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Editorial

The ubiquitin proteasome system and its involvement in cell death pathways

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Following the awarding of the 2004 Nobel Prize in Chemistry to Aaron Ciechanover, Avram Hershko, and Irwin A Rose for the discovery of ubiquitin (Ub)-mediated degradation, *Cell Death and Differentiation* has drawn the attention of its readers to the Ub Proteasome System (UPS) and its involvement in regulating cell death pathways.[1–4] The current set of reviews is an update on this theme.[5–16]

From previous review articles published in *Cell Death and Differentiation*, it was apparent that the UPS has a major mechanistic role in regulating cell death via modification and degradation of key regulatory proteins involved in apoptosis or in related pathways.[17–20] These include cell cycle regulation and DNA damage response, in which p53 protein stabilization has a crucial role.[14–16] Also, substrates cleaved by caspases during apoptosis undergo selective degradation via the N-end rule pathway and other recognition mechanisms.[18] However, a most striking example of cell death regulation by the UPS is highlighted by the finding that critical negative regulators of apoptosis contain domains characteristic of E3 Ub ligases (E3s), such as RING (really interesting new gene 1) fingers. Among these molecules are the Inhibitor of Apoptosis family of Proteins (IAPs), including XIAP, c-IAP-1 and c-IAP-2.[4–7,9,10] IAPs contain between one and three Baculovirus IAP repeats (BIRs), which, together with flanking residues, bind to caspases, thus inhibiting their proteolytic activity. The ligase activity of these proteins enables IAPs to regulate the ubiquitylation and degradation of several downstream players along the apoptotic pathways, including caspase 9, which interacts with BIR3, and caspases 3 and 7, which bind to the flanks of BIR2.[9,10] In addition, cIAPs can modify the activity of Receptor Interacting Protein 1 (RIP1), by catalysing K63-linked Ub chains on RIP1, and thus promoting the assembly of prosurvival complexes. cIAP1 and cIAP2 can also ubiquitylate tumor necrosis factor receptor-associated factor 2 (TRAF2) via their BIR2 and BIR3 domains. Stimulation of the IAP E3 activity can be triggered by binding with IAP antagonists, namely Smac/Diablo in mammals and Hid, Grim and Reaper in insects, or by IAP self-dimerization. IAP-binding motifs can be generated by proteolysis, for example by caspase cleavage; hence, IAPs would recognize N-terminal destabilizing alanine residue at the amino-termini of proteins following their exposure by caspase cleavage. Inactivation of the proteasome following caspase-mediated cleavage may disable the proteasome, interfering with its role in the regulation of key cellular processes and thereby facilitating induction of apoptosis. The noted recent developments show how understanding of these functions is just starting to emerge. For example, why does dIAP1 associate with multiple E2s via its RING finger? Does dIAP1 also interact with the E3 – the F-box protein Morgue, which is a part of an SCF E3 complex? Why does dIAP1, which is an E3, have to interact with other ligases such as the N-end rule UBR1 and an SCF complex? Why does it interact with several E2s? What is the nature of cooperation between these E3s and how do they fine tune the sensitivity to death? All these problems can now be approached experimentally as we enter an exciting era, unraveling yet another layer of mechanistic understanding and regulation of the complex cell death pathways.

During recent years we have seen significant progress in studying death receptor signaling by the UPS, as is elegantly discussed by Wertz and Dixit.[6] TNF receptor type 1-associated death domain (TRADD)-mediated death receptor signaling is regulated by the UPS at several steps.[6] Upon ligand binding, the platform adapter molecule TRADD promotes the assembly of two signaling complexes (Complex I and II) with opposing activities (pro-survival versus pro-apoptotic, respectively). In this respect, the UPS has a crucial role in the survival/death switch and in cell fate determination through RIP-1 ubiquitylation. cIAP1-2- and TRAF2-mediated modification of RIP1 with K63-linked Ub chains within complex I functions as a scaffold for downstream-signaling components, such as transforming growth factor-β activated kinase-1 and its partner protein transforming growth factor-β-activated kinase-1 binding protein-2, leading to IκK activation.[6,8] In addition, K63 polyubiquitylated RIP1 recruits NF-κB essential modulator, thus stimulating IκK activity as well, and ultimately promoting a proliferative cellular response. On the other hand, apoptosis predominates when RIP1 is degraded following K48-linked poly-Ub chain formation, or when its K63-linked Ub chains are removed. The Ub-editing enzyme A20 deubiquitylates K63-linked chains on RIP1, and also promotes K48-linked RIP1 polyubiquitylation. Several regulators of A20 activity have been identified,

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namely Tax 1-binding protein 1 and Itch.[21] RIP1 is the target of other deubiquitylating (DUB) enzymes, including the cellular-zinc-finger anti-NF-κB (Cezanne), and CYLD.[7] The molecular targets of CYLD include RIP1, NF-κB essential modulator, TRAF2, TRAF6, TRAF7, TRAF-interacting protein, Bcl3, polo-like kinase-1, and the transient receptor potential cation channel, TRPA1,[7] but when and how they are differentially regulated is still a mystery. Failure of CYLD to remove K63-based poly-Ub chains results in uncontrolled activation of the NF-κB signalosome complex, which leads to the development of cylindromatosis, scattered benign tumors of the skin appendages.[22]

Recently, a linear, head to tail poly-Ub chain generated by the Hoil/Hoip heterodimeric ligase (linear Ub chain-assembly complex)[20,23] has been shown to be involved in death receptor signaling (Henning Walczak personal communication).

Following DNA damage, but also cellular stress caused by numerous other agents, such as hypoxia, telomere shortening, and so on, p53 has a critical role in determining the fate of the cell—repair of the damage and continued progress along the cell cycle, or cell death. Therefore, regulation of the cell cycle has become an integral part of the broader scene of studies of apoptosis. Bassermann and Pagano[13] analyze the role of Skp1/Cul1/F-box protein (SCF) and anaphase promoting complex/cyclosome—two E3 complexes that are involved in degradation of numerous cell cycle regulators. The dynamic balance of substrate ubiquitylation and degradation is studied via mass spectrometry, attempting to identify DSGxxS—the 'canonical' SCF degradation targeting motif characteristic to most SCF E3s, and its phosphorylation state, which determines whether the substrates will be ubiquitylated. As noted, following damage, p53 has a major role in determining the fate of the cell: progression along the cell cycle or death. Attesting to its importance, it was found that p53 is mutated or functionally inactivated in over two-thirds of human cancers. The crucial role of p53 as a cell fate regulator and as a tumor suppressor is also revealed by the growing number of discovered E3s responsible for its proteasomal degradation or functional regulation. In unstressed cells, p53 steady state protein levels are kept in balance predominantly by the mdm2/mdmX E3s, whereas several other E3s, including P53-induced RING-H2 protein, constitutively photomorphogenic 1, ARF-binding protein 1, and WW domain containing E3 Ub protein ligase 1, participate in its regulation.[14] Mdm2-mediated control of p53 stability is modulated by several cellular inhibitors (p19(Arf), NUMB, P300/CBP-associated factor, Sentrin-specific protease 2, RING1 and Yin Yang 1-binding protein and ribosomal proteins L5, L11), as well as by activators (mdmX, Wild-type p53-induced phosphatase 1, p300 histone acetyltransferase, smoothened, and Yin Yang 1), unveiling a complex regulatory network[15] that potentiates approaches for therapeutic intervention.[5] Ub-independent p53 proteasomal degradation of p53 was also reported, and is mediated via the NAD(P)H quinone oxidoreductase 1; other targets like p21 and p73 were also reported to be targeted by a Ub-independent pathway, but unlike the single best established case of ornithine decarboxylase[16] that is degraded by the proteasome without previous ubiquitylation, the role of this pathway in targeting other substrates is still elusive, requires further substantiation, and its function is not clear.

Having described the basic, yet intricate roles and numerous functions of the UPS in regulating death pathways, the review by Eldridge and O'Brien[5] is dedicated to considerations in drug design and development. Development of reagents that specifically inhibit components of the Ub system are crucial in identifying these components as potential molecular targets in chronic inflammation, cancer therapy, and therapy chemoresistance. Furthermore, understanding how these pathways are regulated in response to DNA damage and how they can be chemically manipulated to augment apoptosis-mediated chemosensitivity, may be crucial to the development of such drugs.

Effective inhibitors of the proteasome have been developed with the initial aim of combating muscle degeneration, but are now used successfully in the clinic to combat multiple myeloma.[24–28] The idea that proteasome inhibitors could become drug candidates emerged from the observation that they can specifically induce apoptosis in different leukemia- and lymphoma-derived cells. Further development and clinical trials ended up in approval by the FDA, in May 2003, of the modified boronic dipeptide ((1*R*)-3-methyl-1-({(2*S*)-3-phenyl-2-((pyrazin-2-carbonyl) amino) propanoyl} aminobutyl) boronic acid (Pyz-Phe-boroLeu; $C_{19}H_{25}BN_4O_4$; Bortezomib, Velcade; known before as PS-341, LDP-341 or MLM341)) as a drug for the treatment of multiple myeloma.[28] It appears that the drug induces apoptosis, probably via several mechanisms. One can be inhibition of the ability of the cell to induce the unfolded protein response, which is essential to combat the stress induced by the misfolded Ig chains made in excess by these malignant B plasma cells. The other can be inhibition of generation of NF-κB, which is a strong anti-apoptotic transcriptional regulator requiring the UPS for many of its generation steps. The new, upcoming proteasome inhibitors appear to be more effective than Bortezomib, as their mechanisms of action are somewhat different. Salinosporamide and Carfilzomib bind to the proteasome irreversibly, not allowing for the fast recovery from the inhibition observed with Bortezomib. In addition, they appear to have different specificities towards the different catalytic sites of the 20S complex. As a result, their effects on cells appear to be different. For example, Salinosporamide is significantly less dependent on Bax and Bak for inducing mitochondria-mediated cell death. On the other hand, Bortezomib relies less on the Fas-associated death domain-caspase 8 signaling axis than Salinosporamide. These differential mechanisms can make the two drugs act synergistically rather than additively, potentiating their effects in a dramatic manner (for a recent review, see Navon and Ciechanover[29]).

It is clear that the proteasome inhibitors represent a new class of anti-cancer agents providing novel efficient therapeutic tools that do not belong to the 'classical' chemotherapeutic agents. However, to achieve more specificity and broaden the scope of diseases that can be treated, and to increase the safety and efficacy of novel therapeutic agents targeting the UPS, an intervention in steps upstream of the proteasome is desirable. In that respect, ubiquitylation by E3s appears to be an ideal targeting step.[5] To date, Nutlin (Roche, Basel, Switzerland), which targets Mdm2,[30] and MLN4924 (Millennium/Takeda, Cambridge, MA, USA;[31]), which targets

Nedd8 E1, are in an advanced developmental stage and evaluation. Reactivation of p53 and induction of tumor cell apoptosis on the other hand binds p53 to prevent Mdm2 binding.[32] Additional Mdm2 inhibitors have been developed by Johnson & Johnson Pharmaceuticals (JNJ-26854165; TDP665759;[33]), and by Karen Vousden and Allan Weissman (HLI98C; HLI98D; HLI98E;[34]). Screening platforms for different E3s have been established by Rigel (Seattle, WA, USA), Proteologics, Celgene, as well as by MRC (Gerry Melino, Aaron Ciechanover, Francesca Bernassola personal communication). Out of ~800 E3s, efficient theoretical targets appear to be members of the HECT (homologous to E6-AP C Terminus) family (reviewed in Bernassola *et al.*[35]). This is because they can accept directly charged Ub from the E2 and transfer it to the substrate. In that sense, and unlike the RING finger E3s, they are regarded as 'classical' enzymes rather than scaffold proteins. Moreover, HECT E3s undergo specific regulation through intermediate conformations, with 100% rotation of the N- and C-lobes on the hinge region, which can be targeted to stabilize a specific conformation intermediate, with a potential allosteric effect on activity. Clearly, the active Cys, the E3/E2, or the E3/substrate interface can also be targeted.[5] The ~600 RING finger E3s are also the subject of intense research as potential drug targets, but specific inhibitors are still not available, suggesting that these targets are more difficult to manipulate. A more straightforward drug development is under way for the ~90 DUBs, and the identification of certain selective and potent compounds further corroborate the hypothesis that DUBs can be targeted by drugs.[5] Considering the relevant role played by DUBs in the regulation of death receptor signaling,[5–8] these drugs have the potential to strongly modulate cell death pathways in chronic inflammation, and by that potentially prevent malignant transformation, provided off-target effects are kept under control.

For those of us engaged in basic research in the field of chronic inflammation and the resulting malignant transformation, along with the identification of novel targets, the development of novel therapeutic approaches is a major motivator. What can we conclude at this stage? The reviews published in this specific and dedicated issue of *Cell Death and Differentiation* take us one step further and higher from basic mechanisms and pathways of cell death, and link them in an intricate manner to the UPS, highlighting their vastly complex regulatory networks. Yet, they also point to novel and potential drug targeting components that can be further developed into a new class of targets for anti-cancer and anti-inflammatory agents, undoubtedly providing the treatment armamentarium against these diseases with valuable tools.

We are indebted to the authors of these reviews in *Cell Death and Differentiation* for giving us the opportunity to provide our readers with a unique and novel view. We hope that our readers will perceive the scientific dimension underpinning these articles, and that it will stimulate enthusiasm and further research into this rapidly evolving and exciting field.

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