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Interferon, restriction factors and SUMO pathways

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ABSTRACT

SUMOylation is a reversible post-translational modification that regulates several cellular processes including protein stability, subcellular localization, protein-protein interactions and plays a key role in the interferon (IFN) pathway and antiviral defense. In human, three ubiquitously expressed SUMO paralogs (SUMO1, 2 and 3) have been described for their implication in both intrinsic and innate immunity. Differential effects between SUMO paralogs are emerging such as their distinctive regulations of IFN synthesis, of IFN signaling and of the expression and function of IFN-stimulated gene (ISG) products. Several restriction factors are conjugated to SUMO and their modifications are further enhanced in response to IFN. Also, IFN itself was shown to increase global cellular SUMOylation and requires the presence of the E3 SUMO ligase PML that coordinates the assembly of PML nuclear bodies. This review focuses on differential effects of SUMO paralogs on IFN signaling and the stabilization/destabilization of ISG products, highlighting the crosstalk between SUMOylation and other post-translational modifications such as ubiquitination and ISGylation.

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1. Introduction

In addition to ubiquitin, a growing list of ubiquitin-like (UBL) proteins [1,2], is emerging that includes small ubiquitin-related modifier (SUMO), neural precursor cell expressed, developmentally downregulated 8 (NEDD8), and Interferon-stimulated gene 15 (ISG15). These modifications regulate a variety of biological processes and are powerful regulators of the cellular antiviral signaling pathway [3–8]. Like ubiquitin, these proteins can be conjugated to protein substrates *via* their own E1-E2-E3 enzymatic cascades, and can modify the same lysine residues targeted by other UBLs.

The covalent modification of SUMO to substrates, termed SUMOylation, is a main regulator of a number of cellular functions involved in response to different stimuli such as interferon (IFN) treatment or viral infection, resulting in a global change of the SUMO proteome [9–17]. IFNs are a family of cytokines that are essential for host defense against viral infection. IFNs were discovered in 1957 by Isaacs and Lindenmann [18] and named for their antiviral property. Subsequently, they have also been shown to have immunomodulatory, anti-proliferative and apoptotic activities [19,20]. Every stage of viral replication may be inhibited by IFNs, including entry, transcription, translation, maturation, assembly and release. IFNs are produced and secreted by cells in response to viral infection, and can confer viral resistance to yet uninfected cells. IFN signaling act *via* the JAK/STAT pathway to induce the expression of hundreds of IFN-Stimulated Genes (ISGs) [21]. Several of these ISG products act as restriction factors capable of inhibiting different steps of the viral replication process to mediate the IFN-induced antiviral process. Some ISGs are constitutively expressed and can provide an intrinsic basal antiviral activity independent of IFN *via* a variety of inhibitory mechanisms. Therefore, an intrinsic antiviral activity may be conferred by constitutively

expressed restriction factors, whose expression levels can be even further enhanced by IFN stimulation.

There is growing evidences indicating that SUMOylation can modulate innate and intrinsic immunity by altering IFN synthesis, JAK/STAT signaling and the expression and function of several restriction factors [7]. Many restriction factors are found to be SUMOylated such as promyelocytic leukemia (PML) [14,22], the double-stranded RNA (dsRNA)-dependent protein kinase (PKR) [23], SAM and HD domain containing deoxynucleoside triphosphate triphosphohydrolase 1 (SAMHD1) [13,16], and the Myxovirus resistance MxA and MxB proteins [13,24]. In addition, IFN α was shown to enhance global cellular SUMOylation in a PML-dependent manner [9], resulting in an increase in IFN α -induced protein ubiquitination and ISGylation that ultimately leads to the destabilization or stabilization of several ISG products [13].

This review will mainly focus on the effect of the different SUMO paralogs on the function and the stabilization/ destabilization of several restriction factors in the absence or the presence of IFN. We will also discuss the inter-relationship between PML Nuclear bodies (NBs), IFN, SUMO, ubiquitin and ISG15 pathways.

2. The SUMO pathway

SUMO is typically attached to acceptor lysine residues of protein substrates harboring a consensus sequence defined by ψ KxE, where ψ corresponds to a large hydrophobic amino acid, K is the modified lysine residue, x is any amino acid and E is a glutamic acid residue [25]. In addition to the canonical four amino acid SUMO consensus motifs, other sequences have been identified including both SUMO consensus motifs and additional elements such as phosphorylation-dependent SUMO motifs and negatively charged amino acid-dependent

SUMO motifs [26,27].

SUMOylation plays an important role in various cellular processes, such as subcellular localization, protein-protein interactions, protein stability, IFN response and antiviral defense [7]. Protein SUMOylation can for instance favor protein interactions *via* SUMO interaction motifs (SIMs), defined by a short core sequence of hydrophobic amino acids (V/I/L)X(V/I/L)(V/I/L) [28,29].

Mammalian cells can express four SUMO paralogs with SUMO1, SUMO2 and SUMO3 being conjugatable and ubiquitously expressed, while SUMO4 expression is restricted to renal, immune and pancreatic cells [30]. In addition, SUMO4 contains a proline residue close to the diglycine motif, which prevents its maturation and conjugation [31]. SUMO1 and SUMO2/3 share 50% of sequence identity, whereas 95 % identity is observed between SUMO2 and SUMO3, making that they cannot be distinguished using currently available antibodies [32,33]. The SUMO conjugation pathway involves a tri-enzyme cascade comprising E1-activating enzymes, E2-conjugating enzyme and E3-ligases [34]. SUMO paralogs are expressed as immature precursors and need to be cleaved by SUMO/Sentrin specific proteases (SENPs) to expose the C-terminal di-glycine that is required for conjugation. Protein SUMOylation is initiated by the SUMO activating enzyme (E1), which forms a high-energy thioester bond with mature SUMO. The activated SUMO is then transferred to the SUMO conjugating enzyme (E2), *via* a trans-thiolation reaction. SUMO conjugation to acceptor Lys residues of protein substrates is finally achieved with or without the aid of an E3 ligase. As SUMOylation is a reversible process, SUMO can be removed from its target substrate by the action of two families of proteases, the UBL-specific proteases and the sentrin-specific proteases (SENPs) [35].

Central to protein SUMOylation is the sole SUMO E2 conjugating enzyme UBC9. Its knockout is lethal in most eukaryotes [36], and mice deficient in UBC9 die early in development [37]. Post-translational modifications of UBC9 can regulate substrate interaction. UBC9 has been reported to be phosphorylated [38], acetylated [39] or SUMOylated [9,40]. Interestingly, SUMOylation of UBC9 at its main acceptor lysine residue (Lys-49) is favored during cell senescence, a modification required for its proper association to PML NBs [41].

While in some cases protein SUMOylation can take place without the assistance of E3 ligases, these enzymes are required to enhance substrate specificity and modification at a non-consensus site [36]. In human cells, there are more than 15 SUMO E3 ligases, including the SP-RING (Siz/Pias) family, RANBP2, the ZNF451 family and members of the TRi partite Motif (TRIM) family, including PML/TRIM19 [36,42]. The mechanisms regulating the expression of SUMO paralogs are not elucidated. To date, only the promoter of *SUMO1* has been identified [43], which has potential NF κ B, FOXP3, p53 and TCF-4E binding sites. SUMO protein levels can also be regulated at post-transcriptional levels. For example, IFN α treatment enhances unconjugated SUMO1 levels without altering *SUMO1* mRNA levels through an miRNA-based mechanism involving the Lin28/let-7 axis [44].

3. Effect of SUMO on IFN signaling and IFN production

The innate immune response is the first line of host defense, triggering IFN synthesis upon viral infection. IFNs are secreted and bind to specific membrane receptors, and activate Signal Transducer and Activator of Transcription (STAT) complexes, before initiating the classical Janus kinase-STAT (JAK-STAT) signaling pathway [20] (Fig. 1). It has been reported that SUMOylation can regulate innate immunity by inhibiting viral-induced IFN and IFN-induced STAT1 phosphorylation [7,45,46].

The effects of SUMO on IFN-activated JAK/STAT signaling and on virus-induced IFN have been recently reviewed [7]. Based on the type of receptor through which they signal, human IFNs are classified into multiple type I species (including IFN α and IFN β), one type II (IFN γ) and four members of type III species (IFN λ) [20]. IFNs act on cells by binding to their respective receptors (IFNAR for IFN α/β , IFNGR for IFN γ or IFNLR for IFN λ) and activating the JAK/STAT pathways to trigger the transcription of more than one hundred ISGs [21]. Briefly, the interaction of IFN α/β or IFN λ with their respective receptor leads to the phosphorylation of STAT1 and STAT2 by activated Tyk2 and JAK1 tyrosine kinases. Phosphorylated STATs heterodimerize (pSTAT1:pSTAT2) or homodimerize (pSTAT2:pSTAT2), and form a complex called IFN-stimulated growth factor 3 (ISGF3) with the aid of the DNA binding protein IRF9 [21,47,48]. ISGF3 translocates into the nucleus to induce ISGs containing an IFN-Stimulated Response Element (ISRE) in their promoters. The binding of IFN γ to its receptor results in the phosphorylation of STAT1 (pSTAT1) by the activated JAK1 and JAK2 kinases. Phosphorylated STAT1 forms homodimers that migrate to the nucleus and bind to a Gamma Activating Sequence (GAS) in the promoter of specific ISGs.

Ubiquitin or UBL proteins such as SUMO and ISG15 have been shown to modify many ISGs and key regulators of IFN signaling [49]. Indeed, in response to IFN α or IFN γ , overexpression of each SUMO paralog leads to endogenous STAT1 SUMOylation and to a decrease in IFN α - and IFN γ -induced STAT1 phosphorylation [45]. This process correlates with both lower levels of STAT1 binding to the GAS and the transcriptional response to IFN γ demonstrating that responses to IFN γ are dominated by the activity of phosphorylated STAT1 homodimers. The IFN α transduction and transcription pathways are unaffected by SUMO, since pSTAT2 homodimers can compensate for the lack of STAT1 phosphorylation [45] (Fig. 1). This is in agreement with studies reporting the existence of alternative STAT2 signaling pathways that

are independent from STAT1 [50,51]. However, it should be noted that the expression of SUMO3, but not of SUMO1, abrogates the increase of several ISG products in response to IFN α due to their RING finger protein 4 (RNF4)-dependent proteasomal degradation [13,45]. Also, there is increasing evidence indicating that SUMOylation decreases IFN synthesis in response to viral infection [7,8]. All known pathways that induce IFN production require the transcription factors IRF3 and IRF7. Interestingly, their SUMOylation was shown to inhibit viral-induced IFN synthesis [52–54]. Recently, it has been shown that the loss of SUMOylation triggers a potent and spontaneous production of IFN in the absence of exogenous stimuli [55,56]. This IFN response is independent of all known IFN-inducing pathways and does not require IRF3 or IRF7 [56]. Taken together, these results show that SUMO expression negatively regulates IFN production.

4. SUMO, restriction factors and antiviral defense

SUMO has been implicated in both intrinsic and innate antiviral activities and several restriction factors namely PML, p53 and PKR require their SUMOylation for their antiviral properties [7]. The following section highlights the intrinsic antiviral activity of SUMO, and focuses on the effects of SUMO paralog overexpression or inhibition of the SUMO pathway by UBC9 depletion on the restriction factors PKR and MxA proteins.

4.1 PKR and SUMO

PKR, a 68 kDa serine/threonine kinase is ubiquitous and constitutively expressed in mammalian cells. PKR is induced by IFN as a monomeric inactive form and subsequently activated by double-stranded RNA (dsRNA) expressed in the cells upon viral infection.

Activated PKR dimerizes and undergoes auto-phosphorylation at distinct residues located on the N-terminal dsRNA binding domain and the C-terminal kinase domain [57–59]. Once active, PKR phosphorylates several substrates, the most studied being the eukaryotic translation initiation factor eIF2 α [60]. This process prevents viral protein synthesis and can confer viral resistance. PKR is an enzyme with multiple cellular functions; playing a role in IFN-induced antiviral defense mechanism as well as in the regulation of the p53, NF- κ B, p38MAPK and insulin pathways [61,62]. In addition to its viral activation, PKR can also be activated by heparin [63], PKR-activating protein (PACT) [64], ISG15 [65] or SUMO1 [66]. PKR is modified by ISG15 [65] and SUMO paralogs [23]. Indeed three SUMO sites have been identified on amino acids K60, K150 and K440. The triple PKR-SUMO deficient mutant was shown to have reduced PKR-dsRNA binding, PKR dimerization and eIF2 α phosphorylation activities [23]. Also, PKR can interact with SUMO in a non-covalent manner. Phosphorylation of tyrosine residue Y162 on PKR modulates both its non-covalent and covalent SUMO interactions [67] revealing a new mechanism of regulation of PKR activity.

Stable expression of SUMO1 and SUMO3 in human cells has differential effects on PKR stability and eIF2 α phosphorylation [66]. Remarkably, expression of SUMO1 results in PKR and eIF2 α phosphorylation with their enhanced activation upon poly (I:C) transfection or viral infection (Fig. 2). Increased PKR and eIF2 α phosphorylation imparted by SUMO1-expressing cells results in a partial resistance to encephalomyocarditis virus (EMCV) infection [66]. In addition, UBC9 depletion in SUMO1-expressing cells correlates with a decrease in global cellular SUMO1 conjugation, resulting in a decrease in both PKR and eIF2 α phosphorylation. The finding that SUMO1 expression is able to activate PKR and eIF2 α in the absence of viral infection, suggests a novel mechanism for PKR activation. Interestingly, PKR

ISGylation at K69 and K159 also results in PKR and eIF2 α phosphorylation in the absence of viral infection [65].

In contrast, SUMO3 expression abrogates PKR and eIF2 α phosphorylation in dsRNA-transfected or virus-infected cells [66]. In addition, infection with EMCV enhances PKR conjugation to SUMO1 and to SUMO3 but induces a caspase-dependent PKR degradation only in SUMO3-expressing cells (Fig. 2). EMCV also enhances the SUMOylation of the PML III isoform leading to its proteasome-dependent degradation [68]. Together, these findings reveal that EMCV has developed strategies by using the SUMO pathway to antagonize the PKR and PML III antiviral activities. Indeed, enhanced global SUMO2/3 modification is observed in the early stage of EMCV infection [68], where the identification of the SUMOylated substrates may lead to important mechanistic insights into viral manipulation of the cellular SUMOylation machinery.

4.2 MxA and SUMO

Human cells harbor two Mx (Myxovirus resistance) genes Mx1 and Mx2 that are induced by type I and type III IFNs *via* the ISRE present in their promoters, encoding MxA and MxB proteins, respectively [69]. Mx proteins belong to the dynamin superfamily of large GTPases [70]. MxA and dynamins share structural and functional homology including domain organization, GTPase activity and oligomerization capacity [69]. Crystallography studies have confirmed that MxA is comprised of three domains. The first domain is represented by the N-terminal GTP-binding domain responsible for GTPase activity, the second is known as the stalk (CID-GED) and is vital for oligomer formation and the third domain is named the Bundle-Signaling Element, also important for oligomerization. MxA protein accumulates in

the cytoplasm and has been shown to inhibit several viruses from different families including Rhabdoviridae, Orthomyxoviridae, Bunyaviridae and Paramyxoviridae [69,71].

Overexpression of SUMO1 or SUMO3 in human cells confers a strong resistance to Vesicular Stomatitis Virus (VSV) in an IFN-independent manner [54]. SUMO expression does not alter VSV entry, but inhibits primary mRNA synthesis, resulting in a 100-fold reduction of viral production, thus protecting cells from VSV-induced cell lysis [54]. Different steps of the VSV cycle have been shown to be inhibited by various ISG products that act as restriction factors [72]. MxA [73,74], like SUMO [54], inhibits primary transcription, whereas the IFN-inducible transmembrane (IFITM) proteins block viral entry [75], the cholesterol-25-hydroxylase (CH25h) impairs the virus cell fusion step by inducing cellular membranes changes [76], ISG20 [77], and PML [78,79] inhibit secondary transcription, PKR [80] and IFN-induced proteins with tetratricopeptide repeats (IFIT) proteins inhibit viral translation [81] and finally Tetherin prevents release of virions from the cell [75]. Interestingly, MxA depletion in SUMO-expressing cells abolishes the inhibition of VSV by SUMO, demonstrating that MxA mediates the SUMO-induced intrinsic VSV resistance [54] (Fig. 3).

The MxA protein is known to self-assemble into oligomers that are essential for its GTPase activity [82]. MxA has been reported to be conjugated to SUMO at K48 [24]. SUMO and UBC9 have been identified as MxA partners by yeast two-hybrid screens. Interestingly, the MxA-SUMO1 and MxA-UBC9 interactions are important for its oligomerization. Accordingly, the monomeric mutant of MxA (L612K) has a reduced interaction with SUMO and UBC9 [24] and is rapidly degraded in cells when compared to wild-type MxA [83], demonstrating that the self assembly of the MxA protein is critical for protein stability. Expression of SUMO1 or SUMO3 highly stabilizes constitutive MxA protein through its oligomerization [54], whereas MxB, which shares 64% sequence identity with MxA, is not stabilized by either paralog [13].

Remarkably, the enhancement of MxA oligomerization by SUMO1 and SUMO3 expression [54] provides a stable intracellular pool of MxA that is available to inhibit invading viruses. Future studies will determine whether other families of replication viruses are inhibited by SUMO *via* the stabilization of MxA or other restriction factors.

5. Links between PML NBs, IFN and the SUMO pathway

5.1 PML, SUMO and IFN

In the nucleus, PML is localized in the nucleoplasm and in the nuclear matrix-associated structures referred to PML NBs [84], that act as nuclear hubs for protein post-translational modifications (phosphorylation, acetylation, ubiquitination and SUMOylation) [85–88]. These structures are also responsible for the protein degradation of SUMO2/3 conjugated PML [89–91] and other PML NB SUMOylated partners such as p53 and the speckled protein of 100 kDa (Sp100) [45,87]. These dynamic assemblies are highly enriched in SUMOylated proteins, and harbor resident proteins such as Sp100, the death domain-associated (Daxx), as well as numerous transient proteins that are stimuli dependent [85,88,92]. The list of cellular proteins recruited to PML NBs, based on colocalization studies, is continuously growing.

The *PML* gene consists of nine exons and alternative splicing yields mainly six nuclear PML isoforms (PML I-VI) and one smaller cytoplasmic isoform (PML VII) missing the nuclear localization signal [89,93]. PML isoforms share the same N-terminal region containing the RBCC/Tripartite motif (that defines the TRIM protein family) but differ in their C-terminal sequences [89,93]. The variability in the C-terminal region of each PML isoform is important for the recruitment of specific interacting partners and therefore for specific functions [79,89,94,95].

The functions of PML nuclear isoforms have been studied mainly in the context of their localization in PML NBs [89]. Although the majority of PML proteins are in the nucleus cytoplasmic PML has been reported to be implicated in TGF- β signaling [96] and apoptosis [97]. In recent years, few comparative studies have been performed with all the PML isoforms and increasing evidence suggests that some PML isoforms exhibit specific functions [89].

PML NBs play key roles in host antiviral defense and mediate the IFN-induced antiviral state since IFN has reduced antiviral activity in the absence of PML (Reviewed in [88,98,99]). PML emerges as a significant coregulator of the IFN pathway. PML acts as a restriction factor implicated in both intrinsic and innate host defenses; Key associations have been reported between PML NBs, IFN, antiviral defense and SUMO pathway, and are summarized below.

- PML is the organizer of NBs that contain two permanent NB-associated partners, Sp100 and Daxx. PML, Sp100 and Daxx are ISG products [100–104] and are found to be conjugated to SUMO [22,105,106].
- PML *via* its RING finger domain directly binds UBC9, resulting in the SUMOylation of several of the PML lysines K65, K160, K209, K226, K337, K380, K394, K400, K401, K476, K478, K487, K490, K497 and K616 [13,14,22]. PML SUMOylation is essential for PML NB functions; it affects its localization, stability and ability to interact with partners. Indeed a PML mutant deficient in the three major SUMO sites K65, K160 and K490 still forms dots but fails to recruit the key PML NB partners Sp100 and Daxx [107,108].
- Nuclear PML positively regulates signaling of type I and type II IFNs [109–111].

- A specific nuclear PML isoform, PML II, promotes transcription factor recruitment to activate IFN β and ISG expression [112].
- A specific nuclear PML isoform, PML IV, is implicated in innate immunity, triggering a dramatic increase of IFN β synthesis *via* a higher virus-induced IRF3 phosphorylation [79]. The positive regulation of IFN β synthesis by PML IV requires its SUMOylation, as a SUMO deficient mutant of PML IV is unable to do so. Mechanistically, SUMOylated PML IV enhances IFN β synthesis by regulating the cellular distribution of Pin1 (peptidyl-prolyl cis/trans isomerase) that was shown to interact with phosphorylated IRF3 and to promote its ubiquitin-mediated proteasomal degradation [113]. PML IV interacts with Pin1 and recruits it within PML NBs where both proteins colocalize. This process prevents the degradation of phosphorylated IRF3, and thus potentiates IRF3-dependent production of IFN β [79].
- In addition to its involvement in innate immunity [7,79,109], PML exerts an intrinsic antiviral activity against several DNA and RNA viruses from different families [88,94,95,99,114,115]. Remarkably, PML IV confers viral resistance by sequestering viral proteins within PML NBs [94,95]. The intrinsic antiviral property of PML also requires its SUMOylation. The antiviral property of PML has been validated *in vivo* with PML knockout mice, where PML deficient mice were more sensitive to infection with the arenavirus lymphocytic choriomeningitis virus (LCMV) and the rhabdovirus VSV [116].
- Many viruses use various strategies to manipulate the SUMO pathway and counteract the antiviral effect of PML by disrupting PML NBs and/or inducing proteasome-dependent PML degradation [68,117,118]. Therefore, targeting PML NBs

likely represents a viral strategy to antagonize both intrinsic and innate immune activities of PML.

- In addition to PML, many PML NB partners are SUMOylated, suggesting that PML NBs are enriched in SUMOylated proteins [92]. Among the SUMOylated proteins that reside within PML NB, there are key players of the SUMO pathway, namely SUMO paralogs, UBC9, SUMO-specific proteases and several SUMO E3 ligases (RANBP2, PIAS1, PIASy) [9,42,92,119,120]. The notion that PML itself is a SUMO E3 ligase [42], further demonstrate that PML NBs function as a dynamic site of SUMOylation.
- IFN α enhances global cellular SUMOylation [9] in wild type, but not in PML $^{-/-}$ cells, highlighting a strong association between PML, IFN and SUMO.

5.2 Enhancement of global cellular SUMOylation by IFNs requires PML

As a result of several technical advances in both sample preparation and instrumentation, mass spectrometry-based approaches have been developed to elucidate the biological importance of SUMOylation in response to different stimuli in a system-wide manner. All SUMO proteomic approaches rely on similar methods, where SUMOylated peptides are purified by SUMO remnant immunoaffinity or chromatography enrichment prior to LC-MS/MS analyses. Large-scale proteomic approaches provide a more comprehensive view of protein SUMOylation compared to conventional Western blotting. Indeed, these workflows garner more information due to their non-targeted and unbiased nature. Proteomics based methods can monitor the abundance of over 10 000 SUMOylation sites in a single experiment. In addition to their higher throughput, proteomics approaches enable the identification of modified residues on target substrates, and the profiling of changes in SUMO protein abundance between experimental conditions. This information is ideal for downstream validation and biological follow-up experiments.

Using these Mass Spectrometry-centric approaches, global changes in protein SUMOylation by SUMO1 and/or SUMO2/3 have been investigated following heat shock [121], DNA damage [122,123], inhibition of the proteasome [15,124], viral infection [12,125] or IFN treatment [9]. Moreover, global cellular SUMOylation is enhanced in response to both type I (IFN α) or type II (IFN γ) IFNs [9]. This process requires the expression of constitutive PML and is orchestrated by PML isoforms III and IV, as evidenced by the introduction of each PML isoform into PML null cells, and where only PML III or PML IV are able to enhance global cellular SUMOylation with a further increase upon IFN α stimulation (Fig. 4). This is consistent with a previous report showing that PML IV acts as an E3 SUMO ligase by promoting the conjugation of SUMO to the model substrates p53 and mdm2 [42].

It is noteworthy that PML null cells have a defect in IFN signaling [109], IFN-induced apoptosis [126], IFN-induced antiviral activity [115] and IFN-induced global cellular SUMOylation [9]. Many restriction factors and key regulators of the IFN pathway are SUMOylated and require this modification for their functions [7]. Therefore, introducing PML III or PML IV into PML null cells restores IFN α -enhanced global cellular SUMOylation and therefore IFN α functions [9] (Fig. 4).

5.3 Identification of proteins conjugated to SUMO3 in response to IFN α

Recent advances in proteomics approaches enabled the identification of SUMO sites in a system-wide manner [15,17,127,128]. Recently, proteins conjugated to SUMO3 were identified in response to IFN α using a proteomics strategy based on stably expressed SUMO mutant 6xHisSUMO-3-Q87R-Q88N (SUMO3m) in human cells and on SUMO remnant immunoaffinity purification [9,13]. This protein was referred to as SUMO3m to indicate the presence of the N-terminal His₆ tag and the insertion of a strategically located tryptic

cleavage site at the C terminus to facilitate the recovery and identification of SUMOylated peptides by affinity enrichment and mass spectrometry. SUMO proteome analysis revealed that various SUMOylation sites are increased in response to IFN α in ISG products including several restriction factors (e. g. PML, ADAR1, Vimentin, SAMHD1, PKR, IFIT1, IRF1, ISG20) and other regulators of IFN signaling or IFN production (e.g. STAT1, IRF1, IRF9 Tif1 α /TRIM24, TRIM28, TRIM33, TBK1) [9,13].

In addition, most of the proteins from the SUMOylation machinery (e.g. UBC9, SUMO1, SUMO2, SUMO3, PIAS1, PIAS2, RanBP2, TRIM28, PML) display increased SUMOylation in response to IFN α [9].

UBC9 is SUMOylated at K14, K18, K48, K49, K65 and K154 [9,40,129]. SUMOylation of UBC9 at K14 was proposed to modulate target selection. While UBC9 SUMOylation does not affect its activity, it has a reduced capacity to SUMOylate RanGAP1 and strongly enhances the SUMOylation of Sp100 *via* its SIM [40]. Out of all UBC9 SUMOylation sites identified only SUMOylation at K49 is enhanced upon IFN α treatment [9]. Furthermore, IFN α induces PML-dependent UBC9 transfer to the nuclear matrix where it colocalizes with PML within the NBs and enhances global cellular SUMOylation [9]. The SUMOylation of UBC9 at K49 promotes its localization to PML NBs [41]. Altogether, these findings demonstrate that SUMOylated UBC9 and PML are key players of IFN-enhanced global cellular SUMOylation.

5.4 Protein stabilized/destabilized by SUMO in response to IFN α : crosstalk between SUMO, ubiquitin and ISG15

It is well established that PML NBs act as sites of proteasomal degradation through targeted ubiquitination of SUMOylated substrates by the E3 ubiquitin ligase RNF4. The most common

example being degradation of PML itself in response to arsenic trioxide [90,91], viral infection [89,99] or IFN treatment [45]. Recently, it has been shown that the expression of SUMO3, but not of SUMO1, in HeLa cells abrogates the IFN α -dependent increase of certain ISG products (such as PML, Sp100 and IFI16) due to their RNF4-dependent proteasomal degradation [13,45].

Mass spectrometry based approaches can also be employed to analyze the impact of SUMO by studying changes in the global proteome, where the modulation of protein abundances can be determined in response to elevated SUMO levels with or without IFN α [13]. Such an approach was developed to collect information on the role of SUMO3 on protein stability. Large-scale SUMO proteome analyses confirmed the role of poly-SUMO3 in promoting protein degradation in both IFN α -dependent and independent mechanisms. In addition, this approach uncovered the novel role of poly-SUMO3 chains in selectively increasing the stability in response to IFN α of several restriction factors [13].

Among the ISG products stabilized, several are implicated in IFN synthesis (RIG-I, MDA5), IFN signaling (STAT1, STAT2, IRF9), antiviral defense (Guanylate Binding Protein 1 (GPB1), GBP5, SAMHD1, Tetherin/BST2 and members of IFITM, IFIT and IFI families) and ISG15 pathway (UBE2L6, TRIM25 and HERC5) [13].

Mechanistically, IFN α increases global SUMO2/3 conjugation, which in turn enhances protein ubiquitination and ISGylation of various proteins (Figs 5 and 6). Accordingly, the fate of SUMOylated substrates depends on their subsequent modification by either ubiquitination or ISGylation, resulting in either RNF4-dependent proteasomal degradation or TRIM25-dependent protein stabilization, respectively [13]. It is noteworthy that the ISG15 ligase TRIM25 is highly stabilized in IFN α -treated SUMO3 cells and its depletion expectedly

impairs protein ISGylation, but also abrogates the upregulation of ISG products suggesting that TRIM25 is a key player in the SUMO3-mediated protein stabilization process.

5.5 SUMO3 enhances IFN α -induced antiviral activities

Importantly, several restriction factors known to confer resistance to Human Immunodeficiency Virus 1 (HIV1) and Herpes Simplex Virus 1 (HSV1) such as GBP1, GBP5, IFITM1, IFITM2, IFITM3, IFIT1, IFIT3, IFIT5, PKR, SAMHD1, Tetherin/BST2 and MxB [130–137] are highly stabilized by SUMO3 in IFN α -treated cells. Therefore, the increase in abundance of these restriction factors in IFN α -treated SUMO3 expressing cells is correlated with an enhancement in IFN α -induced anti-HIV-1 and anti-HSV-1 activities [13]. At the opposite, the loss of SUMOylation reduces the IFN α -mediated resistance to HIV1 [44]. Taken together, these results demonstrate that SUMO3 enhances IFN α -induced antiviral defense. However, further studies are needed to validate and identify the SUMOylation sites of several stabilized ISG products such as GBP1, GBP5, Tetherin/BST2 and members of IFIM and IFIT families. Also, some of the stabilized restriction factors such as MxB and SAMHD1 have been shown to be SUMOylated [13,16] though it is thus far unknown whether their SUMOylation is required for their antiviral activities. Interestingly, it has been shown that SAMHD1 interacts with MxB in IFN α -treated SUMO3-expressing cells [13], suggesting that proteins stabilized by SUMO3 during the IFN α response may be part of several interacting complexes. Therefore, MxB in conjunction with SAMHD1 and SUMO3 could contribute to IFN α -induced antiviral defense.

6. Differential effects of SUMO paralogs

SUMO1 and SUMO2/3 modify in part the same but also distinct substrates, and several reports suggest that SUMO1 and SUMO2/3 may have quite individual functions [138]. The differences between the SUMO paralogs include variable expression levels [32], susceptibility to deSUMOylating enzymes [139] and the ability to form SUMO chains [33]. Similar to ubiquitination, proteins can be mono- or poly-SUMOylated on a target lysine residue. SUMO2/3 contains the consensus SUMO motif and can thus form poly-SUMO chains whereas SUMO1 lacks this motif, and presumably can only be used to mono-SUMOylate a target protein or act as a SUMO2/3 chain terminator [33]. Poly-SUMO chains formed by SUMO2/3 are recognized by RNF4, a SUMO-Targeted Ubiquitin Ligase (STUbL). This protein ubiquitinates SUMO2/3-conjugated PML which result in its proteasomal degradation upon cell treatment with arsenic trioxide [90,91] or IFN α [45]. More recently, it has been shown that the expression of SUMO2/3, but not of SUMO1, is required for enhanced IFN α -induced ubiquitination and ISGylation resulting in the destabilization and the stabilization of several ISG products, respectively [13]. In addition, it has been reported that the loss of all SUMOylation is sufficient to initiate a spontaneous IFN synthesis [55,56]. Deletion of any individual SUMO gene does not result in this IFN production, while the combined disruption of SUMO2 and SUMO3 is enough to yield an IFN response, demonstrating that regulation of IFN synthesis by SUMOylation is redundantly mediated by both SUMO2 and SUMO3, but not SUMO1 [56]. Interestingly, this IFN response does not require the IFN-associated transcription factors IRF3 or IRF7.

Collectively, these findings led to important insights regarding the interplay between IFN, SUMOylation, ubiquitination, ISGylation, PML and antiviral response, which are depicted in

Figure 7.

- The expression of each SUMO paralog increases IRF3 and IRF7 SUMOylation, resulting in a decrease in their viral-induced phosphorylation and therefore in a reduction of IFN production.
- Depletion of SUMO2 and SUMO3, but not of SUMO1, triggers spontaneous IFN synthesis.
- The expression of each SUMO paralog enhances STAT1 SUMOylation, resulting in a decrease in IFN-induced STAT1 phosphorylation that leads to a decrease in type II, but not in type I, IFN transcriptional response.
- In the absence of IFN, the expression of each SUMO paralog stabilizes MxA protein and only SUMO1 induces the activation of PKR.
- SUMO3 expression in IFN α -treated cells alters the localization of some proteins such as UBC9 and TRIM25 that are transferred to the nuclear matrix.
- IFN α enhances global SUMO2/3 conjugation resulting in enhanced IFN α -induced ubiquitination and RNF4-dependent proteasomal protein degradation.
- IFN α enhances global SUMO2/3 conjugation resulting in enhanced IFN-induced ISGylation and TRIM25-dependent protein stabilization.

Conclusion and Perspectives

Post-translational modifications affect a large number of signaling proteins and restriction factors and provide key mechanisms for the regulation of intrinsic and innate immune responses. The actions of UBL proteins on IFN pathways are both dynamic and complex and lead to differential effects on IFN production, IFN signaling, and on the abundance and functions of various restriction factors.

Viruses usurp the ubiquitin, SUMO and ISG15 pathways to modify both viral and cellular host proteins thus counteracting different steps of the IFN antiviral defense. Interestingly, the host cells also use these same post-translational modifications to counteract viral infections. The study of the SUMO pathway is more complex due to the expression of various isoforms, where several lines of evidence indicate that SUMO2/3 have protein targets and functions that are clearly distinct from those of SUMO1. Remarkably, in response to IFN α , increased conjugation of SUMO3, but not of SUMO1, enhances cellular ubiquitination and ISGylation leading to the degradation or stabilization of several ISG products, respectively. System-wide SUMO proteome analyses may bridge the gap between our current understanding and the full biological implications of SUMO during the IFN response. Current workflows enable the quantitative profiling of changes in both protein SUMOylation and protein abundance for thousands of substrates, thereby providing valuable biological insights for subsequent follow-up experiments.

In conclusion, these findings highlight the fast growing functions of poly-SUMO chains. However, a lot remains to be learned about the effects of SUMO during the IFN response, especially its regulation of the restriction factors and its crosstalk with other post-translational modifications. The roles of SUMO in IFN-induced antiviral defense are emerging. Therefore, the mechanisms involved in SUMOylation and the crosstalk among other UBL modifications are of great interest to further understand the cellular response to viral infection.

Legend to Figures

Figure 1: Effect of SUMO1 and SUMO3 on IFN α signaling. The interaction of IFN α with its receptor leads to the phosphorylation of STAT1 and STAT2 by activated Tyk2 and JAK1 tyrosine kinases. Phosphorylated STATs heterodimerize (pSTAT1:pSTAT2) or homodimerize (pSTAT2:pSTAT2) and form with the DNA binding protein IRF9; the ISGF3 complex. ISGF3 translocates into the nucleus to induce ISGs containing an ISRE in their promoters. Although SUMO1 or SUMO3 overexpression leads to an inhibition of IFN-induced STAT1 phosphorylation (pSTAT1), the IFN α -transduction pathway and IFN α transcriptional response are unaffected by this inhibition, because pSTAT2 homodimers can compensate for the lack of pSTAT1.

Figure 2: Differential effect of SUMO1 and SUMO3 on PKR activation and stability. (A) SUMO1 expression induces PKR SUMOylation, enhances PKR phosphorylation and the phosphorylation of eIF-2 α , inhibiting EMCV infection. (B) SUMO3 expression induces PKR SUMOylation, reduces its phosphorylation and the phosphorylation of eIF-2 α upon viral infection. In addition, SUMO3, and not SUMO1, enhances EMCV-induced PKR degradation.

Figure 3: MxA mediates the anti-VSV properties of SUMO. MxA is conjugated to SUMO at lysine 48. MxA protein is highly stabilized in cells expressing SUMO1, SUMO2 or SUMO3. MxA depletion in HeLa cells abrogates SUMO-induced resistance to VSV.

Figure 4: PML is required for IFN α -dependent increase of global cellular SUMOylation. Cell treatment with IFN α results in an increase of global cellular SUMOylation, increase of PML and UBC9 SUMOylation, increase of PML expression and recruitment of SUMOylated UBC9 within PML NBs. PML $^{-/-}$ cells have defects in IFN signaling, in induction of apoptosis and in

an increase of global cellular SUMOylation. Introducing PML III or IV in PML negative cells restores IFN α -enhanced global cellular SUMOylation and IFN functions.

Figure 5: Crosstalk between SUMO, ubiquitin and ISG15 in response to IFN α . IFN α increases PML dependent global SUMO2/3 conjugation, enhances both ubiquitination resulting in the degradation and ISGylation, leading to stabilization of different ISG products.

Figure 6. Interplay between Ubiquitin-like modifiers (UBL) in response to IFN α . Upon IFN α treatment, SUMOylated substrate proteins can either be ubiquitinated and degraded *via the* proteasome-dependent pathway or ISGylated and stabilized.

Figure 7. The multifaceted role of SUMO during the IFN response. SUMO expression increases IRF3 and IRF7 SUMOylation, resulting in a decrease in their viral-induced phosphorylation and in a reduction of IFN production. SUMO2/3 prevents spontaneous IFN synthesis. SUMO expression enhances STAT1 SUMOylation, resulting in a decrease in IFN-induced STAT1 phosphorylation that leads to a decrease in type II, but not in type I, IFN transcriptional response. In the absence of IFN, the expression of SUMO stabilizes MxA, induces the activation of PKR (SUMO1) and alters the localization of some proteins such as UBC9 and TRIM25 that are transferred to the nuclear matrix. IFN α enhances global SUMO2/3 conjugation resulting in enhanced IFN α -induced ubiquitination and protein degradation. IFN α enhances global SUMO2/3 conjugation resulting in enhanced IFN-induced ISGylation and protein stabilization.

Conflict of interest

None

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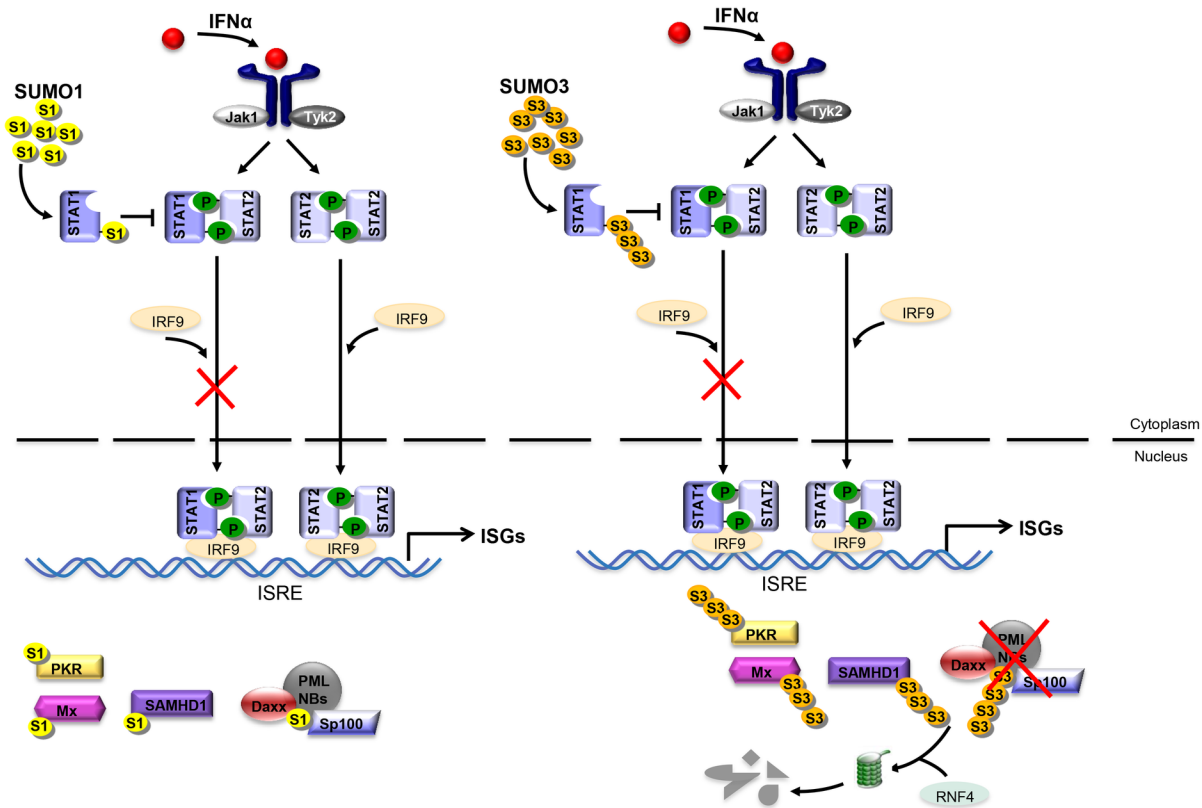
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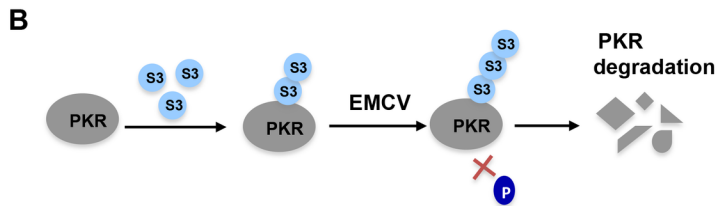
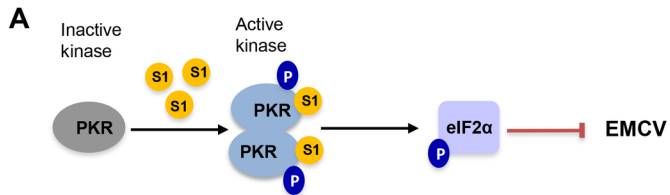
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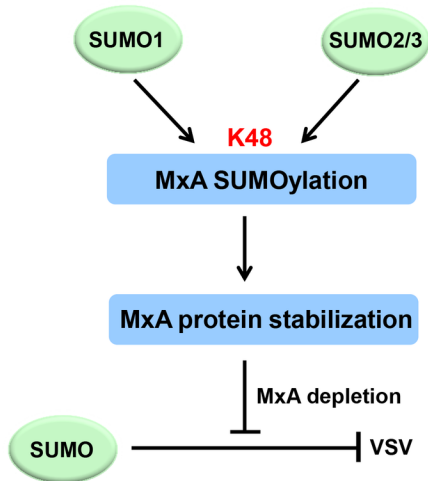
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PML +/+



PML NBs
SUMOylation of PML
SUMOylation of K49 UBC9
Recruitment of UBC9 to PML NBs
Enhancement of global cellular SUMOylation

PML -/-



IFN



Absence of PML NBs
Defect in IFN signaling
Defect in induction of apoptosis
Defect in increase of global SUMOylation

SUMO2/3

