1521-0081/75/5/979–1006$35.00
PHARMACOLOGICAL REVIEWS

dx.doi.org/10.1124/pharmrev.122.000784
Pharmacol Rev 75:979–1006, September 2023

ASSOCIATE EDITOR: GUNNAR SCHULTE

Paradoxes of Cellular SUMOylation Regulation: A Role of Biomolecular Condensates?

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***Abstract*——Protein SUMOylation is a major post-translational modification essential for maintaining cellular homeostasis. SUMOylation has long been associated with stress responses as a diverse array of cellular stress signals are known to trigger rapid alternations in global protein SUMOylation. In addition, while there are large families of ubiquitination enzymes, all small ubiquitin-like modifiers (SUMOs) are conjugated by a set of enzymatic machinery comprising one heterodimeric SUMO-activating enzyme, a single SUMO-conjugating enzyme, and a small number of SUMO protein ligases and SUMO-specific proteases. How a few**

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This work was supported by grants from National Institutes of Health National Institute of General Medical Sciences [Grant R35GM122536], National Eye Institute [Grant R01EY03319], and an award from the American Heart Association (20TPA35410051).

The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

dx.doi.org/10.1124.pharmrev.122.000784.

SUMOylation enzymes specifically modify thousands of functional targets in response to diverse cellular stresses remains an enigma. Here we review recent progress toward understanding the mechanisms of SUMO regulation, particularly the potential roles of liquid-liquid phase separation/biomolecular condensates in regulating cellular SUMOylation during cellular stresses. In addition, we discuss the role of protein SUMOylation in pathogenesis and the development of novel therapeutics targeting SUMOylation.

***Significance Statement*——Protein SUMOylation is one of the most prevalent post-translational modifications and plays a vital role in maintaining cellular homeostasis in response to stresses. Protein SUMOylation has been implicated in human pathogenesis, such as cancer, cardiovascular diseases, neurodegeneration, and infection. After more than a quarter century of extensive research, intriguing enigmas remain regarding the mechanism of cellular SUMOylation regulation and the therapeutic potential of targeting SUMOylation.**

I. Introduction

Small ubiquitin-like modifier (SUMO) was initially discovered around 1996 (Boddy et al., 1996; Matunis et al., 1996; Okura et al., 1996; Shen et al., 1996) and found as a covalent protein post-translational modification (PTM) attached to the Ran GTPase-activating protein (RanGAP1), in a manner similar to that of ubiquitination (Matunis et al., 1996; Mahajan et al., 1997). Protein SUMOylation is a conserved biologic process essential for all eukaryotic organisms (Flotho and Melchior, 2013). While the complete cellular functionalities of protein SUMOylation remain to be elucidated, SUMOylation of a target protein often leads to alterations in its biochemical activity, cellular localization, stability, or capability to interact with other cellular components.

Despite significant technical challenges in site-specific characterization of protein SUMOylation, mainly due to the low stoichiometry of SUMO-conjugation, a large number of SUMOylated proteins have been identified in cultured human cells using various mass spectrometry (MS)-based proteomics approaches (Golebiowski et al., 2009; Becker et al., 2013; Hendriks et al., 2014; Lamoliatte et al., 2014; Tammsalu et al., 2014; Hendriks and Vertegaal, 2016; Hendriks et al., 2017; Lumpkin et al., 2017). A comprehensive mapping of the human SUMO proteome has led to the identification of more than 40,000 SUMO modification sites in 6,747 human proteins (Hendriks et al., 2017), approximately one-third of the entire human proteome. Gene ontology analysis confirms the notion that SUMOylation is highly enriched in the nuclear compartment, with more than 80% of the SUMO protein detected under basal growth conditions localized to the nucleus (Hendriks et al., 2017). Not surprisingly, many SUMOylated proteins are involved in transcriptional regulation, chromatin modeling, DNA damage response, RNA processing, and cell cycle control (Hendriks et al., 2017). Characterization of endogenous SUMOylation in various mouse organs further reinforces the notion that SUMOylation predominantly modifies protein residing in the nucleus and enriched in various nuclear assemblies such as nuclear bodies (NBs), the nuclear pore complex, or at the chromatin (Hendriks et al., 2018). Overall, these analyses have established protein SUMOylation, along with protein phosphorylation and ubiquitination, as one of the most common PTMs essential for various physiologic functions and biological regulations.

II. Basic Machinery of Protein SUMOylation

Like the ubiquitination pathway, protein SUMOylation is accomplished by a cascade of SUMOylation enzymes (Table 1). The initial step in the SUMO conjugation pathway involves the cleavage of the last few C-terminal residues of the SUMO precursors by the hydrolase activity of sentrin-specific proteases (SENPs) to expose the di-glycine residues required for conjugation (Hay, 2007). The mature SUMOs are then activated by a heterodimeric SUMO-activating enzyme (E1). The activation step catalyzed by SUMO E1 proceeds in a two-step reaction that involves the formation of a SUMO adenylate intermediate and, subsequently, a high-energy thioester bond between the C-terminus of SUMO and an active site Cys residue of the E1 SAE2/UBA2 subunit at the expense of ATP hydrolysis (Olsen et al., 2010). This is followed by the transfer of SUMO to UBC9, the only known SUMO-conjugating enzyme (E2). Subsequently, with the help of

ABBREVIATIONS: Aβ, amyloid β peptide; ACE2, angiotensin-converting enzyme 2; AD, Alzheimer's disease; AICD, APP intracellular domain; APP, amyloid precursor protein; AR, androgen receptor; ATF4, activating transcription factor 4; Cdc45, cell division cycle protein 45; DeSI, DeSUMOylating isopeptidases; EBV, Epstein-Barr virus; EPAC, exchange protein directly activated by cAMP; HD, Huntington's disease; Htt, Huntingtin protein; IDR, intrinsically disordered region; ISR, integrated stress response; ISO, isoproterenol; LLPS, liquid-liquid phase separation; LMP1, latent membrane protein-1; MCM, minichromosome maintenance; MHC-I APM, MHC class I antigen-processing and presentation machinery; MS, mass spectrometry; NB, nuclear body; NS, nonstructural protein; PB, processing body; PIAS, protein inhibitor of activated signal transducer and activator of transcription; PINIT, Pro-Ile-Asn-Ile-Thr; PML, promyelocytic leukemia; PTM, post-translational modification; RanBP2, Ran-binding protein 2; RAR, retinoic acid receptor; SAE, SUMO-activating enzyme; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SBMA, spinobulbar muscular atrophy; SENP, sentrin-specific proteases; SERCA2a, sarco/endoplasmic reticulum Ca^{2+}-ATPase 2a; SG, stress granule; SIM, SUMO-interacting motif; SUMO, small ubiquitin-like modifier; UBA, ubiquitin like modifier activating enzyme; UBC9, ubiquitin-conjugating enzyme 9; UFD, ubiquitin-fold domain; USPL1, ubiquitin-specific protease-like 1; XRCC4, X-ray repair cross complementing 4.

TABLE 1
SUMOylation enzymes

Enzyme	Name	SUMOylation	Activity
SUMO E1	SAE1/AOS1:SAE2/UBA2	+	SUMO activation
SUMO E2	UBC9	+	SUMO conjugation
SUMO E3	RanBP2	+	Substrate recruitment
SUMO E3	EGR2	+	Substrate recruitment
SUMO E3	ZNF451/ZATT	+	Substrate recruitment
SUMO E3	ZBED1	+	Substrate recruitment
SUMO E3	NSE2	+	Substrate recruitment
SUMO E3	CBX4/Pc2	+	Substrate recruitment
SUMO E3	PIAS1	+	Substrate recruitment
SUMO E3	PIAS2/PIASx	+	Substrate recruitment
SUMO E3	PIAS3	+	Substrate recruitment
SUMO E3	PIAS4/PIASy	+	Substrate recruitment
SUMO E3	KIAA1586	+	Substrate recruitment
SUMO E3	TRIM28/TIF1B	+	Substrate recruitment
SUMO Protease	SENP1	+	Hydrolase, Isopeptidase
SUMO Protease	SENP2	+	Hydrolase, Isopeptidase
SUMO Protease	SENP3	+	Isopeptidase
SUMO Protease	SENP5	+	Hydrolase, Isopeptidase
SUMO Protease	SENP6	+	Isopeptidase, SUMO chain editing
SUMO Protease	SENP7	+	Isopeptidase, SUMO chain editing
SUMO Protease	DeSI1	+	Isopeptidase, SUMO chain editing
SUMO Protease	USPL1	+	Hydrolase, Isopeptidase

a SUMO E3 ligase, UBC9 covalently attaches the SUMO moiety onto the ε-amino group of a specific lysine residue of the substrate. Additionally, certain lysine residues, for example, K11, of the conjugated SUMO2/3 protein can themselves be SUMOylated, leading to the formation of poly-SUMOylated substrates (Tatham et al., 2001; Hendriks et al., 2014). Akin to phosphorylation and dephosphorylation, protein SUMOylation is reversible and highly dynamic. SUMO-modified proteins can be robustly deconjugated by SENPs, a family of isopeptidases that release the covalently attached SUMO moiety from modified substrates to complete the SUMOylation cycle (Fig. 1). Indeed, with few exceptions, the vast majority of SUMO targets undergo rapid cycles of SUMOylation and de-SUMOylation, leading to a meager fraction of the modified species. It is also important to note that all components of the SUMOylation machinery (Table 1) are known to undergo SUMO modifications themselves (Hendriks et al., 2017), suggesting a potential feedback regulatory mechanism.

A. SUMO Isoforms

Small ubiquitin-like modifiers are small proteins structurally related to ubiquitin, with which they share ∼18% sequence homology. Despite the low sequence homology, SUMO proteins contain a ubiquitin-like β-grasp fold core flanked by a flexible N terminus of approximately 20 amino acids and a short COOH-terminal tail. The N-terminal flexible extremity includes the primary site for poly-SUMO conjugation, especially on the conserved K11 of SUMO2 and SUMO3 (Tatham et al., 2001). While low eukaryotic organisms, such as yeasts, worms, and fliers, only have one SUMO member, five SUMO isoforms, SUMO1 (Boddy et al., 1996; Matunis et al., 1996; Okura et al., 1996; Shen et al., 1996), SUMO2 (Mannen et al., 1996), SUMO3 (Lapenta et al., 1997), SUMO4 (Bohren et al., 2004; Guo et al., 2004), and SUMO5 (Liang et al., 2016), have been reported in the literature. An additional human SUMO6 can also be found in the NCBI Protein Database (Accession: QFR53058). Sequence alignment of the six potential human SUMO isoforms reveals that SUMO1, 5, and 6 are closely related to each other, sharing more than 87% sequence identity. On the other hand, SUMO2, 3, and 4 share more than 83% sequence identity when excluding the 9 extra C-terminal amino acids of SUMO3. Mature SUMO2 and SUMO3 only differ in three amino acid residues at their N-terminal flexible regions and cannot be distinguished by existing antibodies; thus are often referred to as SUMO2/3 in many cell biology studies.

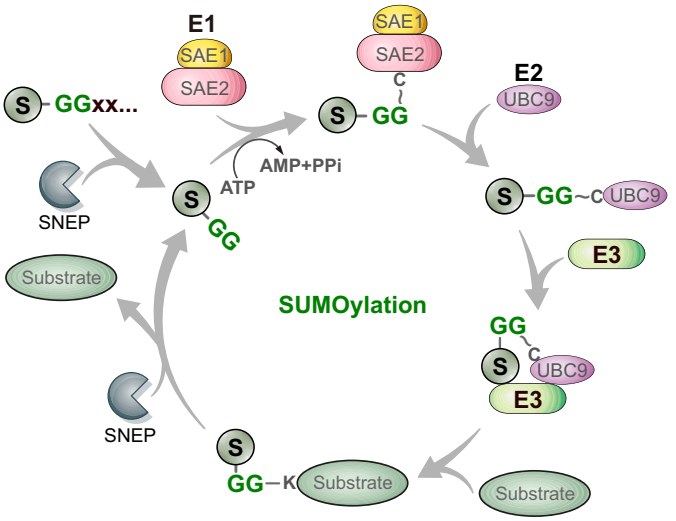

Fig. 1. The SUMO conjugation/deconjugation cascade. SUMO precursors are first processed by SENPs into mature SUMO before being activated by the SUMO E1-activating enzyme to generate a high-energy SUMO-E1 thioester bond, which is then handed over to the SUMO E2-conjugating enzyme. With the assistance of a SUMO E3 ligase, SUMO E2 transfers the SUMO moiety to the ε-amine of a lysine residue on a target substrate. The SUMOylation cycle is completed by SENP isopeptidases that release the covalently attached SUMO moiety from the substrate.

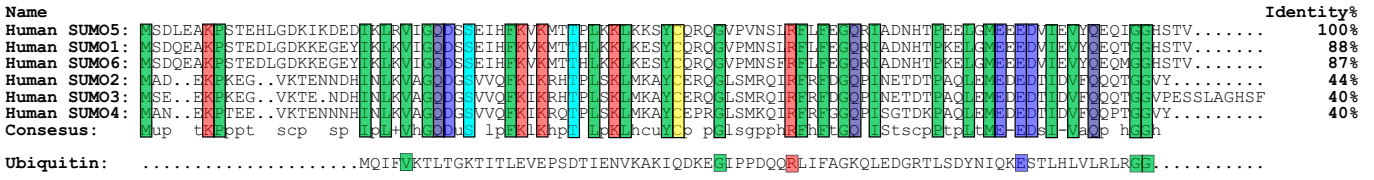

Fig. 2. Human SUMO isoforms. Sequence alignment of putative human SUMO isoforms, SUMO1 (P63165), SUMO2 (P61956), SUMO3 (P55854), SUMO4 (Q6EEV6), SUMO5 (G2XKQ0), SUMO6 (QFR53058), and ubiquitin. Colored boxes highlight identical amino acid residues.

Overall, there is about a 40% sequence identity among all human SUMO isoforms (Fig. 2).

Among six putative human SUMO isoforms, SUMO1, SUMO2, and SUMO3 are well characterized. Mammalian cells express significantly more total SUMO2/3 than SUMO1, which exists mainly in the conjugated form, while the amount of nonconjugated SUMO2/3 is ~50 times greater than that of free SUMO1 (Saitoh and Hinchey, 2000). In addition, in response to environmental stresses, the cellular pool of free SUMO2/3 decreases rapidly with a concomitant accumulation of high molecular mass SUMO2/3 conjugation. In contrast the pattern of SUMO1 conjugation remains relatively constant (Saitoh and Hinchey, 2000). Gene knockout studies reveal that while deletion of SUMO2 leads to severe developmental defects and embryonic lethality (Wang et al., 2014a), SUMO1 and SUMO3 null mice are viable and developmentally normal (Zhang et al., 2008; Wang et al., 2014a). This is likely because SUMO2 is the predominantly expressed SUMO isoform during development (Wang et al., 2014a).

On the other hand, very little is known about SUMO4, SUMO5, and SUMO6. It has been reported that the presence of a proline residue at position 90, adjacent to the C-terminal di-glycine residues, may interfere with the maturation of the SUMO4 precursor and its subsequent SUMOylation (Owerbach et al., 2005). However, subsequent studies show that when cells are under stressed conditions, SUMO4 can be matured by the stress-induced endogenous hydrolase and covalently conjugates with its target proteins (Guo et al., 2005; Wei et al., 2008). SUMO4 expression levels are increased in preeclamptic placentas and models of oxidative stress and hypoxic injury, suggesting that SUMO4-mediated SUMOylation may be involved in the pathogenesis of preeclampsia (Baczyk et al., 2017). Moreover, a functional SUMO4 M55V variant has been described to be associated with susceptibility to type 1 diabetes (Bohren et al., 2004; Guo et al., 2004). To date, it remains controversial if SUMO4 can be processed and conjugated. A recent preprint reported that SUMO4 regulated DNA double-strand break repair and SUMO signaling by promoting the activity of SENP1, independently of conjugation (https://doi.org/10.1101/2022.03.23.485504). While it is believed within the field that SUMO5, also known as SUMO1P1 (SUMO1 pseudogene 1), represents a pseudogene, a 2016 report showed that SUMO5 was transcripted and spliced at high expression levels in testes and peripheral blood leukocytes and likely translated in human lung and spleen tissues. These authors further showed that SUMO5 interacted with the components of SUMOylation machinery, including E1, E2 and formed polymeric SUMO5 chains on K160 of promyelocytic leukemia (PML) protein to facilitate the formation of PML NBs (Liang et al., 2016). However, due to the high sequence identity between SUMO1 and SUMO5, the authors could not generate SUMO5-specific antibodies and failed to detect any endogenous SUMO5 protein expression by MS (Liang et al., 2016). In contrast, neither SUMO6 transcript nor protein expression has been reported in the literature.

B. Small Ubiquitin-Like Modifier-Activating Enzyme

The SUMO E1 is a heterodimeric enzyme composed of two subunits, SAE1/AOS1 and SAE2/UBA2, homologous to the N- and C-terminal halves of monomeric ubiquitin-activating enzyme E1 (UBA1), respectively (Schulman and Harper, 2009). Sequence alignment and structural analyses confirm that SAE1:SAE2 and UBA1 share a similar multidomain architecture, including two evolutionarily related adenylation domains that bind ATP·Mg and SUMO/ubiquitin, a C-terminal ubiquitin-fold domain (UFD) that recruits E2s for thioester transfer, and a catalytic Cys domain that contains the active site cysteine (Lois and Lima, 2005; Lee and Schindelin, 2008). SAE1:SAE2 catalyzes the SUMO activation reaction in two tandem steps. First, in the presence of ATP·Mg, SAE1:SAE2 adenylates the C-terminal di-glycine of SUMOs, releasing pyrophosphate. Subsequently, the SUMO-adenylate intermediate is attacked by a conserved E1 cysteine, resulting in the release of AMP and the formation of a thioester bond between the C-terminal SUMO di-glycine and SAE2 active site cysteine 173.

Chemical syntheses of SUMO derivatives mimicking the SUMO-adenylate and SUMO-E1 thioester intermediates allow the determination of crystal structures of the E1-SUMO adenylate analog or E1~SUMO tetrahedral intermediate analog, respectively. These structures reveal significant conformational changes accompanied by the thioester bond formation half-reaction. The architecture of the SUMO-adenylate intermediate shares many parallels to structures of the SUMO E1 bound to SUMO1-ATP-Mg^{2+} (Lois and Lima, 2005), with similar domain arrangement, including the relative conformations of the UFD and Cys domain (Fig. 3, A and B). On

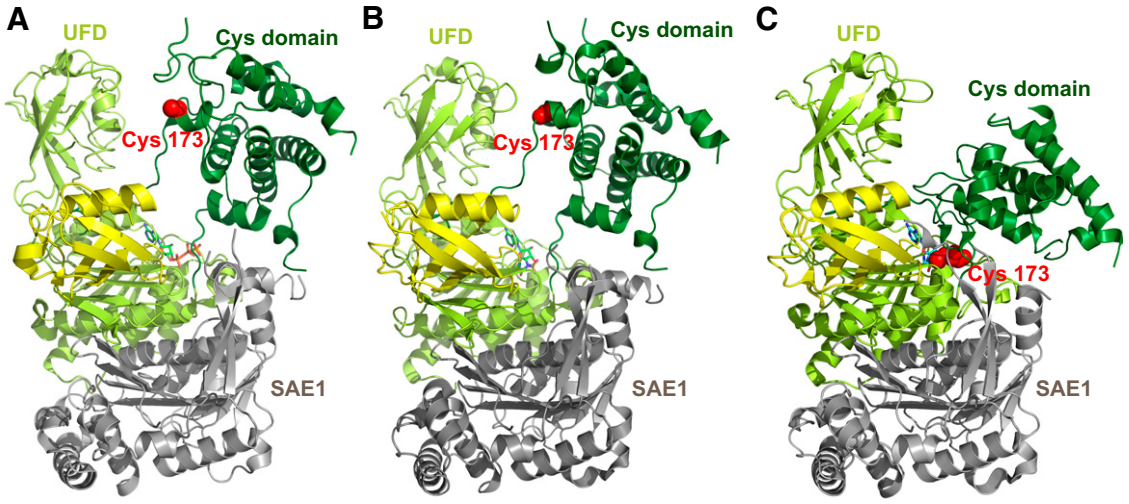

Fig. 3. Conformational changes in SUMO E1 enzyme associated with the adenylate and thioester intermediate formation half-reactions. Cartoon representation for the SUMO E1 in complex with SUMO1-ATP·Mg^{2+} (A, PDB 1Y8R) or a SUMO1-AMP mimic (B, PDB 3KYC) and SUMO E1∼SUMO1-AMP tetrahedral intermediate mimic (C, PDB 3KYD).

the other hand, SUMO-E1 thioester intermediate structure shows several significant differences highlighted by a 130-degree rotation and 3 Å translation of the Cys domain, as well as rearrangements of several key structural elements associated with the adenylation active site and the active site cysteine (Fig. 3C). Such a conformation remodeling results in the total replacement of many of the active site residues required for adenylation with residues from the Cys domain that are essential for thioester bond formation, suggesting that residues important for the adenylation half-reaction are dispensable for the thioester formation half-reaction and vice versa (Olsen et al., 2010).

Based on data from the Mouse Genome Informatics and the International Mouse Phenotyping Consortium databases, SUMO E1 is essential for mouse development as deletion of UBA2 leads to preweaning lethality with complete penetration. SUMO E1 can undergo auto-SUMOylation in cells or biochemically with purified recombinant proteins. Multiple SUMOylation sites have been identified in the UBA2 subunit, and auto-SUMOylation of UBA2 at its C-terminus has been shown to control its nucleocytoplasmic shuttling (Truong et al., 2012).

C. Small Ubiquitin-Like Modifier-Conjugating Enzyme

While there are approximately 30 to 50 mammalian ubiquitin E2s (Zheng and Shabek, 2017), only one SUMO-conjugating enzyme, UBC9, is known to exist (Seufert et al., 1995). UBC9 binds specifically to UBA2's UFD to accept the transfer of SUMO from E1 (Lois and Lima, 2005). It is important to note that UBC9 can directly bind specific SUMO targets with weak affinity. Consequently, many SUMO targets can be SUMOylated by high concentrations of UBC9 in the presence of E1 in vitro (Flotho and Melchior, 2013). Deletion of UBC9 in mice is embryonal lethal. UBC9-deficient embryos die at the early postimplantation stage. Loss of UBC9 leads to significant defects in chromosome condensation and segregation, as well as nuclear envelope dysmorphia and disruption of nucleoli and PML NBs (Nacerddine et al., 2005). Like SUMO E1, UBC9 can be SUMOylated itself. AutoSUMOylation of the mammalian UBC9 at Lys14 regulates target discrimination (Knipscheer et al., 2008). While having no effects on HDAC4, E2-25K, PML, or thymine-DNA glycosylase, SUMOylation of UBC9 impairs its activity toward RanGAP1. On the other hand, SUMOylation of the transcriptional regulator Sp100 is robustly enhanced by UBC9 auto-SUMOylation, which creates an additional interface with the SUMO-interacting motif (SIM) in Sp100 (Knipscheer et al., 2008).

D. Small Ubiquitin-Like Modifier Protein Ligases

Unlike ubiquitination, which requires and is controlled by a large number of E3 ligases (Zheng and Shabek, 2017), SUMO E1/E2 can catalyze the conjugation of SUMO to many target proteins without the presence of SUMO E3 ligases in vitro (Flotho and Melchior, 2013). This has made the identification of bona fide SUMO-specific E3 ligases challenging. So far, only a handful of genuine SUMO-specific E3 ligases have been identified as required for in vivo SUMOylation and annotated as such in the UniProt database (Table 1). While it is not surprising that SUMO E3 ligases contain SIMs (Gareau and Lima, 2010), which likely play essential roles in the recruitment of SUMO substrates, interestingly, all known SUMO E3 ligases are SUMOylation targets themselves (Hendriks et al., 2017).

It is essential to point out that while some of the "SUMO E3 ligases" reported in the literature can stimulate SUMO conjugation under certain experimental conditions, they have not been fully characterized mechanistically to qualify as bona fide SUMO E3 ligases. Three families of SUMO E3 ligases, including RanBP2, SP-RING (PIAS),

and ZNF451, have undergone comprehensive biochemical and structural characterizations. One common characteristic shared by all these genuine SUMO E3 ligases is their ability to align the thioester-bound SUMO on UBC9 in an optimal orientation for the nucleophilic attack by the SUMO-accepting lysine on the target substrate (Pichler et al., 2017).

One of the most well-studied SUMO E3 ligases is Ran-binding protein 2 (RanBP2), a 358 kDa nucleoporin protein localized to the cytosolic side of the nuclear pore complex (Pichler et al., 2002). Structure determination of a protein complex of SUMO–RanGAP1, UBC9, and RanBP2 E3 ligase domain (IR1-M) reveals a model in which RanBP2 acts as an E3 by interacting with both SUMO and UBC9 to position the E2∼SUMO thioester in an optimal orientation for transferring the SUMO moiety to the target substrate (Reverter and Lima, 2005). An alternative model was subsequently proposed in which RanBP2 is quantitatively associated with SUMOylated RanGAP1 and UBC9 in a stable complex. It is this RanBP2/RanGAP1*SUMO1/UBC9 complex, not the free RanBP2, acting as the E3 to recruit a second UBC9 to catalyze the transfer of SUMO to the substrates (Werner et al., 2012).

The protein inhibitors of activated signal transducer and activator of transcription (PIASs) represent another family of well-characterized SUMO E3 ligases (Rytinki et al., 2009). PIAS family SUMO E3 ligases contain an N-terminal SAP domain, a Pro-Ile-Asn-Ile-Thr motif (PINIT) motif, an SP-PING domain, a SIM, and a serine/threonine-rich C-terminal region. While the SP-RING domain and SIM bind UBC9 and SUMO, respectively, the PINIT motif contributes to bringing substrates into proximity to the E2∼SUMO thioester to promote SUMO transfer analogously to ubiquitin RING E3 ligases (Yunus and Lima, 2009). In addition to the PINIT motif, other domains, such as the C-terminal domain, may also be involved in the recruitment of specific substrates. It was proposed that the local concentration of the PIAS E3, rather than a single direct interaction with the substrate, is the major factor in substrate selectivity (Reindle et al., 2006). To date, a wide range of SUMO substrates associated with the PIAS family E3s have been reported, such as P53 (Kahyo et al., 2001), C-Jun (Schmidt and Müller, 2002), phosphatase and tesin homolog (Wang et al., 2014b), AKT (Li et al., 2013), and BRCA1 (Galanty et al., 2009).

The ZNF451 family of SUMO E3 ligases has been more recently discovered. At its N terminus, ZNF451 contains two SIMs connected by a linker containing a PxRP motif. These elements are responsible for ANF451's activity (Cappadocia et al., 2015; Eisenhardt et al., 2015). Structural analyses of the ZNF451 tandem SIM fragment in complex with a SUMO-charged UBC9 reveal that the first SIM of ZNF451 positions the UBC9 SUMO-thioester mimic in a closed active conformation for substrate transfer while the second SIM binds to a scaffold SUMO on the back side of the UBC9. The Arg40 of ZNF451 within the PxRP motif directly interact with Asp19 and His20 of UBC9. Disrupting these critical interactions by mutating these key residues or changing the length of the linker abolishes the E3 ligase activity (Cappadocia et al., 2015; Eisenhardt et al., 2015). These comprehensive biochemical and structural analyses establish ZNF451 as a genuine SUMO E3 ligase.

Our current knowledge of SUMO E3 substrate selectivity and specificity is largely based on studies of individual target substrates. Systematic analyses of the substrate specificity of SUMO E3 ligases are lacking. To address this issue, Zhu and colleagues applied an activity-based methodology, employing a human proteome microarray-based SUMOylation assay with purified recombinant E1, E2, and E3s, to interrogate the global SUMO E3 ligase substrate network (Uzoma et al., 2018). Their studies confirmed that significant fractions (41%) of SUMO substrates could be SUMOylated by high concentrations of E1 and E2 without E3. By optimizing the minimal concentrations of E1 (45 nM) and E2 (12.5 nM) required for nominally detectable SUMOylation signals, they were able to identify more than 1,700 E3 ligase-dependent substrates that are selectively modified with SUMO1 and/or SUMO2. Ligase-specific substrate comparison analysis revealed that while variable degrees of overlap among the substrates were observed for virtually all the pairwise comparisons, more than 1,000 substrates identified only occurred in one unique reaction, indicating a significant amount of specificity within the SUMOylation E3 machinery. RanBP2 showed a preference for SUMO1 at a level of 62.8%, consistent with previous reports (Tatham et al., 2005). On the other hand, PIAS3 and PIAS4 strongly favored modification with SUMO2 (94% and 99%), whereas PIAS1 modified substrates equally with SUMO1 and SUMO2. These observations suggest that certain SUMO E3 ligases play an important role in substrate specificity and SUMO isoform selection (Uzoma et al., 2018). Additional studies are needed to expand the scope and to corroborate if such findings hold in native cellular settings.

E. Small Ubiquitin-Like Modifier-Specific Proteases

As discussed earlier, the SUMOylation cycle would not be able to complete without SUMO proteases that serve two primary functions. The C-terminal hydrolase activity of some of the SUMO proteases is required for the maturation of SUMO proteins. In addition, the isopeptidase activity is required for the SUMO deconjugation of the modified substrates (Hay, 2007). Among the three known subgroups of SUMO cysteine proteases, SENPs are the most studied. In mammalian systems, six SENPs (SENP1, 2, 3, 5, 6, and 7) with distinct subcellular localization and SUMO isoform preference have been reported (Hickey et al., 2012). Whereas SENP1 preferentially deconjugates SUMO1-modified substrates (Shen et al., 2006a), other family members have a preference

for SUMO2/3 (Kolli et al., 2010). SENP6 and SENP7 are proteases with significant deconjugating activity for poly-SUMO2/3 chain editing (Mukhopadhyay et al., 2006; Shen et al., 2009). SENP1, SENP6, and SENP7 are localized in the nucleoplasm, while SENP2 is associated with the nuclear pores (Hickey et al., 2012). SENP3 and SENP5, on the other hand, are found to be enriched in nucleoli (Di Bacco et al., 2006; Gong and Yeh, 2006). These observations are consistent with the findings that SUMO proteases are functional and nonredundant based on knockout studies in mice (Cheng et al., 2007; Kang et al., 2010; Li et al., 2018). SENPs are regulated by PTMs. All SUMO proteases have been detected to undergo SUMOylation (Hendriks et al., 2017). The functional significance of SUMOylation of SUMO proteases has yet to be explored and is unclear.

Besides the SENP family, DeSUMOylating isopeptidases (DeSIs) represent a second class of SUMO proteases (Shin et al., 2012). DeSIs have isopeptidase but not hydrolase for processing SUMO precursors. DeSI-1 can deconjugate both SUMO1 and SUMO2/3 and cleave polymeric SUMO2/3 chains. An additional SUMO-specific protease, ubiquitin-specific protease-like 1 (USPL1), has also been discovered through an activity-based search (Schulz et al., 2012). USPL1 is a low-abundance protein and colocalizes with coilin in Cajal bodies. A recent structural analysis reveals that USPL1 is an atypical ubiquitin-specific protease that employs a unique hydrogen bond network to interact with the SUMO2 C-terminal tail (Li et al., 2022). Gene silencing of USPL1 does not affect global SUMOylation but results in coilin mislocalization, a marked change in Cajal body morphology, and impairment of cell proliferation. Although USPL1 possesses SUMO isopeptidase, as well as some C-terminal hydrolase activity, the aforementioned USPL1 cellular functions are not dependent on its catalytic activity as USPL1 catalytically inactive variants were as competent as the wildtype USPL1 rescuing the cellular defects caused by USPL1 gene silencing (Schulz et al., 2012).

F. Small Ubiquitin-Like Modifier Consensus Site

The observation that protein SUMOylation by SUMO1 is commonly observed on lysine residues immediately surrounded by specific amino acid residues led to the prediction of a canonical SUMO consensus sequence ψKxE (where ψ is a hydrophobic residue and x any amino acid), which is essential for the interaction with the SUMO E2 enzyme UBC9 (Sampson et al., 2001). Interaction with UBC9 can be enhanced by a negatively charged amino acid patch (Yang et al., 2006) or a proline-directed phosphorylation site (Hietakangas et al., 2006) downstream of the SUMO consensus motif. A subsequent MS-based proteomics analysis of endogenously SUMOylated proteins identified 103 SUMO2-targeted acceptor lysines, of which 76 fit the canonical SUMO consensus site while another 8 sites follow an inverted SUMOylation consensus motif, E/DxKψ (Matic et al., 2010). With such knowledge, predicting potential SUMOylation sites for individual proteins is possible. However, it is essential to point out that not all protein sequences containing the consensus sites are SUMOylated, and many experimentally determined SUMO sites do not match with known consensus motifs. Indeed, MS-based proteomics profiling of global endogenous protein SUMOylation shows that 31% or 9% of the SUMO modifications occurred at the canonical motif or the inverted motif, respectively. In comparison, the remaining 60% sites did not fit either but were somewhat enriched with acidic residues (Lumpkin et al., 2017). Proteomics profiling of endogenous protein SUMOylation in mouse organs further revealed that 38.1% of SUMOylated lysine residues resided in the canonical consensus motif, with 50.3% of all SUMOylation occurring on KxE-containing sites (Hendriks et al., 2018).

G. Small Ubiquitin-Like Modifier Interacting Motif

SUMO interacting motif, which was initially proposed to contain an SxS sequence, where S is a Ser residue and x any amino acid, based on a yeast two-hybrid screen for human proteins that interact with p73 and SUMO1 (Minty et al., 2000). However, this putative motif was not tested by direct binding experiments, subsequently shown as not required for binding to SUMO by NMR spectroscopy. Instead, SUMO interaction requires a hydrophobic sequence, [V/I]x[V/I][V/I] (Song et al., 2004; Hannich et al., 2005), which binds to a deep hydrophobic groove in SUMO1 between a β-strand and the α-helix (Song et al., 2005). Free SIMs often adopt disordered conformations and assume an extended β-like conformation when bound to SUMO. Surprisingly, depending on the sequence context, SIM can bind in an opposite orientation to the same groove in SUMO1 (Reverter and Lima, 2005; Hecker et al., 2006). The SUMO and SIM interaction binding affinity is relatively weak in the micromolar or submicromolar range (Song et al., 2004; Hecker et al., 2006). Interaction between SIM and SUMO can be enhanced by flanking amino acid adjacent to the core SIM motif, particularly by negatively charged residues and/or phosphoserines that participate in electrostatic interactions with a positive patch on the SUMO surface. These neighboring residues flanking the SIM determines its specificity in binding to distinct SUMO paralogues (Song et al., 2005; Hecker et al., 2006; Namanja et al., 2012). This paralogue-specific binding property of the SIM within individual SUMOylation target proteins may contribute to their paralog-specific modifications (Hecker et al., 2006). Indeed, in certain SUMOylation targets that contain a SIM motif, the SUMO-binding property of the SIM contributes to substrate recognition and is critical for SUMOylation. For example, mutation of the SIM motif in ubiquitin-specific protease 25 or Bloom syndrome gene product BLM, a RecQ-like DNA helicase, impairs its

SUMOylation by SUMO2/3, respectively (Meulmeester et al., 2008; Zhu et al., 2008).

A recent microarray screening of ~15,000 unique full-length human proteins for receptors of polySUMO2 chains, coupled with carbene footprinting, NMR, and photo-crosslinking analyses, revealed a conserved nonconventional and SUMO2/3-selective SIM (K-[SDE]-[VLI]-[DES]-[FVLI]). Similar to conventional SIMs, this new motif has a hydrophobic patch but an unprecedented positively charged lysine residue at the core (Cabello-Lobato et al., 2022). This motif is found in the N-terminal head domain of X-ray repair cross-complementing 4 (XRCC4), a DNA double-strand break repair protein that preferentially interacts with polySUMO2 over monomeric SUMO (Cabello-Lobato et al., 2022). A putative SIM with a canonical sequence of VITL at position 33-36 (pSIM33) in XRCC4 was previously identified, and its mutation led to the disruption of the binding of polySUMO2/3 (González-Prieto et al., 2021). However, a careful inspection of the crystal structures of XRCC4 reveals that pSIM33 is wholely buried and essential for properly folding the XRCC4 head domain. The apparent loss of interaction with polySUMO2 caused by mutation of pSIM33 is likely due to the loss of structural integrity of the head domain as mutation of pSIM33 also abolished XRCC4's ability to bind XLF, a known binding partner for the head domain. On the other hand, mutation of the novel noncanonical SIM KDVSF at position 102-106 to alanines did not have noticeable effects on the overall structural integrity of the mutated proteins and abrogated binding of XRCC4 to polySUMO2 (Cabello-Lobato et al., 2022). In addition to the short SUMO-interacting motif discussed previously, the ZZ Zinc finger domain of HERC2 has also been shown to bind stoichiometrically with SUMO1 with an affinity of ~3 μM and SUMO2 with a lower affinity, around 60 μM (Danielsen et al., 2012).

Many SUMOylation enzymes, particularly E3 ligases, contain functional SIMs (Lascorz et al., 2021). SIM plays an essential role in the functions of SUMO E3 ligases by anchoring the donor SUMO from the UBC9~SUMO thioester in an optimal orientation for the transfer of SUMO to the accepting lysine residue on the target substrate (Reverter and Lima, 2005; Cappadocia et al., 2015; Eisenhardt et al., 2015; Streich and Lima, 2016; Varejão et al., 2021). Some SUMO E3 ligases also contain additional SIM that binds to a second SUMO located at the backside of UBC9, presumingly contributing to the stabilization of the UBC9~SUMO thioester and/or the enzyme-substrate complex (Cappadocia et al., 2015; Eisenhardt et al., 2015; Streich and Lima, 2016; Lussier-Price et al., 2020; Varejão et al., 2021). In addition to SUMOylation enzymes, SIMs are also presented in the SUMO-targeted ubiquitin ligases, a family of ubiquitin E3 ligases selectively ubiquitinate poly- or multi-SUMOylated proteins and proteins that contain SUMO-like domains via multiple N-terminal SIMs (Prudden et al., 2007; Sun et al., 2007). RNF4, a mammalian SUMO-targeted ubiquitin ligase, is recruited to DNA damage foci along with other SUMOylated DNA repair enzymes and plays a critical role in maintaining genome stability during genotoxic stress (Galanty et al., 2012; Luo et al., 2012; Yin et al., 2012). Additionally, RNF4 is essential for the arsenic-induced degradation of PML, a primary cellular SUMO target (Lallemand-Breitenbach et al., 2008; Tatham et al., 2008). A proteomic analysis aiming for the systematic identification of direct targets of RNF4 has revealed that RNF4 directly targets the SUMO conjugation machinery, SUMO E2 UBC9, and the SUMO E3 ligases PIAS1, PIAS2, PIAS3, ZNF451, and NSE2 (Kumar et al., 2017). These results demonstrate that RNF4 is a crucial nexus for cross-talk between ubiquitination and SUMOylation and plays a critical role in maintaining SUMOylation homeostasis.

A recent proteomic analysis of SUMO-interacting proteins demonstrated that approximately 90% of all SUMO-binding proteins are themselves covalently SUMOylated (González-Prieto et al., 2021). The concomitant presence of SUMO conjugations and SIMs in single proteins facilitates the assembly of large protein complexes/networks among conjugated SUMOs and SIMs to coordinate various cellular processes spatially and temporally. Indeed, multivalent interactions among many multi-SIM-containing proteins and polySUMOylated targets may represent an essential driving force for liquid-liquid phase separation (LLPS) behind the formation of a family of membraneless biomolecular condensates, whose biologic significance has been increasingly appreciated.

III. SUMOylation, Liquid-Liquid Phase Separation, and Biomolecular Condensates

A. Biomolecular Condensates

Eukaryotic cells use numerous membrane-enclosed compartments to attain spatial and temporal control of various cellular processes. While cellular regulation via membrane partitioning or compartmentalization is well understood and accepted in the field of cell biology, accumulating evidence has demonstrated a different type of cellular organization/regulation involving diverse membraneless organelles, collectively referred to as biomolecular condensates (Banani et al., 2017). Some of the first cellular organelles discovered belong to this latter category. For example, the nucleolus, the most prominent NB, was identified around 1835 (Pederson, 2011), more than a half-century earlier than the discovery of the lipid membrane by Ernest Overton (Lombard, 2014). Recent studies have demonstrated that the nucleolus embodies a complex biomolecular condensate, where LLPS drives the formation of many different types of liquid-like droplet substructures (Brangwynne et al., 2011; Lafontaine et al., 2021). Additional well-

characterized cellular condensates include numerous NBs such as Cajal bodies (Gall, 2003), nuclear speckles (Faber et al., 2022), paraspeckles (Fox et al., 2018), and PML NB (Lallemand-Breitenbach and de Thé, 2018), as well as cytoplasmic assemblies such as stress granule (SG) and processing body (PB) (Youn et al., 2019). These diverse membraneless structures play essential roles in various biologic processes and are also increasingly implicated in human pathogenesis (Shin and Brangwynne, 2017).

Biomolecular condensates are dynamic and nonstoichiometric assemblies of concentrated biomolecules with compatible biophysical/biochemical properties distinguishable from the bulk cellular milieu (Banani et al., 2017). A pioneering study by Brangwynne and colleagues demonstrated that, unlike membrane-enclosed organelles, biomolecular condensates, such as the P granules, exhibit liquid-like behaviors, capable of fusion, dripping, wetting, rapidly dissolving, and condensing (Brangwynne et al., 2009). The major components of most biomolecular condensates are proteins and RNA (Roden and Gladfelter, 2021), while DNA and/or small molecule ligands can also be involved (Du and Chen, 2018; Klein et al., 2020). Clustering a subset of biomolecules with common functionality and/or regulatory process allows functional partitioning of the cellular space for more efficient and/or better coordinated biologic processing. For example, by concentrating enzymes, substracts, and/or modulators within the assembly, biomolecular condensates can control biochemical reactions via simple mass action (O'Flynn and Mittag, 2021), biasing enzyme conformation (Tibble et al., 2021), or substrate channeling by facilitating the formation of enzyme complexes associated with a pathway such as the de novo purine biosynthetic pathway (Pedley et al., 2022). In addition, without an enclosing membrane, biomolecular condensates can form, exchange their components, fuse, disassemble, or develop substructures rapidly in response to cellular stimuli. Our understanding of biomolecular condensates is still in its early stage and developing rapidly. The precise compositions of various biomolecular condensates, their biological functions, and their mechanisms of assembly and disassembly remain to be elucidated. However, it is generally accepted that most biomolecular condensates form through LLPS (Alberti et al., 2019).

B. Liquid-Liquid Phase Separation

LLPS is a process in which molecular components in a homogeneous solution demix (separate) into two or more distinct phases with certain molecules enriched. LLPS of macromolecules has long been observed during crystallization trials and has often been viewed as undesirable by structural biologists because LLPS is a metastable state that occurs in the absence of crystal nucleation (Dumetz et al., 2008; Xu et al., 2021). The recognition that LLPS contributes to the formation of biomolecular condensates and serves essential biological functions in the past decade has put LLPS under a new spotlight (Hyman et al., 2014; Boeynaems et al., 2018; Alberti et al., 2019). Using diverse synthetic, multivalent macromolecular components, Li and colleagues demonstrated that the association of concentrated multivalent proteins led to a sharp transition between small complexes and macroscopic lipid droplets, following the thermodynamic principles of phase transition theory of high polymer solution (Li et al., 2012). Around the same time, studies from the McKnight laboratory also demonstrated that recombinant proteins with enriched low-complexity sequences could undergo a concentration-dependent and reversible phase transition to a hydrogel-like state (Han et al., 2012; Kato et al., 2012). Low-complexity sequences are rarely found in folded protein structures and most likely exist in disordered conformations (Huntley and Golding, 2002). It is now well known that many proteins involved in LLPS contain intrinsically disordered regions (IDRs), which contribute to the formation of large protein-protein interaction networks due to their intrinsic flexibility and conformational heterogeneity that allow them to interact with multiple partners (Oldfield and Dunker, 2014).

While the aforementioned pioneering studies have provided an essential framework for dissecting the mechanism of macromolecular LLPS and revealed vital contributing factors, namely weak multivalent interaction and IDRs, for the formation of biomolecular condensates, it is not entirely clear if these underlying principles are sufficient to account for the formation of biomolecular condensates under the physiologic setting in vivo (McSwiggen et al., 2019b). If not, what are the additional or alternative mechanisms (Murray et al., 2017; Rog et al., 2017; McSwiggen et al., 2019a)? The development of novel experimental approaches and new theories capable of assessing the behavior and functions of biomolecular condensates quantitatively under the native conditions of living cells is urgently needed to address these questions (Mittag and Pappu, 2022).

C. Contribution of SUMOylation to Liquid-Liquid Phase Separation

The fact that LLPS is mediated by weak multivalent interactions among conformationally dynamic molecules, especially proteins with IDRs, coupled with the facts that SUMOylation coordinates the plasticity of protein networks by modulating protein-protein interactions, suggest that SUMOylation may play essential roles in regulating the LLPS process. To provide theoretical support for such a notion, Rosen and colleagues engineered a pair of artificial proteins, one with multiple repeats of human SUMO3 (polySUMO) and another with multiple repeats of the SIM from PIASx (polySIM), and observed that recombinant polySUMO and polySIM, when mixed, formed phase-separated droplets in vitro. Furthermore, when tethered recombinant polySUMO-polySIM single peptide chains were expressed in cells,

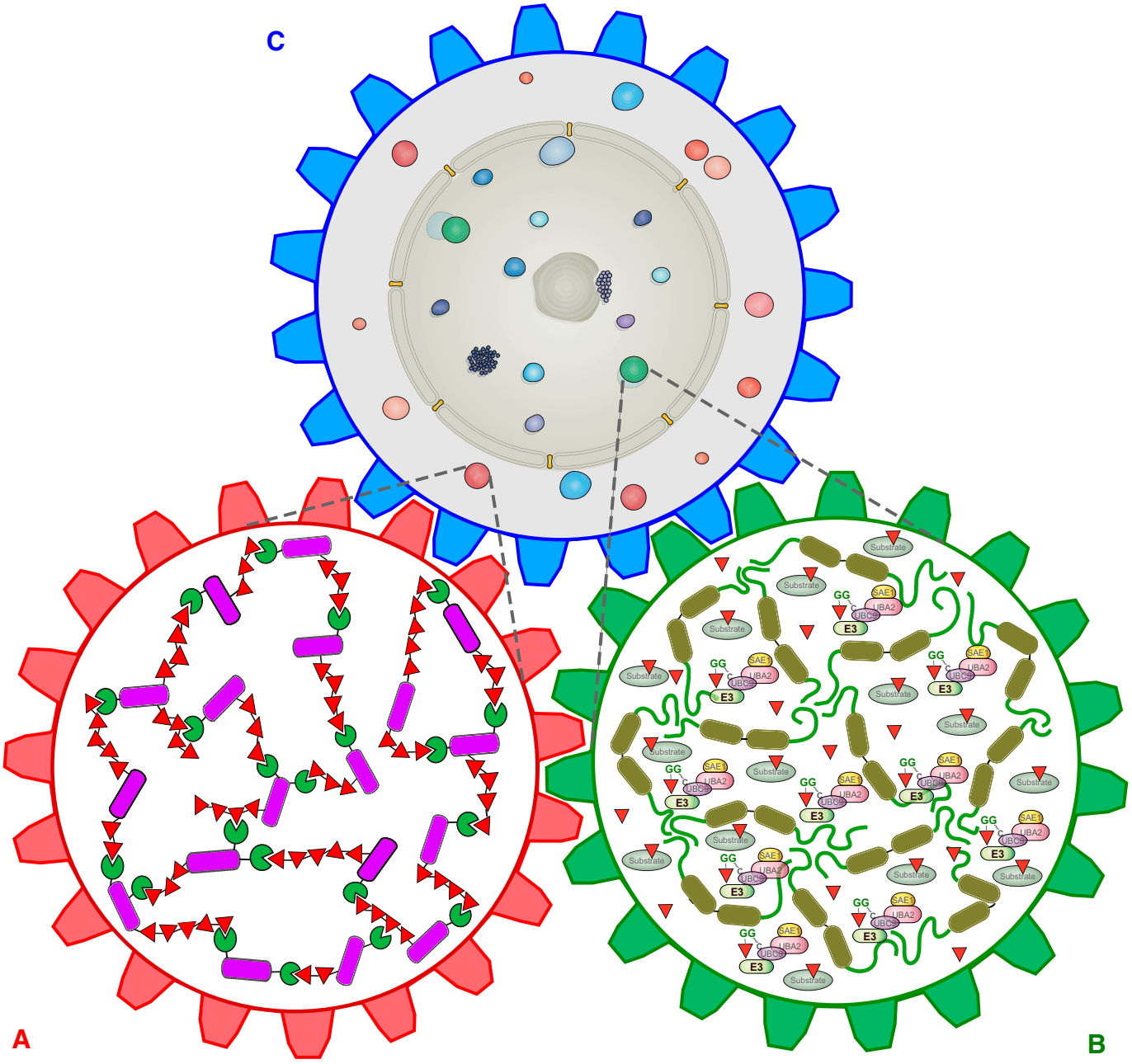

Fig. 4. LLPS and protein SUMOylation work in tandem in biomolecular condensates. (A). Multivalent interactions among SUMOylated proteins and SUMO binding proteins enhance LLPS. (B). LLPS enriches SUMOylation machinery in biomolecular condensates to accelerate cellular SUMOylation. (C). LLPS and protein SUMOylation drive the formation of biomolecular condensates.

they were also able to assemble into liquid-like cellular puncta. On the other hand, ectopically expressed GFP-tagged SUMO or SIM were found to be localized in the PML NBs where SUMO-SIM interactions are known to play an essential role in their formation (Banani et al., 2016). These results provide a conceptual framework in which SUMO-SIM-mediated multivalent interactions function as a driving force for LLPS (Fig. 4A). Subsequent in silico simulation analysis reveals that the same SUMO–SIM interactions of the polySUMO-polySIM system can provide both the strong and weak interactions required for the formation of condensates. While strong interactions drive the formation of zipperlike microstructures, weaker interactions then condense them into a fluid state via cross-linking. Such a hierarchy determines how client recruitment varies with changes in valence and affinity, as well as how the interactions within liquid states can be disordered yet still contain structural features with functionality (Bhandari et al., 2021).

Proteomics and bioinformatics-based analyses show that lysine residues within the disordered regions of proteins are preferentially targeted for SUMOylation (Yavuz and Sezerman, 2014; Hendriks et al., 2017). Since one common feature that many proteins involved in LLPS is the presence of IDRs, which are prone to misfolding and aggregation, SUMOylation may also contribute to the fine-tuning of the dynamics of IDR-mediated LLPS via selective modifications of proteins with IDRs in addition to the involvement of SUMO-SIM interaction.

D. SUMOylation and Biomolecular Condensates: Partners in Stresses?

It has been well documented that many of the components of biomolecular condensates undergo SUMO modifications. Accumulating evidence also suggests that constituents of the SUMOylation machinery are enriched in many membraneless cellular compartments, further strengthening the connection between SUMOylation and biomolecular condensates. Indeed, SUMOylation has been implicated in the formation and/or regulation of numerous biomolecular condensates, such as Cajal bodies (Navascues et al., 2008; Hutten et al., 2014; Schulz et al., 2012; Tapia et al., 2014; Zhang et al., 2021), nucleoli (Mo et al., 2002; Rallabhandi et al., 2002; Tago et al., 2005; Gong and Yeh, 2006; Haindl et al., 2008; Kuo et al., 2008; Yun et al., 2008; Westman et al., 2010; Smith et al., 2014; Capella et al., 2021), nuclear speckle (Kim et al., 1999; Chakrabarti et al., 2000; Weger et al., 2003; Chen et al., 2004; Smolen et al., 2004; Ihara et al., 2008; Yang et al., 2009), and PcG bodies (Kagey et al., 2003; MacPherson et al., 2009; Gonzalez et al., 2014). In this section, we focus our discussion on the role of SUMOylation in PML NBs and SGs, the archetype of nuclear or cytosolic biomolecular condensate, respectively.

1. SUMOylation and Promyelocytic Leukemia Nuclear Bodies. PML NBs are discrete membraneless subcompartments found in most mammalian cell nuclei. They are typically spherical organelles with a diameter between 0.2 and 1 μm and number 1 to 30 bodies per nucleus. PML NBs are dynamic structures that vary in number, size, and position during cell-cycle progression and in response to cellular stresses (Bernardi and Pandolfi, 2007). The major component of PML NBs, PML protein, is a tumor suppressor and a member of the tripartite motif (TRIM)-containing protein family. PML protein is essential for the formation of PML NB by serving as a scaffold for the recruitment of client proteins that permanently or transiently reside in PML NBs (Ishov et al., 1999; Bernardi and Pandolfi, 2007).

The importance of SUMOylation in PML NB assembly has been well documented. It was initially demonstrated that oxidative stress mediated by arsenic trioxide induced a robust SUMO1 modification of PML and simultaneous compartmentalization of SUMOylated proteins in the PML NBs while unmodified PML was localized exclusively in the bulk nucleoplasm (Müller et al., 1998). In addition to SUMO1, PML can be modified by SUMO2 and 3 (Kamitani et al., 1998b) at three major sites: K65, K160, and K490 (Kamitani et al., 1998a). Unlike WT PML, PML mutated at its three SUMO modification sites was unable to nucleate PML-NB formation in $PML^{-/-}$ cells (Zhong et al., 2000). Subsequent studies have established that PML contains SIMs that are required for the nucleation of PML from PML NBs (Shen et al., 2006b). Moreover, many components of PML NBs are also SUMO modified and/or contain SIMs, further suggesting that PML NB assembly occurs through a complex network of noncovalent interactions between modified substrates and SUMO-binding proteins. These findings suggest that PML SUMOylation and noncovalent SUMO-SIM interactions are essential for initiating PML NB formation and subsequent recruitment of SUMOylated proteins and/or proteins containing SIM. On the other hand, other studies suggest that the initial PML NB nucleation is independent of PML SUMOylation or SIM (Ishov et al., 1999; Sahin et al., 2014) but requires the assembly of oxidized PML covalent multimers (Sahin et al., 2014). It is important to note that biomolecular condensates, such as PML NB, are not homogenous. PML NBs consist of an inner dynamic core of transiently associated client proteins enclosed by a shell of stable insoluble PML aggregates. While expendable for outer shell formation, PML SUMO-SIM interactions are indispensable for the recruitment of client proteins and their stable NB association (Lallemand-Breitenbach and de Thé, 2018). Irrespective of the role of PML SUMO-SIM interactions in the nucleation of PML NB, functional connections between protein SUMOylation and LLPS/biomolecular condensates are well established.

PML NBs have been implicated in a plethora of biological processes such as antiviral response, apoptosis, cell cycle control, DNA damage response, DNA replication, senescence, epigenetic control, and transcriptional and post-transcriptional regulation. However, precisely how PML NB regulates these cellular functionalities needs to be clarified. It has been proposed that it may function as a reaction hub to facilitate protein PTMs such as SUMOylation and/or act as a storage/triage compartment in the nucleus during cellular stresses (Bernardi and Pandolfi, 2007). These notions are consistent with the findings that PML NBs are enriched with SUMO E2 enzymes UBC9, which binds to the N-terminal cysteine-rich zinc-binding RING finger domain of PML (Duprez et al., 1999), SUMO isoforms, and SUMO E3 ligases such as RANBP2 and PIASy, as well as several SENPs (Van Damme et al., 2010). Therefore, it is unsurprising that PML NBs are hotspots for SUMOylation, particularly in response to stresses. Moreover, PML NBs are also convergent points of SUMOylation and ubiquitination through the directly recruiting SUMO-targeted ubiquitin E3 ligases, such as RNF11/Arkadia (Erker et al., 2013) or RNF4 as discussed earlier (Lallemand-Breitenbach et al., 2008;

Tatham et al., 2008). Pharmacological inhibition of ubiquitylation leads to changes in SUMOylation of hundreds of newly synthesized proteins and their accumulation in chromatin-associated PML NBs in a UBC9 and PML-dependent manner (Sha et al., 2019). This close coupling between protein SUMOylation in PML NB and ubiquitination, a critical protein quality control pathway, further supports the notion that PML NBs function as protein quality control hubs in the nucleus to maintain proteostasis.

2. SUMOylation and Stress Granules. Stress granules and PBs are two closely related cytosolic membraneless organelles that form through the LLPS of ribonucleoproteins. Whereas SGs and PBs are considered distinct organelles, they share many components of translationally inactive mRNAs and associated RNA-binding proteins that shuttled among them and the cytoplasm. SGs are enriched with translation initiation factors, and PBs are rich in mRNA degradation machinery components (Riggs et al., 2020). Although SGs and PBs are believed to be mainly involved in translational control and RNA metabolism regulation under stress conditions, their precise biologic functions remain poorly understood (Glauninger et al., 2022).

Consistent with the facts that SUMOylation and SG can be triggered by a similar set of stressors, such as heat shock or oxidative stress, emerging evidence suggests that protein SUMOylation may play an essential role in the formation and/or functions of SG and PBs. Many components of SGs are found to be modified by SUMO. For example, the eukaryotic initiation factor 4A1/2 (eIF4A) is found to be modified by SUMO on a single residue, K225/K226. Overexpression of the SUMOylation deficient eIF4A2-K226R mutant leads to a significant reduction in its recruitment to SGs and SG volume (Jongjitwimol et al., 2016). Using multibait proximity labeling proteomics approaches based on an engineered ascorbate peroxidase, a recent study has identified 109 novel SG proteins that include SUMOylation enzymes E1, E2, and E3s and revealed that SGs are enriched with known SUMO target proteins. These findings suggest that SUMOylation of SG proteins likely occurs directly in situ. Doxycycline-inducible depletion of UBC9 in mouse embryonic stem cells led to the complete blockade in SG formation, while inhibition of SUMOylation using siRNA and pharmacological approaches impaired SG disassembly (Marmor-Kollet et al., 2020). These observations, together with recent findings that RNF4 targets SG-associated proteins in the nucleus to facilitate SG disassembly (Keiten-Schmitz et al., 2020; Bennett and La Spada, 2021; Maraschi et al., 2021), provide compelling evidence to support a hypothesis in which mono-SUMOylation promotes SG formation via enhancing SUMO-SIM interactions, whereas poly-SUMOylation contributes to SG disassembly via StUbL-dependent ubiquitination (Marmor-Kollet et al., 2020).

E. Mechanism of Cellular SUMOylation: Liquid-Liquid Phase Separation Comes to Rescue?

As one of the most prevalent PTMs, along with protein phosphorylation and ubiquitination, protein SUMOylation possesses some unique attributes that separate it from the rest. Above all, although ubiquitination and SUMOylation share similar reaction schemes and even common modification sites in specific substrates, ubiquitination is tightly regulated by a large number of ubiquitination enzymes, including at least two E1 ubiquitin-activating enzymes, 30-50 E2 ubiquitin-conjugating enzymes, and more than 600 E3 ubiquitin ligases (Zheng and Shabek, 2017) whereas protein SUMOylation is catalyzed by only one SUMO-activating enzyme (SAE1:SAE2) E1, one SUMO-conjugating enzyme (UBC9) E2, and a small set of validated E3 ligases (Bergink and Jentsch, 2009; Vertegaal, 2022). Additionally, unlike protein phosphorylation, which is frequently triggered by well-defined signals, such as growth factors or second messengers, the biological stimuli that regulate SUMOylation are poorly understood: SUMOylation is regulated by an array of diverse cellular stress signals, such as heat shock or oxidative stress that are more global and less specific in nature (Saitoh and Hinchey, 2000; Zhou et al., 2004; Golebiowski et al., 2009; Yang et al., 2012). Lastly, groups of functionally related proteins often undergo cellular SUMOylation concertedly in response to cellular stresses (Jentsch and Psakhye, 2013; Psakhye and Jentsch, 2012, 2016; Tammsalu et al., 2014). Such group modifications occur topologically at various cellular loci, such as DNA damage/repair sites and PML bodies. It remains an enigma as to how a few SUMOylation enzymes, in response to diversely less defined stimuli, modify a large number of protein substrates in a coordinated fashion.

These distinct features described suggest that cellular SUMOylation is regulated in broad strokes at a more global level, involving either increasing the activity of the conjugation machinery or decreasing the activity of SENPs responsible for deconjugating target proteins. However, the fact that cellular SUMOylation in response to heat shock happens rapidly, reaching maximal within 5 minutes, but their clearance (deSUMOylation) takes place at a much slower pace (~2 hours) suggests a dominant role of the conjugation process during stress responses (7). Moreover, even if SENPs were the significant drivers of dynamic regulation of cellular SUMOylation, we would face the same dilemma as how a handful of SUMO-specific proteases coordinate the global regulation of thousands of SUMOylation targets in response to diverse cellular stimuli.

A recent study from our laboratory suggested that LLPS might represent an essential piece of the puzzle for this unconventional mechanism of SUMOylation regulation. We have long been intrigued by the literature's

lack of connection between cAMP signaling and protein SUMOylation, the two most prevalent stress response pathways. To investigate if cAMP, a universal stress response second messenger, plays a role in regulating cellular SUMOylation, we activated cAMP signaling in human umbilical vein endothelial cells using isoproterenol (ISO), a β-adrenergic receptor agonist and observed an enhanced cellular SUMOylation by SUMO2/3 but not by SUMO1. While pretreatment with H89, a protein kinase A-specific inhibitor, did not affect ISO-induced cellular SUMOylation, treatment of human umbilical vein endothelial cells with a membrane-permeable exchange protein directly activated by cAMP (EPAC)-specific agonist, 8-CPT-2′-O-Me-cAMP-AM recapitulated ISO's SUMOylation promotion effect. Conversely, silencing EPAC1 by EPAC1-specific RNAi decreased cellular SUMOylation. These results suggest that cAMP acts through EPAC1, but not protein kinase A, to promote cellular SUMOylation (Yang et al., 2022). Unexpectedly, EPAC1-induced cellular SUMOylation is not dependent on its canonical exchange activity. To investigate the mechanism of EPAC1-mediated cellular SUMOylation, we performed an unbiased EPAC1-associated proteome analysis via affinity purification and shotgun proteomics and discovered that components of the SUMOylation machinery were highly enriched in EPAC1-associated interactome. Confocal live cell imaging of ectopically expressed EPAC1-EYFP, and super-resolution immunofluorescence microscopic analysis of endogenous EPAC1 revealed that EPAC1 activation stimulated the formation of EPAC1 nuclear condensates that colocalized with components of SUMOylation machinery, including UBA2, UBC9, and SUMO2/3. These findings are consistent with the observation that EPAC1 protein contains multiple IDRs, particularly at its N-terminus, and is capable of undergoing cAMP-dependent LLPS. Notably, Δ(1–148) EPAC1, an EPAC1 deletion mutant without its N-terminal IDR, could not form nuclear condensates. While Δ(1–148)EPAC1 retained all measurable biochemical properties such as cAMP binding, Rap activation, and UBA2 interaction, it could no longer promote cellular SUMOylation as the full-length EPAC1. These results suggest that the ability of EPAC1 to form nuclear condensate is obligatory for EPAC1's cellular activity of promoting SUMOylation (Yang et al., 2022). Our study unveils a novel cellular mechanism whereby LLPS promotes the formation of SUMOylation-activating nuclear condensates where concentrated SUMOylation machinery and substrates accelerate cellular SUMOylation via mass action and/or substrate channeling (Fig. 4B).

The ability of LLPS to enhance catalysis has been well-documented (O'Flynn and Mittag, 2021). Indeed, when the SUMOylation machinery is recruited into artificially engineered condensates generated by LLPS of multivalent scaffolding proteins, the rate of SUMOylation is robustly enhanced (Peeples and Rosen, 2021), providing theoretical support for an LLPS-driven SUMOylation model. While more studies are needed to establish its broad applicability and pertinency, an LLPS-based mechanism of SUMOylation regulation provides several missing pieces of the SUMOylation puzzle. Biomolecular condensates act as depots of a large number of collaborative proteins for specific cellular functions. LLPS-mediated biomolecular condensates, such as PML NBs, offer an ideal platform where a small number of SUMOylation enzymes can modify many functionally and/or topologically related protein group targets. Moreover, LLPS is highly sensitive to changes in the physical and chemical properties of the cellular environment and has been increasingly recognized as a stress survival strategy to promote cellular fitness (Franzmann and Alberti, 2019), making it an excellent medium to bridge diverse cellular stress signals and protein SUMOylation. This reciprocal liaison between protein SUMOylation and LLPS allows them to function feed-forwardly to regulate the dynamics of biomolecular condensates for a robust stress response (Fig. 4C) (Cheng, 2023).

IV. SUMOylation and Human Diseases

Considering the critical roles that SUMOylation plays in various physiologic functions and stress responses, it is not surprising that dysregulation of protein SUMOylation has been implicated in the pathogenesis of various human maladies, such as cancer, cardiovascular diseases, and neurodegeneration, as well as infectious diseases (Chang and Yeh, 2020). Interestingly, many diseases associated with SUMOylation overlap with those related to LLPS (Alberti and Dormann, 2019). The impact of SUMOylation on diseases is complex. Depending upon the specific conditions, SUMO modification has been found to either facilitate or hinder disease progression. Pharmacological interventions specifically targeting the SUMO machinery are currently under development to treat various human diseases, particularly cancers.

A. SUMOylation and Cancer

Many cancer-related proteins, including oncogenes and tumor suppressors, are known SUMO targets. The oncogenic fusion protein PML retinoic acid receptor-α (RARα) responsible for acute promyelocytic leukemia is one of the first known SUMO substrates identified (Müller et al., 1998). Subsequently, some of the most frequently mutated or dysregulated oncogenes and tumor suppressors, such as Akt (Li et al., 2013; Risso et al., 2013), β-catenin (Huang et al., 2014), c-Jun (Muller et al., 2000; Schmidt and Müller, 2002), c-MYC (Kalkat et al., 2014; González-Prieto et al., 2015), cyclin D1 (Wang et al., 2011), Oct4 (Wei et al., 2007; Zhang et al., 2007), PI3K (de la Cruz-Herrera et al., 2016; El Motiam et al., 2021), RAS (Choi et al., 2018a,b), BRCA1 (Morris et al., 2009), p53 (Gostissa et al., 1999; Rodriguez et al., 1999; Kahyo et al., 2001), phosphatase and tensin

homolog (González-Santamaría et al., 2012; Huang et al., 2012), retinoblastoma protein (Meng et al., 2016; Sharma and Kuehn, 2016), and SMAD4 (Lee et al., 2003; Lin et al., 2003a,b; Ohshima and Shimotohno, 2003; Liang et al., 2004; Long et al., 2004), are found to undergo SUMO modifications.

The impact of protein SUMOylation on tumorigenesis is complex and multifaceted. SUMOylation has been shown to play both tumor-promoting and tumor-suppressing roles, depending on the specific proteins, cellular pathways, and cancer types involved. On the one hand, SUMOylation can promote tumorigenesis by crosstalking with oncogenes or tumor suppressors. For example, SUMOylation is essential for MYC-dependent tumorigenesis (Kessler et al., 2012; Hoellein et al., 2014) and is required for KRAS-driven oncogenic transformation (Yu et al., 2015). On the other hand, SUMOylation can have tumor-suppressive effects by regulating DNA repair and genome stability. For example, SUMOylation of BRCA1 promotes its recruitment to sites of DNA damage and enhances overall activity in repairing double-stranded breaks in response to genotoxic stress (Morris et al., 2009). In addition to its effects on specific proteins and pathways, SUMOylation can also have broader effects on cellular processes that contribute to tumorigeneses, such as transcriptional regulation, chromatin remodeling, and protein degradation.

In agreement with SUMOylation's important roles in tumorigenesis, components of the SUMOylation apparatus are often found unbridled in cancers. The expression of the SUMO substrates and SUMO E1 and E2 enzymes, as well as some SUMO E3 ligases, appear to be upregulated in many cancers and presumably lead to enhanced cellular SUMOylation (Seeler and Dejean, 2017). This enhanced expression of cellular SUMO machinery in cancers correlates with poor prognosis and overall survival (Moschos et al., 2007; Chen et al., 2011; Shao et al., 2015; Xia et al., 2022), suggesting functional importance. On the other hand, silencing the expression of SUMO E1 or E2 enzyme leads to reduced cancer cell proliferation in vitro and tumor growth in vivo. Interestingly, activation of oncogenes such as c-MYC and KRAS has been shown to enhance the expression of SUMOylation machinery and promote cellular SUMOylation (Amente et al., 2012; Luo et al., 2022), which is critical for c-MYC and KRAS-dependent oncogenesis (Kessler et al., 2012; Hoellein et al., 2014; Yu et al., 2015). This reciprocal positive interaction between SUMOylation and oncogene activation provides a potential feed-forward mechanism for promoting tumorigenesis.

To date, the role of enhanced SUMOylation as a dependency in cancer is best understood in c-MYC-driven cancer. While the exact mechanism of this SUMOylation-dependency of the C-MYC oncogene is not entirely clear, the ability of cancer cells to adapt to cellular stresses is critical for maintaining viability and growth. Emerging evidence suggests that c-MYC-driven SUMOylation enhancement may contribute to tumor maintenance via buffering of replication stress or proteotoxic stress associated with MYC oncogene activation and represent the Achilles heel for targeting the "undruggable" MYC oncogene (also see discussion in Section V.A).

The c-MYC oncogene-mediated malignant progression induces unrestrained cell growth and proliferation, which may lead to replicative and/or proteotoxic stresses (Dominguez-Sola et al., 2007; Cole and Cowling, 2008; Hart et al., 2012; Rohban and Campaner, 2015). For example, overexpression of c-MYC alters the spatiotemporal program of replication initiation by enhancing the recruitment of cell division cycle protein 45 (Cdc45), a rate-limiting component of the replicative Cdc45-MCM-GINS (CMG) helicases, to replication origins, resulting in abnormal replication-fork progression and DNA damage. This study establishes Cdc45 as a key effector of MYC-dependent DNA replication stress (Srinivasan et al., 2013). Subsequent studies further demonstrate that MYC induces chromatin unfolding and accessibility at targeted genomic sites to promote Cdc45/GINS recruitment to resident minichromosome maintenances (MCMs) for the formation of functional CMG helicases and activation of DNA replication. While MYC-induced chromatin decondensation does not affect MCM distribution, it requires MYC-Box II and Max and is mediated by GCN5 (KAT2A), Tip60 (KAT5), and TRRAP (Nepon-Sixt et al., 2019). It is worth pointing out that the aforementioned Cdc45 (Hendriks et al., 2018), MCM (230), GCN5 (Sterner et al., 2006), Tip60 (Gao et al., 2020), TRRAP (Hendriks et al., 2018), and all four subunits of the GINS complex (Hendriks et al., 2018) are all known SUMO substrates. SUMOylation of the DNA helicase MCM inhibits DNA replication initiation to ensure its accuracy DNA replication (Wei and Zhao, 2016). In addition, several key DNA replication proteins, such as topoisomerases (Sun et al., 2022) and the proliferating cell nuclear antigen (Gali et al., 2012) are modified by SUMO. Enhanced SUMOylation of these key replication proteins may enable C-MYC-driven tumor cells to escape excessive replication stress and proliferate.

By the same token, dysregulated MYC prompts abnormally highlighted protein synthesis and proteotoxic stress that trigger an integrated stress response ISR) for cell survival and tumorigenesis (van Riggelen et al., 2010; Hart et al., 2012). Conversely, excessive proteotoxic stress could also lead to cell death. In this regard, MYC-oncogene needs to adopt a proper level of protein synthesis necessary for cellular survival and tumor progression. For example, a recent study reports that DDX3X, an ATP-dependent RNA helicase important for the translation of mRNAs encoding the core protein synthesis machinery, is frequently mutated in MYC-driven lymphomas and that loss of function DDX3X mutants facilitate MYC-induced tumorigenesis via buffering

oncogene-driven proteotoxic stress (Gong et al., 2021). Similarly, MYC-mediated ISR promotes the translation of activating transcription factor 4 (ATF4). ATF4 induces the expression of eukaryotic translation initiation factor 4E-binding protein 1, a negative translation regulator to balance protein synthesis and relieve MYC-induced proteotoxic stress (Tameire et al., 2019). Interestingly, both DDX3X and ATF4 can be modified by SUMO, although the functional significance of DDX3X and ATF4 SUMOylation has not been explored (Hendriks et al., 2018). In addition, EIF-2 kinases, such as EIF2AK1 (HRI), EIF2AK2 (PKR), EIF2AK3 (PERK), and EIF2AK4 (GCN2), responsible for the phosphorylation of eIF2 and subsequent activation of ATF4, are all known SUMO substrates (Hendriks et al., 2018; Maarifi et al., 2018). While its roles remain to be explored, the SUMOylation of these key regulators of ISR likely plays an integral part in maintaining cellular proteostasis. Indeed, a previous study demonstrates that SUMOylation of chromatin-associated proteins is an integral component of the proteotoxic stress response and plays a vital role in cell survival by contributing to the maintenance of protein homeostasis (Seifert et al., 2015). Therefore, enhanced SUMOylation could cooperate by buffering proteotoxic stress known to be induced by oncogenic MYC to ensure that enhanced translation rates are compatible with survival and tumor progression.

In addition to being directly involved in tumorigenesis, SUMOylation also plays a vital role in innate immune responses and immune surveillance that acts as defensive barriers to the formation and progression of cancer cells, which acquires various counter strategies to escape the blockades. One such strategy involves the employment of SUMOylation. A recent study uncovered a conserved role of SUMOylation in transcriptional silencing of the MHC class I antigen-processing and presentation machinery (MHC-I APM) to allow cancer cells to evade cytotoxic T cell-mediated immunosurveillance in B cell non-Hodgkin lymphomas (Demel et al., 2022). Moreover, pharmacological inhibition of SUMO E1 sensitized the susceptibility of tumor cells to $CD8^+$ T cell-mediated killing via enhancing the presentation of antigens, as well as the direct activation of $CD8^+$ T cells. Mechanistically, silencing of the MHC-I APM was mediated by the scaffold attachment factor B (Demel et al., 2022), whose transcriptional corepressor activity requires SUMOylation (Garee et al., 2011). Loss or downregulation of the MHC-I APM is a common cause of resistance to cancer immunotherapies (Kalbasi and Ribas, 2020). Indeed, a previous study showed that a selective small molecule SUMOylation inhibitor, TAK-981, inhibited tumor growth in mice via activating type I interferon signaling and antitumor immune responses. Notably, a combination of TAK-981 with immune checkpoint blockade, anti-PD1, or anti-CTLA4 antibodies, synergistically improved the survival of tumor-bearing mice in preclinical models (Lightcap et al., 2021).

Overall, the impact of protein SUMOylation on tumorigenesis is complex and context dependent. Despite significant progress in understanding the role of protein SUMOylation in tumorigenesis, there are still several significant gaps in our knowledge, and further research is needed to understand the mechanisms and implications of this process fully. While SUMOylation can have both tumor-promoting and tumor-suppressive effects, the specific effect of SUMOylation on a given protein or pathway in a particular context is not always apparent, partly due to the complexity of the SUMOylation process, which involves multiple enzymes and can occur at different sites on a protein. In addition, SUMOylation can crosstalk with other post-translational modifications, such as phosphorylation and ubiquitination, and these interactions can have important implications for tumorigenesis. However, the specific mechanisms and functional consequences of these interactions are poorly understood. Further research is needed to identify the specific factors that determine the impact of SUMOylation on tumorigenesis in different contexts.

B. SUMOylation and Cardiovascular Diseases

Cardiovascular disease is the leading cause of mortality and morbidity worldwide, and its prevalence continues to rise due to an aging global population, and a rampant epidemic of obesity and type 2 diabetes, which are the significant risk factors of cardiovascular disease development. While the levels of cellular SUMOylation in mouse hearts and brains are relatively low as compared with other organs (Hendriks et al., 2018), many of the proteins necessary for cardiovascular development are modified by SUMO conjugation (Mendler et al., 2016). Similarly, an array of targets associated with various cardiovascular diseases are regulated by SUMOylation. For example, disturbed flow, acting through PKCζ (Heo et al., 2011) and p90 ribosomal S6 kinase (p90RSK) (Heo et al., 2015), induces SUMOylation of ERK5 (Woo et al., 2008; Heo et al., 2013) and p53 (Heo et al., 2011; Takabe et al., 2011), as well as de-SUMOylation and nuclear translocation of MAGI1 (Abe et al., 2019), to promote endothelial dysfunction and atherogenesis. On the other hand, SUMOylation of the nuclear receptor liver receptor homolog 1 enhances its interaction with the corepressor PROX1 and promotes atherosclerosis by suppressing hepatic reverse cholesterol transport (Stein et al., 2014).

In addition to atherosclerosis, protein SUMOylation has also been implicated in heart failure. The Sarco/endoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a) plays a crucial role in the contraction and relaxation of cardiomyocytes by mediating Ca^{2+} reuptake from the cytosol into the sarcoplasmic reticulum during excitation-contraction coupling. Reduced expression and decreased activity of SERCA2a is a hallmarks of heart failure (Meyer et al.,

1995). Normalization of the SERCA2a expression level by gene delivery has proved effective in improving cardiac function in heart failure patients and preclinical animal models (Byrne et al., 2008; Kawase et al., 2008; Jessup et al., 2011). Similar to SERCA2a, SUMO1 was found to be significantly reduced in a murine model of heart failure induced by pressure overload and in a porcine model of heart failure induced by volume overload. Likewise, retroviral rAAV9-based gene delivery of SUMO1 or SERCA2a significantly increased protein levels of SERCA2a and improved mouse cardiac functions to the same degree in a mouse model of heart failure (Kho et al., 2011).

Because of the many parallels between SERCA2a and SUMO1 in heart failure, researchers investigated if there was a direct connection between protein SUMOylation and SERCA2a and discovered that SERCA2a could be SUMOylated by SUMO1 at lysines 480 and 585 and that this SUMOylation is essential for preserving SERCA2a activity and stability. SUMO1 or SERCA2a overexpression in cardiomyocytes isolated from mice after transverse aortic constriction-induced heart failure augmented contractility and accelerated sarcoplasmic reticulum Ca^{2+} reuptake. Furthermore, silencing of SUMO1 using small hairpin RNA decreased SERCA2a activity and enhanced pressure-overload-induced worsening of cardiac function, whereas cardiac-specific, transgene-mediated SUMO1 overexpression increased SERCA2a activity and rescued cardiac dysfunction induced by pressure overload. In contrast, contractile dysfunction resulting from the knockdown of SERCA2a could not be rescued by overexpression of SUMO1, suggesting that the protective effect of SUMO1 expression is largely mediated by SERCA2a (Kho et al., 2011).

The demonstration that SUMOylation is a critical PTM for SERCA2a function provides a platform for the design of novel therapeutic strategies for heart failure. Indeed, AAV1-mediated SUMO1 expression in a swine ischemia-reperfusion heart failure model restores myocardial SUMO1 and SERCA2a expression, rescues left ventricular function, and preserves left ventricular volumes. Notably, combined gene delivery of SUMO1 and SERCA2a leads to additional beneficial effects, providing proof of principle demonstration of the potential clinical utility of targeting SUMOylation for treating heart failure (Tilemann et al., 2013). Building on the promising results based on gene delivery of SUMO1, Hajjar and colleagues developed an in vitro high-throughput assay to screen novel small molecules capable of activating SUMOylation. They identified several hits from large National Cancer Institute libraries of 100,000 compounds. One of the hits, N106, significantly induced endogenous SERCA2a SUMOylation and increased cell contraction in isolated adult cardiomyocytes in a dose-dependent manner. Biochemical characterization, molecular docking, and mutagenesis studies revealed that N106 directly activated the SUMO-activating enzyme E1 by binding to a pocket present only in the active conformation of SUMO E1. With a suitable pharmacokinetic profile, administration of N106 in vivo generated direct beneficial effects on contractility in failing mouse hearts (Kho et al., 2015). While additional preclinical safety studies and experiments in larger animal models are necessary to develop N106 for future clinical applications further, the discovery of a first-in-class small molecule SUMOylation activator provides a novel therapeutic strategy for treating heart failure. Luteolin, a natural flavone, can attenuate myocardial infarct size and plasma LDH level in mice after myocardial ischemia-reperfusion injury by increasing SERCA2a SUMOylation, stability, and activity (Du et al., 2018; Hu et al., 2020). While it is not clear if luteolin promotes SERCA2a SUMOylation via activating the SUMOylation machinery directly in a similar fashion as N106 or via suppressing SUMO protease activity, luteolin appears to upregulate SERCA2a transcription through the transcription factor-specific protein 1 (Hu et al., 2020), and the protective effects of luteolin are mainly dependent on SUMO1 (Du et al., 2018).

C. SUMOylation and Neurodegeneration

Although its precise role remains unclear, protein SUMOylation has been implicated in neuronal development and differentiation (García-Gutiérrez and García-Domínguez, 2021). In rodents, developmental and spatial regulation of the SUMOylation core components in the central nervous system has been observed to support a notion that while SUMO1 modification peaks at the onset of neurogenesis, then declines gradually and reaches a minimum in the adult brain, SUMO2/3 modification follows a biphasic pattern with a second spike right after birth, suggesting a potential role in synaptogenesis (Loriol et al., 2012; Hasegawa et al., 2014). As mentioned earlier, SUMO2 is essential for the development, and deletion of *Sumo2* in mice leads to embryonic lethality (Wang et al., 2014a). A recent study using a conditional knockout mouse line in which *Sumo2* was deleted selectively in forebrain neurons demonstrated that whereas SUMO2 was not essential for neuronal development and neural maintenance in the forebrain, loss of *Sumo2* results in impaired synaptic plasticity and hippocampal-based cognitive dysfunction (Yu et al., 2020).

The involvement of SUMOylation in neurodegenerative diseases is highlighted by the fact that many of the disease-associated proteins are modified by SUMOylation, such as the amyloid precursor protein (APP) (Riley et al., 2005; Zhang and Sarge, 2008), ataxin-1 (Riley et al., 2005), Huntingtin (Steffan et al., 2004; O'Rourke et al., 2013), tau (Dorval and Fraser, 2006; Luo et al., 2014), α-synuclein (Dorval and Fraser, 2006; Krumova et al., 2011; Oh et al., 2011), DJ-1 (Shinbo et al., 2006), Parkin (Um and Chung, 2006), and the transactive response DNA-binding protein 43 (Seyfried et al., 2010).

Considering that many neurodegenerative diseases share the common characteristic of the presence of intracellular inclusion bodies formed by aggregation-prone disease-associated proteins, SUMOylation of these targets may promote or impede the formation of inclusion bodies by altering their stability, solubility, cellular localization, and/or interaction with binding partners. Accordingly, SUMOylation can exert beneficial or harmful effects on individual neurodegenerative diseases, depending on the specific cellular and molecular context associated with these disease-related proteins. It is also sensible to point out that many proteins found in pathologic inclusions associated with various neurodegenerative diseases are known to undergo LLPS (Elbaum-Garfinkle, 2019; Zbinden et al., 2020; Boyko and Surewicz, 2022), further underlining the close relationship between SUMOylation and LLPS in regulating protein aggregation.

Alzheimer's disease (AD) is the most prevailing dementia in the elderly. The accumulation of amyloid β peptide (Aβ) generated from the proteolytic cleavage of APP and tau is one of the major hallmarks associated with AD (LaFerla et al., 2007). It has been reported that the plasma level of SUMO1 is significantly elevated in AD patients (Cho et al., 2015). There is accumulating evidence connecting both APP and tau with SUMOylation. APP can be modified by SUMO1 and SUMO2 on lysines 587 and 595, located immediately adjacent to the site of β-secretase cleavage. SUMOylation of these lysine residues is associated with decreased levels of Aβ aggregates, while overexpression of the SUMO E2 enzyme and SUMO1 results in decreased levels of Aβ aggregates in cells transfected with the familial AD-associated V642F mutant APP (Zhang and Sarge, 2008). In addition to L587 and L595, K43 of the APP intracellular domain (AICD) is also found to be SUMO-modified, facilitated by the SUMO E3 ligase PIAS1. K43 SUMOylation promotes AICD nuclear translocation and increases its association with cyclic AMP-responsive element binding protein and p65 and their DNA binding, leading to transcriptional activation of two major Aβ-degrading enzymes, neprilysin and transthyretin, respectively. As a result, lentiviral mediated AICD or SUMO1 expression in an APP/PS1 mouse model of AD decreases the Aβ level, oligomerization, and amyloid plaque deposits and rescues spatial memory deficits in APP/PS1 mice. In contrast, expression of a SUMOylation deficient AICD-K43R mutant produces opposite effects. Moreover, melatonin is identified as an endogenous factor capable of stimulating AICD SUMOylation. Administration of melatonin dramatically decreases the Aβ level and rescues the reduction of PIAS1, neprilysin, and transthyretin expression in APP/PS1 mice (Liu et al., 2021).

Like APP, tau can be SUMOyated by SUMO1 or by SUMO2/3 to a less extent. Treatment of okadaic acid, a phosphatase inhibitor, increases cellular tau SUMOylation, suggesting tau phosphorylation stimulates tau SUMOylation (Dorval and Fraser, 2006). Conversely, tau SUMOylation mediated by SUMO1 overexpression induces tau hyperphosphorylation at multiple phosphorylation sites. In contrast, SUMOylation deficient mutation K340R or pharmacological inhibition of tau SUMOylation by ginkgolic acid abolishes the effect of SUMO1 overexpression. Tau SUMOylation decreases its solubility and inhibits tau ubiquitination and degradation. In the cerebral cortex of the AD brains, enhanced SUMO immunoreactivity is detected to colocalize with the hyperphosphorylated tau. Moreover, treatment of rat primary hippocampal neurons with Aβ-amyloid prompts a dose-dependent SUMOylation of the hyperphosphorylated tau. These findings suggest a potential role of tau SUMOylation in AD development by reciprocally stimulating tau hyperphosphorylation and suppressing the ubiquitination-mediated tau degradation (Luo et al., 2014).

Huntington's disease (HD) is a dominant neurodegenerative disorder caused by the accumulation of a mutant Huntingtin protein (mHtt) with an abnormal expansion of a polyglutamine (polyQ) repeat (Bates, 2003). It was revealed quite early on that a pathogenic fragment of HTT (Httex1p) could be modified either by SUMO1 or by ubiquitin on identical lysine residues in the N-terminal domain, mostly on K6 and K9. While SUMOylation appeared to stabilize Httex1p, promoting its ability to repress transcription in cultured cells, it aggravated neurodegeneration in a *Drosophila* model of HD. On the other hand, ubiquitination of Httex1p reduced neurodegeneration, whereas mutations that prevent both SUMOylation and ubiquitination of Httex1p also lessened HD pathology, suggesting a complex interplay between mHtt SUMOylation and ubiquitination where the involvement of SUMOylation to HD pathology extends beyond competing with ubiquitination to prevent Htt degradation (Steffan et al., 2004). Subsequent studies demonstrate that Ras homolog enriched in the striatum binds mHtt and augments its neurotoxicity by promoting SUMOylation of mHtt, leading to its disaggregation and augmented cytotoxicity (Subramaniam et al., 2009).

In addition to SUMO1, Htt can also be modified by SUMO2 on the same K6 and K9 residues, with PIAS1 as a putative SUMO E3 ligase. SUMO2 modification increases Htt aggregation and toxicity (O'Rourke et al., 2013). Deletion of Pias1 in HD mice leads to a reduced accumulation of high molecular weight mHtt, a normalization of HD-associated transcriptional dysregulation, and DNA damage repair (Morozko et al., 2021). A recent study reported that SUMO1 deletion in HD mice prevented age-dependent HD-like motor and neurologic impairments and suppressed striatal atrophy and inflammatory response. These phenotypic improvements were accompanied by a drastic reduction in soluble mHtt levels and nuclear and extracellular mHtt inclusions while increasing cytoplasmic mHtt inclusions in the striatum of HD mice. Mechanistically, SUMO1 deletion diminished the mHtt levels by enhancing cellular autophagy flux. Consistent with this

notion, pharmacological inhibition of SUMOylation strongly enhanced autophagy and diminished mHtt levels in human HD fibroblasts. These results establish SUMOylation as a promising therapeutic target in HD (Ramírez-Jarquín et al., 2022).

Another example is spinobulbar muscular atrophy (SBMA), an X-linked recessive neuromuscular degeneration caused by a polyQ expansion in the androgen receptor (AR) (La Spada et al., 1991). PolyQ AR is prone to unfolding and oligomerization, leading to the formation of intracellular aggregates, diminished transcriptional activity, and ligand-dependent proteotoxicity (Beitel et al., 2013). The AR is modified by SUMO1 at K386 and K520, resulting in a suppression of transcriptional activity (Poukka et al., 2000) and reduced polyQ AR aggregation (Mukherjee et al., 2009). To determine if SUMOylation of polyQ AR contributes to the pathology of SBMA, Chua and colleagues generated knockin mice in which the endogenous Ar locus was replaced with either a polyQ AR (AR113Q) or a non-SUMOylatable polyQ AR, in which two SUMOylation lysine acceptors were mutated to arginines (AR113Q-KRKR). While there were no significant differences in disease onset, body weight loss, and grip strength between AR113Q and AR113Q-KRKR groups, disruption of polyQ AR SUMOylation dramatically prolonged survival and rescued exercise endurance and type I muscle fiber atrophy. These findings demonstrate the beneficial effects of enhancing the transcriptional function of the ligand-activated polyQ AR and establishing the SUMOylation pathway as a potential target for therapeutic intervention in SBMA (Chua et al., 2015).

D. SUMOylation and Infection

In light of the crucial role of SUMOylation in cellular stress responses, it is almost as expected that protein SUMOylation plays a significant role in host and pathogen interaction, particularly during viral infection. The involvement of SUMOylation in viral infection was initially reported in a 1999 study showing that the herpes simplex virus ICP0 protein and the human cytomegalovirus IE1 protein both disrupt the PML NB structure by specifically abrogating the SUMO1 modification of PML and Sp100. In addition to disrupting host protein SUMOylation, the human cytomegalovirus IE1 protein itself is found to be modified by SUMO-1, representing the first viral protein found to undergo SUMOylation (Müller and Dejean, 1999). Since then, many viral proteins from across a broad range of viruses, including RNA and DNA viruses, both enveloped and unenveloped, have been shown to undergo SUMO modifications, and, likewise, viral infection is now known to modulate the SUMOylation of a multitude of host proteins, as well as regulating the expression and activity of the components of host SUMOylation machinery (Fan et al., 2022).

Many studies have revealed that viruses can hijack the SUMO pathways to modify both viral and host targets to attain a productive infection. In contrast, the hosts use SUMO modifications as a defense to hinder viral propagation. On the other hand, the relationship between viral infection and SUMOylation is complex, and there is no single consensus effect of SUMOylation on viral infection. Depending upon the specific SUMO targets, SUMOylation can be a double-edged sword for viral infection. For example, the influenza A virus interacts widely with the cellular SUMOylation system during infection. Several influenza virus proteins, including matrix protein M1, nucleoprotein, nonstructural protein 1 (NS1) and 2 (NS2), and the polymerase basic protein 1, are genuine SUMO targets (Pal et al., 2011; Xu et al., 2011) and influenza viral infection leads to a global increase and reprogramming in host cellular SUMOylation (Pal et al., 2011; Domingues et al., 2015). While SUMOylation of M1 (Wu et al., 2011; Guo et al., 2022), nucleoprotein (Han et al., 2014), and polymerase basic protein 1 (Li et al., 2021) is essential for intracellular trafficking, virus replication, growth, or maturation, SUMOylation of NS1 (Santos et al., 2013) and polymerase basic protein 2 (Wang et al., 2022) exerts a negative effect on NS1's interferon-blocking function or viral replication and virulence by reducing the stability of polymerase basic protein 2 and the activity of the viral RNP complex, respectively. A genome-wide CRISPR/Cas9 screen identifies that PIAS3, a SUMO E3 ligase, plays a critical role in the entry and/or fusion stage of the influenza A virus life cycle (Han et al., 2018). Furthermore, the influenza virus can also trigger the loss of SUMO-modified TRIM28, another SUMO E3 ligase, to potentiate canonical cytosolic dsRNA-activated interferon-mediated host innate immune response (Schmidt et al., 2019).

SUMOylation has also been implicated in infection of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the culprit responsible for the current COVID-19 pandemic. A recent study reveals that SUMO E3 ligase PIAS4 promotes SUMO3 modification of angiotensin-converting enzyme 2 (ACE2), the host entry receptor of SARS-CoV-2, while deSUMOylating enzyme SENP3 decreases the SUMOylation level of ACE2. SUMOylation of ACE2 at K187 promotes ACE2 stability by impeding the K48-linked ubiquitination of ACE2 and suppressing its subsequent cargo receptor Toll interacting–protein-dependent autophagic degradation. Administration of SUMOylation inhibitors abrogates the SUMOylation of ACE2 and infection of pseudotyped SARS-CoV-2 in human pulmonary alveolar epithelial cells and suppresses SARS-CoV-2 infection in mice expressing human ACE2. These findings suggest that selective targeting autophagic degradation of ACE2 orchestrated by SUMOylation and ubiquitination is a potential therapeutic strategy to fight SARS-CoV-2 infection (Jin et al., 2022).

In addition to viruses, bacterial pathogens have also been shown to usurp the host SUMOylation machinery.

It was observed that infection with the enteric pathogen *Listeria monocytogenes* resulted in a decrease in the levels of cellular proteins conjugated to SUMO1 and SUMO2/3 both in vitro and in vivo. The decrease in cellular SUMOylation was triggered by the bacterial virulence factor listeriolysin O, which degraded SUMO E2 UBC9 in a proteasome-independent, aspartyl protease-dependent manner. SUMO overexpression led to impaired bacterial infection, countering the pathogen-induced loss of cellular SUMOylation, suggesting that *Listeria* dampens the host response by decreasing the SUMOylation level of proteins critical for infection defense (Ribet et al., 2010). Similar down-modulation of cellular SUMOylation by other bacterial pathogens was reported for *Shigella flexneri* (Fritah et al., 2014) and *Salmonella Typhimurium* (Verma et al., 2015).

V. Pharmacological Tool Compounds and Therapeutics Development Targeting SUMOylation

The close involvement of protein SUMOylation in many pathogeneses suggests that targeting SUMOylation may represent an effective strategy for therapeutic discovery. Indeed, significant efforts have been devoted by the research community to search and develop pharmacological probes and/or therapeutic leads targeting the SUMOylation pathway. These efforts have successfully created a toolbox containing sets of diverse SUMO modulators against various components of the SUMOylation machinery, including E1, E2, and SENPs. Among all known SUMOylation modulators to date, a significant portion of them is aimed at the SUMO E1 enzyme. This is not surprising because we know a great deal about its structure, function, and mechanisms of action. The modular, multidomain structure, its intrinsic conformational flexibility, and allosteric coupling among substrates and regulators, as well as the multistep reaction scheme and the relatively stable thioester intermediate associated with SUMO E1, offers many avenues for probe/therapeutics development (Lois and Lima, 2005; Olsen et al., 2010).

A. SUMO E1 Modulators

The first small molecule SUMOylation inhibitors, ginkgolic acid and anacardic acid, were identified from a screen of a library of 500 plant extracts. Ginkgolic acid and anacardic acid, two structurally related long-chain salicylic acid derivatives, inhibited the in vitro SUMOylation of the C-terminal fragment RanGAP1 with apparent IC_{50} of 3.0 μM and 2.2 μM (Table 2), respectively, by directly binding to SUMO E1 and blocking the formation of the E1-SUMO1 thioester intermediate (Fukuda et al., 2009a). Using an in situ cell-based SUMOylation assay, the same research group identified another SUMOylation inhibitor, kerriamycin B, from 1,839 samples of microbial cultured broth. Kerriamycin B was also shown to inhibit protein SUMOylation by blocking the formation of the E1-SUMO1 intermediate (Fukuda et al., 2009b). Subsequently, several additional natural product SUMOylation inhibitors, including davidiin (Takemoto et al., 2014) and tannic acid (Suzawa et al., 2015), have been discovered to inhibit SUMOylation like ginkgolic acid and kerriamycin B. It is important to note that these SUMOylation inhibiting natural products are structurally diverse. However, all contain thio reactive groups and likely inhibit SUMO E1 by covalently modifying the thiol group of the catalytic cysteine or another cysteine via Michael addition.

A covalent inhibitor (CID 9549553) that binds to an unexpected allosteric site conserved in SUMO E1 enzymes was identified by an HTS of 290,921 compounds using a FRET-based primary assay detecting the formation of SUMO1-RanGAP1 conjugation in the presence of E1 and E2 enzymes. Subsequent lead optimization efforts resulted in COH000, which irreversibly inhibited the adenylation activity of SUMO E1 by covalently modifying the Cys30 residue of UBA2 via Michael addition. COH000 did not inhibit UBC9 and exerted potent anticancer activities both in vitro and in vivo (Li et al., 2019). Because Cys30 is deeply buried in all reported SUMO E1 structures, crystal structures of apo-SUMO E1 and in complex with COH000 were determined to understand the mechanism of action of COH000-mediated inhibition. Indeed, structural analyses reveal that COH000 targets a cryptic binding pocket on SUMO E1 away from the active sites and forms a covalent adduct with Cys30 (Fig. 5A). Docking of COH000 induces both local and long-range conformational changes that include local unfolding and conformation uncoupling of the adenylation and catalytic cysteine domain. These conformational changes result in new networks of intramolecular contacts that lock the enzyme in an inactive conformation by disassembling the SUMO E1 adenylation and thioester bond formation active sites (Lv et al., 2018). Such a structural revelation further supports the notion that SUMO E1 exists in a conformational equilibrium between the adenylation and thioester bond formation active forms, and COH000 exploits this intrinsic multistep catalytic cycle of SUMO E1: disassembly of the adenylation active sites and reorientation of catalytic cysteine domain to position the catalytic cysteine proximal to the SUMO C terminus for subsequent thioester intermediate formation. Structural analyses of canonical ubiquitin-like E1 crystal structures reveal that the corresponding COH000 binding pocket varies significantly among these enzymes despite sharing 71% sequence identity at positions observed to interact with COH000 in SUMO E1 (Lv et al., 2018). These observations explain the selectivity of COH000 against SUMO E1 and also highlight the conformational flexibility of ubiquitin-like E1 enzymes.

Several small molecular inhibitors targeting the ATP binding pockets have been discovered by in silico docking and HTS (Kumar et al., 2013a,b, 2016; Zhou

TABLE 2
Pharmacological probes targeting SUMOylation cascade

Compounds	Targets	MW	IC_{50}/*EC_{50} (μM)	PubChem ID	References
Ginkgolic acid	E1	346.5	3	5281858	Fukuda et al., 2009a
Anacardic acid	E1	348.5	2.2	167551	Fukuda et al., 2009a
Kerriamycin B	E1	844.9	11.7	340932	Fukuda et al., 2009b
Davidiin	E1	938.7	0.15	14682455	Takemoto et al., 2014
Tannic acid	E1	1710.2	12.8	16129778	Suzawa et al., 2015
CID9549553	E1	391.8	0.54	9549553	Li et al., 2019
COH000	E1	419.5	0.2	46835111	Li et al., 2019; Lv et al., 2018
ML-972	E1	551.4	0.0004	86566743	He et al., 2017
TAK-981	E1	578.1	0.001	118628567	Langston et al., 2021
N106	E1	354.4	2.45*	3236395	Kho et al., 2015
Spectomycin B1	E2	714.7	4.4	139587860	Hirohama et al., 2013
Chaetochromin A	E2	546.5	3.7	53277	Hirohama et al., 2013
Viomellein	E2	560.5	10.2	3033108	Hirohama et al., 2013
2-D08	E2	270.24	6	22507438	Kim et al., 2013
GSK145A	E2	454.6	12.5	155905429	Brandt et al., 2013
CPD1	SUMO1	348.4	2.3	332429	Bellail et al., 2021
HB007	SUMO1	328.8	0.85	146255136	Bellail et al., 2021

et al., 2018). The most potent and selective SUMO E1 inhibitor is ML-792, a member of a class of adenosine sulfamate-derived E1 inhibitors developed by Takeda Pharmaceuticals. ML-792 is an AMP mimic that inhibits SUMOylation by forming a SUMO adduct through the reaction of the sulfamate moiety with a cysteine thioester in an ATP-dependent mechanism (Fig. 5B). ML-792 is highly selective with an IC_{50} value of 3 nM or 11 nM for SUMO E1 with SUMO1 or SUMO2 as a substrate, respectively. On the other hand, ML-792 exerts little activity toward closely related NEDD8 E1 or UBA1, with a much high IC_{50} value of 32 μM or $>$ 100 μM, respectively, and does not show significant inhibition against a panel of 366 ATP-utilizing enzymes at a 1 μM concentration. Consistent

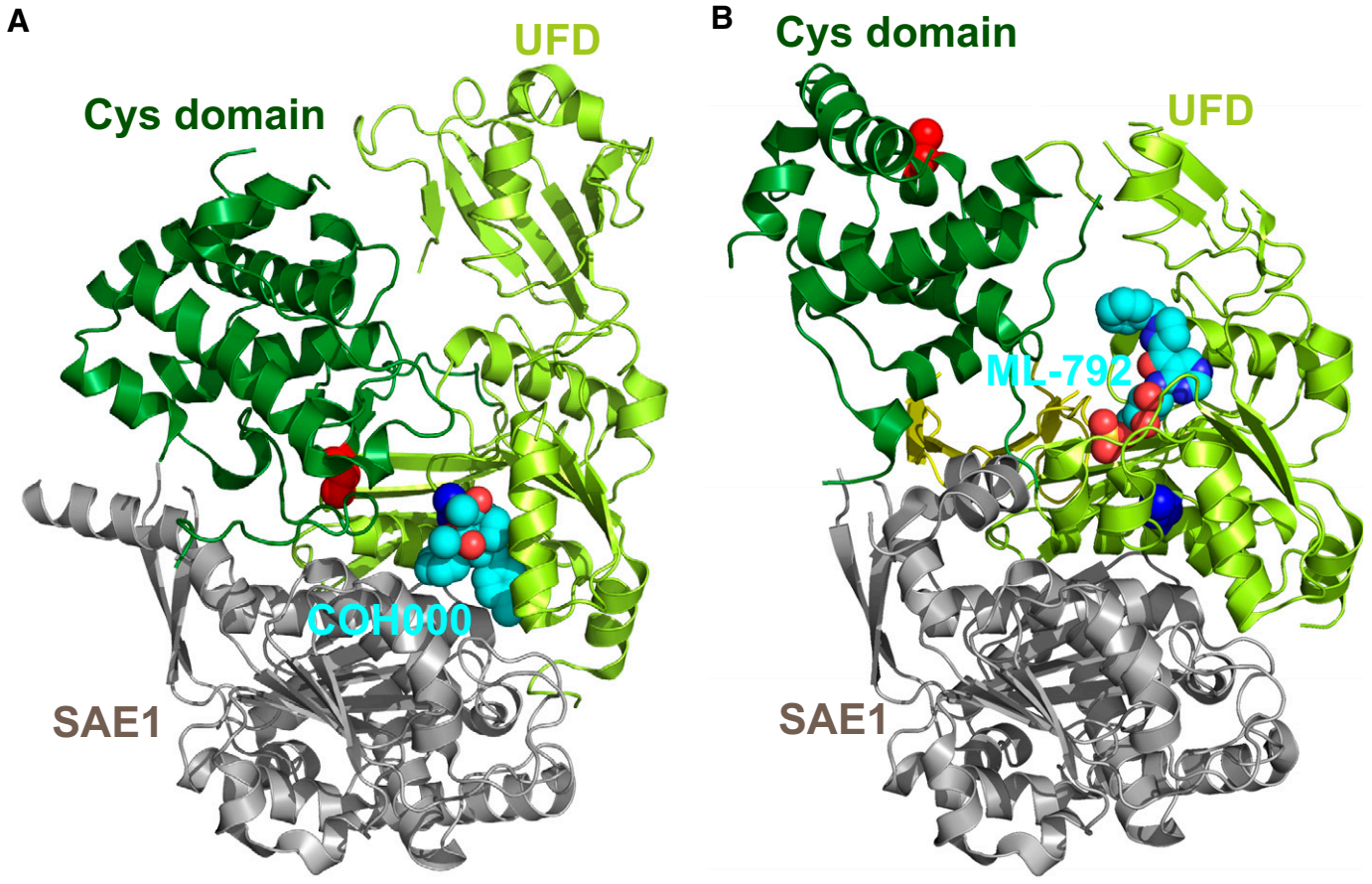

Fig. 5. Small-molecule inhibitors of SUMO E1 targeting an allosteric binding site (A) or the ATP binding pocket (B). Cartoon representation for the SUMO E1 in complex with HB007 (A, PDB 6CWY) and an ML-792 analog (B, PDB 6XOI). The catalytic Cyc173 is highlighted in red while Cys30 is colored in blue.

with its SUMOylation inhibition activity, ML-792 treatment leads to the disruption of PML NBs and the redistribution of PML component DAXX in HCT116 cells (He et al., 2017).

Considering the involvement of SUMOylation in cancer, it is not surprising that ML-792 inhibits cell viability in various cancer cell lines with sub-μM EC_{50} values by inducing cell-cycle/mitotic disruption without significant accumulation of DNA damage and alteration in global gene transcription. Furthermore, in line with the notion that SUMOylation is essential for MYC-dependent tumorigenesis (Kessler et al., 2012; Hoellein et al., 2014), cancer cell viability in response to ML-972 treatment showed an inverse relationship with levels of MYC expression (He et al., 2017). Further supporting the synthetic lethal relationship between MYC and SUMO pathways, a recent study revealed the coactivation of the MYC and SUMO pathways in the basal-like/squamous category of the pancreatic ductal adenocarcinoma, a subtype especially resistant to chemotherapies with a dismal prognosis. Using ML-792 and a related analog ML-93, it was demonstrated that hyperactivation of MYC was connected to an increased sensitivity to pharmacological SUMO inhibition, providing a clear rationale for the future development of SUMOylation inhibitor-based therapies for a pancreatic ductal adenocarcinoma subgroup with coactivation of the SUMO pathway and MYC (Biederstädt et al., 2020). Besides sensitizing cancer cells with hyperactivation of MYC, targeting SUMOylation by ML-792 also sensitizes cancer cells to agents of DNA damage by destabilizing single-stranded DNA-binding protein 1 (Zhou et al., 2020).

Because ML-792 is highly selective and capable of phenocopying the effects of genetic knockdown of SUMOylation, it has now been widely used as a pharmacological tool compound for the interrogation of cellular SUMOylation functions or identification of SUMO targets (Mojsa et al., 2021; Paakinaho et al., 2021; Hirano and Udagawa, 2022). For example, SUMOylation is upregulated in lymphoma tissues in the presence of latent membrane protein-1 (LMP1), the principal oncoprotein of Epstein-Barr virus (EBV). This LMP1-mediated SUMOylation dysregulation contributes to oncogenesis. Using ML-792, a recent study demonstrates that SUMOylation is vital in the maintaining EBV latency and lytic replication following induced reactivation. ML-792 suppressed cellular SUMOylation in multiple EBV-positive B cell lines and EBV-positive nasopharyngeal carcinoma cell lines but was less effective in their EBV-negative counterparts. ML-792 treatment abrogated the oncogenic potential of LMP1 by inhibiting B-cell growth, promoting cell death, modulating LMP1-induced cell migration and adhesion, and decreasing the production of new infectious virus following an induced reactivation and the infection of new cells. These findings suggest that pharmacological inhibition of SUMOylation may represent a potential therapeutic strategy to treat EBV-associated lymphoid malignancies by targeting the EBV life cycle and oncogenesis (Garcia et al., 2021).

Building on the success of ML-792, further medicinal chemistry optimization efforts result in identifying a clinical molecule TAK-981 with a prolonged pharmacodynamic effect and efficacy in preclinical tumor models (Langston et al., 2021). Extensive preclinical studies show that TAK-981 blocks cancer cell cycle progression, promotes antitumor immune responses, and potentiates immune therapies through activation of type I interferon signaling in preclinical models (Lightcap et al., 2021; Hanel et al., 2022; Kumar et al., 2022; Nakamura et al., 2022). In addition, SUMOylation inhibition mediated by TAK-981 enhances multiple myeloma sensitivity to lenalidomide, carfilzomib, or dexamethasone by reducing IRF4 at transcriptional and post-translational levels (Du et al., 2023), by modulating prolyl isomerase PIN1 activity (Heynen et al., 2023), or by decreasing c-MYC and its downstream targets miR-551b and miR-25 (Du et al., 2022), respectively. A phase 0/early phase 1 clinical trial of TAK-981 designed to study the biologic effects within the tumor microenvironment in patients with head and neck cancer was completed on June 20, 2022 (NCT04065555). TAK-981 is currently being evaluated in a combined phase 1 and 2 clinical trials (NCT03648372) as a single agent or phase 1 clinical trial in combination with Rituximab, anti-CD38 monoclonal antibodies, or Pembrolizumab for the treatment of patients with refractory CD20-positive non-Hodgkin lymphoma or multiple myeloma or with select advanced or metastatic solid tumors (NCT04074330, NCT04776018, and NCT04381650).

B. SUMO E2 Inhibitors

Similarly, many SUMO E2 inhibitors have been discovered. Some of the earlier UBC9 inhibitors include several structurally related natural products, spectomycin B1 and structurally related chaetochromin A and viomellein (Hirohama et al., 2013). All these natural products contain reactive cysteine groups and block the formation of SUMO1 thioester intermediate of E2, but not E1, likely by covalently modifying the thiol group of the catalytic cysteine via Michael addition. A trihydroxyflavone derivative, 2-D08, was identified from a gel-shift-based assay to block the transfer of SUMO1/2/3 from E2 thioester to SUMO substrate but did not affect ubiquitination. Since 2-D08 requires the catechol moiety for its SUMOylation inhibition activity, the compound was tested in the presence of 1 mM DTT. It retained its activity, suggesting that nonspecific thiol alkylation is likely not a mechanism for inhibition (Kim et al., 2013).

A high-throughput fluorescence polarization assay was developed to identify SUMOylation inhibitors (Brandt et al., 2013). HTS against 2,268,941 compounds resulted in 728 compounds with reproducible inhibition. After

filtering through counter-screen assays to remove thiol-reactive compounds and E1 inhibitors, 258 UBC9-specific inhibitors were identified. Among these confirmed UBC9 hits, several compounds contained primary amines and acted as competitive substrates. One tool compound, GSK145A, inhibited UBC9 with an IC_{50} of 12.5 uM (Brandt et al., 2013).

C. SUMO1 Degraders

To date, no modulator specifically targeting SUMO E3 enzymes has been reported. On the other hand, small-molecule degraders that induce the ubiquitination and degradation of SUMO1 protein through CUL1 E3 ligase have been identified using a cancer cell-based SUMOylation screening. Screening of the National Cancer Institute diversity set IV libraries of 1596 small molecules for inhibition of the conjugation of SUMO1, but not SUMO2/3 led to the identification of a hit compound, CPD1, capable of blocking SUMO1 conjugation to its substrate proteins and inhibiting cancer cell growth with an IC_{50} of 2.3 μM. CPD1 exhibited a broad activity against various human cancer cell lines. While CPD1 reduced the conjugated and unconjugated SUMO1, but not SUMO2/3 proteins, it did not effect on SUMO1 mRNA expression. Structure-activity relationship studies resulted in identifying a lead compound, HB007, with improved properties and anticancer potency in vitro and in vivo. The SUMO1 degradation activity of CPD1 and HB007 occurred through the 26S proteasome as proteasome inhibitor MG132 pretreatment blocked the activity of CPD1 and HB007. A genome-wide CRISPR-Cas9 knockout screening using a CRISPR-Cas9 knockout library consisting of 123,411 sgRNAs targeting 19,050 genes identified F-box only protein 42 (FBXO42), a substrate recognition component of the S-phase kinase-associated protein 1 (SKP1)–Cullin-1 (CUL1)–F-box protein (SCF)–type E3 ligase complex, as required for HB007 activity. HB007-based affinity pull-down proteomics analysis further revealed the cytoplasmic activation/proliferation-associated protein 1 (CAPRIN1) as the target binding protein. Mechanistically, HB007 likely acts as a molecular glue that induces the interaction between CAPRIN1 and FBXO42, which then recruits SUMO1 to the CAPRIN1-CUL1-FBXO42 ubiquitin ligase complex for polyubiquitination and subsequent degradation. Systemic administration of HB007 in mice selectively degraded SUMO1 in patient tumor-derived grafts, inhibited cancer progression, and increased the survival of the animals (Bellail et al., 2021). Further investigations are necessary to confirm if HB007 indeed functions as a molecular glue by determining the full-length CAPRIN1 structure and its complexes with HB007 and FBXO42, as well as to identify the structural basis of HB007's SUMO1 selectivity. It will also be interesting to test if similar approaches can be applied to discover selective degraders for other SUMO isoforms.

During the past decade, significant advances have been made to develop specific and mechanism-based modulators targeting the SUMOylation machinery. Many pharmacological tool compounds are now available for manipulating the SUMOylation pathway. It is important to emphasize that many of these compounds, especially many of the earlier Micheal acceptor-containing SUMOylation inhibitors such as ginkgolic acid and anacardic acid, have not been carefully characterized, and their specificity is problematic. With the advent of new generations of highly specific and potent SUMOylation inhibitors, these nonspecific SUMOylation inhibitors should be avoided. The current gold standard for inhibiting SUMOylation is the aforementioned ML-792 or TAK-981, which is under clinical development for cancer treatment.

VI. Conclusions and Future Perspective

Protein SUMOylation is one of the most prevalent PTMs and plays essential roles in all aspects of cellular functions. More than a quarter century of extensive studies in the field has revealed much about how the SUMOylation machinery operates at the biochemical level. The enzymatic machinery that regulates the SUMO conjugation-deconjugation process is well characterized. The advancement of MS-based proteomics approaches has led to the identification of tens of thousands of endogenous SUMOylation sites in over one-third of the human proteome. Such a wealth of information provides essential puzzle pieces required to understand cellular functions associated with protein SUMOylation. On the other hand, protein SUMOylation remains one of the most challenging PTMs. The molecular mechanisms of how various biologic stimuli regulate cellular SUMOylation remain poorly understood. How does a small set of SUMOylation enzymes catalyze the cellular SUMOylation of thousands of substrates? How do cells synchronize the SUMOylation of groups of substrates with similar functionalities in response to diverse stress signals? What are the specific functions of individual SUMO E3 ligases in vivo? Do they contribute to the coordinated SUMOylation of functionally related substrate networks and/or play roles in SUMO isoform selectivity? In this review, we discussed intimate connections among protein SUMOylation, LLPS, and biomolecular condensates and how these biologic processes work together to coordinate cellular homeostasis in response to diverse cellular stresses under physiologic and pathophysiological conditions, as well as the current state of drug discovery and development efforts in targeting the SUMOylation pathway. A better understanding of protein SUMOylation's physiologic and pathophysiological functions has been hampered by its overall complexity and technical challenges in monitoring endogenous SUMOylation. With the rapid advances in technologies associated with multiomics analyses and artificial intelligence, our knowledge of protein SUMOylation will grow exponentially in the

next few years. In parallel, therapeutics and/or diagnostic/prognostic tools specifically targeting the SUMOylation pathway will likely enter the clinic shortly.

Authorship Contributions

Participated in research design: Cheng.

Performed data analysis: Cheng.

Wrote or contributed to the writing of the manuscript: Cheng, Yang, Lin, Mei.

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