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Review

Target SUMOylation and other UBL pathways for drug discovery

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ABSTRACT

The ubiquitin-proteasome system (UPS) and UBL pathways, such as SUMOylation and NEDDylation are critical in protein homeostasis and activities *in vivo* and are emerging as the target of new strategies to treat many acute and chronic human diseases, such as infections, inflammation and cancers. Cytokines, including interferons (IFNs) and Toll-like receptors, are the first line of defense for host and have crucial roles in inducing immune responses to the invading pathogens. SUMOylation inhibits the signaling pathways of IFNs and Toll-like receptors, the JAK-STAT and the NFκB pathways, respectively. NEDDylation is required for activating the Cullin family proteins, which mediate protein degradation as part of apoptosis in many solid tumors. Although only one proteasome inhibitor with a novel mechanism has been approved for marketing so far, targeting SUMOylation and other UBLs could lead to a new paradigm for therapeutic agents for a variety of pathological conditions. Recently, the first NEDDylation inhibitor, MLN4924, was shown to have great potential as a novel anti-tumor drug and is in clinical development. A family of other UBLs is emerging as different protein modifiers for different biological processes and may serve as potential drug targets in the future.

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INTRODUCTION

The ubiquitin-proteasome system (UPS) and ubiquitin-like protein (UBL) pathways are emerging as new therapeutic targets for many acute and chronic human diseases, including numerous cancers, infectious diseases, cardiovascular diseases, neurodegenerative disorders and diabetes [1-3]. Consequently, targeting UBL pathways, such as SUMOylation and NEDDylation, is an exciting new strategy for novel drug discovery and development.

A better understanding of the molecular mechanisms in UPS and UBL conjugations will accelerate the development of new therapeutic agents. However, before we can fully realize the therapeutic potential of manipulating protein homeostasis with pharmacological inhibitor(s), we must develop new tools to identify inhibitors and sensitive UBL conjugation assays. These tools include small-molecule inhibitors, small interference RNA (siRNA), crystal structures, and compound screening strategies. For example, the proteasome inhibitor bortezomib targets the UPS, and several drugs in preclinical and ongoing clinical trials involve the proteasome or ubiquitin/NEDD8 ligases. Several highly sensitive and quantitative FRET-based high-throughput screening (HTS) assays have been developed recently to identify

inhibitors of protein-protein interactions *in vitro* and in living cells for SUMOylation and other UBLs.

SUMOylation in innate immunity and inflammation

SUMOylation negatively regulates cytokine signaling in innate and adaptive immunity. Interferons (IFNs), including Types I, II and III, and Toll-like receptors, including tumor-necrosis factor (TNF), are the principle cytokines for anti-infections. IFNs exert their biological roles by inducing the expression of intrinsic antiviral proteins (e.g., protein kinase R (PKR), RNases L, inducible nitric oxide synthase (iNOS)) and induce apoptosis of virus-infected cells and cellular resistance to viral infection [4]. In addition, they activate NK cells and dendritic cells (DC) and induce activation of the adaptive immune system in which IFNγ is also critical for Th1 cell differentiation, MHC class II induction and cytotoxic T-cell activation [5-7]. IFNs and TNF utilize JAK/STAT and NFκB, respectively, as signal transducers to modify transcriptional profiles of host immune-response genes [8].

SUMOylation, mediated by its E3 ligase, the protein inhibitor of activated STAT1 (PIAS1), inhibits both the JAK/STAT and NFκB signal pathways [9, 10] (Fig. 1). PIAS1 was initially identified as a STAT1-interactive protein and later characterized as a SUMO E3 ligase [9, 11, 12]. The physiological functions of PIAS1-mediated SUMOylation in cytokine signaling were elucidated by gene deletion in mice [13]. In agreement with the results of biochemical assays, a null mutation of the mouse PIAS1 gene (*Pias1*$^{-/-}$) is more resistant to viral and bacterial infections than wild-type mice [13]. *Pias1*$^{-/-}$ and wild-type mice were challenged with the same titer of vesicular stomatitis virus (VSV). After 6 days, only 40% of wild-type mice were alive, but all the *Pias1*$^{-/-}$ mice survived. Similar data were obtained in mice challenged with the bacteria *Listeria monocytogenes*. At lower doses, all the *Pias1*$^{-/-}$ mice were alive, but only 70% of wild-type control mice survived. At a higher dose, *Pias1*$^{-/-}$ mice still showed a higher survival rate than wild-type control mice. These discoveries suggest cytokine-responsive genes can be selectively downregulated by specific negative regulators, such as SUMOylation. Similar to the suppressing STAT1 signaling by PIAS1, disrupting *Pias1* resulted in upregulation of a subgroup of NFκB-dependent genes, in response to stimulation with lipopolysaccharide (LPS) or TNF [10]. These experiments suggest a negative role of PIAS1-mediated SUMOylation in IFN- and TNF-mediated innate immune responses. Since STAT and NFκB are important in innate immunity, it will be interesting to test the hypothesis that inhibiting negative regulatory pathways, such as SUMOylation, improves immune responses against microbial infections. However, no specific SUMOylation inhibitor has been reported.

SUMOylation in genome integrity and tumorgenesis

Significant roles of SUMOylation in genome integrity and tumorgenesis have emerged. Like the ubiquitination pathway, SUMOylation is also catalyzed by an E1, E2 and E3 ligase cascade (Fig. 2A). To guard the integrity of its genome from endogenous and exogenous mutagens, cells recruit a highly coordinated response in DNA replication, gene transcription, DNA repair, and cell-cycle checkpoints [14]. Maintaining genome integrity requires activation of the appropriate repair pathways and reversible arrest at cell-cycle checkpoints. The SUMOylation pathway modifies numerous proteins in the genome integrity, such as PCNA, Rad52, Rfa1, Rfa2, and Sgs1 helicase, as well as its human homologues, Bloom syndrome protein (BLM) and DNA helicase WRN, a member of the RecQL family of helicases. Thus, it is important in protecting cells from genome instability [15]. SUMO exerts its functions through various mechanisms, such as affecting protein-protein interactions and regulating enzymatic activity and localization [16, 17].

PML protein is a member of the tripartite motif (TRIM) family, which is often fused with the retinoic receptor alpha (RARα) protein in patients with acute promyelocytic leukemia (APL). In the normal cells, PML localizes in the nucleus and forms puncta that are called PML nuclear bodies (PML-NB). PML-NB formation requires PML SUMOylation and has been implicated in diverse functions, including DNA repair, DNA replication, and DNA transport. Arsenic used to

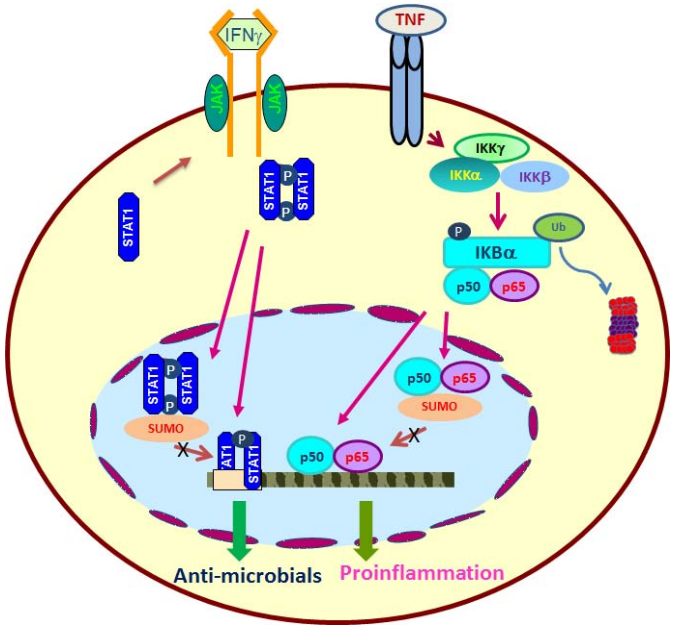

Fig. 1. SUMOylation suppresses IFN and TNF signaling in innate immunity and inflammation. SUMOylation inhibits STAT1- and NFκB-mediated transcription, which leads to an anti-microbial infection response as innate immunity and inflammation response.

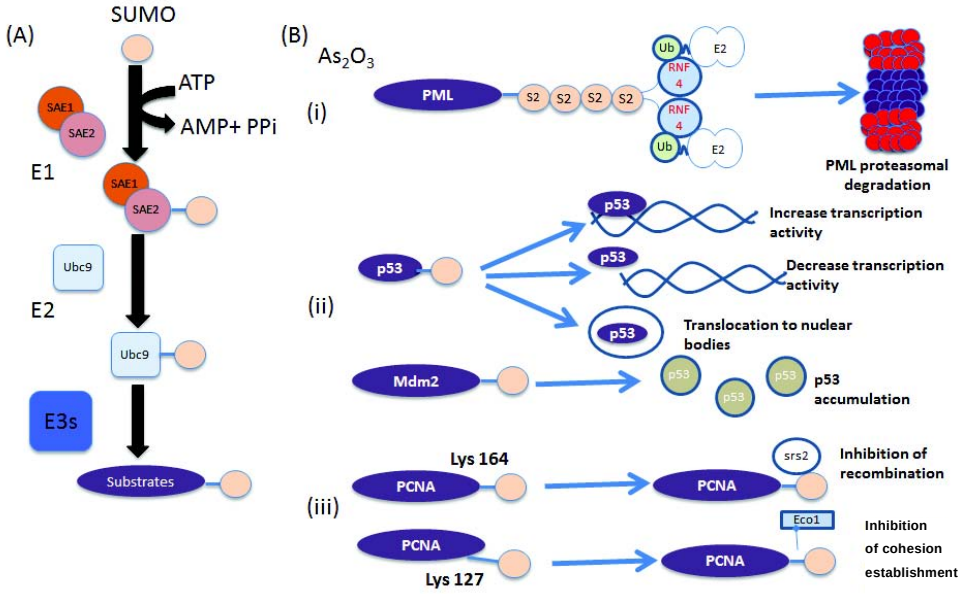

Fig. 2. SUMOylation in genome integrity and cancer development. **A.** Schematic diagram of SUMOylation pathway. SUMO (S) modification is executed by a series of three enzymatic steps: E1 activating enzyme, E2 conjugating enzyme, and E3 ligation enzyme. **B.** Examples of SUMO substrates in genome integrity. (i) In response to arsenic therapy, N-terminal domain of RNF4 binds to poly-SUMO2 chain and mediates ubiquitin degradation of PML proteins. (ii). SUMOylation of p53 can increase p53 transcriptional activity, decrease transcription activity through reduced acetylation and decreased affinity to chromatin, and regulate subcellular localization of p53 to the nuclear bodies. (iii). SUMOylation of PCNA on Lys-164 attracts Srs2 to inhibit recombination during DNA synthesis, whereas SUMOylation on Lys-127 inhibits interaction with Eco1.

treat APL patients induces SUMO-dependent ubiquitin-mediated proteasomal degradation of the PML-RAR fusion protein [18] (Fig. 2B).

SUMO-targeted ubiquitin ligases (STUbLs) are an emerging E3 ubiquitin ligase family that signifies the direct crosstalk between the SUMO and ubiquitin pathways. This family of E3 ubiquitin ligases has been implicated in maintaining genome stability in yeast [19]. Slx5-Slx8 heterodimer ubiquitin ligase, the prototype of STUbLs, contains multiple SUMO-binding domains that specifically recognize a SUMOylated substrate through SUMO interaction motifs (SIMs) [20]. In contrast to the yeast heterodimer STUbL, RING finger protein 4 (RNF4) is a monomer that perform the same STUbL function in mammals [21]. In response to arsenic therapy, N-terminal domain of RNF4 binds to poly-SUMO2 chain and mediates ubiquitin degradation of PML proteins [22]. Structural studies indicate that the RNF4 dimeric RING-type ubiquitin E3 ligases facilitate this catalysis by binding both E2 and ubiquitin and thus activating E2~Ub thioester bond [23].

The p53 protein is essential for the checkpoint control that arrest cells with damaged DNA in G1, hence the term “guardian of the genome” and “cellular gatekeeper”. p53 is an inducible, sequence-specific transcription factor that responds to stress signals, such as DNA damage by regulating cell-cycle progression, apoptosis, DNA repair, cellular metabolism, and autophagy [24]. p53 is regulated by many post-translational modifications, including SUMOylation at a single site K386 (Fig. 2B ii). SUMOylation of p53 is promoted by members of the PIAS family and Topors [12, 25]. Even though p53 is one of the first known substrates of SUMO modification, the significance of SUMOylation on p53 remains to be determined [25, 26].

PCNA is a homotrimeric, ring-shaped protein that encircles DNA and slides freely in both directions [27]. SUMOylation targets residue K164, which is also modified by monoubiquitylation or Lys-63-linked polyubiquitylation, and thus indicates an interaction between these two modifications on PCNA regulation that is often called the “ubiquitin-SUMO switchboard” [28] (Fig. 2B iii). Both ubiquitylation and SUMOylation of PNA occur in S phase, but ubiquitylation specifically occurs when DNA is damaged. PCNA mono-ubiquitylation leads to translation synthesis (TLS) that is error prone. However, polyubituitylation occurs when TLS fails and results in recombination-related error-free pathway. SUMOylation of PCNA recruits Srs2, a helicase-like enzyme, with much higher affinity and strips the recombinase Rad51 from chromatin and prevents unwanted recombination between the newly formed sister chromatids [29]. SUMOylation on Lys-127 inhibits interaction with certain PCNA-binding proteins such as Eco1 [30].

DDR pathways involve both SUMO and ubiquitin modifications in regulating their components, and some of the DDR pathways even require crosstalk between SUMO and ubiquitin to coordinate these complex events [28]. Double-strand breaks (DSBs) are caused mostly by exogenous agents, such as chemotherapy and ionizing radiation and are considered the most lethal form of DNA damage [31]. In response to DNA damage, DDR proteins are recruited and modulated by the post-translational modifications, including phosphorylation, acetylation, methylation, ubiquitination, and poly (ADP-ribosylation) [32]. The two major DNA repair mechanisms that deal with DSB are non-homologous end joining (NHEJ) and homologous recombination. As another example of SUMOylation and ubiquitination pathway interactions, Morris *et al*. identified BRCA1/BARD1 heterodimer as SUMO-regulated ubiquitin ligase (SRUbl) because SUMOylation of this heterodimer greatly increases its activity as a ubiquitin ligase in DNA damage response [33]. Additionally, Morris *et al*. and Galanty *et al*. also identified PIAS1 and PIAS4, SUMO E3 ligases, as a requirement for complete accumulation of dsDNA damage-repair proteins subsequent to RNF8 accrual [33, 34].

DNA damage induced by UV irradiation is predominantly in the form of cyclobutane pyrimidine dimer and (6-4) photoproduct bulky adduct. NER mechanism is the main defense to remove these bulky lesions [28]. In yeast, Rad4, Rad16, Rad7, Rad1, Rad10, Ssl2, Rad3, and Rpb4 are SUMO modified in the presence of Siz1 and Siz2 E3 ligases in response to DNA damage [35].

Development of genomic instability in cancer cells is one of the enabling characteristics that allow cancer cells to acquire different functional capabilities during the course of multistep

carcinogenesis [36]. Genomic instability generates random mutations, including chromosomal rearrangements. SUMO involvement in genomic integrity and its interplay with ubiquitin pathway thus have an important role in carcinogenesis.

Impaired SUMOylation also has been linked to cancers. Microphthalmia-associated transcription factor (MITF) gene mutation (Mi-E418K) inhibits SUMOylation and occurs at a significantly higher frequency in patients with melanoma, renal cell carcinoma (RCC), or both. This gain-of-function mutation has been linked to a fivefold greater risk of developing melanoma, RCC, or both cancers [37].

The SUMO pathway is a suitable target for molecular therapies of cancer because SUMO commonly malfunctions in cancers, and the malfunction state is necessary for the continued maintenance of the cancer. Increased Ubc9 levels are found in a number of human lung adeno-carcinoma [38]. Expression of PIAS3, a SUMO E3 ligase, is altered in a number of different cancer types, such as human breast carcinoma [39] and glioblastoma multiform [40]. Some studies also linked high expression levels of SUMOylation pathway components with poor survival. For example, elevated levels of SUMO E1 enzyme correlated with lower survival rates in patients with hepatocellular carcinoma and multiple myeloma [41].

The de-SUMOylation process has also been linked to carcinogenesis. SENP-3-mediated deconjugation of SUMO2/3 from promyelocytic leukemia has been correlated with accelerated cell proliferation under mild oxidative stress [42]. SENP1 enhances androgen-receptor-dependent transcription through de-SUMOylation of histone deacetylase1 (HDAC1) and thus overcoming the HDAC1 repressive function and reducing HDAC1 activity [43]. Additionally, chronic exposure to a synthetic androgen leads to a fivefold induction of SENP1 mRNA expression in prostate cancer cells, which in turn induces changes in AR-mediated cellular proliferation [44].

Inhibiting NEDDylation is emerging as a new anti-cancer strategy

Similar to ubiquitin and SUMO, NEDD8 is first activated by an E1 enzyme (NEDD8 activating enzyme, a heterodimer of NAE1 and UBA3 subunits), transferred to an E2 enzyme (Ubc12, also known as UBE2M), and conjugated to target substrates [45, 46] (Fig. 3). The best-characterized substrates of the NEDD8 pathway are the ubiquitin E3 ligase Cullin and tumor suppressor gene p53 families of proteins [47-50] (Fig. 2A). NEDDylation is essential for the enzymatic activity of Cullin-RING ligases (CRLs), a large family of ubiquitin ligases (E3s) that targets cellular protein substrates for proteasomal degradation, including several substrates with important roles in cancer, such as p27, Cyclin E, β-catenin [49-54] (Fig. 3), thereby showcasing interconnectivity between ubiquitin and NEDD8 pathways and how NEDD8 regulates ubiquitination of CRL targets. CRL targets many proteins, such as cell-cycle regulators (cycline E, p27), transcription factors (NRF2, c-Jun, β-catenin, NRF2, HIF-1α), inhibitors of transcription (IκBα), regulation of DNA replication (Cdt-1), and growth factor (BimEl, c-Myc) [55]. Deregulation of CRL targets are reported in various cancer developments [49, 50, 55, 56]. p53, MDM2 and pVHL are other well-known targets of NEDD8. NEDDylation of p53 reduces transcriptional activity of p53 [57-59]. Also CRLs effect p53 activity, suggesting different ways NEDD8 regulates p53 [49]. NEDDylation of pVHL (von Hippel-Lindau tumor suppressor protein) is important in fibronectin matrix assembly and, along with HIF (hypoxia-inducible factor) pathway, prevents VHL-associated tumorgenesis [60]. Another important target of NEDD8 is BCA3 (breast cancer-associated protein 3), and NEDDylation of BCA3 results in its interaction with NF-κB and inhibits its transcriptional activity [61]. These targets of NEDD8 indicate its importance in cell processes and highlight how deregulating the function of the NEDD8 pathway may result in cancer development. Also the recent discovery of the drug MLN4924, which inhibits NAE (E1 for NEDD8), is effective as an anti-tumor drug in pre-clinical studies, and now MLN4924 has advanced to clinical trials for treatment for cancer [55, 62], making NEDD8 an important candidate as drug target.

A recently identified NEDDylation inhibitor, MLN4924, acts as selective inhibitor of NEDD8-activating enzyme (NAE). In human tumor cell lines, MLN4924 inhibits the NEDD8ylation of

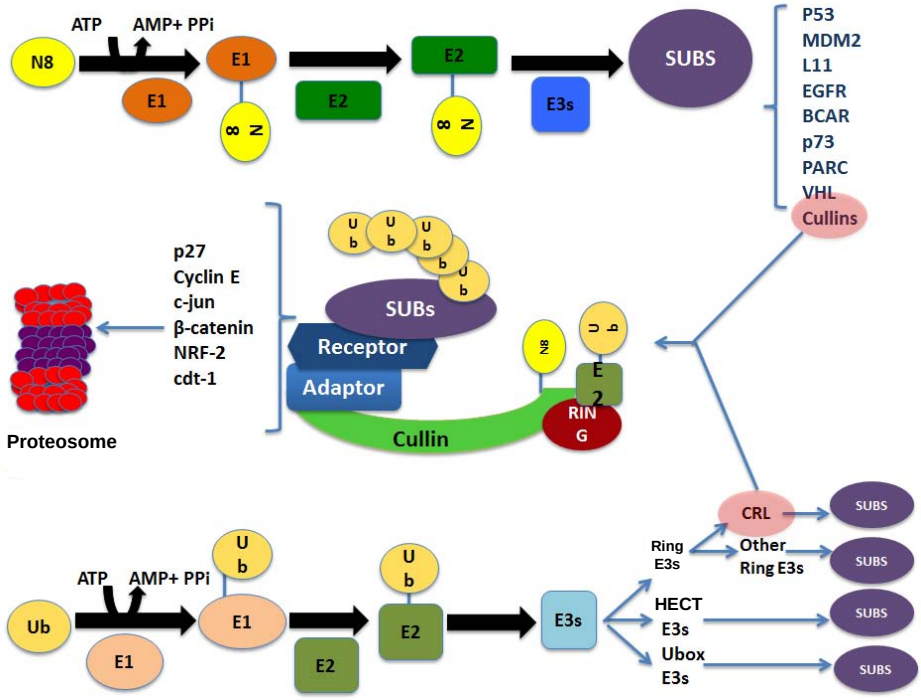

Fig. 3. Schematic representation of major steps involved in the UBL pathway. NEDD8 targets proteins with important roles in various cellular processes. An enzymatic cascade that is homologous to ubiquitination, involving NEDD8's E1 (NAE) and E2 (Ubc12), E3 (DCN1) catalyzes conjugation of NEDD8 to these targets. NEDDylation of Cullins activates CRLs, which is required for ubiquitination of CRL targets. Both substrates of NEDDylation and CRL based ubiquitination are involved in cancer development.

Cullin, resulting in increased levels of Cullin-containing RING-finger E3 substrates [55, 63]. MLN4924 inhibits a variety of tumor cell lines derived from solid tumor (lung and colon) and hematological (myeloma, lymphoma) malignancies. The anti-tumor activity of MLN4924 induces apoptotic death of human tumor cells due to the deregulation of S-phase DNA synthesis and suppresses the growth of human lung and colon tumor xenografts in mice. MLN4924 was discovered from a HTS for E1 ligase in Millennium Pharmaceuticals and is currently in clinic trials.

Other ubiquitin-like modifiers as future therapeutic targets

While ubiquitin has been implicated as a key player of the cell cycle and proteosomal degradation in all eukaryotic cells, a growing list of UBLs have been reported over the past decade. These UBLs are a diverse group of proteins, which are evolutionary conserved in terms of their three-dimensional structure and mode of conjugation to the target substrate [64-68]. However, they are otherwise distinct in terms of enzymes in their respective conjugation pathways, target substrates and their downstream signaling [69, 70]. Importantly, malfunction in any part of UBLs' respective pathways yield the primary basis for many human diseases, such as an array of cancers, cardiovascular diseases, viral diseases and neurodegenerative disorders [64-67]. While few proteins are reported as putative UBLs, 12 UBLs (SUMO1-4, NEDD8, FAT10, ISG15, FUB1, UBL5, URM1, ATG8, and ATG12) have been characterized by their three-dimensional structural homology and their covalent conjugation onto the substrates [67, 70].

CONCLUSION AND PERSPECTIVES

SUMOylation and other UBLs are emerging as a new paradigm for drug discoveries for several

acute and chronic conditions. However, technology developments to accelerate the process of drug discovery and development are needed. A series of FRET-based HTS assays for SUMOylation and NEDDylation inhibitor discovery and characterization have been reported. Förster resonance energy transfer (FRET) is a powerful tool for the study of biomolecular interactions [71-73]. Because it is highly sensitive to the distance between molecules, many attempts have been made to develop FRET assays for HTS [74, 75]. Our general FRET-based HTS strategy uses an engineered FRET pair, CyPet and YPet, that has a much higher fluorescence quantum yield, intensity, and FRET efficiency than their parent pair, ECFP/YFP [76]. This FRET pair enables us to develop robust FRET-based HTS assays for SUMOylation *in vivo* and *in vitro* [3, 77]. Small-molecule inhibitors would be valuable for investigating SUMOylation and NEDDylation. The inhibitors for SUMOylation and other UBLs can be very valuable therapeutic agents for several life-threatening conditions, such as microbial infections and cancers.

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