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SUMO conjugation and cardiovascular development

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1. ABSTRACT

SUMOs (Small Ubiquitin-like modifiers) belong to a superfamily of ubiquitin like proteins (Ubls) that are covalently conjugated to their substrates via enzymatic cascade reactions. The heterodimeric activating complex (SAE1/SAE2, E1) and conjugating enzyme (Ubc9, E2) required for the SUMO conjugation pathway are distinct from those involved in other Ubl pathways, and the presence of ligases (E3) stimulates the conjugation reaction. SUMO is implicated in a variety of physiological as well as pathological processes such as cell division, signal transduction, DNA damage and repair, and cancer development. This review focuses on the fundamental features of SUMO conjugation and its potential implication in cardiovascular development.

2. INTRODUCTION

Posttranslational modifications, such as chemical modifications (phosphorylation, acetylation, methylation), and covalent conjugation of small proteins (ubiquitin and ubiquitin-like proteins, Ubls), constitute dynamic regulatory mechanisms whereby the functions of a wide variety of proteins can be modulated. These modifications are extensively implicated in numerous cellular processes such as cell proliferation and differentiation, apoptosis, organogenesis.

Ubls are special proteins utilized to covalently modify their substrates in a reversible manner. Since the discovery of ubiquitin in the 1970s (1, 2), great strides have been made towards understanding the biochemistry and

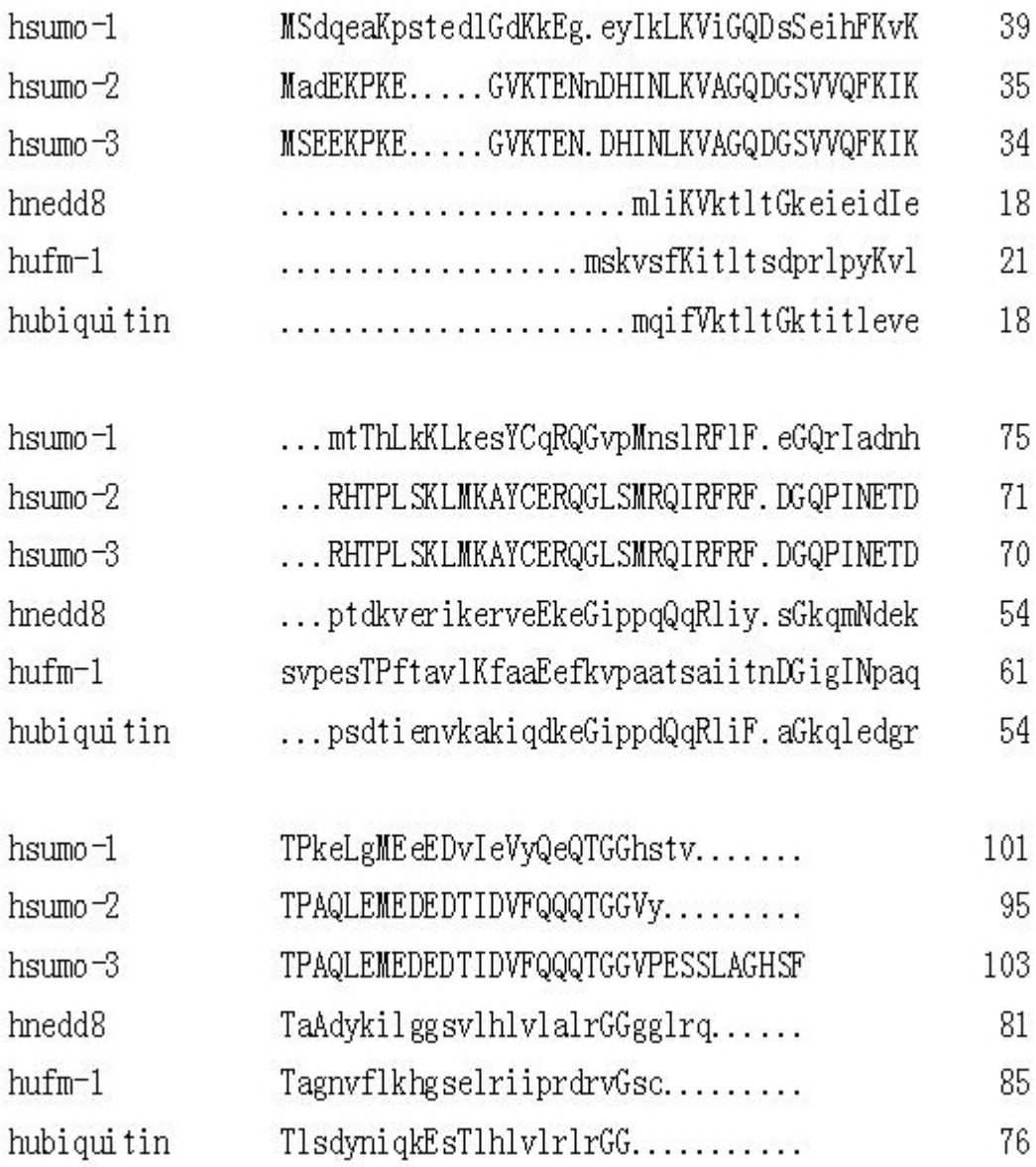

Figure 1. Comparison of amino acid sequences of human SUMO proteins with ubiquitin and some selected Ubl proteins**.**

biological functions of the ubiquitination system. SUMO-1 (Small Ubiquitin-like Modifier-1, also known as sentrin-1, SMT3C, UBL1, GMP-1, PIC1) was discovered by several groups independently in the mid 1990s (3-6). Subsequently, another ubiquitin-like protein, NEDD8 (Neural Precursor Cell-Expressed Developmentally Down-regulated-8, or Rub1), was discovered and shown to function as a ubiquitin-like protein whose major targets are cullin family members (7-11). More recently, another Ubl-related protein coined Ufm1 (Ubiquitin Fold Modifier-1) was identified although its biological function as well as targets remain to be uncovered (12, 13).

Ubiquitin-like proteins exhibit highly conserved three dimensional structures although their primary sequences vary considerably (Figure 1). Furthermore, the similar mechanisms of catalytic cascade reactions apply to all these ubiquitin-like proteins identified to accomplish covalent linkages to substrates although the involved enzymes at each step appear unique for each particular conjugation pathway. The expression patterns and subcellular localizations of ubiquitin-like proteins also vary, indicating mechanisms for control of pathway specificity (8, 14, 15).

As a sophisticated and versatile mechanism for governing the activities of a large pool of target proteins, SUMO modification is emerging as an efficient pathway contributing to a multitude of cellular activities under both physiological and pathological states including mitosis (16, 17), stress response (18, 19), cancer development (20), DNA damage and repair (21), nuclear transport (4),

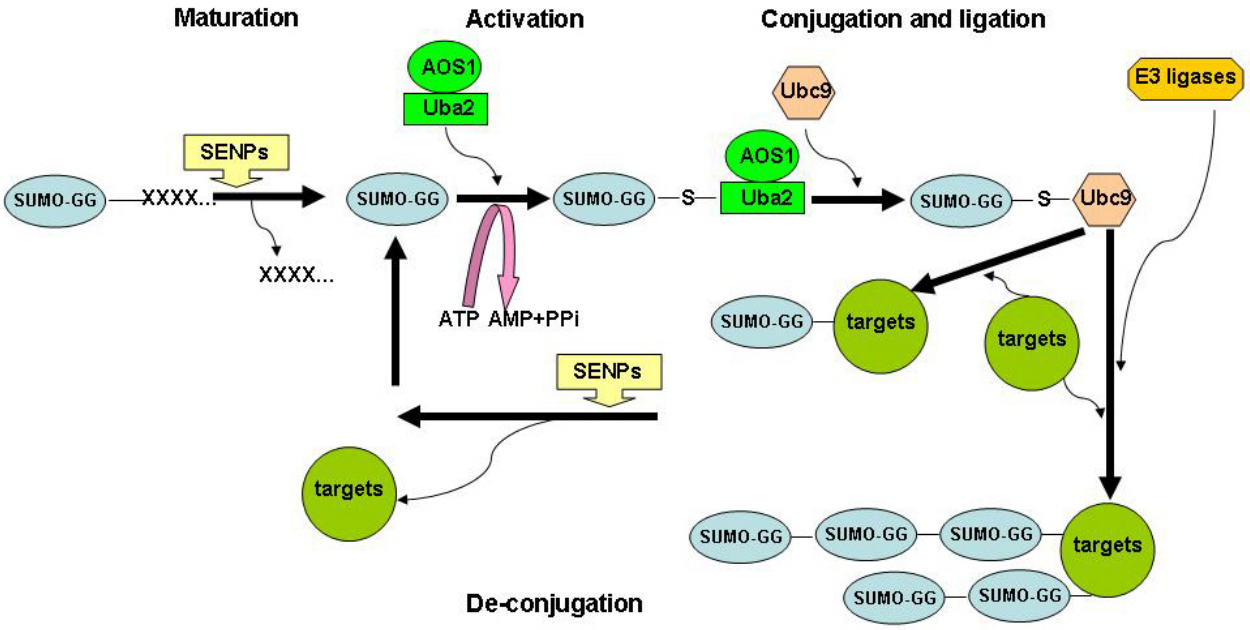

Figure 2. SUMO conjugation pathway. Maturation, SUMO conjugation motif GG is exposed via cleavage by SENPs. Activation, SUMO-GG forms thiol ester bond with Uba2/Aos1 in an ATP consuming way. Conjugation and ligation, SUMO-GG is transferred to its substrates by Ubc9 and/or E3 ligases. Please note that the presence of E3 ligases may promote the formation of poly-SUMO chain or/and activate atypical SUMO attachment site (s). De-conjugation, SUMO-GG is freed from its conjugating state by SENPs and readies for a new round of conjugation.

neurodegeneration (22). The number of proteins identified as SUMO substrates is increasing at a rapid pace. This review briefly discusses the basic features of SUMO conjugation pathway and highlights the recent findings implicating its potential function in cardiovascular development.

3. SUMO CONJUGATION PATHWAY

SUMO (Small ubiquitin-like modifier) is a member of Ubl superfamily. Although only one SUMO protein has been identified in yeast, at least three functional homologs of SUMO proteins have been identified in higher vertebrates (14, 23). SUMO-1 appears to be the most active under physiological conditions (14). Active SUMO-2 and -3 share over 95% similarity at amino acid level but exhibit only ~50% identity with SUMO-1 (Figure 1), which is the most extensively studied among SUMO isoforms. SUMO-4 was first identified involved in pathogenesis of Type I diabetes (23, 24), but later on reported to be ineffective in covalent conjugation due to the presence of a proline-90 amino acid residue that blocks SUMO-4 maturation (25).

SUMO conjugation is accomplished via the following phases: maturation, activation, conjugation/ligation, and de-conjugation (Figure 2). Maturation involves carboxyl-terminal cleavage of the originally synthesized precursor SUMO protein by a number of SUMO-specific hydrolase named SENPs (Sentrin-specific proteases) (15, 26), exposing a diglycine motif required for conjugation. The completion of this phase readies SUMO proteins for entering the subsequent dynamic cycle of the SUMO conjugation process. Activation of SUMO proteins involves the formation of a thioester bond between SUMO proteins and the E1 activating enzyme in an ATP dependent fashion. Activation is completed after transfer of the SUMO protein to a conserved catalytic cysteine in the unique conjugation enzyme (E2)-Ubc9 (27). Thereafter, Ubc9 transfers SUMO protein directly to its substrates. *In vivo* SUMO conjugation and ligation can be modulated by a number of E3 ligases (see below). The last phase, de-conjugation, impinges on freeing conjugated SUMO proteins from conjugating state with substrates by a class of isopeptidase SENPs, which are also involved in the maturation of SUMO proteins (28).

Although the sole Ubc9 identified to date in mammals possesses the capacity to recognize SUMO targets, and *in vitro* reconstituted sumoylation assays reveal that the presence of E1 and E2 suffice to implement SUMO conjugation (29, 30), SUMO E3 ligases do exist *in vivo* that facilitate the SUMO modification and contribute to the specificity of SUMO subtypes and substrates (31-35). PIAS family members, RanBP2, polycomb chromatin-modifying complex component Pc2, Topors, TRAF7 have been reported to serve as SUMO E3 ligases in some particular SUMO substrate modifications (36-40). Interestingly, among these E3 ligases, PIAS proteins link E3 ligase activity to their Ring domains and Topors serves as an E3 ligase for both ubiquitination and sumoylation of p53 (36, 41).

In most cases SUMO-targeted lysine residues are embedded in the consensus sequence ψKXE (ψ stands for a

bulky hydrophobic amino acid and X represents any residue) (42, 43), however, not all proteins harboring this consensus sequence are targeted by SUMO proteins, and atypical SUMO linkage site (s) indeed exists. Of particularly interest, the presence of SUMO E3 ligase like PIAS1 usually promotes formation of poly SUMO chain and /or activates non-consensus SUMO attachment site (s) (30, 44).

4. SUMO MODIFIES SUBSTRATES CRITICAL FOR CARDIOVASCULAR DEVELOPMENT

Cardiovascular malformations are the leading cause of death from congenital defect (45). Normal cardiovascular development is a complex process that requires highly coordinated collaborations among a variety of transcription factors, co-factors and signal transduction pathways. The fact that SUMO conjugation pathway components are abundant in the heart points to the possibility that SUMO may be implicated in cardiovascular development via modifying transcription factors indispensable for normal cardiovascular development (46, 47). Indeed, the work from our laboratory and other research groups has identified several cardiac-enriched transcription factors as SUMO targets.

4.1. GATA4

GATA4 is a zinc finger-containing transcription factor belonging to the GATA superfamily composed of six members with GATA1, 2 and 3 largely restricted to hematopoietic lineage and GATA4, 5 and 6 abundant in heart. GATA4 recognizes the consensus motif (A/T)GATA (A/G) found in regulatory region of the target genes such as α-MHC (48), ANF (49). Gene targeting and null mutation studies have revealed the significant role of GATA4 in the regulation of heart development (50, 51). Posttranslational modifications such as phosphorylation have been shown to regulate GATA4 transcriptional activity (52, 53). We and others revealed that GATA4 is modified by SUMO-1 on lysine residue 366 (30, 54). Mutation of this lysine reduces nuclear occupancy of GATA4 (30). Remarkably, SUMO modification of GATA4 promotes cardiac specific gene expression in pluripotent 10T1/2 cells, indicating that SUMO positively regulates GATA4 transcriptional activity, which was further corroborated by the finding that GATA4 function is elevated by Ubc9, the E2 in SUMO conjugation pathway (55). Furthermore, ~20% of total GATA4 protein purified from cultured cardiomyocytes exhibits SUMO modification (30), suggesting that the SUMO conjugation of GATA4 in cardiomyocytes has physiological relevance.

4.2. SFR (serum response factor)

SRF is a critical factor for mesoderm development and cardiogenesis (56, 57). SUMO-1 is shown to target SRF on its lysine residue 147, but have no significant influence on its DNA binding although lysine 147 is localized in the MADS box crucial for DNA binding activity (58, 59). Conversion of lysine 147 to arginine enhances SRF capacity to activate c-fos promoter (58), but impairs its function to activate cardiac α-actin promoter (manuscript in preparation). Correspondingly, SUMO-1 substantiates SRF transcriptional activity to activate cardiac specified gene promoters. These studies support the notion that the functional consequence of SUMO modification of SRF may be promoter-dependent.

4.3. Myocardin

Myocardin and its related proteins MRTF-A and MRTF-B (60), belong to the SAP superfamily (SAF-A/B, Acinus, PIAS), harboring the chromatin remodeling SAP domain (61). As a co-activator of SRF, myocardin triggers SRF-dependent smooth muscle differentiation program (62, 63). Loss-of-function study reveals severe defect in vascular smooth muscle development (64), demonstrating the indispensability of myocardin in VSMC lineage commitment. Studies performed in *Xenopus* also indicate the importance of myocardin for cardiogenesis during *Xenopus* embryonic development (63, 65). While myocardin alone is not sufficient to activate cardiogenic genes in pluripotent 10T1/2 fibroblast cells (64, 66), SUMO, through linkage to lysine 445 in myocardin, greatly enhances myocardin capacity to activate cardiac specified genes in 10T1/2 fibroblast cells without significantly affecting its ability to induce smooth muscle differentiation (44). Whether endogenous myocardin function is regulated by SUMO modification remains unclear. SUMO modification has no significant effects on myocardin's nuclear localization and its physical interaction with SRF, the major factor through which myocardin functions. It is noteworthy that E3 ligase PIAS1 enhances myocardin transcriptional activity via both stimulation of SUMO modification of myocardin as well as direct physical association, either of which is required to induce cardiogenic gene expression in pluripotent 10T1/2 fibroblast cells (44).

4.4. Myocyte enhancer factors (Mef2s)

Mef2s are transcription factors regulating muscle specific gene activity. Mef2 genes are expressed in early myogenic lineage and knockdown of Mef2 activity suppresses cardiomyocytes differentiation and heart development (67, 68). All four Mef2 genes harbor the SUMO consensus sequence IKSE, but it has only been shown that SUMO targets hMef2A, hMef2C and hMef2D (69). SUMO-site-mutated Mef2s exhibit higher activity than wild-type in the activation of MEF binding sites and promote myogenesis in 10T1/2 cells (70, 71). Interestingly, HDAC4 serves as an E3 ligase to stimulate Mef2 sumoylation, which inhibits Mef2 activity. Since mouse Mef2B is also a potent activator in myogenic lineage commitment (72), it will be of great interest to explore whether Mef2B is SUMO targeted and how its function is regulated.

4.5. Nkx2.5

Nkx2.5, the cardiac specific homeodomain gene (73, 74), is the most recently identified SUMO substrate from our laboratory (manuscript in preparation). Nkx2.5 is a member of the NK-2 class of homeodomain (HD) and required for the normal heart development (75, 76). Nkx2.5 is one of the earliest known markers of cardiac progenitors *in vivo* (73, 74, 77). Nkx2.5 plays important roles in tissue patterning and lineage determination. Homozygous Nkx2.5 null mice die at E9-10 day before heart looping is

completed, but allows for appearance of beating cardiomyocytes (78, 79), supporting the notion that Nkx2.5 plays a central role for early cardiac morphogenesis. Nkx2.5 activity is stimulated via SUMO attachment.

SUMO modification has been shown to affect protein-protein interaction (80, 81). Given the prevalent existence of physical associations among cardiac enriched factors with some of which targeted by SUMO modification, it is possible that SUMO may potentiate the combinatorial actions exerted by protein-protein interaction such as GATA4-Nkx2.5 (82), SRF-GATA4 (83), SRF-myocardin (63), and GATA4-myocardin (84).

5. SUMO TARGETS SIGNAL TRANSDUCTION PATHWAYS INVOLVED IN CARDIOVASCULAR DEVELOPMENT

Normal cardiovascular development requires independent action as well as coordinated crosstalk among multiple signal transduction pathways. Interestingly, SUMO has been shown to target the receptors and effectors of several signaling pathways, hereby modulating the influence of signal transductions in cardiovascular development.

5.1. Steroid receptors:

It has been well established that steroid receptors belonging to the nuclear receptor super-family play important roles in the normal formation of cardiovascular system (85-87). Many steroid receptors, including the androgen receptor, estrogen receptor α (ERα), progesterone receptor, glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), are reported to be SUMO targeted (88). The functional consequences of SUMO modification of these steroid receptors may be positive (activated), like ERα SUMO modification (89), or may be promoter-dependent, like the SUMO modification of androgen receptor and MR as well as GR (90-92). Interestingly, some of these modifications are ligand-dependent, suggestive of the existing crosstalk between SUMO conjugation pathway and the signaling pathway involving steroid receptors.

5.2. Wnt signaling

Wnt signaling pathway is implicated in heart formation both negatively and positively (93, 94). T-cell factor 4 (TCF-4), the downstream effector of Wnt signaling, is SUMO-1-modified on at least one consensus sequence, lysine 297, causing stimulation of TCF-4 transcriptional activity (95). The E3 ligase PIASy potentiates TCF-4 SUMO attachment and its activity. However, PIASy inhibits the transcriptional activity of LEF1, another member of TCF family, via potentiation of its SUMO conjugation (32). These observations indicate that the precise functional consequence after SUMO impinges on Wnt signaling pathway is complex and worth further evaluation.

5.3. Hypoxia-inducible signaling pathway

The transcription factor HIF is a key component in the hypoxia-associated signaling pathway that activates genes involved in angiogenesis. HIF-1 functions as a heterodimer composed of HIF-1α (oxygen-sensitive subunit) and HIF-1β (constitutively expressed subunit, also known as aryl hydrocarbon nuclear translocator). Knockout of either HIF1α or HIF1β results in defects in cardiovascular and vessel development, leading to the embryonic lethality in mice (96, 97). The functions of these two factors are modulated via post-translational modification, including sumoylation. It was previously reported that SUMO targets HIF1α on lysine 391 and 477, located in the oxygen-dependent degradation domain (ODDD), and enhances HIF-1α stability and thereby its activity (98). A recently identified protein named RSUME also stabilizes HIF-1α via sumoylation during hypoxia (99). However, more recently, Cheng J *et al* demonstrated that SUMO serves as a signal for ubiquitination of HIF1α, therefore decreasing its activity (100). SUMO also positively influences HIF-1β activity via affecting its physical association with other factors (101). Given that hypoxia increases SUMO-1 expression in the heart (102), these findings suggest that SUMO may become actively involved in the cellular process associated with the changes in oxygen homeostasis via modulating HIF.

5.4. Transforming growth factor-β signaling (TGF-β)

Many of the TGF-β superfamily proteins are involved in the development and functional maintenance of cardiovascular system (103, 104). The downstream targets activated by TGF-β signaling pathway are SMAD proteins, of which SMAD4 is a common factor implicated in both TGF-β and BMP signaling. SUMO-1 attachment to lysine residues 159 and 113 of SMAD4 increases its transcriptional activity via stabilization (105, 106), however, sumoylation of SMAD4 is also found to inhibit some TGF-β-responsive gene (107), indicating that the net functional effect of SUMO linkage to SMAD4 may be context-dependent. The precise import of SUMO modulation of SMAD4 in cardiac development has yet to be elucidated.

SUMO not only targets transcription factors that directly bind cognate DNA sequences in the specific promoter regions and affects target genes activity, but also modifies co-factors (co-activators and co-repressors) associated with them and then becomes involved in modulating corresponding signaling pathways. Some co-factors affected by SUMO modification are listed in the Table 1.

6. CONCLUSION AND PERSPECTIVE

SUMO covalent conjugation and de-conjugation is a fascinating process that governs the activity of a variety of substrates. Although extensive efforts have been invested into better understanding the biochemical pathways regulated by SUMO and the involvement of SUMO conjugation in physiological as well as patho-physiological processes, the understanding of the role of this pathway in cardiovascular development is just evolving. Normal cardiovascular development necessities orchestrated actions among cardiac-enriched transcription

Table 1. Some co-factors targeted by SUMO modification

Name of co-factors	Activity after sumoylation	Major binding partners	References
SRC	elevated	Steroid receptor	(108)
P300	repressed	Nuclear receptors and many TFs	(109)
CREB binding protein	repressed	Nuclear receptors and many TFs	(110)
CtBP	repressed	HDACs, SMAD6, etc	(86)
HDAC1,4,6	repressed	Multi-protein co-repressor complexes	(111, 112)

TFs: transcription factors

factors, co-factors and signal transduction pathways, and how SUMO conjugation pathway is incorporated into these actions merits exploration. To gain a further insight into the molecular mechanisms underlying the implication of SUMO pathway in cardiogenesis, some critical issues need to be addressed. The temporal and spatial sumoylation pattern, the activity of SUMO pathway components as well as their regulations during the full course of cardiac development need further investigation. Furthermore, SUMO pathway components such as Ubc9 and SUMO-1 may perform some biochemical functions independent of covalent conjugation (113, 114), which raises the question as to how important these non-covalent function of SUMO pathway components is versus the conjugation ability in the physiological context of cardiovascular development. With an expeditious advance in SUMO field, more exciting achievements and better comprehension regarding the contribution of SUMO pathway to cardiovascular development are anticipated.

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