Volume 59(Suppl.2):261-273, 2015
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

REVIEW

# Ubiquitylation: its role and medical significance

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**ABSTRACT** Ubiquitylation is an intracellular chemical reaction in which the small polypeptide, ubiquitin, is covalently attached to proteins to serve as a versatile signal with proteolytic and non-proteolytic functions. Although the importance of ubiquitylation was first recognized in the process of proteasome-mediated protein degradation, its regulatory potential has since been extended considerably. It is now known that through an elaborate and diverse set of ubiquitylating and deubiquitylating enzymes, the ubiquitin-proteasome system affects practically all intracellular processes. Not surprisingly, alterations in the ubiquitylating system have been linked to the development of various human diseases, including neurodegenerative disorders and cancer. Here, we highlight the most important components and processes of the UPS, and then demonstrate in a few examples connections between aberrations in ubiquitylation and the pathogenesis of certain diseases.
**Acta Biol Szeged 59(Suppl.2):261-273 (2015)**

**KEY WORDS**
proteasome
protein degradation
ubiquitin
ubiquitylation

## Introduction

Proteins are basic constituents that ensure normal structure and operation of cells. First, back at the beginning of the 20st century, it was thought that proteins were quite stable, but by the early years of 1940s, Rudolph Schoenheimer showed that proteins maintain a steady state level in the cells through continuous synthesis and degradation (Schoenheimer 1942). Beside the synthesis of proteins, their degradation is also very important, since proteolysis determines their life-span, removes damaged or denatured proteins and replenish the amino acid pool of the cells. Therefore, effective protein degradation is essential for all organisms and indeed, most eukaryotic cells have such mechanisms. One of them is a vacuolar structure called the lysosome which was discovered in the 1950s by Christian de Duve (1953). The lysosomes are membrane-enclosed intracellular organelles that contain about 50 hydrolytic enzymes capable of breaking down just about everything at acidic pH. Another very effective proteolytic mechanism with an intricate specificity assurance is the ubiquitin-proteasome system (UPS). Unlike lysosomes, the UPS is not surrounded by membranes, instead a post-translational modification, the ubiquitylation, serves as a chemical selectivity filter to prevent unwanted destruction (Ciechanover and Iwai 2004). In this pathway, a target protein is first tagged by multimers of a polypeptide called ubiquitin, and then it is selectively recognized and degraded in the "chamber of doom" of the cell, known as the proteasome (Goldberg et al. 2001). The ubiquitylation process was discovered by the groundbreaking studies of Avram Hershko, Aaron Ciechanover and Irwin Rose, starting from the 1970s. We now know that the ubiquitylation is selective that can be modulated or even reversed by a process known as deubiquitylation, and the outcome greatly affects the fate of target proteins. The degradation step in the UPS is confined only to the proteolytic central chamber of the proteasome. It became generally recognized that ubiquitylation-deubiquitylation and the subsequent degradation of nuclear and cytoplasmic proteins are essential in the regulation of many cellular processes, such as intracellular quality control, cell proliferation, apoptosis or gene expression. Since many substrate proteins and many processes are involved in ubiquitylation, it is not surprising that malfunctions of the ubiquitin-proteasome system have been implicated – directly or indirectly – in the etiology of many inherited and acquired human diseases. In this review, we highlight the most important components and processes of the UPS, and then demonstrate in a few examples connections between aberrations in ubiquitylation and the pathogenesis of certain diseases.

Serving as a label for the proteasomal degradation of proteins is, however, clearly not the only role through which ubiquitin and related proteins contribute to regulation of various cellular mechanisms. Following the overview of the

Submitted Apr 30, 2015; Accepted Aug 10, 2015
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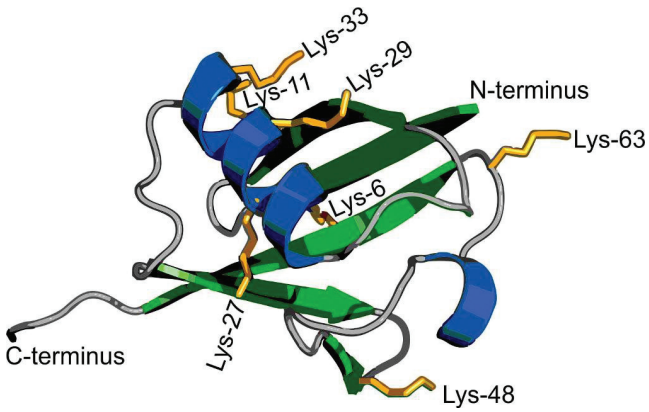


**Figure 1.** Diagram of ubiquitin. Lysine sidechains are shown in orange.

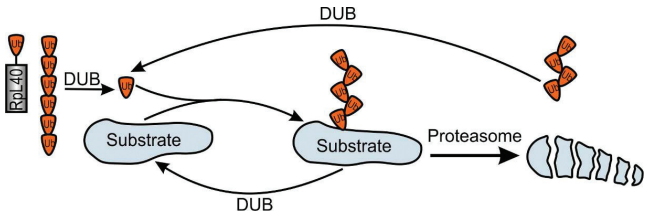


**Figure 2.** The ubiquitylation – deubiquitylation cycle. Ubiquitin is covalently attached to target proteins through ubiquitylation, which is a three-step process involving the E1, E2, E3 enzymes. Deubiquitinating enzymes (DUBs) reverse the ubiquitylation process (middle), process ubiquitin precursors (left) and disassemble unconjugated polyubiquitin chains (right).

UPS system we will discuss briefly other ubiquitin-mediated processes and cover the roles of ubiquitin related proteins (URLs). Due to space limitation we restrict the discussion to general considerations on the versatility of posttranslational modifications by polypeptides and use histone modifications to serve as examples.

## Ubiquitin

The central component of the UPS is a highly conserved essential polypeptide of 76 amino acids, the ubiquitin. Only three of its amino acids differ from yeast to humans, and ubiquitin is present in all tissues of eukaryotes. Ubiquitin is encoded by a multigene family of five genes, three of them coding for tandem polyubiquitin precursors and two of them coding for ubiquitin - ribosomal protein fusion precursors (Nenoi et al. 2000). To generate active free monomers, post-translational cleavage of these precursors is required by deubiquitylating enzymes. When attached to a lysine side-chain of other proteins, ubiquitin serves as a signal that influences the fate, conformation, activity or the localization of those proteins. Ubiquitin itself contains seven lysine residues (Fig. 1) all of which can serve as a site for ubiquitin linkage, the condition for the formation of polyubiquitin chain (Pickart 2001; Xu et al. 2009). Different cells have their own ubiquitin pool in which the ubiquitin exists either as free mono- and polyubiquitin or covalently conjugated mono- and polyubiquitin to other proteins (Kimura and Tanaka 2010). The total ubiquitin content of Human Embryonic Kidney 293 (HEK 293) cells has been estimated as 8 x $10^7$ copies per cell that corresponds to 0.42% (w/w) of total cellular proteins. It is estimated that about a quarter of the ubiquitin accounts for the free monoubiquitin pool. The majority of substrate-conjugated ubiquitin - 63% of the total ubiquitin pool - constitutes the monoubiquitylated forms and about half of these are present in a histone-enriched fraction. About 10% of ubiquitin is linked in polyubiquitin chains (Kaiser et al. 2011; Heride et al. 2014). Considering everything, the level of free monoubiquitin in the cells is determined by ubiquitin synthesis, ubiquitylation and polyubiquitin chain formation, polyubiquitin chain disassembly and ubiquitin degradation.

## The enzymatic cascade of ubiquitylation

Ubiquitylation is a process in which free ubiquitin is covalently linked to target proteins through an isopeptide bond catalyzed by an enzyme cascade of at least three enzymes, termed ubiquitin-activating or E1, ubiquitin-conjugating or E2, and ubiquitin-ligating or E3 enzymes (Fig. 2) (Hershko and Ciechanover 1992; Varshavsky 1997). The isopeptide bond is formed between the carboxy-terminal glycine of ubiquitin and the ε-amino group of a lysine residue of the acceptor protein. The process starts with the activation of a free monoubiquitin (or more precisely, its carboxy-terminal glycine residue) that requires adenosine triphosphate (ATP) as an energy source and catalyzed by the E1 enzyme. This step results in the formation of a high energy thioester bond between the C-terminal carboxyl group of ubiquitin and the sulfhydryl group of the E1 active site cysteine. The activated ubiquitin is then transferred to the active site cysteine of an E2 enzyme via a trans-esterification reaction. In the final step of ubiquitylation an isopeptide bond is formed between a lysine of the target protein and the carboxy-terminal glycine of ubiquitin. In some cases, ubiquitin is transferred directly to target proteins by an E2, but in general, this reaction requires the participation of an E3 ubiquitin ligase, since generally the E3 enzymes interact with substrate proteins, that is, they determine the specificity of ubiquitylation. E3 enzymes have two subtypes: The HECT (Homologous to the E6-associated

protein Carboxyl Terminus) domain containing E3s form a covalent E3-ubiquitin intermediate before transferring the ubiquitin to its substrate protein (Pickart 2001). The RING (Really Interesting New Gene) domain E3 ligases function as docking stations with both substrate and E2 binding modules in such a conformation that the E3 can catalyze the transfer of ubiquitin from the E2 to the substrate protein (Nagy et al. 2012). Following the initial linkage of the first ubiquitin, a polyubiquitin chain can be formed by sequential binding of additional ubiquitins to each other through an internal lysine residue of the previously attached ubiquitin. In some cases, an additional elongation factor, named E4, appeared to be required for efficient polyubiquitylation and proteasomal targeting of a model substrate (Hoppe 2005). The enzymatic cascade of ubiquitylation in mammalian cells includes two E1 enzymes (UBA1 and UBA6), 40 E2s, and more than 600 E3s belonging to one of the above mentioned two families (HECT, RING). A few RING proteins combine with any of seven cullin family members and a variety of adaptor proteins to create a diverse family of cullin-E3 ligases (see in Pickart and Eddins 2004 and references therein).

The fate of ubiquitylated target proteins depends in part on the length of the conjugated ubiquitin chain and on the topology of ubiquitin–ubiquitin linkages within the ubiquitin chain. It is not yet clear how the length of the polyubiquitin chain is regulated, though it must be the result of the balanced action of ubiquitylating and deubiquitylating enzymes. However, it is known, that the lysine (Lys) residue of the ubiquitin on which polyubiquitylation takes place is important in terms of the fate of target proteins. While polyubiquitin chains (with at least four ubiquitin moieties) linked via Lys-11, Lys-29 and Lys-48 will target proteins for proteasomal degradation, linkages via Lys-63 serve as signals for deoxyribonucleic acid (DNA) repair or intracellular signaling (Pickart and Fushman 2004; Welchman et al. 2005; Jin et al. 2008). The functions of other chain topologies are much less known. Monoubiquitylation can also have a variety of effects on protein function that do not include proteasomal degradation.

## Deubiquitylation

Similarly to other post-translational modifications of proteins, ubiquitylation is a reversible process. Ubiquitin is removed from proteins and polyubiquitin chains are processed by deubiquitylating enzymes called DUBs (Fig. 2) (Wing 2003). Since these enzymes attack the isopeptide bond between the ubiquitin and the target protein or another ubiquitin, the DUBs are members of the isopeptidase family of proteases and can be divided into two subfamilies based upon their mechanism of catalysis: cysteine- and metallo-proteases. Cysteine-protease DUBs contain a catalytic amino acid triad of cysteine-histidine-aspartate residues, while the metallo-protease DUBs have a triad of two histidines and an aspartate residue and use a zinc atom for their activity (Ambroggio et al. 2004; Nijman et al. 2005). The cysteine-protease DUBs can be further subdivided into four classes based on their ubiquitin-protease domains: ubiquitin-specific proteases or USPs, ubiquitin C-terminal hydrolases or UCHs, Otubain proteases or OTUs and Machado-Joseph disease proteases or MJDs. All metallo-protease DUBs possess an ubiquitin-protease domain called JAMM. In addition to their catalytic domains, DUBs also contain ubiquitin binding and various protein-protein interaction domains (Ventii and Wilkinson 2008) that have roles in recognition and binding different ubiquitin-ubiquitin linkages and assembly of multiprotein complexes that localize DUBs.

The biological significance of DUBs is manifold. As mentioned above, by removing mono- and polyubiquitin chains from proteins they are able to reverse the effect of ubiquitylation signal. In a coordinated action with the ubiquitylation enzyme cascade, they can edit or modulate the ubiquitylation signal. In addition to this, DUBs are involved in the processing of ubiquitin precursors that are the ubiquitin-ubiquitin and ubiquitin-ribosomal protein fusion products of the ubiquitin genes (Fig. 2). Finally, an equally important function of DUBs is the recycling of free monoubiquitins by disassembling unconjugated polyubiquitin chains (Kovács et al. 2015). Ultimately, all DUBs are involved in ubiquitin recycling, but their relative roles are unclear. It is interesting that in addition to isopeptidase activity, many DUBs have esterase and amidase activities *in vitro* (Pickart and Rose 1985; Wilkinson et al. 1986). These activities could be important *in vivo*, since all cells contain high levels of nucleophile small molecules, like glutathione and polyamines, that can attack the reactive thioester bonds of ubiquitin-E1s and –E2s, and form ester and amid bonds with ubiquitins. Without esterase and amidase activities, the free ubiquitin content of the cells could be sequestered to these small molecules (Pickart and Rose 1985).

In the baker's yeast, *Drosophila* and human genomes, there appear to be at least 21, 45 and 95 genes coding for DUBs, respectively. Their large number and their presence in multiprotein complexes suggest that they have specific effects and may have specific regulatory roles similarly to the ubiquitylating enzymes. Generally, the DUB enzymes are much less characterized than their ubiquitylating counterparts.

## The structure and function of the proteasome

Proteins modified by polyubiquitin chains are recognized and degraded by the proteasome. The proteasome is present

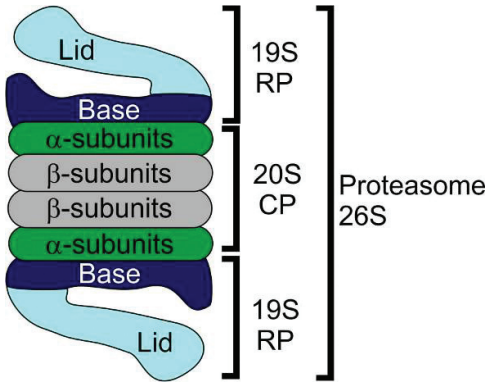


**Figure 3.** Modular structure of the proteasome. The complex contains a 20S core particle (CP) and one or two 19S regulatory particle (RP). The CP has a cylindrical shape and it is composed of four heptameric rings: two outer α-rings and two central β-rings. Three of the seven different subunits of the β rings, β1, β2 and β5 show the distinct enzymatic activities of the proteasome. The RP is divided into two subcomplexes: the lid and the base.

in both the cytoplasm and the nucleus of all eukaryotic cells (Adams 2003). The most prevalent form of the 26S proteasome is a huge multiprotein protease complex consisting of a 20S core particle (CP) that is associated with one or two 19S regulatory particles (RP) (Fig. 3). Each RPs contain 18 different subunits, half of them forming a base and the other half forming a lid subcomplex (Pickart and Cohen 2004). The base subcomplex consists of six ATPases and the polyubiquitin binding Rpn10 subunit, while the proteasome associated deubiquitylases reside in the lid. RPs are capable of binding polyubiquitin chains and removing them from the target protein. The substrate is then unfolded and translocated into the proteolytic chamber of the core particle. The core particle is a cylinder composed of four heptameric rings stacked on each other. The two outer or α-rings interact with the 19S regulatory particles, and form a narrow pore through which only denatured proteins can pass. The catalytic chamber is formed by the two inner β-rings, each of which contains three active sites. These sites differ in their substrate specificity and activity and have been named after enzymes that show similar proteolytic activity or specificity. These active sites are thus termed chymotrypsin-like, trypsin-like, and caspase-like. Proteins are degraded by the core particle in a progressive manner, generating peptides of 3-25 amino acids in length that can diffuse out through the other opening of the chamber.

## The role of ubiquitin-mediated protein degradation in cell cycle regulation

The physiological functions of the UPS are being discovered at an accelerating pace. One of the best known and most important intracellular processes regulated by the UPS is the cell cycle. The cell cycle is an ordered series of unidirectional events by which cells duplicate their content, including their genomic DNA and segregate it into two daughter cells. The cell cycle is regulated primarily by oscillating activities of cyclin-dependent kinases or CDKs. The CDKs, in turn, are regulated by a superimposed complex network of proteins that constitute the cell cycle checkpoints and the UPS. The irreversible nature and precise timing of ubiquitin-mediated degradation of regulatory proteins are the factors that enable key transitions in the cell cycle, and ensure its unidirectionality.

Although several hundred E3 ubiquitin ligases exist in eukaryotic cells, two of them have significant impact in cell cycle regulation: the anaphase promoting complex/cyclosome or APC/C and the Skp1-Cul1-F-box or SCF complex. Both of them are multisubunit enzymes, with similar scaffolding and catalytic modules but different substrate recognition and binding modules (Zachariae and Nasmyth 1999; Deák et al. 2003; Nakayama and Nakayama 2005; Pál et al. 2007). An intriguing feature of the APC/C, that despite having an intricate structure of 13-15 subunits, the complex interacts with two activators, Cdc20 and Cdh1, which interact with the APC/C and contribute to its substrate binding in a cell cycle stage-specific manner. The APC/C is active from prometaphase of mitosis till the end of G1, in which APC/$C^{Cdc20}$ triggers the metaphase-anaphase transition and sets up mitotic exit, APC/$C^{Cdh1}$ regulates mitotic exit and helps to maintain G1 (Vodermaier 2001). The SCF complex stays active throughout the cell cycle (Skaar and Pagano 2009).

Following the start of a new cell cycle, the activity of APC/$C^{Cdh1}$ maintains a stable G1, by ubiquitylating proteins involved in DNA replication, as well as S-phase and mitotic entry. This way proteins like Cdc6, Cdc25A, mitotic Cyclins A and B likewise mitotic kinases kept at low level throughout G1 (Li and Zhang 2009). Any remaining Cyclin-CDK complexes are kept inactive by CDK inhibitors, or CKIs. Toward the end of G1, several mechanisms ensure the gradual inactivation of APC/$C^{Cdh1}$, which is mediated by the ubiquitylation of the APC/C interacting E2 enzyme, UbcH10, and the phosphorylation and SCF-mediated degradation of Cdh1 (Rape and Kirschner 2004; Skaar and Pagano 2009). Stable inactivation of the APC/$C^{Cdh1}$ occurs at the G1-S transition, when the production of the Emi1 inhibitor is induced by the E2F transcription factor, and that lasts till the end of G2 (Hsu et al. 2002). In addition to the activation of the E2F-type transcription factors, the G1-S transition is marked by rising G1/S- and S-specific CDK activities, and inactivation of the Retinoblastoma protein by phosphorylation. In this period, only the SCF complex is active and targets CKIs, such as p21, p27 and p57 for degradation, thus facilitating cell cycle progression through the S and G2 phases and up till the M phase (Tsvetkov et al. 1999; Kamura et al. 2003).

During S phase, the Cyclin E- and CyclinA-Cdk2 complexes are active and direct DNA replication and the duplication of centrosomes. After completion of DNA replication, Cyclin E becomes phosphorylated and tagged for degradation by the SCF complex (Koepp et al. 2001). During G2 progression, the cells get ready for one of the most fateful phase of the cell cycle, mitosis.

The first part of mitosis is directed by the mitotic Cyclin B-Cdk1 complex. Although the Cyclin B-Cdk1 complex starts to accumulate in late G2, it is kept inactive by inhibitory phosphorylation by Wee1-type kinases (Potapova et al. 2011). At the G2-mitosis transition, the SCF complex facilitates mitotic entry by targeting Wee1 for ubiquitylation and fast proteasomal degradation. At the early phase of mitosis, the SCF complex also eliminates Emi1 and as a result, the APC/C becomes active (Margottin-Goguet et al. 2003), and from prometaphase, it becomes the key regulator of mitotic progression. First, the APC/$C^{Cdc20}$ triggers the metaphase-anaphase transition by ubiquitylating the separase inhibitor, securin, and cyclin B1 for proteasomal degradation. In the absence of securin, the protease separase cleaves the cohesin complex and allows sister chromatid separation to occur (Zur and Brandeis 2001). Cyclin B1 degradation results in lowering the activity of Cdk1, which further contributes to anaphase progression (Acquaviva and Pines 2006). Later in anaphase, Cdh1 binds to the APC/C, forming APC/$C^{Cdh1}$, and the two complexes together induce mitotic exit (Kramer et al. 2000) by completely inactivating Cdk1 through ubiquitin-mediated removal of Cyclin B. Following autoubiquitylation and APC/$C^{Cdh1}$-mediated ubiquitylation of Cdc20, only the APC/$C^{Cdh1}$ remains active in G1 (Reus et al. 2006; Foe et al. 2011).

## Ubiquitin beyond proteasome-mediated protein degradation

### *Mono and polyubiquitylation, variations in ubiquitin chain structure and functions*

In addition to protein degradation, ubiquitylation regulates protein function and localization and it is required for basic intracellular processes such as cell cycle control (as discussed above), transcription, DNA repair, apoptosis and several others. Ubiquitin modifications are critical for the formation of protein interaction networks and the activity of protein networks in space and time. Ubiquitin ligation can result in mono-, multi- or polyubiquitylated proteins (Fig. 4). Multiubiquitylation could be the sum of monoubiquitin modifications of the same protein at multiple sites, while polyubiquitylation is the conjugation of one polyubiquitin chain to a single site. Internal lysine residues within the ubiquitin polypeptide allow for chains of ubiquitin to be formed by progressive ubiquitylation events. Polyubiquitin chains may

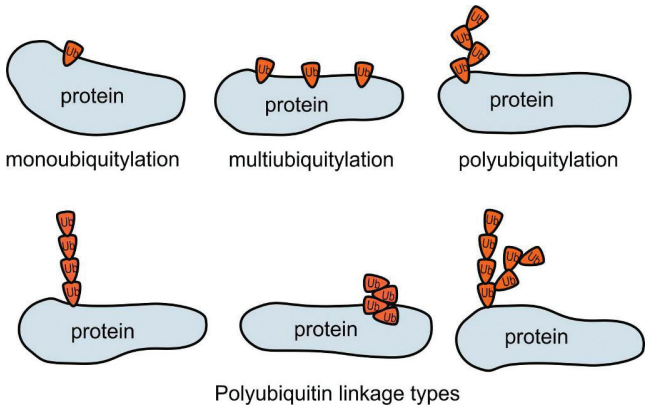


**Figure 4.** Ubiquitylation is variable in length and linkage type. Protein substrates can be monoubiquitylated with a single ubiquitin, multiubiquitylated with two or more monoubiquitins or polyubiquitylated. Polyubiquitins can be formed with different chain topologies – extended, closed or branched – depending on the lysine residues involved in the ubiquitin-ubiquitin linkages.

be constituted from a single or mixed linkage type and also from linkages that allow chain branching. Linear polyubiquitin chains can also be formed by peptide-bond formation between the carboxyl group of the C-terminal glycine of one ubiquitin and the amino-terminal alpha–amino group of the methionine of another ubiquitin (Iwai et al. 2014).

The several types of ubiquitin chains represent a three-dimensional code. Linkages through the different lysine residues diversify the position of neighboring ubiquitin molecules related to each other, thereby offering specific combinations of available surfaces and orientations. Consequently, linkage type and chain length are important parameters that can be interpreted by cellular machineries. Distinct chain types have been linked to specific steps of complex cellular processes; nonetheless the physiological roles of some types of ubiquitin chains are poorly understood. The most extensively studied function is targeting proteins for degradation through the recognition by the proteasome of Lys-48 linked polyubiquitin chains. Although, it is believed, that all chain linkages with the probable exception of Lys63, may contribute to the proteasomal turnover of proteins, other linkage types are involved in other processes: specifically Lys-11 in cell-cycle regulation and Lys-63 in DNA repair and intracellular signaling. Furthermore, for the efficient activation of retinoic acid inducible gene 1 three Lys-63-linked ubiquitins are required. According to a recent report, Lys-33 linked polyubiquitylation promotes post-Golgi transport (Yuan et al. 2014). On the other hand Met1-linked linear ubiquitin chains are involved in nuclear factor–kappaB (NFκB) signaling and in the regulation of tumor necrosis factor (TNF)-mediated apoptosis (Iwai et al. 2014). For further details on atypical ubiquitylation see Kulathu and Komander 2012.

The data above might well illustrate that ubiquitylation serves for a very complex signaling. A further increase in complexity can be achieved by the conversion of ubiquitin signals: a particular chain type can be removed partially and the remaining ubiquitin(s) targeted by another type of linkage(s). Indeed, ubiquitylation plays roles in most of the major cellular signaling pathways. One can view it as a specific code. It is also easily noticeable that this code has an integral quantitative feature as well: longer polyubiquitin chains can offer more binding sites thereby provide stronger signals as compared to shorter ones. The code is written by the ubiquitylating enzymes and erased or modified by the multitude of DUBs. Between these two classes of proteins act the readers of the code, those proteins which recognize the presence of mono and polyubiquitin signals via specific ubiquitin binding domains (UBDs). In the human genome there are at least 20 types of UBDs encoded within hundreds of proteins (Heride at al. 2014). The majority of UBDs recognize a hydrophobic patch in ubiquitin with Ile-44 in the middle. Since free ubiquitin is present in the cell in rather high concentration (10-20 μM range), the affinity of UBDs for monoubiquitin is necessarily low. The affinity for ubiquitin chains is higher, due to the multiplicative effect of more than one binding sites. Indeed frequently ubiquitin binding proteins contain more than one UBD. Associations between UBD-containing proteins also provide more versatility. The combinatorial use of UBDs plays important role in determining ubiquitin chain specificity. Linkage specific avidity can arise from the detection of the distance between the individual molecules of a ubiquitin chain and the orientation of these molecules. The DNA repair protein, Rap80 for example has tandem ubiquitin binding domains separated by seven amino-acid linker regions (Sims and Cohen 2009). This creates the ideal distance for association with ubiquitin molecules connected by a Lys-63 linkage. A shorter linker between the binding domains in ataxin-3 provides binding specificity for Lys-48 linkages. UBDs can be combined with various other domains and associated with multiprotein complexes thereby participating in versatile functions. With combination of DNA binding domains, UBD containing proteins and protein complexes can access chromatin to participate in specific functions as we will discuss in the next chapter. One role of the interaction of UBDs with ubiquitin chains might as well be to protect proteins from proteasomal degradation.

### *Ubiquitylation and chromatin functions: the roles of H2A and H2B ubiquitylation*

In eukaryotic cells octamers of four core histone proteins (H2A, H2B, H3 and H4) wrapped around twice by 146 bp of DNA form nucleosomes, the basic organization units of chromatin. From the arrays of nucleosomes highly ordered chromatin structures are formed through several levels of organization resulting in a high compaction of DNA in order to accommodate it in the restricted space of the nucleus. Interactions among chromatin constituents, however, restrict the accessibility of DNA to the cellular machinery, while on the other hand enable careful regulation of processes occurring on the DNA template. The accessibility is regulated through various mechanisms, including the posttranslational modifications (PTMs) of histones. Most of the histone PTMs take place at the N-terminal tails of core histones, however, in smaller numbers, modifications can occure close to the C-terminal end. Among these are modifications of histone H2A and H2B at lysine 119 (K119) and lysine 120 (K120), respectively (the numbering of lysines refers to their positions in histones of higher eukaryotes). In fact histones are the most abundant ubiquitin conjugates in eukaryotes and histone H2A was the first protein identified to be monoubiquitylated (Goldknopf et al. 1975). In respect of histone modifications, ubiquitylation constitutes a major modification event as 5-15% of H2A and 1-2% of H2B in mammalian cells is found in ubiquitylated form. (In yeast there is no detectable level of UbH2A.) The majority of UbH2A is in monoubiquitylated form, though polyubiquitylated H2A can also be detected. On H2B, only monoubiquitylation is detectable. Besides H2A and H2B histones H1, H2AZ, H3 and H4 have been also reported as substrates for ubiquitin modification but at lower levels than H2A and H2B (Jason et al. 2002; Zhang 2003; Wang et al. 2006).

Despite the fact that histone ubiquitylation was recognized a long time ago and that significant amounts of data have been collected indicating the role of these modifications in DNA repair and both up- and down-regulation of gene expression, a consensus on the role of the different histone ubiquitylations is still missing. There are several possibilities how ubiquitylation might affect chromatin functions. Indirectly, ubiquitin can promote transcription or other chromatin mediated processes by ensuring proteasome-mediated turnover of transcription factors or other regulators acting on DNA. More directly histone ubiquitylation may stabilize nucleosomes or affect higher-order chromatin structure, thereby affecting DNA accessibility. The ubiquitin is about half of the size of core histones, nonetheless nucleosomal particles with normal structure can be assembled when both H2A and H2B are replaced with their ubiquitylated counterparts (Davies and Lindsey 1994). K119 of H2A is in close proximity to the linker histone H1. It is therefore conceivable that ubiquitylation affects chromatin folding at the chromatin fiber level. H2B ubiquitylation has been reported to increase nucleosomal stability *in vivo*. This is consistent with the finding that levels of UbH2B correlate with genome-wide nucleosome occupancy. The enhancement of nucleosomal stability may restrict access of the transcriptional or replication machinery to the

DNA. Furthermore, histone H3 ubiquitylation at multiple sites has been demonstrated to play a role in nucleosome assembly (Han et al. 2013).

Ubiquitylated histones may also serve as signals for the recruitment of regulatory molecules that in turn affect transcription or repair. It was suggested that the dynamic ubiquitylation of H2B creates or destroys a binding site for a protein that acts as a reader of this particular modification. The reader may serve to recruit other proteins. Among these could be other chromatin modifiers. Indeed, UbH2B facilitates H3K4 and H3K79 methylation by Dot1 and Set1 methylases (Sun and Allis 2002). These marks are associated with active transcription. The role of UbH2B in transcription, however, is more than facilitating other histone modifications. It seems that both the addition and removal of ubiquitin are critical for the transcription process. UbH2B deubiquitylation is performed by the USP22 protein that is present in the SAGA complex (Zhang et al. 2008). Inhibition of transcription elongation reduces the global level of UbH2B. This suggests that active transcription is required for maintaining UbH2B. Based on the global distribution of UbH2B it was proposed that UbH2B would repress the assembly of the transcription machinery at gene promoters but favor transcription elongation by promoting nucleosome reassembly in the wake of PolII. This is in accord with an observation that UbH2B was found exclusively at active genes, at the highest level just downstream of the TSS and its level decreases towards the 3' end (Bonnet et al. 2014). The question, however, whether UbH2B in transcribed regions has a direct function in transcription elongation or whether this mark is a byproduct reflecting recent transcription remains to be resolved. The observation that blocking transcriptional elongation results in the loss of UbH2B from active genes, suggests that transcription itself maintains the dynamic state of H2B ubiquitylation. Replication stress also results in the gradual loss of global UbH2B. It is thus possible that DNA replication also maintains dynamic UbH2B. Based on these notions it was suggested that dynamic ubiquitylation results in a fluctuating chromatin environment necessary for transcription and replication. Accordingly, ubiquitylation of H2B may enable the recruitment or retention of the transcription or replication machinery at chromatin, while deubiquitylation is required to enable the machinery to detach and advance along the DNA template (Wright and Kao 2015).

On the contrary to the genome-wide distribution of UbH2B, suppression of H2B ubiquitylation appears to have a mild effect on gene expression. Nonetheless, histone H2B monoubiquitylation is an evolutionarily conserved marker of gene activation. This histone signal is found in association with the transcribed regions of all expressed genes. Several other functions for UbH2B have also been reported. These are related to mRNA processing and cell differentiation, but the data on the exact roles are controversial (Espinosa 2008). Deregulation of UbH2B seems to be involved in cancer development. This is suggested partly by the observation that USP22 that catalyzes the removal of the H2B ubiquitin mark was identified among an eleven-gene signature associated with poor cancer prognosis (Glinsky et al. 2005). The cancer connection is also supported by the fact that RNF20, the E3 enzyme involved in UbH2B formation acts as a tumor suppressor gene. Among others it positively regulates p53 as well (Kim et al. 2005). In mammals the major H2B E2 enzyme is RAD6, and the RING finger containing proteins RNF20 and RNF40 are the major H2B E3 enzymes. Other factors, however, also have roles in UbH2B formation.

H2A K119Ub has also been shown to play important roles in the regulation of essential genomic processes including transcription, cell-cycle progression, and DNA damage response. Importantly H2A ubiquitylation marks silenced genes. The evolutionarily conserved polycomb repressive protein complexes (PRCs) ensure epigenetic repression of gene activity partly through H2A K119 monoubiquitylation. In addition to PRC-mediated homeotic gene repression, X chromosome inactivation is also correlated with H2A K119Ub. The RING1B subunit that is the main E3 ligase responsible for H2A K119Ub formation is a PRC1 complex component (Wang et al. 2004; Scheuermann et al. 2012).

Recently, novel sites, K13 and K15 on the histone H2A and its variant H2AX were shown to be the target of polyubiquitylation during DNA damage response (Mattiroli et al. 2012). Response to DNA double stranded breaks (DSB) activates a signaling cascade involving phosphorylation, ubiquitylation and SUMOylation events. The process is initiated by ATM-mediated phosphorylation of the histone variant H2AX and results in the recruitment of key repair factors to the breaks. At least six different ubiquitin ligases feature in this cascade. The modification events at the site of a DSB provide specificity to the localization of relevant repair factors and contribute to fine-tuning the amplitude of the response. There is a positive feedback built into the system which operates through the waves of ubiquitylation mediated by ubiquitin-binding E3 complexes. This results in an amplification of the signal.

## Ubiquitin-like proteins and their functions

Ubiquitin is an evolutionary conserved prototype of a family of proteins that display remarkably similar structures, but variable sequences. As we mentioned above, the primary structure of ubiquitin differs only 3 of 76 positions between yeast and humans. Prokaryotes have no molecule that functionally analogous to ubiquitin, but the ubiquitin fold is present in enzymes which participate in cofactor insertion into enzymes (ThiS and MoaD). In eukaryotes the ubiquitin

family encompasses nearly 20 proteins that are involved in the posttranslational modifications of various macromolecules (van der Veen and Ploegh 2012). The ubiquitin-like proteins (ULPs) that are part of this family adopt the β-grasp fold that is characteristic of ubiquitin. ULPs are very little related in sequence to ubiquitin but most importantly they display a highly similar three-dimensional structure. ULPs regulate a diverse set of cellular processes, including nuclear transport, proteolysis, translation, autophagy, antiviral pathways and many more. Most ULPs are conjugated to proteins via an enzymatic cascade that resembles ubiquitylation. The common biochemical mechanism of the ULP-mediated signaling is an isopeptide bond formation between the terminal glycine of the modifier and an amino group of the target protein. The general mechanism of action is thus the following: conjugation factors select the target, the modification then changes the properties of the substrate mostly by affecting protein-protein interactions, finally downstream effectors recognize the modified substrate and transmit the signal of the modification. Intriguingly, a certain type of ULP modification often affects enzymes participating in the formation of a different type of ULP modification.

The closest in sequence to ubiquitin is NEDD8, they share 58% identity. NEDD stands for neural precursor cell expressed, developmentally downregulated. The origin of the name stems from the observation that NEDD8 is one of ten, highly expressed proteins in embryonic mouse brain. NEDD8, similarly to the other extensively studied ULP, small-ubiquitin-related modifier (SUMO), is universally distributed in eukaryotes. In most eukaryotes neddylation is an essential protein modification pathway. Neddylation follows the routine of ubiquitylation: the NEDD8 activating enzyme is NAE, the E2 enzymes are Ubc12 and Ube2F, and there are several known NEDD8 E3 ligases (Rabut and Peter 2008). Despite the homology between NEDD8 and ubiquitin, NAE and Ubc12 are strictly specific. Under certain conditions, however, (NEDD8 overexpression, low ubiquitin level,) atypical neddylation can happen by ubiquitylating enzymes. Neddylation is mostly mononeddylation, although polyneddylation can also take place. The function of the latter is unknown. Removal of NEDD8, the deneddylation, is done by zinc-dependent metalloenzymes (van der Veen and Ploegh 2012).

The best studied targets of neddylation are the cullin family proteins. These are scaffold proteins for the assembly of RING E3 ligase complexes (CRLs: cullin RING E3 ligases) that are involved in targeting substrates for proteasomal degradation. Many CRL targets are cell cycle regulators. Mononeddylation stimulates the ubiquitylation activity of CRLs (Watson et al. 2011). The tumorsuppressor p53 can also be neddylated. Several pathogens interfere with the neddylation pathway (*Chlamydia*, *Plasmodium*, *Escherichia coli*) (van der Veen and Ploegh 2012). Since the targets of neddylation, the cullin proteins are important cell cycle regulators; neddylation can be a target for controlling cancer cell proliferation. A selective inhibitor of NAE inhibits the growth of several tumors and, at present, it is in clinical trial (Soucy et al. 2009).

The small-ubiquitin-related modifier (SUMO) shows less sequence similarity to ubiquitin than NEDD8: human SUMO1 is only 20% identical to human ubiquitin. Although SUMO is also an evolutionarily conserved protein as the human SUMO is 52% identical to the yeast SUMO. In vertebrate genomes, there are four SUMO genes. The products of these differ in their cellular localization. The paralogs are specific to a subset of targets. Hundreds of SUMO substrates have been identified. Many of these contain a consensus acceptor site (ψKχE, where ψ is large hydrophobic and χ any amino acid residue), and can form polymeric K11-linked chains (Sampson et al. 2001). SUMO chains are recognized by the SUMO-interaction motifs (SIMs). These form a beta-strand that can be inserted between the alfa-helix and beta-strand of SUMO. Many proteins contain SUMO binding sites that conform to this consensus motif. SIMs are also present in several SUMO substrates, such as, the transcription factors promyelocytic leukemia protein (PML) and death domain associated protein (Daxx), and the base excision repair enzyme thymine DNA glycosylase (TDG) (van der Veen and Ploegh 2012). SIMs are also found in SUMO enzymes: SUMO E3 ligases (PIAS, RanBP2) and the SUMO E2 enzyme Ubc9. Tandem SIMs are found in the ubiquitin E3 ligase RNF4. Interactions between the four SIMs of RNF4 and SUMO are essential for the ubiquitylation and degradation of SUMOylated PML and poly(ADP-ribose) polymerase (PARP-1) (Tatham et al. 2008). The ubiquitin polymer can be linked directly to the substrate or to the poly SUMO chain. RNF4 was the first recognized SUMO-targeted ubiquitin ligase. It can regulate intracellular localization of proteins, as it is in the case of HTLV transcriptional regulator Tax (Fryrear et al. 2012). SUMOylation also regulates nucleo-cytoplasmic transport and cell cycle progression by modulating the localization and activity of the substrates. Furthermore, SUMOylation regulates nuclear organization, transcription, chromatin remodeling, DNA repair and ribosomal biogenesis. SUMOylation of a protein can modify its DNA-binding properties. SUMO exerts mostly but not exclusively a repressive effect on gene activity. This can be achieved for example by recruiting co-repressors such as histone deacetylases. In the context of DNA repair, ubiquitin and SUMO interplay with each other in several pathways, such as in double-strand break repair by homologous recombination, non-homologous end joining, nucleotide and base excision repair and the bypass of replication fork-stalling lesions during DNA replication (Ulrich 2014). Oxidative and hypoxic stress also results in increased SUMO conjugation. Several pathogens interact

with the host SUMOylation pathway. In many cases, however we still do not know the functional consequences of substrate modification by SUMO. The proteases which remove SUMO moieties are the sentrin-specific proteases (SENPs).

Interferon-stimulated gene 15 (ISG15) is one of the first ULPs identified. It is also called ubiquitin cross-reactive protein (UCRP). The linear diubiquitin analog ISG15 expression is induced by bacterial lipopolysaccharides (LPS), viral dsRNA and interferons alpha and beta (Jeon et al. 2010). ISGylation also involves an enzyme cascade resembling ubiquitylation. ISG15 is present in higher eukaryotes which produce interferons. ISG15 conjugation plays a role in normal development and interferon-mediated responses to viral infection. ISG targets are both cellular and viral proteins. ISG deficient mice are more susceptible to viral infection (van der Veen and Ploegh 2012). ISGylation also effects infection by influenza, HIV and ebola viruses. ISGylation of 10% of HPV capsid protein decreases the viral infectivity by 70%. ISGylation seems to be less target-specific than ubiquitylation and it is supposed that it takes place co-translationally (Durfee et al. 2010).

In addition to the ULPs discussed above, there are several less well characterized ULPs. Some of these are single domain proteins others are linear diubiquitin analogs like ISG15. Furthermore, new members of the ubiquitin family have just been discovered recently (Urm1 and Hub1) (van der Veen and Ploegh 2012). Some ULPs are found only in mammals and play roles in cytokine induced apoptosis. The function of others, however, remains unknown. It is interesting to note that the evolutionarily newer functions of some of these ULPs are in accord with their recent appearance in evolution.

## Medical significance of ubiquitylation

Ubiquitylation and ubiquitin mediated degradation of proteins are involved in many essential intracellular processes. Therefore, it is not surprising that inherited or acquired disruptions of the ubiquitin-proteasome system and/or the ubiquitin related protein-mediated signaling systems have been linked to many inherited and acquired human diseases. Most of these abnormalities can be linked to loss of function mutations, however, gain of function mutations were also identified. Concerning tumor development, depending on the nature of the substrate a ubiquitin ligase can act both as an oncogene and as a tumor suppressor. Therefore ubiquitin ligases are attractive drug targets as they are the specificity-conferring components in the ubiquitylation process. Increasing evidences suggest that there is a crosstalk between the ubiquitin family members. Examples can be seen for this in that the conjugation of SUMO to certain substrates can defend them against modification by ubiquitin. Or that NEDD8, the closest relative of ubiquitin modifies cullin E3 ligases and thereby stimulates these enzymes to become more active in the conjugation of ubiquitin to cognate substrates. Malfunction of several components of the ubiquitylation pathway are directly linked to pathological states. Here two of the UPS related diseases, a neurodegenerative disorder and a malignancy, are presented briefly.

Parkinson's disease is a progressive neurodegenerative disorder. It is characterized by tremor, locomotory difficulties and stiffened posture. The main neuronal phenotype seen in patients with Parkinson's disease is the loss of dopaminergic neurons in the substantia nigra. This results in a severe depletion of dopamine, and the appearance of characteristic eosinophil inclusions known as Lewy bodies in the degenerating dopaminergic neurons (Forno 1996). Evidence implicating a direct role for the UPS in Parkinson's disease came from the association of genetic mutations in the parkin gene with a special form of familial Parkinson's disease. Parkin is an E3 ubiquitin ligase that, in normal cells, tags specific proteins with polyubiquitin, targeting them for degradation in the proteasome. One of the proteins that Parkin normally ubiquitylates for destruction is α-synuclein, a neuronal protein with a not well understood function. Loss of function parkin mutations results in failure of proteasomal degradation of α-synuclein, and the subsequent increase in α-synuclein stability and concentration can promote aggregation of α-synuclein leading eventually to the death of dopaminergic neurons. A more complex involvement of the UPS in Parkinson's disease was further highlighted in case studies that revealed mutations in other components, such as the UCH-L1 deubiquitylating enzyme as causative agents (Leroy et al. 1998).

Von Hippel-Lindau syndrome is an inherited disease characterized by the formation of hemangioblastomas and retinal angiomatosis which are tumors of the blood vessels that can form in many different parts of the body (Neumann et al. 1995). These tumors can be either cancerous or noncancerous, but even in noncancerous forms, they can cause serious, sometimes life-threatening complications. The gene disrupted in Von Hippel-Lindau syndrome is a tumor suppressor gene that encodes a subunit of an E3 ubiquitin ligase termed the VHL-complex. The primary function of the VHL complex is to regulate the stability of the HIF (hypoxia inducible factor) transcriptional complex by targeting HIF subunits for ubiquitylation and rapid proteasomal degradation under normal oxygen levels (Gossage et al. 2015). At low oxygen concentration that is in hypoxic conditions, the HIF transcription factor is stabilized and upregulates the expression of several genes involved in cell proliferation, angiogenesis, and the production of red blood cells, such as vascular endothelial growth factor (VEGF) and transforming growth factor alpha (TGF-α) (Wizigmann-Voos et al. 1995). When

VHL function is lost, the VHL-complex cannot target the HIF subunits for degradation, and as a result, HIF can accumulate in cells. Excess HIF brings about abnormal cell proliferation and undesired production of new blood vessels. Rapid and uncontrolled cell division, along with the abnormal formation of new blood vessels, can lead to the development of tumors in patients with von Hippel-Lindau syndrome.

In accord with key functions ubiquitin and related protein modifications fulfill, drugs interfering with them are studied extensively to modulate these functions. The proteasome inhibitor bortezomib has been used to treat tens of thousands of cancer patients. MLN-4924, an inhibitor that blocks an E1 for the ubiquitin-like molecule NEDD8, is also very promising in clinical trials. Thalidomide binds to celebron a component of a cullin E3 ligase complex. It is used to treat multiple myeloma (Heride et al. 2014).

## Conclusions

Ubiquitin and ubiquitin-like modifiers are key components of very versatile mechanisms by which multitudes of protein functions, protein-protein interactions, intracellular protein localization, and protein turnover are regulated in eukaryotic cells. The basic features of the mechanism of protein modification by linkage to other protein(s) seem to be evolutionarily conserved. The numbers of used polypeptide moieties, the variations of linkage types and resulting polymer chain structures are increasing with evolution and newer types of signals serve more recent functions. Polypeptide chains as posttranslational signals offer great variability and specificity for the signaling pathways that use them and include features permitting qualitative responses and signal amplification. The importance of the ubiquitin-proteasome system and the central role of ubiquitylation in several aspects of cell life are well documented. Concerning the role of other related modifiers our knowledge is more limited. To accumulate further information on them is highly warranted as both the proteasome-mediated protein degradation and the ubiquitin and related proteins mediated signaling pathways offer plenty of specific targets for inventions in pathological cases.

## Acknowledgments

This work was supported by the TÁMOP-4.1.1.C-13/1/KONV-2014-0001 program entitled „Practice-oriented, student-friendly modernization of the biomedical education for strengthening the international competitiveness of the rural Hungarian universities".

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