirs in the cells.

1.1.4 STRUCTURE OF UBIQUITIN:

The key player ubiquitin is a small, compact, globular protein consisting a polypeptide chain of 76 amino acid residues of 8565 Da. Structure of yeast ubiquitin was described by Vijay kumar et. al. in 1985 using X-ray crystallography primarily at 2.8Å (Vijay Kumar et al., 1985) and later it was refined at 1.8Å (Vijay Kumar et al., 1987) (Figure 1.3). Ubiquitin, a protein devoid of any cysteines, metal ions or cofactors, has secondary structural features which include 3.5 turns of α-helix spanning residues 23 to 34, a short piece of 3_{10}-helix involving 56 to 59 residues, with five strands of mixed β-sheet characterized by a particular fold denoted as SSHSSS. Residues 1 to 7 and 64 to 72 of β-sheet are parallel whereas residues 10 to 17, 40 to 45 and 48 to 50 run in an antiparallel direction. This characteristic structural arrangement is called ‘β-grasp fold’ or ‘ubiquitin fold’.

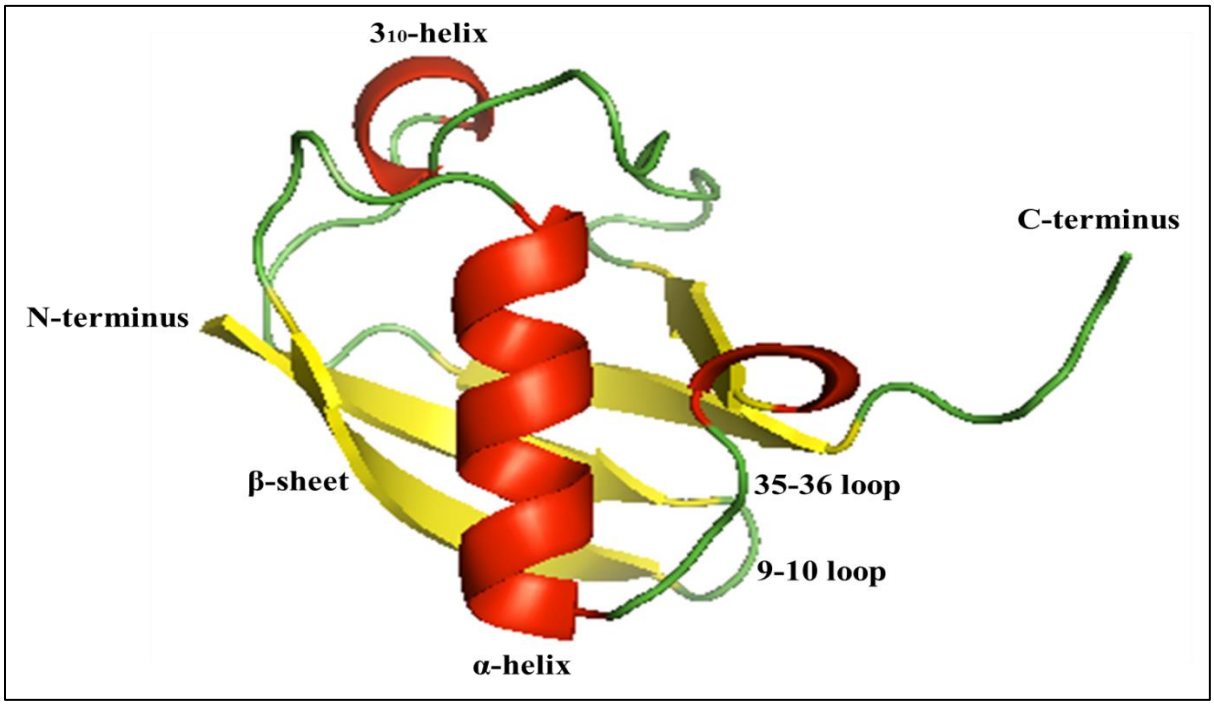

Figure 1.3: Cartoon structure of ubiquitin.

Secondary structure of the Ub is stabilized by hydrogen bonding. Marked stability of ubiquitin is imparted by the hydrophobic core which results due to fine grasping of α-helix in the concavity formed by the left-handed twisted β-sheet (Vijay kumar et al., 1985). NMR studies showed histidine, tyrosine and phenylalanine are buried in the hydrophobic core of the molecule (Cary et al., 1980; Jenson et al., 1980). Conformational fluctuations necessary for enzyme action involve swinging of helix in and out by >3 Å with a simultaneous reorientation of the C-terminal segment, which takes ten microsecond time range for the "open-to-closed" and the "closed-to-open" transitions (Kitahara et al., 2005). Ub molecule has nine reverse turns, with four reverse turns crowding the polypeptide stretch from Phe45 till Ser65 known as the "turn-rich" region and residues 56 to 59 form 3^{10}-helix. Ub also has two β-bulges in its structure. One is situated at the N-terminal end between antiparallel β-sheet strands involving Gly10, Lys11 and Thr7 residues, which would correspond to positions 1, 2 and X. Gly10 of this bulge participates in type I turn as 4^{th} residue. Second β-bulge is situated at the C-terminal end between two parallel β strands involving Glu64 (1), Ser65 (2) and Gln2 (N). C-terminal β-bulge is unusual among parallel β-bulges as it contains glutamate instead of glycine at 1^{st} position. Presence of Glu64 is supported by unusual Φ and Ψ angles, which are not commonly to Glu. Further these angles are observed with Gly (Vijay-Kumar et al., 1987). The N-terminal methionine is unexposed to solvents due to the

hydrogen bonding with the Lys63 and hence cannot be readily alkylated. The di-glycine motif at the C-terminal end is crucial for Ub-substrate conjugation as COOH terminus is protruding outwards without any bonding with rest of the polypeptide and thus available for considerable movements for interacting with other substrates (Vijay kumar et al., 1987).

1.1.5 FOLDING OF UBIQUITIN:

Activity in proteins is associated with conformational changes in their structure. Residues 4 and 66 of ubiquitin were mutated to cysteines to study if there are any conformational changes in it when conjugated to substrate proteins as a tag for proteolysis. Disulfide bond formation between the residues led to 70-80% decline in ubiquitin mediated proteolysis, implying that intramolecular motions plays important role in carrying out various biological functions by ubiquitin (Ecker et al., 1989). Ubiquitin structure was studied using circular dichroism (CD) in different solvents as a function of dielectric constant, and the results show a stabilized, partially folded state of ubiquitin in aqueous solvent and alcohol mixtures at pH 2 and room temperature, which is called the "A" state of ubiquitin (Wilkinson and Mayer, 1986).

In the A-state the C-terminal half of ubiquitin loses secondary structure corresponding to seven reverse turns and 3_{10} helix, as they form random coil. Residues 24-34 form partially structured α-helix interacting with hydrophobic surface of the β-sheet formed by first two β-strands and partially formed third β-strand. The A state is much more flexibility than the N-state (Harding et al., 1991). In A state of Ub, the stretch of residues Thr^{9}-Glu^{18} forms stable structure displaying least dynamicity, while the segment corresponding to Gly^{53}-Gly^{76} is in random coil (Hoerner et al., 2005). It was initially reported that peptides of Ub 1-21 and 1-35 attain β-hairpin conformation in aqueous methanol (Cox et al., 1993). Later studies indicated that they can fold autonomously to attain native like structure even in the absence of any organic solvent although to a lower extent (Zerella et al., 1999).

Studies on folding of Ub by H-D exchange pulse labelling and 2D NMR revealed that the amide protons of N-terminal β-sheet, α-helix and protons involved in H-bonding at the interface were protected early in folding process, whereas amide protons of C-terminal indicated slower protection. Residues 59, 61, and 69 display slower folding rates as they form a surface loop. Cis-trans isomerization in the intermediate state at peptide bonds

involving Pro37 and Pro38 slow down the folding kinetics of Gln-41 and Arg-42. Ub exhibits rapid folding kinetics as it is devoid of slow folding moieties (Briggs and Roder, 1992).

1.1.6 STABILITY OF UBIQUITIN:

Several structural features in ubiquitin contribute to its structural stability over wide ranges of pH and temperature. Ubiquitin has a compact hydrophobic core. The extensive secondary structure made up of α-helix, β-sheets and 9 β-turns impart stability through hydrogen bonding. All ionizable groups in ubiquitin are completely exposed to the solvent in native form suggesting that the Gibbs free energy contributions associated with the change of the environment of the charged groups upon folding is negligible (Rashin and Honig, 1984). Besides, the characteristic β-grasp fold or ubiquitin fold holding α-helix across the concavity formed by five β-strands reinforces the overall structure. The α-helix is of three full turns with N and C- capping interaction making it stable which has backbone-backbone hydrogen bonding between Gly35 and Gln31 and hydrophobic interaction between Ile30 and Ile36 (Thomas and Makhatadze, 2000). Thermal unfolding of ubiquitin is reversible, obeying the two-state, equilibrium model (Wintrode et al., 1994; Makhatadze et.al., 1998; Ibarra-Molero et al., 1999). It is a thermostable protein at neutral pH as electrostatic factors play prominent role in stabilizing the molecule (Makhatadze et.al., 1998).

The antiparallel β-bulge formed by Gly10, Lys11 and Thr7 residues of ubiquitin is important in stabilizing the transition state as N-terminal residues (1-17) function as one of the nucleation centres for its folding. Deletion of Gly10 slows down the refolding and unfolding rate by about one half (Chen et al., 2001). Ubiquitin has a parallel β- bulge formed by Gln2, Glu64 and Ser65. In order to understand its contribution to structure and stability of ubiquitin, mutants of β-bulge were generated in our laboratory. Structural studies on the mutants Glu64Gly, Ser65Asp and Gln2Asn showed that they could form stable structures at room temperature. Replacement of residues which have preference for same kind of secondary structure did not change the structure of the molecule much (Mishra et al., 2009; Mishra et al., 2011; Sharma and Prabha, 2011; Sharma and Prabha, 2015). The pair of oppositely charged surface residues i.e. K11 and E34 form a salt bridge, which partly contributes to the thermal stability of the protein (Makhatadze et al., 2003).

1.2 LYSINE LINKAGE: DECODING UBIQUITIN

Ubiquitination, affects protein stability, cell signalling, response to DNA damage, intracellular trafficking and many other vital activities of eukaryotic cell. Unlike other post-translational modifications, ubiquitination has a greater complexity for mediating cellular responses. This versatility is provided by seven lysine residues namely, Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63 or the N-terminal Met, which can serve as acceptors of ubiquitin molecules, leading to formation of eight structurally and functionally distinct polymers (Kulathu and Komander, 2012). Distinct cellular functions are mediated based upon the various polyubiquitin topologies and lysine linkages (Figure 1.4).

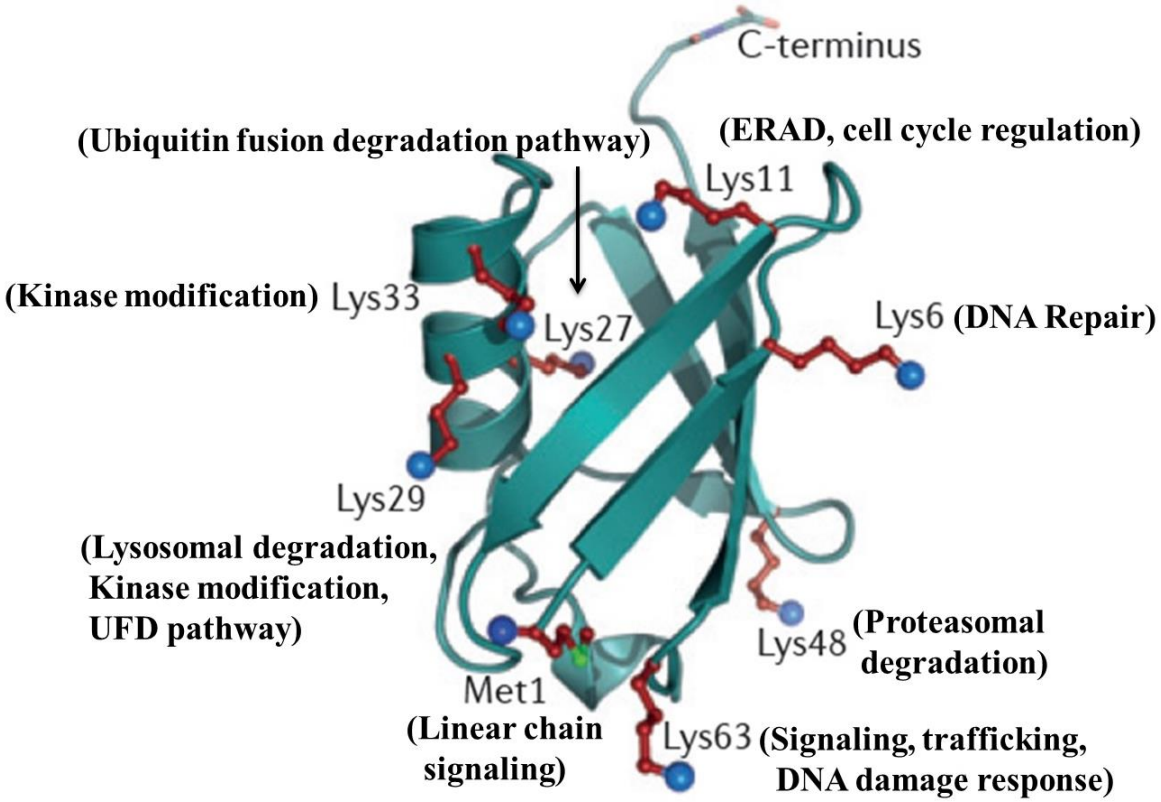

Figure 1.4: Ubiquitin lysine linkages along with facilitated functions.

Ubiquitination is classified based upon ubiquitin linked to the substrate protein i.e. monoubiquitination where one Ub is attached to substrate protein, multiubiquitination where more numbers of Ub are bound to substrate at different sites and polyubiquitination which comprises of chain of Ub molecules attached to target protein. Further, the ubiquitin polymer

can be either of homotypic Ub chain, where similar lysine linkages are involved or heterotypic Ub chain, which consists of alternating linkage or branched chains (forked chains: as a result of a single ubiquitin undergoing ubiquination at two or more sites) (Pickart, 2001; Reyes-Turcu et al., 2009; Winget and Mayor, 2010; Husnjak and Dikic, 2012) (Figure 1.5). Ubiquitin binding domains (UBDs) recognize ubiquitinated substrates by interacting with various surface patches of ubiquitin. Deubiquitin enzymes (DUBs) hydrolyze linkages between substrate or ubiquitin or between two ubiquitin molecules (Hurley et al., 2006; Komander et al., 2009). Hydrophobic surface patches act as docking sites for UBDs for chain assembly on proximal lysine residues. For instance, most UBDs involved in cell division and also the proteasome bind to the Ile44 patch (Shih et al., 2000; Sloper-Mould et al., 2001; Dikic et al., 2009). The Phe4 patch is essential for vegetative growth of yeast, protein trafficking, interaction with UBAN domain and ubiquitin specific proteases and DUBs (Sloper-Mould et al., 2001; Hu et al., 2002; Rahighi et al., 2009). A ‘‘TEK-box’’ in Ub facilitates the formation of K11-linked polyUb by APC E3 and UbcH10 E2. Moreover, two hydrophobic patches Ile44 and Ile36 present on ubiquitin are recognized by HECT E3s (Kamadurai et al., 2009), DUBs (Hu et al., 2002) and UBDs (Reyes-Turcu et al., 2006; Wang et al., 2006; Jin et al., 2008).

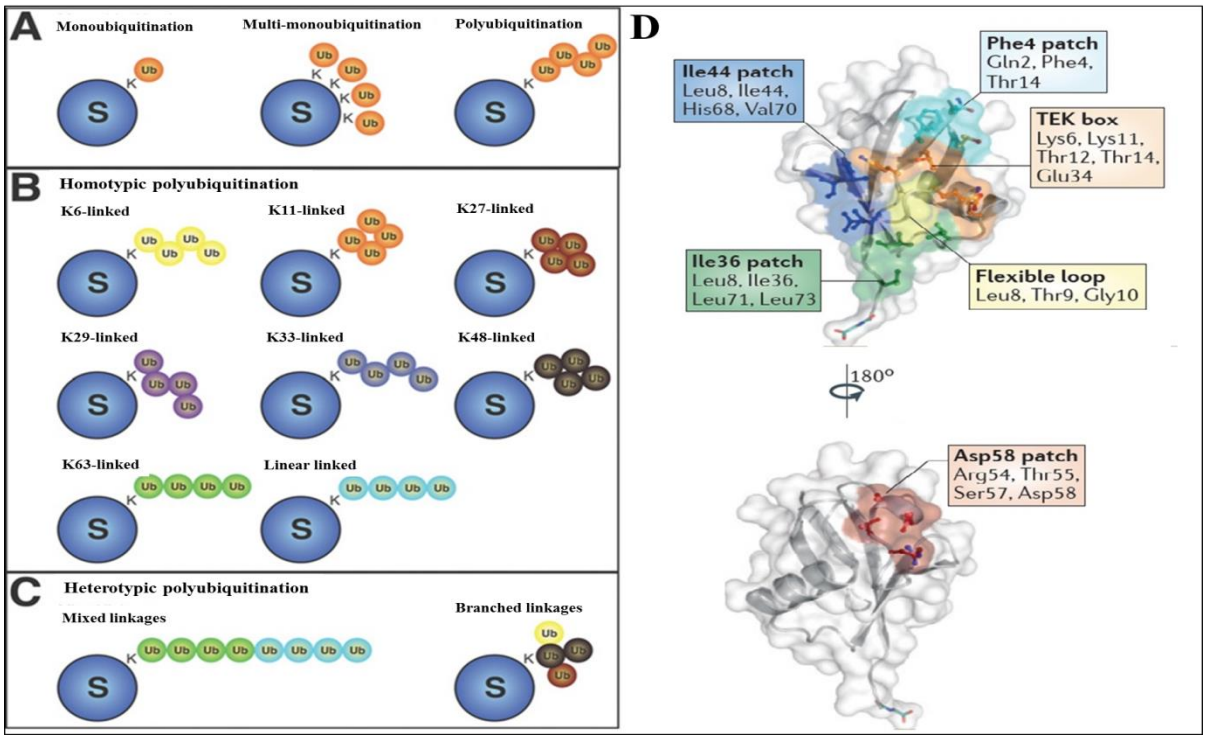

Figure 1.5: Forms of ubiquitination.(A) mono-ubiquitination, multi-mono-

ubiquitination and polyubiquitination (B) homotypic and heterotypic polyubiquitination (C) heterotypic polyubiquitination further classified in to mixed linkages and branched or forked polyubiquitin chains (D) various hydrophobic patches of ubiquitin (Komander, 2009).

Till date, the term polyubiquitin is mostly used for Lys48 and Lys63 linkages. It is well established that substrate protein which is linked through Lys48 chain is targeted for proteasomal degradation by the 26S proteasome (Chan et al 1989; Finely et al., 1994; Hochstasser et al., 1991) whereas those which are linked with Lys63 trigger a range of other processes, like DNA repair (Spence et al., 1995; Ulrich et al., 2002), inflammatory response (Sun et al., 2004), protein trafficking (Hicke et al., 2003), and ribosomal protein synthesis (Spence et al., 2000). However, in the last decade knowledge accumulated on the participation of Lys6, Lys11, Lys27, Lys29, Lys33 or Met1 in polyubiquitin formation. Met1-linked Ub chains have various nonproteolytic roles (Chen and Sun, 2009). K11 linked polyUb is believed to play role as proteasomal signal (Chau et al., 1989; Wickliffe et al., 2011; Baboshina and Haas, 1996; Jin et al., 2008; Kim et al., 2007). Moreover, K11 linkage-specific substrates, including Ubc6 involved in endoplasmic reticulum-associated degradation (ERAD) (Xu et al., 2009). It also plays role in the mammalian cell cycle (Jin et al., 2008). K6 linkage is known to be involved in DNA repair which is catalysed by BRCA1/BARD1 E3 ligase. BRCA1, tumor suppressor protein forms polyUb via K6 linkages (Nishikawa et al., 2004). K29 linkage is principal signal for targeting protein degradation by ubiquitin fusion degradation pathway (Johnson et al., 1995). Finally, K27 and K33 linkages, which are involved in stress response, are formed by U-box type E3 ligases (Hatakeyama et al., 2001).

1.3 UBIQUITIN PROTEASOME PATHWAY

The energy-dependent protein breakdown in reticulocytes, and probably all eukaryotic cells, proceeds through a multi-enzyme proteolytic system. Post-translational modification of proteins by the covalent attachment of ubiquitin to target proteins serves as an obligatory signal for their degradation (Ciechanover et al., 1978; Hershko et al., 1979; Ciechanover et al., 1980; Hershko et al., 1980; Wilkinson et al., 1980; Haas and Rose, 1981). Accumulating evidence explains the central role of ubiquitin/proteasome-dependent proteolysis in various biological processes including rapid and selective degradation of proteins (Belle et al. 2006). The ubiquitin-proteasome system includes multi-protein complexes and protein-protein

interactions. It comprises of ubiquitin, ubiquitin activating enzyme (E1), Ubiquitin conjugating enzyme (E2), Ubiquitin ligase (E3), proteasome and deubiquiting enzymes (DUBs).

1.3.1 UBIQUITIN

Various aspects of ubiquitin protein i.e. structural features, stability, conservation, evolutionary aspects, conservatory aspects, ubiquitin coding gene families, various lysine linkages along with possible observed topologies, significance of surface residues and its hydrophobic core residues are discussed in detail in above sections.

1.3.2 UBIQUITIN ACTIVATING ENZYME (E1)

The protein conjugation cascade is initiated by activation of ubiquitin molecule, which is catalysed by the ubiquitin activating enzyme (E1). First step involves adenylation of ubiquitin molecule at C-terminus in Mg^{2+} and ATP dependent process leading to formation of an ubiquitin adenylate intermediate. In the second step ubiquitin is transferred to E1 by formation of thioester bond between a conserved catalytic cysteine and ubiquitin. Usually E1 is loaded with two ubiquitins, one at adenylation site and other at thioester formation site. ATP-dependent conformational changes of E1 enhance accessibility of an ubiquitin binding site, which otherwise shows less affinity for ubiquitin. Finally, the E1-Ub thioester complex recruits an E2 to facilitate transfer of ubiquitin to conserved E2 cysteine via transthioesterification (Haas and Rose, 1982; Haas et al., 1982; Hershko et al., 1983).

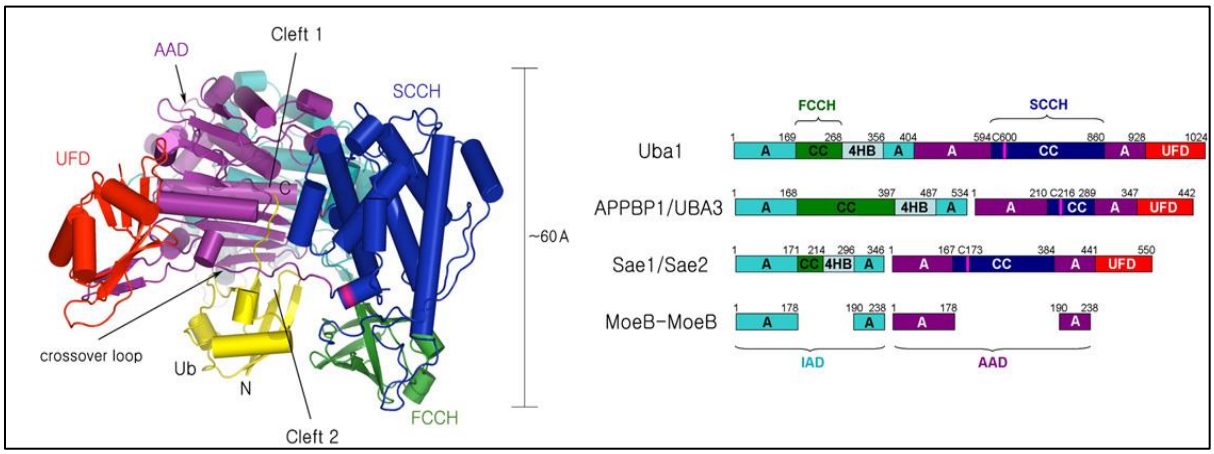

Figure 1.6: 3D structure of ubiquitin activating enzyme E1. Different colours display E1

domains.

Ubiquitin activating enzyme (E1) is monomeric protein of 110 KDa. In *S. cerevisiae* E1 is encoded by UBA1 gene (McGrath et al. 1991). E1 comprises of two-fold repeat of a domain, which is homologous to bacterial MoeB and ThiF proteins. E1 enzymes meant for activation of ubiquitin like proteins (NEDD8 and SUMO) are heterodimeric, containing the domains on separate subunits (Johnson et al., 1997). Ub-E1complex comprises of the following domains: the inactive adenylation domain (IAD), active adenylation domain (AAD), first catalytic cysteine half-domain (FCCH), second catalytic cysteine half-domain (SCCH), four-helix bundles (4HB) and Ubiquitin-fold domain (UFD). AAD facilitates binding of ATP and ubiquitin (Figure 1.6) (Lake et al., 2001; Lois and Lima, 2005; Walden et al., 2003b). SCCH contains active site cysteine (Szczepanowski et al., 2005). UFD present at the C-terminal and facilitates recruitment of E2s (Huang et al., 2005, 2007; Lois and Lima, 2005). Several temperature sensitive mutants of E1 serve as platforms to understand the crucial role played by E1 in the cell. For instance, cell line derived mutants, ts20 and ts85 arrest cells at S/G2 transition (Kulka et al., 1988). Other two, Uba1-204 and Uba1-206 inhibit ubiquitin proteasome system which leads to the rapid depletion of ubiquitin conjugates and stabilizes multiple substrates (Ghaboosi and Deshaies 2007).

1.3.3 UBIQUITIN CONJUGATING ENZYME (E2)

The next step in the conjugating cascade is transfer of the activated ubiquitin from the active site cysteine of E1 residue to active site cysteine of E2. E2 catalyzes transfer of ubiquitin to ubiquitin ligases (E3s), if the E3 enzymes belong to HECT group of E3s. Subsequently the E3s transfer ubiquitin to their cognate substrates. (either HECT E3 or RING E3). The hierarchical enzymatic cascade comprises of a single E1, 13 E2s in *S.cerevisiae*, ~40 E2s in higher organisms and 650-700 cognate E3s which recognize a set of substrates (Hershko and Ciechanover, 1998; Hochstrasser, 1996; Stewart et al., 2016). Gene family encoding E2 enzymes has been identified from species ranging from yeast to plants and mammals (Jentsch, 1992b). Structurally, all E2 proteins contain core catalytic domain (UBC domain) consisting of 150 conserved residues. It has active site cysteine residue necessary for accepting ubiquitin through thioester bond formation. This domain adopts α/β-fold usually with four α-helices, a short 3_{10} helix, and four stranded antiparallel β-sheet. The E2 active site and loop regions form part of the E3-binding site (Stewart et al., 2016). Residues around the

active site are also found to be conserved.

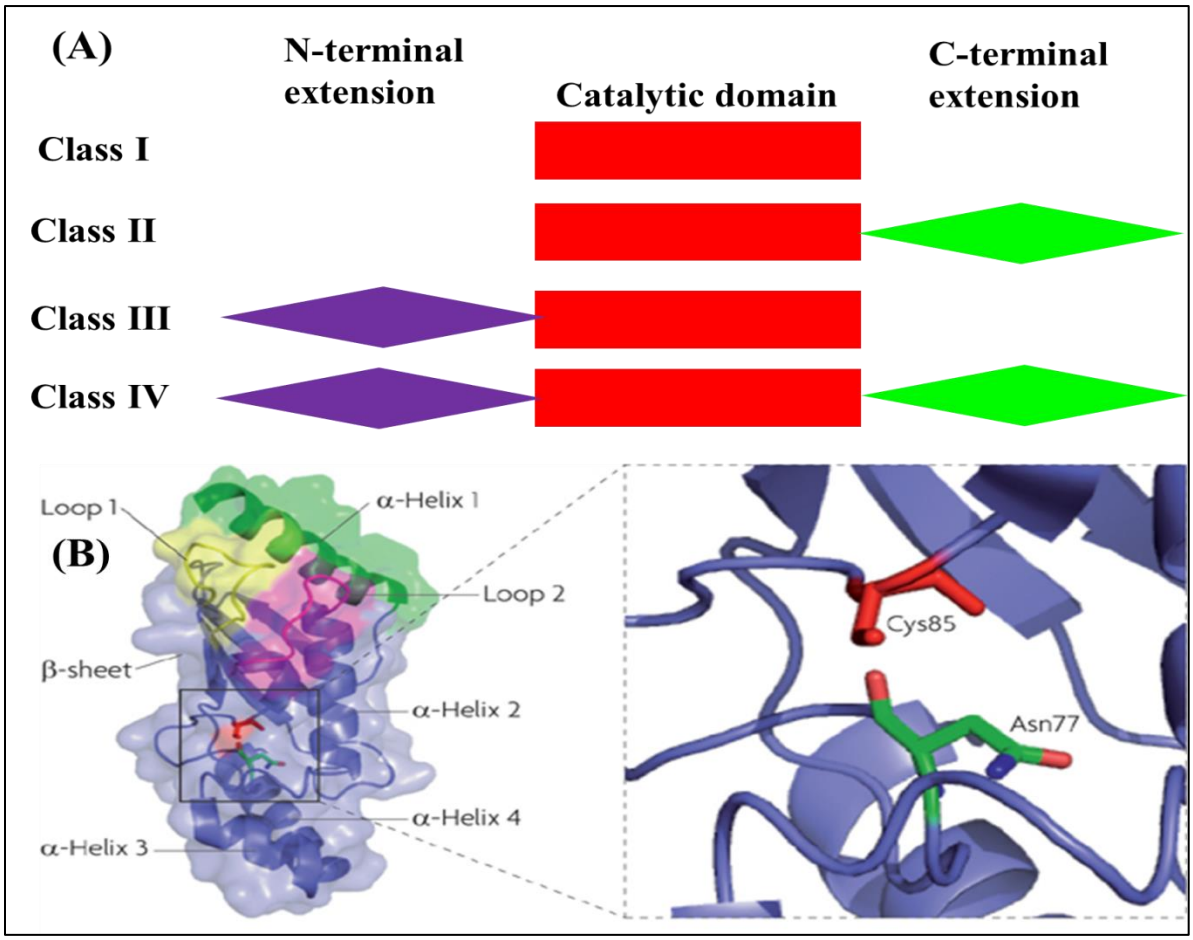

Figure 1.7: Structure of ubiquitin conjugating enzyme E2. (A) classification of ubiquitin conjugating enzyme (B) 3D image showing E2 structure along with the catalytic domain and active cysteine site (Ye and Rape, 2009).

Depending on the location and presence of unrelated sequences, E2 enzymes are classified in to four classes. E2s belonging to class I are the smallest, as they have only the core catalytic domain. E2s consisting of a C-terminal extension to the catalytic domain are classified as class II, while those containing N-terminal extension are the class III enzymes and class IV contain both N- and C- terminal extensions (Figure 1.7) (Ye and Rape, 2009). Usually, E2s are engaged in transthiolation (transfer from a thioester to a thiol group) and aminolysis (transfer from a thioester to an amino group) reactions, although additional types of reactivity of E2s have also been reported. C-terminal ubiquitin carboxylate is conjugated to the E2 active site cysteine in an E1-catalyzed reaction. The E2~Ub conjugate reacts with the side chain of a Cys, Ser/Thr, Lys, or N-terminus on a substrate by forming thioester, oxyester,

isopeptide or peptide bond in order to form the diverse ubiquitin linkages (Ye and Rape, 2009).

The accuracy of biological functions depends on specific E2 utilized and the number of E2s involved to complete the reaction. For instance, mammalian E2 protein E2-25K is capable of assembling polyubiquitin chains without E3 enzyme which is like Ubc1, Ubc3 (Cdc34) built chains, required for labelling short-lived protein and subsequent degradation (Chen and Pickart, 1990; Gwozd et al., 1995; Hodgins et al., 1996). There are substrates whose ubiquitination requires multiple E2s. For example, degradation of yeast MATα2 transcriptional repressor requires collective action of four different E2s, including Ubc6 and Ubc7 and ubiquitination involves endoplasmic reticulum localized E3s (Chen et.al., 1993; Sommer and Wolf, 1997; Swanson et.al., 2001). Moreover, one of the E2s, UbcH5 when paired with RING E3, synthesizes forked ubiquitin chain comprising all types of isopeptide linkages. Whereas when paired with HECT E3, UbcH5 synthesizes homogenous linkage with either lys48 or lys63, where the E3 enzymes are E6AP and Nedd4 respectively (Hyoung et.al., 2007).

1.3.4 UBIQUITIN LIGASE ENZYME (E3)

The last step in ubiquitination cascade is catalyzed by E3 ligases. They transfer ubiquitin from E2 to substrate. As a result a covalent bond is formed between the C-termianl carboxyl of ubiquitin and side chain of lysine. The dynamic process of ubiquitination depends on successful interactions of E3 with two cognate proteins viz. the ubiquitin conjugating enzyme and the target protein. Interestingly, cellular E3 levels are kept under self-surveillance by autoubiquitination and further regulated by ubiquitin mediated proteolysis (Michael and Oren, 2002; Ardley and Robinson, 2004; Wasch and Engelbert, 2005; Robinson and Ardley, 2004). It is important to note that substrate specificity is imparted mostly by different E3s as well as through E2-E3 interactions. Aberrations caused in E3 lead to alteration in the functions catalysed, resulting in several diseased conditions. Ubiquitin ligases are classified into four distinct classes, which catalyze ubiquitin transfer by either direct or indirect means. They are (1) HECT (homologous to E6-associated protein C-terminus) (2) RING finger (really interesting new gene) (3) U-box (a modified RING motif without the full complement of Zn^{2+} binding ligands) E3s and (4) RIR (RING in between RING–RING) domain and multi-protein complex E3s e.g. [CRL (Cullin-RING E3)] (Ardley

and Robinson, 2005).

1.3.4.1 HECT domain E3s:

Mammals comprise ~30 HECT domain E3s. Structurally, these E3s have conserved C-terminal HECT domain consisting ~350 amino acids. The N-terminal domains are diverse to target various substrates. The HECT domain is bi-lobed. Its N-terminal region interacts with E2 and C-terminal region interacts with ubiquitin by forming thioester bond as C-terminal segment contains the active site cysteine (Huang et al., 1999). Both the lobes of HECT domain are connected through flexible hinge region that increases accessibility for successful ubiquitin transfer. It is evident that chain linkage specificities depend on the last 60 resides of HECT domain C-lobe (Kim and Huibregtse, 2009). The HECT E3s play prominent roles in protein trafficking, immune response, and in various signalling pathways that regulate growth and proliferation of cells (Figure 1.8) (Rotin and Kumar, 2009).

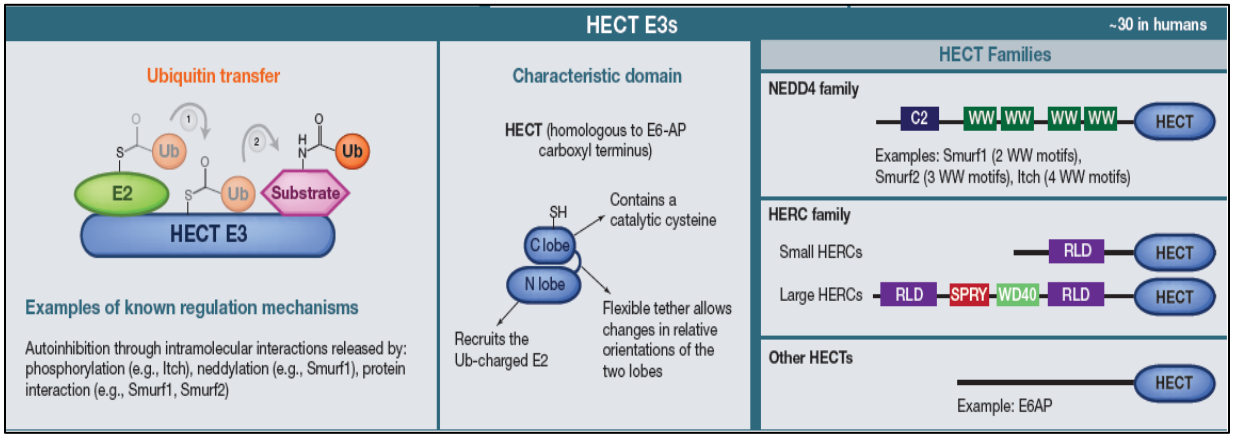

Figure 1.8: Mechanism of HECT E3s and domains involved in various HECT families (Morreale and Walden, 2016).

1.3.4.2 RING domain E3s:

The mammalian genome encodes the largest group of E3s comprising more than 600 potential RING finger E3s (Borden and Freemont, 1996; Jackson et al., 2000; Li et al., 2008). RING finger E3s do not have catalytic role unlike HECT domain E3s (Figure 1.9). Rather they act as scaffolds to bring together E2 and substrate for ubiquitination. A canonical RING finger domain is zinc chelating domain consisting of several cysteine/histidines residues, which facilitates either protein-protein or protein-DNA interactions. It is denoted as Cys^{1}-Xaa_{2}-Cys^{2}-Xaa_{9-39}-Cys^{3}-Xaa_{1-3}-His^{4}-Xaa_{2-3}-$Cys/His^{5}Xaa_{2}$-Cys^{6}-Xaa_{4-48}-Cys^{7}-Xaa_{2}-Cys^{8}

(where Xaa can be any amino acid residue) (Borden and Freemont, 1996; Lorick et al., 1999). Two zinc ions conjugate with cysteine/ histidine residues in 'cross-brace' manner, offering proper folding of RING domain. Substitution of cysteine [5] for histidine[5] hampers E2-E3 binding suggesting that Cys[5] or His[5] play crucial part for E2 recognition (Ardley et al., 2001).

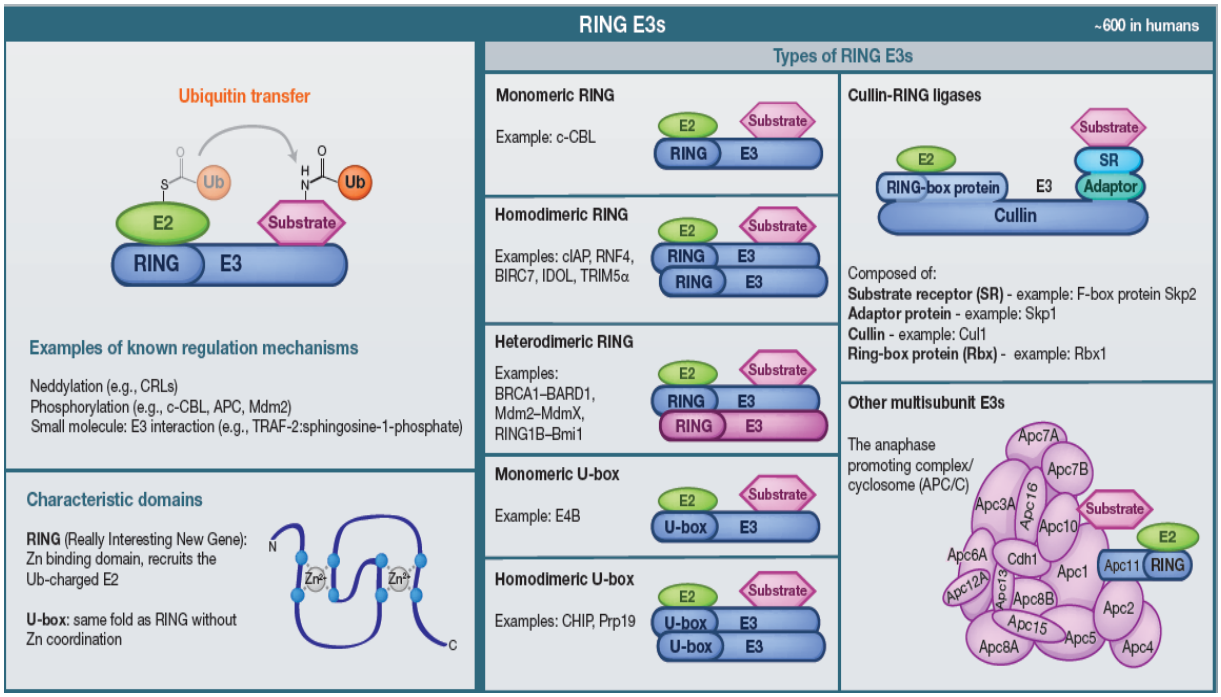

Figure 1.9: Ubiquitin transfer mechanism of RING E3s accompanied by monomeric, homodimeric and heteromeric RING and U-box RING E3s. Multi-subunit RING E3s, APC and Cullin-RING E3 ligase are also shown (Morreale and Walden, 2016).

1.3.4.3 RIR (RING-IBR-RING) domain E3s:

It consists of complex E3s. The mechanism of these E3s is analogous to HECT E3s, wherein ubiquitin is first transferred to the catalytic cysteine residue of E3 followed by conjugation to substrate. RIR encompasses two RING domains viz. RING1 and RING2 separated by the presence of in-between RING domain (IBR) (Figure 10). RING1 domain facilitates recruitment of ubiquitin charged E2, RING2 domain has catalytic cysteine (Rcat = required-for-catalysis) and RING3 is structurally similar to RING2 except for catalytic cysteine (BRcat = benign-catalytic) (Morett and Bork, 1999; Capili et al., 2004; Marin et al., 2004).

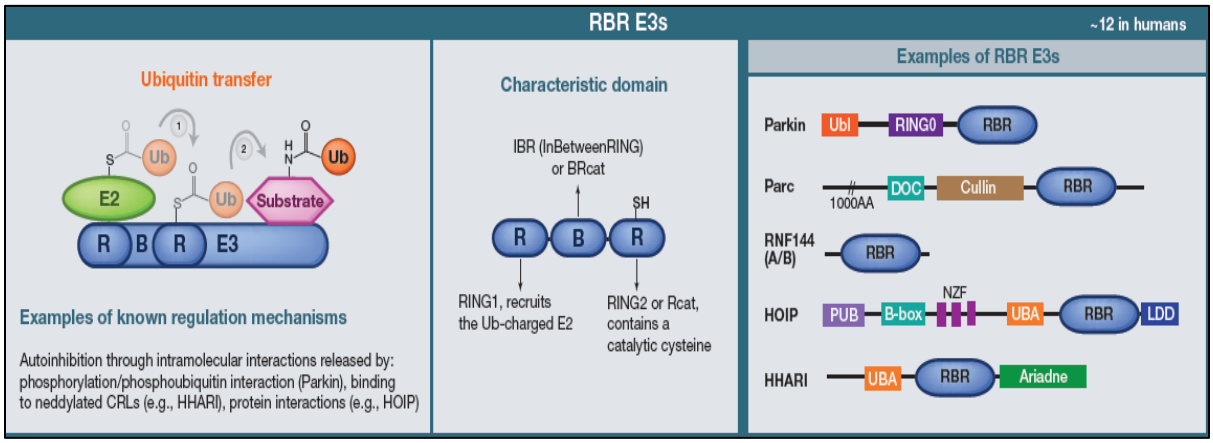

Figure 1.10: Ubiquitin transfer mechanism of RIR (RING-IBR-RING) domain E3s. Proteins containing RIR domain are also displayed (Morreale and Walden, 2016).

1.3.4.4 U-box E3s:

The U-box domain is structurally similar to RING finger domain comprising 74 amino acid residues except that it does not chelate any metal ion. The U-box domain utilizes salt bridges to maintain structural integrity. It is also known as E4. U-box E3s can act alone and as part of multi-protein complexes with their mechanism similar to RING E3s. The best example for U-box domain proteins is C-terminus of Hsc70 Interacting Protein (CHIP U-box protein) (Cyr et al., 2002; Hatakeyama and Nakayama, 2003).

1.3.4.5 Multisubunit RING E3s:

SCF E3s (Skp1-Cullin-F-box protein), and the APC (Anaphase Promoting Complex) are examples of this E3 class marked by presence of at least one Cullin and one RING protein. RING finger protein is the crucial component of these E3s. SCF contains RING finger domain known as Rbx1 made up of approximately 100 residues and plays essential role in recruiting cognate E2s. The Cullin proteins (CUL1, CUL3 and CUL2A) act as scaffold as explained above. The Skp1 (F-box protein) interacts with N-terminal sequence serving as receptive site for substrate. Reports established that Cul1, Rbx1 and cognate E2 displayed robust ligase activity that resulting either in autoubiquitination of cognate E2 or formation of free polyubiquitin chains (Peters et al., 1998; Deshaies, 1999; Huang et al., 1999; Zheng et al., 2000; Hochstrasser, 2000; Borden, 2000). Similarly, APC contains subunit APC2 which is a cullin-like domain and APC11 which is a RING finger protein that facilitates binding of E2 (Wasch & Engelbert, 2005). However, activation of APC requires

interchangeable regulatory subunits Cdc20 (cell-division cycle 20) or Cdh1 (Cdc20 homologue 1; also known as fizzy), which provide substrate specificity by behaving like F-box proteins. Other subunits are involved in scaffolding (Vodermaier et al., 2003; Passmore et al., 2003).

1.3.5 DEUBIQUITINATING ENZYMES (DUBs):

Ubiquitination is a dynamic and reversible post-translational modification. Deubiquitinating enzymes (DUBs) are key proteases which disassemble polyubiquitin chains. Basically, deubiquitnases hydrolyze the isopeptide linkage between a lysine ε-amino group and the C-terminal carboxylate of ubiquitin to release a ubiquitin monomer. Certain DUBs display specificity towards lysine linkages, while other DUBs can act on multiple chain types (Reyes-Turcu et al., 2009; Komander et al., 2009). Catalytically efficient conformation is attained by accurate substrate binding and scaffolding (Liz and Sousa, 2005). DUBs are also known to be modular proteins, as in addition to catalytic domain they also possess ubiquitin binding domain (UBD) and protein –protein interaction domains which are essential for recognizing various lysine linkages (Reyes-Turcu and Wilkinson, 2009).

Key functions of DUBs include generating free ubiquitin monomers by processing of ubiquitin precursor i.e. ubiquitin fused to ribosome protein or ubiquitin linear fusion products i.e. polyubiquitin. The functions of DUBs include rescueing ubiquitin from proteasomal degradation by disassembling it from substrate (Welchman et al., 2005; Chen and Sun, 2009), modulating levels of plethora of important molecular targets for maintaining cellular homeostasis viz. apoptosis, gene expression, cell cycle, DNA repair and cytokine signalling and generating various ubiquitin signals by trimming polyubiquitin chains (Ikeda and Dikic, 2008; Chen and Sun, 2009; Wilkinson, 2009).

Mammalian genome encodes ~100 DUBs. These are classified in to five families based on their structural features (Nijman et al., 2005; Kirisako et al., 2006). (1) The ubiquitin specific proteases DUBs (USPs) (2) the Ubiquitin C-terminal hydrolases (UCHs) (3) the Ovarian Tumor DUBs (OTUs) (4) The Josephin domain DUBs (MJD) (5) The JAB1/MPN/MOV34 (JAMM/MPN+) DUBs. Except JAMM/MPN DUBs, all others are thiol proteases that have mechanistic resemblance to papain, a plant cysteine protease (Storer et al., 1994; Johnston et al., 1997). It comprises Cys-His-Asp/Asn triad where Asp/Asn orients His

for nucleophilic attack on the isopeptide bond, which connects substrate and ubiquitin.

DUBs belonging to UCH family have 230 residues with catalytic domain placed at N-terminal segment. In few cases it contains extensions at C-terminal end which are found helpful in protein-protein interactions. The UCH-L1 and UCH-L3, smaller DUBs, facilitate cleavage of small groups that are connected by ester, thioester or peptide bonds to the C-terminus of ubiquitin, while UCH37 and BAP1, large DUBs, disassemble polyubiquitin chains (Lam et al., 1997; Larsen et al., 1998; Misaghi et al., 2009; Machida et al., 2009).

DUBs of USP family contain catalytic domain of 295-850 residues, six conserved motifs having N and C-terminal extensions with few insertions. DUBs act directly with substrate proteins, and sometimes with the help of scaffold or with substrate adapters by variable sequences (Nijman et al., 2005; Ye et al., 2009; Reyes-Turcu and Wilkinson, 2009).

Identification of OTU family DUBs is based on sequence homology they bear with ovarian tumor gene that is involved in development of fruit flies ovaries (Makarova et al., 2000; Goodrich et al., 2004). Structurally core domain consists of helical domains surrounding five β-strands (Nanao et al., 2004; Messick et al., 2008; Komander and Barford, 2008). These DUBs are marked by lysine linkage specificities. For example, OTUB1 and A20 recognise K48 linked chains, OTUB2 recognises chains with both K63 and K48 (Wang et al., 2009; Edelmann et al., 2009), Cezanne identifies K11-linked chains, and TRABID prefers K29 and K33-linked chains (Komander and Barford, 2008; Bremm et al., 2010; Licchesi et al., 2012).

DUBs belonging to Josephin domain (MJD) family have ~180 residues long Josephin domain e.g. Ataxin-3, Ataxin-3L, Josephin-1 and Josephin-2. Of these Attaxin-3 DUBs have been studied in depth (Tzvetkov and Breuer, 2007; Weeks et al., 2011). Structurally they resemble UCH domain DUBs. However, they are C-terminally extended with two-three repeats of ubiquitin-interacting motifs (UIMs) and a poly-Gln stretch. The UIMs in these DUBs promote ubiquitin binding whereas residues of their active sites facilitate editing of K63 linked chain (Mao et al., 2005; Berke et al., 2005; Winborn et al., 2008). Moreover, expansion of poly-Gln stretch leads to the Machado-Joseph disease (spinocerebellar ataxia type 3), a neurodegenerative disorder (Matos et al., 2011).

JAMM domain DUBs are metalloproteases, with two Zn ions, one promoting

hydrolysis of isopeptide bond and the other involved in recognizing proximal end of K63 linked diubiquitin (Maytal-Kivity et al., 2002; Tran et al., 2003). Ubiquitin residues Lys63, Gln62 and Glu64 are precisely contacted by AMSH-LP JAMM domain DUB core (Sato et al., 2008). One report suggested involvement of these DUBs in membrane receptor trafficking (Williams and Urbe, 2007), DNA repair (Dong et al, 2003; Cooper et al, 2009), deneddylation (Cope et al, 2002).

1.3.6 PROTEASOME: MACHINERY FOR DEATH SENTENCE

The proteasome is an ATP-dependent chambered protease, which recognizes and degrades proteins that are covalently modified by attachment of ubiquitin using E1, E2 and E3 enzymatic cascade (Coux et al., 1996; Pickart and Cohen, 2004). It is a gated protein chamber made up of tightly regulated ATP dependent proteolytic machinery (Figure 1.11).

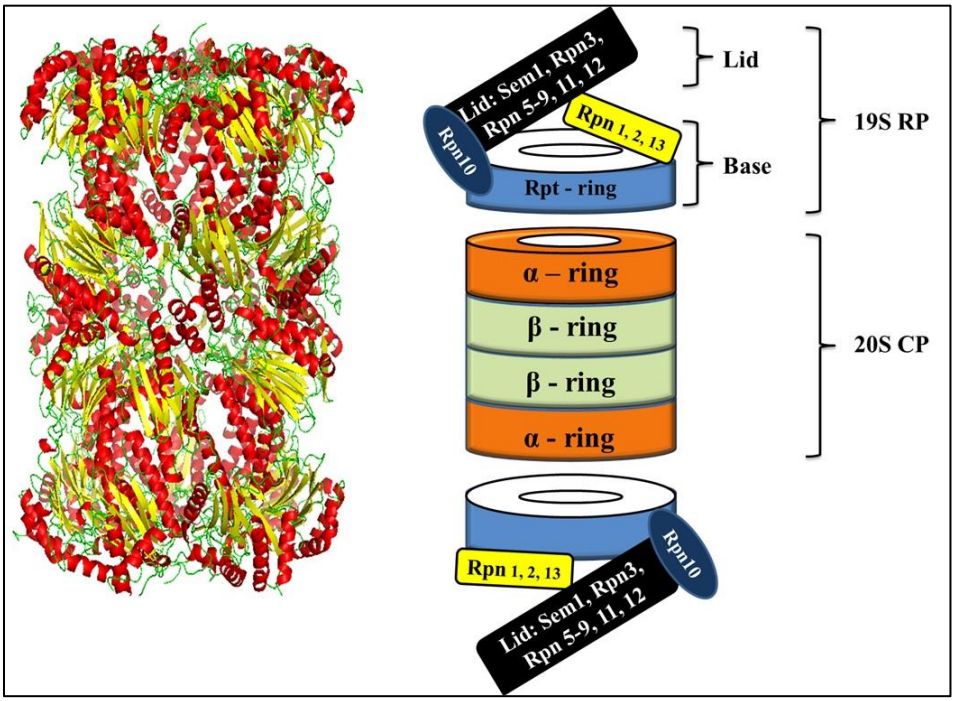

Figure 1.11: 3D structure of 26S proteasome showing catalytic particle (CP) and regulatory particle (RP) subunits.

The 26S proteasome comprises of 20S core particle (CP) and a 19S regulatory particle (RP, it is also known as PA700). The 20S CP subunit consists of four stacked heteroheptameric rings i.e. two outer α-rings and two inner β-rings, giving a barrel-shaped look to proteasome (Tomko and Hochstrasser, 2013). Outer α-rings are composed of seven α-

subunits (α1-α7) arranged to form ring like structures with a central pore, which acts as gate for selective entrance of substrates and elimination of degraded products. The N-terminus of the α3-subunit is essential for activation and organization of gate as its deletion leads to a constantly open pore (Groll et al., 2000). Reports suggest that α-subunits harbour nuclear localization signal (NLS), which directs subcellular localization of proteasome (Tanaka et al., 1990; Nederlof et al., 1995; Ogiso et al., 2002). Similarly, the inner β-rings are composed of seven different β-subunits (β1-β7) which are synthesized as precursors, having propeptides placed N-terminally, which are eliminated during proteasomal maturation process in order to expose N-terminal catalytic Thr. Moreover, β1, β2 and β5 are responsible for peptidyl-glutamyl-hydrolyzing or caspase-like, the trypsin- like and the chymotrypsin-like activities respectively (Groll et al., 1997; Dick et al., 1998). The 19S RP (Regulatory Particle) modulates identification, binding, deubiquitination, unfolding and translocation of substrates by opening α-ring gate of CP. Collectively, it contains 19 subunits which are divided in two sub-complexes viz. the "base" and "lid". The base comprises Rpt1-Rpt6 AAA ATPase subunits which are arranged into a ring, while Rpn1, Rpn2, Rpn10 and Rpn13 (Adrm1) are non-ATPase subunits (Díaz-Villanueva et al., 2015). Rpn1, Rpn10 and Rpn13 act as ubiquitin receptors and recognize substrates which are destined for proteasomal degradation (Fu et al., 1998; Husnjak et al., 2008; Shi et al., 2016). The lid is made up of nine distinct Rpn subunits i.e. (Rpn3, Rpn5-9, Rpn11, Rpn12 and Rpn15 (Dss1/Sem1)). Amongst these Rpn11, Uch37 and Ubp6/Usp14 are known deubiquitinases (Lam et al., 1997; Verma et al., 2002; Yao and Cohen, 2002; Leggett and Hanna, 2002; Maytal-Kivity et al., 2002). The processes carried out by 19S subunit are energy dependent (Gallastegui and Groll, 2010; Tomko and Hochstrasser, 2013).

1.4 THE MULTITUDES OF CELLULAR FUNCTIONS OF UBIQUITIN

1.4.1 PROTEASOMAL DEGRADATION

Various protein concentrations are kept under surveillance of ubiquitin proteasome system in order to maintain homeostasis of cell and to maintain proper functioning of cellular machinery. Substrate proteins which are destined for degradation are modified by covalent attachment of ubiquitin in ATP dependent reaction. This step requires involvement of three enzymes i.e. ubiquitin activating enzyme (E1), Ubiquitin conjugating enzyme (E2) and ubiquitin ligase enzyme (E3) (Figure 1.12). Functions and mechanisms of these enzymes are

discussed in above sections in detail. Ubiquitin-substrate complex forms polyubiquitin chain by going through ubiquitination reactions catalyzed by the same enzymatic cascade for several times. Proteasome recognizes polyubiquitin signal on the substrate and degrades it. The ubiquitin molecules are recycled with the help of deubiquitinases (Hershko, 1983; Pickart, 2004). The peptides formed are used for antigen presentation on MHC class I molecules (Lecker et al., 2006). Ubiquitin consists of seven lysine residues that can be conjugated to other ubiquitin moieties to form polyubiquitin chains of various linkages. It is established that polyubiquitin chain linked by means of K48 linkage follows ubiquitin dependent proteolytic pathway (Ikeda and Dikic, 2008).

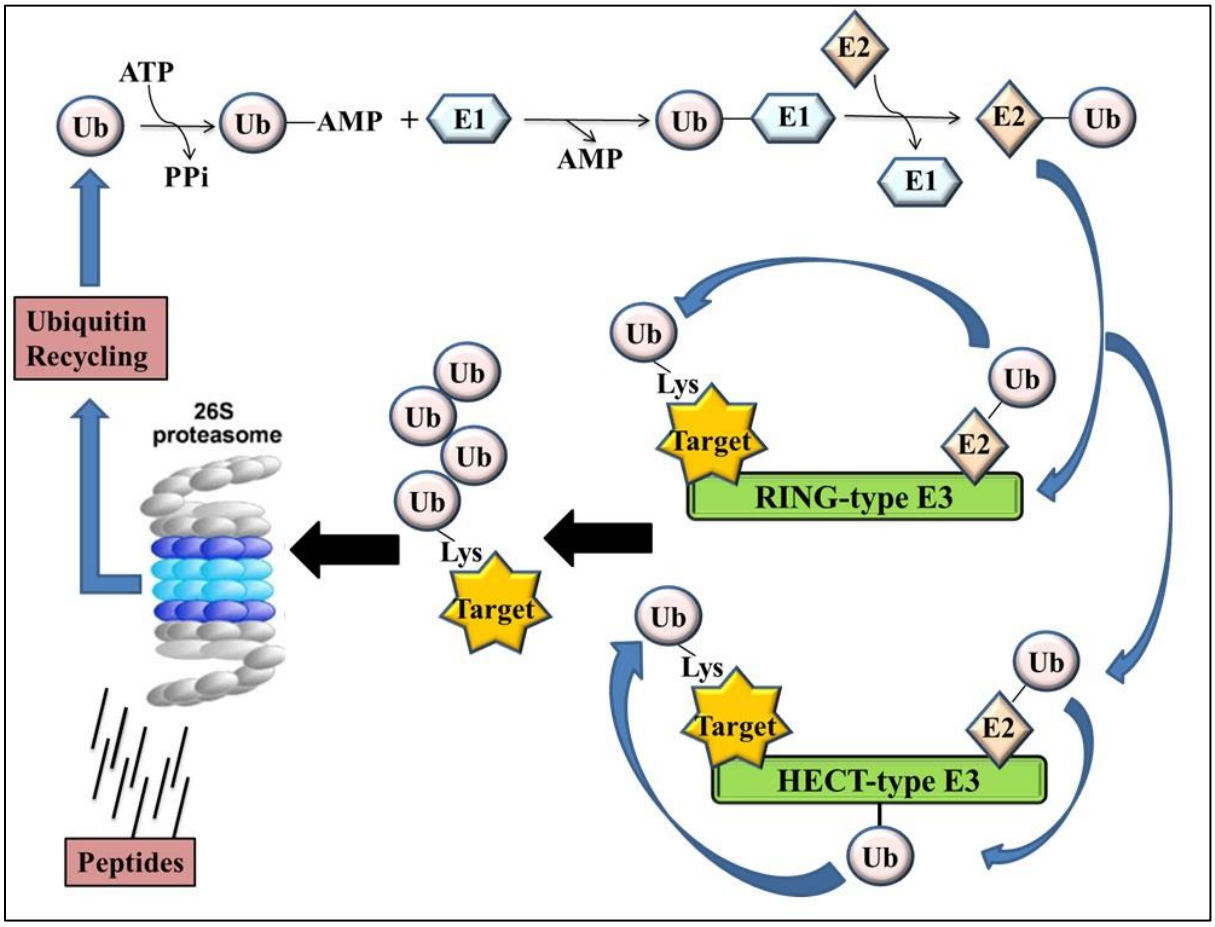

Figure 1.12: The enzymatic reaction of ubiquitin proteasome pathway.

Cell proliferation in normal cells is a well regulated process occurs in response to specific developmental or mitogenic signals which is organised in a stepwise manner, the cell cycle. Major events of the controlled cell cycle include synchronized appearance and disappearance of the cyclin, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase

inhibitors (CKIs) (Park and Lee, 2003). Ubiquitin proteasome pathway accomplishes the irreversible cell cycle progression events by degrading the above mentioned cellular regulators with the help of two ubiquitin ligase complexes, one is the Skp1-Cullin-F-box protein (SCF) complex, and the other is the anaphase-promoting complex/cyclosome (APC/C) (Nakayama and Nakayama, 2006; Mocciaro and Rape, 2012).

1.4.2 NON-PROTEOLYTIC FUCNTIONS OF UBIQUITIN

Apart from proteasome mediated degradative functions, ubiquitin is involved in various regulatory processes. Different types of lysine linkages and topologies of polyubiquitin play pivotal role in facilitating such non-proteasomal functions. Few of the non-proteolytic functions are addressed here:

Inflammatory response of cell requires activation of pro-inflammatory transcriptional activator, NF-κB whose transport to nucleus is depended on IκB degradation. Degradation of IκB is mediated by the E3 β-TRCP (β-transducin repeat containing protein) dependent ubiquitination (Lecker et al., 2006). Further, ubiquitin also modulates IκBα kinase activation through TAK1 kinase and the Ubc13/ Uev1a ubiquitin-conjugating enzyme (Ben-Neriah, 2002) wherein TAK1 kinase is activated by K63-linked polyubiquitin chains on the RING finger ligase TRAF6. The cellular stress like DNA damage can also lead to activation of NF-κB signalling cascade by modification of NEMO by ubiquitin -like molecule, SUMO (Huang et al., 2003). In addition, regulation of AMP-activated protein kinase (AMPK)-related kinases is found to be assisted by Lys29/Lys33 linked mixed ubiquitin chains. These polyubiquitin chains further block kinase activation by interfering with phosphorylation of the activation-loop residues, the examples for which include AMPK family member 5 (ARK5/NUAK1) and MAP /microtubule- affinity-regulating kinase (MARK) 4 kinases (Al-Hakim et al, 2008).

Ubiquitination is majorly involved in gene regulation by modifying H1, H2A, H2B and H3 through mono-ubiquitination or by forming short ubiquitin chains (Jenuwein and Allis, 2001). These are essential for regulating meiosis (Robzyk et al., 2000), silencing telomere (Muratani and Tansey, 2003) and nucleosome structure/ chromatin remodelling by methylating H3 at multiple sites and by H2B ubiquitination (Dover et al., 2002; Ng et al., 2002; Sun and Allis, 2002; Jason et al., 2002). In addition, activity of p97/Cdc48 ATPase is partly controlled by protein adapters Ufd1-Npl4 and p47. Ubiquitination regulates action of

these adapters to carry out endoplasmic reticulum-associated degradation, reassembly of the nuclear envelope and the Golgi apparatus (Lord et al., 2002; Meyer et al., 2002).

Maintaining genome integrity is another crucial task of a cell. In the nucleus, ubiquitination is widely involved in varying affinity or interaction of proteins for other proteins and DNA. Proliferating cell nuclear antigen (PCNA) is a homotrimeric complex, serving as a sliding clamp that encircles DNA and provides a platform to recruit necessary proteins involved in DNA replication and repair (Hoege et al., 2002). In response to DNA damage, the RAD18 (RING E3 ligase) stalls replication fork via RPA (single-strand DNA-binding protein) (Davies et al., 2008), which is then followed by mono-ubiquitination of PCNA by RAD18 and RAD6 on K164 (Hoege et al., 2002). This modification is recognized by UBDs of translesion synthesis (TLS) polymerases, leading to switching on of the high-fidelity replicative polymerases (Guo et al., 2006; Parker et al., 2007). After bypassing the lesion, ubiquitin-specific protease 1 (USP1) eliminates ubiquitin from PCNA (Huang et al., 2006) followed by recruiting the error-free, processive polymerase. Further, it is known that Ubc13 mediated K63 polyubiquitination orchestrates the recruitment of numerous DNA repair proteins to the DSB foci (Bennett and Harper, 2008).

In proliferating cells, besides taking part in proteolytic functions ubiquitin also plays important non-proteolytic role. During cell cycle progression, L28, a component of the large ribosomal subunit is found to be ubiquitinated during S phase. This modification is facilitated by Lys63 linked polyubiquitin chain. *K63R* mutant displays inhibited ubiquitination of L28. Moreover, cells expressing mutant ubiquitin show defects in ribosomal functions as well as hypersensitivity to translational inhibitors (Spence et al., 2000).

Plasma membrane proteins tagged by either monoubiquitination or Lys63 linked chain are directed for lysosomal degradation (Mukhopadhyay and Riezman, 2007). For example, the Uracil permease, a membrane permease of *S. cerevisiae*, required for the transport of extracellular uracil into the cell. When uracil is in excess, the uracil permease is down regulated. It is modified by the HECT E3 Rsp5 (Terrell et al., 1998) and undergoes Lys63 linked ubiquitination , for recognition by early endosomes. Ubiquitin tag is cleaved off by ubiquitin hydrolase Doa4 and the uracil permease is directed for degradation (Blondel et al., 2004). Ubiquitinated protein aggregates are coupled to autophagosomes by adaptor molecules wherein substrate binding moderately prefers Lys63-linked chains (Kirkin et al.,

2009). Ubiquitination also regulates protein localization in cells. For example, Lys63 modification of carboxypeptidase S (CPS) mediated by Rsp5 E3 ligase leads the protein to endosomal vesicle. Recycling of EGFR to plasma membrane is facilitated through deubiquitination by Usp8 or AMSH (Mizuno et al., 2005; Clague and Urbe, 2006).

1.5 ROLE OF UBIQUITIN PROTEASOME SYSTEM IN DISEASES

Proteomic balance or proteostasis of the cell and regulation of many cellular processes are accomplished by ubiquitination pathway. Aberrations in the system, directly or indirectly, lead to pathogenic conditions. Mutations in ubiquitin, enzymes of UPS or substrate recognition motifs result in wide array of diseases. The UPS related pathologies occur either due to loss of function by stabilizing substrates / ubiquitin-substrate conjugates or due to gain of function causing accelerated degradation of cellular proteins. Well studied diseases related to dysregulation of ubiquitin proteasome system include various types of malignancies due to alteration in E3 and DUBs, neurodegenerative disorders like Parkinsonism, Alzheimer's diseases, Liddle's syndrome, cystic fibrosis, Angelman syndrome and others. Recent studies reveal involvement of UPS in cardiomyopathies (Gilda and Gomes, 2017), diabetes and obesity (Wing, 2008), muscle wasting (Solomon et al., 1998; Jagoe and Goldberg, 2001). Some of the diseases and the component of UPS responsible for diseased condition are listed in table 1.2.

Associated diseases	**Substrate**	**Responsible UPS component**	**Biological functions hampered by dysregulation**	**References**
Malignancies				
Infection by high-risk HPV in cervical, anal, genital, head and neck carcinomas	p53, p27	E6-AP	Cell cycle control, apoptosis	Scheffner et al, 1993 Scheffner, 1998 Mishra et al., 2009
One third of sarcomas, breast and lung cancer	p53	MDM2	Cell cycle control, apoptosis	Quesnel et al., 1994 Haupt et al., 1997 Momand et al., 1998 Iwakuma and Lozano, 2003
Breast and ovarian Cancer	CtIP, NPM,	BRCA1	DNA damage response, cell	Scully, 2000 Sato et al., 2004

	RNA polymerase II		cycle control	Starita et al., 2005 Yu et al., 2006
Lung, glioma, gastric, prostate, ovarian, breast and colon cancer	p27	SCF^{skp2}	Cell cycle control	Sutterluty et al., 1999 Shapira et al., 2005 Traub et al., 2006 Frescas and Pagano, 2008
Cholangiocarcinomas, T-cell acute lymphocytic leukemia, endometrium, colon and stomach cancer	c-Myc, c-jun, Notch, mTOR and cyclin E	SCF^{fbw7}	Cell cycle control, cell growth, cell division and cell differentiation	Oberg et al., 2001 Koepp et al., 2001 Yada et al., 2004 Nateri et al., 2004 Akhoondi et al., 2007 Mao et al., 2008
Renal cell carcinoma of the clear cell type, Pancreatic cancer, cerebellar and retinal hemangioblastomas	HIF-1α	Von Hippel–Lindau tumor suppressor	Tumor vascularization	Maxwell et al., 1999 Lonser et al., 2003
Breast and prostate Cancer	KLF5, p53, p63, ErbB4	WWP1	Transcription activity, apoptosis, cell cycle control	Laine and Ronai, 2007 Chen et al, @#*2007 Li et al., 2008 Li et al, 2009
Esophageal squamous cell cancer	Smad1, Smad2, TGFβ Receptor 1	Smurf2	Cell growth, cell migration, metastasis	Lin et al., 2000 Kavsak et al., 2000 Zhang et al., 2001 Fukuchi et al., 2002
Prostate cancer	FAS, MDM2, MDMX	USP2a	Apoptosis	Graner et al., 2004 Priolo et al., 2006 Stevenson et al., 2007 Allende-Vega et al., 2010
Colon and breast cancers	c-MYC, CHK2, 53BP1	USP28	Cell cycle control, apoptosis, DNA	Zhang et al., 2006 Popov et al., 2007

			damage response	
Colorectal Tumors	β-Catenin	$SCF^{\beta\text{-}TrCP}$	signal transduction and differentiation of the colorectal epithelium	Giles, 2003
Membrane protein associated diseases				
Liddle's syndrome	proline-rich (PY) motif of α, β and γ subunits	NEDD4	kidney Epithelial Na+ Channel (ENaC)	Rotin, 2000 Debonneville et al., 2001
Cystic fibrosis (CF)	CFTR mutation	-	chloride ion channel	Kopito, 1999 Roomans, 2001 Kaufman, 2002
Neurodegenerative disorders				
Autosomal recessive juvenile parkinsonism (AR-JP)	-	Mutation in Parkin E3	Dopaminergic neural cells	Kitada et al., 1998 Shimura et al., 2000
Alzheimer's disease	-	Ub^{+1} frame shift mutation (Ubiquitin)	Central nervous system	Lam et al., 2000 Lindsten et al., 2002 Bardag-Gorce et al., 2002
CAG expansion (poly Q) diseases e.g. Huntington's disease (HD)	5'-CAG repeat at N-terminal e.g. Huntingtin	Probable role of UPS	Central nervous system	Kalchman et al., 1996
Angelman syndrome	-	Mutation in UBE3A/E6-AP E3	Brain	Kishino et al., 1997 Cummings et al., 1999

Table 1.2: Diseases associated with ubiquitin proteasome pathway.

1.6 DRUG DEVELOPMENT TO TARGET UBIQUITIN PROTEASOME SYSTEM

With the enormous involvement in maintaining cellular homeostasis through selective protein degradation, the ubiquitin and ubiquitination system are crucial to existence of eukaryotic cell. Its activity is carried out by collective action of the ubiquitin protein and the

enzymes viz. E1, E2, E3, proteasomes and DUBs. As described in above section, UPS has a crucial role in the onset of various pathophysiological conditions. UPS represents a hierarchical system with precise substrate specificity and hence it can be regulated at multiple levels for desired outcome. Several scientific groups are trying to develop strategies to exploit UPS and its regulators for treating malignancies (Liu et al., 2014), neurodegenerative disorders, infectious diseases (Edelmann et al., 2011) and cerebrovascular diseases (Di Napoli et al., 2005). Several inhibitors of enzymes ubiquitin conjugation and deubiquitination were evaluated for their therapeutic potential either singularly or in combination with other drugs. Few novel UPS modulators are shown in Figure 1.13. Bortezomib came up as the first proteasome inhibitor drug which is approved by FDA for treating multiple myeloma patients with relapsed/refractory multiple myeloma and mantle cell lymphoma (Chen et al., 2011).

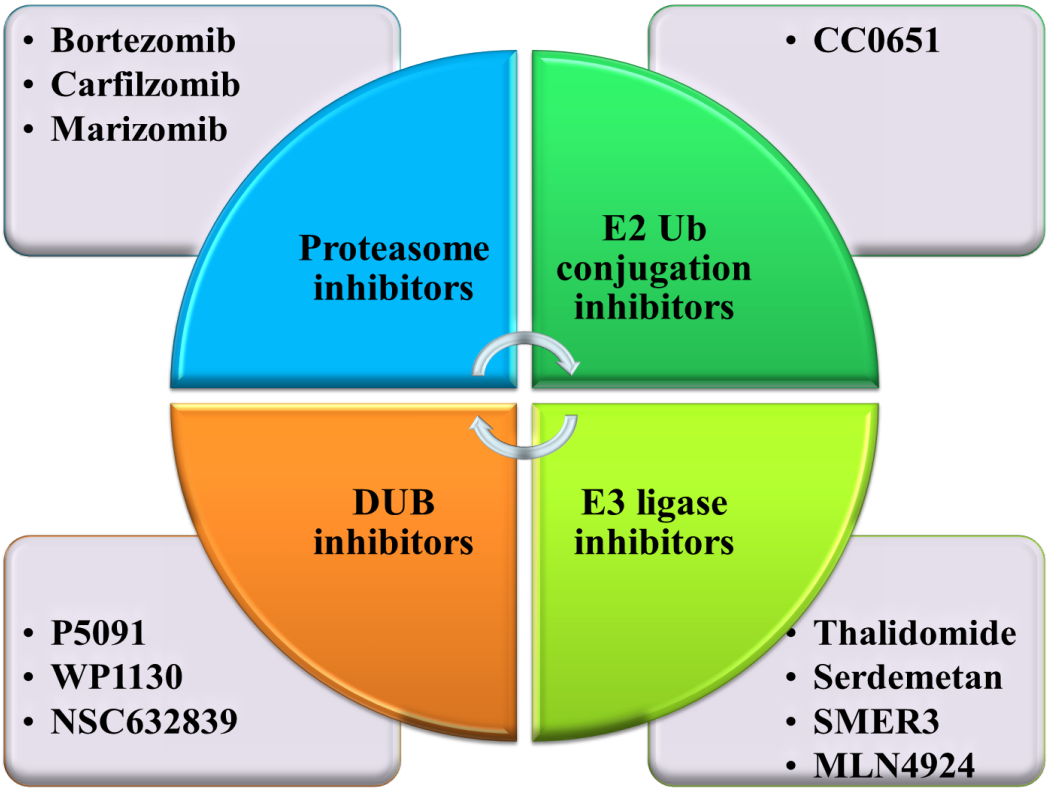

Figure 1.13: Drugs targeting ubiquitin proteasome pathway.

1.7 HYPOTHESIS AND OBJECTIVES OF PRESENT STUDY

Ubiquitin is a highly conserved protein with only three residues replaced from yeast to human with rest of the protein sequence remaining invariant. The compact structure of

ubiquitin comprises of β-grasp fold with a flexible six-residue C-terminal tail, hydrophobic core made up of almost all rigid residues, a flexible β1/β2 loop containing Leu8 which is essential for recognition by ubiquitin-binding proteins. Ubiquitin plays integral part in several major events in the eukaryotic cell and interacts with number of protein partners, which suggests that there is high evolutionary pressure to conserve the ubiquitin structure. Our laboratory aims to understand the structural and functional significance of the conserved residues of ubiquitin. With the help of *in vitro* evolution approach, several ubiquitin mutants were generated in our laboratory, of which UbEP42 turned out to be lethal to *S.cerevisiae* cells at permissive conditions. Sequencing of the mutated gene encoding UbEP42 revealed four amino acid residue replacements viz. S20F, A46S, L50P and I61T (Prabha et al., 2010). Present work aimed at characterizing the structural and functional changes caused by UbEP42 and the constitutent mutations individually in *S.cerevisiae, C.albicans* and human breast cancer cell line MCF-7. Brief account of each chapter is as given below;

Chapter 1 includes detailed survey of literature on ubiquitin protein, its evolutionary lineage, structural features, folding kinetics, and protein stability. The chapter describes the proteolytic role of ubiquitin in ubiquitin proteasome syatem along with the structural features and functional roles of its components namely, ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), ubiquitin ligase (E3), proteasome and deubiquitinases (DUBs) and proteasomes. Ubiquitin accomplishes various functions through ubiquitin proteasome pathway. Ubiquitin consists of seven lysine residues which are conjugated to substrate proteins to direct them for degradartion or for accomplishing other functions. Apart from the major task i.e. proteasomal degradation of target proteins, ubiquitin also carries out several nonproteolytic functions which are presented here. This chapter also describes several well studied diseases which are caused due to aberrations in the components of ubiquitin proteasome system and highlights the strategies to target UPS for efficient drug development.

Chapter 2 presents studies on UbEP42, UbS20F, UbA46S, UbL50P and UbI61T using *Saccharomyces cerevisiae* as model organism in order to understand the significance of conserved residues Ser20, Ala46, Leu50 and Ile61 in maintaining the functional integrity of ubiquitin. All the studies were carried out in SUB60 strain of *S.cerevisiae* which lacks *UBI4*, polyubiquitin encoding gene. Since, UBI4expression is essential for survival of the organism under various stresses, SUB60 shows hypersensitivity to stress. Expression of wild type

ubiquitin (UbWt) from plasmid has been shown to help the strain overcome the stress conditions successfully. SUB62 strain with its *UBI4* gene intact and SUB60 cells expressing UbWt were used as positive controls for the experiments. All ubiquitin mutants were expressed extra-chromosomally under metal ion inducible promoter *CUP1*, whose expression is driven by $CuSO_4$. It is evident from previous studies of our laboratory that UbL50P and UbI61T showed phenotypes similar to UbEP42 i.e. overexpression of these mutants displayed dosage dependent lethality. SUB60 cells expressing UbEP42, UbL50P and UbI61T showed slowing of growth with increase in doubling time, decreased viability, inability to withstand elevated temperature and heightened sensitivity to translational inhibitor, cycloheximide. Moreover, these three mutants hindered ubiquitin fusion degradation (UFD) pathway. UbS20F and UbA46S did not show any detrimental effects on SUB60 cells (Doshi et al., 2014). Present study was focused on understanding effects of these mutants on SUB60 cells in detail. Cell cycle progression was studied using FACS analysis. Cell morphology and bud size were observed using confocal microscopy. Cell proliferation assay was carried out using BrdU to see START (of replication) and cell cycle progression through S phase. To investigate the reason for delay in transition from G0/G1 to S phase in cells expressing UbEP42, UbL50P and UbI61T, the levels of Cdc28 protein kinase were monitored and found to be diminished compared to the positive controls and cells expressing UbS20F and UbA46S. Increased levels of Fus3, a type of MAPK showed opposite trend in the above cells. Cdc28 and Fus3 MAPK mRNA levels were checked and found to be almost identical, suggesting that the differences in their levels appeared at protein stage. However, cells incubated with proteasomal inhibitor MG132 did not show anydifference in the levels of either of the substrates ruling out involvement of proteasome mediated degradation. The transcript levels of G1 cyclin, CLN3 and a molecular chaperone Cdc37 were also checked and found to be unaffected. Reactive oxygen species levels were measured using DCFDA dye. Membrane protein uracil permease is ubiquitinated and degraded in the lysosomes. Here, to study the effect of ubiquitin mutants on lysosomsl degradation of membrane proteins, uracil permease levels were monitored in SUB60 cells expressing ubiquitin mutants, using western blotting and fluorimetric analysis. Carboxypeptidase S is a resident protease of lysosomes. In order to study protein sorting into lysosomes gene fusion of green fluorescent protein and carboxypeptidase S was constructed (GFP-CPS) and trnasfected into SUB60 cells expressing mutant ubiquitins. Endosomal sorting of carboxypeptidase S was observed using

confocal microscopy. The profiles of polyubiquitination with Lys48 and Lys63 linkages were analyzed in cells expressing mutants of ubiquitin using western blot analysis. Effect of ubiquitin variants over ubiquitin fusion degradation pathway was studied using ubiquitin–β–galactosidase fusion protein, where N-terminal of β – galactosidase was Met, Pro, Asn, Glu, Gly, Leu or Arg. Degradation of fusion proteins was monitored by western blotting and enzyme assay. Complementation ability of mutant ubiquitin under antibiotic stress was observed using various antibiotics like hygromycin, gentamicin, geneticin, canavanine and tunicamycin. For this study cycloheximide was used as reference. Our results displayed that UbEP42, UbL50P and UbI61T affected all the above aspects negatively, establishing the reason for observed lethality. Expression of the other two mutants UbS20F and UbA46S did not have any detrimental effects over SUB60 cells under the conditions tested (Doshi et al., 2014; Doshi et al, 2017).

Chapter 3 deals with the study of structural alterations caused by amino acid replacements in ubiquitin mutants, utilizing spectroscopic techniques. Ubiquitin has only one week fluorophore Phe45 and no other strong flurophore in its sequence. Hence to facilitate fluorescence spectroscopic studies, UbF45W mutant was constructed, wherein phenylalanine was replaced with tryptophan at 45^{th} position. UbF45W is also known as "pseudo-wild type ubiquitin" as the structural and biological functions remain unaltered and thus it can also be considered as wild type ubiquitin for structural study purpose. This chapter used UbF45W for introducing the mutations viz. S20F, A46S, L50P and I61T by site directed mutagenesis. Purification of UbF45W, UbEP42, UbS20F, UbA46S, UbL50P and UbI61T was carried out using standard methods and purity was established by checking on SDS-PAGE. Tertiary structural characterization was done by Near-UV circular dichroism (CD) spectroscopy and fluorescence spectroscopy and secondary structural changes were studied by Far-UV circular dichroism (CD). Results showed that as compared to UbF45W, in UbEP42, UbL50P and UbI61T β-sheet content increased significantly with decrease in helical content. However, UbS20F and UbA46S did not show any noticeable alteration in protein structure (Doshi et al., 2017).

Chapter 4 describes influence of ubiquitin variants namely UbEP42, UbS20F, UbA46S, UbL50P and UbI61T on *Candida albicans*. The observations from *S. cerevisiae* and structural studies led to the question of what would be the influence of ubiquitin variants over the pathogenic organism. We used *C. albicans* as model organism as it is an opportunistic

pathogen and it shares common ancestors with *S. cerevisiae*. The ubiquitin mutant protein was expressed extra-chromosomally from yeast expression vector under *CUP1* promoter. CAI4 strain of *C. albicans* was used for the study which consists of the original copies of ubiquitin genes in the genome. Studies included in this chapter are the effects of over expression of ubiquitin mutants on *C.albicans* and when expressed at sublthal levels the effects seen on growth kinetics, percentage survival, cell cycle progression, Cdc28 protein kinase levels, polyubiquitination with Lys48 and Lys63 linkages, thermotolerance, ability to withstand heat and antibiotic stresses. From the results it is evident that the mutants UbEP42, UbL50P and UbI61T cause lethality when overexpressed. Sublethal levels of expression of the mutants have negative effects over all the above aspects of the organism. The other two mutants UbS20F and UbA46S had no significant effects over growth and survival of *C. albicans*, similar to the results observed with *S. cerevisiae*. *C.albicans* cells display yeast-to-hyphae morphogenesis, which is essential for causing pathogenesis in host. Evidence from literature supports that ubiquitin is actively involved in yeast-to-hyphae formation. Therefore, the effect of ubiquitin mutants on morphogenesis was studied here and the results revealed that none of the mutations allow morphogenesis.

Chapter 5 presents functional alteration caused due to the introduction of ubiquitin mutations in breast cancer cell line MCF-7. Some of these mutant forms have hindered important biological functions in *S. cerevisiae* and *C. albicans* leading to adverse effects on cells. Thus, it was hypothesized that if these mutations impart similar effect to cancer cells, then the results would be laying roads for the development of novel therapeutic approach. We have utilized MCF-7 as model cell line in this study. Mammalian ubiquitin varies from yeast ubiquitin with three amino acid replacements. Therefore, we have used mammalian ubiquitin gene and mutant forms of ubiquitin viz. *HUbEP42*, *HUbS20F*, *HUbA46S*, *HUbL50P* and *HUbI61T* were generated by site-directed mutagenesis. These were expressed extra-chromosomally in the mammalian expression vector under tetracycline inducible promoter. The ubiquitin variants were transfected in MCF-7 cells and stable cell lines were generated. Initially, inducer concentration and duration of expression were standardized. Later similar conditions were used for other experiments. Effect of overexpression of ubiquitin mutants was monitored on MCF-7 cells and growth kinetics was followed. Cell proliferation and colony forming ability of mutant expressing cells were studied using MTT assay and clonogenic assay respectively. The effect of mutant forms of ubiquitin on the survival of

MCF-7 cells was observed in the presence of tunicamycin, which triggers ERAD pathway and translational inhibitor canavanine, which is an arginine analogue. Moreover, to study localization pattern of ubiquitin variants, GFP tagged mutant ubiquitin coding constructs were prepared and transfected MCF-7 cells were selected for stable expression of GFP-ubiquitin mutants. Studies using confocal microscopy established that there is no significant difference observed as far as localisation is concerned and the mutant ubiquitin proteins are found ubiquitously present throughout the cell. From the results it was concluded that UbEP42, UbL50P and UbI61T led to lethal phenotype in MCF-7 cells and their expression at sublethal levels slowed the growth. Cell proliferation and survival upon exposure to antibiotics were also found to be reduced. HUbS20F and HUbA46S expressing cells remain unaffected.