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Chapter 5

Proteasome Activator 28γ: Impact on Survival Signaling and Apoptosis

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.74731

Abstract

This chapter aims to explain the functional impact of the proteasome activator (PA)28γ protein in cellular survival. The mechanistic complexity of this proteasome activator, encoded by the PSME3 gene and overexpressed in tumors in correlation with the degree of severity, is attracting growing attention. Taking anti-apoptotic properties of PA28γ into account, a simple view might explain therapy resistance of tumors by the presence of high concentrations of this proteasome regulator. A more sophisticated approach would consider functional parameters such as subcellular distribution, competition with other proteasome regulators, and factors affecting heptamer assembly, proteasome binding, and activation. Recently, PA28γ has been attributed as a proteasomal recognin, particularly for intrinsically unstructured proteins (IUPs), targeted by ubiquitin-independent proteasomal protein (UIPP) degradation. Other reports demonstrated inhibitory or stimulatory effects of PA28γ on turnover of substrates of the ubiquitin- and ATP-dependent proteasome system (UPS). Since the understanding of functional implications of PA28γ on diverse signaling processes has grown exponentially and the orchestration of proteolytic systems within apoptosis is fairly complex, this article summarizes the recent developments in PA28γ biology with emphasis on cell survival signaling pathways such as DNA repair and apoptosis.

Keywords: acetylation, apoptosis, autophagy, ATM kinase, caspase, cancer, DNA double-strand break (DSB), DNA damage response (DDR), B-cell lymphoma-extra-large ($BclX_L$), PSME3 gene, proteasome activator 28 gamma (PA28γ; REGγ), proteasome activator 700 (PA700), inhibitor of apoptosis (IAP), inflammation, melanoma-associated antigen 1 (MART-1), metabolism, NF-κB signaling, nuclear body (NB), nuclear speckle (NS), Cajal body (CB), c-Myc, p53, promyelocytic leukemia (PML), sirtuin (SirT), ubiquitin (Ub)

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1. Introduction

Posttranslational modification and regulated intracellular proteolysis are intimately linked with regulation of proliferation, differentiation, and apoptosis. Aforementioned survival processes depend on the steady-state levels of proteins acting as molecular switches of signaling networks and regulated proteolytic systems activating or removing such switches. Proteasomal protein degradation bifurcates into ubiquitin-dependent pathways, namely, the ubiquitin proteasome system (UPS) and ubiquitin-independent proteasomal protein (UIPP) degradation pathways [1]. Due to the modular structure of the proteasomal proteolytic system, composed of 20S proteasomes, 19S regulatory particles (RP), and 11S proteasome activator 28 (PA28) subtypes, it is challenging to identify definite relations between 20S proteasomes, regulator-associated proteasomes, and their substrates.

Among 11S regulators, proteasome activator 28γ (PA28γ) has earned remarkable attention, due to its pronounced overexpression in cancer and its functional relation to tumor biology. Several evidences indicate that PA28γ, in addition to its role as a 20S proteasome activator and as a putative substrate recognition module, might also act as a modulator of E3 ubiquitin ligases. Since the first contributions of PA28γ to UIPP have been reviewed a decade ago [2], a reevaluation of proteasome biology with a focus on the intrinsic pathway of apoptosis seems justified. Growing knowledge about the remarkable efforts of various proteasomal proteolytic systems in cell survival will improve the mechanistic knowledge for target definition and specific drug design to counterstrike cancer [3].

2. Ubiquitin proteasome system (UPS)

The discovery of the ubiquitin proteasome system seems to be a never ending story of surprise, starting with the first observations of non-lysosomal, ATP-dependent protein degradation [4], recognizing ubiquitin as a barcode label for proteins dedicated for proteasomal degradation [5] and, finally, realizing that ubiquitin labeling is not an absolute requirement for proteasomal recognition, unfolding, and decay [6].

2.1. Ubiquitin conjugation and barcode properties

The enzymatic machinery-conjugating ubiquitin (Ub) or ubiquitin-like proteins (UBLs) to lysine is based on hundreds of enzymes, activators, inhibitors, and substrate adaptors, establishing an information-based system for multiple purposes in signal transduction. Fifteen human E1 enzymes (ubiquitin-activating enzymes (UBAs)) initiate the conjugation cascade either specifically using ubiquitin or one of the ten different UBLs as substrate. Thioester-linked E1-Ub/UBL can be transferred to about 80 E2 enzymes (ubiquitin-conjugating UBC) via transesterification to the thiol group of an active site cysteine. Finally, more than 600 monomeric or multimeric E3 enzymes (ubiquitin protein ligases), either harboring RING or HECT domain motifs, constitute molecular scaffolds catalyzing the substrate-specific transfer

of thioester-linked Ub/UBL mostly to lysine residues of substrate proteins. Since ubiquitin contains seven putative conjugation sites, linear or branched Ub conjugates can be formed by isopeptide bonds creating individual barcodes. Processing of conjugates, either as a requirement for substrate channeling into proteasomes or for fine tuning the barcode information, is performed by cysteine proteases of the deubiquitinase (DUB) protein family [7]. The resulting barcodes are guiding proteins into different fate decisions, namely, affecting subcellular locations (UBL:SUMO), enzymatic activity (UBL:NEDD8), or 26S/30S proteasomal turnover (UBL: ubiquitin) [3–5, 8].

Ubiquitin-K48 conjugates are mostly used as degradation signal [5] that can be recognized by 19S regulatory particles (PA700) attached to one or both ends of 20S proteasomes, either forming 26S or 30S proteasomes, respectively [9].

2.2. 20S proteasomes

2.2.1. Core proteases of UIPP and UPS

The term "20S proteasome" encompasses variant isoforms of compartmentalized, barrel shaped 700 kDa core proteases (CP) of $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ stoichiometry [10]. Six active site β-subunits of the catalytic chamber are processed from zymogenic β1, β2, and β5 precursor subunits during standard (s20S) proteasome assembly, establishing the amino-(N)-terminal threonine nucleophiles central to the catalytic mechanism [11]. Proteasomes reveal cleavage site preferences for hydrophobic, basic, and acidic P1 residues resembling specificities of prototypic proteases (chymotrypsin-like (CHYT), trypsin-like (TRYP), caspase-like (CASP). Typically, for a compartmentalized protease, access to the active sites is regulated by gated pores, formed by the amino-terminal chains of α-ring subunits [12]. The narrow orifice formed by the α-rings restricts access of most native proteins. Therefore, unstructured protein regions or protein unfolding is a prerequisite for substrate channeling, conducted by 19S 11S proteasome activators [12–15].

Due to tissue-specific or cytokine-inducible expression of proteasomal active site subunits [16, 17], a high diversity of 20S proteasome complexes is principally available to associate with proteasome activators (PA) such as PA700 (19S), PA28αβ, or PA28γ (11S). As reviewed elsewhere [9, 10], proteasomes have been purified and biochemically characterized from various sources, most recently discovering the unique properties of 20S proteasomes from thymus (t20S) [18], a discovery highly relevant for the understanding of positive selection of T cells [19]. Constitutively, expressed standard 20S proteasomes (s20S) and immune proteasomes (i20S) have been compared with respect to their different assembly kinetics, half-lives, and catalytic capabilities [20].

The pro-inflammatory cytokine interferon-γ (IFN-γ) modulates the composition of the immunoproteasome (i20S), due to the expression of zymogens (pro-subunits) of inducible active site subunits (iβ1, iβ2, iβ5). During POMP-dependent proteasome assembly in proximity to the ER, active site subunits of constitutively expressed proteasomes (s20S) are replaced by the inducible subunits, forming immunoproteasomes (i20S). The i20S complexes reveal faster assembly (about 21 versus 80 min) and shorter half-lives (27 versus 133 h) and differ in

activity and cleavage site preferences from s20S proteasomes [21, 22]. In concert with other intracellular proteases, combinations of both 20S isoforms, associated with 11S/19S activator complexes, contribute uniquely to processing of peptides for MHC class I antigen presentation of self or foreign antigens [23].

2.3. 19S regulatory particle and 26S/30S proteasomes

Degradation of ub-labeled proteasome substrates depends on 26S/30S proteasomes. These protease complexes contain either one or two 19S regulatory particles (RP) associated with the α-ring subunits of s20S or i20S CPs. The 19S RP (proteasome activator PA700) is composed of base and lid sub-complexes that reveal defined pathways of chaperone-assisted assembly [9]. The base ATPase sub-complex (subunits Rpt1–Rpt6) harbors a chaperon-like unfoldase activity [24] and is involved in substrate channeling into 26S or 30S proteasomes. The lid sub-complex enables ub-conjugate binding, as well as deubiquitination for ubiquitin recycling and substrate channeling. Assembly, composition, and topology, as well as specific biological aspects of 26S/30S proteasome genesis and function, have been reviewed comprehensively by others [20, 25, 26].

2.4. Proteasome activator 28$\alpha\beta$ and MHC class I antigen presentation

The proteasome activator (PA) 28 protein family is encoded by three PSME genes. The IFNγ-inducible PSME1 and PSME2 genes accompany immunoproteasome expression. However, preferential association with i20S versus s20S proteasomes has not been supported by in vitro kinetics of proteasome activation [14]. Apart from their ability to form homo-heptameric proteasome-activating complexes in vitro, the PA28α and PA28β subunits prefer assembly into PA28$\alpha_4\beta_3$ hetero-heptamers as revealed by kinetic and structural analysis [14, 15]. PA28$\alpha_4\beta_3$ activates 20S proteasomes by channeling, regulating the width of the narrow orifice, and excluding natively folded protein substrates. The control of substrate import and export of peptide products, possibly as a molecular sieve, or due to allosteric regulation of proteasomes by PA28$\alpha_4\beta_3$ may affect proteasomal processivity and quality of peptide products [27, 28].

Double knockouts of the IFNγ-inducible members of the PSME1 and PSME2 genes [29] were examined with respect to immunological functions and immunoproteasome assembly. Substantiating the role of so-called hybrid proteasomes (PA28$\alpha_4\beta_3$-20S proteasomes) [30, 31], it was found that association of 26S proteasomes with PA28$\alpha_4\beta_3$ increased proteasomal activity and presentation of particular antigens [29]. However, PA28$\alpha\beta\gamma$-null mice showed normal antigen presentation with some antigens and impairment with others. Based on these observations, it has been stated that PA28$\alpha\beta\gamma$ is not a prerequisite for antigen processing in general, but seems to be essential for the processing of certain CTL epitopes [23].

2.5. Biochemical properties of proteasome activators 28γ

The third member of the PA28 protein family, proteasome activator (PA)28γ, encoded by the PSME3 gene, was discovered as nuclear Ki autoantigen about four decades ago [32]. Biochemical key features of the heptameric 11S regulator PA28γ [33–39] are summarized in **Table 1**.

http://dx.doi.org/10.5772/intechopen.74731

PA28γ Protein	Cellular Context & Biochemistry	Cellular or Biochemical Activities and Experimental Evidences	Refs
Biochemical Properties	Monomer/Heptamer: Assembly into $PA28\gamma_7$ $PA28\gamma_7$-20S- $PA28\gamma_7$-UIPP Protease Activates proteasomal TRYP-like activity	Purification of 11S regulator Characterization of PA28α/β/γ PA28α/β/γ: Regulation by IFNγ Properties of nuclear PA28γ Gelfiltration/Ultracentrifugation	[33] [34] [35] [39]
Sequence/ Structure	$PA28\gamma_7$ structure not available; structural information from other members of the PA28 protein family	First human PSME3 cDNA sequence $PA28\alpha_7$: first heptameric structure 11S-20S proteasome structure Structure of mammalian $PA28\alpha_3b_4$	[36] [37] [38] [15]
Subcellular Localization **Nuclear Dynamics**	PA28γ is majorly localized to the nucleus, but cytosolic shuttling has been observed. Specific functions in nuclear bodies	Biochemical evidence: purification Immunhistochemistry PA28γ and 20S colocalize in NS CBs: PA28γ-coilin interaction DSB: ATM-dep. PA28γ-20S recruitment Maintenance of chromosome stability	[39] [50] [40] [41] [42] [71]
Phosphorylation by /Association with Protein Kinases	ATM MEKK3 CHK2	PA28γ-20S recruited to DSB sites PA28γ apparently stabilized PML and CHK2 association of PA28γ	[42] [43] [69]
Acetylation	CBP acetylase SirT1 deacetylase	K195 acetylation increases heptamer stability and 20S association	[45]
Sumorylation	PIAS1 E3	PIAS1 deficiency: proteasomal degradation of p21 impaired	[44]
PA28γ Turnover	Half-life: 24 h	Pulse chase experiment	[46]
	Caspase 3/7	PA28γ harbors DGLD cleavage site	[47]

Table 1. Cellular biochemistry of PA28γ.

Particularly, roles in nuclear dynamics [40, 41], posttranslational modification [43–45], or contributions in apoptotic signaling are emphasized [46, 47].

Application of PA28γ as a diagnostic marker in inflammatory autoimmune or neoplastic diseases has been proposed [48, 49]. Initially identified as a nuclear autoantigen [32, 39, 50],

context-dependent cytosolic presence has also been reported. In breast cancer, PA28γ accumulates in nuclei, whereas in pancreatic cancer cells, PA28γ emerges in the cytosol [51], indicating that cellular context generates diverse scenarios with respect to subcellular localization and function of PA28γ. Nuclear-cytosolic shuttling, mediated by posttranslational modification, depends on cell types and conditions [44, 52, 53]. Sumoylation of amino-terminal lysine residues K6/14/12 by PIAS1 (E3) enhances cytosolic localization and increases stability, presumably prerequisites for proteasome association and substrate recognition [44]. Contrarily, acetylation at K195 by CREB-binding protein (CBP) favors assembly of nuclear PA28γ. Deacetylation by sirtuin 1 (SirT1) has been demonstrated in mammalian cells. Since K6 and K14 residues can be acetylated as well, competition with sumoylation cannot be excluded [45]. Furthermore, phosphorylation of PA28γ by protein kinase MEKK3 resulted in a concomitant increase of PA28γ levels in Cos cells [43]. Stress kinases Chk2 and ATM have been reported to phosphorylate PA28γ, thereby affecting protein-protein interactions (PPI) and functions in DNA damage response [42].

2.6. Overexpression of PA28$_\gamma$ in cancer

Several authors showed the association of PA28γ with tumorigenic pathways and correlation of expression with malignancy and metastasis. In skin carcinogenesis, PA28γ is involved in modulating WNT signaling [54]. The improved survival properties of PA28γ overexpressing cells [46] and the well-documented overexpression in tumors and cancer cell lines raise the question, how PSME3 gene expression is regulated on the transcriptional and translational level.

PA28γ-mediated mechanisms supporting cell survival in cancer cells are of high relevance for cancer therapy, particularly in the light of profoundly increased levels of PA28γ in cancer cell lines and in tumor tissue. Highly increased steady-state level of PA28γ was observed majorly in epithelial and mesenchymal tumors such as thyroid neoplasm [55]; breast tumors, particularly those with poor prognosis [56–58]; colorectal cancers [48]; hepatocellular carcinoma [59]; and oral squamous cell carcinoma (OSCC) [60].

3. The PA28γ scaffolding hub in tumor biology

A particular interest of this article is the question, how UPS and UIPP are involved in regulating cell survival pathways intimately linked with tumor biology and therapy resistance (**Figure 1**). Cyclin-dependent protein kinases (CDKs) are one regulatory implement of proliferative control. As indicated in **Figure 1A**, the PA28γ-dependent degradation of several CDK inhibitors (CDKIs) has been observed [61]. Surprisingly, expression of thermo-labile E1 enzymes in ts20TG or tsBN75 cells did not impair degradation of p21 [62]. Furthermore, contribution of SCF E3 ligases in p21 degradation could be excluded, since ts41 cells harboring a temperature sensitive mutation in the NEDD8-activating enzyme did not reveal an increased p21 half-life at the restrictive temperature. However, $p21^{Cip1}$ half-life, and similarly that of lysine-less $p16^{INK4A}$ or $p19^{Arf}$, was extended in embryonic fibroblasts from PA28γ knockout

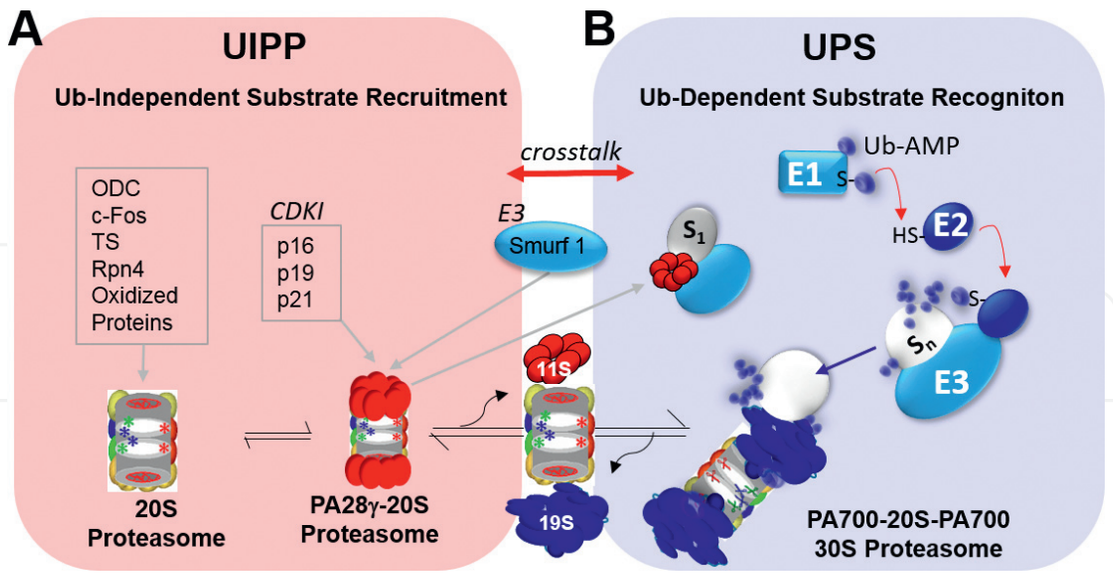

Figure 1. Mechanistic diversity of PA28γ-mediated protein turnover. (A) Typical protease complexes and substrates of UIPP. (B) Core components of UPS. Note, that UIPP and UPS are connected by cross talk: 11S and 19S regulators compete for binding to the 20S core protease. Increased assembly of PA28γ complexes is supposed to reduce the relative amount of 30S proteasomes. In cells overexpressing PA28γ, certain proteasomal substrates of PA28γ-20S proteasomes may be recognized by increased turnover, while others may accumulate due to UPS inhibition. The second mode of cross talk takes place, if UIPP degrades E3 enzymes or components required for their activation. As the third mode, PA28γ might increase PPI between E3s (Mdm2) and their substrates (S1, p53), thereby increasing UPS-dependent substrate turnover.

mice [61]. Noteworthy, aforementioned CDKIs had measurable slow rates of proteasome-dependent turnover after depletion of PA28γ, indicating that PA28γ-20S proteasome UIPP is more efficient than the PA28γ-independent pathway. Furthermore, inhibition of amino-terminal ubiquitination via acetylation or mutagenesis of internal lysine residues did not impair degradation of p21 [62]. Degradation of the three mentioned CDKIs could be performed by 20S proteasomes, but not by 30S proteasomes in vitro [61].

PA28γ-mediated effects can be assigned mechanistically to distinct categories (**Figure 1**). Firstly, central to its role in UIPP, PA28γ recruits substrates for degradation by the 20S proteasome. CDKIs revealing features of intrinsically unstructured proteins (IUP) are prototypic for this mode [61].

Secondly, a cross talk between UIPP and UPS is established, since 11S and 19S regulators compete for binding to 20S proteasomes. Interestingly, overexpression of PA28γ correlates with the increase of some UPS substrates such as c-Myc [63], ERα [58], $BclX_L$, or MART-1 [46]. Such stabilization may indicate inhibitory effects on specific E3 enzymes, UIPP degradation of auxiliary UPS factors, or reduction of the active 30S proteasome pool. Perhaps, putative 30S substrates may escape UPS degradation due to activation of DUBs. Thirdly, UPS-mediated degradation might be affected either by degradation of specific E3 enzymes (Smurf1; [64, 65]) or by enhancing the interaction of specific E3s with their substrates, as has recently been shown for Mdm2:p53:PA28γ [66].

Here, PA28γ promotes the interaction of p53 and Mdm2 (**Figure 1B**), thereby reducing total p53 levels in UV-C-radiated cells via the ubiquitin-dependent proteasomal degradation pathway.

Silencing of the PSME3 gene, as well as application of Nutlin-3, an inhibitor of Mdm2:p53 interaction, resulted in exaggerated stabilization of p53 in HCT116 or A549 cells, respectively [66].

In general, proteasomal contribution to degradation of regulatory proteins has been confirmed by using proteasome inhibitors in an adequate concentration range, thereby stabilizing either the protein of interest (POI) or its ubiquitin conjugates. Ubiquitin conjugates of specific POIs have been demonstrated after transient expression of HA-tagged ubiquitin, HA-pulldown techniques, and consecutive immunoblot analysis. Cellular systems with thermosensitive E1 enzymes were used to demonstrate dispensability of ATP-dependent ubiquitination for some proteasomal substrates. Depletion of subunits crucial for 19S assembly by siRNA silencing and in vitro degradation assays based on purified proteasome complexes either with or without regulators are alternative approaches to demonstrate UIPP or UPS involvement [1].

3.1. PA28γ in DNA damage response

DNA double-strand breaks (DSBs) may lead to chromosomal rearrangements, dysfunctional gene expression, or even aneuploidy, if not properly being targeted by the DNA damage response (DDR). The DDR is a tightly regulated nuclear process, organized majorly by post-translational modification (PTM) signals, implemented by certain stress protein kinases. It has been presumed that DSB mobility and nuclear chromatin dynamics may serve to support DNA repair in nuclear sub-compartments [67]. Major DNA repair pathways are carried out within a highly organized three-dimensional nuclear environment. Homologous recombination (HR) occurs throughout the cell cycle, while nonhomologous end joining (NHEJ) is restricted to S and G2 phases.

Recently, ATM- and PA28γ-dependent recruitment of 20S proteasomes to sites of DSB has been demonstrated by live cell imaging [42]. PA28γ depletion enhanced the focal retention of proteins of the DNA repair machinery (MDC1, 53BP1, RNF8, or BRCA1) at DNA damage sites, whereas early accumulation of focal proteins and initial formation of modified histone γH2AX were not affected. PA28γ silencing moderately reduced the NHEJ pathway, whereas the HR pathway was markedly enhanced. PA28γ seems to assemble proteasomes at the sites of DSB during early stages of the DNA repair pathway [42].

3.2. PA28γ in nuclear dynamics

While varying levels of chromatin compaction result in euchromatin and heterochromatin, nuclear bodies structure interchromatin as well [68]. PA28γ and 20S proteasomes have been found to be physically associated with nuclear structures such as nuclear speckles [40], Cajal bodies (CBs) [41], and PML bodies [69, 70] or have been associated with nuclear survival functions and chromosomal stability [71].

Nuclear speckles as subnuclear interchromatin domains are enriched in components of the pre-mRNA splicing machinery. 20S proteasomes and PA28γ co-localize in such NS structures [40]. PSME3 silencing affects NS organization and recruitment of splicing factors of the SR family to transcription sites. Proteasome inhibitors promote the accumulation of SC35 in NS.

Contributions of UIPP and/or UPS to NS protein dynamics seem to be crucial for nuclear speckle function.

CBs are sites of assembly of small nuclear ribonucleoproteins and small noncoding RNA traffic [72]. They are specific nuclear targets of the cellular stress response [41]. It has been shown that UV-C irradiation induces a stable association of PA28γ with coilin, the intrinsically disordered marker protein of CBs. The accumulation of PA28γ correlates with the disruption of CBs. Apart from its canonical targeting via Mdm2, the unstructured nature of coilin and its association with PA28γ might be the first evidence for coilin targeting via UIPP.

3.3. PA28γ in infection and inflammation

PA28γ and NF-κB signaling constitute activation loops affecting inflammatory processes [73] and bacterial infection [74]. Since inflammatory states are preceding neoplastic transformation, the molecular link between PA28γ and NF-κB biology might be of relevance in tumor biology. The PSME3 gene is under control of the transcription factor NF-κB. Since inhibitory proteins of NF-κB signaling are substrates of a PA28γ-mediated UIPP, NF-κB-mediated transcription depends on PSME3 expression and vice versa. This positive feedback regulation between PSME3 gene regulation and NF-κB-directed transcriptional regulation has been observed in two different models. First, IκBε degradation prevents the removal of NF-κB from sites of transcription, promoting constitutive activation of inflammatory pathways in the gastrointestinal mucosa. A DSS colitis model demonstrated the requirement of PA28γ for the pathological process. PSME3 knockout resulted in impairment of the DSS colitis pathology [74]. The second target of PA28γ in NF-κB signaling is KLF4, a negative regulator of NF-κB. Targeting of KFL4 by the PA28γ-proteasome releases the negative modulatory effect on NF-κB, resulting in a higher PSME3 expression and further activation of the inflammatory response to counterstrike bacterial infection [73].

Accessing sera of autoimmune and cancer patients, we observed an increased level of extracellularly localized PA28γ in patients suffering from a diverse spectrum of autoimmune diseases. In patients with rheumatoid arthritis, the level correlated with the disease state and responded to treatment with abatacept [49]. Since PA28γ has been associated with sepsis [75] or wound healing [76], development of clinical applications beyond autoimmune or cancer diagnosis is an emerging future perspective.

3.4. Transcriptional regulation of metabolism

Transcription factor c-Myc, a highly unstable protein [63, 77, 78], acts as a heterodimer with Max, recruiting coactivators through CACGTG-binding motifs to promotors of target genes involved in the regulation of cell growth, proliferation, metabolism, and apoptosis. Interestingly, c-Myc appears to be targeted by diverse PTMs and proteolytic mechanisms. UPS-dependent degradation of c-Myc depends on several E3 enzymes, F-Box proteins, and antagonizing pathways [79]. Using gene set enrichment analysis, PA28γ was shown to reprogram energy metabolism via the c-Myc-glycolysis axis, affecting glucose utilization and lactate production in cancer cells. PSME3 gene silencing reduced c-MYC levels, whereas the increased levels of

PA28γ in cancer cells increased Myc. High levels of PA28γ protein in the cytosol and nucleus repress UPS-mediated c-Myc decay and reprogram pancreatic tissue and pancreatic cancer cell lines metabolically [80]. These results were surprising, since overexpression of PA28γ in Hela cells promoted degradation of c-Myc, while depletion of PA28γ markedly increased the protein stability of c-Myc [51]. These contradictory observations indicate contextual variability of c-Myc turnover in pancreatic tumors versus other models and underline the requirement for studies focusing on cross regulation of UIPP and UPS.

3.5. PA28γ: Impact on autophagy and metabolism

Highly selective proteolytic systems such as UPS or UIPP, responsible for the selective and regulated degradation of proteins, are supplemented by autophagosomes and ub-based substrate labeling for cargo selection. Ubiquitin-like proteins and a corresponding conjugation system are initially involved in phagophore formation, a process, which integrates phosphatidylethanolamine-linked LC3-II into both leaflets of the autophagosome. Cargo receptors for ubiquitinated proteins such as p62 are recruited to autophagosomes via LIR domains (LC3-interacting region (LIR)). Finally, degradation of bulky aggregates or organelles can be conducted by lysosomal proteases during basal autophagy. Under environmental stress conditions, as an adaptive survival strategy, autophagy can be gradually upregulated, avoiding excessive activity of autophagy which may lead to cell death. The regulation of this sophisticated machinery for bulk degradation has recently been reviewed [81]. Interestingly, since Bcl-2 family proteins are closely linked to cytoprotective responses, regulation of metabolism, and apoptosis, the involvement into regulation of autophagy is not surprising. Generally, anti-apoptotic members such as $BclX_L$ can inhibit autophagy, whereas pro-apoptotic BH3-only proteins may induce autophagy. The principal behind anti-apoptotic or anti-autophagic roles of $BclX_L$ may be considered simply the same, while complexity in cross talk between autophagy and apoptosis majorly results from $BclX_L$ phosphorylation and subcellular localization. $BclX_L$ has been reported to be localized in the cytosol, the nuclear envelope, the endoplasmic reticulum, and the outer mitochondrial membrane, establishing $BclX_L$ pools of different functionalities. In anti-apoptotic signaling, $BclX_L$ antagonizes pro-apoptotic proteins via physically interacting with Bax, cytochrome c, or cytosolic p53, preventing MOMP [82, 83]. Binding of Beclin 1 to $BclX_L$ prevents assembly and activation of class III phosphatidylinositol 3-kinase (PI3K) complex, which is involved in autophagosome function.

Similarly, multifaceted as pro-survival factor $BclX_L$ [82], anti-apoptotic PA28γ protein reveals a plethora of PPIs and functional versatility. Targeting of deacetylase SirT1 for PA28γ-20S proteasome-mediated UIPP degradation was shown to regulate liver autophagy [84]. Deficiency of PA28γ as well as energy starvation dissociates the REGγ-SirT1 interaction and releases SirT1 to deacetylate components of the autophagy machinery, thereby stimulating autophagy.

Analyzing metabolic parameter of PA28γ knockout mice, a reduction of ATP consumption, possibly due to an inhibition of rDNA transcription, has been demonstrated. Under starvation, nucleolar deacetylase SirT7 was negatively regulated by PA28γ. Consistently, depletion of PA28γ induced increased Sirt7 levels, as well as increased ATP consumption. These observations

might be of relevance for cancer therapy, since PA28γ depletion sensitizes tumors to treatment with hexokinase inhibitor 2-desoxy-glucose [85].

3.6. Cross talk of UIPP and UPS in the regulation of apoptosis

3.6.1. Stoichiometry of 20S proteasomes and regulators in cellular systems

The stoichiometry of the key regulators of UPS and UIPP, namely, 19S and 11S regulators, is of particular interest to further understand how cross talk of these two proteolytic systems is affected by PA28γ. Since each biochemical process is restricted kinetically by its rate limiting step, the assembly of proteasomal complexes should be a matter of serious debate. Only few studies so far covered this question due to methodological limitations. Therefore, a recent proteomic approach is remarkable, elaborating the stoichiometry of 20S proteasome-associated regulators in detail [86, 87]. Using formaldehyde cross-linking in combinations with 20S-complex directed affinity purification on MCP21-coupled sepharose, and applying mass spectrometric quantification, proteomic analysis compared the stoichiometry of 20S proteasome complexes in nine different cell lines. Interestingly, 19S and 20S particles reached 1:1 ratio, but PA28γ-associated proteasomes occurred as minor species, occupying about 1–0.2% of proteasomes [86]. All investigated cellular systems had in common that about 20–40% of 20S proteasomes persisted in a regulator-unbound state, establishing a pool of proteasomes directly available for UIPP tasks or for dynamic behavior in the case of expressional alterations in the activator population [86].

As central pathways of protein degradation, UPS and UIPP participate in regulating the delicate balance of pro- and anti-apoptotic proteins and cell cycle regulators [88, 89]. The contribution of proteasomal degradation in controlling crucial steps of the mitochondrial pathway of apoptosis has been reviewed comprehensively [89]. Here, we are focusing on the PA28γ-mediated mechanisms, affecting the balance between pro- and anti-apoptotic regulatory proteins (**Figure 2**).

3.6.2. Discovery of anti-apoptotic properties of PA28γ

Initial studies on PA28γ-deficient mice suggested a role for PA28γ as a regulator of cell proliferation and body growth [90]. Lack of PA28γ did not affect expression of other PSME family members such as PA28α or PA28β and resulted in smaller body size. Entry into S phase was impeded and number of G1 cells increased. MEFs depleted in PA28γ revealed increased spontaneous apoptosis during logarithmic growth.

Recently, we demonstrated a correlation between cellular PA28γ levels and the sensitivity of cells toward apoptosis in different cellular contexts, thereby confirming a role of proteasome activator PA28γ as an anti-apoptotic regulator [46]. We investigated the anti-apoptotic role of PA28γ upon UV-C stimulation in B8 mouse fibroblasts stably overexpressing the PA28γ-encoding PSME3 gene and upon butyrate-induced apoptosis in human HT29 adenocarcinoma cells with silenced PSME3 genes. Interestingly, our results demonstrate that PA28γ has a strong influence on different apoptotic hallmarks, especially the levels of transcriptionally active phosphorylated p53, $BclX_L$, and active effector caspases (**Figure 2**) [46].

3.6.3. Connecting the p53 activity status and PA28γ biology

The biology of p53, a key regulator of DDR and apoptosis, appeared recently being intimately connected to PA28γ. Our data on UV-C-induced apoptosis in murine fibroblasts overexpressing PA28γ revealed an increase of phosphorylated nuclear p53, paralleled by cytosolic disappearance of p53. Kinetic experiments of apoptosis induction showed that protein levels of pro-apoptotic Bcl-2 family member Bax, cell cycle inhibitor p21, and ubiquitin E3 ligase MDM2 were transcriptionally upregulated in a time-dependent manner, indicating an increased level of transcriptionally active p53 in a pro-apoptotic context.

Furthermore, it was observed in endometrial cancer that mutant $p53^{R248Q}$ promoted the upregulation of the PSME3 gene [91]. That the PSME3 gene locus is under inhibitory transcriptional control has been reported too. Wild-type p53 inhibits the PA28γ-20S UIPP pathway by repressing PSME3 gene transcription, whereas mutant p53 was unable to repress PSME3 transcription [92].

Among other cellular survival functions, the p53 tumor suppressor protein particularly regulates key decision points of DNA repair and apoptosis via affecting transcriptional regulation of pivotal regulators of these two processes. Under normal conditions, the p53 protein is constitutively degraded via Mdm2-mediated ubiquitination and the 30S proteasome (**Figure 2D**). Under non-apoptotic conditions, PA28γ facilitates cytosolic redistribution of p53 by enhancing its nuclear export via a mechanism involving mono-ubiquitination [53]. Cytosolic p53 can activate MOMP via Bax, a mechanism prevented or balanced by the p53:$BclX_L$ and BAX: $BclX_L$ interactions [82]. Similarly, a release of cytochrome c into the cytosol, triggering apoptosome formation (**Figure 2A**), can be antagonized by Cyt c:$BclX_L$ [83] or p27:Cyt c interaction (p27, small heat shock protein), while other heat shock proteins counteract via interaction with Apaf-1 (**Figure 2A**). One key regulator in this picture is BclXL, which responds transcriptionally to p53 activation and is posttranscriptionally downregulated by PARK2, the specific E3 ubiquitin ligase preparing $BclX_L$ for UPS-mediated degradation (**Figure 2B**). Another anti-apoptotic regulator of MOMP, Mcl-1, is similarly targeted for degradation by Mule E3 ubiquitin protein ligase (**Figure 2B**) [82, 89].

DNA damage-induced ATM protein kinase (**Figure 2D**) activates p53 by phosphorylating p53 and Mdm2, thereby regulating Mdm2 oligomerization and processivity [93]. As a result of site-specific phosphorylation (human p53 ^{Ser15P} or mouse $p53^{Ser18P}$), p53 becomes stabilized and translocates into the nucleus. Phosphorylated wild-type p53 tetramers are capable of promotor and coactivator/corepressor binding [94].

In healthy cells, $p53^{wt}$ proteins cooperate with Smad3 in repressing the PMSE3 promotor [92]. Contrarily, tumor cells reveal high levels of $p53^{mut}$ activating the PMSE3 promotor via derepression and/or activation with unknown coactivators. Consistently, elevated PA28γ levels have been detected in several types of cancer and tumor cell lines [92]. These PA28γ-overexpressing cells, hence, reveal higher resistance to apoptotic stimuli and increased proliferation.

In cancer cells, mutant p53 ($p53^{mut}$) variants reveal alternative promotor selectivity. This might explain how irreparable DNA damage in healthy cells results in transcriptional amplification

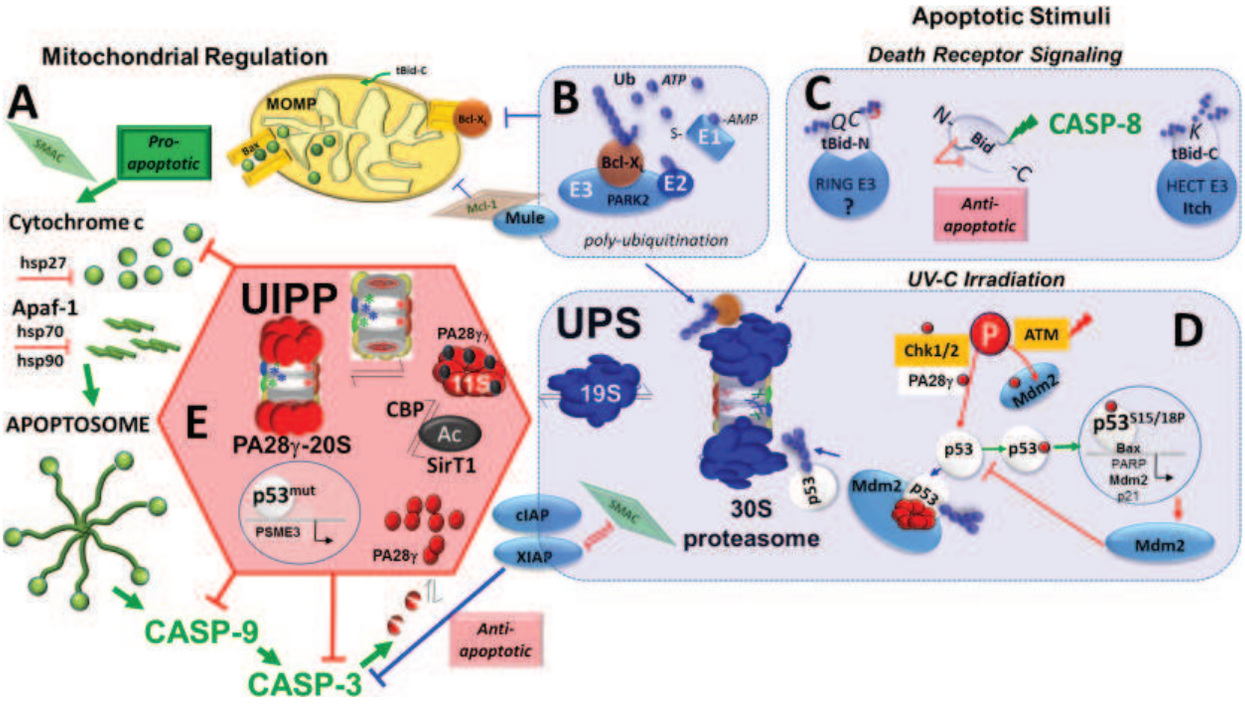

Figure 2. Impact of PA28γ on UIPP- and UPS-mediated regulation of apoptosis. (A) Mitochondrial regulation of apoptosis via MOMP, apoptosome assembly, and caspase activation. (B) Anti-apoptotic role of $BclX_L$ and its control via the UPS. (C) Death receptor signaling and extrinsic activation of apoptosis via caspase-8 (CASP-8) depend on the intriguing UPS-mediated regulation of the bid protein (reviewed by [89]). CASP-8 cleavage of bid generates amino- and carboxy-terminal peptide fragments (N-tBid; t-bid-C) that act antagonistically. Anti-apoptotic N-tBid still bound to pro-apoptotic tBid-C prevents apoptosis. Noncanonical ubiquitination at Gln and Cys residues initiates UPS-mediated degradation of N-bid. N-bid degradation is required for activation of pro-apoptotic tBid-C. Inactivation of t-bid-C is realized by itch E3 ubiquitin ligase, initiating its UPS-mediated degradation. This prevents mitochondrial outer membrane pore formation and activation of apoptosis. (D) Induction of stress kinases ATM and Chk2 by UV-C irradiation induces phosphorylation of p53, Mdm2, and PA28γ, thereby disconnecting p53 from its constitutive restriction through E3 ubiquitin ligase Mdm2. Phosphorylated nuclear p53 activates transcription of pro-apoptotic Bax, and cytosolic p53 can activate Bax-mediated pore formation directly. It has been observed that PA28γ reduces p53 levels by enhancing Mdm2:p53 interaction [66]. (E) Our model proposes a central role of anti-apoptotic PA28γ-20S UIPP in cancer cells [46]. High levels of PA28γ might be based on altered promotor selectivity of mutant p53, which has been shown to increase PSME3 transcription, whereas wild-type p53 represses PSME3 gene expression (D) [91, 92]. High PA28γ levels and acetylation are favoring heptamer assembly and proteasome activation. UIPP, either based on 20S proteasomes alone or in association with PA28γ, may reduce the level of released cytochrome c or activated caspases (CASP-9; CASP-3). If PA28γ levels are low, restriction of caspases by UIPP is released, and monomeric PA28γ is targeted by effector caspases (CASP-3). Of note, experimental evidences support the proteasomal degradation of effector caspases [46, 95], but the precise role of PA28γ in this process remains to be investigated in detail.

of apoptosis due to increasing expression of pro-apoptotic genes, while in cancer cells an anti-apoptotic scenario prevails.

3.6.4. Feedback regulation: PA28γ inhibits effector caspase activity

Overexpression of the PSME3 gene was accompanied by an increased resistance to apoptosis induction. Even with elevated levels of $BclX_L$ and a partially impaired cytochrome c release in PA28γ-overexpressing cells, we observed execution of the caspase-9/caspase-3 activation cascade upon UV-C stimulation [46]. Surprisingly, PA28γ overexpression correlated with significantly

decreased active effector caspase levels. Consistently and vice versa, PMSE3 miRNA-mediated PA28γ downregulation was accompanied by increased sensitivity to butyrate-triggered apoptosis of HT-29 cells and increased levels of active caspase-3/caspase-7 [46]. Reduced caspase activities were not due to transcriptional but posttranslational regulation.

The anti-apoptotic impact of high PA28γ levels could not solely be explained by enhanced PA28γ-mediated degradation of pro-apoptotic p53 as suggested by others [66]. Our current findings support a model (**Figure 2A** and **E**), where high PA28γ levels inhibit effector caspase-3/caspase-7, while at low PA28γ levels, caspase-3/caspase-7 activity and PA28γ turnover are increased. As a reasonable explanation, the high level of PA28γ favors heptamer stability, whereas at low concentrations, monomeric PA28γ may be more susceptible to cleavage by activated effector caspases [46, 47]. Furthermore, we found that proteasome inhibition stabilized active caspase-3 levels, indicating that the PA28γ-dependent degradation of caspase-3/caspase-7 is indeed proteasome-dependent [46]. Since the RING domain of IAP proteins ubiquitinates caspase-3/caspase-7 [95], the canonical UPS is certainly involved in regulating effector caspase activity. If PA28γ conducts restriction of caspase-3/caspase-7 activity through enhancement of physical interaction between IAPs and caspases, or directly via the PA28γ-20S proteasome UIPP route, has to be clarified in the future.

4. Concluding remarks

In light of the fact that the apoptotic potential of the cell is finally restricted by caspase levels and their execution efficiencies [96], the importance of check and balance for controlling caspase activity appears to be obvious. Recently, the impact of posttranslational modification on activity and stability of caspase-3 has been reviewed [97]. Apart from inhibitory and stimulatory phosphorylation, ubiquitination plays an important yet not a fully understood role. However, cIAP1-dependent ubiquitination of a processing intermediate of caspase-3 was followed by proteasome-dependent degradation of caspase-3. Interestingly, proteasome inhibitors stabilizing majorly active caspase-3 among other effector caspases enhanced apoptosis [97].

The integration of the recent knowledge on proteasomal contribution to regulation of apoptosis via UIPP and UPS might lead to a refined concept in system biology of apoptosis [98]. Despite of the remaining questions, experimental evidences indicate that tumor survival and resistance to therapeutic approaches may crucially relate to a plethora of new roles of PA28γ in the regulation of apoptosis, autophagy, inflammation, and metabolic adaptation.

Acknowledgements

This work has been supported by grants from the Ministry for Science, Research and Culture, MWFK, Brandenburg, via BTU Cottbus-Senftenberg (project no. c90110631) and via Health Campus Brandenburg, Cluster “Consequences of age-associated cell and organ function” (project no. 92106360).

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