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Review

The ubiquitin system: pathogenesis of human diseases and drug targeting

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Abstract

With the many processes and substrates targeted by the ubiquitin pathway, it is not surprising to find that aberrations in the system underlie, directly or indirectly, the pathogenesis of many diseases. While inactivation of a major enzyme such as E1 is obviously lethal, mutations in enzymes or in recognition motifs in substrates that do not affect vital pathways or that affect the involved process only partially may result in a broad array of phenotypes. Likewise, acquired changes in the activity of the system can also evolve into certain pathologies. The pathological states associated with the ubiquitin system can be classified into two groups: (a) those that result from loss of function-mutation in a ubiquitin system enzyme or in the recognition motif in the target substrate that lead to stabilization of certain proteins, and (b) those that result from gain of function-abnormal or accelerated degradation of the protein target. Studies that employ targeted inactivation of genes coding for specific ubiquitin system enzymes and substrates in animals can provide a more systematic view into the broad spectrum of pathologies that may result from aberrations in ubiquitin-mediated proteolysis. Better understanding of the processes and identification of the components involved in the degradation of key regulatory proteins will lead to the development of mechanism-based drugs that will target specifically only the involved proteins.

Keywords: Ubiquitin system; Human disease; Drug targeting

1. Diseases related to aberrations in the ubiquitin system

1.1. Malignancies

Alterations in ubiquitination and deubiquitination reactions have been directly implicated in the etiology of many malignancies (Fig. 1). In general, specific cancers can result from *stabilization* of oncoproteins/growth promoting factors, or *destabilization* of tumor suppressors. Some of the natural substrates for degradation by the proteasome are oncoproteins that, if not properly removed from the cell, can induce malignant transformation. For instance, ubiquitin targets N-myc, c-Myc, c-Fos, c-Jun, Src, and the adenovirus E1A proteins. Destabilization of tumor suppressor proteins such as p53 and p27 has also been implicated in the pathogenesis of malignancies.

1.1.1. p53

It was noted that the level of the tumor suppressor protein p53 is extremely low in uterine cervical carcinoma tumors caused by high-risk strains of the Human Papillomavirus (HPV). Detailed studies both in vitro and in vivo have shown that the suppressor is targeted for ubiquitin-mediated degradation by the HPV oncoprotein E6 coded by high risk strains of the virus (E6–16 or 18, for example). Low risk strains that encode slightly different E6 proteins (such as E6–11) do not transform cells and do not target p53 for degradation [1]. Degradation is mediated by the HECT domain E3 enzyme E6-Associated Protein, E6-AP, where E6 serves as an ancillary protein that allows recognition of p53 in *trans* [2] (reviewed recently in Ref. [3]). E6 associates with both the ubiquitin-ligase and the target

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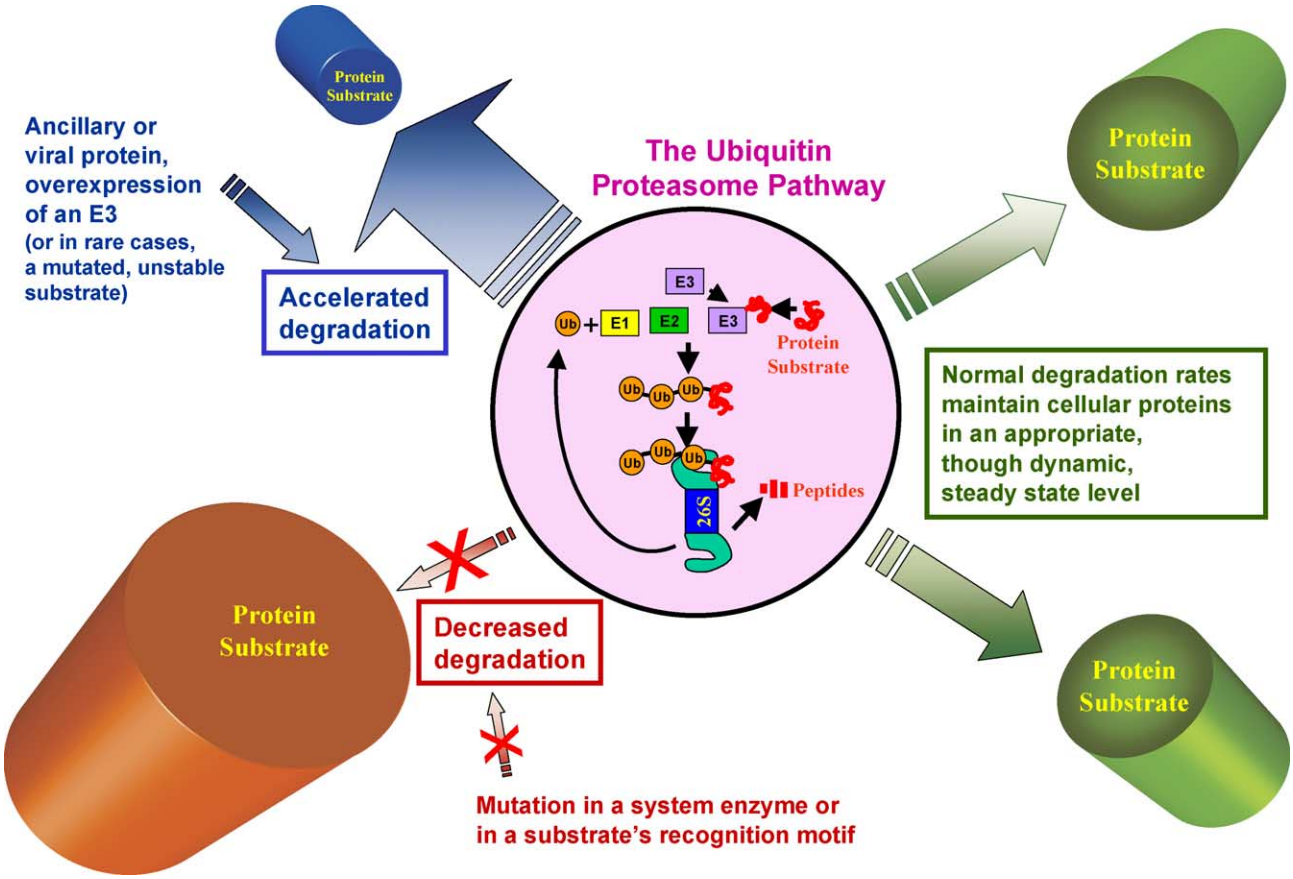

Fig. 1. Aberrations in the ubiquitin–proteasome system and pathogenesis of human diseases. Normal degradation of cellular proteins maintains them in a steady state level, though the steady state can be dynamic and change under various pathophysiological conditions (upper and lower right side). When degradation is accelerated due to an increase in the level of an E3 (Skp2 in the case of p27, for example) or overexpression of an ancillary protein that generates a complex with the protein substrate and targets it for degradation (the Human Papillomavirus E6 oncoprotein that associates with p53 and targets it for degradation by the E6-AP ligase, or the cytomegalovirus-encoded ER proteins US2 and US11 that target MHC class I molecules for ERAD), the steady state level of the protein decreases (upper left side). A mutation in a ubiquitin ligase [such as occurs in Adenomatous Polyposis Coli—APC, or in E6-AP (Angelman syndrome)] or in the substrate's recognition motif (such as occurs in β-catenin or in ENaC) will result in decreased degradation and accumulation of the target substrate.

substrate and brings them, most probably, via the generation of a ternary complex, to the necessary proximity that is assumed to allow catalysis of conjugation to occur. Removal of the suppressor by the oncoprotein is probably an important, though not the only, mechanism used by the virus to transform cells.

p53 is short-lived even in cells that are not infected with HPV. It is only following infliction of stress, such as DNA damage, that the protein is stabilized. Under normal conditions, p53 is targeted by the ubiquitin ligase Mdm2. In certain tumors it was found that Mdm2 is overexpressed, often as the result of gene amplification (see for example Ref. [4]; reviewed in Ref. [5]). It is possible that removal of p53 in these cells plays a role in their transformation.

It should be noted, however, that in many malignancies, p53 is mutated and consequently inactive, and the resulting pathology is not related to changes in its stability.

1.1.2. $p27^{Kip1}$

Similar to the case of p53, low levels of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ have been demonstrated in colorectal, prostate and breast cancers [6–8]. p27 acts as a negative growth regulator/tumor suppressor and plays an important role in proliferation of mammalian cells. It binds and negatively regulates the activity of CDK2/cyclin E and CDK2/cyclin A complexes and thus does not allow cell cycle progression from G1 and entrance into the S phase. Its level is high in quiescent cells, but following mitogenic stimuli, it is rapidly degraded by the ubiquitin system, allowing the CDK/cyclin complexes to drive the cell into the S phase. As noted, the level of p27 is markedly reduced in several cancers, including breast, colorectal, and prostate carcinomas, and in many of these cases there is a strong correlation between the low level of p27 and the aggressiveness of the disease—tumor grading, clinical staging and poor prognosis of the patients. Levels of p27 have become an important and novel prognostic factor for survival, recurrence, and evaluation of therapy, where extremely low expression predicts poor prognosis. Dissection of the mechanism that underlies the decrease in p27 revealed that the rapidly degraded p27 is of the WT species, and it is probably abnormal activation of the ubiquitin system that leads to accelerated degradation of

the suppressor. Detailed mechanistic analysis revealed that the low level of p27 correlates directly with increased level of Skp2, the F-box protein involved in p27 ubiquitination, and that ectopic overexpression of Skp2 in experimental animals is oncogenic [9].

1.1.3. β-Catenin

β-Catenin plays an important role in signal transduction and differentiation of the colorectal epithelium, and aberrations in ubiquitin-mediated regulation of its levels may play an important role in the multi-step development of colorectal tumors [10]. These tumors develop in 50% of the Western world population by the age of 70, and in 10% of these individuals (5% of the population) the tumors progress to malignancy. Fifteen percent of these patients have a genetic predisposing defect leading to the development of the malignancy. In the absence of signalling, glycogen synthase kinase 3β (GSK3β) is active and promotes last phosphorylation step necessary for degradation of β-catenin via the ubiquitin–proteasome pathway [11]. Signalling promotes dephosphorylation, stabilization and subsequent activation of β-catenin via complex formation with T Cell Factor (TCF) and Lymphocyte Enhancer Factor-1 (LEF-1), otherwise inactive subunits of transcription factor complexes. β-Catenin interacts with the 300-kDa tumor suppressor Adenomatous Polyposis Coli (APC) and Axin to generate a complex that appears to regulate, in a yet unknown manner, its intracellular level. It appears that β-catenin must be bound to the complex in order to be phosphorylated by Casein Kinase I (CKI; see below) and GSK3β, and then recognized by the ubiquitin ligase $SCF^{\beta\text{-}TrCP}$ complex. The APC–Axin complex acts most probably as a scaffold complex that enables ubiquitination to occur. Aberrations in degradation of β-catenin lead to its stabilization, accumulation, and subsequent oncogenic activation, and can result from two distinct mechanisms: (a) mutations in the phosphorylation/recognition motif of the protein by GSK3β/β−TrCP, and (b) mutations in the targeting machinery. Mutations in the GSK3β/β-TrCP phosphorylation/E3 recognition domain of β-catenin, which is similar to other recognition sites of the kinase and ligase, is composed of two Ser residues interspaced by three other amino acids [DS(P)GψDS(P); where S(P) denotes phosphorylated Ser and ψ an hydrophobic residue], have been described in several colorectal carcinomas, and also in malignant melanomas [12]. These mutations, which typically affect the Ser residues, result in a protein species that cannot be phosphorylated, and therefore cannot be recognized by the ubiquitin ligase. Interestingly, mutations in the more downstream Ser45 which resides in a Casein Kinase I (CKI] site, have also been s described in several malignancies. Recently, it has been demonstrated that phosphorylation in this site primes phosphorylation by GSK3β [13]. As for ubiquitination, colon cancer cells that do not express APC, or that harbor APC proteins that are mutated in one of the Catenin-binding clusters, do not associate with β-catenin. Here too, the protein accumulates in the cytosol and is translocated into the nucleus where it acts as part of an active transcriptional complex. Expression of full-length APC in these cells leads to degradation of the excessive β-catenin and to abrogation of the *trans*-activation effect.

1.1.4. c-Jun

c-Jun, but not its transforming counterpart v-Jun, can be multi-ubiquitinated and rapidly degraded in cells. The stability and lifetime of v-Jun are therefore greatly enhanced, which can result in malignant transformation. Mechanistic analysis of the differential sensitivity to the ubiquitin system revealed that the δ domain of c-Jun, an amino acid sequence that spans residues 31–57 and that confers instability upon the normal cellular protein, is missing in the retrovirus-derived molecule. Deletion of this domain stabilizes c-Jun [14]. Interestingly, the δ domain does not serve as a the ubiquitination site of the molecule as it lacks Lys residues. It may serve, however, as an anchoring site to the specific Jun ubiquitin-ligase. The loss of the δ domain during retroviral transduction is yet another example to the diverse mechanisms evolved by viruses to exploit the ubiquitin system in order to ensure replication and continuity of infection. While mutated Jun has been identified in several tumors, the mutation has not been implicated directly with the pathogenesis of any known malignancy.

1.1.5. pVHL

Mutations in components of the ubiquitination machinery can also cause malignancies. Mutations in one germ line copy of *VHL* predisposes individuals to a wide range of malignancies, including in more then 80% of sporadic cases of renal cell carcinoma, pheochromocytoma, cerebellar hemangioblasomas, and retinal angiomas. A hallmark of $VHL^{-/-}$ tumors is a high degree of vascularization that arises from constitutive expression of hypoxia inducible genes including the master switch transcription factor hypoxia-inducible factor-α (HIF-α) and the crucial vascular endothelial growth factor (VEGF) (reviewed recently in Refs [15,16]). It has been recently shown that pVHL is a subunit in a ubiquitin ligase complex that is similar to the SCF complexes, and contains, in addition, Cullin 2A, Rbx1/Hrt1, Elongin B (a ubiquitin-like molecule) and Elongin C (a molecule related to Skp1) [17,18] that is involved in targeting of HIF-α for ubiquitin- and proteasome-mediated degradation [19,20]. The HIF-α subunits, HIF-1α and HIF2α, belong to the basic helix-loop-helix (bHLH) family of proteins. These regulated proteins heterodimerize with the constitutively expressed HIF-1β to generate the active transcription factors. Under normoxic, and obviously hyperoxic, conditions, HIF-1α is hydroxylated specifically on Pro residue 564 to generate a hydroxyproline derivative [21,22], in a reaction that involves molecular oxygen and soluble prolyl-4-hydroxylase [23]. This hydroxylated Pro residue is recognized by the pVHL E3 complex that targets the molecule for ubiquitination and subsequent degradation

[21,22]. Under hypoxic conditions, HIF-1α is stable, as the efficiency of the hydroxylation reaction under these conditions is extremely low. Loss of VHL function stabilizes HIF-1α which can explain the stimulation of vascular growth in tumors in which VHL is mutated or lacking. Since overexpression of VEGF alone or of many of the other known target proteins of HIF does not lead to malignant transformation, and since WT VHL can restore normal growth control in these malignant cells, researchers assume that pVHL and/or HIF must have additional, yet unknown, substrates. Interestingly, microtubule stability was also shown to be regulated by pVHL [24], and it is possible that this function is related, in a yet unknown mechanism, to cell division. However, the regions in the VHL protein responsible for that function overlap with those that were shown to be involved in susceptibility to hemangioblastoma and phaeochromocytoma, but not to renal cell carcinoma, supporting the hypothesis that aberration in the degradation of several distinct substrates of pVHL may underlie the pathogenesis of different malignancies.

1.1.6. c-Cbl and Hakai

Another oncoprotein is c-Cbl/Sli-1, which attenuates cell proliferation by serving as an E3 for membrane tyrosine kinase receptors, the EGF-R for example [25–27] (reviewed recently in Ref. [28]). Following ligand binding and autophosphorylation at Tyr residue 1045, the receptor is rapidly endocytosed as a complex along with CIN85 and endophilin, and is ubiquitinated by c-Cbl that recognizes only the modified receptor. It is then either recycled to the cell surface membrane or transported for degradation in the lysosome via a series of vesicles; the most distal ones are multivesicular bodies. This process is mediated by the ESCRT-1 (Endosomal Sorting Complex Required for Transport) complex, which contains, among its components, TSG101, a ubiquitin-conjugating enzyme variant protein (Vsp23 in yeast) that is involved in recognition of ubiquitinated membrane proteins [29]. Mutant, inactive species of c-Cbl cannot properly terminate signalling, causing abnormal cell fate (see for example Ref. [30]). While it will not be surprising to find, mutations in c-Cbl have not been described in defined human malignancies. A cellular homologue of c-Cbl, Hakai, is mediating the down-regulation of E-cadherin [31].

1.1.7. Deubiquitinating enzymes

The human oncogene tre-2 has been shown to encode a deubiquitinating (DUB) enzyme that disassembles multi-ubiquitin chains from peptides still bound to the proteasome [32]. Since the ubiquitin pathway rapidly degrades many proto-oncoproteins, increasing the levels of such proteins that may result from inactivating mutations in deubiquitinating enzymes, such as tre-2, that lead to deficiency in free ubiquitin, can potentially be tumorigenic. It should be noted, however, that no malignancies have been associated or attributed to mutations in *tre-2*. Over-expression of another DUB of the ubiquitin proteases (UBP) family, Unp, has been found in lung carcinoma in humans and in mouse tumors [33]. Unp may have a role in regulating the degradation of specific, yet unidentified, substrates in these tissues, though it does not seem to be crucial for general proteolysis [34]. An interesting deubiquitinating enzyme is CYLD, which is involved in deubiquitinating K63-Ub from NF-κB Essential Modifier (NEMO), Tumor Necrosis Factor Receptor (TNFR)-Associated Factor 2 (TRAF2) and TRAF6 (see for example Ref. [35]). Ubiquitination of NEMO [a regulator of the IκB Kinase (IKK) signalling complex] and the TRAF proteins (which are ubiquitin ligases), and generation of polyubiquitin chains linked via Lys63 of the ubiquitin moiety, does not target these proteins for degradation, but results in their activation. Thus, inhibition of deubiquitination of NEMO, by mutation in CYLD for example, may lead to uncontrolled activation of the IKK complex with increased activity of NF-κB. Indeed, CYLD was found to be mutated in Familial Cylindromatosis, a rare pathology characterized predisposition to multiple tumors of the skin appendages such as the salivary gland [36].

1.1.8. BRCA1 and BARD1

BRCA1 and BARD1 generate a heterodimeric E3 complex that, like many RING-finger containing ligases, has an autoubiquitinating activity [37,38] (reviewed in Ref. [39]). This activity can be involved in regulating the level of the enzyme via auto-destruction, but it has also been reported that it leads to a significant activation of the enzyme [40]. It also catalyzes monoubiquitination of several histones, such as H2A, H2AX, H2B, H3 and H4 [40], though the physiological relevance and significance of this activity/modification is not clear. Importantly, inactivating mutations of BRCA1 have been reported in an extremely high frequency in breast carcinoma [41] (reviewed in Ref. [42]). The target substrate(s) that is possibly accumulated in these cases, and its increased level may be tumorigenic, has not been identified. However, an interesting linkage has been reported in Fanconi Anemia (FA). FA is an autosomal recessive disorder characterized by cancer susceptibility, multiple congenital anomalies, and bone marrow failure. Cells of patients are sensitive to Mitomycin C. Six FA genes have been cloned, and the encoded proteins function in a novel pathway that is required for the normal cellular response to DNA damage. Following DNA damage, the pathway is activated, leading to monoubiquitination of the FA protein FANCD2, and to its subsequent translocation, along with BRCA1, to subnuclear foci. Mutations in the machinery involved in the monoubiquitination of FANCD2 disrupt its translocation to the nuclear foci following DNA damage [43]. While it was suggested that BRCA1 is the FANCD2 monoubiquitinating ligase [43], recent evidence suggests that the modification can occur independently of BRCA1, although the ligase is still required for the translocation of FANCD2 [44]. Mutations in the FANCD2

pathway lead, most probably, to aberrations in DNA repair, resulting in the cellular and clinical abnormalities observed in FA (reviewed recently in Ref. [45]).

1.2. Membrane proteins

1.2.1. Liddle's syndrome

An interesting syndrome involves aberration in the regulation of the kidney Epithelial Na^+ Channel (ENaC). The channel, which is composed of three homologous subunits α, β and γ, is a short-lived protein complex that is degraded by the ubiquitin system and is recognized via a proline-rich (PY) motif by NEDD4, a member of the HECT domain family of E3s. Binding of NEDD4 is mediated via a WW domain in the ligase [46]. A mutation in the PY motif leads to stabilization of the channel complex, as it does not associate anymore with the ligase, accumulation of its subunits, excessive reabsorption of Na^+ and H_2O, and, consequently, a severe form of early-onset hypertension. An interesting finding relates to the regulation of the degradation pathway of ENaC. It has been shown that NEDD4-2 is phosphorylated by the aldosterone-induced Sgk1 kinase [47] on specific Ser residues. This phosphorylation weakens the interaction between Nedd4-2 and ENaC, leading to elevated ENaC cell surface expression.

1.2.2. Cystic fibrosis (CF)

The CF gene encodes the CF transmembrane regulator (CFTR) which is a 1480-amino-acid-residue chloride ion channel. Normally, only a small fraction of the WT protein matures to the cell surface, whereas most of the protein is degraded from the ER by the ubiquitin system. The ubiquitin system enzymes involved in this process have not been identified. Although more than 600 mutations have been described, the most frequent one (>70%) is ΔF508. The mutation leads to an autosomal recessive inherited multisystem disorder characterized by chronic obstruction of airways and severe maldigestion due to exocrine pancreatic dysfunction. Despite normal ion channel function, when inserted into liposomes, for example, $CFTR^{\Delta F508}$ does not reach the cell surface at all, and is retained in the ER from which it is degraded. The rapid and efficient degradation in the ER results in complete lack of cell surface expression of the ΔF508 protein, suggesting that it contributes to the pathogenesis of the disease (reviewed in Ref. [48]). However, the utilization of proteasome inhibitors does not lead to increased expression of mutated CFTR on the cell surface. Rather, the protein aggregates and precipitates in a detergent insoluble form, suggesting that the proteasome substrate is not in direct equilibrium with intermediates on the folding pathway (reviewed in Refs. [48,49]). Thus, pharmacological trials to increase the expression of the protein on the cell surface are not aimed at inhibition of the ubiquitin system but rather at folding the protein properly or stimulating its expression. Glycerol, D_2O and amines such as betaine can be used as "chemical chaperones". These compounds have protein stabilizing properties that are due to their ability to increase protein hydration. Treatment of ΔF508 CFTR expressing cells with glycerol increases the expression of the functional channel on the cell surface. This is due to increased maturation of the core glycosylated protein to a post-ER compartment and to decreased degradation which is due to enhanced folding of the protein [48]. Since the ΔF508 CFTR folding defect is leaky, cell surface expression of the molecule can be also increased by stimulating the synthesis of the protein. Addition of butyrate that stimulates the transcription of the CFTR mRNA or of 8-cyclopentenyl-1,3-dipropylxanthine (CPX, an adenosine A1 receptor agonist), which also increases synthesis of mutated CFTR, results in increased cell surface expression of the ion channel (reviewed in Refs. [48–50]).

1.3. Neurodegenerative diseases

Accumulation of ubiquitin conjugates and/or inclusion bodies associated with ubiquitin, proteasome, and certain disease-characteristic proteins has been reported in a broad array of chronic neurodegenerative diseases, such as the neurofibrillary tangles of Alzheimer's disease (AD), brainstem Lewy bodies (LBs)—the neuropathological hallmark in Parkinson's disease (PD), LBs in LB dementia, Bunina bodies in Amyotrophic Lateral Sclerosis (ALS), and nuclear inclusions in CAG repeat expansion (polyglutamine extension) disorders such as Huntington's disease (HD), *S*pinoc*c*erebellar *A*taxias (SCAs), and Spinobulbar Muscular Atrophy (Kennedy's Syndrome). However, in all these cases, a direct pathogenetic linkage to aberrations in the ubiquitin system has not been firmly established. One factor that complicates the founding of such a linkage is the realization that many of these diseases, such as Alzheimer's and Parkinson's, are not defined clinical entities, but rather syndromes with different etiologies. Accumulation of ubiquitin conjugates in Lewy inclusion bodies in many of these cases may be secondary, and reflect unsuccessful attempts by the ubiquitin–proteasome system to remove the damaged/abnormal proteins. While the initial hypothesis was that inclusion bodies are generated because of the inherent tendency of the abnormal proteins to associate with one another and aggregate, it is now thought that the process maybe more complex and involves active cellular machineries [51–53]. The pathogenetic significance of these aggregates has remained enigmatic. Recent findings demonstrate that the aggregated proteins can inhibit the ubiquitin system [54]. Yet, an emerging concept is that the sequestration of the aggregated proteins from the cytosol and nucleoplasm and their concentration in defined inclusion bodies separates them from sensitive cellular machineries, such as the transcriptional apparatus, and is therefore protective: it is the soluble fraction of the aggregated proteins that is toxic. In any event, the appearance of the

inclusion bodies that contain the aggregated, disease-specific proteins has emerged as a common but poorly understood mechanistic theme in sporadic and hereditary neurodegenerative disorders.

1.3.1. Parkinson's disease

The case of PD highlights the complexity of the involvement of the ubiquitin system in the pathogenesis of neurodegeneration, as several, apparently independent aberrations linked to defects in the ubiquitin–proteasome system have been described in various forms of hereditary PD.

An important player in the pathogenesis of PD is Parkin. Parkin is a 465-amino-acid-residue protein with moderate similarity to ubiquitin at the amino terminus and a RING-finger motif at the carboxy terminus. Mutations in the gene appear to be responsible for the pathogenesis of autosomal recessive juvenile parkinsonism (AR-JP), one of the most common familial forms of PD [55]. Interestingly, this disease is characterized by lack of LBs, the pathognomonic hallmark of sporadic forms of the disease. Later studies have identified Parkin as a ubiquitin-protein ligase that acts along with the ubiquitin-conjugating enzyme UbcH7 and UbcH8, and have shown that mutant Parkins from AR-JP patients display loss of the ubiquitin-protein ligase activity [56]. Like all other known RING finger-containing ligases, Parkin has an autoubiquitinating activity. Yet, an important problem is the identification of its exogenous, native cellular substrates. The hypothesis is that a defect in Parkin results in accumulation of this protein(s) which is toxic to the dopaminergic neurons. Several substrates have been identified, yet it is not clear whether it is the accumulation of one or several of these proteins that underlies the pathogenesis of this familial form of PD. One of these substrates is Cell Division Control related protein-1 (CDCrel-1), a synaptic vesicle-enriched septin GTPase of 44-kDa molecular mass that has been implicated in the inhibition of exocytosis through its interactions with syntaxin [57]. For example, upon transfection into HIT-T15 cells, CDCrel-1 inhibits secretion of growth hormone [58]. It is possible that CDCrel-1 is also involved in regulating transmitter release, and its accumulation perturbs the process. A second important substrate is the Pael receptor, a putative G protein-coupled transmembrane polypeptide [59]. When overexpressed in cells, this receptor becomes unfolded, aggregates, and ubiquitinated. The insoluble Pael elicits the unfolded protein response (UPR) which induces, after some time, apoptosis. Parkin specifically ubiquitinates the Pael receptor and promotes its degradation. Overexpression of Parkin can rescue dopaminergic cells in *D. melanogaster* from neurotoxicity induced by excess the Pael receptor [60]. Importantly, the insoluble form of Pael accumulates in the brains of AR-JP patients that have mutated Parkin. Another substrate of Parkin is a novel, 22-kDa form of *O*-glycosylated α-synuclein [61]. While mutations in the non-glycosylated, 14-kDa form of α-synuclein have been linked to the pathogenesis of PD (see below), to date, they have not been linked with Parkin-associated AR-JP. The function of this newly discovered form of synuclein, as well as of its modification, is yet to be elucidated. Parkin also ubiquitinates Synphilin-1, a protein of hitherto unknown function that contains a coiled-coiled domain and an ATP/GTP-binding motif, and that associates with α-synuclein [62]. Overexpression of synphilin-1 with α-synuclein results in the formation of protein inclusions, yet the function of synphilin in this process as well as the role of its ubiquitination are still elusive. Parkin has been reported also to associate with actin filaments but not with microtubules [63]. Parkin has not been demonstrated to modify actin, yet, it is possible that the ligase regulates actin activity by ubiquitinating an actin-associated protein, which in turn affects the function of actin and movement of synaptic vesicles. Thus, while mutations in Parkin have been associated with a certain form of a relatively rare hereditary PD, the discovery of Parkin, elucidation of its role as a ubiquitin ligase, identification of certain protein substrates that are targeted by the ligase, and the finding that all mutations found in Parkin in AR-JP patients inactivate its ubiquitin ligase function are of utmost importance, as they point clearly towards a direct etiological role of this enzyme in the pathogenesis of the disease.

Recent findings in a German family with PD have revealed mutations in the gene encoding the ubiquitin carboxyl-terminal hydrolase, UCH-L1 [64]. While the mutation did not lead to complete inactivation of the enzyme, the enzyme was clearly less active than its WT counterpart. The hypothesis is that the mutation results in shortage of free ubiquitin required for degradation of certain—yet unidentified—proteins, that when accumulate, are toxic to neurons. Interestingly, intragenic deletion in the gene coding for UCH-L1 in mice leads to Gracile Axonal Dystrophy (GAD syndrome). This is a recessive autosomal mutation that displays sensory ataxia at an early stage, followed by motor ataxia at a later stage [65]). It is not clear, however, if the mouse model represents faithfully the human disease, as it does not display a Parkinsonian phenotype. Also, neuronal loss in the GAD mice occurs in the gracile tract and not in the substantia nigra, where it occurs typically in PD. The mutation in the human UCH-L1 was identified as I93M. It has been reported recently that while the monomeric form of UCH-L1 catalyzes deubiquitination, the dimers display a ubiquitin ligase activity that generates ubiquitin–K63 bonds [66]. Mono- and di-ubiquitinated α-synuclein were polyubiquitinated by the enzyme, suggesting that it acts as an E4, a ubiquitin ligase involved in elongating polyubiquitin chains rather than in initiating their formation [67]. The ligase activity was diminished significantly in a S18Y mutant of UCH-L1. Interestingly, mutation in this site is a polymorphic variant that protects the carrier population from PD. Thus, it appears that it is the activity of the ligase that plays a pathological role in the UCH-L1 I93M hydrolase mutation, as decreased activity of the hydrolase results in a net increased activity of

the ligase. It should be noted, however, that the physiological role of this activity as well as the function of the mono-, di-, and polyubiquitinated α-synucleins has remained elusive. In particular, the pathogenetic role of polyubiquitinated, Lys63 α-synuclein is not known.

A third important player that links aberrations in the ubiquitin system to the pathogenesis of PD is α-synuclein. α-Synuclein is a small, 140-amino-acid-residue protein that is thought to regulate/participate in dopamine neurotransmission/release via effects on vesicular storage. This function is impaired in familial forms of PD where mutant α-synuclein is found. In the late 1990s it was reported that two mutations in the N-terminal domain of α-synuclein, A30P and A53T, are associated with a rare form of autosomal dominant familial PD (reviewed recently in Ref. [68]). In parallel, the protein was shown to be a major component of LBs and Lewy neurites in sporadic PD, dementia with LBs (DLB), and the LB variant of AD. The autosomal dominant nature of the disease associated with these mutants strongly suggests that a gain, rather than a loss, of function underlies the mechanism of disease formation. Overexpression of WT, and in particular mutant α-synuclein, in many, but not in all, cell types induced apoptosis or sensitized the cells to toxic agents, including to proteasome inhibitors (see for example Ref. [69]). Overexpression of WT Parkin rescued the cells from the toxic effects of α-synuclein [70], suggesting that the two proteins share a common pathway involved in cell death of dopaminergic neurons. An important characteristic of all synuclein proteins is their ability to generate fibrils, where the mutant proteins generate polymers at lower concentrations then the WT species. Thus, it was not surprising to find that α-synuclein is an important, if not the main, component of LBs. As noted above, however, it is not clear whether the inclusion bodies that contain the aggregated proteins are toxic or protective: recent studies in several neurodegenerative disease models, including PD, suggest that it is the soluble forms of the disease characteristic proteins that are responsible for their toxicity (for α-synuclein, see Refs. [70,71]). The connection between α-synuclein and the ubiquitin system is also not clear. AR-JP is characterized by lack of LBs and α-synuclein aggregates. One can argue that the polyubiquitinated synuclein precipitates preferentially and the protein that is not modified by ubiquitin is more soluble, but that has not been demonstrated. The finding that it is the glycosylated form of α-synuclein that is targeted by Parkin may solve part of the enigma: it is possible that the accumulation of this form in patients with AR-JP that cannot degrade it (due to a mutation in the Parkin protein; see above) is toxic. However, this cannot explain synuclein toxicity in most sporadic PD patients that have a functional and active Parkin. An important finding, however, in that respect is that aggregated and even monomeric α-synuclein bind to the S6′ proteasomal protein and inhibit proteasomal function [72]. It is possible that this aggregation, which is the primary event, leads to a secondary damage by inhibiting the ubiquitin system [54]. Whether polyubiquitination plays a role in α-synuclein degradation is not clear. While α-synuclein is found in LBs along with ubiquitin, it is not clear how much of it is ubiquitinated and to what extent. It has been shown that the protein is targeted by the proteasome [73], but it is not clear whether it has to be ubiquitinated prior to its destruction, whether mutant forms of α-synuclein are less susceptible, and whether aberrations in this process play a role in the pathogenesis of PD. Thus, a pathogenertic linkage between the ubiquitin system and α-synucleinopathies has yet to be demonstrated.

1.3.2. Alzheimer's disease

AD is characterized by accumulation/association of ubiquitin with the phosphorylated form of Tau in neurofibrillary tangles and senile plaques, two lesions that are characteristic to the neuronal abnormalities associated with the disease. They are also present in LBs characteristic to some forms of the disease. However, the role of Tau and other putative target proteins in the pathogenesis of the disease is still not clear. Sporadic AD is also characterized by amyloid plaques that contain the β-amyloid, erroneously processed peptide Aβ. Additional and probably distinct players in the pathogenesis of AD are the Presenilins 1 and 2 (PSs) that are involved in processing of the amyloid precursor protein, APP, by the γ-secretase proteolytic complex. Numerous mutations causing early-onset AD have been identified in the PS genes, particularly the PS1 gene. Both PS1 and PS2 are targeted by the proteasome [74,75]. Furthermore, SEL10, the human homologue of the yeast F-box protein Cdc4, interacts with PS1 and enhances its ubiquitination [76], though the significance of this interaction and modification is not clear as this interaction leads also to increase in the level of the Aβ peptide. Such an increase would not have been expected if SEL10 would destabilize PS1. A more direct, relationship between the ubiquitin system and pathogenesis of AD was established with the discovery of a frameshift mutation in the ubiquitin transcript which leads to extension of the molecule with 20 amino acid residue [Ub(+1)], and which has been selectively observed in the brains of AD patients, including those with late-onset, non-familial disease [77]. Ub(+1) is an efficient acceptor for polyubiquitination, though it cannot be activated by E1 (as it lacks the essential G76 residue) and be transferred to a substrate or to another ubiquitin moiety. The resulting polyubiquitin chains are refractory to disassembly by deubiquitinating enzyme, in particular Isopeptidase T [78] which requires for its activity an exposed G76 residue at the proximal ubiquitin moiety. The accumulated polyubiquitin chains block proteasomal degradation [79] which results in neuronal apoptosis [80]. Thus, expression of Ub(+1) in the brain, which increases apparently with aging, can potentially result in dominant inhibition of the ubiquitin–proteasome system, leading to accumulation of

toxic proteins with neuropathologic consequences. Since (Ub(+1)) was described also in other disorders such Down's Syndrome [77] or supranuclear palsy [81], it is clear that it is not entirely specific to AD, and a major problem of how the mutation leads to distinct pathologies in different patients remains unsolved.

1.3.3. CAG expansion (poly Q) diseases

Another group of genetically inherited neurodegenerative disorders is caused by genomic instability that leads to an expanded 5′ -CAG repeat which is translated to an N-terminal polyglutamine repeat extension. In the case of Huntington's disease (HD), the affected gene is *Huntingtin*, which codes for a protein with unknown function. Similarly, in a series of diseases known as Spinocerebellar Ataxias (SCAs) 1–3, the affected proteins are Ataxins 1–3, respectively, while in Spinobulbar Muscular Atrophy, the affected protein is the androgen receptor [82–84]. The polyglutamine-containing proteins aggregate and accumulate in intranuclear inclusion bodies that are stained also for ubiquitin, though the extent of modification of these proteins by ubiquitin is not clear [82–86]. Huntingtin [87] and Ataxin 1 [88] are probably targeted by the ubiquitin system. Furthermore, at least for Ataxin 1, it has been shown that the degradation of the polyglutamine-containing and mutated protein in vitro is somewhat slower than that of the WT protein [88], suggesting that the inability of the system to remove the mutated protein may underlie its accumulation and possible aggregation. While it is clear that the known catalytic sites of the proteasome cannot cleave within the polyglutamine repeat, it is not known whether the aberration in the system leads to accumulation of the intact proteins or long polyglutamine stretches that are released from them. Also, it is not clear whether the aggregated protein and/or the inclusion bodies are toxic, and their generation underlies the pathogenesis of the disease. Here too, initial experimental evidence suggests that the formation of inclusion bodies may serve as an scavenger mechanism to store proteins that cannot be removed by the ubiquitin system. "Solubilization" of these proteins via expression of chaperones or removal of E6-AP (one putative ligase of Ataxin 1), manipulations that do not allow the proteins to precipitate in either their free or ubiquitin-conjugated form, aggravates the disease symptoms [88].

1.3.4. Angelman syndrome

The ubiquitin system is probably involved in human brain development. A defect in the gene coding for the E3 ligase E6-AP has been implicated directly as the cause of Angelman syndrome characterized by mental retardation, seizures, out of context frequent smiling and laughter, and abnormal gait [89,90]. The target brain protein(s)—that is most probably accumulating as it becomes stabilized—has not been identified. Unlike E6-mediated targeting of p53 (see above), the degradation of this putative protein(s) is E6-independent, and it is probably one of the native cellular substrates of E6-AP. Interestingly, the disease displays genomic imprinting where a subset of mammalian genes can be expressed monoallelically in a parent-of-origin manner (uniparental disomy—UPD). During gametogenesis, the imprinting process marks alleles, in an epigenetic manner, according to their parental origin. In the case of Angelman syndrome, the imprinted gene is localized to chromosome 15q11–q13 where the deletions in the patients were identified. Confirmatory studies in mice with UPD of the mouse homologue (Ube3a) show marked reduction in expression of the protein within Purkinje cells and hypocampal neurons, thus strongly establishing imprinting of brain E6-AP in Angelman syndrome pathogenesis [91].

1.4. Immune and inflammatory response

Peptide epitopes presented to cytotoxic T cells (CTLs) on class I MHC molecules are generated in the cytosol from limited processing of antigenic proteins. It is generally accepted that processing of most known MHC class I antigens is mediated by the ubiquitin–proteasome pathway [92,93]. An interesting finding is that the cytokine γ-interferon (γ-IFN) stimulates antigen presentation and leads to induction and exchange of three proteasomal subunits in human cells that result in alteration of the cleavage site preferences of the proteasome. The changes in activities probably lead to generation of peptides that terminate mostly with basic and hydrophobic residues, similar to the vast majority of known peptides presented on MHC class I molecules and bound to the T cell receptor. The ubiquitin system degrades, in a nondiscriminatory manner, both intracellular ("self") as well as foreign ("non-self") proteins such as viral proteins. Peptides from both populations are presented to CTLs, but those that are derived from "self" proteins do not elicit a T cell response. It can be hypothesized that aberrations in processing of these proteins may lead to presentation of differently cleaved "self" peptides that will be recognized now as "non-self". Presentation of "self" antigen as "non-self" can potentially underlie the pathogenesis of autoimmune diseases.

A wide array of immune and inflammatory disorders can also be caused by untoward activation of the immune system key transcription factor NF-κB. Activation of the factor stimulates transcription of many cytokines, adhesion molecules, inflammatory response and stress proteins, and immune system receptors. The factor is activated by the ubiquitin system via a two-step proteolytic mechanism: (a) limited processing of the precursor protein p105 to yield the active subunit p50, and (b) signal-induced phosphorylation and subsequent degradation of the inhibitor IκBα that enable translocation of the factor into the nucleus where it initiates specific transcriptional activity (recently reviewed in Refs. [94,95]). An interesting case in that respect involves mutations in NF-κB Essential Modifier (NEMO [96]). NEMO is an essential component in the signalling complex that contains also IκB kinases (IKKs) 1 and 2 that

phosphorylate IκB, a step necessary for its ubiquitination and degradation, and of the precursor proteins p100 and p105, which is necessary for their processing to the active subunits p50 and p52, respectively. Mutations in the protein lead to a series of diseases that affect the skin, among them Incontinentia Pigmenti (IP), hypohidrotic/anhidrotic exto-dermal dysplasia, but also, as expected, immune deficiency [97] (reviewed recently in Refs. [98,99]). IP is an X-linked dominant genodermatosis which is lethal in males. The mutation in NEMO results in undetectable NEMO protein and almost complete lack of active NF-κB. Hypohidrotic/ anhidrotic ectodermal dysplasia (HED/EDA) is attributed to defects of at least three genes, but also involves defective NF-κB activation. Hypomorphic *NEMO* mutations have been found to cause anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID), while stop codon mutations cause a more severe phenotype that includes, in addition to EDA-ID, also osteopetrosis and lymphoedema (OL-EDA-ID). The immunological and infectious features observed in patients result from impaired NF-κB signalling, including cellular response to LPS and a variety of cytokines.

As described above, the HPV evolved a mechanism for proteolytic removal of p53 that enables continuous replication and propagation of the virus under conditions of DNA damage that normally would have ended with p53-induced apoptosis. Two other viruses evolved mechanisms that also utilize the ubiquitin system, here to escape immune surveillance. In one case, the Epstein–Barr Virus (EBV) Nuclear Antigen 1 (EBNA1) persists in healthy virus carriers for life, and is the only viral protein regularly detected in all EBV-associated malignancies, such as Burkitt's lymphoma. Unlike EBNAs 2–4 that are strong immunogens, EBNA1 is not processed and cannot elicit a CTL response. The persistence of EBNA1 contributes, most probably, to some of the pathologies caused by the virus. An interesting structural feature common to all EBNA1 proteins derived from different EBV strains is a relatively long and unusual Gly-Ala repeat at the N-terminal domain of the molecule. Transfer of a strong antigenic epitope from EBNA4 to EBNA1 prevented its presentation, while its insertion into an EBNA1 mutant that lacks the Gly-Ala repeat results in its presentation to the appropriate CTL. Similarly, insertion of the Gly-Ala repeat to EBNA4 inhibited CTL recognition of EBNA4-derived antigenic peptides. Thus, the Gly-Ala repeat constitutes a *cis*-acting element that inhibits antigen processing and subsequent presentation of potential antigenic epitopes. It has been shown that while EBNA4 is degraded in an ATP-, ubiquitin- and proteasome-dependent manner, EBNA1 is resistant to proteolysis. EBNA1 is degraded, however, by the ubiquitin/ proteasome system if the Gly-Ala repeat is deleted. Thus, the evolution of the Gly-Ala repeat enabled the virus to evade proteolysis and subsequent presentation to the immune system [100] (reviewed recently in Ref. [101]). An additional interesting observation involves the pathobiology of the human Cytomegalovirus (CMV). The virus genome encodes two endoplasmic reticulum (ER) resident proteins, US2 and US11, that down-regulate the expression of MHC class I heavy chain molecules. The MHC molecules are synthesized, transported to the ER where they are glycosylated, but shortly thereafter, in cells expressing US2 or US11, are transported back to the cytosol, de-glycosylated, and degraded by the proteasome following ubiquitination [102]. It appears that the viral products bind to the MHC molecules and escort/dislocate them to the translocation machinery where they are transported back into the cytosol. The detailed mechanism of action of the viral proteins is not known. They may diffuse laterally in the membrane and interact with the emerging nascent MHC chain, an interaction that does not allow insertion of the stop-transfer signal and proper anchoring of the molecule in the membrane. Alternatively, they may compete with binding of the ER chaperone BIP that may be necessary for proper folding of the MHC molecule. Alternatively, they may compete with MHC molecules on binding to the ER chaperone BIP, binding that maybe necessary for proper folding of the MHC molecules. In any event, the US2/11-induced proteolytic removal of the MHC molecules inhibits presentation of the viral antigenic peptides, thus enabling the virus to evade the immune system.

Thus, it appears that evolution of many viruses has involved intimate recognition with a variety of proteolytic processes. This enabled the evolution of viral mechanisms that enhance the function- via subversion of the normal proteolytic machinery- of the viral replication and propagation machinery.

1.5. Muscle wasting

The ubiquitin system plays major roles in pathophysiological processes in the muscle. Muscle degeneration that follows long-term immobilization, denervation, and severe catabolic states, such as occurs in sepsis and cancer-induced cachexia, leads to activation of the ubiquitin pathway and induction of many of its enzymatic components. This in turn results in massive degradation of muscle proteins [103–105]. Interestingly, it appears that N-end rule pathway plays an important role in stress-induced muscle proteolysis [106]. It is not clear how and via what mechanism(s) the major muscle proteins, actin and myosin, are converted into N-end rule pathway substrates, as clearly they are not native substrates of the pathway. Their conversion, if happens, must occur upstream to the entry point of the ubiquitin system. An important development in this area is the discovery of two, degeneration-induced ubiquitin ligases, Murf1 (Muscle RING Finger) and Murf2 [107]. Murf1 was discovered independently by another group and was designated Atrogin 1 [108]. It should be noted, however, that the substrates of these induced E3s have not been identified. Also, the nature of signalling mechanisms involved in regulating muscle hypertrophy and atrophy is

still obscure, and it appears that cytokines such as TNFα and IL-6 are not involved, at least not directly. It appears that Akt/mTOR (mammalian Target Of Rapamycin) signalling pathway is up-regulated during muscle hypertrophy and down-regulated during atrophy. In agreement with these findings, genetic activation of the Akt/mTOR pathway induced hypertrophy and prevented atrophy in vivo, while genetic blockade of this pathway inhibited hypertrophy [109].

1.6. Diseases associated with animal models

The utilization of animal models in which single genes along the ubiquitin–proteasome pathway are manipulated can be used as powerful tools to study the effects of such manipulations on the development and pathophysiology of the mature organism. They can also be used to study disease models and the activity of novel therapeutic modalities. Indeed, studies in the fruit fly *D. melanogaster* turned out to reproduce faithfully the pathologies observed in mammals in a variety of neurodegenerative disorders, such as PolyQ expansion diseases (reviewed recently in Ref. [110]). It will be impossible within the frame of this review to describe in a comprehensive manner all the animal models developed along the years to study the functions of the ubiquitin system. However, we shall highlight some of them.

Mutations in the *D. melanogaster*'s *Bendless* gene that codes for an E2 enzyme result in impairment in formation of neuronal networks, due most probably to a defect in neuronal guidance [111]. Inactivation of the fly's *Ariadne-1* that codes for a RING finger E3 results, in most cases, in embryonic lethality. The few survivors demonstrate a severe defect in the development of the central nervous system [112]. Targeting of the *Fat Facets* (*faf*) gene that codes for a deubiquitinating enzyme results in impaired development of the *Drosophila's* eye with increased number of omatidia [113].

In mammals, three interesting pathological states have been described that may also have implications for human diseases. Inactivation of HR6B, one of the mammalian homologues of Ubc2/Rad6, the E2 involved in DNA repair and in targeting "N-end rule" pathway and other protein substrates, leads to a single defect, male sterility, due to defects in spermatogenesis. The target substrate proteins may be histones, as their degradation is critical for postmeiotic chromatin remodeling that occurs during spermatogenesis [114]. Another interesting case is that of the *Itch* locus in mice that encodes an E3 enzyme [115]. Defects in the locus result in a variety of syndromes that affect the immune system. Some animals develop inflammatory disease of the large intestine. Others develop a fatal disease characterized by pulmonary interstitial inflammation and alveolar proteinosis, inflammation of the stomach and skin glands that results in severe and constant itching and scarring, and hyperplasia of the lymphoid and haematopoietic cells. Itch was reported recently to target the intracellular domain of the transcription factor Notch [116]. However, it is not clear whether accumulation and subsequent excessive transcriptional activity of Notch, which is due to inactivation of Itch, leads to the Itchy syndrome, or whether it is a different target protein of Itch that accumulates and leads to the observed phenotype. In a different case, mice were developed that lack Ntan1, the gene that codes for asparagine-specific N-terminal amidase. The enzyme hydrolyzes the amide group of N-terminal asparagine and converts it to aspartate, a strong N-end rule "destabilizing" residue. The aspartate residue is modified by origine and as a result, the protein is recognized by site I of E3α and is targeted for rapid ubiquitination and degradation. The Ntan1(−/−) mice were fertile and outwardly normal, but differed from their congenic wild-type counterparts in spontaneous activity, spatial memory, and a socially conditioned exploratory phenotype that has not been previously described with other mouse strains [117]. The inactivation of another N-end rule enzyme, Arg-tRNA-protein transferase, that mediates N-terminal arginylation of acidic N-termini and possibly of Cys, rendering them susceptible to recognition by E3α, leads to embryonal death with defects in heart development and in angiogenic remodeling of the early vascular plexus [118]. In both these cases, however, the target substrates have not been identified. A mutation in the *ax* gene that codes for a deubiquitinating enzyme (Usp14) in mice results in the development of a complex neuronal syndrome characterized by severe tremors at 2–3 weeks of age followed by hindlimb paralysis and death by 6–10 weeks [119]. Usp14 cannot process polyubiquitin, and its physiological substrate may contain a monoubiquitin side chain. Importantly, in contrast to other neurodegenerative disorders such as PD and SCAs in humans and GAD in mice, neither ubiquitin-positive protein aggregates nor neuronal cell loss is detectable in the central nervous system of *ax* mice. Instead, they display defects in synaptic transmission in both the central and peripheral nervous systems.

1.7. Drug development for targeting aberrant activity of the ubiquitin system

Because of the central role the ubiquitin system plays in such a broad array of basic cellular processes, development of drugs that modulate the activity of the system may be difficult. Inhibition of enzymes common to the entire pathway, such as E1 or the proteasome, may affect many processes nonspecifically, although a narrow window between beneficial effects and toxicity can be identified for a short-term treatment. Recent experimental evidence strongly suggests that proteasome inhibitors may indeed be beneficial in certain pathologies, such as in cancer [120], asthma [121], brain infarct [122], and autoimmune encephalomyelitis [123]. As a matter of fact, for multiple myeloma, a malignancy of the immune plasma cells, a specific proteasome inhibitor [Bortezomib; PS341] was approved for use in patients [124–126]. In malignancies,

the drugs may act via inhibition of degradation of different cell cycle inhibitors or via inhibition of the anti-apoptotic transcriptional regulator NF-κB, whereas in neuroprotection they may act via inhibiting activation of NF-κB, which in this case elicits the inflammatory response. In autoimmune diseases, they may act by inhibiting presentation of "self" peptides, but also by interfering with signal transduction along cellular immune cascades. A completely different approach to drug development can be the development of small molecules that bind and inhibit specific E3s. For example, specific phospho-peptide derivatives that span the phosphorylation targeting domains in different substrates can serve as "baits" to the respective E3s [127,128]. This approach can turn, however, into a double-edged sword. In the case of p27 and IκBα, where phosphorylation destabilizes negative regulators, inhibition of the E3 can control dysregulated cell cycle and decrease untoward activity of the immune system. Thus, compounds that exert such activity can be thought of as potential drugs for the treatment of certain forms of malignancies and uncontrolled inflammatory states, respectively. However, the similarity between the phosphorylation sites of IκBα and β-catenin may also lead to stabilization of β-catenin, which is a transcriptional activator, and its excessive transcriptional activity can result in malignant transformation of benign cells. A better approach may be the development of small molecules that are substrate-specific and bind, preferably, to specific substrates or to their ancillary proteins rather then to an E3. When accelerated degradation of a tumor suppressor results in sensitization of cells to malignant transformation, selective inhibition of the recognition machinery can potentially reverse the malignant phenotype. Peptides that bind specifically to HPV-E6, and prevent its association with p53, may interfere with p53 targeting. Such peptides were able to induce p53 in HPV-transformed cells with subsequent reversal of certain malignant characteristics or induction of apoptosis [129]. Treatment directed at increasing the level of $p27^{Kip1}$ resulted in regression of the malignant phenotype in experimental models. While it is not clear that they act via the ubiquitin system, IL-6 [130] and phenylacetate [131], for example, lead to G1 arrest by increasing the level of p27.

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