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Chapter 8

Targeting the Ubiquitin Proteasome System in Cancer

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Abstract

The ubiquitin proteasome system is involved in a myriad of biological functions including cell cycle progression, intracellular signaling and protein degradation. As such, it is not surprising to find many components of the system misregulated in cancer. The clinical success of Bortezomib for treatment of multiple myeloma proves that targeting the ubiquitin proteasome system is valid and feasible. Here, a detailed examination of the strategies used to target the ubiquitin proteasome system in cancer is discussed. The inhibitors available, its targets, the cancer type and the developmental stage it is in are discussed.

Keywords: ubiquitin, proteasome, E1, E2, E3, ubiquitin proteasome system, cancer, deubiquitinase, DUBs inhibitors

1. Introduction

The function and activity of most proteins can be partially modulated by posttranslational modifications (PTMs). In particular, ubiquitination has emerged as one of the most versatile PTMs over the past few decades. Ubiquitination is a process that attaches ubiquitin, a short polypeptide of 76 amino acids, for its covalent link to proteins. It is a highly conserved process that mostly targets unwanted proteins for degradation either through proteasome-mediated or by directly sorting proteins to the lysosome and thus helps to maintain cellular homeostasis [1]. However, ubiquitination may also play a crucial role in other non-proteolytic regulatory functions such as protein activation, interaction, and translocation [2].

Ubiquitination is a multistep process and requires the sequential action of three enzymes, the E1 activating enzyme, E2 conjugating enzyme, and E3 ligase [3, 4] (**Figure 1**). The process of ubiquitin attachment commences when E1 recruits free ubiquitin in the cell through its active cysteine residue. The C-terminal glycine residue of ubiquitin is activated through ATP-dependent

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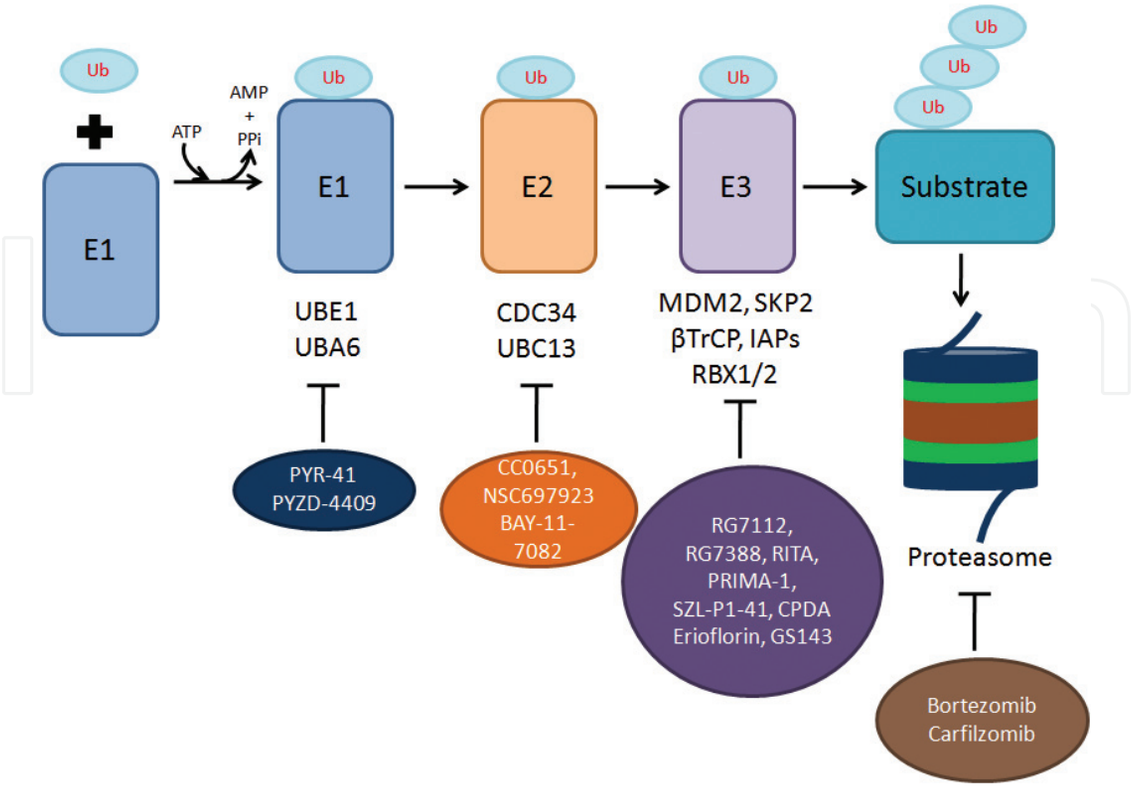


Figure 1. Schematic representation of ubiquitin conjugation cascade and inhibitors targeting specific cascade component.

adenylation and thioester bond formation catalysed by E1, resulting in attachment by non-covalent linkage to the E1 cysteine residue [5–7]. Activated ubiquitin is then transferred from E1 to a cysteine residue of the E2 conjugating enzyme linked through a thioester bond [4, 8].

The E3 ligases are responsible for substrate recognition and facilitates transfer of ubiquitin to the substrates from E2 resulting in covalent attachment of ubiquitin to the substrate's lysine residue [4, 9, 10]. The two major classes of E3s are the RING and HECT domain E3s which transfer ubiquitin through different mechanisms [9, 10]. HECT domain ligases accept ubiquitin from E2 through its catalytic cysteine residue and act as an intermediate entity capable of transferring ubiquitin to its recruited substrate [10]. RING domain ligases, instead of directly transferring ubiquitin, function as scaffolds and allow ubiquitin transfer from the E2 directly to the substrate [9]. In addition, other E3 classes such as ring-between-ring E3s are not discussed here [11].

Moreover, the ubiquitin molecule itself contains seven intrinsic lysine residues (K6, K11, K27, K29, K33, K48, and K63) and Met1 that can be further ubiquitinated allowing for the formation of various types of ubiquitin chains [12]. These come in the form of linear, branched, forked, homotypic, heterotypic kinds of monoubiquitin, multi-monoubiquitin, and polyubiquitin chain types. Each type could be associated with distinct cellular functions. For example, one of the best-known polyubiquitinations is K48-linked ubiquitination which acts as a degradation signal targeting substrate for proteasomal degradation [13].

The degradation of polyubiquitinated proteins is subsequently carried out in the 26S macromolecular proteasome complex which is present in both the cytosol and nucleus of eukaryotic

cells [14]. These complexes keep the proteins under quality checks and help cells to degrade misfolded/unwanted proteins. The proteasome is an approximately 2.5 MDa proteinase complex containing the catalytic active 20S core particle and the regulatory 19S particles [15, 16]. The 20S core particle is a barrel-shaped structure containing four stacked rings with two outer α-rings and two inner β-rings [17]. Each ring is composed of seven distinct α (α1–α7) or β (β1–β7) subunits [17]. The outer α-ring serves as the "gate" for entry of substrates, while the β-rings contain the catalytic activity. Namely, β1, β2, and β5 subunits confer the peptidyl-glutamyl-hydrolysing or caspase-like, the trypsin-like, and the chymotrypsin-like activity, respectively [17].

The 19S subunit can be separated into the "base" and "lid." The base contains ATPase subunits (RPT1–6) and four non-ATPase subunits (Rpn1, 2, 10, 13) [17]. The non-ATPase subunits are ubiquitin receptors that identify ubiquitinated substrates [17]. The lid contains nine subunits (Rpn3, 5–9, 11, 12, 15) and two proteasome-associated deubiquitinating enzymes (UCHL5/Uch37, Ubp6/Usp14) [17, 18]. Together with Rpn11/PSMD14, UCHL5/Uch37 and Ubp6/Usp14 carry out the deubiquitination of substrates before it moves on to the 20S core for degradation [17]. Although it is generally assumed that ubiquitinated proteins end up degraded by the proteasome, a recent review highlighted the strict requirements needed for proteasomal degradation wherein certain ubiquitinated substrates which do not meet these requirements escape from the proteasome and survive degradation [18].

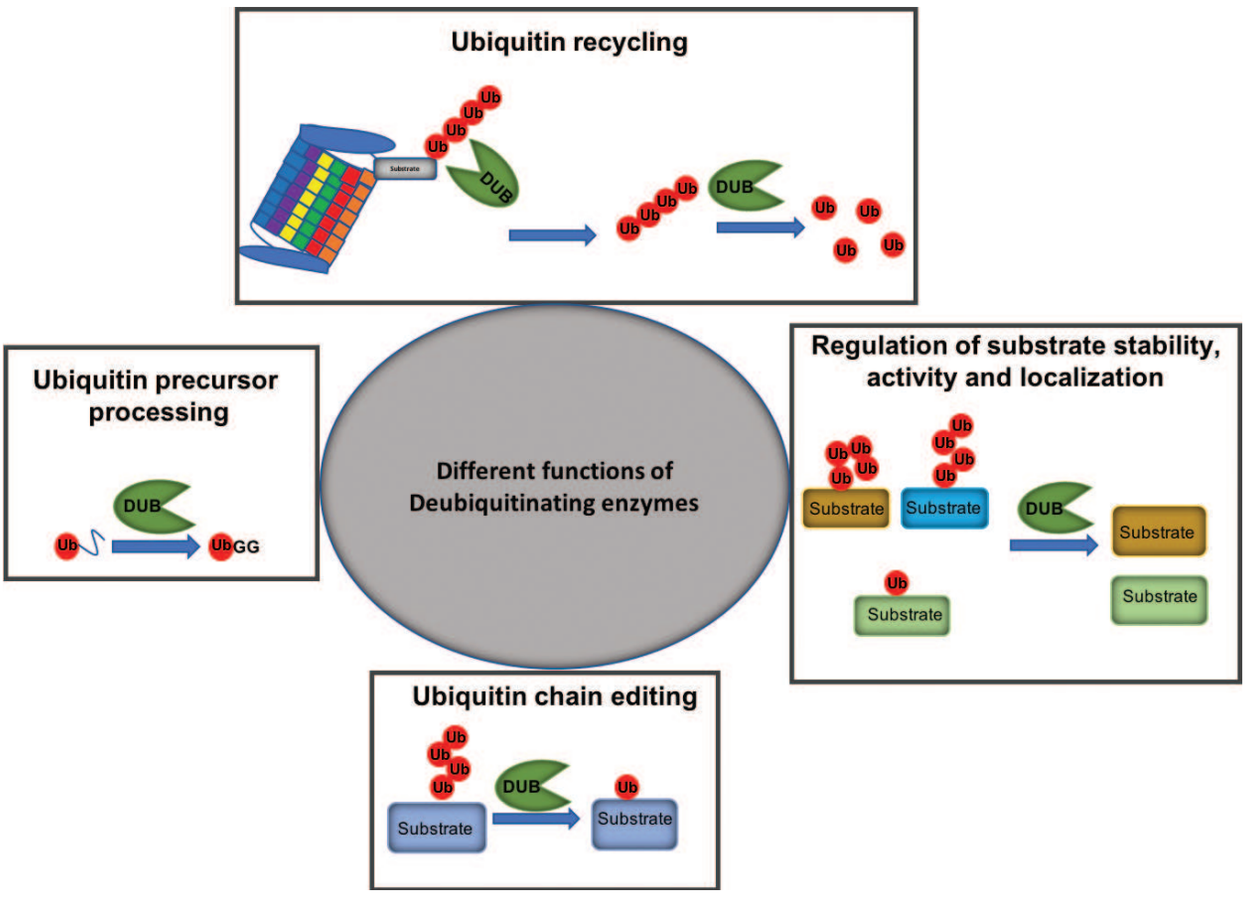


Figure 2. Pictorial representation for involvement of DUBs in different functions.

The ubiquitination process is antagonized by another set of enzymes that specifically removes ubiquitin moieties and counteracts ubiquitin-mediated function of a protein. These specific enzymes are called deubiquitinating enzymes (DUBs). As the name suggests, DUBs are responsible for cleaving the isopeptide bond between protein and ubiquitin. Other than regulating stability and function of its substrates, DUBs are also involved in ubiquitin precursor processing, ubiquitin recycling, and ubiquitin chain editing (**Figure 2**). By conducting the process of removing ubiquitin from its target, DUBs are mostly involved in opposing the effect of ubiquitination on substrates and thus leave a remarkable impact in the field of protein biology.

2. History of the ubiquitin proteasome system

The 2004 Nobel prize for Chemistry was awarded to Avram Hershko, Aaron Ciechanover, and Irwin Rose for the discovery of ubiquitin-mediated protein degradation [19]. Remarkably, the ubiquitin proteasome system (UPS) has been implicated in multiple cellular processes such as cell cycle, stress response, and DNA damage repair [20]. In 1978, Hershko and Ciechanover for the first time showed that ATP-dependent degradation required more than one component [21]. Using reticulocyte lysate and a DEAD cellulose column, they separated 2 fractions that individually do not catalyze ATP-dependent degradation but when combined, restored proteolysis [21]. Shortly after, the 2 fractions were identified. Fraction 1 contained ATP-dependent proteolysis factor 1 (APF-1) which was later identified to be ubiquitin [21–23]. Together with Irwin Rose, Aaron Ciechanover and Avram Hershko identified fraction 2 by further separating it into 2 other fractions containing a 450 kDa protein unknown at that time the proteasome, and the protease system containing E1, E2 and E3 enzymes [24]. It should be noted that prior to this, ubiquitin was first identified by Goldstein in 1975, as a universally present polypeptide, although its function was unknown at that time [25]. Prior to these findings, two reports in 1977 had characterized histone H2A covalently tagged with a single ubiquitin. Although not for degradation, the finding implied that ubiquitin could be used for tagging [26, 27]. Subsequently, a series of papers from the Nobel laureates characterized and defined the multi-step ubiquitin-tagging model for protein degradation through the E1, E2, and E3 enzymatic cascade [4–7, 28, 29]. Additionally, multiple ubiquitin could be tagged to a single molecule of lysozyme showcasing the first polyubiquitin chain [28].

Up till this point, the remaining piece of the puzzle was to identify the downstream protease(s) responsible for degradation of the tagged proteins. In order to characterize the protease(s) responsible, two large multi-subunit proteinase complexes were purified from reticulocytes [15, 16, 30]. One of which requires ATP to degrade the tagged protein (~1500 kDa), while the other is ATP independent (~700 kDa). It was later discovered that these were the 26S proteasome and the 20S core catalytic subunit of the proteasome, respectively [15, 31, 32]. Apart from the ATP-dependent E1 ubiquitin activation step, the process of degradation by the protease was also ATP dependent although the mechanism was unknown [33, 34]. This was resolved when it was found that the assembly of the 26S proteasome from the 20S catalytic core and 19S regulatory subcomplex is ATP dependent, explaining the reliance of energy for substrate degradation by the proteasome [31, 35].

The first papers to show a biological role for the ubiquitin cascade were in 1984 [36, 37]. In a mutant mouse cell line (ts85) that is conditionally lethal and temperature sensitive, monoubiqutinated H2A disappears at high temperatures, suggesting defects in the ubiquitin cascade [38, 39]. As it turns out, the E1 enzyme in ts85 was temperature sensitive, resulting in defects in ubiquitination at high temperatures [36, 37]. Additionally, the cells were arrested at G2 at higher temperature, indicating a role of the UPS in cell cycle regulation. These two papers set the stage for further discovery of biological roles played by the ubiquitin cascade in the coming years [20].

The first observation of deubiquitinating activity was in fact, in the very paper that the first scheme of ATP-dependent degradation was proposed [28]. Specifically, removing ATP from the ^{125}I-labeled ubiquitin-tagged lysozyme in the presence of endogenous proteins reversed the ubiquitin tagging, implying the presence of a DUB which the authors described as an amidase [28]. Subsequently, the first deubiquitination assay was developed and showed the deubiquitinating activity of mammalian ubiquitin C-terminal hydrolase L3 (UCHL3) and its yeast homolog Yuh1, which represented the first DUBs identified and characterized [40, 41]. The work of Varshavsky and colleagues ensued, identifying DUBs in yeast and up till today, there are a total of ~80 known DUBs [42, 43].

3. Ligases in cancer

Since ubiquitination occurs through a multi-step cascade, it can be inferred that multiple proteins along the cascade can be targeted. In particular, inhibitors targeting all three (E1, E2, and E3) classes of enzymes are utilized both in research and clinics. For an overview, the inhibitors that are about to be discussed in this section and the class of enzyme (E1, E2, and E3) which is targeted are summarized in **Figure 1**.

3.1. E1 enzymes

UBE1 and UBA6 are the only two E1 enzymes that are known in humans [44]. Till date, there are only two UBE1 inhibitors, PYR-41 and PYZD-4409 [45, 46]. Among the two, PYR-41 has been shown to inhibit the nuclear factor κB(NF-κB) pathway by regulating the stability of inhibitor of NF-κB (IκB). Additionally, it also prevents the degradation of the tumor suppressor p53 resulting in increased transcriptional activity of p53 [45]. On the other hand, PYZD-4409 was specifically shown to induce ER stress-induced apoptosis in cancer cells and, in a mouse model of leukemia, delayed tumor cell growth [46]. Although these results suggest the potential of targeting E1 in cancer treatment, none of these are currently in clinical trials, perhaps due to off-target effects or poor pharmacokinetic properties.

3.2. E2 enzymes

There are ~38 E2 enzymes in the human genome implying that they serve as more specific targets than E1 [8]. CC0651 is an allosteric inhibitor of CDC34, the common E2 enzyme for

Cullin ligase complexes. Treating cancer cells with CC0651 results in the accumulation of the tumor suppressor p27 and inhibition of proliferation, which suggests that CC0651 could be a potential inhibitor for clinical use [47]. However, development of this compound has met with great difficulties due to pharmacokinetic reasons [48]. Another potential target in cancer is the E2 enzyme UBC13-UEV1A, an important regulator of NF-κB pathway induction through the formation of ubiquitin K63-linked chains. The inhibitor NSC697923 has been shown to inhibit the formation of K63-linked chains by UBC13 *in vitro* and is effective in inhibiting the proliferation and survival of diffuse large B-cell lymphoma (DLBCL) [49]. BAY-11-7082 is a well-known inhibitor of the NF-κB pathway and has been thought to inhibit the IκB kinases [50]. However, it was found to inhibit the E2 UBC13 by preventing ubiquitin conjugation to it, thereby preventing K63-linked chain formation in the same way as NSC697923 [50]. Likewise, it was shown that BAY-11-7082 induces cell death to DLBCL HBL-1 cells [50]. Although E2 inhibitors show immense potential for cancer treatment, so far, E2 inhibitors are present only in preclinical stages.

3.3. E3 enzymes

Amongst enzymes in the ubiquitin conjugation cascade, E3s are the most abundant in number with ~700 ligases identified so far [48]. Due to the large number, targeting E3 will likely increase the specificity and decrease side effects. Due to space limitation, we will be discussing a few of the E3 ligases that are implicated in cancer and refer the readers to the following review about E3 ligases family [51].

3.3.1. Mouse double minute 2 homolog (MDM2)

Termed the guardian of the genome, p53 is frequently upregulated in stress conditions and functions to activate the expression of genes involved in apoptosis and cell cycle arrest to prevent cellular transformation [52]. MDM2 is an E3 ligase of p53 responsible for its degradation and is frequently upregulated in cancer [53, 54]. Thus, targeting MDM2 could be useful for cancer treatment. To this end, several MDM2 inhibitors are available. In particular, the Nutlin family of cis-imidazoline inhibitors shows the greatest potential [55]. One of the latest developed Nutlin inhibitors, RG7112, has been tested in phase I clinical trials and shows activity against relapsed leukemia [56]. Although it showed good clinical outcomes, a high dose was required and it caused gastrointestinal side effects [56, 57]. A more potent pyrrolidine-based MDM2 inhibitor, RG7388 is currently in clinical trial and might be able to overcome these issues [58]. In preclinical setting, RG7388 showed potent tumor inhibition specifically in p53 wild-type xenograft neuroblastoma indicating its possible use in neuroblastoma treatment where majority of tumors are p53 wild-type at diagnosis [59].

A majority of MDM2 inhibitors bind to MDM2 itself to prevent it from binding to p53. RITA (reactivation of p53 and induction of tumor cell apoptosis), however, binds to p53 and prevents MDM2 from interacting [60]. In this case, the mechanism of stabilization might be MDM2 independent and could possibly be used to treat MDM2-independent p53-destabilized cancers. Thus far, the mentioned MDM2 inhibitors aim to restore p53 levels. Given that p53 is known to be mutated in ~50% of all cancers, these therapies are severely limited to a subpopulation of p53 wild-type tumors [48, 61]. An ingenious way to overcome this is through the use

of drugs, which restore mutant p53 function. One example of such an approach is the drug PRIMA-1 which alkylates the thiol groups of mutant p53, correcting protein folding and enabling p53 to carry out its tumor suppressive function [62].

3.3.2. S-phase kinase associated protein 2 (SKP2)

SKP2 is a F-box protein which functions as the substrate recognition subunit of the SCF (SKP1/Cullin/F-box) RING E3 ligase complex [63]. In particular, its role in ubiquitinating and degrading cell cycle regulators, p27 and p21 makes it a potential target in cancer [64, 65]. Additionally, SKP2 is upregulated in several different cancers and serves as a prognostic marker for cancer patient survival [66–68]. Particularly, a structural pocket formed by SKP2 and its neighboring subunit CKS1 within the SCF complex is important for binding and degradation of p27. This outlines a potential vulnerability which could be targeted in cancer therapy. As such, using *in silico* screening to identify inhibitors for this structural pocket, four compounds were shown to increase p27 levels and arrest cells at G1 [69]. Another mechanism that could be utilized to inhibit SKP2 could be by targeting its association with the SCF complex through inhibiting SKP1-SKP2 binding. SZL-P1-41was identified to block SKP1-SKP2 interaction and shows strong antitumor effects against lung and prostate tumor xenograft in mouse models with concomitant increase of p27 [70]. Lastly, CPDA is another compound identified due to its ability to inhibit *in vitro* ubiquitination of p27 by SCF complex [71]. Although its mechanism is unknown, it has been shown to induce cell cycle arrest specifically in leukemic cells but not marrow components [71].

3.3.3. Beta-transducin repeat containing E3 ubiquitin protein ligase (βTrCP)

Like SKP2, βTrCP is a component of the SCF-Cullin E3 ligase complex. It utilizes its N-terminal F-box domain to bind to SKP1 and its C-terminal WD40 domain to bind to substrates including pro-caspase-3, IκB, p53, CDC25, and WEE1. In most cancers, it is upregulated and acts as an oncogene [72]. Erioflorin and GS143 are two βTrCP inhibitors that block the interaction of βTrCP with its targets, PDCD4 and IκB, respectively, leading to their stabilizations [73].

3.3.4. RING box protein 1/RING box protein 2 (RBX1/RBX2)

Both RBX1 and RBX2 are important subunits of the SCF complex and function to physically bring the activated E2 closer to the substrate for ubiquitination [74]. Increased expression of RBX1 is seen in breast, liver, kidney, and lung cancer indicating an oncogenic function [75]. An exception is in melanoma where RBX1 is higher in nevi than in melanomas [76]. Likewise, RBX2 is overexpressed in many human cancers and targets IκB, c-Jun, HIF-1α, and NF1 for degradation [75]. Although its precise mechanism in cancer progression is not well studied, depletion of RBX2 induces apoptosis, decreases tumor growth, and sensitizes cells to DNA damage [77, 78].

3.3.5. Inhibitor-of-apoptosis proteins (IAPs)

The IAPs are RING E3 ligases that inhibit caspases and thereby block apoptosis which makes them putative targets in cancer [79]. During apoptosis, the second mitochondria-derived activator of caspase (SMAC) is released from the mitochondria and binds IAPs which releases

caspases to perform their pro-apoptotic function. IAPs such as c-IAP1 and c-IAP2 are reported to have genomic amplifications in a variety of cancers like hepatocellular carcinoma, cervical, pancreatic and esophageal cancers. SMAC on the other hand gave a better prognosis in breast, colorectal, and bladder carcinomas [80]. Mimicking the SMAC binding region, a few inhibitors were shown to bind IAPs and activate apoptosis in cancer. These are currently in phase I clinical trials [80].

4. Ubiquitin proteasome system in virus-induced cancers

The idea of viral oncoproteins hijacking the cellular degradation system to degrade potential tumor suppressors is exemplified by the early papers showing human papillomavirus (HPV) oncoproteins E6 and E7 utilizing E6-associated protein (E6-AP) and Cullin 2 RING ligase to target p53 and retinoblastoma for degradation, respectively [81–85]. By developing small molecule inhibitors, targeting these ligases in virus-induced cancers holds great potential for cancer therapy. Additionally, depleting E6-AP expression has been shown to increase p53 protein levels and inhibit growth in HPV-positive cells [86]. So far, inhibitors identified that abalte E6-AP and E6 binding using binding assays have been disappointing as they show low efficacy in inducing cell death in culture [86–88]. It is suggested that more structural data are required in order to design better inhibitors [86]. On the other hand, the small molecule RITA mentioned earlier was shown to block the binding of E6 to p53 thereby preventing E6-mediated degradation degradation of p53 [89]. Cervical carcinoma xenografts showed substantial growth suppression when treated with RITA, suggesting its potential use in cervical cancer [89].

Another important tumor suppressor targeted by the HPV E6 protein is Tat-interactive protein 60 kDa (TIP60) [90]. In addition to HPV E6, adenovirus oncoproteins were also reported to target TIP60, implying an important tumor suppressive role played by TIP60 in virus-induced cancer [91]. The mechanism of degradation in HPV-positive cells involves the use of E3 identified by Differential Display (EDD1) to ubiquitinate and target TIP60 to the proteasome [92]. Importantly, overexpression of TIP60 or depletion of EDD1 in cervical cancer mouse xenografts inhibited tumor growth implying that EDD1 could be a novel target in cervical cancer therapy [92]. In addition, EDD1 is also upregulated in ovarian, breast, and pancreatic adenocarcinoma, as such an EDD1 inhibitor could be extended to these cancers [93].

Latent membrane protein 2A (LMP2A) is one of the 9 proteins expressed from Epstein-Barr virus transformed genome and is involved in viral latency and persistence [94]. In order to perform its function, it recruits neural precursor cell-expressed developmentally down-regulated 4-like (NEDD4-like) ligase to facilitate degradation of Lyn, a tyrosine kinase [94, 95]. This in turn blocks signal transduction of B-cell receptor. Although the mechanism is not completely understood, it increases our understanding of how cellular ligases are utilized at different stages of viral-induced cancers [95].

Apart from individual ligases utilized by viral proteins to degrade cellular substrates, there have been many cases reported where viral oncoproteins interact with the proteasome and hijacks it for their own purposes [95]. For example, through binding to the 20S proteasome and the NF-κB precursor p105, Tax, which is the human T-cell leukemia virus (HTLV) oncoprotein,

enhances the proteolytic activation of NF-κB, which sustains T-cell proliferation [95–97]. In particular, this represents a potential susceptibility using proteasome inhibitors in HTLV-infected T-cell leukemia treatment. Indeed, treatment with proteasome inhibitor Bortezomib was investigated in mouse models with mixed results [98, 99]. In HTLV-1-associated xenograft models, Bortezomib inhibited tumor growth and the mice showed prolonged survival [98, 99]. However, heterogeneity in response was seen between tumors treated with vehicle or Bortezomib derived from Tax transgenic mice [98]. More studies need to be conducted before proteasome inhibitors could be used for HTLV infected T-cell leukemia.

Hepatitis B virus X-antigen (HBX) is another oncoprotein known to interact with proteasome subunits PSMA7 and PSMC1 [100]. In the presence of HBX, two well-defined proteasomal substrates had increased half-lives suggesting that HBX can block proteasomal activity [100]. The importance of proteasome inhibition by HBX is shown in its ability to regulate HBV virus replication. Particularly in cells infected with mutated HBV not expressing HBX, proteasomal inhibitors MG132 and Epoxomicin were able to rescue virus replication back to wild-type levels supporting HBX's role in inhibiting proteasome [101, 102].

Apart from the proteasome, HBX also binds to cellular DDB1, a subunit of the Cullin 4 RING ligase (CRL4) complex [101]. Rather than being degraded by the CRL4 complex, it is stabilized and has been suggested to alter CRL4 specificity by displacing DDB1-CUL4-associated factors (DCAFs), which are proteins that confer substrate specificity to the CRL4 complex [103, 104]. Indeed, two recent papers identified structural maintenance of chromosomes 5/6 (SMC5/6) as novel degradation targets of the CRL4-HBX complex [105, 106]. Since SMC5/6 complex is essential for inhibiting the extrachromosomal HBV gene expression, identification of SMC5/6 as CRL4-HBX targets solves the long-standing question of how HBX-DDB1 interaction is important in HBV virus replication [101, 107, 108]. From these data, one plausible strategy would be to design inhibitors to block the interaction between HBX and DDB1 or the proteasome.

5. Families of deubiquitinating enzymes

There are approximately 80 functional DUBs known in humans [109]. These DUBs are mainly divided into six different classes based on their structure and active site homology: ubiquitin specific proteases (USPs), ubiquitin carboxyl-terminal hydrolases (UCHs), ovarian tumor proteases (OTUs), Machado-Joseph disease protein domain proteases (MJDs), JAMM/MPN (JAB1/MPN/MOV34 metalloenzyme) domain associated metallopeptidases (JAMMs), and monocyte chemotactic protein-induced protein (MCPIP) [110] (**Figure 3**). All DUB families belong to cysteine proteases with the exception of JAMMs family of DUBs, which are zinc-dependent metalloproteases. The mechanism of action for cysteine-dependent DUBs is through nucleophilic attack on the isopeptide linkage of an ubiquitinated lysine residue by the catalytic cysteine, which is facilitated by a nearby histidine side chain that helps to decrease the pKa of the cysteine. A third residue, aspartic acid or asparagine, helps in this whole process. This residue aligns and polarizes the catalytic histidine. Some enzymes which do not have this third residue use other means to polarize histidine [111, 112]. On the other hand, the mechanism of action for JAMMs which are metalloproteases is facilitated by two zinc ions which are present

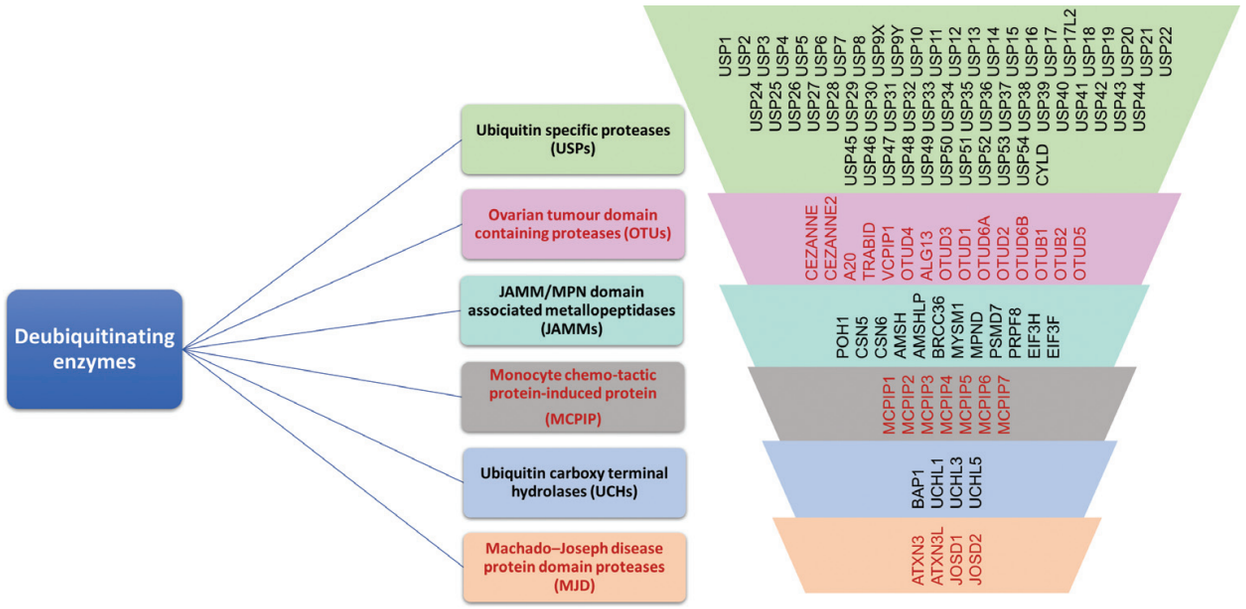


Figure 3. Schematic representation of different families of deubiquitinating enzymes and their members.

within its catalytic site and coordinated by invariant histidine, aspartic acid, and serine side chains [113]. This zinc ion activates a water molecule to form a hydroxide ion which in turn attacks the carboxyl carbon in the isopeptide link [114].

Out of the six classes of DUB families, the USP family is the largest with more than 50 members. These proteins belong to cysteine protease family (clan CA, family C19) [115]. USPs are characterized by the presence of a catalytic core involving histidine and cysteine boxes [116]. DUBs from the USP family contain a highly conserved USP domain characterized by three subdomains which form the palm, thumb, and fingers of a right hand [117]. The active site cysteine is present between the palm and thumb while the finger is used for interaction with ubiquitin. CYLD (cylindromatosis D) is the only USP which does not have the finger domain but possesses an additional domain known as B-box domain [118]. The presence of additional domain and terminal extensions has also been seen in several other USPs, which plays critical roles in conferring specificity to DUBs. For example, USP3, USP5, USP39, USP44, USP45, USP49, and USP51 have zinc finger USP domain, USP25 and USP37 contains ubiquitin-interacting motif, USP5 and USP13 possess ubiquitin-associated domain, USP4, USP11, USP15, USP20, USP33, and USP48 have the domain in USPs (DUSP), and USP52 has the exonuclease III domain. Moreover, several USPs such as USP4, USP7, USP14, USP32, USP47, and USP48 have the ubiquitin-like domain which can be found within and outside of the catalytic domain [115, 119].

UCHs are another family of DUBs, which contain four members in humans, UCHL-1, UCHL-3, UCHL-5, and BAP1. This class of DUBs was the first to be structurally characterized. In particular, UCHs have a short catalytic domain of approximately 200–300 amino acids [109] and can only target short peptide from the C-terminus of ubiquitin because of the presence of a confined loop which prevents polyubiquitin chain recognition and large protein processing. A well-studied member of the UCH class of DUBs is UCHL-1, which is one of the shortest DUBs, having only 223 amino acids [120]. UCHL-1 was initially known to be involved in ubiquitin

maturation by cleaving single amino acids or short peptides from the C-terminus of ubiquitin precursors to generate mono-ubiquitin rather than cleaving ubiquitin from proteins [121].

In UCHL-5 and BAP1, there is the presence of additional C-terminal extension of about 100 and 500 amino acids, respectively. The additional extension at the C-terminus of UCHL-5 directs it to proteasome and helps in trimming polyubiquitin chain from conjugated protein as they are degraded [122]. However, the additional extension of BAP1 contains a nuclear localization signal and helps it to interact with the N-terminal ring finger of BRCA1 (a ubiquitin ligase) [122, 123].

Due to space limitation, we have summarized the targets of different USPs and UCHs in cancer in **Table 1**.

DUBs family	DUBs	Important targets (direct/indirect)	Mechanism/pathway	Relevance to neoplasm
Ubiquitin specific proteases (USPs)	Cylindromatosis (CYLD)	TNFR-associated factor 2 (TRAF2) and TRAF6 [197]	Promotes apoptosis [198]; negatively regulates NFκB signaling [197]	Downregulated in lung cancer [199], liver cancer [200], colon cancer [200] and multiple myeloma [201]
	USP1	Fanconi anaemia complementation group D2 (FANCD2) [202]; proliferating cell nuclear antigen (PCNA) [203]	Involved in DNA repair and DNA-damage response pathways [202]	Overexpressed in hydatidiform mole [204]
	USP2	MDM2 [205], MDMX [206], Cyclin D1 [207]	Indirect regulation of tumor suppressor p53; increase cell proliferation [208]	Associated with bladder cancer and increase in proliferation, invasion and migration in bladder epithelial cells [209]; overexpressed in prostate cancer [210]
	USP4	TGFβRI [211]	Regulates TGFβ signaling pathway [211]; important player mediating crosstalk between TGFβ and PI3K signaling pathway [211]	Upregulated in human hepatocellular-carcinoma samples and has been suggested to induce aggressive phenotype [212]; downregulated in small cell lung cancer cell lines [213]
	USP5	p53 [214]	Inhibits accumulation of free unanchored polyubiquitin chains	
	USP7	p53 [187], PTEN [188], IRS1/2 [215], Chk1 [216], Claspin [217]	Regulates stability of p53 and MDM2 [218]; reported to induce IGF signaling [215]; modulates ATR-Chk1 pathway [216, 217]	Overexpressed in prostate cancer [188]
	USP8	EGFR [219]; ERBB2, ERBB3 and MET [220]	Regulates endosomal ubiquitin dynamics and required for RTK downregulation following internalization [221, 222]	Gain-of-function mutation in Cushing disease [223]; depletion of USP8 leads to selective death of Gefitinib resistant non-small cell lung carcinoma (NSCLs) cells [220, 224]
	USP9X	SMAD4 [225], β-catenin [226]	Regulates signaling pathway such as TGFβ [225] and MAPK pathway [227]	Overexpressed in breast cancer [228], ERG-positive prostate tumors [229] and osteosarcoma cell line SaOS2 [230] and its

DUBs family	DUBs	Important targets (direct/ indirect)	Mechanism/pathway	Relevance to neoplasm
				increased expression has been correlated with ill prognosis outcomes in multiple myeloma patients [231] and esophageal squamous cell carcinoma [232]
	USP10	p53 [233], T-box transcriptional factor (T-bet) [234]	Regulates ATM-p53 and mismatch repair (MMR) [235, 236]	Overexpressed in breast cancer [228], glioblastoma [237] and in metastatic melanoma [147]
	USP11	TGFβRII [238], p53 [239]	Regulates TGFβ signaling pathway [240] and BRCA2 mediated damage response [241]	High expression of USP11 has been observed in murine lung tissue [238]
	USP12	Androgen receptor (AR) [242]		Suggested as a putative regulator of progression and metastasis in prostate cancer [242]
	USP13	PTEN [243]	Implicated in PI3K signaling	Important for melanoma growth in soft agar assay and nude mice [244]
	USP15	TGFβRI [245], E6 (human papilloma virus (HPV) protein) [246]	Enhances TGFβ signaling pathway [245]	Overexpressed in glioblastoma, ovarian and breast cancer [245, 247]
	USP16	Histone H2A	Regulates progression of cell cycle and gene expression [248]	Regulates stem cell self-renewal and pathologies associated with Down syndrome [249]
	USP17	Ras-converting enzyme 1 (RCE1) [250]	Important for chemotaxis and chemokinesis, and have a crucial role in cell migration [251]	USP17 is amplified in tumors and found to regulate G1/S cell cycle advancement and proliferation [252]
	USP18	EGFR [253]	Involved in interferon signaling [254]	Implicated in regulation of viral disease and malignancies; identified as anticancer target in acute promyelocytic leukemia (APL) [254, 255]
	USP19	KPC (Kip1 ubiquitination-promoting complex) [256]	Regulates cell growth [256]	Putative target for inhibiting proliferation [256]
	USP20	HIF1α [257], Claspin [258]	Promotes transcription of hypoxic response genes [257]	Decreased expression in gastric cancer cells and negative correlation with tumor size and tumor invasion [258]
	USP21	GATA3 [259], Histone H2A [260], EZH2 [261]	Activates transcription [260]	Upregulated in bladder carcinoma [261], breast carcinoma [262, 263] and cancer stem-like cells (CSCs) of renal cell carcinoma cell lines [263] and its expression was correlated with tumorigenic behavior of cells such as tumor size, proliferation, metastasis and invasion
	USP22	Histones H2A and H2B [264]; c-MYC [265]	Regulates epigenetic modulations that support neoplastic change [264, 266]; involved in regulation of various	Elevated expression of USP22 has been reported to be associated with ill prognosis of several cancer like breast [268],

DUBs family	DUBs	Important targets (direct/ indirect)	Mechanism/pathway	Relevance to neoplasm
			tumor associated processes such as cell cycle, proliferation, and apoptosis [267]	colorectal [269] and esophageal squamous cell carcinoma [270]
	USP25	Tankyrases (TNKS1 and TNKS2) [271]	Regulates Wnt signaling pathway [271]	The upregulated mRNA and protein level of USP25 was observed in NSCLC patients which was linked to metastasis [272]
	USP28	c-MYC [273, 274], Chk2 [275], LSD1 (lysine-specificdemethylase1) [276]	Involved in DNA damage response [274, 275]	Somatic mutation has been observed in case of lobular breast cancer [277]; overexpressed in colon [278] and breast cancer [273]
	USP29	p53 [279], Claspin [280]	Involved in regulation of p53 and ATR-Chk1 pathway	
	USP30			
	USP33	Interact with Robol [281]; CP110 (centriolar protein) [169]	Required for Slit signaling [281]; involved in regulation of centrosome duplication and genomic stability [169]	Overexpressed in pediatric acute lymphoblastic leukemia [282]
	USP34	RNF168 [283], AXIN [284]	Regulate genome stability [283]; positively regulates Wnt signaling pathway [284]	
	USP42	p53 [285]	Supports "protect and repair function" of p53 without altering its basal level [285]	
	USP44	Mad2-Cdc20 [286], H2B [287]	Regulates mitotic spindle checkpoint [286]	Overexpression in human T-cell-leukemia [288]; defects in chromatin segregation has been observed with USP44 depletion [286]
	USP47	Polβ [289]	Regulates base excision repair (BER) [289]	USP47 is suggested to be possible therapeutic target as USP47 depletion upregulated level of Cdc25A and decreased cell survival [290]
	USP50	Cdc25B Wee1 [291]	DNA damage response signaling pathway [292]	
Ubiquitin C-terminal hydrolases (UCHs)	UCHL-1	p53 [293]	Involved in ubiquitin maturation [294] and activation of AKT signaling pathway [295]	Linked to several types of cancer including Breast [296], lung [297], colorectal [298] and pancreatic [299]
	BRCA1-associated protein1 (BAP1)	Host cell factor 1 (HCF-1) [300]	Participate in epigenetic regulation in tumor and regulation of histone stability [301]	Mutated in melanoma [302] and implicated in lung and breast cancer [123]
	UCHL-5	Smad2 and Smad3 [303]; NFRKB [304]	Promotes TGFβ signaling [303]; regulates DNA double-strand breaks (DSBs) resection and repair by homologous recombination [304]	Overexpressed in epithelial ovarian cancer [63] and hepatocellular carcinoma [175]

Table 1. USP and UCH family of DUBs, their targets and relevance in cancer.

Ovarian tumor (OTU) represents a superfamily of proteins which are characterized by the presence of an ovarian tumor domain (OTUD) [124]. This domain was first described in the ovarian tumor gene in fruit flies which is involved in the development of ovaries [125]. In 2003, some members of the OTU superfamily were identified to have active cysteine protease site and were described as deubiquitinating enzymes [126]. Based on its characteristics, this class is further subdivided into four groups: Otubains, A20-like OTUs, OTUDs, and OTULIN like OTUs [127]. According to recent studies, the OTU core domain is suggested to consist of five β-strands placed between two αhelical domains. The helical domains vary in sizes among OTU DUBs [128–130]. Like USPs, OTU members also possess additional domains. For example, A20 has A20-type Zn fingers, TRABID has NP14-type Zn fingers, OTUD1 and OTUD5 have ubiquitin-interacting motif, and CEZANNE contains ubiquitin-associated domain [118]. In humans, there are 14 DUBs which belong to the OTU family of DUBs [124]. These DUBs are able to cleave different linkages of ubiquitin chains. For example, OTUB1 and A20 specifically remove K48-linked ubiquitin chains, CEZANNE is specific for K11-linked chains, and TRABID cleaves K29- and K33-linked chains [131].

OTUB1 has a crucial role in DNA damage repair through regulating the RNF8/168 pathway. Recently, OTUB1 is reported to be overexpressed in non-small-cell lung carcinoma (NSCLC) and promotes RAS activation by inhibiting RAS monoubiquitination [132]. Moreover, high expression of OTUD1 is also seen in thyroid carcinoma signifying its oncogenic nature [133]. On the other hand, OTUD5 is linked to apoptosis and is involved in stabilization and activation of p53, suggesting a possible tumor suppressive role [134].

A20 and CEZZANE take part in the negative regulation of NFκB signaling, whereas TRABID positively regulates Wnt signaling pathway [135–137]. A20 is unique and known to have activity of both an E3 ligase and a DUB [138]. A20 cleaves K63-linked ubiquitin chains from RIP1 (receptor interacting protein 1) and negatively regulates the NFκB pathway [137]. A20 genes have been reported to be mutated/deleted in lymphoma suggesting it to be a tumor suppressor [139]. On the other hand, increased A20 is associated with poor outcome in glioma patients [140] and Tamoxifen resistance in breast cancer [141]. Overall, the widespread involvement of these DUBs in a variety of tumorigenic processes makes them potential targets for cancer treatment.

The Josephin family of DUBs is named after a neurodegenerative disease known as Machado-Joseph disease. Particularly, genetic mutations of ATXN3, a member of MJD class of DUBs, are linked to the cause of Machado-Joseph disease [142]. There are four DUBs in humans that form the MJD class: Josephin domain-containing protein 1 (JOSD1), JOSD2, ATXN3-like and ATXN3. ATXN3 can cleave both K48- and K63-linked chains with a higher preference for K63 chains. ATXN3 controls protein folding and stability by editing polyubiquitin chains [143]. The other three members of the Josephin family (JOSD1, JOSD2, and ATXN3L) have highly conserved catalytic triad formed by one cysteine and two histidine residues. An additional domain such as ubiquitin-interacting motif has been identified in ATXN3 and ATXN3L, indicating probable interaction between two distal ubiquitins in a polymer [144]. It has been reported that all Josephin family DUBs especially ATXN3 inhibits PTEN transcription in lung cancer and inhibition of these DUBs induces PTEN expression [145]. In light of these observations, ATXN3 could be a putative target for PTEN repressed tumors. JOSD1 is a membranous DUB and is involved in

regulating membrane dynamics and endocytosis [146]. Moreover, JOSD1 was found to be significantly overexpressed in NSCLCs but its function remains to be elucidated [147].

As mentioned earlier, the JAMM family of DUBs has zinc metalloprotease activity. The crystal structure of AMSH-LP (associated molecule with SH3 domain-like proteases), a DUB from the JAMM family, bound to K63-linked diubiquitin, assisted the understanding of catalytic mechanism of JAMM family [148]. The members of the AMSH family are involved in specific removal of K63-linked polyubiquitin chains and regulate vesicle trafficking and receptor recycling. The domain of AMSH-LP consists of a JAMM core and two conserved insertions. JAMM proteases which do not have AMSH-specific inserts show no specificity for K63-linked polyubiquitin.

There are 12 JAMM proteins along with AMSH-LP that are encoded by human genome. Seven out of the 12 JAMM proteins have isopeptidase activity for ubiquitin or ubiquitin-like proteins while the rest are catalytically inactive. The JAMM proteins with isopeptidase activities are: AMSH-LP, AMSH/STAMBP, BRCC36 (BRCA1/BRCA2-containing complex subunit 36), POH1/PSMD14 (26S proteasome-associated PAD1 homolog 1), MYSM1 (Myb-like with SWIRM and MPN domains 1), MPND (MPN domain-containing protein), and CSN5/JAB1 (COP9 signalosome subunit 5). The high degree of similarity between POH1, AMSH, and AMSH-LP sequences indicates a common mechanism for ubiquitin recognition and catalysis for these JAMMs [148].

BRCC36 belongs to the JAMM class of DUBs whose overexpression has been observed in breast cancer cell lines and tumors [149, 150]. EIF3H and COP6S are other examples of DUBs that belong to JAMM class. COP6S is amplified in breast cancer [151] and EIF3H is amplified in breast and prostate cancer [152].

MCPIP1 proteins possess a domain with deubiquitinating activity which suggests the presence of a sixth family of DUBs in the human genome [153]. This family is suggested to have seven members according to bioinformatics analysis of a recent study [110]. The interaction of MCPIP1, which is the founding member of this family with ubiquitinated proteins, is carried out by ubiquitin-associated domain placed at the N-terminus. However, this domain is not essential for its DUB activity. The other domains of MCPIP1 proteins include N-terminal conserved region, a conserved CCCH-type zinc-finger domain in the middle region of the protein, and a proline-rich domain at its C terminus. The domains that are required for activity of the MCPIP1 proteins are the N-terminal conserved region and zinc finger. In addition, similar to cysteine proteases, the catalytic domain of MCPIP1 also consists of cysteine and aspartic acid boxes but lacks histidine in the catalytic core. However, possibility of histidine outside the core cannot be ruled out [153].

6. Targeting proteasome in cancer

The 26S proteasome is a 2.4 MDa multi-subunit complex responsible for the degradation of intracellular proteins [154]. Currently, there are two FDA-approved proteasome inhibitors

namely Bortezomib (Velcade) and the more potent Carfilzomib (Kyprolis) (**Figure 1**). The FDA initially approved Bortezomib in 2003 for relapsed multiple myeloma (MM) patients [155]. Now, its use has been extended to new MM patients as well as for the treatment of mantle cell lymphoma [156]. Generally, there are three well-accepted models. These are NF-κB inhibition through stabilization of IκB, activation of the unfolded protein response by proteasome inhibition due to high endoplasmic reticulum (ER) stress, and stabilization of pro-apoptotic proteins such as BAX and NOXA [48, 155, 157, 158]. Carfilzomib was approved by FDA in 2012 for relapsed and refractory MM patients, who had previously been treated with Bortezomib [156, 157]. It binds irreversibly to proteasome and inhibits its function by up to 80% resulting in nonfunctional proteasomes as such, it is used for Bortezomib-resistant MM patients [48].

6.1. Proteasome associated DUBs as therapeutic target

Although Bortezomib and Carfilzomib have shown great promise in the clinic [159, 160], it also exhibits side effects [161]. As such, targeting proteasome-associated DUBs might present better specificity by minimizing off-target toxicity attributed to inhibiting the entire proteasome complex. These DUBs play two critical roles in the UPS system. First, by cleaving the attached ubiquitin molecules, it promotes the entry of polyubiquitinated substrate to the 20S catalytic portion of the proteasome. Second, the cleaved ubiquitin would then be available to be recycled as free ubiquitin [162]. Considering the fact that DUBs are intrinsic part of ubiquitin-proteasome system and majority of cancers demonstrate altered expression of DUBs which might drive a number of cancer-associated pathways, targeting DUBs may be considered as a reasonable approach for regulating UPS and is current area of research. There are three DUBs which are associated with the proteasome: PSMD14 (or POH1), USP14, and UCHL5.

6.1.1. PSMD14

PSMD14 is a JAMM metalloprotease. Other than recycling ubiquitin, it is also essential for the structure and function of the 26S proteasome [163]. The importance of PSMD14 in cancer is seen in MM, where its level has been shown to be negatively correlated with the overall patient survival [164]. Depletion of PSMD14 showed decrease in cell viability in multiple myeloma cells. Moreover, upregulation of nuclear PSMD14 is reported in hepatocellular carcinoma and correlates with E2F transcription factor 1 (E2F1) expression and cancer prognosis [165]. PSMD14 is also known to deubiquitinate and modulate the stability of ERBB2 [166]. In addition, PSMD14 has been reported to promote cellular responses to DNA double-strand breaks through homologous recombination. In light of these observations, targeting PSMD14 could lead to better therapeutics in cancer patients.

6.1.2. USP14

Another DUB which is important for ubiquitin recycling is USP14 and has been shown to be involved in delaying protein breakdown by the proteasome and thus, inhibits proteasome activity [167]. USP14 perhaps does so by preventing deubiquitination of proteasome substrate by PSMD14. Although USP14 depletion in mammalian cells has no detectable effect on the accumulation of polyubiquitin [122], it has been shown to inhibit proteasome through its deubiquitinating

activity. USP14 also assists substrate degradation by increasing 20S gate opening [168]. USP14 is overexpressed in NSCLC [169] and in ovarian cancer cells [170]. The expression of USP14 in NSCLC is associated with poor overall survival of patients and tumor cell proliferation, which further strengthens the evidence of USP14 as a tumor-promoting factor in NSCLCs, and a promising therapeutic target. Moreover, USP14 expression in colorectal cancer has been found to be associated with liver and lymph node metastases [171]. It is also implicated in several important signaling pathways [172, 173]. The small molecule inhibitor of USP14, IU1 was shown to stimulate proteasome degradation, further proving its role in proteasome inhibition. This inhibitor specifically binds and inhibits proteasome-bound USP14 [167].

6.1.3. UCHL5

Similar to USP14, UCHL5 is involved in removing ubiquitin from the distal tip of polyubiquitin chains. However, in contrast to USP14, UCHL5 can only release mono-ubiquitin [174]. Clinically, UCHL5 is overexpressed in epithelial ovarian cancer [63] and hepatocellular carcinoma [175]. It has been shown to be associated with poor clinical outcomes in epithelial ovarian cancer [63] and promotes cell migration and invasion in hepatocellular carcinoma [175], implying that it could be a novel predictor of hepatocellular carcinoma reoccurrence. A small molecule compound WP1130 has been shown to inhibit UCHL5 and is expected to functionally block proteasome [176].

Another small molecule compound b-AP15, which was initially identified in cell-based screen, was found to increase the accumulation of polyubiquitin in the cells. Later b-AP15 was identified as an inhibitor of USP14 and UCHL5 [177]. Utilized in solid tumor and MM, b-AP15 showed considerable anti-cancerous effect in animal models. Thus, inhibiting these DUBs by b-AP15, IU1 or related inhibitor may be of therapeutic benefits.

7. DUBs inhibitors

By regulating ubiquitin homeostasis, DUBs have been implicated in tumorigenesis as both its overexpression or loss may drive oncogenesis. Hence, it is not surprising that deregulation of DUBs can lead to severe pathological conditions. To target DUBs, a number of inhibitors either specific for a single DUB or pan-enzyme inhibitors have been identified and are currently explored for its risk-free use in patients. Another approach to target DUBs would be to identify an antagonist that can bind to the DUB's substrate for cancer therapy. DUBs show a great degree of substrate specificity and have a well-defined active site such as the catalytic cysteine which makes DUBs attractive targets for small molecule drug discovery. The active site catalytic cysteine of DUBs is very reactive toward electrophiles. A majority of the DUBs inhibitors are compounds with Michael acceptors such as α, β-unsaturated ketones which are capable of forming covalent adducts with free thiols of nucleophilic cysteine which in turn blocks the DUBs activity [178]. A diverse number of compounds ranging from synthetic small molecules to natural compounds with inhibitory properties for DUBs have been identified and studied. Several strategies can be used to target DUBs and we have summarized them in **Figure 4** and **Table 2**.

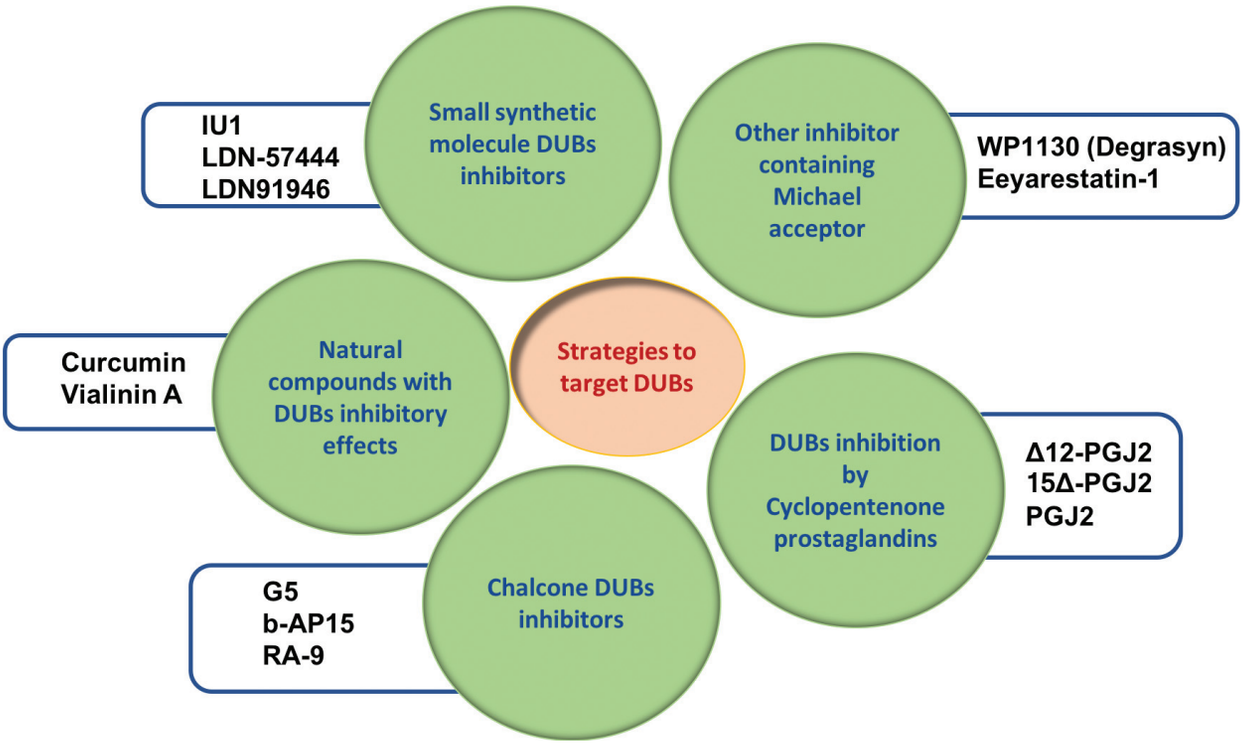


Figure 4. Pictorial representation of different strategies to target DUBs.

7.1. Cyclopentenone prostaglandins

The induction of polyubiquitinated proteins in cells by prostaglandins of the PGJ2 class was first reported by Fitzpatrick and coworkers [179]. DUB activity was shown to be inhibited by prostaglandin PGJ2 which contains α, β-unsaturated ketones. PGJ2 is then further metabolized to Δ12-PGJ2 and 15Δ-PGJ2 [179, 180] which show inhibitory effect toward UCHL3 and UCHL1, respectively [167].

7.2. Chalcone compound with DUB inhibitory effect

A chalcone is an aromatic ketone and an enone that is centrally essential for a broad range of biological compounds. These compounds have cross-conjugated α, β-unsaturated ketones and accessible β-carbons that are important for inhibiting DUBs [181]. These compounds act as either relatively specific or broad-spectrum inhibitors. For example, b-AP15 and its analogue VLX1570 are relatively specific to USP14 and UCH37, whereas another chalcone compound G5 possesses broad inhibitory effect [182, 183].

7.3. Other DUB inhibitors containing Michael acceptors

A small molecule, WP1130, which was derived from a compound with inhibitory activity for Janus-activated kinase 2 (JAK2) kinase was reported to selectively inhibit the activity of USP5 along with USP9X, USP14, and UCH37 [176].

S. no.	Inhibitor	Target (DUB)	Major attributes	Developmental stage
1.	LDN-57444 [195]	UCHL-1	A potent active site directed inhibitor for UCHL-1 [195]; cell permeable inhibitor; decreases proteasome activity	Preclinical
2.	LDN-91946 [196]	UCHL-1	Is able to inhibit UCHL-1 in a noncompetitive manner [196]	Preclinical
3.	15Δ-PGJ2	UCHL-1	Is a metabolite of prostaglandin, PGJ2 that was identified to retain inhibitory effects towards UCHL-1 by affecting overall structure and thus activity [305, 306]	
4.	AM146, RA-9, and RA-14 [307]	UCHL-1	Are chalcones which act as partially selective DUBs inhibitor and can inhibit UCHL-1 activity [307]	
5.	LS1 [308]	UCHL-3	Inhibits UCHL-3, identified in FRET-based screen [309]	
6.	NSC112200 and NSC267309 [310]	TRABID	Inhibited the growth of colorectal tumor cell lines HCT-116 and SW480 [310]	
7.	b-AP15 [177]	UCHL-5 and USP14	Anti-cancerous effect against solid tumor and multiple myeloma *in vivo*	
8.	WP-1130 [176]	USP9X, USP5, USP14, UCH37, UCHL-5	A small molecule, WP1130 serves as a pan DUBs inhibitor which was derived from AG490 (JAK2 inhibitor) and reported to inhibit activity of several DUBs [176]; elicits apoptosis of tumor cells	Preclinical
9.	Pimozide [311] and ML323 [312]	USP1	Works by blocking complex formation between USP1-UAF1, which in turn inhibits USP1activity. ML323 and related N-benzyl1-2-phenylpyrimidine-4-amine derivatives shows higher selectivity and inhibitory potency towards USP1/UAF1 than Pimozide [312]	Preclinical
10.	ML364 [313]	USP2	Is a small molecule inhibitor, which has been identified to enhance Cyclin D1 degradation in colorectal cancer and lymphoma model [313]	
11.	Vialinin A [185]	USP4 and USP5	A natural compound isolated from Chinese mushroom *Thelephoravialis* and has been shown to inhibit enzymatic activity of USP4 and USP5 [185, 186]	Preclinical
12.	P5091 [191]	USP7	Selective inhibitor of USP7, triggers apoptosis in multiple myeloma cells [191]	Preclinical
13.	P22077 [192]	USP7 and USP47	A specific inhibitor of USP7 identified by Progenra [190]	Preclinical
14.	Cpd14 [192]	USP7 and USP47	Resulted in increase in p53 and induction of p21 protein in HCT-116 cells upon treatment [192]	
15.	HBX41, 108 [193]	USP7	HBX 41,108 is an noncompititive reversible inhibitor and it allosterically modulates the catalytic reaction of USP7 [193]	Preclinical
16.	HBX19, 818 [194]	USP7	Binds selectively to the active site of USP7 [194]	Preclinical
17.	HBX28, 258 [194]	USP7	Selective inhibitor for USP7	
18.	HBX90397 [314]	USP8	Specifically target USP8 [116, 314]; inhibited cancer cell growth	
19.	Spautin1 [315]	USP10 and USP13	Induce Vps34 PI3K complex degradation [315]	Preclinical

S. no.	Inhibitor	Target (DUB)	Major attributes	Developmental stage
20.	Mitoxantrone [316]	USP11		Preclinical
21.	IU1 [167]	USP14	Cell permeable; reversible; encourages ubiquitin dependent protein degradation *in vitro*	Preclinical
22.	GSK2643943A [317]	USP20	Identified by GSK from a screen involving compounds targeting USP20/Ub-Rho. It has an IC_{50} of 160 nM [317]	Preclinical
23.	15-oxospiramilacetone [318]	USP30	15-oxospiramilacetone is the only inhibitor for USP30 defined so far which can be used in case of some mitochondrial dysfunctions [318]; natural compound from spiramine A; induce mitochondrial fusion	Preclinical
24.	PR619 [319]	Broad range DUBs inhibitor	Nonselective, noncompetitive, reversible; results in accumulation of ubiquitinated proteins	
25.	1,10-phenanthroline [320, 321]	JAMM type isopeptidase	Chelates active site Zn^{2+}	

Table 2. Different inhibitors and their target DUBs.

7.4. Natural products with DUB inhibitory effect

A number of natural compounds have been identified to have DUB inhibitory effect. One of such is Curcumin, which is a yellow pigment isolated from the *Curcuma longa.* Curcumin possesses two α, β-unsaturated ketones moieties and has been linked with suppression of tumorigenesis and various other diseases. It was reported that Curcumin accelerates polyubiquitinated protein accumulation at concentrations of 40 μM [184]. USP4 has been reported to be targeted by a small natural compound known as Vialinin A. Vialinin A is isolated from the Chinese mushroom *Thelephoravialis* and has been shown to inhibit the enzymatic activity of USP4 and USP5 [185, 186].

7.5. Synthetic small molecule DUB inhibitors

Several inhibitors have been developed to target the multifunctional deubiquitinating enzyme USP7. USP7, also known as HAUSP, is probably the most attractive DUB in the field of cancer biology. USP7 has been reported to regulate the function and stability of at least three important tumor suppressor p53 [187], PTEN [188] and TIP60 [189]. Progenra identified P022077 as a specific inhibitor for USP7 [190]. Other inhibitors of USP7 are P5091 and Cpd14, which triggers apoptosis in MM cells and inhibits tumor growth [191, 192]. Other Hybrigenics compounds which could inhibit USP7 function are HBX41108 [193], HBX19818 [194], and HBX28258 [194]. An isatin O-acyl oxime, LDN-57444 is a most potent active site directed inhibitor for UCHL-1 [195]. LDN-91946 is another compound which was identified as a hit in an *in vitro* screen for identifying blockers of Ub-AMC activity and was able to inhibit UCHL-1 in a noncompetitive manner [196].

Despite the multitude of inhibitors identified to target DUBs, so far, no DUB inhibitors are approved for clinical use. Only a few of these inhibitors, such as VLX1570, are in clinical trial for

cancer therapy. Out of 98 DUBs, only several DUBs have been explored structurally providing a platform for understanding, identifying, and validating various DUB inhibitors for clinical usage.

8. Conclusion

The UPS is implicated in several human diseases such as neurodegenerative disease, inflammation, bacterial and viral infection and most importantly, in cancer. The type of ubiquitin linkages formed/cleaved with the help of a cascade of enzymes (E1, E2, E3/DUBs) intensifies biological complexities. Hence, it is important to discover and identify the targets for therapeutic intervention. One of the strategies that can be used is targeting components of the UPS. Over the past 35 years, our knowledge and understanding of the UPS has significantly increased and it is evident that the UPS plays critical roles in various important cellular functions and can regulate both structural and functional behavior of cells. The success of Bortezomib provides a proof-of-concept to expand the use of other inhibitors targeting different components of UPS system in cancer. However, the results were not satisfying due to challenges in bringing these inhibitors to clinic. This is mostly because E3 ligases and DUBs have multiple substrates which makes it complicated. Therefore, it is critical to find the right target(s) for a specific cancer, to understand how the target functions and eventually find the finest way to effectively manipulate these targets for treatment intervention.

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