Chapter 1

Introduction

1.1. Post-translational Modifications and Ubiquitination

Almost everything that occurs in the cell involves one or more proteins. The process of protein synthesis does not directly result in the generation of functionally and structurally complete macromolecules. Most of the proteins undergo chemical modifications after translation, before becoming functional in different cells. These modifications are known as Post Translational Modifications (PTMs). The activities of many proteins are controlled by the rapid interplay between modifying and unmodifying enzymes. These PTMs are regulators of activities like protein folding, stability, conformation, function and degradation. PTMs of proteins are known to play a crucial role in signaling, gene expression and protein-protein interactions (Geiss-Friedlander and Melchior, 2007; Morrison et al., 2002), finally dictating the course of cellular activities such as cell survival, proliferation, differentiation and death (Grotenberg and Ploegh, 2007). The most common modifications are the specific cleavage of precursor proteins, the formation of disulfide bonds and covalent addition or removal of chemical groups and molecules, thus leading to modifications like phosphorylation, glycosylation, ubiquitination, nitrosylation, methylation, acetylation, lipidation and others (Gilbert et al., 2016).

Approximately 200 different types of PTMs have been reported (Minguez et al., 2013; 2012), amongst which more than 40 PTMs have been identified and related to diseases such as diabetes, heart disease, cancer and neurological disorders (Gyorgy, 2006). It has been found that mutations of the post-translational target sites led to a disease condition. For example, loss of acetylation sites in the Androgen Receptor (AR) has been associated with Kennedy's disease, an inherited neurodegenerative disorder (Thomas et al., 2004).

Ubiquitination or ubiquitylation is an important PTM of eukaryotic cells, in which a small protein, ubiquitin is added to substrate proteins. Ubiquitination can affect the stability, interactions, localization or activity of thousands of proteins, thereby providing specific signals that are widely used in cellular control (Yau and Rape, 2016).

1.2. Protein Degradation

Degradation of cellular proteins is a highly complex and tightly regulated process, necessary to allow their levels to change quickly in response to external stimuli or specific intracellular signals. Tight regulation of the cellular proteome is critical for executing faultless interplay of different proteins necessary for normal cellular function, survival, and proliferation. Furthermore, defective or damaged proteins need to be recognized and rapidly degraded in cells to eliminate protein aggregation.

In eukaryotic cells, protein degradation involves two major pathways—the Ubiquitin Proteasome Pathway (UPP) and lysosomal autophagy. Autophagy is a process in which cellular components are engulfed by an intracellular membrane and then delivered to the lysosome for degradation by lysosomal proteases, whereas in the ubiquitin-proteasome pathway, proteins are selectively targeted for degradation. In all tissues, the majority of intracellular proteins are degraded by UPP (Rock et al., 1994). Though, extracellular proteins and some cell surface proteins are engulfed by endocytosis and degraded within lysosomes. Some cytosolic proteins are engulfed in autophagic vacuoles that fuse with lysosomes and undergo degradation (Baehrecke, 2005; Lardeuxs et al., 1987).

It is important for a cell to be able to select specific proteins for degradation to avoid degrading proteins important for cell functioning, to

maintain the balance that exists between the proteins in a regulated fashion and finally to meet the cell's ever-changing protein requirements.

1.3. Ubiquitin Proteasome System (UPS)

1.3.1. A Brief History

Researchers Avram Hershko, Aaron Ciechanover and Irwin Rose won Nobel Prize in Chemistry in 2004, for their work on the ubiquitin system, which was published between 1978-1980 (Hunt et al., 1977; Hershko et al. 1979; Wilkinson et al., 1980). They worked on rabbit reticulocytes, which lack lysosomes and hence were devoid of lysosomal proteases. They investigated protein degradation *in vitro* using lysates of rabbit reticulocytes, which led to some interesting observations. The first observation was that degradation of substrates used energy in the form of ATP, second, proteolysis required the presence of small protein molecules, which were heat stable (Ciehanover et al., 1978) and third, substrates became *larger* prior to degradation. Initially, this heat stable proteinaceous molecule was named APF-1 (ATP-dependent Proteolysis Factor 1) for its properties (Ciechanover et al., 1980). Further investigation revealed that APF-1 had already been identified in lymphocyte differentiation *in vitro* (Goldstein et al., 1975) and because of its omnipresent distribution in tissues and across species, the protein was renamed ubiquitin (Wilkinson et al., 1980), a protein that is "universally present".

1.3.2. Ubiquitin

Ubiquitin is 76 amino acids long protein and is conserved throughout in Eukaryota, however, it is absent in prokaryotes. Even though fungi and humans are separated by approximately one billion years of evolution, yeast

ubiquitin differs only in three amino acid positions from human ubiquitin. This strict evolutionary conservation of ubiquitin emphasizes the significance of the ubiquitin in basic cellular physiology.

Reasonably, ubiquitin is structurally conserved as well and is nearly identical between different organisms (Vijay-Kumar et al., 1987). Ubiquitin is folded into a tightly packed globular β-grasp conformation consisting of a hydrophobic core surrounded by five strands of β-sheet and 3.5 turns of α-helix along with a short 3_{10} helix, as depicted in **Fig. 1.1** (Vijay-Kumar et al., 1987). In addition to the hydrophobic core, there is extensive intramolecular hydrogen bonding. Altogether the properties of tight packing, a large hydrophobic core and extensive hydrogen bonding seemingly contribute to structural stability, explaining its heat-stable properties.

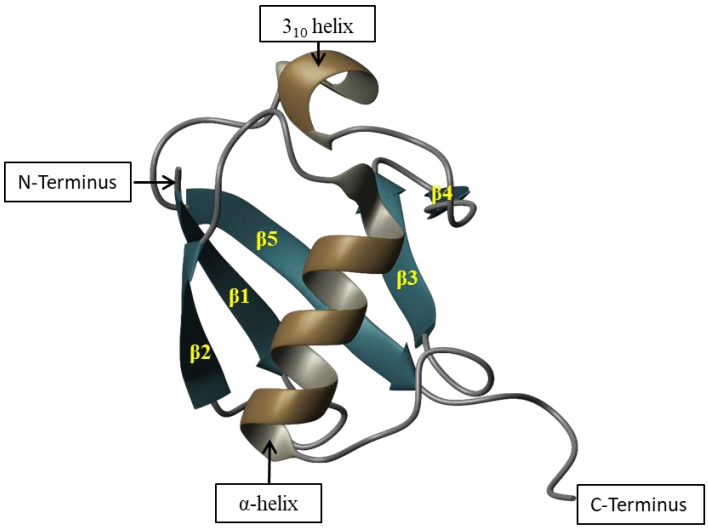

Fig. 1.1. Structure of Ubiquitin (PDB:1UBQ). Five anti-parallel β-sheets are shown in blue color, brown color displays the presence of α-helix and a 3_{10} helix.

One more striking feature of ubiquitin is the hydrophobic patch present on the surface made of Ile44, Leu8, Val70, and His68. The Ile44 patch binds to proteasome and most Ubiquitin Binding Domains (UBDs) and has been proved to be essential for cell division (Dikic et al., 2009; Sloper-Mould et al., 2001). A second hydrophobic surface is made up of Ile36, Leu71 and Leu73; the last two residues belong to the ubiquitin tail region. The Ile36 patch is involved in interactions of multiple ubiquitin molecules in the chain, and it is also recognized by HECT E3s (Kamadurai et al., 2009), DUBs (Hu et al., 2002) and UBDs (Reyes-Turcu et al., 2006). The Phe4 patch present on the surface formed along with Gln2 and Thr12 is involved in cell division in yeast. This patch might also be involved in trafficking (Sloper-Mould et al., 2001), and interaction with the Ubiquitin-Specific Protease (USP) domain of DUBs (Hu et al., 2002).

1.3.2.1. Ubiquitin Linkages - Diverse Cellular Signals

The most important features of ubiquitin are its N-terminus, di-glycine in its C-terminus and its seven lysines (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and lys63), which are the attachment sites for ubiquitin chain assembly. These residues are present all over the surface of ubiquitin and point into distinct directions as we can see in **Fig. 1.2**. Lys6 and Lys11 are located in the most dynamic region of ubiquitin that may undergo conformational changes in the context of a chain or upon association with UBDs. As Lys27 is buried, conformational changes may be required in ubiquitin for linkage assembly through this residue. Under normal cellular conditions, Lys48 covers 29% of all ubiquitin–ubiquitin linkages, Lys63 makes 16%, Lys11 makes 28%, Lys6 covers 11%, Lys27 makes 9% linkages, whereas Lys29 and Lys33 make up for 3% each (Xu et al., 2009).

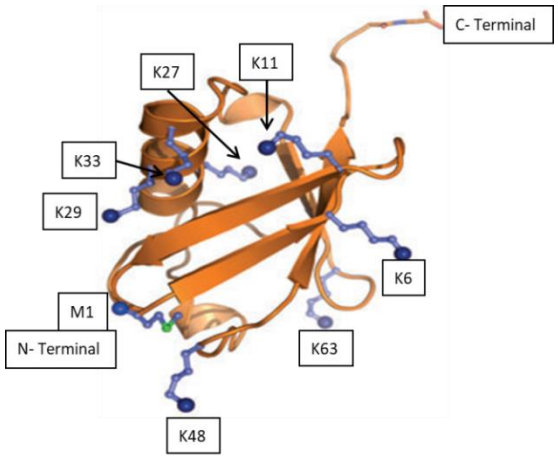

Fig. 1.2. Lysine residues of Ubiquitin (PDB: 1UBQ).

Lys11-linked chains are generally produced during cell division targeting cell cycle regulators for proteasome-mediated degradation (Castañeda et al., 2013; Meyer and Rape, 2014). Lys6 linkages are observed during the removal of damaged mitochondria from cells (Ordureau et al., 2015) and they may be involved in DNA repair (Nishikawa et al., 2004). Both Lys27 and Lys33 linkages may be assembled by U-box-type E3 ligases during stress response (Hatakeyama et al., 2001). Lys29 linked chains may be involved in ubiquitin fusion degradation (Johnson et al., 1995). Met1-linked chains (**Fig. 1.3**) play essential roles in immune signaling and NF-κB activation (Ikeda et al., 2011; Gerlach et al., 2011) and regulation of interferon production (Inn et al., 2011).

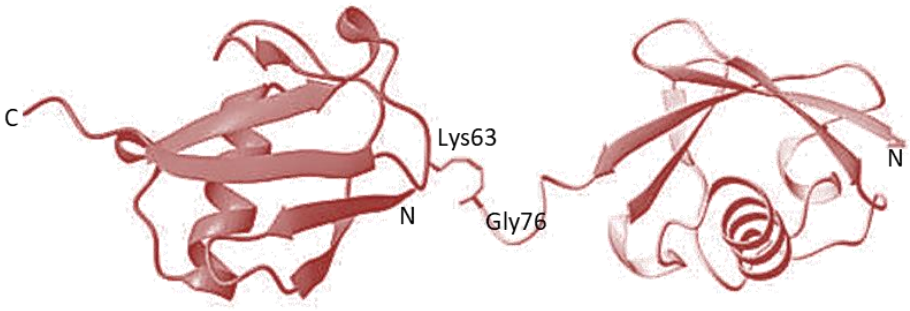

Fig. 1.3. Met1-linked linear diubiquitination (PDB: 2W9N).

Lys63 and Lys48 are two very important and well-studied linkages. Lys63 linkages act as non-proteolytic signals in several intracellular pathways (**Fig. 1.4A**). They are known to regulate activation of transcription factors of NF-κB, DNA repair, inflammatory responses, ribosomal protein synthesis, clearance of damaged mitochondria and protein sorting (Pickart and Fushman, 2004; Ordureau et al., 2015; Lauwers et al., 2009). Lys48 linkages are the most abundant linkages involved mostly in targeting proteins to 26S proteasomes for degradation (**Fig. 1.4B**).

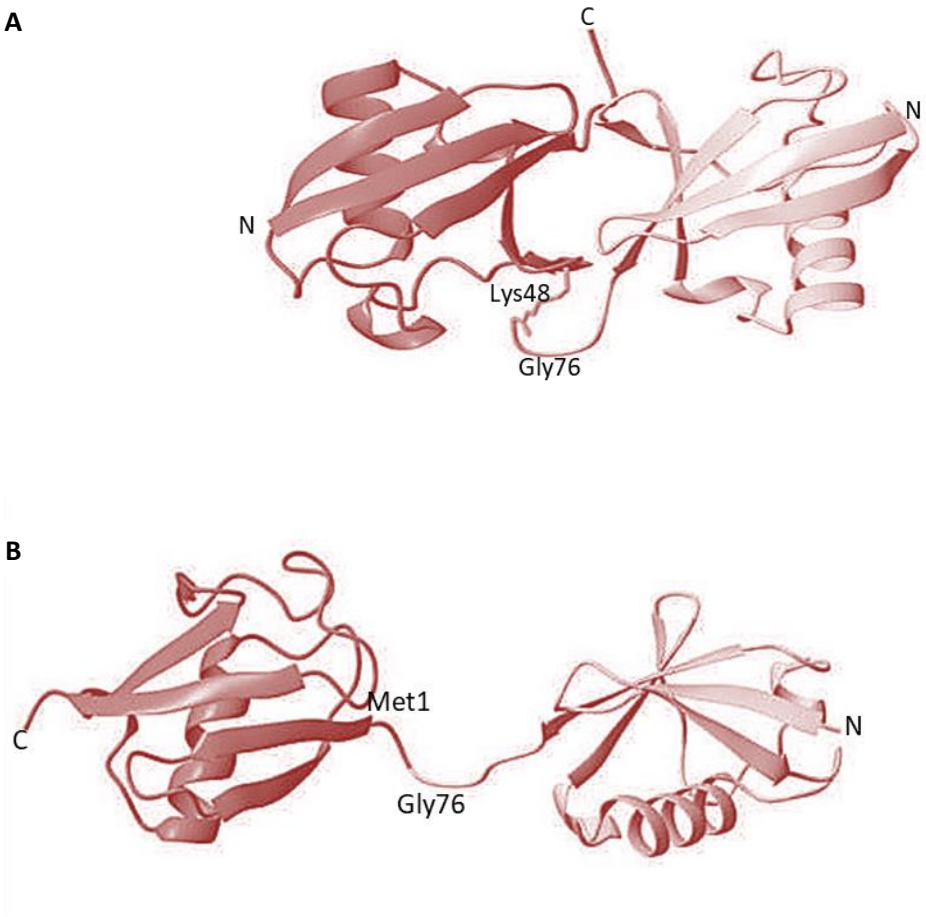

Fig. 1.4. A) Lys-63 linked diuniquitination (PDB: 2JF5), B) Lys-48 linked diubiquitination (PDB: 1AAR).

1.3.2.2. Ubiquitin Chains

Ubiquitin is a common molecular signal in the cell because of its ability to modify a large number of substrate proteins. The fate of ubiquitinated substrates largely depends on the type of ubiquitin linkage and the length of the ubiquitin chain (Pickart and Fushman 2004). Attachment of a single ubiquitin moiety to a single lysine on a substrate results in monoubiquitination. Attachment of several monoubiquitins on different lysine residues of a protein is called multi-monoubiquitination. Monoubiquitination does not target proteins for proteasomal degradation but acts as a molecular

recognition signal in membrane trafficking and regulation of endocytic machinery (Staub and Rotin, 2006). Several ubiquitin conjugates to preceding ubiquitin moieties and consequently form different types of ubiquitin chains are called polyubiquitination. Polyubiquitinated proteins are frequently destined for degradation by the proteasome (**Fig. 1.5**).

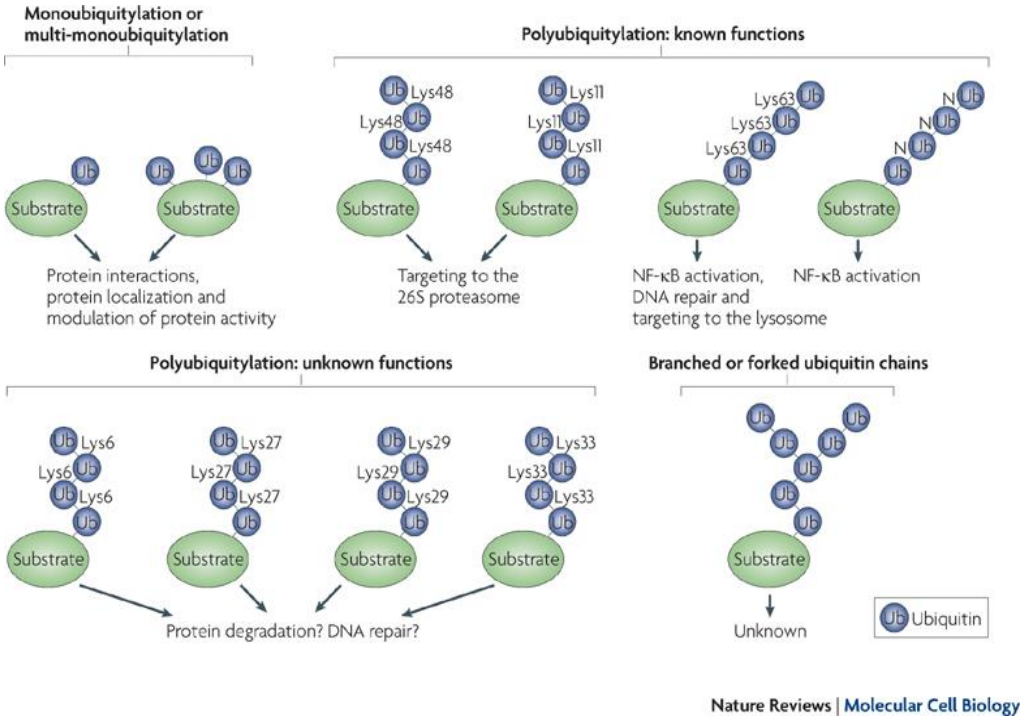

Fig. 1.5. Chains of ubiquitin with different branching patterns and their functions. (Ye and Rape, 2009)

Structural studies have revealed significant differences between ubiquitin chains of Lys48 and Lys63 and of linear linkages. Lys63-linked and linear di-ubiquitins show no contact between two ubiquitin moieties and adopt open conformations (Komander et al., 2009). However, Lys48 linked tetraubiquitin moieties pack against each other and alternate between closed

packed structure and an extended open conformation. Though recently it has been found that Lys48-linked di-ubiquitin shows closed conformation, whereas Lys63-linked di-ubiquitin has an extended conformation, thus implying their selective recognition by different types of UBDs (Raasi et al., 2005; Varadan et al., 2004; 2005).

1.3.3. Ubiquitination/Ubiquitin Proteasome Pathway

Both ubiquitination and Ubiquitin Proteasome Pathway (UPP) are highly complex, temporally controlled and strongly regulated, playing important roles in various basic cellular processes. Ubiquitin protein is at the core of the ubiquitination pathway, operating both in the cytosol and the nucleus. This pathway enables precise control of several thousand target proteins through the cascade of three different enzyme assemblies. These targets include the cell cycle and growth regulators, components of signal transduction pathways, enzymes of metabolic pathways and mutated or post-transnationally damaged proteins.

The ubiquitin conjugation machinery shows a hierarchical organization. The first piece of the puzzle, Ubiquitin activating enzyme (E1/Uba1) was identified in 1981, when it was shown that E1 covalently binds to ubiquitin in presence of ATP via a thioester bond (Ciechanover et al., 1981; Hershko et al., 1981). Later in 1983, ubiquitin conjugating (E2/Ubc enzymes) and ubiquitin ligase (E3) enzymes were isolated and identified by affinity purification through ubiquitin-sepharose column (Hershko et al., 1983). The action of these enzymes along with ubiquitin brings about protein degradation (**Fig. 1.6**).

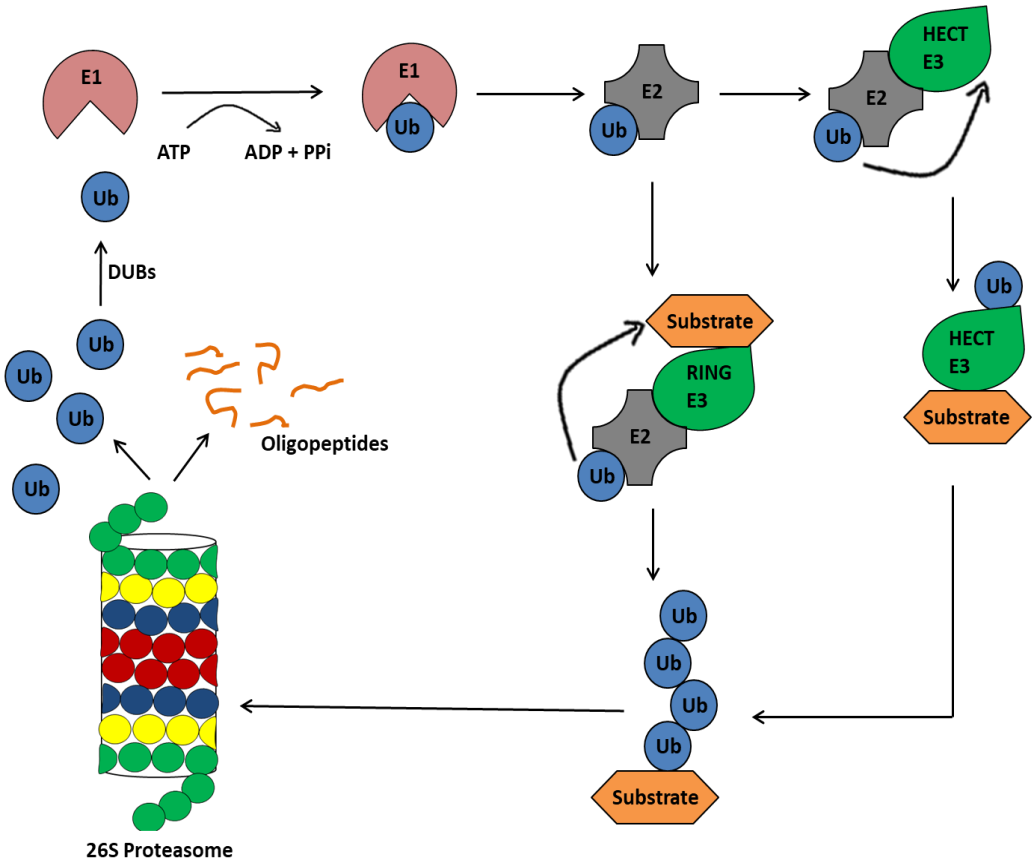

Fig. 1.6. Substrate protein degradation by ubiquitin proteasome pathway.

The ubiquitin-activating enzyme E1 initiates the reaction by adenylating the C-terminal carboxyl group of ubiquitin. This reaction requires ATP to generate a high-energy ubiquitin–adenylate intermediate that lets the ubiquitin to be transferred to a cysteine residue of the E1 (Schulman and Harper, 2009). Activated ubiquitin is then transferred from E1 to a cysteine residue of E2 ubiquitin-conjugating enzymes to form ubiquitin-E2 conjugate (Ye and Rape, 2009). The final stage of the ubiquitin transfer cascade is catalyzed by E3 ligases. E3 enzymes are divided into two groups based on the mechanism of ubiquitin transfer from E2 enzymes to target proteins. The first group is also known as HECT family of E3 enzymes receive ubiquitin from

E2 enzyme and form thioester bond with it, prior to transferring ubiquitin to a target protein. The second group of E3 enzymes, comprising of RING family and U-box family, act as bridges between ubiquitin carrying E2 enzymes and target proteins bound on them, facilitating the transfer of ubiquitin to a target protein. Typically, ubiquitin forms an isopeptide bond between its C-terminal carboxyl group and the ε-NH_2 group on substrate lysyl residues of a target protein. Interestingly, ubiquitination machinery consists of one or two E1s, multiple E2s, and a large family of E3s (Hicke et al., 2005).

The target conjugated ubiquitin serves as the "acceptor" for the addition of subsequent ubiquitins to form a polyubiquitin chain on target protein (Behrends and Harper, 2011). Polyubiquitin chains are formed by forging isopeptide bonds between the ε-amino group of one of the Lys residues (generally Lys48) of target conjugated ubiquitin and the carboxyl group of Gly76 of incoming ubiquitin. This polyubiquitin chain present on substrate protein is recognized as a degradation signal by the regulatory particle of 26S proteasome. Ubiquitin C-terminal hydrolase present in regulatory particle removes the polyubiquitin chain from the target protein. The target protein is unfolded by the ATPase activity present in RP and subjected to proteolytic cleavage by a large, cylindrical multisubunit complex called core particle of the proteasome (Fu et al., 2010), releasing smaller peptide fragments. Meanwhile, intact ubiquitins are released by de-ubiquitinating enzymes (DUBs) for another cycle of ubiquitination (Wing, 2003).

1.3.4. Aftermath of Ubiquitination

1.3.4.1. Regulation of Protein Turnover

Protein degradation is an irreversible process, unlike most regulatory mechanisms. It brings about complete, rapid and permanent change in cell

proteome. The rapid degradation of specific proteins permits adaptation to new physiological conditions.

1.3.4.2. Regulation of Gene Transcription

Ubiquitination affects transcription as many transcription factors are ubiquitinated and degraded by the proteasome (Muratani and Tansey, 2003). Degradation of activators by ubiquitination may even stimulate transcriptional activity by removing already "Used" activators leading to restarting a promoter for further rounds of transcription (Lipford et al., 2005). Further, removal of inhibitors and repressors may initiate transcription of a new set of genes.

1.3.4.3. Cellular Quality Control

Many times abnormally folded or damaged proteins are generated within the cell either by missense or nonsense mutations, biosynthetic errors, or damage by oxygen radicals or by denaturation (especially at high temperatures). The UPP selectively eliminates these misfolded proteins and removes the junk. For e.g. many misfolded proteins within the ER (Endoplasmic Reticulum) are retrotranslocated out of ER compartment into the cytosol by a series of ER membrane-associated ubiquitin conjugating proteins; these proteins are targeted to cytosolic proteasomes for degradation subsequently, this process is known as ERAD (Endoplasmic Reticulum-Associated protein Degradation) (Meusser et al., 2005).

1.3.5. Ubiquitin Activating Enzyme (Uba1)

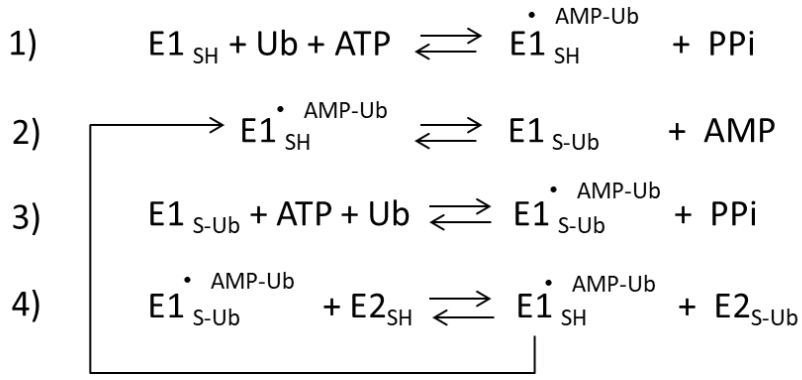

Fig. 1.7. Reaction scheme of E1 enzyme. Non-covalent complexes are indicated with a dot, and covalent complexes are indicated with a hyphen. SH refers to the free form of the catalytic cysteine, S-Ub refers to the thioester between the catalytic cysteine of E1 and Ub (ubiquitin) C-terminus, and PPi refers to inorganic pyrophosphate.

As it was already discussed, ubiquitin-activating enzyme Uba1 (E1) is at the apex of the ubiquitin-proteasome degradation pathway, which activates the ubiquitin and directs to the downstream pathway. E1 activates ubiquitin in a two-step process (Ciechanover et al. 1982; Hershko et al., 1981). At first, ATP is hydrolyzed and C-terminus of ubiquitin is adenylated to form ubiquityl-AMP intermediate and PPi is released (Reaction 1). Ubiquitin adenylate binds non-covalently at the adenylating site of E1. In the second step, ubiquitin moiety is transferred forming a covalent high-energy thioester linkage between the C-terminus of ubiquitin and the active-site cysteine residue of E1, while AMP is released (Reaction 2). Meanwhile, one more ubiquitin is recruited and adenylated (Reaction 3), in the adenylation site of E1. Therefore, a fully loaded E1 contains two ubiquitin molecules, one in the form of adenylate and another as a thioester. Coupling the second adenylation

reaction with ubiquitin transfer to E2 might make the cascade energetically or conformationally favorable, or might prevent the E1 from becoming trapped in an unfavorable conformation. At this point, other enzymes in the pathway become involved, and the ubiquitin, which is covalently attached to E1 is transferred to catalytic cysteine of E2 to form a thioester complex via transthioesterification (Reaction 4) (**Fig. 1.7**), which is then followed by the eventual transfer of ubiquitin to a target protein (Pickart and Rose, 1985). So, in a way, a single activating enzyme supports the multiple conjugation pathways defined by mutually specific E2-E3 pairs.

1.3.6. Distant Relatives of E1 and Ubiquitin

Although presences of post-translational modification pathways are well known in eukaryotes, they exist in prokaryotic organisms too. Interestingly, analogous chemical reactions found in prokaryotes suggest the origin of ubiquitin and E1 of eukaryotes from the proteins involved in sulfur metabolism (Lake et al. 2001; Duda et al. 2005; Iyer et al., 2006) and thiamine biosynthesis. The bacterial proteins subunit 1 of molybdopterin converting factor (MoaD) and thiamine biosynthesis protein S (ThiS) are structural homologues of ubiquitin (Wang et al., 2001; Duda et al., 2005). MoaD and ThiS undergo adenylation at their carboxyl termini, in a reaction reminiscent of ubiquitin adenylate formation. The adenylation reactions of MoaD and ThiS are catalyzed by larger bacterial enzymes MoeB and ThiF, respectively (Taylor et al., 1998; Leimkühler et al., 2001). The formation of an Ubl~adenylate intermediate is the most ancient reaction in ubiquitin-like protein pathways. The MoeB and ThiF enzymes are preceded by a still earlier MccB enzyme that synthesizes bacterial antibiotic microcin C7 (Regni et al., 2009). MoeB, MccB, and ThiF share sequence homology with the domain of eukaryotic E1, which is responsible for ubiquitin binding and adenylation

underlining the conservation of a common mechanism through ancestry (**Fig. 1.8**).

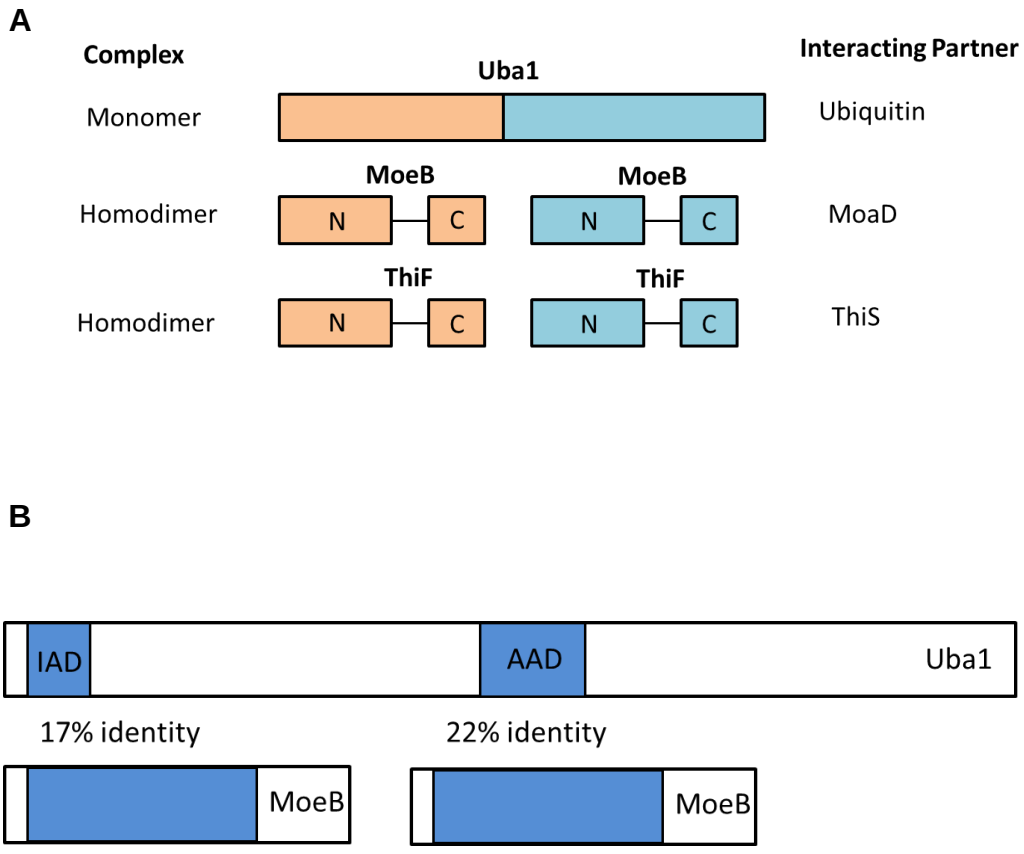

Fig. 1.8. A) Comparison of ubiquitin activating enzyme (UBA1) with bacterial ancestor MoeB and ThiF proteins, B) Schematic diagram of sequence relationships between MoeB and UBA1, with conserved sequence motifs indicated by colored blocks. *E. coli* MoeB and residues 1-250 and 400-660 of human Uba1 are 17% and 22% identical, respectively.

The crystal structures of the MoeB-MoaD and ThiF-ThiS complexes have provided insights into the mechanism of ubiquitin activation (Lake et al., 2001; Lehmann et al., 2006). The MoeB and ThiF are homodimeric structures

with two symmetric catalytic centers. Each contains a four-stranded β-sheet, with a hydrophobic surface that binds the region of MoaD or ThiS. The binding sites of these proteins correspond to the residues of Ile44 hydrophobic patch of ubiquitin (Sloper-Mould et al., 2001). MoeB and ThiF exist as homodimers to provide a binding site for MoaD and ThiS respectively. The interaction between MoaD and MoeB is shown in **Fig. 1.9**. The C-terminal tails of MoaD and ThiS protein extend towards ATP that is located in the nucleotide-binding site. The active site of MoaD and ThiS contain conserved ATP-binding Arg finger contributed by the opposite monomer in the complex, besides a conserved Asp which coordinates Mg^{2+}. These two structural features relieve the electrostatic repulsion that may arise when the α-phosphate of ATP·Mg^{2+} complex is attacked by the C-terminal carboxylate oxygen of ubiquitin. The side-chains belonging to basic residues of MoaD or ThiS would stabilize the negative charge developing from the pentacovalent phosphate intermediate and lead to the formation of MoaD or ThiS~adenylate and PP_i. However, these ancestral proteins of E1 present in bacteria do not catalyse a step analogous to E2 transthioesterfication of ubiquitination pathway unique to eukaryotes.

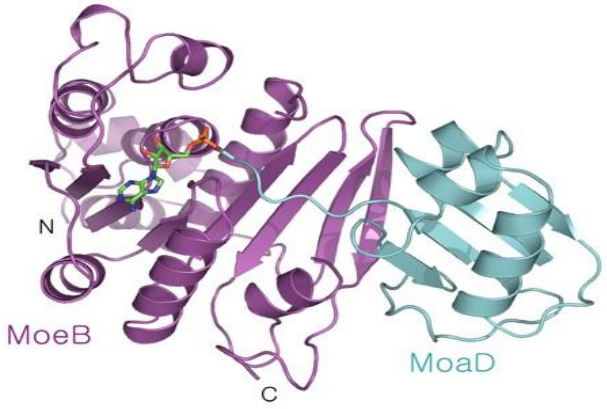

Fig. 1.9. Structure of MoeB-MoaD complex (PDB: 1JWA). The N-terminal subdomain of MoeB adopts a variation of the Rossmann fold and is involved in ATP (in Green) binding and catalyzes the adenylation reaction. The C-terminal subdomain contains an antiparallel, four-stranded β- sheet, which makes direct contact with the globular domain of MoaD (Lake et al., 2001).

1.3.7. Alleles of UBA1

Mutant alleles of UBA1 have been instrumental in understanding the functional significance of many aspects of the ubiquitin-proteasome system. The first E1 mutant identified was temperature sensitive (ts) mutant of mammalian Uba1. The ts85 Uba1 cells were arrested in a G2/M phase transition of the cell cycle and failed to degrade short-lived proteins *in vivo* under restrictive conditions (Finley et al., 1984). Another mammalian E1 mutant, E36-ts20, was later identified in hamster lung fibroblast cells. This mutant displayed similar defects in ubiquitin-protein conjugation and cells were arrested in the late S/early G2 phase (Kulka et al., 1988). One more temperature-sensitive mutation reported carried a substitution at amino acid M256I in the catalytic domain of Uba1 of Chinese Hamster cells, exhibited chromosomal instability and cell-cycle arrest in the S to G2 phases with decreased DNA synthesis at the non-permissive temperature, 39°C (Sugaya et

al., 2014). These studies establish that temperature-sensitive mutations resulting in loss of Uba1 function, lead to an overall reduction in the levels of ubiquitinated proteins and protein degradation, causing cell cycle arrest. Uba1 is almost entirely present in the nucleus during G1 and G2 phases, while it is present in both the nucleus and the cytoplasm in other phases of mitosis (Grenfell et al., 1994).

In UBA1 knockdown, charging of the two E2s CDC34A and CDC34B with ubiquitin were halted (Jin et al., 2007). UBA1, being the fountainhead of the ubiquitination pathway, is most crucial in the regulation of a range of cellular and molecular pathways. Studies showed that inhibition of UBA1 leads to increased miniature and spontaneous synaptic currents at both excitatory and inhibitory synapses in cultured hippocampal neurons (Rinetti and Schweizer, 2010).

X-Linked infantile Spinal Muscular Atrophy (XL-SMA) is a disease of rare occurrence associated with mutations of UBE1 (homologue of UBA1 in humans). Missense mutations and synonymous variations of the UBE1 gene affect ubiquitin system-mediated protein degradation in spinal motor neurons (Ramser et al., 2008). *In vivo* suppression of UBA1 was found to produce effects similar to the motor neuron axon defects observed in SMA-model Zebra fish (Wishart et al., 2014). These observations revealed targeting UBA1 was sufficient to generate a pronounced motor neuron defects which precipitate decreased survival (Liu and Pfleger, 2013).

HMW mHtt (High Molecular Weight mutant Huntington) levels are found to be increased by inhibition of ubiquitin-activating enzyme E1 (Ube1) in the unaffected tissues. With aging, the Ube1 level is found to be lower in brain tissues than in other tissues, causing increased accumulation of mHtt in the brain and neuronal nuclei (Wade et al., 2014). This finding suggests a decrease in the degradation of misfolded Htt by UPS, resulting in the accumulation of toxic forms of mHtt in the brain.

Increased levels of UBA1 has been found to result in slow Wallerian degeneration (Wld^s) phenotype in mouse (Wld^s) mutation, which selectively protects axons and synapses in the central and peripheral nervous systems from degeneration-inducing stimuli caused by a variety of traumatic and disease related conditions (Wishart et al., 2008, 2007).

UBA1 activity manipulation at different developmental stages of *Caenorhabditis elegans* revealed a variety of roles for ubiquitination, like in sperm fertility, control of body size, and sex-specific development. Ubiquitin-mediated proteolysis is known to regulate meiosis. Mutations in uba1 have been shown to cause a delay in meiosis in the early stages of the mouse embryo (Kulkarni and Smith, 2008). Several UBA1 mutant alleles have also been identified in *Drosophila melanogaster*. It is found the reduction of Uba1 during development in flies led to tissue overgrowth, suggesting a possible role for Uba1 in tumor suppression (Lee et al., 2008; Pfleger et al., 2007), whereas complete loss of Uba1 leads to cell cycle arrest (Lee et al., 2008). Uba1 was also shown to be essential for the repair of double-strand DNA breaks (Moudry et al., 2012).

UBA1 accumulation in spermatogonia and spermatids led to the conclusion that protein ubiquitination involving UBA1 contributes to sperm function during fertilization (Yi et al., 2012).

1.3.8. Ubiquitin Conjugating Enzyme

The second step in ubiquitination cascade is the transfer of activated ubiquitin to ubiquitin conjugating enzyme (E2) from the E1 enzyme. E2 accepts thioester-linked ubiquitin via transthioesterification reaction from E1 to form a thioester bond with its catalytic cysteine. E2 then transfers ubiquitin either directly to a substrate, which is bound to RING E3 or to a cysteinyl residue of the HECT E3. The large size of the E2 family caters to a larger

group of E3 enzymes, which in turn supplies ubiquitin to an even larger number of substrates. The cascading effect seen in this system is meant to increase specificity and regulation at each step.

E2 enzymes interact with both E1 and E3 and act as mediators in the unidirectional transfer of ubiquitin from E1 to the substrate. E2s have a common binding site for both E1 and E3, which prevents E2s from recharging with ubiquitin while bound to E3s. Therefore, a single binding site forces the dissociation of E2 from E3 first, before proceeding for the next round of conjugation (Eletr et al., 2005). Moreover, E1 has a higher affinity towards uncharged E2s than loaded E2~ub conjugated forms (Hershko et al., 1983; Pickart and Rose 1985). Similarly, E3s show a better affinity for E2~Ub conjugates than for the uncharged E2s, which helps in directional ubiquitin transfer (Siepmann et al., 2003; Saha and Deshaies 2008).

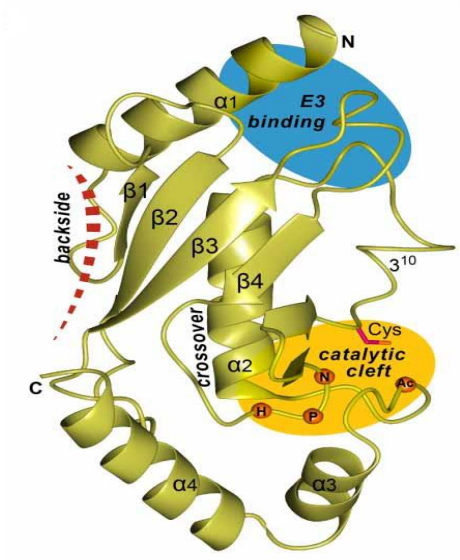

Fig. 1.10. Structure of conserved UBC domain of E2 (PDB: 2ESK) (Alpi et al., 2016).

All E2 enzymes possess a ~150 amino acid catalytic domain referred to as the UBC domain (Stewart et al., 2016). This domain adopts an α/β-fold typically with four α-helices and a four stranded β-sheet (**Fig. 1.10**). Important loop regions form part of the E3-binding site and E2 active site. The active site cysteinyl residue is present within this region, which forms the thioester bond. A conserved tripeptide HPN (His-Pro-Asn) is present about 10 residues N-terminal to the active site cysteine in the UBC domain of almost all E2s. The asparagine acts as a catalyst by stabilizing the temporary oxyanion formed during isopeptide bond formation (Wu et al., 2003). Histidine plays a structural role in stabilizing these residues along with proline (Cook and Shaw, 2012). E2s have polypeptide chain extensions on the N-terminus or C-terminus or on both sides of the core catalytic UBC domain. These extensions may contribute to regulating association with E3, intrinsic E2 activity or substrate recognition (Pickart, 2001). The extensions are intrinsically disordered, however, a few of these extensions adopt a secondary structure as they bind the UBC domain (Schelpe et al., 2016).

E2 enzymes are also important for protein quality control pathways outside the nucleus. Ubc4 and Ubc5 are highly redundant in function to conjugate ubiquitin to abnormal proteins in the cytosol for proteasome degradation (Seufert and Jentsch, 1990). Ubc1, Ubc6, and Ubc7 are involved in the degradation of misfolded proteins from the endoplasmic reticulum. Ubc1 can create a polyubiquitin chain on its own, a unique feature not shared with any other yeast E2 (Rodrigo-brenni and Morgan, 2007). Its C-terminal ubiquitin associated (UBA) domain binds ubiquitin to form a polyubiquitin chain (Merkley and Shaw, 2004). Few E2s have some specific functions for example; Ubc2 is required for DNA repair, and Ubc3 is required for cell cycle transition from G1 to S-phase (Hochstrasser, 1996; Jentsch, 1992).

In our laboratory, studies have been carried out to check complementation of function between Ubc4 and Ubc5, as we already discussed these two enzymes are redundant in function (Seufert and Jentsch, 1990). These enzymes are identical with only 11 amino acid differences. They show functional complementation as well during the protein degradation process, especially during stress conditions. Though, the presence of two identical proteins raised the question for its need in the cell. There might be some specific functions to them under selective conditions. To understand the purpose of residues difference between both the proteins, mutations were introduced in the UBC4 gene to generate single residue variants by swapping with codons from UBC5. Results showed the variants were functionally active in Δubc4Δubc5 strain of yeast. In comparison to UBC4, these variants showed reduced growth under normal conditions, altered survival under heat stress and antibiotic stress. This study indicated the UBC5 doesn't have any advantage over UBC4 because of differences in residues. Hence, UBC5 might be evolved to meet cell stage-specific needs (Raimalani et al., 2019).

1.3.9. Ubiquitin Ligase

As mentioned above, ubiquitin ligases catalyze the transfer of activated ubiquitin from an active-site cysteine residue of an E2 to a lysine residue of a substrate protein. This reaction occurs in two distinct steps: (i) binding of E3 to the substrate after recognizing the ubiquitination signal and (ii) the covalent ligation of one or more ubiquitins to the substrate. Usually, the polyubiquitin chain with K48-linkage is required on a substrate for undergoing degradation by the proteasome. The type of linkage in ubiquitination depends on interactions of the E3 with two protein elements, the E2 and the signal present on the substrate, thus providing chances to regulate the conjugation reaction at the level of protein-protein interactions. In certain cases, it was reported that the E3 may need to undergo modification before interacting with one of them

(Lahav-Baratz et al., 1995) or the expression of a cognate E2 may need to be induced (Rajapurohitam et al., 1999; Wefes et al., 1995).

The first E3 to be discovered was E3α (Ubr1). It was originally defined as a third enzyme component required for the ligation of ubiquitin to some specific proteins, in addition to E1 and E2 (Hershko et al., 1983). The presence of a large number of E3s is a necessity for regulating the ubiquitination of diverse substrates (Hershko and Ciechanover, 1998).

E3s fall into two broad classes depending on their structure: (i) E3 enzymes homologous to HECT (Homologous to E6-AP Carboxy-Terminus) family proteins or RING (Really Interesting New Gene) family, which includes U-Box family of proteins (Jackson et al., 2000). The HECT and RING domains both show the biochemical property of E2 binding.

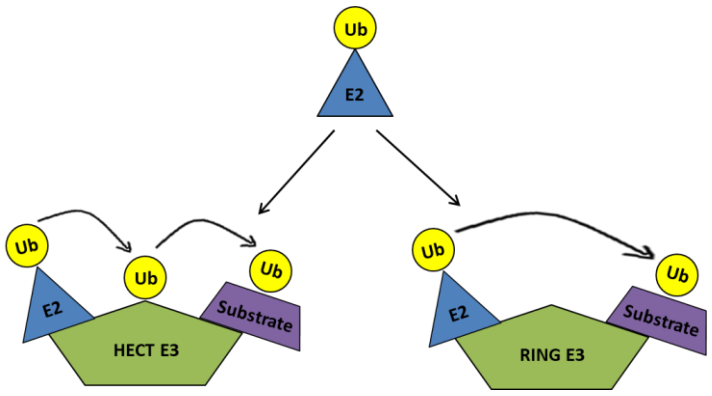

Fig. 1.11. HECT and RING E3 ubiquitin ligases.

RING and HECT domain E3s follow different mechanisms of catalyzing ubiquitination (**Fig. 1.11**). The HECT domain E3 receives ubiquitin from its cognate E2 and forms a thioester between active site cysteine present within the HECT domain and ubiquitin. Subsequently, the ubiquitin molecule bound to E3 is transferred to the substrate (Scheffner et al.,

1995). Whereas RING E3s do not form thioester bond with ubiquitin, instead they transfer ubiquitin by positioning the charged E2~Ub in proximity to the substrate.

1.3.9.1. HECT Domain E3s:

First HECT E3 identified was E6 Associated Protein (E6AP). HECT E3s are identified by their ~350 residues long C-terminus region. E3s which have homologous C-terminus with E6AP's C-terminus are categorized under the HECT E3 family. In the HECT domain, in the conserved region is a strictly conserved cysteine residue positioned ~35 residues upstream of the C terminus. The properties of this domain were first characterized while studying the conditional degradation of the p53 tumor suppressor. p53 levels were selectively brought down by UPS in cells infected by oncogenic forms of human papillomaviruses (HPVs), (Scheffner et al., 1990). The N-terminal part of the HECT domain protein binds to cognate E2, and the C-terminal part containing the active site cysteine forms a thioester intermediate with ubiquitin. The N-terminal and C-terminal parts are connected by a flexible hinge region (Huang et al., 1999). All HECT proteins contain a conserved active site cysteine residue near the C-terminus. Variable N-terminal regions of the different HECT proteins indicate the possibility that N-terminal domains may be involved in the recognition of specific protein substrates (Scheffner et al., 1995). Most HECT-domain proteins could be E3 enzymes or parts of multiprotein complexes that contain E3-like activities.

One of the HECT proteins found in yeast is Rsp5, which ubiquitinates several cellular proteins, including the large subunit of RNA polymerase II. *in Vitro* experiment, showed the N-terminal domain of Rsp5 binds to polymerase subunit while the C-terminal (HECT) domain does not show any interaction, establishing the N-terminal domain plays role of substrate binding (Huibregtse et al., 1997). Furthermore, yeast Rsp5 contains several WW domains, which

are believed to be involved in an interaction with proline-rich sequences containing the XPPXY (or PY) motif (**Fig. 1.12**) (Henry et al., 1996). Rsp5 is an active E3 involved in the ubiquitination of a large number of substrates and regulation of different biological pathways (Gupta et al., 2007; Rotin and Kumar, 2009).

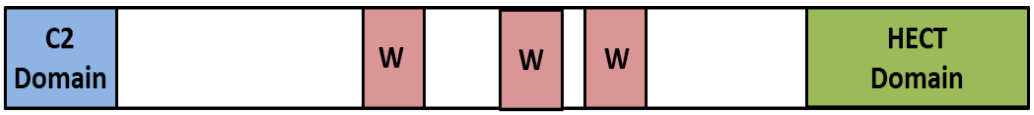

Fig. 1.12. Domain architecture of yeast Rsp5 protein. N-terminus contains a C2 domain followed by several WW domains. The HECT domain is situated on C-terminus, which has catalytic cysteine to form a bond with ubiquitin.

1.3.9.2. RING Finger E3s

RING finger proteins belong to the larger superfamily of zinc-finger proteins. The zinc ions and their ligands are catalytically inactive in these enzymes. Even though the primary structure does not show much conservation, the spacing of the amino acid residues that chelate zinc ligands is conserved in the protein sequence of the RING family (Borden, 2000).

RING finger E3s can be monomeric enzymes or multisubunit complexes. The RING domains bind E2 enzymes (Zheng et al., 2000) stimulate ubiquitin transfer by subtle structural changes (Ozkan et al., 2005). Substrates are recruited either at substrate binding domains within the same polypeptide chain as the RING domain (single subunit RING E3s) or by specialized substrate receptors that form multisubunit RING E3s (Deshaies and Joazeiro, 2009). The RING domain consists of a cysteine and histidine residues rich short motif, which coordinate two zinc ions (Borden, 2000). The RING domain shows a globular conformation because of a distinctive cross-brace arrangement of the zinc interacting residues. It has a central α-helix and

several small β-strands which are separated by loops of variable-length (**Fig. 1.13.A**) (Zheng et al., 2000). The important character is none of the RING domain side chains come closer than ~15Å to the E2 active site cysteine indicating the main role of RING E3 is to bring substrate lysine and the E2~Ub intermediate in close proximity (VanDemark and Hill, 2002).

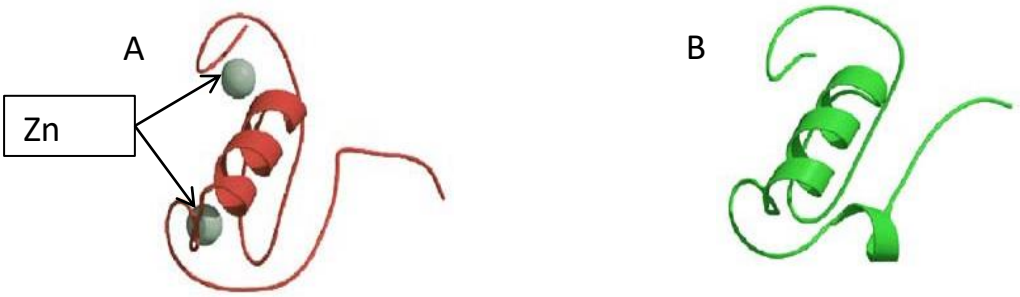

Fig. 1.13. A) RING domain, B) U-Box domain. (Pickart and Eddins, 2004)

The "U box" is a motif related to the RING finger. Here, the domain is stabilized by salt bridges and hydrogen bonds instead of zinc ions. It is believed, U box might have evolved from the RING finger domain replacing zinc ions by salt bridges (**Fig. 1.13.B**). U-box is quite stable because of its hydrophobic core, along with two "internal interaction centers" comprising multiple hydrogen bonds and salt bridges (Ohi et al., 2003). The spacing of the molecules involved in the interaction is also similar to that of the zinc-coordinating residues of the RING domain (Ohi et al., 2003). The folding of the U-box creates a shallow groove on one face of the domain which is largely hydrophobic, like the RING domain.

MDM2 (E3 ubiquitin-protein ligase Mdm2) is a RING E3 ligase, which facilitates the proteasomal degradation of the tumor suppressor p53 (cellular tumor antigen p53), whereas E3 ubiquitin-protein ligase CHIP (CHIP) is a homodimeric U-box E3 ligase (Wu et al., 2011), which suppresses apoptosis by targeting heat shock proteins (Paul and Ghosh, 2014).

1.3.10. 26S Proteasome

The proteasome is a large subcellular, self-compartmentalized, hollow cylinder-shaped protease that carries sites for proteolytic activities on its inner surface (Voges et al., 1999). Substrate delivery to the proteasome is facilitated by Ub-dependent chaperones and shuttling factors (Stolz et al., 2011). The proteasome is found in all eukaryotes and is highly conserved during evolution.

The proteasome is present in different sites in the cytosol and nucleus of a eukaryotic cell (Peters et al., 1994). The 26S proteasome is composed of two major parts; the 19S regulatory particle (RP) and the 20S core particle (CP). RP recognizes substrates to be degraded, while CP contains the proteolytic active sites. The proteolytic sites are present on the inner surface of the CP to restrict the access of the substrates to proteolytic sites which minimizes uncontrolled proteolysis.

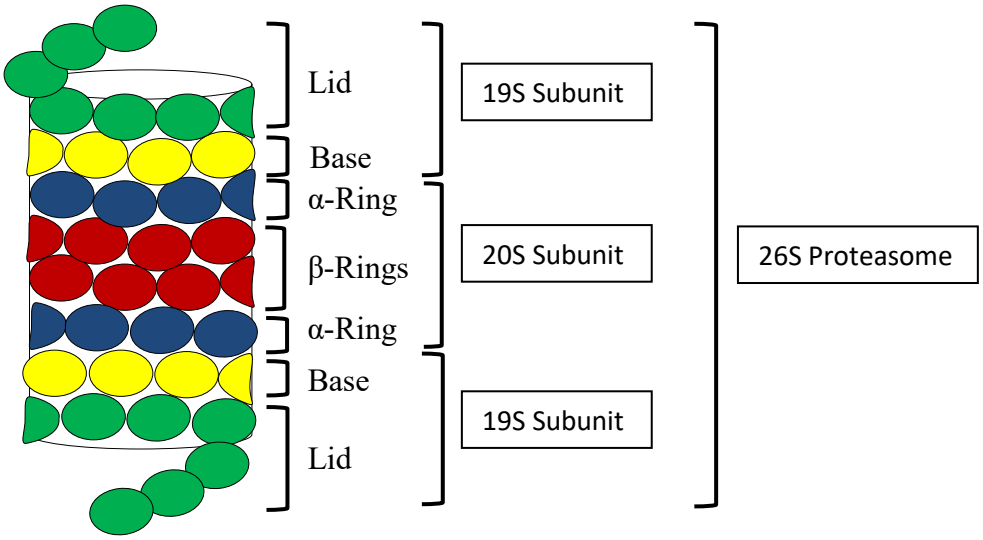

Fig. 1.14. Structure of 26S proteasome.

The CP is also referred to as 20S proteasome and is a 720 kDa cylindrical complex. It contains four stacked heptameric protein rings (Groll et al., 1997). The two outer rings made up of seven different α-subunits (α1-α7) are known as α-rings, whereas the two inner rings made up of seven β-subunits (β1-β7) are known as β-rings (**Fig. 1.14**). The proteolytic activity of the proteasome is because of the two β-rings (Groll et al., 1997), which contain trypsin-like, chymotrypsin-like and postglutamyl-like hydrolytic activities (Wilk and Orlowski, 1983). The two α-rings sandwich the β-rings. The α-subunits block the nonspecific entry of proteins into the proteolytic chamber by extending their amino termini at the orifice of the proteolytic chamber by leaving only narrow pores on either end of the cylinder (Groll et al., 1997; Wenzel and Baumeister, 1995).

The RP is also known as 19S particle, acts like a gate present at either end of the 20S particle. The 19S regulatory particle, which is present on both sides of the 20S subunit, is made of two substructures, a lid and base (Fig. 14) (Lupas et al., 1993). The lid contains 9-subunits whereas the base is composed

of the 10-subunits. The main role of the 19S subunit is in substrate selection, preparation, and protein translocation into the catalytic 20S chamber for degradation (Glickman et al., 1998; Larsen and Finley 1997). Six ATPases present on the 19S subunit are responsible for the unfolding of the protein substrate and its translocation into the 20S subunit with the help of ATP hydrolysis (Glickman et al., 1998). The outer-lid subcomplex of the 19S component is involved in the recognition of ubiquitin chain processing before substrate translocation and degradation (Deveraux et al., 1994, 1995). Therefore, the 19S particle is equally essential for the specific degradation of target proteins because the 20S particle by itself can degrade small peptides only (Glickman et al., 1998).

26S proteasome hydrolyzes a single protein at a time (Akopian et al., 1997). Substrate protein is cleaved to peptide products of 6 to 10 amino acids in length. Further hydrolysis of the peptides to individual amino acids occurs in the cytosol (Wenzel et al., 1994).

1.3.11. De-ubiquitinating Enzymes

An important step in the ubiquitination pathway involves the recycling of ubiquitin. De-ubiquitinating Enzymes (DUBs) are a large group of proteases, which show specificity towards ubiquitin conjugates and ubiquitin chains. They catalyze the hydrolysis of the isopeptide bonds that link ubiquitin to its targets and other molecules (Francisca et al., 2009). Release of ubiquitin is important for two processes: firstly, ubiquitin biosynthesis and secondly, protein degradation.

At the time of proteasomal degradation, polyubiquitin is released from Lys residues of the substrate protein and polyubiquitin chains are disassembled. The release of free ubiquitin molecules involves specific enzymatic cleavage between the covalently joined ubiquitin molecules. DUBs

can either assist proteolysis or inhibit it. By removing ubiquitin moieties from mistakenly tagged proteins they inhibit proteolysis. There is one more important function of the DUBs is to recycle ubiquitin by recovering it from ubiquitin protein conjugates before the target protein is degraded. Defects in this process lead to reduced ubiquitin levels and pleiotropic stress sensitivity (Swaminathan et al., 1999).

Specific enzymatic cleavage between the fused residues releases free ubiquitin molecules. Ubiquitin is encoded by many genes and is translated in a variety of structurally distinct forms. One of them is a linear, head-to-tail polyubiquitin precursor (Ozkaynak et al., 1984). The last ubiquitin moiety in many of these precursors is encoded with an extra C-terminal residue that has to be removed in order to expose the active C-terminal Glycine. In some precursors, ubiquitin is synthesized as an N-terminal fused extension of two ribosomal proteins L40 and S31 (Finley et al., 1989). DUB activity is necessary to release ubiquitin from these precursor forms and make it available for ubiquitination.

In yeast, DUBs are divided into 4 families: the Usp (ubiquitin-specific proteases) family, the Otu (ovarian tumor proteases) family, the JAMM (Jab1/Mov34/Mpr1 Pad1 N-terminal+ domain) and Uch (ubiquitin C-terminal hydrolases) families.

1.3.12. Significance of the Ubiquitin Proteasome System

Ubiquitination is diverse. The addition of one or more ubiquitins in different configurations gives the system flexibility and diversity for different consequences. Ubiquitination affects the stability, interactions, localization or activity of thousands of proteins, by forming conjugates of distinct topologies, in a way providing specific signals that are widely used in cellular control (Yau and Rape, 2016). It has been shown that it plays important role in

maintaining cellular homeostasis by cell cycle regulation, DNA repair, sodium channel function, regulation of immune reaction, inflammatory response and cellular response to stress (Ciechanover, 1998; 2006; Malik et al. 2006). Although, abnormalities in this pathway cause numerous human disorders including malignancies, neurodegenerative diseases and systemic autoimmunity (Nalepa et al., 2006). Considering the wide range of cellular substrates and processes that are controlled by ubiquitin-proteasome pathway, the components of this pathway have become attractive targets for therapeutic intervention.

1.3.13. Diverse Functions of the Ubiquitin Proteasome System

1.3.13.1. Development

There is evidence that links the ubiquitin system to the developmental processes of the Central Nervous System (CNS). The *Drosophila bendless* (*ben*) gene encodes an E2 that appears to be restricted to the CNS during development. Morphological abnormalities within the visual system are caused by mutations in this gene which lead to impairment of synaptogenesis between photoreceptor cells and other elements of the system (Muralidhar and Thomas, 1993; Oh et al., 1994). This suggests that the significance of the gene product is extensive and it might be playing role in other developmental processes of the neuromuscular system as well. Moreover, defect in a gene coding for the E3 enzyme E6-AP results in Angelman syndrome in humans, a disorder resulting in mental retardation, seizures and abnormal gait (Kishino et al., 1997; Matsuura et al., 1997).

The UPS is also found to be involved in the formation and maintenance of neuronal polarity. Hippocampal neurons of rat that were grown in cell culture showed remodeling before their final polarization.

Neuron development from soma includes several processes and forms neurites, only one of the neurites forms a single axon, whereas the others develop into dendrites (Craig et al., 1994). Axon growth and maintenance are determined by AKT (serine/threonine kinase) protein levels which are present at the tip. AKT protein was found to be stable in neurites that develop into axons but undergoes degradation in a ubiquitin-dependent manner in neurites that differentiate into dendrites (Yan et al., 2006).

Drosophila faf facets is another gene found to be involved in the development process is a de-ubiquitinating enzyme from the UBP family. The *faf* gene is involved in process of eye development, and mutant *faf* flies have more than eight photoreceptors in each of the compound eye units, the facets (Huang et al., 1995, 1996).

Yeast E2 RAD6/Ubc2 has human homolog HR6B which is involved in DNA repair and targeting of N-end rule substrates. Inactivation of HR6B leads to male sterility. It affects the development of sperm and interferes with the general process of meiosis (Roest et al., 1996). The hyperplastic disc (*hyd*) gene is another ubiquitin system related gene involved in sex differentiation, oogenesis, or spermatogenesis. It belongs to the HECT family of E3 enzymes and plays a major role in *D. melanogaster* development. The null phenotype appears to be lethal at an early embryonic stage (Mansfield et al., 1994).

During the gestation period in human, ubiquitin protein was found in cytotrophoblast, in both free and conjugated form but lacked in syncyiotrophoblast layer (Bebington et al., 2000). Mice missing the UbcM4 gene encoding E2 enzyme, showed multiple developmental abnormalities like intrauterine growth retardation and perinatal death, indicating the importance of UPS in placental development (Harbers et al., 1996).

1.3.13.2. Apoptosis

Polyubiquitin gene was one of the first genes found to be involved in apoptosis in the hawk-moth *Manduca sexta*. It is up-regulated during the metamorphosis *(Schwartz et al., 1990).* In moth during metamorphosis Decline in the molting hormone 20-hydroxyecdysone leads to the induction of ubiquitin conjugation and degradation pathways. Further, the polyubiquitin gene is induced leading to an increase in the quantity of ubiquitin, nearly 10-fold increase in ubiquitin-conjugate levels, with an associated increase in the activity of E1, several E2 enzymes, and E3 enzymes (Haast et al., 1995).

Further, the involvement of ubiquitin in apoptosis came from studies involving human lymphocytes exposed to γ-irradiation. It has been found that the levels of ubiquitin mRNA and ubiquitinated nuclear proteins increased after the exposure to γ-irradiation. The apoptotic phenotype can be suppressed by expressing ubiquitin sequence-specific antisense oligonucleotides (Delic et al, 1993). Deprivation of Nerve Growth Factor (NGF) induces apoptosis in sympathetic neurons. Inhibition of the ubiquitin system has been shown to save the neurons from apoptosis, establishing the role of UPS in programmed cell death (Sadoul et al., 1996). However, apoptosis is stimulated by inhibition of UPS in leukemic cells (Drexler, 1997), activated T-cells (Cui et al., 1997) and some neuronal cells (Lopes et al., 1997).

The transcription factor p53 is a part of the network of proteins with diverse functions involved in a variety of stress signaling pathways (Caspari, 2000). The activation of p53 in response to such signals prevents tumor formation in cells either by apoptosis or cell-cycle arrest, in an attempt to maintain the integrity of healthy cells. Under normal conditions, levels of P53 are quite low mainly due to its rapid turnover. The ubiquitin proteasome pathway is responsible for regulating its levels in cells (Ciechanover et al., 1991; Maki et al., 1996). But this tumor suppressor p53 has been found as one of the most frequently mutated genes in many types of cancers. MDM2 is one

of the major regulators of p53, which gets induced by its substrate p53 (Haupt et al., 1997). The transactivation domain of p53 present on its C-terminus is bound by MDM2, which results in inhibition of p53 transcriptional activity. Moreover, MDM2 bound p53 proceeds for ubiquitination and MDM2-P53 complex eventually undergoes degradation by proteasome-mediated proteolysis.

Basically, UPS produces different effects in different cellular environments. In some cases, inhibition of the system may lead to the accumulation of abnormal proteins with the possible induction of apoptosis, whereas in others the system may play a direct role in the destructive process and its inhibition leads to inhibition or a delay in the onset of the apoptotic chain of events.

1.3.13.3. Immunity

Ubiquitin proteasome system is involved in generating antigenic peptides from cytosolic proteins for presenting to MHC class I molecules (Rock and Goldberg, 1999). Antigenic proteins are subjected to limited processing by ubiquitin proteasome pathway and the peptide epitopes generated are presented to MHC class I molecules on cytotoxic T lymphocytes (CTLs) (Groettrup et al., 1996). The peptides corresponding to epitopes can also be generated by proteasome-independent mechanisms (Vinitsky et al., 1997).

Proteasome inhibitor lactacystin (Craiu et al., 1997) was found to reduce antigen presentation because the accumulated MHC class I molecules continuously bind with TAP (Transporter associated with antigen processing) in the ER (endoplasmic reticulum) in the absence of proper antigenic peptides (Rock et al., 1994). The cytokine γ-interferon (γ-IFN) that stimulates antigen presentation leads to induction and exchange of three proteasomal subunits in

human cells: LMP2 for X, LMP7 for Y, and MECL1 for Z (Driscoll et al., 1993). These proteolytic activity profiles of new subunits are different and they most likely lead to a more efficient generation of antigenic peptides recognized by the MHC class I molecules.

1.4. Hypothesis and Objectives of the Present Research Thesis

Inhibitors of ubiquitin proteasome system are used in certain terminal cancers (Bedford et al., 2011). The inhbitors targeted against E1 and E3 enzymes are investigated for their potential to treat cancers (Harper and King, 2011). Hence, it would be crucial to study the domains of E1 as independent units to understand if isolated domains can retain the native structure and to what extent they enjoy functional independence. The main focus of this work, therefore is to characterize structural and functional aspects of the four domains of E1, i.e. FCCH, SCCH, 4HB and UFD as independent units. Also, β-bulge mutants were previously created in the laboratory, further studies were done to improve understanding on the effect of the mutations on both structure and functiona of the protein.

Chapter 1 presents introduction to ubiquitin proteasome system (UPS), the structural and functional aspects of various proteins belonging to UPS. In this chapter, the process of ubiquitination and its various purposes have been discussed. Ubiquitin protein is involved in various pathways, regulating many cellular processes. The types of bonds formed and the number of ubiquitin molecules attached to target proteins determine their destiny of following pathways. Here, different components of the ubiquitin proteasome pathway have also been described in brief. There are three groups of enzymes mainly involved in the pathway namely, E1, E2 and E3. The function and structure of these enzymes have been introduced to get a detailed

understanding of UPS pathways. Mutations in this system lead to many diseases and disordered conditions. The chapter ends with a brief outlay of organization of the thesis.

Chapter 2 deals with domains of ubiquitin activating enzyme E1. It is a 110 kDa molecular weight multidomain protein. E1 comprises six domains namely, Inactive Adenylation Domain (IAD), Active Adenylation Domain (AAD), First Catalytic Cysteine Half-domain (FCCH), Second Catalytic Cysteine Half-domain (SCCH), Four Helix Bundle (4HB) and Ubiquitin Fold Domain (UFD). Main focus of this chapter was the structural characterization of FCCH, SCCH, 4HB and UFD domains as independent units. All four domains were cloned in a bacterial expression vector to purify the peptides. Structural characterization was carried out by CD and fluorescence spectroscopy techniques. Results obtained from the far-UV-CD spectra indicated the presence of secondary structure in all four peptides. Domains 4HB and SCCH were mostly helical in structure. Domains FCCH had more of β-sheet content and UFD showed mixed α/β structure. Upon exposing these domains to denaturation by guanidine hydrochloride, all four domains showed changes in fluorescence intensity and red shift in λ_{max} wavelength indicating a change in the environment of aromatic amino acids, suggesting the presence of tertiary structure. An extrinsic fluorophore ANS was added to check the position of hydrophobic residue. Domains FCCH, 4HB and SCCH indicated the presence of hydrophobic residues in core whereas UFD showed the presence of hydrophobic residues on the surface. Comparison of the results to structural details obtained from X-ray crystallographic structure of whole protein indicated at least three of the peptides namely FCCH, SCCH and 4HB folded to attain native like structures. With UFD eventhough the secondary structure appeared to be similar to that of native state, it is difficult to draw any conclusion on its. tertiary structure

Chapter 3 probes into functional efficiency of the domains of E1 i.e., FCCH,4HB, SCCH and UFD by expressing them *in vivo* and looking at the

effect of their presence in cells. Domains were expressed in cells to test whether they have the potential to interact with any binding partners and interfere with cellular machinery by blocking the binding site. Domains were overexpressed in cells under various stress conditions like heat stress and antibiotic stress. Results showed that the presence of SCCH and UFD domains slowed cell growth under antibiotic and heat stress conditions. The presence of rest of the two domains FCCH and 4HB had no effect on cell growth under stress.

Chapter 4 deals with β-bulge of ubiquitin which is made up of residues Gln2, Glu64 and Ser65. These residues are highly conserved in evolution throughout the eukaryota in the ubiquitin protein. To understand the importance of these residues, mutants i.e., Q2N, E64G and S65D were already constructed in our laboratory (Mishra et al., 2009, 2011). Results from previous studies suggested these mutations caused minor effects on the structure of ubiquitin with significant functional effects under stress conditions. However, previously all functional studies with β-bulge of ubiquitin were carried out *in vivo* in yeast cells, which have wild type ubiquitin expressed from UBI1, UBI2 and UBI3 genes. In the present work these mutations of ubiquitin were tested *in vitro* for their potential to be activated by ubiquitin activating enzyme E1. An *in vitro* ubiquitin activation assay was carried out to get a clearer picture of the working efficiency of ubiquitin mutants. All three mutants Q2N, E64G and S65D were found to be equally efficient in forming a covalent bond with E1. Moreover, as ubiquitin is a very stable protein, the stability of β-bulge mutants under extreme pH conditions was also checked to understand the effect of mutations on structural stability. Results suggested β-bulge mutants were found to be retaining structure at extreme pH conditions.