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SUMOylation in skeletal development, homeostasis, and disease

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ABSTRACT

The modification of proteins by small ubiquitin-related modifier (SUMO) molecules, SUMOylation, is a key post-translational modification involved in a variety of biological processes such as chromosomes organization, DNA replication and repair, transcription, nuclear transport, and cell signaling transduction. In recent years, emerging evidence has shown that SUMOylation regulates the development and homeostasis of the skeletal system, with its dysregulation causing skeletal diseases, suggesting that SUMOylation pathways may serve as a promising therapeutic target. In this review, we summarize the current understanding of the molecular mechanisms by which SUMOylation pathways regulate skeletal cells in the physiological and disease contexts.

KEYWORDS

SUMO; MSC; osteoblast; chondrocyte; osteoclast; signaling pathway; arthritis; osteosarcoma; developmental disorders

Introduction:

The emergence of skeletal system was a leap forward in evolution, for it created a strong framework for the vertebrate body, protecting vital organs, facilitating movement, establishing a niche for hematopoiesis, and serving as a mineral reservoir.

The skeletal system develops from mesenchymal cells originated from the ectoderm and mesoderm through one of two types of ossifications processes: intramembranous or endochondral ossification. In intramembranous ossification, mesenchymal cells directly differentiate into osteoblasts to generate flat bones of the skull and lateral clavicles[1]. Whereas endochondral ossification, which gives rise to the bones at the base of skull and the long bones, starting from mesenchymal condensation followed by primary and secondary ossification[2]. The condensed mesenchymal cells first undergo chondrogenic differentiation to form cartilage templates[2-4]; next, chondrocytes in the center of the cartilage templates mature and differentiate into hypertrophic chondrocytes that secrete factors to promote vascular invasion[2-5]. This brings in hematopoietic cells from the blood and osteogenic progenitors from the perichondrium[2-5]. Next, osteoblasts, derived from either osteogenic progenitors or hypertrophic chondrocytes, produce bone matrix to replace the cartilage templates generated by the apoptotic hypertrophic

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chondrocytes[2-7]. At the same time, bone-absorbing osteoclasts derived from the hematopoietic lineage remodel the bone and form the bone marrow cavity[8]. Secondary ossification areas form at the center of the cartilage at both ends of long bones in a process similar to primary ossification[8,9], dividing cartilage into two parts: the growth plate, which contains growth plate chondrocytes (GPCs); and articular cartilage, which consists of articular cartilage chondrocytes (ACCs). The finely controlled, directional chondrocyte proliferation and differentiation in the growth plate propels the elongation of the bone. The coupling between osteoblast-mediated bone formation and osteoclast-mediated bone resorption continues throughout life to maintain bone tissue homeostasis[10,11].

The development and homeostasis of the skeletal system requires diverse and responsive signaling and cell-cell communication, which heavily rely on dynamic posttranslational modifications (PTMs) systems. PTMs expand the proteome size tremendously without needing *de novo* protein synthesis, allowing cells to regulate complex cellular processes dynamically and efficiently. PTMs participate in every aspect of cell homeostasis, and their dysregulation often leads to disease[12]. PTM pathways are common drug targets for disease treatments, for they are reversible and dependent on enzymatic activity. SUMOylation is a branch of ubiquitination-like (Ubl) PTMs that conjugate SUMO (an ~100 aa protein tag) to target proteins and has a strong connection with stress responses and aging. Below, we summarize the contribution of SUMOylation pathways to skeletal physiology and disease.

A. SUMO and SUMOylation

SUMOylation is a highly dynamic and reversible PTM that attaches SUMO proteins onto target proteins. Five SUMO paralogues (SUMO1, 2, 3, 4, and 5) have been identified in mammals, each exhibiting unique expression patterns and levels of homology[13-16]. SUMO1-3 are ubiquitously expressed in all tissues, whereas SUMO4 is mainly found in kidney, spleen, and lymph nodes, and SUMO5 expression is restricted to several tissues, with exceptionally high expression levels in testes and peripheral blood leukocytes[14-17]. In humans, SUMO2 shares 97%, 86%, 50% and 48% amino acid sequence homology with SUMO3, 4, 5 and 1 respectively[14,15,18]. SUMO5 is 88% identical to SUMO1[14].

SUMO modifications are attached to a single or multiple lysine residue(s) of target proteins (mono-SUMOylation and multi-SUMOylation, respectively). SUMO2 and 3 contain several lysine residues that are themselves SUMOylated, allowing for polymeric and branched SUMO chain formation (polySUMOylation)[14,19-21]. Generally, SUMO1 modifications tend to occur

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under normal physiological conditions, while SUMO2 and 3 conjugations are more prominent in response to stress[22], with some exceptions[23-27]. SUMO4 and 5 are not well characterized and their functions remain unknown.

SUMOylation involves a series of enzymatic reactions with E1, E2, and E3 ligases[28] (**Figure 1**). First, the SUMO precursor protein is cleaved by the Sentrin-specific proteases (SENPs), a family of SUMO-specific C-terminal hydrolases, to expose its C-terminal di-glycine (GG) motif. This mature SUMO is then activated by the E1 complex, which consists of SUMO activating enzyme subunit 1 (SAE1) and SAE2 (UBA1), by forming a thioester bond at the cysteine of SAE2 via an ATP-dependent reaction[29]. Next, the activated SUMO group is transferred to the sole SUMO E2 enzyme, UBC9 (SUMO ubiquitin-conjugating enzyme 9). Finally, UBC9, with or without the help of SUMO E3 ligases, conjugates the SUMO group to the epsilon-NH2 of a lysine in the target protein. SUMOylation substrate specificity is determined by UBC9 or SUMO E3 ligases. UBC9 recognizes consensus motifs, typically ψKxE (ψ represents a hydrophobic amino acid; K, lysine; x, any amino acid; and E, glutamic acid)[28,30]. SUMO E3 ligases facilitate the transfer of the SUMO molecule from UBC9 to the substrate proteins.[28,29,31]. Unlike the ubiquitylation system, where hundreds of distinct E3 ligases have been identified, there are only a few known SUMO E3 ligases, including members of the protein inhibitor of STAT (PIAS) family[32-35]. The SUMO E3 ligase activity of PIAS proteins reflects only one aspect of their function[36].

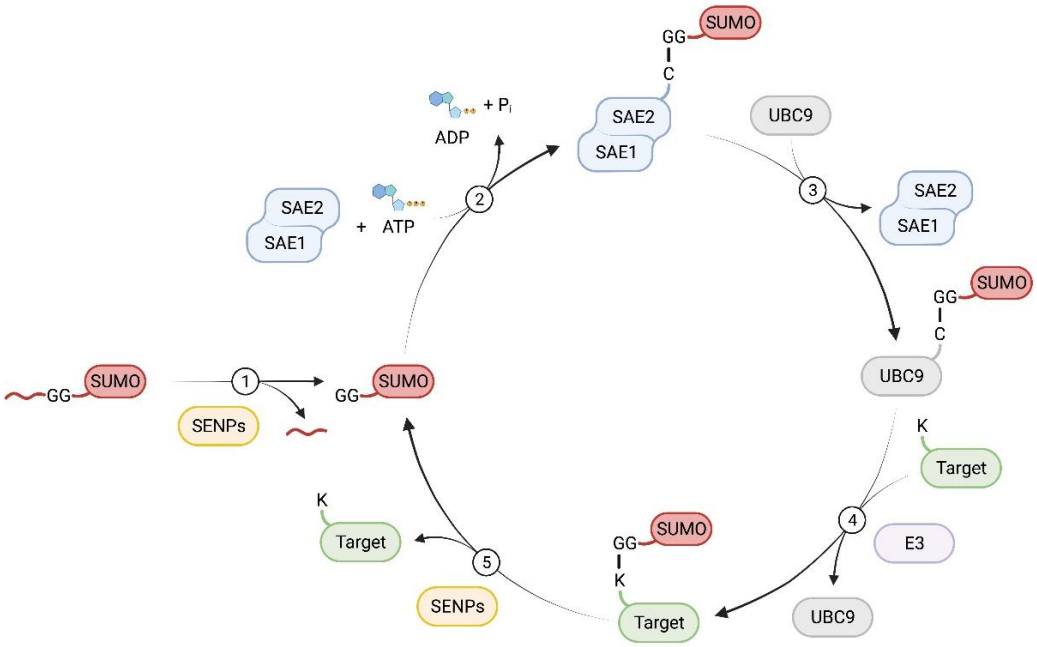

Fig. 1 The enzymatic process of protein sumoylation and desumoylation.

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In addition to proteolyzing the SUMO precursor, SENPs can also remove SUMO proteins from their targets, a process known as deSUMOylation[31]. Seven SENP proteins have been identified in humans (SENP1-3, SENP5-7, and SENP8[19]). SENP1, 2, 3, and 5 catalyze both SUMO maturation and deconjugation, whereas SENP6 and 7 do not catalyze SUMO maturation, but instead have poly-SUMO chain-editing function[28,37,38]. Besides the SENP family, three additional SUMO proteases have been identified in humans: desumoylating isopeptidase 1 and 2 (DeSI1 and DeSI2)[39], and ubiquitin-specific protease-like 1 (USPL1)[40]. These desumoylases share little sequence homology with the SENP proteases, and their functions are less well characterized[41].

The effects of SUMO modifications on their target proteins are diverse and are mainly classified into three categories[13] : first, the attachment of the SUMO group can mask binding sites of the target protein, thus impairing its interaction with other molecules[13,42]; second, SUMOylation can introduce novel binding sites within the target protein, thus conferring novel molecular interactions[13,42]; finally, SUMO can change the structure of the target protein, thereby affecting its function[13,42]. The SUMOylation/deSUMOylation equilibrium regulates many cellular processes, including DNA damage response, mitochondrial dynamics, cell growth, proliferation, senescence, and apoptosis. Disruption of this SUMOylation/ deSUMOylation balance is associated with many diseases, including cancer, neurodegenerative diseases, heart disease, and skeletal diseases, such as osteoarthritis (OA) and rheumatoid arthritis (RA)[29,43-45].

B. SUMOylation in Skeletal Cell Differentiation, Homeostasis, and Disease

Osteoblasts, chondrocytes, and osteoclasts are the major cell types of the skeletal system and cooperate seamlessly to regulate bone development and homeostasis[46-48].

B.1 SUMOylation in osteogenesis, osteoblast homeostasis, and bone mass regulation

SUMOylation regulates key signaling pathways, transcription factors, hormones, and epigenetic regulators of osteogenesis and osteoblasts; the requirement for this PTM is demonstrated by the dysregulation of bone development and homeostasis when SUMOylation is disrupted (selected examples are illustrated in **Figure 2**).

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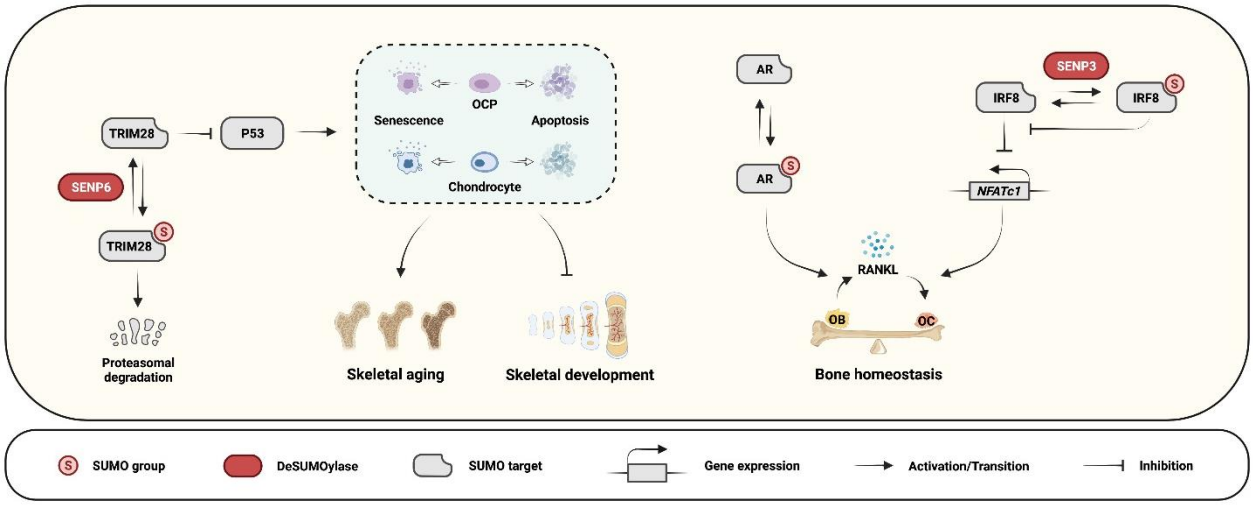

Fig. 2 Exemplary sumoylation pathways in skeletal physiology.

Signaling pathways. We reported that postnatal and ubiquitous loss of SENP6 leads to kyphosis, a sign of premature skeletal aging[49]. Furthermore, mice with OCP-specific *Senp6* knockout have small skeletons and decreased trabecular bone mass and cortical thickness, as well as delayed secondary ossification center formation[49]. OCP-derived cell lineages undergo severe apoptosis and cellular senescence. Mechanistically, *Senp6* loss results in excessive SUMOylation of the multifaceted protein TRIM28, which is involved in chromatin silencing, transcriptional repression, and p53 inhibition. SUMOylation destabilizes TRIM28 and weakens TRIM28-mediated p53 repression, leading to OCP/chondrocyte apoptosis and senescence[49].

Importantly, SUMOylation regulates TGF-β/BMP signaling, a fundamental and diverse signaling network that controls embryonic skeletal development and postnatal bone homeostasis[50-53]. TGF-β/BMP superfamily ligands interact with their heteromeric receptor complexes and transmit extracellular signals to the nucleus via SMAD proteins[50-53]. In the human Saos-2 osteosarcoma cell line, SMAD4 interacts with and is SUMOylated by UBC9. Knockdown of *Ubc9* decreases the levels of SMAD4 protein and phosphorylated SMAD1, prevents the nuclear accumulation of SMAD1 and 4, and decreases the expression of osteogenic transcription factors downstream of BMP (*Runx2*, *Dlx5*, *Msx2*, and *Osx*)[54]. Conflicting data in C2C12 mouse myoblasts and ST2 mouse bone-marrow derived stromal cells (BMSCs), has demonstrated that *Ubc9* knockdown can elevate BMP signaling and enhance osteogenic differentiation[55]. Mutation of the SMAD4 SUMOylation site (K158R) increases SMAD4 transcriptional activity[55]. One explanation for the contradictory findings is that the

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osteosarcoma cells are a transformed cancer cell line that likely has distinct signaling machinery from the BMSC and C2C12 myoblasts.

Transcription factors. Essential transcription factors for osteoblast differentiation, including the RUNX family members RUNX1, 2, 3 and Osterix[56-61], are also regulated by SUMOylation[62-64]. The SUMO E3 ligase PIAS1 promotes SUMOylation at K144 of RUNX1, K181 of RUNX2, and K148 of RUNX3[62]. Increased RUNX2 SUMOylation leads to RUNX2 degradation, and PIAS1-mediated SUMOylation inhibits RUNX3 transcriptional activity[62]. Osterix is SUMOylated by SUMO1 in C2C12 cells[64]. Knockdown of the SUMO E3 ligase, PIASxβ, in MC3T3-E1 mouse osteoblastic cells inhibits osteogenic differentiation and matrix mineralization[65]. PIASxβ expression, but not expression of a PIASxβ-SUMOylation-defective mutant, enhances the transcriptional activity of Osterix, suggesting that Osterix SUMOylation increases its activity[65].

Our own studies have shown that inhibition of SUMOylation can yield profound effects on BMSC fate determination between osteogenesis and adipogenesis. We reported that *ginkgolic acid*, a SUMOylation inhibitor that binds to E1 ligase to prevent the formation of the SAE1-SUMO intermediate, inhibits the expression of RUNX2 and Osterix while promoting the expression of the adipogenic transcription factors PPARγ and CE/BPα[66]. Consistent with our findings, PPAR-γ SUMOylation inhibits PPAR-γ transcriptional activity in BMSCs. When stimulated with GDF11 (a TGFβ family member), PPAR-γ SUMOylation attenuates adipogenesis in favor of osteogenesis[67].

Hormones. Hormones and their receptors, especially the Androgen receptor (AR), are important regulators of skeletal development. AR knockout dramatically reduces trabecular and cortical bone mass[68]. SUMOylation of ARs is necessary for bone mass maintenance, as mutations (K381R and K500R) within the AR SUMOylation site result in significantly decreased trabecular bone and cortical bone mass[69]. Of note, while loss of AR SUMOylation decreases osteoblast numbers, the number of osteoclasts is unaffected[69].

Epigenetic regulators. SUMOylation is also implicated in the epigenetic regulation of osteogenesis. In human dental follicle stem cells, SENP3 binds to and deSUMOylates RBBP5, an important component of several histone methyltransferase complexes[70-72]. This facilitates the formation of active MLL1/MLL2 histone methyltransferase complexes that methylate H3K4 residues on the promoters of *DLX3* (an osteogenic transcription factor) and a subset of other HOX genes, thus enhancing osteogenic differentiation[73].

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B.2. SUMOylation in chondrogenesis, chondrocyte homeostasis, and osteoarthritis

Chondrocytes of healthy cartilage are formed by the differentiation of skeletal progenitor/stem cells (SSCs) into GPCs through an intermediate and bipotent osteochondroprogenitor, or into ACCs via a multipotent joint progenitor[4,74,75]. GPCs proliferate and produce the extracellular matrix template for subsequent ossification, thus allowing for fast elongation of bone elements [4,74,75]. In contrast, ACCs are mostly quiescent, but secrete and maintain extracellular matrix to sustain the cartilage integrity in response to outside stimuli and tissue damage and to provide a smooth and lubricated surface for articulation[76,77].

SUMOylation regulates the function of chondrogenic transcription factors. SOX9, the master regulator of chondrogenesis and cartilage development[78-80], is a SUMO target protein. SUMOylation of SOX9 has been detected in COS-7, chick neural crest cell, U2OS osteosarcoma cells and 293T cells, however, the consequences of SOX9 SUMOylation varies in these contexts[81-84]. A link between chondrogenesis and SOX9 SUMOylation was observed in a mouse model with OCP-specific deletion of *Shp2*[85], a protein-tyrosine phosphatase required for activating the Ras/ERK pathway[86,87]. The knock-out OCPs have increased chondrogenesis but decreased ossification[85]. Total Sox9 protein, phosphorylated SOX9, and SUMOylated SOX9 were all upregulated in SHP2-deficent chondrocytes, in addition to the SOX9 target genes *Acan* and *Col2a1*[85]. This supports the notion that SUMOylation regulates chondrogenesis through SOX9.

SOX6 and NKX3.2 are two other chondrogenic transcription factors regulated by SUMOylation[88,89]. SOX6 is a downstream target of SOX9. In 293T cells, SUMOylation represses SOX6 transcriptional activity[88]. When SUMOylation is reduced, via mutations of two SOX6 SUMOylation sites, *UBC9* knockdown or loss of function mutations, or SENP2 overexpression, SOX6 transcriptional activity increases[88]. NKX3.2 regulates chondrocyte viability and differentiation, while preventing chondrocyte hypertrophy[89]. In the ATDC5 chondrogenic cell line, HDAC9-dependent deacetylation of NKX3.2 triggers its SUMOylation[89]. This leads to SUMO-targeted NKX3.2 ubiquitylation and degradation, causing hypertrophy and apoptosis of ATDC5 cells[89].

SUMOylation also likely regulates the maintenance of heterochromatin structure in articular cartilage. For instance, DGCR8 – which maintains heterochromatin through interactions with TRIM28 and HP1γ – is stabilized to prevent its degradation via the ubiquitin-proteasome

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pathway by SUMO1 modification at the K707 residue[90,91]. We know that DGCR8 is important for chondrocyte differentiation, maintenance, and cartilage regeneration[92,93]; future studies are needed to confirm a direct role for DGCR8 SUMOylation in chondrocyte homeostasis. Another heterochromatin regulator, CLOCK, the core component of the mammalian circadian machinery, prevents stem cell aging and promotes chondrogenesis by stabilizing heterochromatin via TRIM28[94,95]. CLOCK transcriptional activity is increased by SUMOylation at residues K67 and K851[94,95]. Again, future experiments assessing whether CLOCK SUMOylation is required for chondrocyte differentiation and homeostasis are needed. *In vivo* studies where SUMO specific regulatory proteins are knocked out specifically in chondrocytes or chondrocyte progenitors will clarify the role of SUMOylation in the development of chondrocytes and homeostasis of articular chondrocytes.

Osteoarthritis (OA) is characterized by progressive loss of cartilage, the formation of bone spurs, and chronic synovial inflammation[96]. OA severely impairs joint function and often causes joint pain[96]. The onset and progression of OA are highly associated with various risk factors, including gender, genetic predisposition, obesity, joint malalignment, sports injury, and aging[96]. Several lines of evidence suggest that enhanced SUMOylation promotes OA pathogenesis. A large genome-wide association analysis in Europe identified the rs9350591 C/T single nucleotide polymorphism (SNP) located upstream of the *SENP6* locus as one of the most strongly OA-associated SNPs[97]. SENP6 expression is significantly decreased in OA cartilage even in the absence of rs9350591, suggesting that a deficiency in SENP6 desumoylase activity may be a widespread phenomenon in OA[98]. Moreover, IL-1β treatment of human articular chondrocytes induces the SUMO1 modification of S100A4 (a member of the Ca^{2+}-binding S100 proteins that modulates p53 transcriptional activity), resulting in S100A4 nuclear translocation and activation of MMP13 (a major OA-promoting protease that degrades cartilage) expression by binding to the *MMP13* promoter region[99].

In contrast, several studies suggest that SUMOylation decreases OA marker expression. A high-throughput screen of primary human ACCs identified *SENP3* as a pro-OA gene[100]. SENP3 overexpression up-regulated several OA markers, including *MMP13*, *COX2* (cyclooxygenase-2), *iNOS* (inducible nitric oxide synthase), and *AGG1* (aggrecanase-1)[100]. Also, SUMO1 modification of interferon regulatory factor 1 (IRF-1) was induced by the antioxidant alpha-lipoic acid in human ACCs[101]. This modification decreased the transcriptional activity of IRF-1, thus inhibiting the IL-1β-induced expression of OA marker genes, including *MMP3* and *MMP13*[101]. Furthermore, in human primary ACCs, basic fibroblast growth factor

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(bFGF) increases ETS-like-1 protein (ELK-1) phosphorylation but decreases ELK-1 SUMOylation. Decreased ELK-1 SUMOylation enhances its transcription of MMP13, thus promoting cartilage matrix degradation[102].

B.3. SUMOylation in osteoclastogenesis and osteoclast function

Osteoclasts differentiate from the hematopoietic cell lineage upon induction by cytokines, such as m-CSF and RANKL, present in the bone and bone marrow microenvironment[103,104]. Osteoclast progenitors differentiate, fuse, and form multinucleated mature osteoclasts, which produce acid and matrix-degrading proteases and serve as dedicated bone-resorbing cells of the skeletal system[103,104] .

Recent studies revealed the regulatory role of SUMOylation in osteoclast formation and function. For instance, SENP3 suppresses osteoclastogenesis. Mice with the *Lyz2*-Cre-mediated *Senp3* deletion in bone marrow-derived monocytes exhibit decreased bone mass[105]. These knockout mice also have aggravated bone loss after ovariectomy due to overactivation of osteoclasts. Mechanistically, *Senp3* deletion increases SUMO3 modification of IRF8 and weakens the ability of IRF8 in suppressing *NFATc1* gene expression[105]. In addition, transgenic mice overexpressing the SUMO E3 ligase PIAS3 exhibit an osteopetrotic phenotype caused by impaired osteoclast differentiation[106]. PIAS3 overexpression in RAW264.7 cells inhibits *c-Fos* and *Nfatc1* expression, thereby blunting RANKL-induced osteoclastogenesis[106]. In a bone marrow monocyte–osteoblast co-culture system, PIAS3 overexpression in osteoblasts downregulated IL6-induced RANKL expression and inhibited osteoclast formation. Conversely, downregulation of PIAS3 in osteoblasts increased RANKL expression[106]. Thus, PIAS3 inhibits osteoclastogenesis either by intrinsically inhibiting osteoclast differentiation or by indirectly suppressing the expression of osteoclastogenic cytokines, such as RANKL, from osteoblasts. However, as PIAS3 has other functions besides SUMO E3 ligase activity, it is still unclear whether the activity of PIAS3 in osteoclastogenesis depends upon its E3 ligase function or not.

B.4 SUMOylation in developmental diseases

Split hand/split foot malformation (SHFM). SHFM is a rare limb malformation characterized by clefts in the middle of the hands and feet, as well as syndactyly, aplasia/hypoplasia of phalanges, metacarpals and metatarsals[107]. P63α mutations are associated with SHFM[108,109]. The C-terminal domain of P63α binds to UBC9, which conjugates

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SUMO1 to K549 and K637 of P63α[110,111]. The SHFM-associated P63α mutation Q634X disrupts the interaction between P63α and UBC9. K549E and K637E mutations of P63α, both of which block P63α SUMOylation, markedly increase the transcriptional activity of TAP63α (an isoform of P63α containing the N-terminal transactivation domain)[111]. At the same time, these mutations inhibit the dominant-negative effect of the naturally occurring N-terminus truncated isoform of P63α, ΔNP63α. Cells expressing mutant P63α lacking the two SUMOylation sites have decreased expression of genes related to bone and tooth development, such as *Runx2* and *Mint*[111]. Furthermore, both SUMOylation and ubiquitylation are required for the efficient degradation of ΔNP63α[112]. These data indicate the functional importance of P63α SUMOylation in limb development.

Craniofacial disorders. Craniofacial disorders are one of the most common human birth defects. Cleft lip and palate are the most frequent types of craniofacial disorders[113]. Several studies have linked SUMO1 to cleft lip and palate. First, a balanced chromosomal translocation 46,XX,t(2;8)(q33.1;q24.3) that results in *SUMO1* haploinsufficiency was identified in a patient with isolated cleft lip and palate[114]. Second, a 4-SNP SUMO1 haplotype was found significantly associated with non-syndromic cleft lip with or without cleft palate (NSCLP) from a study of 181 patients and 162 healthy controls of Han Chinese origin[115]. Other studies have related SUMO1 to cleft lip with or without cleft palate, cleft palate only, or NSCLP in Poland[116], Ireland[117], and western China[118]. In addition, transcription factors such as TBX22, MSX1, SATB2, P63, PAX9, TRPS1, and EYA1, which contribute to the development of the lip and palate, have all been identified as substrates of SUMO modification[119]. For example, SUMOylation regulates the subnuclear localization, stability, and transcriptional activity of SATB2[120,121], affects subnuclear localization of MSX1[122,123], modulates the transcriptional activity and stability of P63 (see above section on SHFM), facilitates the transcriptional repressor activity of TBX22[124], and regulates the transcriptional suppression function of TRPS1[125]. In summary, the formation of lip and palate appears to be particularly sensitive to changes in SUMOylation[119].

B.5 SUMOylation in rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic systemic, inflammatory disease characterized by joint stiffness and destruction[126,127]. Synovial inflammation is a hallmark of RA and the main driver of cartilage degradation. The main cellular features of RA include synovial hyperplasia, increased vascularity, and inflammatory cell infiltration[126,127].

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A direct relationship between the SUMOylation pathway and RA was first reported in 2000[128]. SUMO1 mRNA was found to be highly expressed in synovial specimens from RA patients, predominantly in the synovial fibroblasts of the lining layer and at the sites where cartilage is invaded by synovium[128]. The expression of SUMO1 in RA synovial fibroblasts (RASFs) is over 30 times higher than that of OA synovial fibroblasts or normal fibroblasts[128]. A recent study found that SUMO1 knockdown inhibits the migration and invasion of RA fibroblast-like synoviocytes (RAFLSs), and RAFLS expression of *MMP1* and *MMP3.* Mechanistically, SUMO1 deficiency suppresses the activity of the Rac1/PAK1 pathway, which normally promotes cell motility[129]. Furthermore, the expression of PIAS3 is increased in RAFLSs and RA synovial tissues[130]. PIAS3 promotes the SUMOylation of Rac1 and activates the expression of Rac1 downstream targets, such as PAK1 and JNK[130]. Decreased PIAS3 expression can inhibit the invasion and migration of RAFLSs and the expression of *MMP3*, *MMP9*, and *MMP13*[130].

SUMO E1 conjugating enzymes SAE1 and SAE2 are also increased in FLSs and synovial tissues of RA patients[131]. Knockdown of SAE1 or SAE2 by siRNA results in a less aggressive phenotype and reduced inflammation of RAFLSs[131]. SAE1 and SAE2-mediated SUMOylation of pyruvate kinase M2 (PKM2), thereby promoting its phosphorylation and nuclear translocation, results in the suppression of pyruvate kinase activity, which contributes to synovial glycolysis and joint inflammation[131].

In line with these reports, the expression of the SENP1 desumoylase is decreased in RA synovial fibroblasts (RASFs)[132,133], indicating the anti-RA function of SENP1. Further mechanistic studies have revealed that overexpression of SENP1 can desumoylate nuclear promyelocytic leukemia (PML) nuclear bodies and inhibit the recruitment of DAXX, a FADD (Fas-associated death domain)-interacting protein, to PML nuclear bodies, thus promoting the Fas-mediated apoptosis of RASFs[132]. In addition, SENP1 suppresses *MMP1* expression by promoting HDAC4 binding to the *MMP1* promoter, further weakening the invasiveness of RASFs[133].

These studies show that increased SUMOylation is positively related to RA, suggesting that down-regulation of SUMOylation may have therapeutic benefits. In support of this, in a mouse collagen-induced arthritis model, down-regulation of UBC9 using siRNA can reduce arthritis intensity scores and joint destruction[134]. RA-related markers, including serum levels of anti-collagen (CII) antibodies, VEGF-A, MMP3, and MMP9, were also decreased. Moreover, down-regulating UBC9 expression in *ex vivo* human RAFLS cultures inhibits TNF-α-stimulated secretion of VEGF-A, MMP-3, and MMP-9 and blocks RAFLS proliferation and migration[134]. The

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expression of SUMO2 in RA tissue or RASFs is significantly higher than that of OA tissues and is increased in the synovium and synovial fibroblasts of human TNF-transgenic (hTNFtg) mice, a common RA model[135]. TNF-α treatment promotes the expression of SUMO2 *in vitro*, while *SUMO2* knockdown significantly increases the expression of *MMP3* and *MMP13* induced by the TNF-α- and IL-1β-stimulated NF-κB pathway[135], suggesting the anti-inflammatory function of SUMO2. Most of these studies show that gross alteration of SUMOylation in the joint contributes to the development of OA and RA. Although the detailed mechanisms are still not well understood, some insight may be derived from studies in other disease conditions or cell types, which suggest that SUMOylation regulates inflammation by modulating the NFκB pathway, the PPARγ pathway, among others[67,136-139].

B.6 SUMOylation in osteosarcoma

Osteosarcoma is the most common cancer type in the human skeletal system. It occurs in humans in a biphasic pattern, i.e., with a peak in adolescents and another in patients over 60 years of age[140,141]. SUMOylation of proteins has a crucial role in regulating the cell cycle, genome stability, and the expression of oncoproteins and tumor suppressors[142,143], and has been linked to the development of osteosarcoma[83,144-152]. However, there is no consensus view on whether SUMOylation is pro- or anti-tumorigenic in osteosarcoma, as this is likely dependent on the specific proteins modified, and the individual effects of the SUMO PTM on each protein.

B.6.a Studies supporting a pro-tumorigenic effect of SUMOylation: Several studies have linked increased SUMOylation to osteosarcoma. For example, *UBC9* is overexpressed in osteosarcoma tissues and cell lines[144]. *UBC9* knockdown inhibits the proliferation and migration of osteosarcoma cells and markedly increases the sensitivity of these cells to the combination treatment of herpes simplex virus thymidine kinase/ganciclovir (HSV-TK/GCV)[144]. The integrity of gap-junction-mediated intercellular communication (GJIC) is required for the HSV-TK/GCV-induced tumor repression. Ubc9 knockout decreases SUMO1 modification and increases the free protein level of connexin 43 (CX43), which is important for GJIC[144]. Thus, UBC9 deficiency sensitizes osteosarcoma cells to chemotherapy by reconstructing and promoting GJIC[144].

In addition, SENP1 expression is decreased in osteosarcoma tissues, cell lines, and osteosarcoma stem cells compared to non-cancer cells and stem cells[145]. Low SENP1 is essential for maintaining the stemness of osteosarcoma stem cells, and overexpression of SENP1 markedly decreases the stemness of osteosarcoma cells while sensitizing them to

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apoptosis induced by HSV-TK/GCV combination treatment[145]. This shows the potential of using SENP1 activation for the treatment of osteosarcoma. SENP2 expression is also significantly decreased in osteosarcoma compared with adjacent normal tissue[83]. SENP2 overexpression inhibits osteosarcoma cell proliferation, migration, and invasion, while SENP2 knockout by CRISPR-Cas9 has the opposite effect[83]. Mechanistically, SENP2-mediated deSUMOylation promotes SOX9 ubiquitylation and degradation[83]. SOX9 knockdown greatly reduces the proliferation and invasiveness of the SENP2 knockout osteosarcoma cells[83]. This study suggests that SENP2 acts as an osteosarcoma suppressor by destabilizing SOX9.

Talin is a key component of focal adhesions[153] and can be modified by SUMOylation in U2OS osteosarcoma and MDA-MB-231 breast cancer cells. Using ginkgolic acid (GA) to inhibit SUMOylation increases the number and size of talin-containing focal adhesions[146]. Inhibition of SUMOylation can significantly reduce the migration of MDA-MB-231 breast cancer cells, but this effect was not studied in U2OS cells[146]. Cumulatively, these studies indicate that SUMOylation has a positive role in promoting osteosarcoma proliferation, invasion, and migration, and that targeting it may be a relevant point of therapeutic intervention.

B.6.b Studies supporting an anti-tumorigenic effect of SUMOylation: In contrast to what was presented above, several studies suggest that SUMOylation can have *anti-osteosarcoma* effect. For example, the desumoylase SENP5 is highly expressed in osteosarcoma cells and tissues[147]. Silencing SENP5 expression in two osteosarcoma cell lines, U2OS and Saos-2, significantly inhibits growth and colony formation and promotes apoptosis[147]. This tumor-suppressor effect of SENP5 silencing may be via the regulation of apoptosis and cell cycle genes, as *SENP5*-knockdown in U2OS and Saos-2 cells increases caspase-3/-7 activity (apoptosis activators), and decreases the expression of cyclin B1[147].

The expression of the E3 ligase PIASxα is lower in osteosarcoma compared to adjacent tissue[148]. Notably, PIASxα overexpression can significantly inhibit osteosarcoma cell proliferation and increase apoptosis[148], whereas PIASxα silencing in U2OS cells increases the expression of cyclin D kinase genes. Moreover, PIASxα overexpression weakens the tumorigenic potential of U2OS cells in nude mice[148]. Again, given the pleiotropic functions of PIASxα, further studies are needed to determine whether the anti-tumor effects observed depends on SUMO-E3 ligase activity of PIASxα.

As another example, all-trans-retinoic acid (ATRA), is an anti-cancer drug that can induce osteosarcoma cell differentiation, which is used as a prognostic indicator of weakened osteosarcoma malignancy and tumor progression[149]. SUMO1 is required for the differentiation

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effect of ATRA, as SUMO1 deletion blocks the anti-osteosarcoma efficiency of ATRA [150] . In addition, retinoic acid receptor α (RARα), the ATRA target, can be stabilized by SUMOylation at K399[150]. Mutation of K399 inhibits SUMO1 modification of RARα and impairs ATRA-induced osteosarcoma cell differentiation[150]. These suggest that SUMO1 acts as an anti-osteosarcoma molecule by targeting RARα.

In a hypoxic environment, the human osteosarcoma cell line MG-63 expresses high amounts of SENP1[151]. SENP1 inhibition reduces the expression of two major hypoxia-induced genes, *HIF1α* and *VEGF* (vascular endothelial growth factor). In turn, blockage of HIF1α normalizes hypoxia-induced SENP1 expression[151]. SENP1 knockdown accelerates apoptosis by decreasing *Bcl-2* expression while increasing *Bax* expression, and weakens cell invasiveness by suppressing epithelial-mesenchymal transition (EMT) genes under hypoxic exposure[151]. These findings suggest a positive feedback loop between SENP1 and HIF1α in regulating proliferation, invasion, and EMT of osteosarcoma cells in hypoxic conditions. In a more recent study, presence of SENP1 expression was found more often in osteosarcoma tissue than in adjacent normal tissue (53/60 vs. 28/60)[152]. Levels of SENP1-derived from plasma exosomes correlate with osteosarcoma tumor size and location, necrosis rate, pulmonary metastasis, and surgical stage[152]. Patients with higher plasma exosome derived SENP1 levels had worse disease-free and overall survival. The prognostic value of plasma exosome derived SENP1 levels in osteosarcoma was found to be better than plasma SENP1[152]. This finding contrasts with the previous finding in which SENP1 expression was lower in osteosarcoma tissue.

SUMOylation is also associated with malignant tumors that form from bone cartilage, known as conventional chondrosarcoma[140,154]. SUMO1 and SUMO2/3 expression are positively correlated with increased aggressiveness of chondrosarcomas, and patients with high SUMO2/3 expression have poorer survival outcomes[154]. While there is no simple generalization that SUMOylation is always associated with tumor suppressor or promoter activity, these studies clearly demonstrate that SUMOylation as a PTM must be considered as an important factor in the regulation of cancer cell survival, invasion, and tumor progression.

C. Summary and Future Perspectives

In summary, PTM by SUMOylation regulates signaling pathways and transcription factors that are crucial for skeletal cell differentiation, development, and homeostasis. Dysregulation of SUMOylation is associated with skeletal diseases such as OA and RA,

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craniofacial defects, and bone tumors (**Figure 3**). Thus, targeting SUMOylation/deSUMOylation pathways is a promising strategy for the development of new treatments for these disorders. However, this requires a better characterization of the SUMOylation/deSUMOylation processes, especially in a tissue- and disease-specific manner. The establishment of related mouse genetic models will be a valuable resource to achieve this goal. In addition, future studies will need to focus on dissecting the functions of the components of the SUMOylation/deSUMOylation machinery, identifying the regulators and effectors (substrates) of SUMOylation/deSUMOylation, and discovering therapeutic molecules that can specifically target this machinery. SUMOylation is involved in regulating signal transduction, stress response, epigenetics, and senescence, which are closely associated with age-related and degenerative diseases. Further studies dissecting the relationship between SUMOylation and aging will bring forth new perspectives to promote skeletal health.

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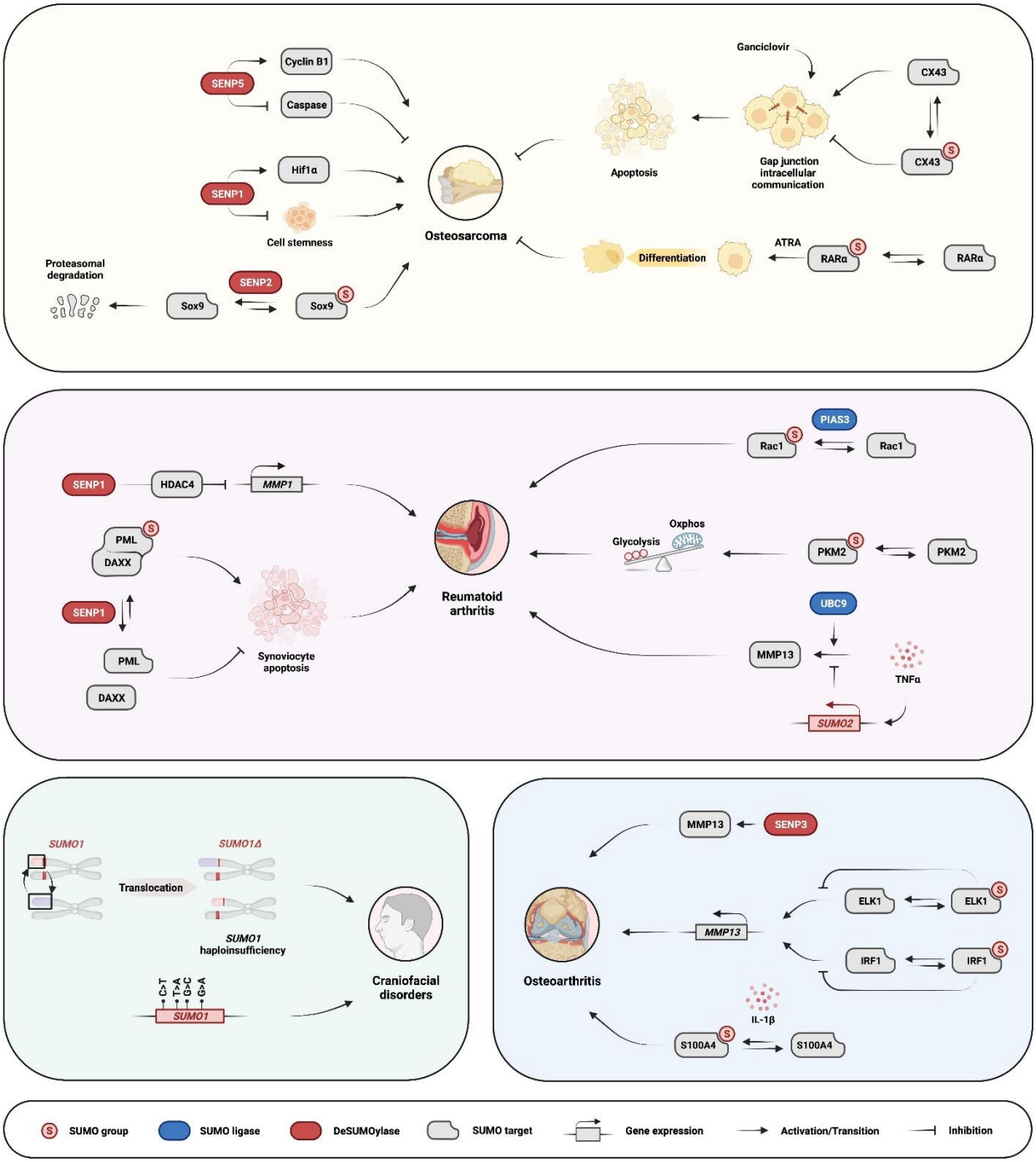

Fig. 3 Exemplary sumoylation pathways in skeletal diseases.

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