Review

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The Ubiquitin Code in Disease Pathogenesis and Progression: Composition, Characteristics and its Potential as a Therapeutic Target

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Conjugation of substrate proteins with ubiquitin (Ub), a 76 amino acid protein, was discovered as the first major translational modification responsible for protein degradation. Ubiquitination occurs as a cascade among ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligases (E3) enzymes that transfer and covalently conjugate Ub to the lysine (Lys) residue and α-amino group in methionine (Met) residues of substrates. Following the initial conjugation, Ub itself then undergoes ubiquitination via its seven lysine residues (K6, K11, K27, K29, K33, K48, and K63) and N-terminal methionine (M1). These possible sites of Ub polymerization/assembly result in a significantly diverse and numerous set of linkage types and lengths, including homotypic, mixed and/or branched chains, which provoke distinct cellular responses via their proteolytic and non-proteolytic functions. We overview here the multiplicity of ubiquitin code with a particular focus on linkage-specific roles in biological processes, especially in the pathogenesis and progression of diseases such as cancer, neurodegeneration, and immune disorders. We will also discuss the possibility and ongoing efforts of modulating the ubiquitin code as a therapeutic strategy in drug development, including targeted protein degradation (TPD).

Keywords: ubiquitin code; neurodegenerative disease; cancer; immune disorder; targeted protein degradation

Introduction

Ubiquitin (Ub), a 76 amino acid protein, was first discovered in 1975 and has since then emerged as the first major post-translational modifying (PTM) degradation signal (degron) for intracellular and extracellular proteins [1–3]. Ubiquitination/ubiquitylation occurs when ubiquitin is covalently linked to lysine (Lys) residues on substrate proteins and is orchestrated by a cascade of enzymatic reactions involving the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligases (E3). The E1 enzyme activates the C-terminal glycine (Gly) of Ub in an adenosine triphosphate (ATP)-dependent manner, forming a ubiquitin-adenylate intermediate. Activated Ub is then transferred to a cysteine residue on the E2 enzyme, after which E3s facilitate the transfer of Ub from the E2 to the lysine residues or N-terminal methionine of target substrates through an isopeptide bond [2,4]. Approximately 600 distinct human E3s, each of which recognizes a selective clientele confer substrate specificity [5]. Similar to other PTMs, ubiquitination exhibits reversibility via a diverse family of deubiquitinases (DUBs) that cleave the Ub-substrate isopeptide bond [6,7]. Mammalian cells can express approximately 100 DUBs, categorized as either cysteine proteases or zinc metalloproteases based on their catalytic mechanisms [8]. Like their E3 counterparts, each DUB exhibits specificity in recognizing and cleaving particular Ub linkages and thus maintains the balance of the ever-changing ubiquitome within cells, particularly under conditions of cellular stress [7].

Ubiquitination exhibits remarkable versatility by forming a multitude of monomeric and polymeric chains via its seven internal lysine residues (K6, K11, K27, K29, K33, K48, and K63) and the N-terminal methionine (M1). The combination of distinct lysine-chain linkages, collectively referred to as the Ub code, includes monoubiquitylation, multi-monoubiquitylation, homotypic polyubiquitylation (comprising a single type of linkage), and heterotypic polyubiquitylation (consisting of mixed or branched linkages), with each type resulting in distinct cellular outcomes (Fig. 1) [9]. Emerging evidence suggests that more complex ubiquitin structures play critical roles as regulatory signals

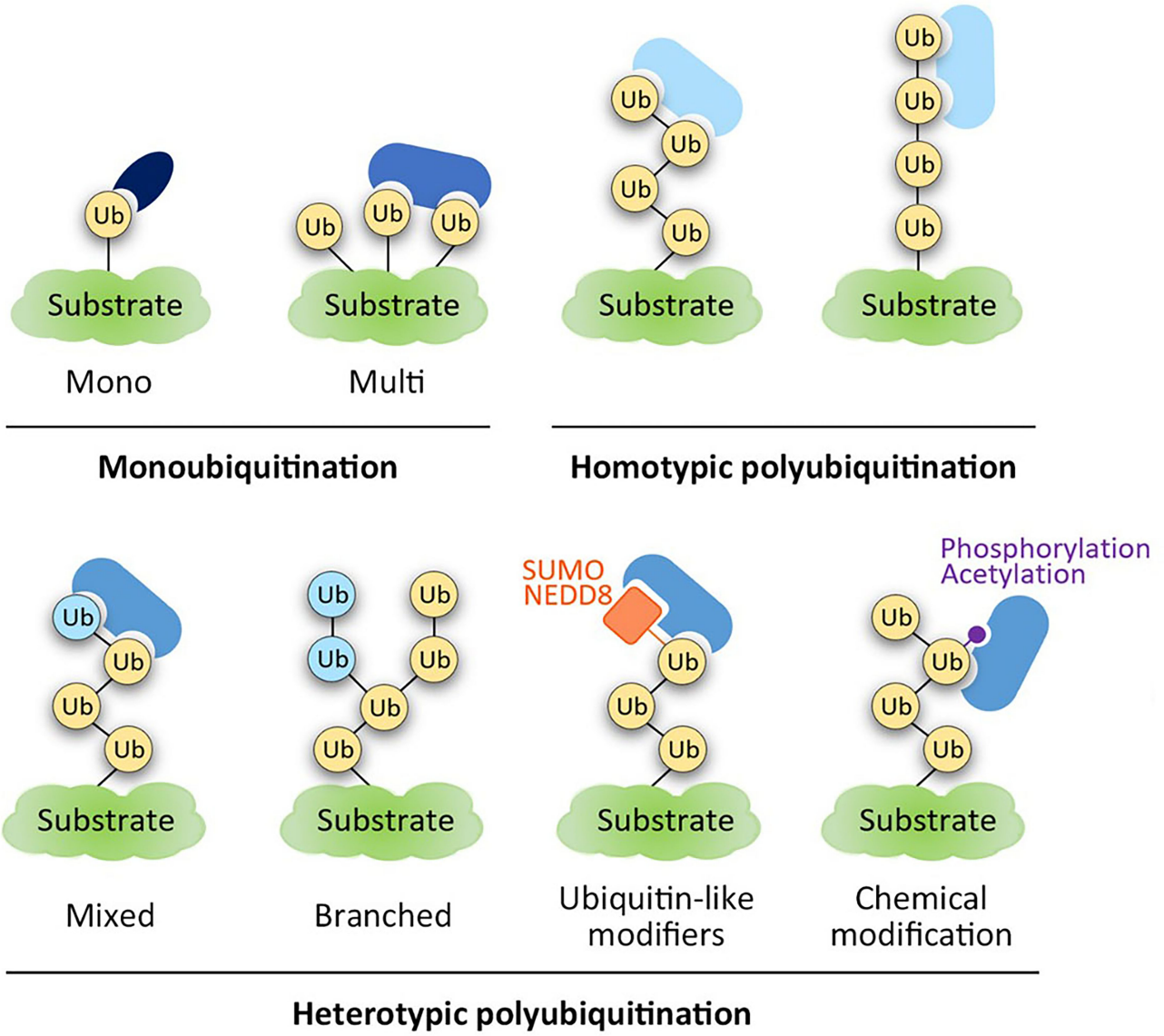

Fig. 1. The various topologies of ubiquitin linkages. The diverse modification of ubiquitin chains can lead to different biological outcomes and distinct linkage specificity by binding with ubiquitin-binding domain (UBD)-containing effector proteins. Multiple shades of blue proteins respectively represent effectors recognizing single and multiple types of Ub linkages. The ubiquitin modifies protein substrates with various arrangements of lengths, and structures. A single or multiple Ub/s are attached to mono- or multi-ubiquitination, respectively. The further attachment of ubiquitin leads to polyubiquitination, which can be classified into homotypic- and heterotypic-ubiquitination. The homotypic ubiquitination comprises one type of ubiquitin linkage (K6, K11, K27, K29, K33, K48, and K63), whereas heterotypic chains are uniform with multiple types of linkages. The heterotypic chains are subdivided depending on the number of acceptor sites, or the types of modifiers other than ubiquitin protein such as ubiquitin-like proteins or chemical modification proteins. Ub, ubiquitin; SUMO, Small Ubiquitin-like Modifier; NEDD8, Neural precursor cell expressed developmentally Down-Regulated 8. Created with PowerPoint (Microsoft Office Professional Plus 2019, Microsoft, Redmond, WA, USA).

across various biological pathways. Notably, these include ubiquitin-like modifiers such as SUMO (Small Ubiquitin-like Modifier) and NEDD8 (Neural precursor cell expressed developmentally Down-Regulated 8), as well as PTMs such as acetylation and phosphorylation [10,11]. Each Ub code influences cellular processes not only by controlling protein degradation through the proteasome and lysosome but also by modulating non-proteolytic functions, including DNA repair, transcriptional regulation, apoptosis, and immune signaling [12–14]. Given the above complexity, it comes as no surprise that the functions of a specific type of Ub are not limited to any one biological pathway.

Linkage-specific polyubiquitin chains have significant implications for pathogenesis and progression in al-

most all diseases. Particularly, Ub signaling has been implicated in a range of diseases, including proteinopathies, neurodegenerative disorders, cancer, and immune dysregulation [14–16]. Dysregulation of ubiquitination can lead to the accumulation, aggregation, and propagation of damaged/misfolded proteins, directly generating causative pathological agents such as in Alzheimer's and Parkinson's disease [15,17]. In cancer, aberrant ubiquitination has been linked to tumor progression, with mutations in E3s or DUBs disrupting critical pathways that control cell proliferation and apoptosis [16]. In immune disorders, modulating ubiquitin signaling pathways has emerged as an attractive therapeutic strategy to regulate immune responses and inflammation [18].

In this review, we examine the complexity of the ubiquitin code, including its basic composition, arrangement, and structural features, and discuss how these aspects are involved in disease pathogenesis. Specifically, we highlight recent advances in understanding how specific ubiquitin linkages contribute to the progression of diseases such as neurodegeneration, cancer, and immune disorders. Moreover, we discuss the therapeutic potential of targeting the ubiquitin system for drug development, exemplified by targeted protein degradation (TPD) and other platforms aimed at modulating the ubiquitin landscape.

Homotypic Chains

In homotypic ubiquitin chains, ubiquitin molecules are continuously linked through the same lysine residue [19]. With respect to protein degradation or signal transduction pathways, the most well-studied homotypic chains are linked via K48 or K63, termed canonical ubiquitination. K48-linked chains typically signal for proteasomal degradation, while K63-linked chains are associated with autophagic degradation as well as signaling pathways, such as the regulation of the nuclear factor Kappa B (NF-κB) pathway [9]. The structural simplicity of homotypic chains allows for straightforward recognition by specific ubiquitin-binding domains (UBDs), facilitating targeted cellular responses. Non-canonical ubiquitination refers to ubiquitin chains linked through atypical lysine residues (e.g., K6, K11, K27, K29, K33) or the M1, which regulated diverse cellular processes beyond proteasomal degradation, including cell signaling, stress responses, and organelle-specific functions [20]. Understanding the formation, recognition, and regulation of homotypic ubiquitin chains is crucial for deciphering their role in maintaining cellular homeostasis. Below, we discuss the current landscape on homotypic Ub chains and their involvement in the pathogenesis and progression of various diseases (Fig. 2; Table 1, Ref. [21–43]).

Canonical Homotypic Chains

K48-Linked Polyubiquitin Chains

K48-linked polyubiquitin chains are typically associated with the degradation of proteinaceous substrates via the ubiquitin-proteasome pathway [44]. They show a compact conformation with sequestered hydrophobic patches at the interdomain interface [45]. K48-linked ubiquitination plays a crucial role in regulating key proteins involved in cell cycle progression, apoptosis, and stress responses. The K48-Ub chains are recognized by the 26S proteasome, which facilitates the degradation of a wide multitude of proteins including GPX4 (glutathione peroxidase 4), ULK1 (unc-51-like autophagy-activating kinase 1), NLRP3 (nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3), and TDP-43 (transactive response DNA binding protein 43) [23–26,46]. This process is essential for removing misfolded, damaged, or excess proteins, thus preventing cellular toxicity and promoting survival.

Over the last decade, an increasing number of studies have shown the dysregulation of K48-linked ubiquitination and subsequent proteolysis in neurodegenerative diseases, tumor development, and immune responses. Key examples include neuronally-expressed NLRP3, whose hyperactivation contributes to the pathogenesis of Parkinson's disease (PD), is degraded by Parkin-mediated K48-linked polyubiquitination [26]. Huntingtin (HTT) undergoes K48-ubiquitination via UBE3A (ubiquitin-conjugating enzyme E3A), a K48-specific E3s that is increasingly downregulated in aged mice brain samples [47]. Similarly, brain samples from Alzheimer's disease (AD) patients largely showed K48-ubiquitination of tau neurofibrillary tangles (NFTs) [48]. In cancer, the Cullin 3 (CUL3) E3s mediates K48-linked ubiquitination of Beclin 1 (BECN1), a core protein of phagophore biogenesis in macroautophagy that has been shown to play both oncogenic and tumor suppressive roles [21]. In especially breast and ovarian cancers, CUL3 enhances tumor cell proliferation and is a marker for poor patient prognosis [21]. The mechanistic target of rapamycin complex 1 (mTORC1) undergoes K48 ubiquitination and is subsequently stabilized in a FBXW7 (F-box and WD repeat domain-containing 7)-dependent manner [16]. The stability of both adenylate-activated protein kinase alpha (AMPKa) and protein kinase B (AKT) is mediated by K48 ubiquitination via the E3s TRIM28 (tripartite motif containing 28) and SKP2 (S-phase kinase associated protein 2), respectively [49,50]. In immune responses and inflammation, the prototypical IκBα (NF-κB inhibitor alpha) is phosphorylated by IKK (IκB) for K48 ubiquitination and proteasomal degradation resulting in nuclear translocation of NF-κB dimers [14] K48-linked ubiquitination of TAB2 (TGF-β activated kinase 1 (MAP3K7) binding protein 2) mediated by the RNF99 (ring finger protein 99) E3s suppresses toll-like receptor (TLR)-associated downstream signaling via prote-

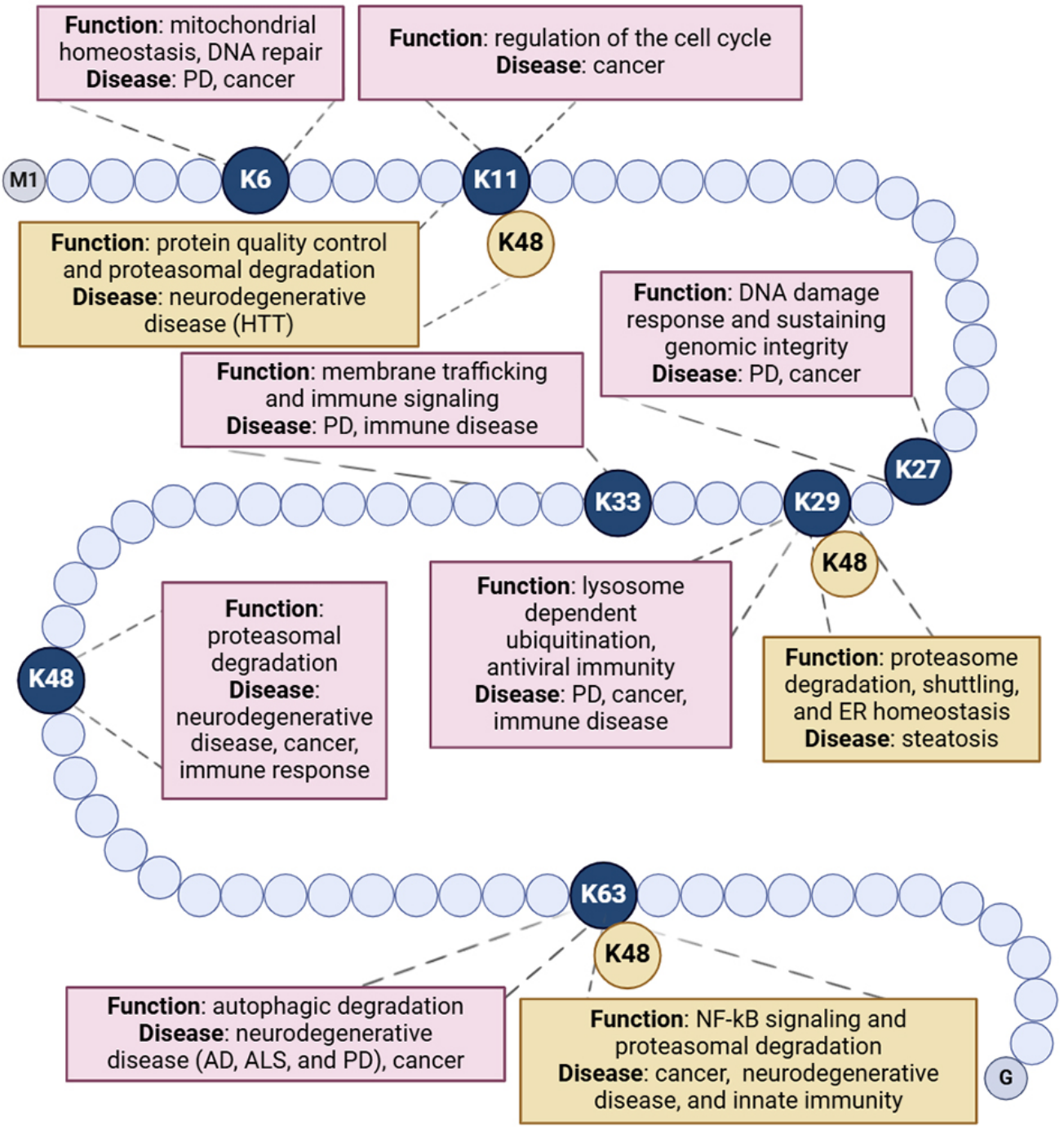

Fig. 2. Overview of different ubiquitin linkage types and their biological roles. Ub is a small, 76-amino-acid protein highly conserved across eukaryotic species. It contains seven lysine residues (K6, K11, K27, K29, K33, K48, and K63) and an N-terminal methionine (M1), which serve as key sites for forming distinct Ub chains. Additionally, the Ub code consists of homotypic chains and heterotypic branched ones (K11/K48, K29/K48, and K48/K63). These Ub chains enable proteins to perform specific cellular functions and are associated with various diseases. NF-κB, nuclear factor Kappa B; PD, Parkinson's disease; HTT, huntingtin; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; ER, endoplasmic reticulum. Created with BioRender (https://www.biorender.com/).

olysis of TAB2 [22]. Additionally, the Nrdp1 (neuregulin receptor degradation protein-1 (FLRF)) E3s catalyzes K48-linked poly-Ub chains on Myd88 for its proteasomal degradation [51].

K63-Linked Polyubiquitin Chains

In contrast to their K48-linked counterparts, K63-linked Ub chains exhibit an extended conformation without hydrophobic ubiquitin contacts [52]. Such structural

Table 1. Chains and linkages types of ubiquitination: E3s, substrates, function, disease.

Types of chains		Types of linkages	E3s	Substrate	Function	Disease	Refs
Homotypical chains	Canonical chains	K48	CUL3	BECN1	Proteasome degradation	Cancer	[21]
			RNF99	TAB2	Suppress TLF-mediated inflammatory immune response	Neurodegenerative disease, cancer, immune response	[22]
			TRIM25	GPX4, TDP-43	Proteasome degradation	Cancer	[23,24]
			TRIM27	ULK1			
			Parkin, RNF220	TDP-43		Parkinson's disease	[25]
			TRIM31	NLRP3			[26]
		K63	CHIP	Tau	Autophagic degradation	Alzheimer's disease	[27]
			NEDD4L	α-synuclein	Trafficking, degradation	Parkinson's disease	[28]
			TRIM27	cGAS	DNA damage repair	Neurodegenerative disease, amyotrophic lateral sclerosis	[29]
	Non-canonical chains	K6	SKP2	FOXA1	DNA repair	Parkinson's disease, cancer	[30,31]
			TRAF6	DJ-1, α-synuclein		Parkinson's disease, Huntington disease	[32]
		K11	cIAP1/2	RIPK1	Proteasomal degradation	Immune response	[33]
			APC/C	UBE2S		Amyotrophic lateral sclerosis	[34]
		K27	RNF168	Histone H2A	DNA damage response	Parkinson's disease, cancer	[35]
			TRAF6	DJ-1, α-synuclein		Parkinson's disease, Huntington disease	[32]
		K29	NEDD4L	FOXA1	Antiviral immunity	Cancer, immune disease	[30,31]
			LRRK2	WSB1	Lysosome depend-ent ubiquitination, antiviral immunity	Parkinson's disease, cancer, immune disease	[36]
			SKP1-Cullin-Fbx21 (SCF) complex	ASK1		Infection	[37]
			TRAF6	DJ-1, α-synuclein		Parkinson's disease, Huntington disease	[32]
		K33	Parkin	PINK1	Membrane traffick-ing, immune signaling	Parkinson's disease, immune disease	[32]
			Nrdp1, Cblb	Zap70		Autoimmune disease	[38]
		M1	TRIM HOIP	α-synuclein Nemo	NF-κB signaling, antiviral immunity	Neurodegenerative disease	[31,32] [39]

Table 1. Continued.

Types of chains		Types of linkages	E3s	Substrate	Function	Disease	Refs
Heterotypical chains	Branched chains	K11/K48	APC/C, UBR4, UBR5	Nascent protein, huntingtin	Cell cycle, protein quality control	Neurodegenerative disease	[34]
		K48/63	NEDD4L, cIAP1/2, HECTD3	TRAF3	NF-κB signaling, proteasomal degradation	Antiviral innate immunity	[30]
			HUWE1, TRAF6	TAB2	NF-κB signaling	Inflammation, cell survival, tumor growth	[40]
			ITCH, UBR5	TXNIP	Proteasomal degradation, proapoptosis	N/A	[41]
			TRAF6	ATG9A	Autophagic degradation	Neurodegenerative disease, cancer	[42]
		K29/48	UBE3C	VPS34	Autophagy, liver metabolism	Steatosis	[30]
			TRIP12	BRD4, CRABP2, TRIM24	Proteasome degradation	N/A	[43]

E3, ubiquitin ligases; CUL3, Cullin 3; BECN1, Beclin 1; RNF99, ring finger protein 99; TAB2, TGF-β activated kinase 1 (MAP3K7) binding protein 2; TRIM25, tripartite motif containing 25; GPX4, glutathione peroxidase 4; TDP-43, transactive response DNA binding protein 43; ULK1, unc-51-like autophagy-activating kinase 1; NLRP3, nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3; TLF, trypanosome lytic factors; CHIP, carboxyl terminus of the HSC70-interacting protein; Tau, tubulin associated unit; cGAS, cyclin GMP-AMP synthase; SKP2, S-phase kinase associated protein 2; FOXA1, forkhead Box Protein A1; TRAF6, TNF receptor-associated factor 6; DJ-1, protein deglycase; cIAP1/2, cellular inhibitor of apoptosis protein 1/2; RIPK1, receptor-interacting protein kinase 1; APC/C, anaphase promoting complex/cyclosome; UBE2S, ubiquitin-conjugating enzyme E2S; LRRK2, leucine-rich repeat kinase 2; WSB1, WD repeat and SOCS box containing 1; ASK1, apoptosis signal-regulating kinase 1; PINK1, PTEN-induced kinase 1; Nrdp1, neuregulin receptor degradation protein-1 (FLRF); Cblb, casitas B-lineage lymphoma-b protoncogene; Zap70, zeta-chain-associated protein kinase 70; HOIP, HOIL-1-interacting protein; Nemo, NF-κB essential modulator; UBR4, ubiquitin protein ligase E3 component n-recognin 4; HECTD3, HECT domain-containing E3 ubiquitin-protein ligase 3; HUWE1, UBA and WWE domain containing E3 ubiquitin protein ligase 1; ITCH, itchy E3 ubiquitin protein ligase; TXNIP, thioredoxin-interacting protein; ATG9A, autophagy related 9A; VPS34, Vacuolar Protein Sorting 34 (class III PI3kinase); TRIP12, thyroid hormone receptor interactor 12; BRD4, bromodomain containing 4; CRABP2, cellular retinoic acid binding protein 2; N/A, not available; NEDD4L, Neural precursor cell expressed developmentally Down-Regulated 4-like.

differences are also mirrored in functionality, with K63-linked polyubiquitination generally associated with the autophagy-lysosome system as opposed to the ubiquitin-proteasome system. K63-linked chains have also been involved in DNA repair, internalization of plasma membrane proteins, and endosomal/exosomal trafficking [12, 53]. In terms of their degradative roles, K63-linked Ub chains enable identification and macroautophagic targeting of specific cargoes ranging from soluble monomeric proteins and their high-molecular insoluble aggregates (aggrephagy) to subcellular organelles (organellophagy), pathogens and other foreign materials (xenophagy) [12, 54]. Moreover, HIF1α (hypoxia-inducible factor 1-alpha) has been confirmed as a substrate of chaperone-mediated autophagy by K63-linked polyubiquitination via the STUB1 (STIP1 homology and U-box containing protein 1) E3s [54]. K63-linked poly-Ub chains on substrates targeted for autophagic/lysosomal degradation are recognized by various autophagic receptor proteins. These receptors, such as p62 (sequestosome 1), NDP52 (nuclear dot protein 52 kDa), OPTN (optineurin), and NBR1 (neighbor of BRCA1 gene 1), simultaneously contain the UBDs or ubiquitin-associated (UBA) domains and LC3 (microtubule-associated proteins 1A/1B light chain 3B)-interaction region (LIR) domains, allowing the receptors to function as intermediaries [55,56].

Particularly in neurodegeneration K63-linked polyubiquitin chains are implicated in the activity, interaction, propagation, and aggregation of several hallmark proteins [27]. K63-linked soluble tau oligomers accumulated at higher levels in the brains of AD patients compared to age-matched controls. At the cellular level, K63-linked tau oligomers are associated with enhanced seeding activity and propagation, suggesting the role of K63 ubiquitination

in the formation, accumulation, and pathological propagation of tau aggregates [27,57]. In stable cell lines, ubiquitination of α-synuclein via K63-linked chains was four-fold more abundant when compared to that of the proteasome-associated K48- or K11-linked chains [28]. Additionally, the cytoplasmic aggregates of TDP-43 C-terminal fragments are progressively K63-ubiquitinated and accumulated in mammalian brains due to age-dependent elevated levels of UBE2N, an E2 Ub-conjugating enzyme [58]. Additionally, K63-linked ubiquitination can attenuate the pathogenesis of several neurodegenerative diseases via proteolysis of cyclin GMP-AMP synthase (cGAS) following its K63-linked ubiquitination by the E3 TRIM27 [29]. cGAS activation, triggered by TDP-43 abnormalities in amyotrophic lateral sclerosis (ALS), DNA damage in AD, and α-synuclein aggregates in PD, pathologically drives neuroinflammation. Interestingly, the formation of K63-linked chains often seems to compete against that of their K48 counterparts, especially in a number of substrates associated with neurodegeneration and autophagy [59,60]. Recent emerging evidence shows that K63-linked ubiquitination plays a crucial role in tumor initiation, progression, metastasis, drug resistance, response to therapy, as well as the immune and inflammatory response [13,61]. Select few examples of key substrates that undergo K63-ubiquitination include phosphoinositide 3-kinase (PI3K)/AKT signaling pathway, c-myc (cellular myelocytomatosis oncogene), JNK (c-Jun n-terminal kinase), YAP (yes-associated protein), p53 (tumor protein p53), and Wnt/β-catenin (wingless-related integration site/β-catenin signaling pathway) which are modulated by K63-linked ubiquitination [61].

Non-Canonical Homotypic Chains

K29-linked polyubiquitin chains regulate the control of TLR2 part from K48- and K63-linked polyubiquitin chains, the formation and functions of the remaining non-canonical Ub chains (K6, K11, K27, K29, and K33), remain relatively less understood. Poly-Ub chains formed via K6 and K11 are structurally compact, and typically associated with proteasomal degradation [32]. The methionine 1 (Met1) ubiquitination results in linear linkages that result in extended and flexible chains that allow the ubiquitinated proteins to interact with other signaling molecules, such as NF-κB [32,33,36]. Similarly, other non-canonical linkages have been shown to carry out specialized functions in cellular regulation, immune responses, and DNA repair [35]. K27-linked Ub chains, which show an open conformation, are selectively assembled by E3s such as RNF168 during DNA damage response and sustaining genomic integrity [30]. K29-linked ubiquitination, associated with lysosome-dependent ubiquitination, promotes antiviral immunity rather than protein degradation as exemplified by NEDD4L (Neural precursor cell expressed developmentally Down-Regulated 4-like)-dependent regulation of FOXA1 (forkhead Box Protein A1) for carcinogenesis, progression and lineage specification. K33-linked ubiquitination regulates membrane trafficking and immune signaling via CUL3- and Cblb/ITCH (casitas B-lineage lymphoma-b protoncogene/itchy E3 ubiquitin protein ligase)-mediated ubiquitination of Coronin7 and TCR (T-cell receptor), respectively [38,62–64].

In neurodegenerative diseases such as PD, non-canonical ubiquitin linkages play crucial roles in pathological gain-of-function protein aggregation, accumulation, and prion-like propagation [36]. K6-, K27-, K29-, and K33-linked polyubiquitination of α-synuclein, the primary component of Lewy bodies, and DJ-1 (protein deglycase), another PD-associated protein, promotes the formation of insoluble aggregates, disrupting normal protein homeostasis and contributing to cellular toxicity [32,65]. DJ-1 undergoes atypical polyubiquitination (K6, K27, and K29) and subsequent aggregation via the E3 ubiquitin ligase TRAF6 (TNF receptor-associated factor 6) [32]. LRRK2 (leucine-rich repeat kinase 2), a multifunctional protein kinase essential for PD, is modified by K27- and K29-linked ubiquitination, further promoting its aggregation and linking these non-canonical ubiquitin chains to PD pathogenesis [66]. Moreover, in Huntington's disease, mutant huntingtin (mHTT) is selectively mediated by K6- and K9-linked ubiquitination, promoting its early aggregation and fibril nucleation [32]. In cancer, K6-linked ubiquitination maintains mitochondrial homeostasis, and its dysregulation is linked to the early stages of carcinogenesis via defects in mitochondrial quality control and DNA repair [65,66]. K11-linked ubiquitination in particular is closely linked to the cell cycle in cancer cells. K11-linked poly-Ub chains conjugated on RIPK1 (receptor-interacting protein kinase 1) [33] by cIAP1/2 (cellular inhibitor of apoptosis protein 1/2) and linear ubiquitin chain assembly complex (LUBAC) inhibits cell proliferation by serving as anchors that recruit distinct ubiquitin-binding proteins, including TAB2/3 and NEMO (NF-κB Essential Modulator), to restrict RIPK1 activation and subsequently promote NF-κB signaling [33,67,68]. Additionally, the master cell cycle regulator anaphase promoting complex/cyclosome (APC/C) ubiquitinates K11-linked chains on various cell cycle regulators such as Cyclins A and B, as well as securing, FOXM1 (forkhead box protein M1), Aurora kinases A and B (AURKA and AURKB), Geminin, Cdc6 (cell division cycle 6 protein) among others for their proteasomal degradation to ensure proper mitotic progression [33,69,70]. Moreover, dysregulation of K27-linked ubiquitination has been implicated in abnormal DNA damage responses in cancer, wherein RNF168 targets histones H2A/H2A.X (histone H2A/histone H2A variant X) for chromatin ubiquitination and thus regulates faulty DNA repair signaling pathways [30].

In the immune system, K29- and K33-linked ubiquitination play essential roles in regulating immune signaling. K29-linked ubiquitination, mediated by NEDD4L, pro-

motes antiviral innate immunity by modifying TRAF3, distinguishing it from other forms of ubiquitination that mark proteins for degradation [33]. Viral infections and replication, including VSV (vesicular stomatitis virus) and HSV-1 (herpes simplex virus type 1), are also regulated by K29-linked ubiquitination of ASK1 (apoptosis signal-regulating kinase 1) through the SKP1-Cullin-Fbx21 (SCF) E3 complex [37]. K29-linked polyubiquitin chains play a regulatory role in TLR2 signaling. Upon activation, TLR2 triggers immune responses, including cytokine and chemokine production, by enhancing NF-κB signaling. This involves TRAF6 ubiquitination, which is promoted by the E3 ubiquitin ligase Trim13. However, K29-linked ubiquitin chains inhibit Trim13-mediated TRAF6 polyubiquitination, fine-tuning the immune response. Another E3, Trim1, is upregulated in macrophages upon TLR2 ligand activation and contributes to modulating the TLR2 pathway. Together, TRAF6 and Trim13 activate TLR2-mediated immune responses, with K29-linked ubiquitin chains acting as critical regulators [71,72]. K33-linked ubiquitination of Zap70 (zeta-chain-associated protein kinase 70) by Nrdp1 and Cblb is involved in membrane trafficking and immune regulation, with dysregulation leading to autoimmune disorders due to improper signaling regulation [14]. K33-linked polyubiquitin chains inhibit protein-DNA interactions and regulate NF-κB signaling in a linear N-terminus to C-terminus manner. K33 chains bind RNF167 via Tollip at K235, triggered by tumor necrosis factor-alpha (TNF-α) stimulation. This activation subsequently induces NF-κB and MAPK (mitogen-activated protein kinase) pathways. NF-κB and MAPK signaling can be inhibited by regulating Tollip through TNF-α. The ubiquitination of Tollip by RNF167 modulates its function, affecting the inhibition of Tollip activity in response to TNF-α-induced NF-κB and MAPK activation [71]. These non-canonical modifications highlight the diverse and critical roles of ubiquitin linkages which play across various cellular functions, from neurodegeneration and cancer to immune responses.

Heterotypic Branched Chains

Compared to homotypic chains, heterotypic chains have been relatively less studied. Approximately 20% of all ubiquitin chains are estimated to exist as branched forms, with a much greater number of heterotypic chains. Various forms of heterotypic polyubiquitin chains, such as mixed, branched, or combined structures, generate a theoretically exponential number of cellular signals [73]. Recently, the unique functions of heterotypic chains have been uncovered and differentiated from their homotypic counterparts. The formation of heterotypic chains occurs through the linking of ubiquitin at multiple lysine sites on the target substrates or ubiquitin itself, which are characterized as either mixed or branched. In mixed chains, more than two distinct types of homotypic linkages are generated on a given substrate. Branched chains, on the other hand, consist of at least one proximal ubiquitin molecule linked to two or more distinct lysines via distal ubiquitin molecules. The formation of branched chains is facilitated by unique combinations of acceptor sites, with branch points capable of emerging from distal, proximal, or internal ubiquitins within the chain. However, due to the lack of methods to monitor their assembly, the molecular mechanisms behind the formation of heterotypic chains remain largely unclear.

The diversity of biological information conveyed through ubiquitylation signals is broadened by the significant increase in complexity provided by branched ubiquitin chains (Fig. 2). Various branched ubiquitin chains exhibit differences in their length, linkage types, and overall structures, allowing them to sense and respond to a myriad of cellular stress conditions. For example, in U2OS cells, branched K48/K63 chains that comprise approximately 20% of all K63 linkages significantly increase under doxycycline-treated conditions [40]. Under proteotoxic stress, the accumulation of K11/K48 chains is increased in HEK293T and HeLa cells. These findings may be a hint that degradation signals from branched chains are typically more effective than those from unbranched chains, creating a distinctive coding signal that regulates the interpretation of the ubiquitin code (Table 1).

K11/K48-Linked Polyubiquitin Chain

Branched ubiquitin chains constitute about 10–20% of all ubiquitin chains [74]. Increasing evidence highlights distinct physiological roles for linkages such as K11/K48, K48/K63, and K29/K48. These branched chains are more effective in directing substrates for proteasomal degradation [75,76]. The K11/K48 branched chain is one of the most extensively studied heterotypic ubiquitin chains. These chains play a crucial role in maintaining physiological processes by regulating protein quality control and facilitating proteasomal degradation. K11/K48 chains conjugated at sites of DNA damage by unknown E2 ligases but not UBE2N during p97 inhibition accumulate in the cytoplasm and at the ER (endoplasmic reticulum) membrane surface, where they are recognized by p97 before being targeted for proteasomal degradation [77]. During the ER-associated degradation (ERAD) process, p97 bounds to K11/K48 polyubiquitinated substrates, a process regulated by DUBs that inactivate an E3 cofactor YOD1. The disruption of K11/K48 ubiquitination leads to the accumulation of specific substrates, such as CD3δ (homodimer-forming type 1 transmembrane (TM) protein), at the ER membrane, resulting in a failure to maintain ER homeostasis. This accumulation highlights the critical role of K11/K48 ubiquitination in regulating the degradation of misfolded or excess proteins during the ERAD process, ensuring proper protein turnover and ER function. Boughton *et al.* [78] provided structural insights into how K11-linked ubiquitin chains enhance the function of K48-linked chains.

The addition of K11 linkages to a K48-linked chain increases the chain's stability, protecting it from DUB activity. Furthermore, a high-affinity interaction was observed between the branched K11/K48-linked tri-ubiquitin complex and the proteasomal subunit Rpn1 (regulatory particle non-ATPase 1) (ubiquitin-conjugating enzyme E2S). This interaction enhances the local concentration of substrates near the proteasome, facilitating their recognition and subsequent degradation. This phenomenon suggests that this interdomain interface plays a key role in promoting substrate recognition and degradation [78].

The specific substrates and pathological roles of K11/K48 branched chains, such as mitosis-related regulators and aggregate-prone proteins involved in neurodegenerative diseases, have been well characterized through cell-based studies [34,75]. The APC/C assembles K11/K48-branched chain, by cooperating with UBE2S and UBE2C to ubiquitinate Nek2A (NIMA-related kinase 2A) kinase and cyclin A, thereby improving the efficiency of proteasomal substrates recognition by APC/C [34,75]. Additionally, K11/K48-branched chains specifically control the protein degradation machinery of neurodegeneration-associated hallmark proteins, as exemplified by ubiquitin protein ligase E3 component n-recognin 4 (UBR4)- and UBR5-mediated ubiquitination of aggregation-prone mutant HTT in neurons [34,75]. Specifically, the aggregate-prone form of HTT, 73Q-HTT, is labeled with K11/K48-branched ubiquitin chains, which not only target HTT for degradation but also increase the levels of K11/K48-linked ubiquitination on other unknown substrates. Similar phenomena are observed in cancer cells, embryonic stem cells, and differentiated neurons, suggesting a conserved role for these ubiquitin linkages in various cellular contexts [75]. The K11/K48-linked ubiquitin chains conjugated to HTT facilitate its interaction with key degradation machinery components, including BAG6 (BAG cochaperone 6), p97 (valosin-containing protein (VCP/p97)), UBQLN2 (ubiquilin 2), and p62/SQSTM1 (Sequestosome 1). These interactions direct HTT toward either autophagic or proteasomal degradation pathways, thereby mitigating the toxic effects of its aggregation [75]. These results suggest the significance of K11/K48-branched ubiquitin chains in maintaining protein quality control and cellular homeostasis, particularly in diseases like neurodegeneration and cancer.

K29/K48-Linked Polyubiquitin Chain

K29/K48 branched chains have been shown to interact with the proteasome and are also linked to the shuttling factors Rad23 (Radiation Sensitivity Protein 23) and Dsk2 (Ubiquitin-Binding Scaffold Protein 2) in yeast [79]. In this process, Ufd4p (E3 of the UFD pathway), an E3, assembles the K29-linked ubiquitin chain where Ufd2p, ubiquitin chain elongation factors (E4) conjugates K48-linked ubiquitin. Moreover, loss-of-function and rescue experiments have demonstrated that TRABID (TRAF-binding protein domain (a deubiquitinase), was destabilized via HECTD1 (HECT domain-containing E3 ubiquitin-protein ligase 1) protein levels, identifying this novel DUB-E3 pair as an essential regulator of K29-linked polyubiquitination [80]. UBE3C preferentially assembles K29-linked ubiquitin chains, while HECTD1 predominantly generates mixed K29/K48-linked ubiquitin chains. These mixed chains serve as critical signals for managing the degradation of ERAD clients, ensuring the proper turnover of misfolded or aberrant proteins to maintain ER homeostasis. Additionally, K29/K48 ubiquitination controls the cellular fate of VPS34 (Vacuolar Protein Sorting 34 (class III PI3kinase) by increasing its affinity for the proteasome. During ER stress, the activity of the ubiquitin ligase UBE3C targeting VPS34 is reduced, aiding in the maintenance of proteostasis [81]. In conditions of steatosis, the deubiquitinase enzyme TRABID plays a vital role in stabilizing VPS34, which is crucial for lipid metabolism [43]. Moreover, the deubiquitinase enzyme TRABID plays a vital role in stabilizing VPS34, which is crucial for lipid metabolism. TRABID prevents the K29/K48-linked polyubiquitination of VPS34, thereby protecting it from proteasomal degradation. Inhibition of TRABID disrupts this protective mechanism, resulting in the polyubiquitination and subsequent degradation of VPS34. This loss of VPS34 impairs lipid metabolism, contributing to the development and progression of steatosis.

Additionally, K29/K48-branched ubiquitin chains induce the collaboration of the HECT-type E3 ubiquitin ligase TRIP12 (thyroid hormone receptor interactor 12) with Cullin-RING ligase (CRL) complexes to promote PROTAC (proteolysis-targeting chimera)-induced degradation of neo-substrates. In this system, the degradation of BRD4 (bromodomain containing 4) is facilitated through the cooperative actions of TRIP12 and CRL2VHL (Cullin-RING ligase 2 with von hippel-lindau tumor suppressor). TRIP12 initiates the process by assembling K48-linked ubiquitin chains on BRD4, which serve as a platform for further modification. Subsequently, CRL2VHL adds K29-linked ubiquitin chains onto the pre-existing K48-linked chains, forming K29/K48-branched ubiquitin structures. These branched chains synergistically accelerate BRD4 degradation through PROTAC-induced and CRL2VHL-mediated mechanisms, ensuring efficient protein turnover. These branched ubiquitin chains have been proposed as a specific ubiquitin code for targeted protein degradation induced by chimeric compounds or molecular glues, which will be discussed later [43].

K48/K63-Linked Polyubiquitin Chain

K48/K63-branched chains comprise approximately 20% of all K63-linked ubiquitin chains [40,41]. These chains have been credited with enhancing NF-κB signaling while also promoting proteasomal degradation of

TAB2 in response to interleukin-1β [40,41]. In reaction to interleukin-1β, K63 chains conjugated by TRAF6 are modified by the E3 ubiquitin ligase HUWE1 (UBA and WWE domain containing E3 ubiquitin protein ligase 1), which generates K48 branches. This K48-K63 branched linkage not only enables TAB2 recognition but also safeguards the K63 linkages from deubiquitination by CYLD (cylindromatosis tumor suppressor), ultimately enhancing NF-κB signaling. Through this dual functionality, these branched chains not only amplify NF-κB signaling but also highlight a mechanism by which K63 linked ubiquitin contributes to proteasomal degradation. This finding underscores a new layer of cross-talk between distinct ubiquitin modifications. Furthermore, under pathological oxidative stress found in cancer and neurodegenerative diseases, the TRAF6 E3 generates K48/K63-linked branched chains that influence the interaction of ATG9A (autophagy related 9A) with Beclin 1 and the assembly of the VPS34-UVRAG (Vacuolar Protein Sorting 34–UV Radiation Resistance-Associated Gene Complex) complex. Notably, the lysine residues K581 and K838 of ATG9A are critical for TRAF6-mediated ubiquitination, highlighting their essential role in promoting autophagy under these stress conditions. Beyond its role in autophagy, ATG9A also regulates innate immune signaling, inflammatory responses, and NLRP3 inflammasome activation in response to LPS (lipopolysaccharide)-induced oxidative stress. These findings underscore a dual regulatory mechanism in which TRAF6-mediated ubiquitination and A20-mediated deubiquitination dynamically modulate ATG9A activity, revealing the molecular pathways that govern oxidative stress-induced autophagy [42]. K63-linked polyubiquitylation is also associated with the targeting of proapoptotic regulator TXNIP (thioredoxin-interacting protein) toward ubiquitin ligase ITCH-mediated proteasomal degradation [41]. In addition, K63 ubiquitin chains enable the subsequent formation of K48/K63-branched ubiquitin chains by recruiting ubiquitin ligases that assemble K48 chains, ultimately guiding substrates to the proteasome for degradation. Once K48/K63-branched chains are conjugated, the K48 linkages extended from K63 chains are likely recognized by shuttle proteins like Rad23, which guide the substrates to the proteasome [82]. In line with this, previous studies have shown that Rad23 can recognize the K48 linkage within K48/K63-branched ubiquitin trimers *in vitro* [83]. Given that TXNIP is a key regulator of apoptosis, these findings underscore the critical role of the TXNIP–ITCH–UBR5 axis in regulating cell fate through targeted degradation. In conclusion, these results highlight that K63 ubiquitylation promotes proteasomal degradation by acting as a "seed" for K48/K63-branched chains, offering new insights into the ubiquitin code and its impact on cell fate regulation [41]. NEDD4L forms a direct interaction with TRAF3 and facilitates the K29-linked ubiquitination of two cysteines, Cys56 and Cys124, which are part of the zinc finger structure [30]. This modification leads to a strengthened association between TRAF3 and the E3s cIAP1/2 and HECTD3, as well as an increase in K48/K63-linked ubiquitination of TRAF3 to promote antiviral innate immunity [30].

Therapeutic Applications of the Ubiquitin Code: Targeted Protein Degradation

Given that ubiquitination alters the molecular functions of tagged substrates, affecting their turnover, biological activity, subcellular localization, or protein-protein interactions, the pathogenesis and progression of various diseases almost always involve its dysregulation [84,85]. This is most likely attributed to the sensitive and fragile equilibrium between ubiquitination and deubiquitination, whose disruption is caused by genetic mutations, abnormal expression, or malfunctions in E3 ubiquitin ligases, and DUBs. Consequently, significant research efforts over the past two decades have focused on unraveling the mechanisms of ubiquitination regulation, with the aim of developing small-molecule ligands for E3s or DUBs [86]. Below, we summarize key examples of drug development strategies that harness ubiquitination. Specifically, we explore previous and ongoing approaches that directly affect the ubiquitination of a substrate, as well as those that facilitate ubiquitin-dependent proteasomal targeted degradation by the proteasome. Finally, insights into the role of ubiquitination in autophagy-based targeted protein degradation will be explored to highlight the multifaceted role of ubiquitination in cellular homeostasis and its potential as a target for innovative therapeutic interventions (Fig. 3; Table 2).

Pharmacological Modulation of E3s and DUBs

The ubiquitin-proteasome system (UPS) presents various potential drug targets, including two E1 enzymes, around 40 E2 enzymes, nearly 100 DUBs, approximately 600 E3s, and 32 proteasome subunits. In contrast to E1 and E2, E3 and DUBs exhibit high specificity through their direct interaction with selective substrates, thereby reducing the risk of off-target effects upon their pharmacological modulation [87]. Therefore, regulating individual E3s or DUBs has emerged as an efficient targeted therapeutic strategy from drug discovery to development and clinical trials, some of which are discussed below.

Currently, the only FDA (food and drug administration)-approved drugs targeting E3s is thalidomide, lenalidomide, and pomalidomide for the treatment of multiple myeloma, mantle cell lymphomas, and/or myelodysplastic syndromes [88–90]. These modulators enhance the binding of Ikaros and Aiolos transcription factors to the CRL4CRBN (Cullin-RING E3 ubiquitin ligase 4 cereblon complex) E3 [91]. Surprisingly, however, the development of small-molecule ligands that target other E3s has remained notoriously difficult. Among E3 families, the SCF E3 complexes are the largest and have

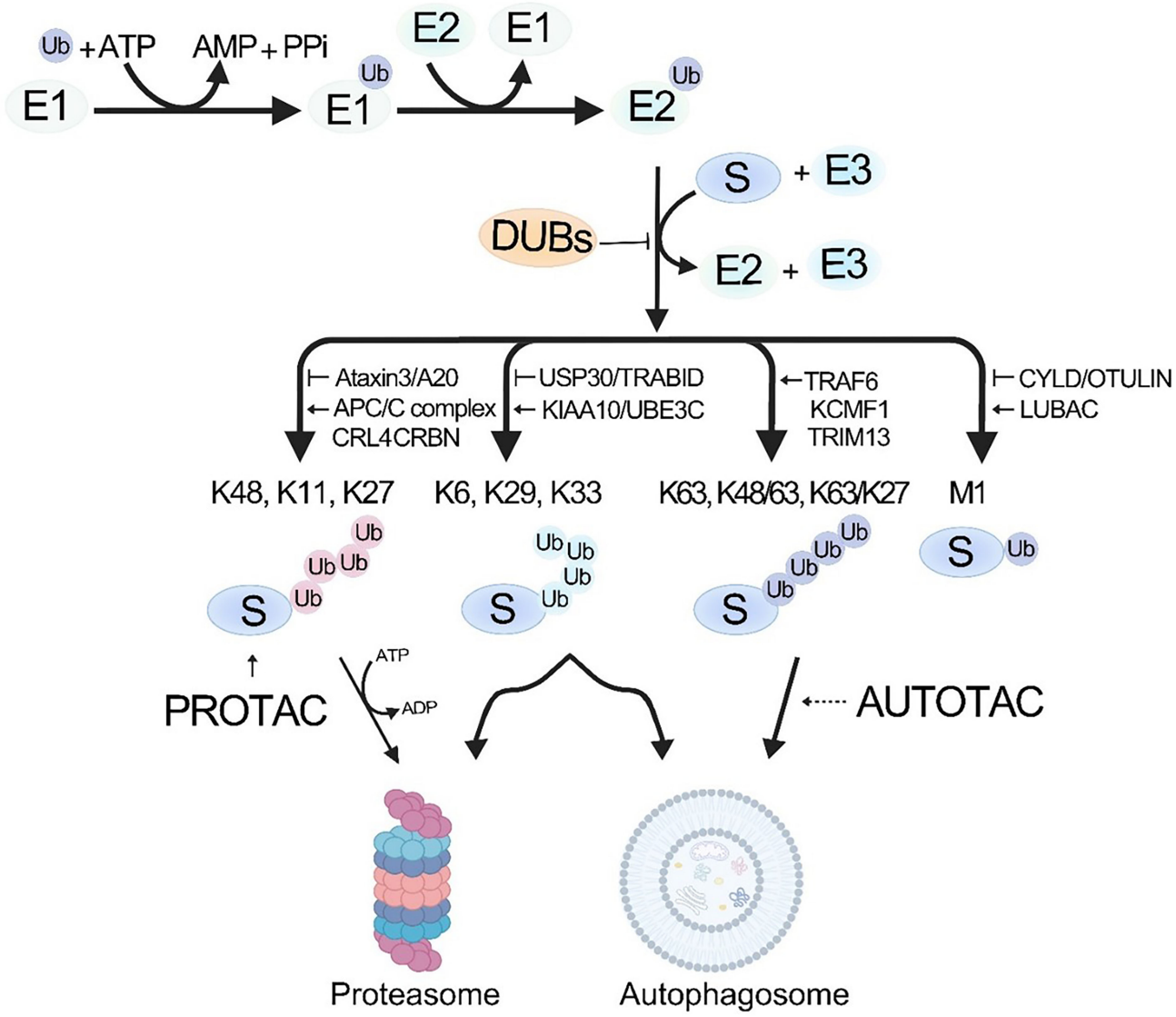

Fig. 3. Therapeutic applications of the ubiquitin code of targeted protein degradation. Ubiquitination binds ubiquitin to a specific protein via E3s, making it for degradation or other cellular process. Conversely, deubiquitinase (DUB) remove ubiquitin to prevent the targeted protein from being broken down, allowing it to be recycled. E3s and DUBs that participate in conjugating each Ub code are represented. PROTAC uses a ubiquitin-proteasome pathway to degrade targeted proteins by simultaneously binding the E3 ubiquitin ligase, facilitating proteasomal degradation. AUTOTAC, a newly developed approach, functional/powerful autophagy to degrade proteins by simultaneously binding the target protein and an autophagy receptor, allowing the target protein to be captured by the autophagosome and degrade in the lysosome. ATP, adenosine triphosphate; AMP, adenosine monophosphate; CRL4CRBN, Cullin-RING E3 ubiquitin ligase 4 cereblon complex; USP30, ubiquitin specific peptidase 30; TRABID, TRAF-binding protein domain (a deubiquitinase); CYLD, cylindromatosis tumor suppressor; LUBAC, linear ubiquitin chain assembly complex; PROTAC, proteolysis-targeting chimera; AUTOTAC, autophagy-targeting chimera. This figure is created with BioRender (https://www.biorender.com/) and Photoshop (Adobe Inc., San Jose, CA, USA).

attracted the most attention in basic science, drug discovery and development [92]. In particular, SKP2, β-TrCP (β-transducin repeat-containing protein), and FBXW7 and their structure-function relationship have been extensively researched, including their over-expression in numerous types of cancers [93]. A variety of anti-tumor inhibitors specifically targeting the SCF E3 complex, such as small-molecule ligands BC-1215 and GS143 (A chemical inhibitor targeting β-catenin signaling) that respectively target Fbxo3 (F-box protein 3) and β-TrCP, have shown pre-clinical efficacy but all failed in clinical trials [94,95]. For example, NRX-2663 strengthens the interaction between β-catenin and its E3 SCFβ-TrCP, although its effectiveness is reduced *in vivo* due to mutations or phos-

Table 2. Therapeutic applications of the ubiquitin code: treatment, targets, diseases, and functions.

Treatment	Targets	Diseases	Functions
Thalidomide, lenalidomide, pomalidomide	CRL4CRBN	Multiple myeloma, mantle cell lymphomas, myelodysplastic syndromes	E3 inhibitor
BC-1215, GS143	Fbxo3 & β-TrCP components	Cancers	
NRX-2663	SCFβ-TrCP	Cancer	
Pevonedistat (MLN4924)	NAE	Myelodysplastic syndrome, acute myeloid leukemia, chronic myelomonocytic leukemia	
Mezigdomide (CC-92480), iberdomide (CC-220), eragidomide (CC-90009)	Cereblon	Relapsed/refractory multiple myeloma, acute myeloid leukemia	
KPG-818	CRL4	Hematological cancers	
GlueTAC	Varies (direct degradation without ubiquitination)	Neurodegeneration, cancer	
AbTACs	PDL1 via RNF43	Cancer	
MTX325	USP30	Parkinson's disease	DUB inhibitor
USP inhibitor	USP30, USP14, USP7, USP1, USP2, USP28, UCHL1, BAP1	Neurodegeneration, cancers	
KSQ-4279	USP1	Advanced solid tumors	
PCAF	CRBN	Inflammatory responses	Proteasome targeting
BTK PROTACs (NX-5948, NX-2127)	CRBN	B cell malignancies, autoimmune diseases	
IRAK4 PROTAC (KT-474)	CRBN	Autoimmune diseases	
ARV-110	Androgen receptor	Prostate cancer	
ARV-776			
ARV-471	Estrogen receptor	Breast cancer	
USP7 PROTAC	USP7	Cancer	
MD-224	MDM2	Cancer (leukemia xenograft tumor regression)	
KT-333	STAT3	Refractory hematologic malignancies	
ARV-102	LRRK2	Parkinson's disease	
LYTACs	Varies (direct degradation without ubiquitination).	Neurodegeneration, cancer	Proteasome/autophagy targeting
Serdemetan	HDM2	Human cell lymphoma, leiomyomas	Autophagy targeting
AUTACs	BRD4, MetAP2, FKBP12, mitochondria	Cancer, etc.	
ATTECs	Varies (direct degradation without ubiquitination).	Neurodegeneration, cancer	
AUTOTACs	Mutant tau, etc.	Neurodegeneration, cancer	

Fbxo3, F-box protein 3; NAE, ND8 E1 Ub activating enzyme; GlueTAC, covalent nanobody-based degrader; AbTAC, antibody-targeting chimera; PDL1, programmed cell death-ligand 1; PCAF, P300/CBP-associated factor; IRAK4, interleukin-1 receptor-associated kinase 4; ARV, androgen receptor; MDM2, murine double minute 2 proto-oncogene; STAT3, signal transducer and activator of transcription 3; LYTACs, lysosome-targeting chimera; HDM2, human homolog of MDM2; AUTAC, autophagy-targeting chimera; MetAP2, methionyl aminopeptidase 2; FKBP12, FK506-binding protein 12; ATTECs, autophagosome-tethering compounds; β-TrCP, β-transducin repeat-containing protein.

phorylation issues [96]. If anything, targeting NAE (ND8 E1 Ub activating enzyme) using pevonedistat (MLN4924) as part of a combinatorial treatment has shown clinical efficacy for myelodysplastic syndrome, acute myeloid leukemia and/or chronic myelomonocytic [97].

Alternatively, a new generation of modulators for the cereblon E3 such as mezigdomide (CC-92480), iberdomide (CC-220), and eragidomide (CC-90009) have shown promising pre-clinical efficacy and have entered phase 1/2 clinical trials for relapsed/refractory multiple myeloma or acute myeloid leukemia [91,98]. Additionally, new small molecule inhibitors targeting MDM2 (murine double minute 2 proto-oncogene) have been discovered, and KPG-818 which interacts with the CRL4, is in Phase I trials for hematological cancers [99]. Serdemetan inhibits p53 degradation by blocking HDM2 (human homolog of MDM2) ubiquitination and is being studied for human cell lymphoma and multiple leiomyomas [100,101]. In neurodegeneration, E3 modulators have been relatively less investigated as a viable drug development strategy.

Compared to that of E3 modulators, pre-clinical development – and entry into the clinical stage – of DUB inhibitors as novel drugs is relatively new. However, DUB inhibitors may, in theory, be relatively easier to develop given that inducing ubiquitination of a neo-native substrate, as opposed to inhibiting its de-ubiquitination, may be more complex from a spatiotemporal and kinetic standpoint [43]. Additionally, hallmark proteins responsible for the pathogenesis of cancer or neurodegeneration more often than not exhibit dysfunctional or absent ubiquitination to prevent their degradation [102,103]. As a result, inhibitors against the USP (ubiquitin specific peptidase) family (USP30, USP14, USP7, USP1, USP2, and USP28), UCHL1 (ubiquitin C-terminal hydrolase L1) and BAP1 (BRCA1 associated deubiquitinase 1) have been investigated as novel drug candidates [102–106]. In neurodegeneration, these inhibitors have been used to enhance peroxisome turnover and mitophagy potentially by accelerating PINK1 (PTEN-induced kinase 1)-dependent generation of phospho-Ser86ubiquitin (USP30), accelerating tau degradation (USP14) or inhibit its aggregation UCHL1, or rescue mutant SOD1 (superoxide dismutase type 1)-mediated neurocytotoxicity (USP7) [107,108]. One such inhibitor (MTX325) has entered a first-in-human phase 1 clinical trial for Parkinson's disease [109]. In cancer, anti-migration, pro-apoptosis and anti-proliferation/cell-cycle properties were observed in cell and murine models of myeloma and SCLC (UCHL1), HCT116 colon tumor (USP7), mutant BRCA-expressing cancer (USP1) and Mino cells (USP2) among others [110–112]. However, despite the abundance of pre-clinical candidates, clinical trials with anti-cancer DUB inhibitors are only just beginning to gain speed. A USP1 inhibitor (KSQ-4279) is undergoing phase 1 trials for patients with advanced solid tumors such as ovarian and triple-negative breast cancers [113].

Targeted Protein Degradation via the Ubiquitin-Proteasome System and Autophagy-Lysosome System

Targeted protein degradation (TPD) represents a next-generation drug development platform designed to overcome the limitations of conventional agonists and ligands by enabling event-driven, rather than occupancy-driven, pharmacology. This innovative approach employs heterobifunctional molecules that intrinsically eliminate target proteins by linking a target-binding moiety to a degradation-inducing component, making it a promising strategy for eradicating disease-associated proteins, including those previously considered undruggable [114]. Heterobifunctional chimeric degraders that comprise a target-binding warhead connected via an intermediate linker to a degradation-inducing ligand/moiety have shown excellent *in vitro* and *in vivo* efficacy in targeted proteolysis of a wide variety of substrates in both cancer and neurodegeneration, and offer an exciting and alternative means to overcome drug resistance, decrease off-target toxicity and side-effects, and expand the pool of druggable targets [115,116]. Below, we review select TPD platforms that are reliant upon ubiquitination for their proteasome-based mechanism of action and discuss the possible roles of ubiquitination in autophagy-based TPD platforms.

PROTAC induces spatiotemporal proximity between an E3 Ub ligase and a target neo-native substrate protein for its ubiquitination and proteasomal degradation [117]. In addition to the advantages that the TPD platform confers, PROTACs in theory can promiscuously match a given E3s with a neo-native substrate and thereby induce its ubiquitination [118]. For example, an E3 Cereblon (CRBN)-based PROTAC targets P300/CBP-associated factor (PCAF) and GCN5 (general control non-depressible 5), reducing inflammatory responses in LPS-stimulated macrophages and dendritic cells. Additionally, PROTACs targeting BTK (NX-5948) and interleukin-1 receptor-associated kinase 4 (IRAK4) (KT-474) via CRBN are in Phase 1 and 2 clinical trials for B cell malignancies and autoimmune diseases [119,120]. In addition to inflammation, research on PROTACs is particularly active in cancer treatment. Androgen receptor (ARV)-471 is PROTAC targeting the estrogen receptors, currently in 3 clinical trials for breast cancer. ARV-110 and ARV-766 are PROTACs as a treatment for metastatic castration-resistant prostate cancer, which is in phase 1/2 and 3 trials [121,122]. KT-333, a STAT3 (signal transducer and activator of transcription 3) degrader that regulates cell growth and division, is revealed in phase 1a/b trials that it is well tolerated for refractory hematologic malignancies and solid tumor patients [3,4].

Additionally, the first PROTAC-based DUB degrader targets USP7 via its ubiquitination and degradation in a CRBN (E3s)-dependent manner for regulating the MDM2/p53 pathway in various cancers [104]. The PROTAC MD-224 promotes the swift degradation of

MDM2 following its CRBN/cullin4 E3 complex-dependent ubiquitination, resulting in tumor regression in an *in vivo* model of leukemia xenografts [123]. Other PROTACs, including another BTK PROTAC (NX-2127) are in Phase I trials, along with a BCL-xL (B-cell lymphoma extra large) degrader (DT2216) [124].

For neurodegeneration, PROTACs targeting hallmark aggregation-prone proteins such as tau or α-synuclein in a CRBN or VHL (von hippel-lindau)-dependent manner have been proposed in a proof-of-concept, with several degraders having entered pre-clinical development [125,126]. However, due to the intrinsic limitations of the proteasome in degrading protein-protein complexes, oligomers, and aggregates, it remains to be seen whether PROTACs will be able to degrade pre-formed fibrils that are often found in neurodegeneration [127]. On the other hand, PROTACs targeting kinases and other regulator proteins that modify the hallmark aggregation-prone proteins have arguably seen more successful and rapid development. The ARV-102 PROTAC targeting LRRK2, whose phosphorylation of ASK1 has been pathologically associated with neuronal cell death in Parkinson's disease, has entered phase 1 trials [128–130]. However, given that the only viable E3s until now have been limited largely to VHL or CRBN, questions remain on whether E3s can truly be promiscuous, or if there is a designated clientele of neo-native substrates. To that end, further mechanistic elucidation and the structure-function relationship of E3s for ubiquitination remain ever-important issues.

In other TPD modalities, the role of ubiquitination remains an open question. Antibody-targeting chimera (AbTAC) recruits membrane-bound E3 RNF43 to degrade transmembrane PDL1 (programmed cell death-ligand 1) by acting as a bi-specific antibody for cell surface proteins [131]. While the RNF43-AbTAC-PDL1 complex is internalized into the cell and degraded within the lysosome, it is not confirmed whether RNF43 ubiquitinates PDL1 for its endocytosis [131,132]. Autophagy-targeting chimera (AUTAC), the first reported autophagy-dependent TPD modality, harnesses the interaction between ubiquitin chains and autophagy cargo receptors to selectively degrade proteins and organelles. AUTAC utilizes the nucleotide 8-nitro-cGMP (S-guanylation) (8-Nitroguanosine 3′,5′-cyclic monophosphate-S-guanylation) as a degradation-inducing tag, facilitating K63-linked poly-ubiquitination and subsequent macroautophagic/lysosomal hydrolysis of oncoproteins such as BRD4, MetAP2 (methionyl aminopeptidase 2) and FKBP12 (FK506-binding protein 12), as well as organelles including mitochondria [133]. The K63-linked Ub chains conjugated on the target cargoes have been shown to recruit autophagy cargo receptors such as p62/SQSTM1 and NBR1, which directly bind the LC3 (microtubule-associated protein 1A/1B light chain 3) protein on autophagic membranes [134]. However, it is not clear what E3s are specifically being recruited by the S-guanylation tag, and whether a similar lack of promiscuity as seen with E3-target protein combinations in PROTACs is observed with AUTACs. In lysosome-targeting chimera (LYTACs), autophagosome-tethering compounds (ATTECs) and a covalent nanobody-based degrader (GlueTAC), target proteins-of-interest are directly transferred to the autophagosome or the lysosome and so far do not seem to require ubiquitination [135].

Autophagy-targeting chimera (AUTOTAC) presents a novel concept where the degradation-inducing moiety is not only a ligand (as is the case for other TPD modalities) but also an agonist for the archetypal autophagy cargo receptor p62/SQSTM1. This is made possible by a peptidomimetic agonist (termed autophagy-targeting ligand; ATL) that mimics the binding mode of N-terminal arginine residue (Nt-Arg) to the p62-ZZ domain within the autophagic Arg/N-degron pathway [136,137]. Upon Nt-Arg binding to its ZZ domain, p62 undergoes a conformational change wherein its PB1 domain is freed from a regulatory linker, facilitating cis- and trans- self-oligomerization. Consequently, p62 undergoes a biological activation resulting in autophagosome biogenesis and increased delivery (flux) of ubiquitinated cargoes (recognized by its UBA domain) to autophagic membranes [136,137]. Proof-of-concept of the AUTOTAC platform using stably-expressed mutant tauP301L showed that the UBA domain of p62 is not required for AUTOTAC-induced complex formation between p62 and pathological tau [138]. This suggests that the ternary complex formation between the degradation-inducing protein and the target protein-of-interest, which is critical to E3-dependent TPD modalities, is not required for AUTOTACs (i.e., each warhead need only bind its intended target for AUTOTAC to function). Based on these results, ATC102 has entered Phase 1 clinical trials in South Korea as a first-in-class tau aggregate degrader for tauopathies, including Alzheimer's disease and progressive supranuclear palsy (PSP). However, it will be interesting to see if conformational and biological activation of p62 via its ligandable ZZ domain can enhance UBA domain-dependent recognition of Ub chains. If so, and with enough spatiotemporal proximity between p62 and the target protein, AUTOTACs may facilitate recognition of Ub chains on target proteins-of-interest for a secondary (but not required) measure of target engagement.

Conclusion

The ubiquitin (Ub) code has been researched for more than 30 years since its first discovery in 1975. As one of the most complex and far-reaching PTMs, ubiquitination boasts an impressive array of not only target substrates and key regulators but also various ubiquitin chains, including monoubiquitylation, multi-monoubiquitylation, homotypic polyubiquitylation, and heterotypic polyubiquitylation. Such diversity is only compounded by the independent molecular structures of each ubiquitin linkage, rang-

ing from open conformations assembled by unique E3s to compact configurations with sequestered hydrophobic patches at the interdomain interface. Deciphering the different roles of these different ubiquitin linkages will not only deepen our understanding of the ubiquitin-proteasome system and the autophagy-lysosome system, but also pave the way for its pharmacological modulation for drug development against neurodegenerative diseases, cancer, and immune disorders among others. Particularly, the roles of homotypic Ub linkages excluding K48 and K63 in the pathogenesis and progression of diseases remain relatively less clear. Elucidating the circumstances and mechanisms of if and how certain types of Ub linkages promote either proteasomal or lysosomal hydrolysis of degradation-tagged proteins may provide clues and new druggable targets. Similarly, heterotypic chains are even less well-understood and most likely represent the biggest challenge due to their sheer complexity and possible conformations. Investigating the precise molecular principles and the identity of E3s responsible for heterotypic ubiquitination may facilitate the first-in-class development of next-generation E3 modulators. Such efforts will synergize with the development and characterization of current E3 modulators, DUB inhibitors and especially molecule glues and targeted protein degraders.

Availability of Data and Materials

Not applicable.

Author Contributions

SBK, YTK, and CHJ contributed to the conceptualization and supervision of the manuscript. HYK, SJL, JSL, and SBK designed the graphical illustration. JSL created a summary table. All authors were involved in the drafting. All authors contributed significantly to editorial changes of important content. All authors have read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

Yong Tae Kwon and Chang Hoon Ji are consultants/proctors for AUTOTAC Bio Inc. All other authors have declared no conflicts of interest related to the content of this paper.

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