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Emerging Roles of SUMO Modification in Arthritis

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Abstract

Dynamic modification involving small ubiquitin-like modifier (SUMO) has emerged as a new mechanism of protein regulation in mammalian biology. Sumoylation is an ATP-dependent, reversible post-translational modification which occurs under both basal and stressful cellular conditions. Sumoylation profoundly influences protein functions and pertinent biological processes. For example, sumoylation modulates multiple components in the NFκB pathway and exerts an anti-inflammatory effect. Likewise, sumoylation of peroxisome proliferator-activated receptor γ (PPARγ) augments its anti-inflammatory activity. Current evidence suggests a role of sumoylation for resistance to apoptosis in synovial fibroblasts. Dynamic SUMO regulation controls the biological outcomes initiated by various growth factors involved in cartilage homeostasis, including basic fibroblast growth factors (bFGF or FGF-2), transforming growth factor-β (TGF-β) and insulin-like growth factor-1 (IGF-1). The impact of these growth factors on cartilage are through sumoylation-dependent control of the transcription factors (e.g., Smad, Elk-1, HIF-1) that are key regulators of matrix components (e.g., aggrecan, collagen) or cartilage-degrading enzymes (e.g., MMPs, aggrecanases). Thus, SUMO modification appears to profoundly affect chondrocyte and synovial fibroblast biology, including cell survival, inflammatory responses, matrix metabolism and hypoxic responses. More recently, evidence suggests that, in addition to their nuclear roles, the SUMO pathways play crucial roles in mitochondrial activity, cellular senescence, and autophagy. With an increasing number of reports linking SUMO to human diseases like arthritis, it is probable that novel and equally important functions of the sumoylation pathway will be elucidated in the near future.

1. INTRODUCTION

Arthritis encompass over 100 different forms of arthritis, among which osteoarthritis (OA) and rheumatoid arthritis (RA) are the most common forms and well studied. OA is

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characterized by progressive loss of cartilage, osteophyte formation, and frequently chronic synovial inflammation, which compromise joint function and often cause pain. Despite poor understanding on its etiology, numerous studies have identified a variety of risk factors contributing to the onset and progress of OA, including genetic predisposition, aging, joint malalignment, trauma and obesity. In osteoarthritic state, chondrocytes shift their metabolism to mostly catabolism, and are partially functionally impaired in response to anabolic stimuli, resulting in unsuccessful tissue repair. Well-established players participating in cartilage degeneration and inflammation include matrix metalloproteinases (MMPs), pro-inflammatory cytokines (e.g., IL-1, TNF- α), and reactive oxygen species (ROS). A series of anabolic factors in cartilage biology has also been identified, notably including TGF- β , bone morphogenetic protein (BMP), and IGF-1. These growth factors have been demonstrated to facilitate cartilage healing and repair in various *in vivo* studies and clinical scenarios. RA is a systemic inflammatory disease leading to joint destruction and frequently functional impairment. In contrast with OA, synovial inflammation is the hallmark of RA, which often drives cartilage degradation. Synovial hyperplasia, increased vascularity and inflammatory cell infiltration constitute prominent features of RA. Incoming cells and resident synovial fibroblasts and macrophages produce destructive cytokines, such as IL-1, TNF- α , and IL-6, in a paracrine or autocrine manner. Secreted cytokines then stimulate synovial fibroblasts and chondrocytes to unduly express degrading proteases and to offset anabolic endeavors by chondrocytes, which exacerbate the pathological condition. Current therapies against RA consist of drugs targeting the inflammatory pathway and destructive proteases, such as non-steroidal anti-inflammatory drugs, MMP inhibitors, TNF antagonists, and IL-1 inhibitors.

Post-translational modification is a collective term for biochemical reactions on a protein after its translation. Moieties differing in chemical nature can be added to protein in an enzymatic or non-enzymatic fashion to affect its property and function. In general, post-translational modification can regulate any aspect of a protein, such as stability, conformation, activity, localization, and protein-protein interaction. Like other post-translational modifications, such as acetylation, phosphorylation and ubiquitination, sumoylation occurs within the cell in response to intracellular and extracellular stimuli in a fast and very efficient way, and is utilized as a molecular mechanism to modulate cell signaling cascades. Although its functions are as diverse as its substrates, one generalization is that modification of substrate by SUMO often alters its interactions with other proteins and DNA molecules. At any given time, only a very small proportion of a substrate is modified, usually less than 1 %, which may account for the technical difficulty in detecting SUMO-modified forms of substrate proteins. Dynamic SUMO modification has emerged as a new mechanism of protein regulation in mammalian cell biology. Indeed, a great many proteins are modified by SUMO species and a number of pathways are altered through sumoylation.

In this review, we summarized recent discoveries and our current knowledge on SUMO modification, in order to establish its potential biological connection with human arthritis. We particularly focused on the hypothetical role of SUMO in cellular processes implicated in the pathogenesis of OA and RA. Since the functional impacts of sumoylation have mostly been demonstrated in cell lines or cells types not present in the joint, we have extrapolated these findings to conjecture possible roles of SUMO modification in primary chondrocytes and synovial fibroblasts.

2. OVERVIEW OF SUMO MODIFICATION

2.1 THE MAMMALIAN SUMO MACHINERY

SUMO represents a category of protein moieties of approximately 12 kDa in size which is conjugated to certain proteins in a manner analogous to ubiquitination. Mammalian cells primarily express three SUMO isoforms, designated SUMO-1, -2, and -3. SUMO-2 and -3 belong to the same subfamily and share 50% of primary structure with SUMO-1. SUMO-1 is actively involved in dynamic conjugation processes, whereas SUMO-2 and -3 exist in free form abundantly, and are utilized during cellular stress. In addition, despite the conjecture that SUMO-4 is a pseudogene, SUMO-4 has been shown to be expressed in kidney cells and immune tissues with undefined functions.

SUMO-1, -2 and -3 are expressed as precursors and must be proteolytically processed to become active. Cleavage of the precursors reveals the conserved double-glycine motif within the C-terminal region. To date, three members of the sentrin-specific protease (SEN) family are known to participate in SUMO maturation in human cells. SENP1 possesses stronger proteolytic activity for SUMO-1 than for SUMO-2. The catalytic domain of SENP1 is sufficient to determine the substrate specificity towards SUMO-1, -2 and -3. In contrast, SENP2 processes SUMO-2 with higher efficiency than SUMO-1. Both SENP1 and SENP2 have weak proteolytic activity for SUMO-3, whereas SENP5 specifically catalyzes SUMO-3 maturation.

Sumoylation takes place *via* a series of enzymatic steps. In human cells, SAE1/SAE2, a heterodimeric SUMO-activating enzyme (E1), first catalyzes the formation of an intermediate in which the C-terminal carboxyl group of SUMO is covalently linked to the sulphhydryl group of a cysteine residue in SAE2 in an ATP-dependent fashion. In the second step, SUMO is transferred to a catalytic cysteine residue (C93) of Ubc9, the only SUMO-conjugating enzyme (E2) utilized in humans. Ubc9 is capable of recognizing substrate proteins directly and transferring SUMO to the ϵ -amino group of the lysine residue within a SUMO conjugation motif. A well-characterized core consensus motif subjected to sumoylation is ψ KxE/D (ψ stands for a hydrophobic amino acid residue; x stands for any amino acid residue). However, wider consensus sequences have been identified including the phosphorylation-dependent sumoylation motif (PDSM) and the negatively-charged amino acid-dependent sumoylation motif (NDSM). Both of these motifs have residues located downstream from the core recognition motif which are negatively charged in either a constitutive manner (ie Asp and Glu residues) or in an inducible manner (ie phosphorylation of Ser or Thr residues). Notably, not all proteins possessing such a consensus sequence are subject to sumoylation, suggesting other factors, such as the steric environment surrounding the motif and sub-cellular localization, may also play a role. Conversely, many proteins are modified by SUMO on sites which do not contain the core consensus motif. The mammalian SUMO machinery also utilizes a group of enzymes termed SUMO ligases (E3) to enhance the efficiency of sumoylation. To date members of PIAS (Protein Inhibitors of Activated STAT) family, RanBP2 (Ran-binding protein 2), HDAC4 (Histone Deacetylase 4) and Pc2 (Polycomb2) have been identified as E3 ligases in human cells, with PIAS family being the largest subgroup. The SP-RING (Siz/PIAS-really interesting new gene) domain within PIAS proteins has been demonstrated to play a critical role in enhancing protein sumoylation. SUMO E3 ligases provide loose specificity for substrate proteins, because some E3 ligases seem to promote sumoylation of multiple unrelated proteins both *in vitro* and *in vivo*.

An important facet of the dynamics of SUMO modification is desumoylation mediated by SENPs. In addition to their role in SUMO maturation, SENPs also cleave the isopeptide bonds between SUMO and its target proteins. Interestingly, SENP family members display distinctive patterns of cellular localization. SENP1 is localized in the nucleoplasm but not in

the nucleolus ; SENP2 is primarily associated with the nuclear envelope and undefined nuclear speckles ; both SENP3 and SENP5 have been shown to localize to the nucleolus ; despite a discrepancy in literature, SENP6 seem to localize in the nucleoplasm ; SENP7 was shown to localize to the nucleoplasm, but its catalytic activity remains to be ascertained. The nuclear import/export signals within each SENP suggest that localization of SENPs can be regulated by extrinsic factors. Indeed subnuclear shuttling of SENP3 from the nucleolus to the nucleoplasm has been shown to occur in the presence of mild oxidative stress (Huang et al., 2009). With regard to specificity, SENP1 and SENP2 preferentially target SUMO-1, -2 and -3 conjugates, while SENP3, SENP5 and SENP6 possess enzymatic activities for SUMO-2 and -3.

More recently, accumulating evidence unveiled the transcriptional and post-translational regulation of SENPs. The expression level of SENPs is controlled by environmental factors. In the case of *SENP1*, its transcription is upregulated by androgen and interleukin-6, and its ubiquitin-mediated degradation is partially controlled by hypoxia. The binding of SENPs is also regulated by various stimuli. The formation of an intramolecular disulfide bond between Cys603 and Cys613 of SENP1 following H₂O₂ treatment renders SENP1 inactive, but also protects it against further irreversible oxidative damage.

2.2 FUNCTIONAL SIGNIFICANCE OF SUMOYLATION

The biological consequences of sumoylation of any newly identified target are difficult to predict. Sumoylation can influence any aspect of a target protein, including localization, activity and stability; it also participates in organization of chromatin structure, transcriptional regulation, and shuttling *via* the nuclear pore complex. Sumoylation frequently confers novel interaction properties on the target protein. It has been documented that SUMO itself can participate in protein-protein interaction. A short hydrophobic motif, named SIM/SBM, has been identified as a SUMO-interacting module in a few proteins, including PML and Daxx. It should also be noted that, in spite of the enormous body of literature focusing on the roles of nuclear SUMO, sumoylated proteins actually have been discovered in all sub-cellular compartments. Intriguingly, for most target proteins identified to date, the sumoylated form only occupies a small fraction of the whole pool at steady state. Nevertheless, such low-level sumoylation often elicits dramatic effects. A plausible explanation for this paradox comes from the highly dynamic nature of SUMO modification. Certain biological processes may only require the presence of sumoylated proteins for a short period of time, so a small amount of sumoylated proteins would be sufficient to carry out the functions through rapid sumoylation and desumoylation. For example, studies have implicated HDACs and Daxx in gene repression mediated by SUMO. It is possible that, after recruiting such chromatin-remodeling factors with repressive activities, target genes will remain silenced even in the absence of sumoylation on interacting partners, which allows the recycling of sumoylated proteins.

Transcription factors take up a considerable proportion of currently known SUMO substrates. Sumoylation of a given transcription factor mostly leads to transrepression, but transcriptional activation after SUMO modification has also been observed in a several substrates. The current paradigm suggests that sumoylation regulates the activities of transcription factors *via* two main types of mechanism. First, sumoylation of a transcription factor could influence its binding affinity to specific binding elements within promoters, which causes changes in either transactivation or transrepression. Second, sumoylation could change the repertoire of interacting proteins of a transcription factor substrate, leading to recruitment of differential cofactors, which would impact differently upon target genes. Additionally, several enzymes participating in epigenetic events are also subjected to sumoylation, which could work in concert with modified transcription factors to regulate

gene expression. Reviews dedicated to SUMO regulation of transcriptional activity can be found elsewhere.

2.3 SUMO PATHWAYS IN RA AND OA

Biological pathways which control the functions of chondrocytes and synovial fibroblasts are interrelated, and deregulation of one or several pathways often leads to pathogenesis in the joint, most notably, RA and OA (Figure 1). The initial observation that *SUMO-1* is preferentially expressed in the RA synovium, rather than the normal or OA-derived synovium, is fairly intriguing. *SUMO-1* is overexpressed in both outer and inner lining cells of the RA synovium, particularly at points of synovial invasion. Franz and colleagues, utilizing co-immunolocalization of cell specific markers, demonstrated that *SUMO-1* is specifically expressed in synovial fibroblasts rather than monocytes, macrophages, or endothelial cells in the RA synovium. High levels of SUMO-1 persist in RA synovial fibroblasts for as long as 60 days in a SCID mouse model, despite the absence of inflammatory stimuli. By contrast, SENP1 levels decreases in RA synovial fibroblasts. Thus, though inflammation might be required for the onset of *SUMO-1* expression in synovial fibroblasts, it is not required for maintaining this expression.

Perturbed chondrocyte homeostasis and enhanced release of destructive proteases contribute to the destruction of extra-cellular matrix in OA. OA is characterized by synovial inflammation, cartilage degeneration, osteophyte formation, and impairment of sub-chondral bone. In a recent functional genomic screen for OA-specific pathogenic molecules in chondrocytes, *SENP3* is one of the 12,000 annotated clones selected from 50,000 clones in an OA chondrocyte-derived cDNA library. *SENP3* was subsequently cloned into a retroviral vector and transfected into primary chondrocytes. Overexpressed *SENP3* results in the transcription of *iNOS* (inducible nitric oxide synthase), *COX2* (cyclooxygenase-2), *ADAMTS-4* and *MMP13*, which increase by more than four fold compared to basal levels. This study provides a preliminary indication for a role of the SUMO pathway in suppressing gene expression in OA. Interestingly the same study identified bFGF as well as fibroblast growth factor receptor 1 (FGFR1) as mediators of OA, which will be discussed below.

3. REGULATION OF INFLAMMATION BY SUMOYLATION

3.1 SUMO REGULATION OF NFκB PATHWAY

Deregulation of the NFκB pathway substantially contributes to RA and OA pathogenesis. In the RA joint, NFκB-driven increases in the expression of proinflammatory mediators, adhesion molecules, as well as matrix degrading enzymes, integrate synovial hyperplasia with cartilage matrix degeneration. NFκB is highly activated within RA synovial fibroblasts, and its activation in RA appears to be stronger than that in OA. In both synovial fibroblasts and articular chondrocytes, NFκB is involved in the induction of *MMP-13*, a major player in cartilage degradation. *MMP-13* transcription is regulated by NFκB in response to several stimuli, including bFGF, IL-1, fibronectin fragments, hyaluronan (HA) oligosaccharides, retinoid acid : retinoid receptor (RA:RXR) heterodimer, as well as innate immune responses in the OA joint. Results from studies using animal models of inflammatory arthritis further corroborate the active role of NFκB in the onset and progression of arthritis. In a murine collagen-induced arthritis (CIA) model, NFκB expression correlates with *MMP-3* and *MMP-13* expression levels, and NFκB activation was observed before clinical manifestation of arthritis. Ectopic expression of *IKKβ* enhances NFκB binding to DNA and causes marked arthritis in rats. Therefore, NFκB plays a consequential role in the inflammatory pathways in RA and OA, which entails joint destruction. It is now known that the NFκB signaling is in part regulated by SUMO modification of its components, which is illustrated in Figure 2 with simplification for the sake of clarity.

One emerging regulatory mechanism of NF κ B pathway is the preferential sumoylation of the inhibitory I κ B α on Lys21, and to a lesser extent, Lys22, in a serine (Ser32, Ser36) phosphorylation-independent manner. Because they are also sites for ubiquitination, these two post-translational modifications on Lys21 and Lys22 are competitive. Furthermore, the N terminus of I κ B α interacts with SUMO-specific conjugating enzyme, Ubc9. Thus, sumoylation competes off the phosphorylation-dependent, ubiquitination-mediated proteosomal degradation, thereby stabilizing I κ B α and repressing NF κ B-mediated transcription. The same research group subsequently demonstrated that TNF- α , IL-1, as well as okadaic acid enhance NF κ B-responsive reporter activity, which is significantly inhibited by *SUMO-1* and/or *Ubc9* overexpression *in vitro*. In accordance, gene profiling studies have indicated an important repressive role for PIAS family members with E3 SUMO ligase activity (PIAS1 and PIASy) in NF κ B-mediated transcription. More detailed reviews on the sumoylation in NF κ B signaling are currently available elsewhere.

A recently identified hypoxia inducible component of the SUMO pathway, termed RWD-containing sumoylation enhancer (RSUME), was found to increase noncovalent binding of SUMO-1 to Ubc9 and enhance Ubc9 thioester formation. More importantly, RSUME causes increased I κ B α sumoylation *in vitro* and *in vivo*. RSUME-mediated I κ B α sumoylation has a repressive effect on NF κ B-mediated transcription of *IL-8* and *COX2*. Interestingly, we found *RSUME* is expressed in normal knee chondrocytes with undefined functions (Im et al 2009, unpublished finding).

Among the upstream signal activators of NF κ B, the IL-1 receptor (IL-1R) is of utmost significance within arthritic joints. The Toll/IL1R (TIR) domain of IL-1R, which is responsible for receptor activation, is sumoylated at Lys504, Lys507, Lys509 and Lys527 by SUMO-1 conjugation. Sumoylation of IL-1R mediates IL-1R associated kinase (IRAK)-dependent downstream signal activation. Of the four IRAK isoforms and known splice variants, IRAK1 has been shown to be a SUMO substrate. Sumoylation appears to trigger IRAK1 nuclear translocation, and it is speculated that sumoylation may compete with ubiquitination for its functional regulation. Recruitment of an IRAK-Toll interacting protein (Tollip) occurs in response to IL-1R activation as well as toll-like receptor 2 (TLR2) and TLR4 activation. Tollip associates with Ubc9, PIAS E3 ligases, Daxx and SUMO-1. Furthermore, Tollip itself is sumoylated *in vitro*. In co-immunoprecipitation experiments, tagged Tollip was shown to bind both unmodified and sumoylated RanGap1. The implication for this association may extend to a role for Tollip in directional nuclear localization of its associated IL-1R signaling binding partners. Aside from sumoylation, increased levels of Tollip have been identified as a negative regulator of NF κ B signaling, suggesting its anti-inflammatory function. However, how sumoylation of Tollip modulates its biological function remains to be explored.

Given the prominent role of NF κ B pathway in RA and OA biology, it is worth investigating how sumoylation regulates the activity of NF κ B as well as other pathway components in chondrocytes and synoviocytes. Hypothetically, SUMO modification stabilizes I κ B α , which leads to repression of NF κ B-dependent genes. Yet sumoylation of other pathway components, such as IL-1R and IRAK1, might not cause signaling attenuation like I κ B α . It is possible that sumoylation exerts opposite effects upon different substrates in the NF κ B pathway, allowing for fine-tuning of signaling. A hypothetical model based on current knowledge is presented in Figure 2.

3.2 SUMO pathway in PPAR γ -mediated transcriptional regulation

Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear hormone receptors (NHRs) involved in energy metabolism, insulin sensitivity and adipogenesis. Three isoforms of PPARs, termed PPAR α , PPAR β/δ and PPAR γ , have been identified to

date. Upon ligand binding, PPAR heterodimerizes with retinoid X receptor (RXR) and subsequently binds to peroxisome proliferator response elements (PPREs) within enhancers of regulated genes. Activation of PPAR γ elicits anti-inflammatory responses in several cell types such as monocytes and macrophages. Furthermore, PPAR γ is present in major cell populations in joints, including chondrocytes, synovial fibroblasts and endothelial cells. The activation of PPAR γ results in transrepression of pro-inflammatory cytokines (*TNF- α* , *IL-1*), inflammatory mediators (*iNOS*, *COX-2*) and MMPs (*MMP-1*, *MMP-13*), which contributes to attenuation of catabolic activities within the joint. Such a potent anti-inflammatory property renders PPAR γ a promising therapeutic candidate for inflammatory joint diseases. PPAR γ agonists have shown encouraging effectiveness in a series of independent studies. For instance, in RA synovial fibroblasts, a synthetic agonist troglitazone inhibits production of *TNF- α* , *IL-6*, *IL-8* and *MMP-3* without causing apoptosis. In adjuvant-induced murine arthritis, administration of rosiglitazone or pioglitazone significantly inhibits expression of *iNOS* and formation of nitrotyrosine. In RAW 264 cells, a murine macrophage cell line, rosiglitazone and pioglitazone inhibit lipopolysaccharide and *TNF- α* -induced expression of *iNOS*, *COX-2*, *ICAM-1* and nitrotyrosine formation. In murine CIA model, administration of agonist CLX-090717 reduces the proliferation of arthritic lymphocytes and *TNF- α* release.

Sumoylation of PPAR γ mainly occurs at Lys107 within its activation function 1 (AF1) domain. PIAS1 and PIASx β function as E3 ligases for PPAR γ , and conjugation of SUMO-1 to PPAR γ represses its transcriptional activity. Phosphorylation at Ser112 facilitates the sumoylation of PPAR γ 2 at Lys107. Evidence also suggests that sumoylation of PPAR γ at multiple sites, including Lys107, increases its stability. One mechanism through which sumoylation regulates PPAR γ -mediated transrepression was conspicuously demonstrated in the case of *iNOS* regulation, as demonstrated in Figure 3. In the basal state, the nuclear receptor co-repressor (NCoR)/histone deacetylase-3 (HDAC3) complex occupies the promoter of *iNOS* to repress its transcription. Upon stimulation, the PPAR γ ligand-binding domain becomes sumoylated, which allows PPAR γ to bind to the NCoR/HDAC3 complex which is already assembled on promoters. This binding prevents the recruitment of the ubiquitylation/19S proteasome machinery which usually mediates the clearance of the repressive complex from the promoter region. Consequently, *iNOS* is maintained in a repressed state. Similarly, after stimulation with apoptotic cells, PPAR γ sumoylation prevented LPS-induced clearance of NCoR from the NF κ B site within *TNF- α* promoter in murine macrophages. The binding partner of PPAR γ , RXR α , is also modified by SUMO-1 *in vitro* and *in vivo*. Sumoylation of Lys108 leads to a decrease in transcriptional activity of RXR α , as well as the RXR α /PPAR γ heterodimer. In a chondrosarcoma cell line, a synthetic RXR ligand induces sumoylation of RXR, which may serve as a mechanism for the inhibition of IL-1 β -driven *MMP-1* and *MMP-13* expression. Recently, PGC-1 α , a co-activator for PPAR γ , was demonstrated to be sumoylated at Lys183 by SUMO-1. Sumoylation appears to decrease the transcriptional activity of PGC-1 α through enhancing its interaction with a co-repressor RIP140. In the context of PPAR γ -dependent transcription, abolishing sumoylation of PGC-1 α by mutation augments its activity. So far evidence suggests that sumoylation positively regulates the transrepression of PPAR γ -dependent genes *via* directly sumoylating PPAR γ and modifying its interacting partners, which is likely to be the case in synoviocytes and chondrocytes (Figure 3). PPAR γ -regulated *iNOS* expression may play a critical role in arthritis pathogenesis, considering the established detrimental role of deregulated *iNOS* activity in RA and OA. Thus, the aforementioned accumulating evidence strongly implies a significant role of SUMO-dependent regulation of the PPAR γ pathway in joint homeostasis, and studies specifically focusing on its role in cell populations within the joint are warranted.

4. REGULATION OF APOPTOSIS BY THE SUMO PATHWAY

4.1 SUMO MODIFICATION IN SYNOVIAL FIBROBLAST APOPTOSIS

It has been reported that Fas-mediated apoptosis is preferentially observed in RA rather than OA synovial fibroblasts. Apoptosis may serve as a mechanism for the clearance of proliferative synovial fibroblasts which express Fas antigen, thus promoting tissue homeostasis. However, in RA, synovial fibroblasts often evade apoptosis, which leads to synovial hyperplasia, pannus formation and ensuing cartilage degeneration. Death receptors on the RA synovial fibroblasts include Fas/CD95, TRAIL-R1 and R2, and TNFR1. Their shared death domain (DD) is known to interact with several adaptors and signal-responsive kinases and phosphatases, which further activate the apoptosome in the extrinsic pathway. SUMO-1 was first identified as a novel apoptosis-inhibiting molecule, deriving its name 'sentrin', from 'sentry' that interacts with the cytoplasmic domain of Fas receptor and TNFR1 DD. The function of SUMO-1 appears to be protective against Fas-/TNFR1-induced apoptosis within RA synovial fibroblasts. Meinecke and colleagues demonstrated a role for SUMO-1 in mediating the resistance to Fas-induced apoptosis and conversely, a role for desumoylation by SENP-1 in increasing FasL-mediated apoptosis within the RA synovial fibroblasts. Their model implies an enhanced sumoylation of nuclear promyelocytic leukemia (PML) protein results in the 'trapping' of Daxx, a DD-interacting protein, in PML bodies, thus inhibiting receptor-mediated apoptosis. This model is further validated functionally by a gene silencing experiment in which knockdown of *SUMO-1* had no effect on basal apoptosis, but increased FasL-mediated apoptosis. It is well known that SUMO-1 is covalently attached to a subpopulation of PML in a reversible and phosphorylation-dependent manner. The unmodified form of PML is found in the soluble nucleoplasmic fraction, whereas the SUMO-1-modified form is compartmentalized exclusively in punctuate PML bodies, which are thought to act as storehouses for sumoylated proteins within the nucleus. Similar evidence of a role for sumoylation in RA synovial cells comes from another study on 'prosthesis loosened' synovial fibroblasts, in which *SUMO-1* is overexpressed and similarly confers resistance to Fas-induced apoptosis.

Recently bFGF was shown to decrease the sensitivity of RA synovial fibroblasts to Fas-mediated apoptosis. Fas ligation with its agonistic monoclonal antibody induces pronounced cytotoxicity in TNF- α -treated, but not bFGF-treated, RA synovial fibroblasts. This is particularly relevant since a high level of bFGF has been observed in the hyperplastic RA synovium, as well as at the cartilage-pannus interface. The expression pattern is indicative of its role in mediating pannus formation and synovial invasion within the epiphysis. The apoptotic mechanism appears to rely on the activation of caspase 8 (FLICE). FLICE inhibitory protein (FLIP) interferes with the interaction between FADD and FLICE, thus attenuating the apoptotic signaling. Interestingly, caspase 8 can be sumoylated and this modification does not interfere with its activation. Sumoylation also causes caspase 8 to localize in the nucleus, thereby possibly leading to cleavage of its nuclear substrates. Whether caspase 8 sumoylation affects its interaction with FADD or FLIP is worth examining, and future findings will shed more light on bFGF-mediated apoptotic resistance in RA synovial fibroblasts.

Intriguingly, PPAR γ activation has been associated with synovial fibroblast apoptosis. In RA synovial fibroblasts, three PPAR γ agonists, thiazolidinedione, troglitazone and 15d-PGJ₂, induce apoptosis *in vitro*. Intraperitoneal administration of troglitazone and 15d-PGJ₂ suppresses pannus formation and mononuclear cell infiltration in a rat adjuvant-induced arthritis model. In sharp contrast with RA synovial fibroblasts, 15d-PGJ₂ exerts a strong anti-apoptotic effect on OA synovial fibroblasts, suggesting the role of PPAR γ activation in synovial fibroblast apoptosis may be disease-dependent. However, although SUMO-

dependent regulation of PPAR γ in synovial fibroblast apoptosis has not been investigated, such a role appears likely due to the profound effects sumoylation has on PPAR γ activity.

4.2 SUMO MODIFICATION IN CHONDROCYTE APOPTOSIS

Studies have shown the presence of cell death in normal and diseased cartilage, and cell death appears to be more extensive among OA and RA chondrocytes. Although frequencies vary dramatically across previous studies, chondrocyte death is reckoned as one contributing factor of arthritis pathogenesis. Interestingly, chondrocyte death does not bear all classical features of apoptosis; rather, chondrocytes seem to undergo a variant program termed chondroptosis *in vivo*. Since cartilage does not possess vascularity or resident phagocytes, remnants of chondroptosis stay within the lacunae due to lack of clearance. Structural evidence suggests that membranous vesicles form following chondrocyte disintegration. Contents released from these vesicles may facilitate cartilage degradation and matrix mineralization.

A series of regulators has been implicated into chondrocyte death, including extracellular matrix components, p53 and death receptors. Among matrix components, type II collagen is critical for chondrocyte survival *in vivo*. In chondrocytes, the $\alpha 1\beta 1$ integrin is the major collagen receptor. Accordingly, inactivation of the *ITGA1* leads to increased apoptotic frequency in murine cartilage. In chicken chondrocytes, disruption of integrin-mediated matrix binding impairs mitochondrial oxidative phosphorylation. Aside from type II collagen, hyaluronan exerts an anti-apoptotic effect on FAS-induced chondrocyte apoptosis by binding to its specific receptors including CD44. In RA and OA cartilage lesions, p53 expression correlates with the number of apoptotic cells. Hydrostatic pressure and shear strain both induce chondrocyte apoptosis and the mechanism seems to be p53-dependent. Ectopic expression of wild-type p53 in chondrocytes further enhances nitric oxide (NO)-induced apoptosis, while expression of a dominant negative form blocks it, suggesting p53 plays an essential role in NO-induced apoptosis. In addition, death receptor Fas is expressed in both normal and OA chondrocytes. However, unlike RA synovial fibroblasts, Fas activation only induces limited cell death in human chondrocytes. Although the above-mentioned experimental conditions may not accurately mimic those promoting chondroptosis *in vivo*, these studies still provide us with valuable data concerning important regulators.

In order to transmit integrin-induced intracellular signal, focal adhesion kinase (FAK) needs to localize to sites of integrin receptor clustering, which is mediated by its C-terminal focal adhesion targeting (FAT) domain. The N-terminal domain of FAK can interact with $\beta 1$, $\beta 3$ and $\beta 5$ integrin, but the roles of these interactions have not been well established yet. A crucial step during FAK activation is autophosphorylation on Tyr397, which creates a high affinity docking site for downstream molecules. The N-terminal domain of FAK interacts with PIAS1, resulting in its sumoylation at Lys152. Sumoylation of FAK appears to occur predominantly in the nucleus, suggesting the involvement of active molecular trafficking in this regulatory process. Sumoylation remarkably elevates the efficiency of FAK autophosphorylation *in vitro* and *in vivo*. However, sumoylation does not depend on the catalytic activity of FAK. Whether sumoylation of FAK promotes its activation and thus integrin-dependent chondrocyte survival is worth studying.

Sumoylation of p53 primarily occurs at Lys386, and a secondary site appears to play a minor role. In the case of p53, current evidence suggests that SUMO-1 and ubiquitin do not compete for the same Lys residue. All PIAS isoforms interact with p53 *in vitro*, and their forced expression enhances p53 sumoylation. However, whether all isoforms are involved in SUMO modification in physiological conditions remains to be determined. Neither is it clear which member(s) of the SENP family acts as a deconjugase of p53 at physiological levels,

though ectopic expression of SENP1 reduces p53 sumoylation. The impact of sumoylation on p53 transcriptional activity remains debatable as the case stands. One line of evidence indicates that sumoylation contributes to p53-mediated transactivation. Co-expression of *Ubc9* and *SUMO-1* with *p53* increases p53-mediated activation of a *p21*-luciferase reporter or reporter harboring canonical *p53* elements. Mutation of the SUMO acceptor site compromises p53-induced pro-apoptotic effects. Moreover, PIASy-mediated p53 sumoylation causes activation of p53 target genes in human fibroblasts. Nevertheless, another line of evidence suggests an opposite role of sumoylation. In a lung carcinoma cell line, PIASy inhibits DNA-binding of p53 and induction of target genes including *p21*. A supporting piece of evidence comes from the finding that PIASy-mediated sumoylation of p53 facilitates its nuclear export. Closer examination is required to resolve these contradictions. Different experimental conditions could give rise to discrepancies across studies, and overexpression as utilized in some of those studies could enhance sumoylation of interacting regulators of p53 and bring interfering factors into data interpretation. In addition, PIAS1 and PIASx β promote p53 sumoylation and thus repress its transcriptional activity. Yet in another study PIAS1 activates p53 target gene expression in a sumoylation-independent manner. This inconsistency again suggests that the role of p53 sumoylation may be contextual. PIAS expression exhibits a tissue-specific pattern, as previously demonstrated. Therefore, identification of isoforms present in chondrocytes should precede in-depth analyses of their functions.

5. SUMOYLATION IN MATRIX METABOLISM

5.1 SUMO REGULATION OF bFGF SIGNALING IN CHONDROCYTES

Basic FGF (bFGF) is a key growth factor in joints, where its effects depend on cellular context, developmental stages, and differential *FGFR* expression. bFGF is synthesized by chondrocytes, stored in cartilage extracellular matrix, and released upon injury or excessive load. bFGF significantly increases chondrocyte proliferation, but the proliferative chondrocytes form clusters with very little pericellular matrix, primarily due to decrease in PG synthesis. bFGF counteracts the PG deposition mediated by bone morphogenetic protein 7 (BMP-7) and insulin growth factor 1 (IGF-1). bFGF also upregulates *IL-1 β* , thereby upregulating *MMP-13* in chondrocytes *via* autocrine and/or paracrine mechanisms. It has been shown that bFGF and *IL-1 β* synergistically enhance *MMP-13* expression in human chondrocytes. Several studies have delineated bFGF signaling within chondrocytes. bFGF preferentially binds to and activates FGFR1 in human articular chondrocytes. As a result, the ERK1/2 MAPK, p38 MAPK and JNK pathways all become activated. Other upstream activators of the MAPK pathways such as purine rich tyrosine kinase (PYK), focal adhesion kinase (FAK), and PKC δ are also involved in the bFGF-FGFR1 signaling axis in human chondrocytes. Among all the MAP kinases, ERK1/2 phosphorylation is the most robust response and remains detectable at least 24 hours after bFGF stimulation. One of the downstream events of ERK1/2 phosphorylation is the activation of ETS-like transcription factor-1 (Elk-1). Because many inflammatory signaling pathways converge on MAPK and NF κ B activation, and the MAPK pathways have Elk-1 as a common target, Elk-1 may play a central role in chondrocyte homeostasis. Im and colleagues have demonstrated that Elk-1 activation through phosphorylation at Ser383 occurs after bFGF-mediated ERK1/2 activation and is responsible for *MMP-13* induction in human chondrocytes.

Elk-1 is a member of the ternary complex factor (TCF) subfamily and contains two domains critical to its activity. The C-terminal transcriptional activation domain (TAD) contains several phosphorylation sites, including Ser383, which are targeted by MAP kinases. Independent studies have demonstrated the role of Ser383 phosphorylation in Elk-1 activation through this domain. Another domain, named R motif, plays a role in transcriptional repression. This motif maintains a low basal activation status of Elk-1 in

order to prevent unnecessary gene induction, because in the absence of the R motif, the Elk-1 activation domain exhibits a basal activity comparable to the activity of intact Elk-1 upon MAP kinase-mediated phosphorylation. The R motif was discovered to possess two sumoylation sites, Lys230 and Lys249. Sumoylation of this motif is essential to Elk-1 repression, as mutation of the acceptor lysines leads to elevated Elk-1 activity. Interestingly, the transcriptional activity of Elk-1 is reciprocally regulated by phosphorylation and sumoylation. ERK activation leads to loss of sumoylation concomitant with acquiring phosphorylation of Elk-1, thereby conferring a dynamic nature upon Elk-1 regulation. This trade-off mechanism also governs bFGF-mediated *MMP-13* induction in human articular chondrocytes (Figure 4). Phosphorylation of Elk-1 by ERK is accompanied by desumoylation of Elk-1. Phosphorylated Elk-1 transactivates *MMP-13*, while sumoylated Elk-1 exerts the opposite effect. This novel finding associates dynamic SUMO modification with chondrocyte gene regulation, and the role for sumoylation may extend to regulation of an array of biological processes in chondrocytes, which calls for further investigation. More in-depth dissection of the Elk-1/SUMO pathway revealed that sumoylation of the R motif promotes the recruitment of histone deacetylase (HDAC), primarily HDAC-2, and subsequent transrepression. Activation of the MAP kinase pathway results in HDAC release from the repressive complex. Unexpectedly, PIAS α functions as a signal integrator *via* differentially regulating Elk-1 in response to the upstream signaling inputs. In response to ERK activation, PIAS α facilitates the removal of HDAC-2 from sumoylated Elk-1 in an E3 ligase activity-independent manner. In response to p38 MAP kinase activation, however, PIAS α inhibits HDAC and SUMO loss. More recently, SENP1 was identified as the major deconjugase for Elk-1. Thus, the molecular details of the dynamic interplay between the MAP kinase pathway and the SUMO pathway on Elk-1 activity are becoming clearer. Based upon our current knowledge, we proposed a working hypothesis for SUMO regulation in bFGF-mediated *MMP-13* expression in chondrocytes (Figure 4). As different MAP kinases are activated in arthritic disease, it will be interesting to determine their actions with respect to the SUMO pathway activity towards relevant transcription factor substrates.

Activator protein-1 (AP-1), a downstream transcription factor in the MAP kinase pathway, plays an essential role in cartilage homeostasis. The activation of AP-1 initiates the expression of critical proteins, including collagenases and stromelysin. Moreover, selective inhibition of c-Fos/AP-1 resolves arthritis in a CIA model. Among all AP-1 components, c-Jun was first discovered as a sumoylation substrate. SUMO-1 targets Lys229 and Lys257 on c-Jun, and sumoylation appears not to interfere with its ubiquitination. Rather, JNK activation leads to decreases in sumoylation, and conversely, abrogating JNK phosphorylation sites enhances sumoylation. Importantly, in common with other transcription factors, sumoylation negatively regulates c-Jun activity. Downregulation of activity mediated by SUMO was also observed in c-Fos. The Lys265 of c-Fos can be conjugated to SUMO-1, -2 and -3. Sumoylated c-Fos displays an altered nuclear distribution, which may be linked to decreased transcriptional activity. Similar to c-Jun, phosphorylation of c-Fos at Thr232 antagonizes its sumoylation, but c-Fos sumoylation is not downregulated by ERK1/2 activation. In HEK293 cells, c-Fos is also the substrate of SUMO-4, and this modification reduces AP-1 activity at least under overexpression conditions. Another member JunB is known to transactivate various cytokine genes, including *IL-4* and *IL-10*. This is notable as one of the few examples for a potential role for SUMO-4. Interestingly, sumoylation of JunB appears not to modulate its intrinsic transactivation capability; rather, it positively regulates JunB-dependent cytokine transcription, including *IL-2* and *IL-4*, suggesting sumoylation of JunB changes its interactions with other transcription factors or cofactors. To date, the impact of AP-1 SUMO modification upon chondrocyte biology has remained unexamined. Given the central role of AP-1 in chondrocytes, future research in this area will likely produce significant insights.

5.2 SUMO MODIFICATION IN SMAD SIGNALING

Several members of the TGF- β superfamily have well established roles in cartilage development, anabolism and catabolism. Smad signaling pathway is pivotal to TGF- β /BMP-mediated biological effects, especially anabolic activities in chondrocytes. BMPs activate receptor-regulated Smad (R-Smad) 1, 5 and 8, whereas TGF- β signals *via* Smad2 and 3, and contextually, Smad1, 5 and 8. Activated R-Smad complexes with Smad4 (co-Smad) and translocalizes into the nucleus to regulate transcription. Several strategies are utilized by the cell to terminate Smad signaling, including Smurf-mediated proteosomal degradation of receptors, inhibition of receptor activity and R-Smad/co-Smad complex formation by inhibitory Smads (I-Smads), and dephosphorylation mediated by a group of phosphatases.

Smad4 was first identified as a substrate for sumoylation. Smad4 associates with SUMO-1 and Ubc9, and sumoylation occurs at Lys113 within the evolutionarily conserved N terminus, and Lys159 in the linker region. The consequence of Smad4 sumoylation, however, is not clear yet. Several groups have reported distinct effects of sumoylation on Smad4 activity. Enhanced transcriptional activity of sumoylated Smad4 is supported by several findings. First, sumoylation does not appear to interfere with the formation of Smad4-dependent transcription activating complex, but it significantly attenuates ubiquitin-mediated degradation of Smad4, suggesting its stimulatory role in TGF- β signaling. Additionally, SUMO-1 conjugation to Smad4 is strongly elevated by TGF- β -activated p38 MAP kinase pathway, *via* upregulation and stabilization of PIAS β , and by a yet unknown TGF- β -activated pathway, *via* the E3 ligase activity of PIAS1 for Smad4. In contrast, another group reported an inhibitory effect of *Ubc9* and/or *SUMO-1* overexpression on TGF- β -induced reporter activity. Subsequently, Daxx was found to interact with SUMO conjugated to Smad4, leading to transcriptional repression. Such dramatic discrepancies may be due to different cell lines and assays utilized in these studies, and should be interpreted with great caution. For example, the conclusion that sumoylation enhances Smad4 transcriptional activity was in part derived from overexpression of *Ubc9* and/or *SUMO-1*. The global overexpression of these proteins could lead to increased sumoylation of a plethora of targets, including Smad-associated coactivators and corepressors, which might interfere with the readouts of transcriptional activity. A large body of literature has demonstrated that the outcome of TGF- β signaling is highly contextual. Therefore, the specific role of sumoylation on Smad4 needs to be further explored.

To date, among all R-Smads, only Smad3 has been demonstrated to be sumoylated. Smad3 interacts with PIASy through its MH2 domain, and SUMO-1 conjugation to Smad3 requires the ligase activity of PIASy. Furthermore, *PIASy* expression is dramatically upregulated by TGF- β stimulation, and PIASy inhibits Smad3-dependent gene transcription providing a negative feedback loop. A subsequent study revealed that Smad3 sumoylation mediated by PIASy causes decreased DNA-binding affinity of Smad3, which is responsible for the suppression of TGF- β signaling. In addition, coexpression of *Smad3* with *PIASy* and *SUMO-1* stimulated the nuclear export of Smad3. Notably, in a comparative gene expression profiling study between synovial fluid mononuclear cells from patients with extended-to-be oligoarticular juvenile idiopathic arthritis (JIA) and those from patients with persistent condition, *Smad3* expression levels are significantly different. Moreover, another profiling study shows highly differential expression of *Smad3* between peripheral blood mononuclear cells from healthy individuals and those from patients with polyarticular JIA. These findings suggest that Smad3 may participate in the progression of JIA, but whether sumoylation plays a role in this process remains unknown.

More recently, a particularly interesting study revealed that type I transforming growth factor- β receptor (T β RI) is also a target of sumoylation. In response to TGF- β stimulation, T β RI becomes sumoylated primarily at Lys389, resulting in the facilitation of Smad3

recruitment and phosphorylation. The sumoylation of T β RI occurs on a Lys residue which is not located in the consensus ψ KxE/D motif, and requires the kinase activities of both T β RI and T β RII. The consequence of sumoylation on T β RI may also include other regulatory aspects of TGF- β signaling, such as facilitation of binding of Smad co-activators, regulation of non-Smad signaling pathways, and endocytosis and recycling of TGF- β receptor complexes.

5.3 A POSTULATED MECHANISM OF SUMO-DEPENDENT CROSS-TALK BETWEEN THE SMAD AND MAPK PATHWAYS

bFGF inhibits the anabolic activity of BMP-7 in human articular chondrocytes and in addition, bFGF counteracts at least some of TGF- β -mediated effects, such as upregulation of *COL2A1* and *DCN*. These observations suggest the existence of interactions between bFGF- and TGF- β /BMP-activated pathways. Indeed, the Smad signaling pathway is able to cross-talk with the MAP kinase pathway, as previously demonstrated in fibroblasts. In rat kidney fibroblasts, both the JNK and ERK pathways interfere with the Smad pathway, leading to differential outcomes. The JNK cascade appears to coordinate with Smad signaling to induce *Smad7* transcription in an AP-1-dependent manner. The ERK pathway, on the contrary, negatively regulates *Smad7* expression *via* inhibiting the translocation of Smad complex into the nucleus. Recently, bFGF was shown to mediate Smad1 linker phosphorylation within the PXSP consensus motif primarily through the ERK pathway in human articular chondrocytes. In addition, we have evidence showing that bFGF-mediated upregulation of *Smad6* and *Smad7* also requires ERK activation in human articular chondrocytes (Im et al, unpublished data). We also observed a decrease in the DNA binding of Smad complex after prolonged bFGF stimulation in chondrocytes, indicative of indirect mechanisms such as production of I-Smads or compromised Smad4 stability. Interestingly, OA chondrocytes express lower level of *Smad4* and exhibit higher basal activity of the bFGF-FGFR1-ERK-Elk-1 pathway compared to healthy chondrocytes, which appear to hinder matrix production mediated by TGF- β superfamily members (Im et al, unpublished findings). Based upon these observed phenomena, we hypothesize that upregulated bFGF expression in damaged cartilage activates the MAP kinase pathway to attenuate the Smad pathway. In this hypothetical model, bFGF through FGFR1 activates ERK, which in turn activates Elk-1 *via* reciprocal phosphorylation and desumoylation. Phosphorylated Elk-1 could then transactivate the *Smad6* and *Smad7*, which would subsequently inhibit Smad signaling. Alternatively, other downstream transcription factors in the ERK and SUMO pathway might be involved. Another aspect of this hypothesis is that the bFGF-activated MAP kinase pathway also downregulates Smad4 stability *via* desumoylation and hence causes degradation of Smad4 through a ubiquitin-dependent pathway. Additionally, bFGF stimulates the production of MMP-13 *via* Elk-1 activation, which further inhibits extracellular matrix accumulation. Thus, bFGF counteracts the effects mediated by TGF- β superfamily members on three levels—upregulating I-Smads, downregulating co-Smad and enhancing *MMP-13* expression. Together those could profoundly effect OA pathogenesis.

5.4 SUMO-DEPENDENT REGULATION OF IGF-1 SIGNALING

IGF-1 is one of the main anabolic factors in articular cartilage. IGF-1 increases the synthesis of collagen and PG. IGF-1 also slows PG degradation in a dose-dependent fashion. Although nonresponsiveness to IGF-1 in OA cartilage has been reported, application of IGF-1 in cartilage repair has achieved considerable success. For instance, in an equine model, local administration of IGF-1 through fibrin clots significantly improves the quantity and quality of tissue repair. Enhanced continuity and consistency of tissue repair was also acquired using cell-based repair techniques. Results from these studies underscore the importance of IGF-1-mediated effects in cartilage biology.

Insulin-like growth factor 1 receptor (IGF-1R) consists of two ligand-binding α subunits and two transmembrane β subunits. Upon ligand binding, dimeric β subunits transphosphorylate each other at tyrosine residues within their tyrosine kinase domains. Then the phosphorylated tyrosines serve as docking sites for other molecules to transmit the signal. IGF-1R has been shown to be sumoylated at three evolutionarily conserved residues – Lys1025, Lys1100, and Lys1120 – in the β subunit upon IGF-1 binding. None of these residues resides within the canonical sumoylation motif ψ Kx(D/E). Despite the absence of an obvious nuclear localization sequence, sumoylated IGF-1R actively translocalizes into the nucleus. After its nuclear import, IGF-1R is rapidly desumoylated and then directly or indirectly binds to enhancers, functioning as a transcriptional cofactor. It is known that IGF-1R is also subjected to ubiquitination after its autophosphorylation. Whether a competitive relationship exists between ubiquitination and sumoylation of IGF-1R remains not understood, but it is certainly worth investigating.

6. SUMO MODIFICATION IN THE HIF-1 PATHWAY

Normal articular cartilage is an avascular tissue. Thus, oxygen supply to resident articular chondrocytes is highly limited and largely depends on oxygen binding of the synovial fluid. It has been demonstrated that oxygen tension gradually decreases from the articular surface to the deep zone in healthy cartilage. To adapt to this chronic hypoxia, chondrocytes utilizes cell-specific mechanisms to promote tissue homeostasis, in which hypoxia-inducible factors (HIFs) play a pivotal role.

Human HIF family consists of six members, namely HIF-1 α/β , HIF-2 α/β and HIF-3 α/β . The stability of HIFs is regulated by hydroxylation of specific amino acid residues, mediated by several members of iron-/oxoglutarate-dependent hydroxylase family. In the presence of sufficient oxygen, hydroxylation leads to two possible outcomes. Hydroxylation of an Asn residue within the C-terminal domain of HIF-1 α prevents its interaction with coactivator p300, thus reducing the transactivation of target genes; proline hydroxylation leads to proteosomal degradation of HIF-1 α . Conversely, with limited oxygen supply, hydroxylation of HIF-1 α is inhibited, enabling it to heterodimerize with HIF-1 β . The complex then translocates into the nucleus, binds to hypoxia responsive elements, and transactivates a series of genes, such as *VEGF*, *iNOS* and *LEP*. In chondrocytes, HIF-1 α is indispensable in promoting cell survival. Inactivation of HIF-1 α results in massive cell death in murine growth plate and articular chondrocytes. Recent work further demonstrates HIF-1-mediated protection against induced apoptosis in chondrocytes. In the deep zone of intact cartilage, HIF-1 α is stored in the perinuclear region of chondrocytes, indicating these cells have a special phenotype for hypoxia adaptation. Aside from its role in healthy cartilage, HIF-1 also mediates cellular responses under osteoarthritic condition. Oxygen partial pressure in OA synovial fluid is even further decreased compared to normal conditions. Accordingly, both synovial fibroblasts and chondrocytes express high levels of *HIF-1* and associated target genes, including *iNOS* and *VEGF*. Pro-inflammatory cytokines and mechanical loading appear to activate *HIF-1* and HIF-1-dependent gene expression.

The precise role of HIF-1 in OA cartilage, however, is still debatable. Although it seems that HIF-1 mediates catabolic effects in response to low oxygen abundance and noxious stimuli, mounting evidence also reveals its contribution to tissue repair. In OA joints, reduced synovial oxygen levels accelerate the hydroxylation of type II collagen *via* an HIF-1 pathway, hence increasing the synthesis of type II collagen. The murine *Sox9* promoter contains a hypoxia responsive element (HRE). During hypoxia, recruitment of HIF-1 α to this site gives rise to *Sox9* induction. Then *Sox9* activates the transcription of genes encoding major matrix molecules, such as *ACAN* and *COL2A1*. Furthermore, differentiated fetal chondrocytes express higher levels of *HIF-1 α* compared to dedifferentiated cells. It is

worth studying whether HIF-1 α still conduces to phenotype maintenance in adult chondrocytes, because altered phenotypes are characteristic of OA chondrocytes and possibly account for the compromise of tissue integrity and responses to anabolic factors.

It has been demonstrated that HIF-1 α undergoes modification with SUMO-1, -2 and -3 in HeLa cells. Hypoxia upregulates the expression of SUMO-1, which participates in HIF-1 α sumoylation. One study showed that sumoylation negatively regulates the transcriptional activity of HIF-1 α , because SUMO-deficient HIF-1 α increases HRE-dependent transcription. This contradicts with an earlier report claiming that sumoylation increases HIF-1 α -mediated transcription in the same cell line. The earlier finding, however, appears questionable, in that global overexpression of *SUMO-1* was used to examine the impact of SUMO modification of HIF-1 α . Excessive abundance of SUMO-1 may elevate sumoylation of other potential substrates, thereby indirectly interfering with HIF-1 α -mediated effects. Yet another independent study demonstrated that RWD-containing sumoylation enhancer (RSUME) mediates the sumoylation of HIF-1 α , thus increasing its transactivation activity during hypoxia. Discrepancies also exist on the role of sumoylation in regulating HIF-1 α stability. One study showed that sumoylation of HIF-1 α promotes its degradation *via* a proteosomal pathway, suggesting cooperation between sumoylation and ubiquitination. In stark contrast, several other groups demonstrated that SUMO modification stabilizes HIF-1 α . Another group showed that HIF-1 α turnover rate is not affected by sumoylation *in vivo*. More detailed analyses are required to resolve these controversies. In addition, coactivators which cooperate with HIF-1 α , such as poly(ADP-ribose) polymerase 1 (PARP1) and p300, are also subjected to SUMO regulation. Modification of these cofactors will indirectly modulate HIF-1 α -dependent transcription.

Another member of the HIF family, HIF-2 α , appears to be essential to hypoxic induction in human articular chondrocytes. Hypoxia-induced matrix synthesis and *Sox9* expression can be abolished by HIF-2 α depletion. It was then hypothesized that HIF-2 α upregulates *Sox9*, which in turn transactivates key cartilage genes. Recently, HIF-2 α was demonstrated as a SUMO substrate. Sumoylation mainly occurs at Lys394 and reduces HIF-2 α transcriptional activity. SENP1 specifically mediates HIF-2 α desumoylation, which does not alter its subcellular localization. SUMO-targeted ubiquitin ligase RNF4 and VHL negatively regulate sumoylated HIF-2 α , suggesting a cooperative mechanism between the SUMO pathway and the ubiquitin-dependent proteosomal pathway, reminiscent of the similar cooperation between these two pathways observed in HIF-1 α .

7. SUMO REGULATION IN CHONDROCYTE SENESENCE

Chondrocytes are under the influence of aging. Compared to cartilage in young individuals, aged cartilage shows increased surface fibrillation, decreased aggrecan size, decreased proliferation, and decline in anabolic activities. Aging itself does not cause OA, but aging-related functional changes in chondrocytes are likely to render cartilage vulnerable to pathogenesis. Several cellular processes, such as oxidative damage and mitochondrial dysfunction, have been proposed as contributors to chondrocyte senescence. Mitochondrial respiratory activity declines with age, due to a progressive decrease in the number of mitochondria and disruptions of the electron transport chain. As a result, free radical production becomes elevated, which aggravates mitochondrial damage and leads to either senescence or apoptosis. In turn, ROS is able to induce senescence. Therefore, based upon the intertwined relationship between oxidative stress and mitochondrial impairment, it is likely that chondrocytes are subjected to replicative and stress-induced senescence.

NO has been established as one of the oxidative stress inducers in chondrocytes. NO is capable of inducing desumoylation globally. The underlying mechanism is that NO targets PIAS3 and causes S-nitrosation at Cys459, which eventually leads to PIAS3 degradation. Inducible nitric oxide synthase (iNOS) is a major intracellular source for NO production. C/EBP β , a SUMO-1 substrate, is required for maximal *iNOS* transcription. SUMO-1 modifies C/EBP β , reduces its activity, and thus reduces *iNOS* induction. Dynamin-related protein 1 (Drp-1), which is required for mitochondrial fission, is also a SUMO substrate. Drp-1 is modified by SUMO-1, -2 and -3. Mitochondrial-anchored protein ligase (MAPL) was identified as an enzyme for Drp-1 sumoylation, and its specificity may extend to a wide array of mitochondrial targets. Sumoylation possibly stabilizes Drp-1, but contradictory evidence also exists. In particular, SUMO-2 and -3 modification are significantly enhanced by oxidative stress, suggesting their role for adaptive and maladaptive mitochondrial responses. Sumoylation may interplay with Drp-1 GTPase activity, because a GTPase deficient Drp-1 mutant shows higher level of sumoylation. SENP5 catalyzes the removal of SUMO-1 from numerous mitochondrial substrates. Overexpression of *SENP5* reverses SUMO-1-induced mitochondrial fragmentation partially due to *Drp-1* downregulation. Hence, it appears that sumoylation leads to enhanced Drp-1-mediated mitochondrial fission, and proper level of sumoylation is needed for structural integrity of mitochondria. SENP5 also seems to dampen free radical production, but the underlying mechanism has yet to be explored. Collectively, this emerging evidence adds an additional layer of regulation in cellular stress response and mitochondrial function, both of which are associated with cell senescence. Taken together, despite the absence of direct evidence, SUMO modification probably participates in chondrocyte senescence *via* modulating oxidative stress responses and mitochondrial activities, and it provides a new direction for future investigations into cartilage aging.

8. SUMO MODIFICATION IN CHONDROCYTE AUTOPHAGY

Autophagy is a physiologic mechanism which targets dysfunctional macromolecules and organelles for lysosomal degradation and recycling. It appears that autophagy is a homeostatic mechanism in normal chondrocytes. The expression of several critical regulators in the autophagy pathway declines with age and in OA, compared to young and healthy chondrocytes, respectively. The proposed term chondroptosis also includes an autophagy component. In a rat OA model, the progression of cartilage degeneration in the superficial zone and partial middle zone is driven by chondrocyte autophagy in addition to cell death. As yet the mechanistic aspects of autophagy in OA pathogenesis have not been unveiled, but HIF appears to actively function in this process. When *HIF-1* expression is silenced in chondrocytes, *BECN1* and microtubule-associated protein 1 light chain 3 (*MAP1LC3*) expression are significantly suppressed. Because both Beclin 1 and LC3 are required to execute the autophagy program, this finding suggests that HIF-1 serves to promote autophagy. By contrast, HIF-2 seems to function as a negative regulator of the autophagic pathway. *HIF-2* silencing elicits a robust autophagic response in chondrocytes, and *HIF-1* expression is upregulated concomitantly. Accordingly, in *HIF-2 α ^{-/-}* mice, elevated autophagic activity was observed in growth plates. *HIF-2* expression seems to diminish in OA chondrocytes, implying deregulation of the autophagic pathway. As discussed above, both HIF-1 α and HIF-2 α are SUMO substrates. Therefore, SUMO regulation of HIF may influence not only the hypoxic response, but also autophagy in chondrocytes.

Autophagy also intertwines with cellular senescence and death in various contexts, which further complicates its currently obscure role in chondrocyte biology. Again, HIF-1 may act as a node which interconnects these pathways. Evidence suggests that HIF-1 promotes chondrocyte apoptosis *via* activation of caspase 8 besides enhancing autophagy. It would be

very intriguing to examine how post-translational modifications including sumoylation modulate HIF-1 properties in chondrocytes.

Conclusion

Our knowledge on SUMO modification has been burgeoning for over a decade since its discovery. The biological outcomes elicited by SUMO modification are diverse, and the SUMO pathway actively cross-talks with other post-translational modifications, which enables fine-tuning of functions of its target proteins. As discussed in this review, many significant players in joint homeostasis have been directly or indirectly associated with dynamic sumoylation. Dynamic sumoylation hypothetically regulates matrix metabolism, inflammation, survival, hypoxic responses, senescence and autophagy in cell populations within the joint. In joint biology, some players such as I κ B α and PPAR γ are well characterized, while the others such as HIF-1 and p53 require further examination to clarify their biological roles. It is worth noting that those biological processes extensively cross-talk with each other, so certain SUMO substrates may function in other pathways than currently known ones (Figure 1). Hence, those discoveries imply a potential contribution of deregulation of SUMO modification to the pathogenesis and progression of arthritis. However, few of the hypothesized links between SUMO modification and joint biology, as discussed in this review, has been substantiated experimentally. With the development in research tools for studying sumoylation, intense efforts should be invested to squarely delineate its roles in joint homeostasis and arthritis. Many proteins and biological pathways which are regulated by SUMO modification are also targets of drugs and therapies against arthritis. Therefore, research on SUMO modification in joint biology will not only lead to a deeper understanding of their modes of action, but also will provide us with potential targets for novel drug design.

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Figure 1.
Summary of relevant SUMO substrates in chondrocyte and synovial fibroblast biology.
Arrows indicate functional connections. The dash-line box indicates intertwined relationship between individual biological pathways.

Figure 2. Hypothetical model for SUMO regulation in NFκB pathway. Sumoylation of IκBα mediated by Ubc9 and RSUME competes off the ubiquitination pathway, which stabilizes IκBα and inhibits NFκB signaling. IL-1R is also sumoylated upon stimulation, which conduces to signal transduction. The question marks indicate processes which have not been fully substantiated experimentally.

Figure 3.

Hypothetical model for SUMO regulation in PPAR γ -mediated anti-inflammatory effects. Sumoylation of PPAR γ mediated by PIAS1 or PIASx β prevents the clearance of transcriptionally repressive complex from promoters of target genes such as *iNOS* and *TNF*, resulting in maintained repression of target genes. TF stands for the transcription factor which NCoR binds to in *iNOS* or *TNF* regulation.

Figure 4.

Working hypothesis for SUMO regulation in bFGF-FGFR1-MAPK-Elk-1 signaling axis in chondrocytes. In the basal state, sumoylated Elk-1 recruits putative repressive cofactors (CoR) and HDAC to the promoter of *MMP-13* to inhibit its transcription. Activation of FGFR1 upon bFGF stimulation leads to ERK1/2 activation and subsequently, Elk-1 desumoylation and concomitant phosphorylation. Phosphorylated Elk-1 then transactivates *MMP-13*. In contrast, p38 activation may counteract the impact of ERK1/2 via inhibiting HDAC and SUMO loss. The question marks indicate processes which have not been fully substantiated experimentally.