

Chapter

Functional Significance of the E3 Ubiquitin Ligases in Disease and Therapeutics

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Abstract

E3 ubiquitin ligases of which there are >600 putative in humans, constitute a family of highly heterogeneous proteins and protein complexes that are the ultimate enzymes responsible for the recruitment of an ubiquitin loaded E2 ubiquitin-conjugating enzyme, recognise the appropriate protein substrate and directly or indirectly transfer the ubiquitin load onto the substrate. The aftermath of an E3 ligase activity is usually the formation of an isopeptide bond between the free carboxylate group of ubiquitin's C-terminal Gly76 and an ϵ -amino group of the substrate's Lys, even though non-canonical ubiquitylation on non-amine groups of target proteins have been observed. E3 ligases are grouped into four distinct families: HECT, RING-finger/U-box, RBR and PHD-finger. E3 ubiquitin ligases play critical roles in subcellular signalling cascades in eukaryotes. Dysfunctional E3 ubiquitin ligases therefore tend to inflict dramatic effects on human health and may result in the development of various diseases including Parkinson's, Amyotrophic Lateral Sclerosis, Alzheimer's, cancer, etc. Being regulators of numerous cellular processes, some E3 ubiquitin ligases have become potential targets for therapy. This chapter will present a comprehensive review of up-to-date findings in E3 ligases, their role in the pathology of disease and therapeutic potential for future drug development.

Keywords: classification, disease, E3 ligases, dysfunction, mechanism, therapeutics, ubiquitin

1. Introduction

1.1 E3 ubiquitin ligases in Ubiquitylation

E3 ubiquitin ligases play crucial roles in ubiquitin conjugation to substrates and therefore ubiquitin signalling. Protein ubiquitylation (also referred to as ubiquitination) is a dynamic multifaceted post-translational modification in which ubiquitin is covalently attached to a specific protein target in a three-step enzymatic cascade involving the action of an E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and E3 ubiquitin ligase [1]. An E3 ubiquitin ligase is the ultimate enzyme which directly or indirectly catalyses the transfer and subsequent ligation of an ubiquitin monomer to a specific target protein (i.e., the substrate). The aftermath

of ubiquitylation is usually the formation of an isopeptide bond catalysed by the E3 ligase between the free carboxylate group of ubiquitin's C-terminal Gly76 and an ϵ -amino group of the substrate's Lys [2]. However, non-canonical ubiquitylation in which ubiquitin is conjugated to the target protein's N-terminal amino group of Met has been observed in more than 22 proteins including ubiquitin itself [3–5]. There is also increasing evidence of ubiquitylation on non-amine groups of target proteins including the thiol groups of Cys [6] and the hydroxyl groups of Thr, Ser and probably Tyr [7].

In the initial ATP-dependent activation step of ubiquitylation, E1 catalyses the acyl-adenylation of ubiquitin's C-terminus for conjugation by forming an ubiquitin-adenylate intermediate (**Figure 1**). In the second step, ubiquitin is transferred to the active site Cys residue resulting in the formation of a thioester linkage between the C-terminal carboxyl group of ubiquitin and the E1 Cys sulphhydryl (-SH) group with a consequential release of AMP [8]. Ubiquitin is then transferred from the ubiquitin-adenylate intermediate in the subsequent transthiolation reaction to the -SH group of the catalytic Cys of an E2 enzyme [9]. In the final ligation step, E3 binds both the target protein and the ubiquitin-charged E2 and facilitates the transfer of ubiquitin from E2 to the ϵ -amino group of a Lys in the target protein. E3 enzymes thus function as the substrate recognition modules and consequently determine substrate specificity of ubiquitin conjugation, while the E2s determine the chain type of polyubiquitylation [10, 11]. Human cells possess two E1 enzymes (UBA1, UBA6), approximately 40 E2 enzymes and more than 600 putative E3 enzymes [12–15]. This notwithstanding,

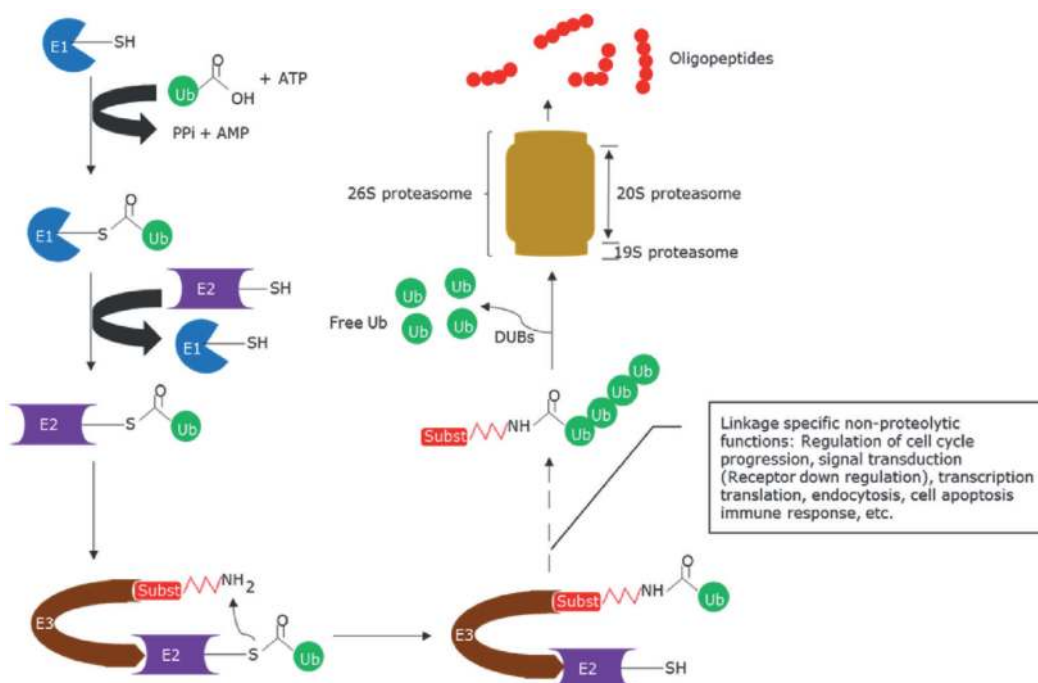


Figure 1.

The ubiquitin-mediated proteasome proteolytic pathway. Ubiquitin modification is an ATP-dependent process carried out by three classes of enzymes, E1, E2 and E3 which specifically target proteins to either change their activity or their location, or in some cases earmark target proteins for proteasome-mediated degradation. E1 forms a thioester bond with ubiquitin which allows subsequent transfer of ubiquitin to E2, followed by the E3 ligation of an isopeptide bond between the carboxyl-terminus of ubiquitin and a Lys residue on the substrate protein. Isopeptide bonds between ubiquitin and ubiquitin or ubiquitin and other target proteins can, however, be hydrolysed by deubiquitylating enzymes (DUBs).

ubiquitin-chain elongation factors (E4 enzymes e.g., the mouse double minute 2 homologue (mdm2)) which extend pre-existing polyubiquitin chains on substrate proteins (e.g., the tumour suppressor p53) have also been reported [16, 17].

1.2 Cellular functions of ubiquitylation in eukaryotes

Ubiquitin's versatility in the regulation of cellular processes is by virtue of its ability to covalently modify other proteins [18]. Post-translational modifications, which are usually covalent and reversible, may alter the properties and therefore, the functions of the modified protein. A major function of ubiquitin is regulation of the degradation of other specific proteins, literally referred to as '*the molecular kiss of death*' [19–21]. The 26S proteasome is responsible for degradation and recycling of unwanted, short-lived, inactive, oxidised, unfolded and/ or misfolded proteins (**Figure 1**) [22, 23]. Proteasomal proteolysis enables the cell to rid itself of these misfolded or damaged proteins and re-adjusts the concentration of essential proteins so that cellular homeostasis is maintained [24]. For a condemned protein to be recognised by the 26S proteasome, a polyubiquitin chain of at least four ubiquitin molecules must be covalently attached to a substrate Lys residue [25]. The proteasome is a barrel-shaped multisubunit protein complex, consisting of two chambers within which proteolysis occurs. The eukaryotic 20S proteasome is the catalytic portion of the 26S proteasome. The 19S regulatory complex mediates substrate recognition and substrate unfolding (**Figure 1**). Exploration of the ubiquitin system in eukaryotes has shown that the chemical modification of proteins by ubiquitin is an incredibly important post-translational event that is crucial to numerous complicated cellular processes beyond the ubiquitin proteasome system. Ubiquitin conjugation also plays a wide variety of roles that are independent of proteasomal degradation [2, 18]. The ubiquitin code modulates cell cycle progression, differentiation, signal transduction, protein–protein interactions, and intracellular protein trafficking. Ubiquitin regulates subcellular localisation of proteins where they control other protein function and cell mechanisms. Transcription, autophagy, inflammatory signalling, modulation of enzymatic activity, DNA repair, heat shock responses, chromatin structure, embryogenesis, cell apoptosis, virus budding, vacuolar protein sorting, inflammatory response and receptor endocytosis are also regulated by ubiquitin-mediated signalling [26, 27]. The cellular environment must constantly maintain homeostatic conditions. The ubiquitin-proteasome system is the major ubiquitin-mediated process recognised as the cellular quality control system [28]. Deubiquitylating enzymes (DUBs) cleave isopeptide bonds releasing free ubiquitin residues from protein substrates. As such DUBs are also implicated in the regulation of cellular events by trimming (poly) ubiquitin conjugates and recycling ubiquitin monomers.

1.2.1 Ubiquitin modifications

The functional consequences of ubiquitylation vary because of recognition by different ubiquitin-binding modules which can distinguish different polyubiquitin modifications. Monoubiquitylation is the conjugation of a single ubiquitin molecule to a single Lys of the target protein. Multimonoubiquitylation occurs when a target protein is tagged with more than one single molecule of ubiquitin. In polyubiquitylation, the target protein is tagged with an ubiquitin chain linked through the C-terminal Gly of each ubiquitin unit and a specific internal Lys of the previously attached ubiquitin through sequential rounds of ubiquitylation (**Figure 1**). The presence of N-terminal

Met including seven Lys residues per ubiquitin moiety on which polyubiquitylation occurs, empowers ubiquitin with potential to exhibit diverse and highly complicated linkage specific post-translational modification of target proteins [29]. Unlike homogenous chains of ubiquitin which contain a single ubiquitin-ubiquitin linkage type, heterogeneous polyubiquitin chains contain more than one linkage type. In mixed polyubiquitin chains therefore, one linkage type can be extended by a second type, forming a non-branched structure. In branched polyubiquitin chains however, different linkage types form one or more branches (i.e., multiple Lys residues in the same ubiquitin) [29].

Monoubiquitylation has been implicated in the endocytic trafficking of certain cargo proteins, e.g., small GTPases and receptors (e.g., Epidermal Growth Factor Receptor, EGFR) to specific cellular compartments at different stages of the endocytic pathway. Monoubiquitylation has also been implicated in gene expression and DNA repair [30–33]. Multimonoubiquitylation is important for receptor endocytosis [34]. Lys48-linked polyubiquitylation (i.e., polyubiquitin chains linked via Lys48 of the proximal ubiquitin to the next ubiquitin moiety in the chain) has predominantly been linked to targeting proteins for proteasomal degradation [35]. Lys63-linked polyubiquitin chains function as scaffolds to assemble signalling complexes e.g., activation of transcription factor NF- κ B involved in inflammatory and immune response, DNA damage tolerance, the endocytic pathway and ribosomal protein synthesis [36]. It has been demonstrated that unanchored (substrate-free) Lys63-linked polyubiquitin chain assembled via UBE2N/UBE2V1 (E2) and TRAF6 (E3) enzymes activate the NF- κ B pathway by activation of TAK1 which in turn phosphorylates and activates I κ B kinase (IKK) [37]. Linear linkage via *N*-terminal Met is also reported to regulate NF- κ B signalling [38–40].

2. Types of E3 ubiquitin ligases

Based on considerations of structure, chemistry, and mechanisms by which ubiquitin is transferred to the substrate, four families of E3 ubiquitin ligases are distinguishable. These include homologous to the E6AP carboxyl terminus (HECT), Really Interesting New Gene (RING)-finger/U-box, RING-between RING (RBR) and the recently characterised plant homeodomain (PHD)-finger ligases.

2.1 RING-finger E3 ligases

The RING E3s constitute the largest family of E3 ligases. RING-finger E3 family of ligases include the U-box ligases. Although able to promote the formation of polyubiquitin chains, RING-Finger/U-box E3s lack a catalytic site and hence, do not participate directly in catalysis. RING-type E3s are characterised by the presence of the canonical Cys3HisCys4 amino acid motif (i.e., the RING domain) and consequently mediate the direct transfer of ubiquitin from E2 ~ ubiquitin complex to the substrate (**Figure 2a**). Each canonical Zn finger (Cys3HisCys4) type domain binds two Zn²⁺ ions which are critical to its stability. RING E3 ligases can exist and act as single-subunits e.g., CHIP/Stub1, mdm2, RNF4, RNF114, UBE4B (**Figure 2a**) or may be assembled on a Cullin scaffold to form multiple subunits. Multi-subunit RING E3s contain a substrate receptor (responsible for substrate specificity) at the *C*-terminus, an adaptor(s), a Cullin- and a RING-box at the *N*-terminus (**Figure 2b**). The APC (anaphase-promoting complex) and the SCF (Skp1-Cullin-F-box protein) complex are examples of multi-subunit RING

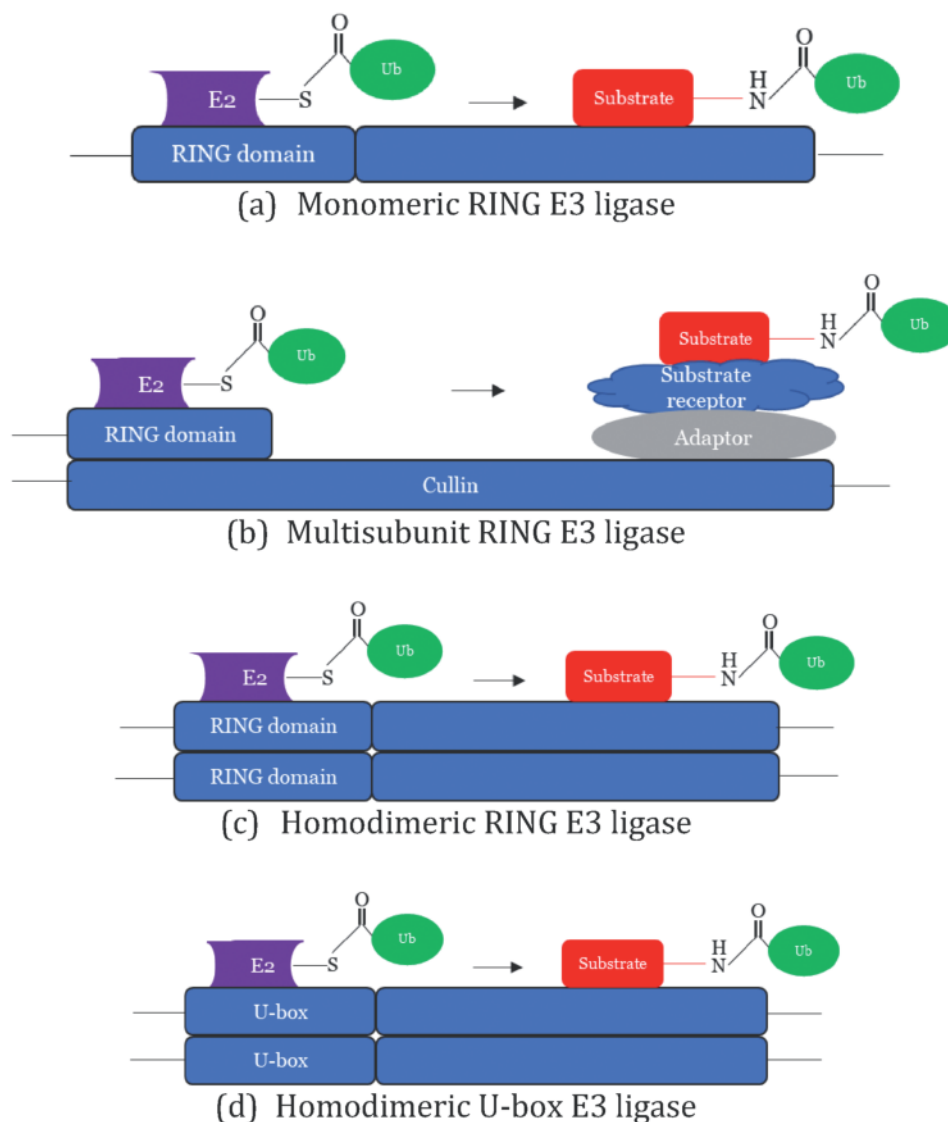


Figure 2.
Illustration of RING/U-box E3 ligase types and their mechanism of ubiquitin transfer to target proteins.

E3s involved in substrate recognition and are the most abundant type of RING E3s [41, 42]. RING-finger E3 ubiquitin ligases regulate several cellular processes, including cell metabolism, cell proliferation, apoptosis, differentiation, and DNA repair making them potential targets for anti-cancer drug development. There is evidence to suggest that RING-finger domains can also allosterically activate the E2 enzymes [43]. RING E3 ligase activity is often regulated by neddylation, phosphorylation and protein-protein interactions with small molecules among others.

2.1.1 U-box E3 ligases

The U-box proteins contain a U-box domain of ~70 amino acids which lacks the characteristic Zn chelating Cys and His residues in RING-finger domain and are characteristically stabilised by a network of H-bonds within each loop, flanked by a central α -helix [44–46]. U-box E3s are more abundant in plants than animals [47].

Both RING and U-box domains are responsible for binding the ubiquitin-charged E2 and stimulating the transfer of ubiquitin to substrate (**Figure 2**). Additionally, RING and U-box E3 ligases can function as monomers (**Figure 2a**), homodimers (**Figure 2c and d**), or heterodimers. In a homodimer, each monomer can bind an E2, but apparently not the case in the heterodimeric RINGs.

2.2 PHD-finger E3 ligases

Another RING-related family of E3s are the PHD E3 ligases. Unique sequence and structural signatures that distinguish the PHD-finger from RING fingers have been demonstrated indicating that the PHD-fingers function primarily as E3 ligases that promote protein degradation and constitute a distinct class of E3 ligases. The PHD or leukaemia-associated-protein (LAP) domain resembles the RING finger domain [48–50]. It also has the eight conserved metal binding ligands, Cys4HisCys3 consensus, with similar spacing [51] however, it represents a variant of the RING finger. An example of a PHD domain E3 ligase is Mekk1 kinase. The second *N*-terminal Cys-rich domain of Mekk1 kinase has been shown to exhibit E3 ubiquitin ligase activity toward ERK1/2 and is involved in the down regulation of the MAP kinase cascade [52]. The PHD domain is found in many proteins involved in chromatin-mediated transcriptional regulation [53], however, very little is known about their precise functions.

2.3 HECT E3 ligases

Unlike the RING-finger E3s, the HECT type E3s instead contain a conserved C-terminus HECT domain which consists of a larger *N*-terminal bi-lobe architecture encompassing the E2-binding site and a smaller *C*-terminal lobe which comprises the active-site Cys residue (**Figure 3a**). Their reaction cycle consists of three steps; binding to an E2 ~ ubiquitin, transiently loading ubiquitin on themselves via formation of a covalent ubiquitin-thioester linked intermediate with the catalytic Cys, before transferring the ubiquitin molecule onto the target protein [54]. These two lobes are connected by a flexible hinge region, which is critical for juxtaposing the catalytic Cys residues of the E2 and E3 during ubiquitin transfer (**Figure 3a**). A conformational change involving an alteration in the relative orientation of the two lobes is thought to facilitate the transthiolation reaction [54–56]. Whereas the *C*-terminal HECT domain is responsible for E3 catalytic activity, the *N*-terminal portion is highly variable and determines the substrate specificity. HECT domain containing E3 ligases are estimated at 30 in mammals and 28 in humans out of the over 600 E3s [57]. Human HECT E3s can further be categorised into subfamilies based on the mode of protein–protein interactions of their *N*-terminal domain extensions which determine their substrate specificity. These include the Nedd4 family, which are characterised by the presence of tryptophan-tryptophan (WW) motifs, the HERC (HECT and RCC1-like domain) family, which contain one or more regulators of chromosome condensation 1 (RCC1)-like domains (RLDs), and the SI (ngle)-HECT/“other” HECT E3s lacking either WW or RLDs domains but contain various other domains. These subfamilies have been extensively reviewed [58]. HECT domain E3s play several roles. They determine the specificity of ubiquitylation and mediate the trafficking of many receptors. They are also regulators of immune response and several signalling pathways in cell proliferation [59]. Naturally, the intrinsic catalytic activity of HECT E3s is normally folded together in an autoinhibited state by intramolecular interactions between domains which can be released to an active form of the enzyme in response to various signals by unfolding to expose the catalytic Cys.

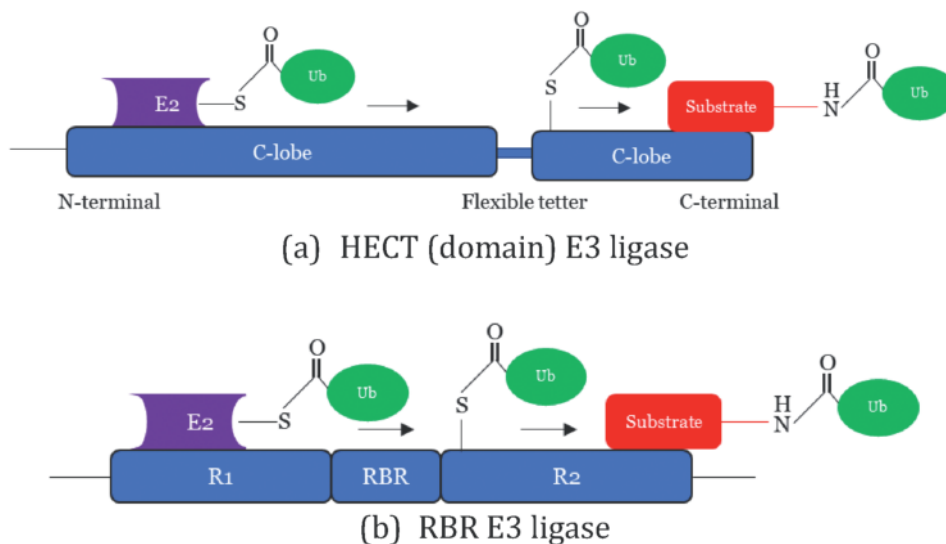


Figure 3.
Mechanism of ubiquitin transfer to target protein by (a) HECT and (b) RBR E3 ubiquitin ligases.

2.4 RBR E3 ligases

RBR E3 ligases employ the characteristic RING-HECT hybrid mechanism [60–65]. Like the HECT E3 ligases, the RBR E3 ligases catalyse the transfer of ubiquitin from E2 to the substrate through a three-step reaction where the RBR E3 first binds the E2, transfers the ubiquitin load to its catalytic Cys and subsequently to the substrate (**Figure 3b**). RBR E3 ubiquitin ligases differ from RING-type E3 mainly because they possess an active site which is absent in other RING-type E3s. However, like RING E3s, the RBR E3 ligases have four RING Zn²⁺ finger domains. Each of these domains coordinates two Zn²⁺ ions through His and Cys residues. They include the canonical Cys3HisCys4-type RING (named RING1) domain as in RING E3s, that binds the E2 enzyme followed by; in-between-RING (IBR) domain and RING2 domain which contains the active site Cys residue (**Figure 3b**). The name RBR was derived from the presence of two predictable RING1 and RING2, separated by an IBR (i.e., the RING1-IBR-RING2 module). Though RING2 domain possesses the catalytic Cys (**Figure 3b**), it does not conform to the canonical RING E3 structure, and it has also been called Rcat (required-for-catalysis) domain. The IBR domain is conserved among RBR E3 family of ligases. Its specific function remains elusive. The IBR domain adopts the same structural fold as RING2 domain, however, it lacks the essential catalytic Cys residue and is sometimes referred to as the BRcat (benign-catalytic) domain [66]. The Zn²⁺ ions bound within RING domains are also reported to be essential for structural stability and proper regulation of its intrinsic enzymatic activity. Their removal from parkin for instance, result in near complete unfolding of the protein [67, 68]. HHARI and Parkin were initially characterised to have the hybrid mechanism [64]. TRIAD1, RNF144A, HOIP, and HOIL-1 L have later been characterised to employ the same RING-HECT hybrid mechanism [69]. RBR RING domains are also normally involved in intramolecular interactions between amino acids of different domains that keep the enzyme in a coiled autoinhibited state. Through different molecular mechanisms such as phosphorylation and protein-protein interactions, the uncoiling of closed-compact or folded autoinhibited states of RBR E3 ligases may be triggered thereby exposing the catalytic sites and increasing the intrinsic E3 ligase activity.

3. Implication of the E3 ubiquitin ligases in disease and therapy

3.1 Role of E3 ligases in disease

Aberrations of ubiquitin signalling are often associated with pathogenesis of several diseases and genetic disorders [58, 70–73]. Errors in ubiquitin signalling processes result in defective autophagy and mitophagy, DNA repair mechanisms, NF- κ B signalling, etc. [74]. Subsequently, associated diseases including Parkinson's, Amyotrophic Lateral Sclerosis, Alzheimer's, cancer, systemic lupus erythematosus, rheumatoid arthritis, and inflammatory bowel diseases (e.g., Crohn's disease and ulcerative colitis) among several others may ensue [75, 76]. Ubiquitin E3 ligases, most notably RING finger and RING finger-related E3s are fundamental to the specificity of the ubiquitin proteasome system. Many RING finger E3s are implicated in either the suppression or the progression of cancer and cancer chemoresistance [58, 75, 77, 78]. Due to limited space only some key E3-linked diseases have been explained below.

Parkin, a RBR E3 ligase functions in the covalent attachment of ubiquitin to specific substrates e.g., outer mitochondrial membrane proteins – Mfn1, Mfn2, and Miro GTPases [62]. Parkin is involved in protein degradation, collaborating with the ubiquitin-conjugating enzyme, UbcH7 [79, 80]. Even though much of the aetiology of Parkinson's disease remains largely unknown, malfunctioning of PINK1 and/or parkin causes accumulation of damaged mitochondria, which trigger familial parkinsonism. Parkinson's disease (named after Dr. James Parkinson, AD1783–1824) is a neurodegenerative movement disorder caused by the progressive death of dopamine producing neurons in the substantia nigra pars compacta of the mid-brain [81]. The characteristic symptoms of Parkinson's disease include muscle tremor, muscle rigidity, slowness of movement (bradykinesia) and postural instability [82–85]. Parkinson's disease is the second most common neurodegenerative disease after Alzheimer's, affecting predominantly, individuals above 65 years even though earlier onset have been reported [79, 84, 86, 87]. Early-onset Parkinson's disease (EOPD - onset in individuals before 50 years) which account for approximately 5–10% of all cases of Parkinson's disease are attributable to monogenic causes [88, 89]. Mutations in coding regions of *PARK2* gene are often implicated as the most common cause of autosomal-recessive juvenile Parkinsonism (AR-JP) (EOPD), followed by *PINK1* gene variants resulting in a loss of kinase function which apparently, is required for parkin phosphorylation and subsequent activation [84, 90–93]. This renders PINK1 and parkin proteins vital targets for drug development. Greater than 120 pathogenic Parkinson's disease mutations are spread throughout parkin's four domains, indicating the critical functions for each of the individual domains [94].

The multi-functional pathways regulated by RING-type E3 ubiquitin ligases in inflammatory signalling and consequential inflammatory bowel disease have been expansively reviewed by several researchers [73]. Inflammatory bowel disease is characterised by inflammation of the digestive tract and patients present with anomalies in gut microbiota composition e.g., increased levels of harmful bacterial strains, reduced levels of bacterial diversity and protective probiotics. These trigger proinflammatory intestinal pathogenic immune responses which in turn induce intestinal mucosal inflammation. Examples of implicated RING E3 ligases in inflammatory bowel diseases include, TRAF5, TRAF3, TRAF2, UHRF1, RNF183, RNF40, RNF20, RNF170, and RNF186 [73]. For instance, RNF170 E3 ligase ubiquitylates TLR3 for proteasomal degradation. TLR3 is a pattern recognition receptor which recognises pathogen-associated molecular patterns (PAMPs) of lipopolysaccharides,

flagellin, and microbial nucleic acids and triggers activation of downstream effectors in innate immune responses. Proteasomal degradation of TL3 therefore suppresses TLR3-mediated innate immunity in macrophages thereby promoting inflammatory diseases [95]. Several sites of the NF- κ B pathway are regulated by ubiquitylation. NF- κ B constitutes a family of conserved transcription factors well known to regulate several cell processes particularly inflammatory responses, cell proliferation and apoptosis. NF- κ B signalling is therefore associated with several diseases such as asthma, arthritis, cancer, etc. [96]. For instance, RNF183 upregulates proinflammatory responses via NF- κ B signalling by ubiquitylating I κ B α - the inhibitor of c-Rel/p50 heterodimer, for proteasomal degradation which may induce intestinal mucosal inflammation [97]. Receptors that stimulate the NF- κ B pathway following various stimuli include CD30, CD40, RABK, TNF- α , TCR, and TLRs. In contrast, TRAF2 and TRAF3 catalyse Lys48-linked ubiquitylation of c-Rel and interferon regulatory factor 5 thereby signalling their degradation by the proteasome. This in turn inhibits biosynthesis of proinflammatory cytokines, thereby down regulating immune responses in macrophages [98, 99].

The roles of E3 ubiquitin ligases in the RTK pathway (e.g. EGFR) and MAPK pathway and other oncogenic/tumour suppressive signalling pathways in glioblastoma have been expounded including validation of the potential of E3 ligases as future therapeutic interventions for glioblastoma treatment [72]. Glioblastoma is a malignant brain tumour which is characterised by a mutation in the metabolic enzyme isocitrate dehydrogenase 1 with limited treatment options [100]. In glioblastoma, 67% of cases have mutation in at least 1 RTK and about 20% of classical tumours express a truncated form of EGFR (EGFRvIII). RTK signalling is down regulated by several E3 ligases such as Cbl, Chip and parkin [72]. Ubiquitylation of EGFR by Cbl E3 ligase results in clathrin-mediated internalisation of the receptor and subsequent sorting into lysosomes where the receptor is degraded and therefore reduces EGFR signalling in glioblastoma. LZTR1 is the substrate recognition domain of a Cul3 E3 ubiquitin ligase complex. Mutations in LZTR1 are associated with schwannomatosis and Noonan syndrome in which loss of LZTR1 function drives de-differentiation and proliferation of cells. LZTR1 is also mutated or deleted in about 4% or 20% of glioblastoma cases respectively, where mutations of LZTR1 increase Ras-dependent proliferation of cells coupled with increased resistance to tyrosine kinase inhibitors (glioblastoma chemotherapy) because of enhanced MAPK signalling [101, 102]. The MAPK pathway is a commonly mutated pathway in human cancers. It upregulates cellular phenotypes such as proliferation, differentiation, migration, and invasion.

The BRCA1 RING-type E3 ubiquitin ligase is a human tumour suppressor gene and plays critical roles in DNA repair. Mutation of BRCA1 is associated with the inherited predisposition for breast and ovarian cancers [102]. The E3/E4 ubiquitin ligase, mdm2 is an important negative regulator of the p53 tumour suppressor gene as mentioned earlier. p53 protein regulates the cell cycle, DNA repair and induces cell apoptosis, hence it functions as a tumour suppressor. Mdm2 serves as an E3 ubiquitin ligase of p53. This implies that increased activity of the mdm2 oncogenic protein via augmented mdm2 expression induces tumorigenicity especially those 50% that retain wild-type p53. In addition, inactive mdm2 results in increased cellular levels of p53, which is detrimental to cells and may accelerate the ageing process by excessive apoptosis [103]. It has also been reported that SIAH2 is a RING-finger E3 ligase which contributes to the progression of various malignant tumours, including breast carcinoma, lung and prostate tumours, and oral cancer [104–106]. Furthermore, the E3 ligase, c-Cbl has also been demonstrated to be frequently dysregulated in

myelodysplastic myeloproliferative neoplasms and additionally associated with myelodysplastic syndromes, myeloid neoplasms, and primary colorectal cancer [75].

Multiple studies have implicated several E3 ubiquitin ligases in ovarian cancer chemoresistance which hamper improvement of ovarian cancer patient outcome through degradation of various chemoresistance-related substrates in ovarian cancer. Among several studies, [107] demonstrated that NEDD4-2 protein (an E3 ligase that regulates endocytosis and lysosomal degradation of ENaC and other channels) expression is reduced in invasive ovarian epithelial cancer tissues compared with non-cancer ovarian tissue suggesting an important role of NEDD4-2 in the regulation of chemoresistant ovarian cancer [58]. Indeed Nedd4-1 and Nedd4-2 E3 ligases have been demonstrated to mediate numerous pathophysiological processes [108]. Briefly, Nedd4-1 mediates endosomal trafficking of receptor tyrosine kinases, such as EGFR and fibroblast growth factor receptor (FGFR), by ubiquitylating endocytic or vesicle sorting proteins. Nedd4-1 is overexpressed in lung epithelial cells and is associated with lung cancer progression [109]. NEDD4-1 can promote Alzheimer's disease by weakening synaptic strength through ubiquitylation of AMPAR (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) and cytoplasmic internalisation. Nedd4-2 knockout mice reportedly die perinatally due to failure of the pups to breathe resulting from increased ENaC expression, extensive fibrosis, and infiltration of inflammatory cells in the alveolar spaces [110]. This indicates the important regulatory functions of Nedd4-2 in the respiratory system. Ubiquitylation of ENaC by Nedd4-2 E3 ligase through direct binding to its conserved proline-rich PY motif in the C-terminals results in sodium current decrease and associated lung oedema [111]. UBR5/EDD is an E3 ligase that mediates the accumulation of ubiquitylated-H2A and -H2AX at DNA damage sites, and G2 checkpoint control. UBR5 is reported to enhance cell survival and cisplatin resistance by regulation of expression of the pro-survival protein myeloid cell leukaemia sequence 1 (Mcl-1) and is an undesirable prognostic factor for patients with serous epithelial ovarian cancer [112]. UBR5 influences ovarian cancer cell cisplatin resistance by mediating MOAP-1 ubiquitylation and degradation through cooperation with Dyrk2 kinase [113].

BCL6 proto-oncogene is the transcription factor implicated in the pathogenesis of human B-cell chronic lymphocytic leukaemia. Deregulation of BCL6 expression or increased degradation are pathogenic events in many lymphomas. Mutation of the multi-subunit SCF FBXO11 E3 ligase is associated with the development of B-cell lymphomas. FBXO11 is the substrate recognition component of the ligase and functions as a tumour suppressor by targeting BCL6 transcription factor for proteasomal degradation [114, 115]. BCL6 binds to specific DNA sequences and down regulates transcription of a variety of genes involved in B-cell development, differentiation and activation including the transcription of STAT-dependent IL-4 responses of B-cells [116].

FANCL is a multisubunit E3 ubiquitin-protein ligase that monoubiquitylates FANCD2 and FANCI, a key step in the DNA damage repair pathway. FANCL contains a RING finger-like PHD domain with E3 activity [78, 117]. Mutations in 8 out of 13 components of the FANCL complex cause Fanconi anaemia (FA). Individuals with this condition experience severe multiple congenital and haematological abnormalities including predisposition to development of a variety of cancers [118]. Individuals may experience congenital skeletal anomalies especially of thumb and forearm. They also display endocrine abnormalities, short stature correlating to growth hormone production and hyperthyroidism among several other anomalies [119].

Mutations in von Hippel–Lindau (VHL), the substrate recognition component of Cullin RING E3 ubiquitin ligase (CRL2VHL), results in an autosomal-dominant familial VHL syndrome. This implies that a mutation in just one copy of the VHL gene in each cell is enough to increase risk of developing VHL disease. Substrates for CRL2VHL E3 ligase tumour suppressor include the hypoxia-inducible factor (HIF) family of transcription factors (HIF1-3 α) which bind the VHL subunit when hydroxylated on two proline residues by prolyl hydroxylase. This results in ubiquitylation of HIF1-3 α and subsequent proteasomal degradation which would otherwise accumulate and cause inappropriate upregulation of hypoxia-inducible genes such as Transforming growth factor alpha (TGF- α), Vascular endothelial growth factor A (VEGF-A), etc. leading to hyperangiogenesis in VHL mutant individuals [120]. VHL disease is characterised by benign and malignant tumours mostly clear cell kidney and lung carcinomas [121]. Hemangioblastomas that develop in the brain and spinal cord cause headaches, vomiting, weakness, and a loss of muscle coordination (ataxia). If left untreated, hemangioblastomas may result in blindness, permanent brain damage or death. Pheochromocytomas develop and affect the adrenal glands, which may produce excess hormones thereby causing high blood pressure.

Angelman syndrome is a complex genetic disorder caused by a disruption of the UBE3A gene, which encodes the wild-type ubiquitin ligase E6 associated protein (E6-AP/UBE3) [122]. The disease occurs in individuals with a loss of expression or mutations in E6-AP. Angelman syndrome is characterised by intellectual disability, severe speech impairment, a tendency to jerky movement (ataxia), recurrent seizures (epilepsy) and a small head size [123, 124]. Even though investigations have established that E6-AP plays an essential role in the proteasome-dependent degradation of several identified cellular substrates and therefore promote Angelman syndrome, to date, the molecular mechanisms behind the disease pathology is poorly understood [125]. Identified E6-AP substrates include Sox9, C/EBP α , α -synuclein, p27, promyelocytic leukaemia (PML) tumour suppressor, annexin A1, amplified in breast cancer 1 (AIB1), hHR23A, etc. [126]. This indicates that a functionally defective E6-AP mutant cannot initiate degradation of substrates thereby promoting development of Angelman syndrome.

3-M syndrome is a rare autosomal-recessive growth retardation disorder associated with mutations of the Cullin7 (Cul7) E3 ligase [127]. 3-M syndrome is characterised by severe pre- and postnatal growth retardation, large head circumference, facial dysmorphism and skeletal abnormalities including dwarfism even though affected individuals exhibit normal intelligence [128]. Cul7 is a member of the Cullin family of proteins, which function as scaffolds in the formation of numerous E3 ligases with RING proteins, adaptor proteins and substrate recognition receptors [129]. The specific role and substrates of Cul7 are mostly unknown. Recently however, evidence of CUL7's involvement in pivotal growth-regulatory signalling have begun to emerge. Cul7 interacts with both Skp1-Fbx29 heterodimer and the ROC1 RING-finger protein to form the Cul7 E3 ligase complex which ubiquitylates proteins for proteasomal and lysosomal degradation [130]. The insulin receptor substrate 1 (IRS-1), a critical mediator of the insulin/insulin-like growth factor 1 signalling has been identified as a proteolytic target of the Cul7 E3 ligase [131]. Additionally, mammalian Eag1 potassium (Eag1 K⁺) channels, which are widely expressed in the brain, are novel targets of Cul7 E3 ligase [130]. Mutant Eag1 K⁺ channels are associated with congenital neurodevelopmental anomalies. Cul7 E3 ligase has also been implicated in the proteasomal degradation of cyclin D1 [132]. Cyclin D1 proto-oncogene is a vital regulator of cell

cycle progression from G1 to S phase in several different cell types. Accumulation of cyclin D is associated with development and progression of cancer and deregulation of its expression is linked to resistance to hormone therapy in breast cancer [133]. Consequently, in many cancers, impaired activity of Cul7 is essentially responsible for cellular elevated levels of cyclin D1.

Ubiquilin (UBQLN), a ubiquitin binding protein, is a ubiquitin-like protein that shares a high degree of sequence similarity with ubiquitin across several species. UBQLNs contain both *N*-terminal ubiquitin-like and *C*-terminal UBA (ubiquitin associated) domains and are capable of functionally recruiting E3s and linking to the ubiquitylation machinery to proteasomal degradation of targeted proteins [134, 135]. Mutations in UBQLNs may be associated with lesions in Alzheimer's and Parkinson's disease. UBQLNs have also been shown to modulate accumulation of presenilin proteins, which play an important role in the generation of β amyloid precursor protein (APP). Presenilin is the sub-component of gamma secretase whose role is endoproteolysis of APP [136]. UBQLN-1 is also a molecular chaperone for APP. Therefore, its presence prevents aggregation of APP [137]. Consequently, low levels of UBQLN-1 increase malformation of APP thereby triggering Alzheimer's disease [138].

The list of diseases associated with E3 ligase dysfunction is not exhaustive. More insights are available in the literature of which each apparently addresses a specified disease in more detail. It is anticipated that new discoveries in this regard will emerge in the future.

3.2 Potential therapeutic benefits of E3 ligases

There remains a significant unmet need for novel therapeutic strategies for genetic disorders such as Alzheimer's, Amyotrophic Lateral Sclerosis, Arthritis, cancer, etc. Owing to the critical cellular signalling role of the ubiquitin system in protein degradation, activation, subcellular localization and beyond, various targets of the ubiquitylation pathway are being earmarked to have potential for development of drugs to treat malignant cancers among several other diseases. Among these targets include the E3 ubiquitin ligases [41]. So far, some E3 ligase inhibitors including Velcade (bortezomib), Ninlaro (ixazomib), and Kyprolis (carfilzomib) have proved effective and have been approved by the US FDA for the treatment of multiple myeloma suggesting that specific E3 inhibitors are promising in anti-cancer therapy and beyond. This breakthrough has inspired researchers to probe more aspects of the ubiquitylation system for therapeutics [139]. Ideally, a potential cancer treatment target should be playing an essential role in carcinogenesis, it should overexpress in cancer cells and its activity inhibition or expression should induce growth suppression and/or apoptosis in cancer cells [140]. In addition, an ideal druggable candidate should be an enzyme or a G Protein Coupled Receptor (GPCR) and therefore druggable. More importantly, it is either not expressed or is only expressed at low levels in normal cells and its inhibition has minimal effect on normal cell growth and function. Inhibition of such a target would achieve a maximal therapeutic index with minimal toxicity [140]. Being enzymes and mostly natural tumour suppressors, many E3 ligases function within these criteria and are therefore attractive targets for therapeutic intervention of cancers. Several studies have explored and validated the therapeutic potential of E3 ligases [58, 71, 72]. In this section, the rationale for selected E3 ligases with pharmacological potential have been reviewed.

Firstly, one therapeutically significant pathway is the PINK1-parkin cascade for mitochondrial quality control. PINK1 and parkin appear to offer multiple therapeutic

targets for the treatment of Parkinson's disease and several other neurodegenerative disorders [141, 142]. Unidentified and putative members of this cascade may also be therapeutically relevant. Parkin displays low basal activity under normal physiological conditions [82]. Any means by which wild-type parkin activity may be increased for instance by development of small molecule activators could be significant in promoting mitophagy to downregulate progression of Parkinson's disease and other PINK1-parkin pathway-mediated neurodegenerative diseases. In *PINK1* mutant patients, PINK1 by-pass biomolecules that either mimic phospho-Ser65-ubiquitin or can disrupt the serpentine autoinhibited tertiary structure of parkin could augment its E3 ligase activity and hence, its neuroprotective functions [143]. Investigations revealed that primary neuron cells and post-mortem brain tissue from Parkinson's disease patients carrying pathogenic mutant PINK1 were largely devoid of phospho-Ser65-ubiquitin signal because phosphorylation of ubiquitin at Ser65 is dependent on PINK1 kinase activity [144]. Their data further indicated that phospho-Ser65-ubiquitin accumulates with stress, disease or age in individuals bearing functional PINK1 and therefore highlight the significance of phospho-Ser65-ubiquitin as a potential candidate for the development of biomarkers and therapeutics. *In vitro* mutagenic investigations have shown that mitochondrial depolarisation and PINK1-dependent recruitment of parkin to mitochondria is significantly enhanced by mutations at Trp403 in repressor element (REP) or Phe463 in RING2 of parkin [145]. This knowledge is pharmacologically relevant. The REP linker of parkin is anchored through Trp403 with RING1 and prevents E2 binding to its site on RING1. REP must dissociate from RING1 to enhance E2 binding. In addition, RING0 must dissociate from RING2 to expose the active site Cys431 [62, 146]. Therefore, any biomolecule capable of binding tightly to the pockets occupied by the amino acid side chains can disrupt parkin's autoinhibited structural state and induce UbcH7 binding and discharging thereby enhancing E3 ligase activity. Scientists have also shown that the DUBs, USP30 and USP15 function antagonistically to the PINK1-parkin dependent mitophagy making inhibitors of these enzymes prime candidates for designing drugs that will enhance PINK1-parkin dependent mitophagy [147, 148]. In contrast, USP8 promotes parkin-mediated mitophagy and thus agonists of this DUB will enhance mitophagy and neuroprotection [149]. Mutations of PINK1's kinase domain are also observed with Parkinson's disease patients where parkin translocation to mitochondria or mitochondrial aggregation does not occur [95].

As previously mentioned in Section 3.1, mdm2 is a direct downstream target of p53. P53, is a well-studied tumour suppressor which is often mutated in more than 50% of human cancers. This is because p53 induces growth arrest and apoptosis upon activation by different stimuli e.g., DNA damage [150]. Upon induction by p53, mdm2 in turn acts as an E3 ubiquitin ligase to ubiquitylate p53 for proteasomal degradation. This action reduces p53 levels and consequently inhibits p53-mediated cell apoptosis [151]. As such, inhibition of mdm2 E3 ligase activity is a potential approach to increasing p53 levels in order to induce cell apoptosis in human cancer cells harbouring wild-type p53. Mdm2 is usually expressed in low levels in normal cells but is overexpressed in several human cancers such as breast carcinomas, soft tissue sarcomas, oesophageal carcinomas, lung carcinomas, glioblastomas and malignant melanomas and will represent an excellent pharmacological candidate for further research [152].

Pirh2 has recently been found to be a major E3 ligase partnering mdm2 to target (tetrameric)p53 for proteasomal degradation. Further, the p53-Pirh2 complex promotes Twist1 degradation leading to inhibition of epithelial-mesenchymal transition

in ovarian cancer [153]. Epithelial-mesenchymal transition is critical in cancer metastasis and chemoresistance implying that Pirh2 specific inhibition might be therapeutically relevant. Some IAPs (Inhibitor of Apoptosis Proteins) that are overexpressed in most common human cancers (correlating chemoresistance) also represent important therapeutic targets for drug development [154]. Examples of IAPs include XIAP, cIAP-1, cIAP-2, Ts-IAP, NAIP, Survivin, Livin/ML-IAP, and Apollon/Bruce. IAPs are characterised by the presence of BIR (baculoviral IAP repeat) domain(s) required for suppression of apoptosis. Additionally, some members of the family may contain a RING finger domain at the C-terminus essential for ubiquitylation and subsequent proteasomal degradation of the apoptosis inducer proteins, caspases and Smac [155]. Indeed, it has been demonstrated that overexpression of IAPs suppress apoptosis [156]. Therefore, IAPs contribute to their anti-apoptosis function by promoting proteasomal degradation of pro-apoptotic caspases and Smac proteins. Targeting specific IAPs' E3 ubiquitin ligase activity inhibition thus, has potential for anti-cancer drug development [140]. Additionally, research has shown overexpression of components of SCF of RING E3 ligases in several human cancers. For instance, Cul4A is reported to be overexpressed in breast cancers [157]. Skp2 is likewise overexpressed in other human cancers e.g., squamous, colorectal, gastric, and prostate carcinomas, small cell lung carcinoma, and breast cancer. Skp1-Cullin-F Box components are the substrate recognition sites of the RING E3 ligases and recognise a variety of substrates involved in critical cellular processes such as the cell cycle. Examples of SCF targets for ubiquitin-mediated proteasomal degradation are p27 and cyclin E which are down regulators of different sites of the cell cycle [44]. Furthermore, the role of Nedd4-1 in lung cancers has been studied (see 3.1 above). NEDD4-1 expression is up-regulated in lung adenocarcinoma compared with normal cells. It has also been demonstrated that NEDD4-1 silencing reduces non-small cell lung cancer cells *in vitro* as well as tumour growth *in vivo* [158]. Overexpression of Nedd4-1 is associated with lymph node metastasis and chemoresistance, as such NEDD4-1 is a potential drug target since specific inhibitors of NEDD4-1 will likely promote cancer cell apoptosis. NEDD4-1 E3 ligase activity inhibition therefore has potential for lung cancer treatment [108].

Cbl E3 ligase (Section 3.1) ubiquitylates EGFR thereby reducing EGFR signalling in glioblastoma. Specific inhibitors of Cbl could helpfully target upregulating EGFR signalling in anti-glioblastoma therapy. As the MAPK pathway is commonly mutated in many cancers resulting from increase in cellular proliferation, differentiation, migration, and invasion, specific inhibitors of E3 ligases of the MAPK pathway (e.g., TRIM9, SCFFBXO31, KBTBD7, LZTR1) may have potential for anti-cancer therapy [72].

Research suggests that ubiquitylation of *Plasmodium falciparum* proteins play essential roles in parasite development. Recent data indicate ubiquitylation of several essential proteins (e.g., merozoite pellicle proteins involved in erythrocyte invasion, exported proteins, and histones) of the human malaria parasite, *Plasmodium falciparum*, which suggest potential for the use of small-molecule inhibitors of the ubiquitin-mediated degradation machinery for the development of anti-malarial drugs [159]. The data further showed that some commercially available inhibitors of the ubiquitylation process e.g., the UBA1 inhibitor MLN7243, is a potent inhibitor and blocked schizont differentiation into merozoites by interrupting nuclear division and intracellular structural formation. Identification of the associated E3 ligases will predictably present a varied number of druggable targets with potential for malaria treatment [159]. More research will unveil the most relevant E3s to target in this regard.

Finally, besides small-molecule E3 ligase inhibitors, PROTACs (PROteolysis-TARgeting Chimeras) appear to have significant therapeutic potential. Reports indicate that small-molecule inhibitors have limitations [160]. For instance, small-molecule inhibitors are limited to molecules that have an active site (enzymes and receptors e.g., RTKs). The PROTAC technology has emerged to overcome these limitations and to facilitate the 75% of human proteome e.g., transcription factors, scaffolding proteins, and non-enzymatic proteins which are signal effector proteins but lack active sites and are thus undruggable. The emerging characteristics of PROTACs such as induction of substrate selectivity, rapid, profound, and sustained proteasomal degradation and consequential induction of robust inhibition of downstream signals coupled with overcoming resistance to small molecule inhibitors have been extensively reviewed [72, 160]. A PROTAC is a small heterobifunctional molecule consisting of an E3 binding domain and a substrate binding domain covalently joined together by a linker. This spatial arrangement enhances recruitment of the E3 enzyme in proximity with the specific substrate (e.g., an oncoprotein) for ubiquitylation and subsequent proteasomal degradation thereby inhibiting downstream signals and subsequently down regulating the cell cycle or inducing apoptosis. PROTAC technology therefore will utilise the E3-mediated ubiquitin proteasome mechanistic pathway to treat disease. It is a very promising alternative technique where E3 inhibitors are limited or will present less efficiency.

4. Conclusion and perspectives

The biological importance of E3 ubiquitin ligases cannot be overemphasised because protein ubiquitylation is crucial in the regulation of numerous cellular processes. E3 ubiquitin ligases have therefore recently emerged as significant future therapeutic opportunities for drug development for treatment of several human diseases associated with ubiquitin-mediated signalling. Regrettably, the mechanisms by which the ubiquitin system regulates cellular signalling and pathogenesis remain largely unknown. Many questions remain unanswered considering the number of E3 ligases (over 600) in the human genome and lack of the most relevant technologies to assess these principles, coupled with the extreme complexity of ubiquitin signalling processes. Blocking protein–protein interactions is problematic, yet it is apparently the most effective treatment option for utilising the ubiquitin system. This option relies on blocking the E3 ligase at specific substrate recognition sites. Hypothetically, targeting rapid screening of small specific molecular inhibitors of E3s which have potential to selectively stabilise specific downstream cellular proteins regulated by specific E3s while avoiding unwanted effects on other cellular proteins will achieve less toxicity. Therefore, a complete understanding of the mechanisms involved in protein substrate recognition by E3 ligases and functions, as well as easy identification of aberrant entities within the ubiquitin pathway will be instrumental in understanding the aetiology of associated diseases. With the current advances in proteomics technology, more E3 substrates are being identified and more insights to E3 regulatory roles in many diseases are being better understood. Though new technologies such as the siRNA, for validation of many E3s, the Fluorescence Resonance Energy Transfer (FRET) for High throughput (HTS) assays for screening inhibitors of ubiquitin transfer from E2s to E3s, and electrochemiluminescence (ECL)-Based HTS for screening inhibitors of the ubiquitylation machinery have facilitated screening of compounds against E3s and improved research in this aspect so far, these methods

are themselves not devoid of challenges. Development of more efficient, cheaper, and simpler techniques will fast-track understanding of the ubiquitin system and the drug discovery process. Predictably, E3 ubiquitin ligases will present one of the most efficacious targets for anti-cancer drug discovery and for other diseases in the future.

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Conflict of interest

The authors declare no conflict of interest.

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