PML requirement for interferon-induced

global cellular SUMOylation

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Abbreviations:
IFN, interferon; SIM, SUMO-interacting motif; SUMO, small ubiquitin-related modifier; PML, promyelocytic leukemia protein; NBs, nuclear bodies; PTM, post-translational modification; MEFs, mouse embryonic fibroblasts; LC-MS/MS, liquid chromatography-tandem mass spectrometry; FDR, false discovery rate; UBC9, ubiquitin conjugating enzyme; Ni-NTA, nickel-nitrilotriacetic acid; SCX, strong cation exchange; AGC, automatic gain control; TCE, total cell extract; PSM, peptide spectrum match; DMP, dimethyl pimelimidate.
SUMMARY

We report that interferon (IFN) α treatment at short and long periods increases the global cellular SUMOylation and requires the presence of the SUMO E3 ligase promyelocytic leukemia protein (PML), the organizer of PML nuclear bodies (NBs). Several PML isoforms (PMLI-PMLVII) derived from a single PML gene by alternative splicing, share the same N-terminal region but differ in their C-terminal sequences. Introducing each of the human PML isoform in PML-negative cells revealed that enhanced SUMOylation in response to IFN is orchestrated by PMLIII and PMLIV. Large-scale proteomics experiments enabled the identification of 558 SUMO sites on 389 proteins, of which 172 sites showed differential regulation upon IFNα stimulation, including K49 from UBC9, the sole SUMO E2 protein. Furthermore, IFNα induces PML-dependent UBC9 transfer to the nuclear matrix where it colocalizes with PML within the NBs and enhances cellular SUMOylation levels. Our results demonstrate that SUMOylated UBC9 and PML are key players for IFN-increased cellular SUMOylation.

Key words: SUMO, PML, UBC9, LC-MS, MS, Interferon
INTRODUCTION

Interferons (IFNs) have been first recognized for their antiviral properties, but also have important immunomodulatory, anti-proliferative and apoptotic activities (1-3). They act on cells by interacting to their respective receptors, activating the JAK/STAT pathways and inducing the transcription of IFN-stimulated genes (ISGs) (3, 4). IFNs are classified in three types (1-3). In humans, type I IFN includes IFNα, IFNβ, IFNγ, IFNκ and IFNω, type II IFN comprises only IFNγ, while type III IFN contains IFNλ1 to IFNλ4. Although type I and type III IFNs bind to different cell surface receptors, they initiate the same signal transduction pathway leading to the activation of the JAK tyrosine kinases, Tyk2 and JAK1, that in turn phosphorylate STAT1 and STAT2, which heterodimerize and form, with the DNA binding protein IFN regulatory factor 9 (IRF9), a complex called IFN-stimulated growth factor 3 (ISGF3) (3). ISGF3 then translocates to the nucleus and binds to ISG promoters containing an IFN-stimulated response element (ISRE). There are also growing evidences that type I IFN activates a STAT2/IRF9 complex that forms an ISGF3-like complex in the absence of STAT1, and is sufficient to activate ISRE-driven transcription (5, 6). The interaction of type II IFN to its specific receptor induces the phosphorylation of STAT1 by JAK1 and JAK2, the homodimerization of activated STAT1, their migration to the nucleus and binding in the promoter regions of ISGs to an element termed gamma-activated sequence (GAS).

It has been established that many of the ISGs or key regulators of IFN signalling are modified by ubiquitin or ubiquitin-like modifiers such as Small Ubiquitin-like Modifier (SUMO) or ISG15 (7, 8). Protein modification by SUMO termed SUMOylation involves the covalent binding of SUMO to a target protein lysine residue that is regulated by a unique SUMO E2-conjugating enzyme named UBC9. There are five SUMO family members; the best studied being SUMO1, which shows ~50% sequence homology with SUMO2 and SUMO3 that are themselves 97% identical to each other. While SUMO1, 2 and 3 have a broad tissue distribution, the expression of SUMO4 is limited to certain organ types (9) and contains a proline residue close to the diglycine motif, which prevents its maturation and
SUMOylation (10). SUMO5, a novel tissue-specific member of the SUMO family has been shown to facilitate the formation of PML nuclear bodies (NBs) in human cells, but does not appear to be expressed in mice (11).

Although SUMO1 and SUMO2/3 are conjugated via the same set of enzymes, they can preferentially target different substrates and demonstrate distinct dynamics and distribution within the cell (12). SUMO2/3 is more abundant in a large free pool compared to SUMO1 and is also more dynamically conjugated and deconjugated from substrate proteins (13). Also, the covalent modification of proteins with SUMO2/3 may have varying consequences compared to SUMO1 modification (7, 14). In addition to being covalently attached to substrates, SUMO can interact in a non-covalent manner via the SUMO interaction motifs (SIMs) (15).

It has been reported that STAT1 is conjugated to SUMO1 at Lys703 and that mutations in the SUMO attachment site in STAT1 result in increased transcriptional activity in response to IFNγ (16). More recently, we reported that the stable expression of the different SUMO paralogs leads to STAT1 SUMOylation and to a decrease in IFNα- and IFNγ-induced STAT1 phosphorylation that result in an inhibition of IFNγ transcriptional response without affecting the IFNα pathway (7). Also, protein SUMOylation contributes to the antiviral effects of IFNα against HSV1 or HIV, though only a limited number of SUMO targets are presently known (17). So far, some ISG products (STAT1, IRF-1, IRF-2, IRF-3, IRF-7, PML, Sp100, p53, TRIM5α) have been shown to be SUMOylated in transfected cells (18), and cell treatment with IFN for 24h was previously reported to increase SUMOylation (17). However, it is unknown whether IFNα treatment for short or long periods could alter the SUMOylation of proteins that regulate IFN pathway. Therefore, we sought to identify proteins conjugated to SUMO3 in response to IFNα using a novel proteomics approach based on SUMO remnant immunoaffinity purification (19). Remarkably, Kinetics studies revealed that cellular SUMOylation was enhanced at short (0.75 h) and long (16 h) periods following IFNα treatment, and necessitated the presence of promyelocytic
leukemia (PML) protein, the key organizer of NBs (20). Also, we found that several SUMO sites were regulated in response to IFNα including UBC9 K49. These observations support the notion that the recruitment of UBC9 to PML NBs upon IFN treatment cooperated with PML to enhance global cellular SUMOylation.

RESULTS

Enhancement of global cellular SUMOylation in response to IFNα - Initially, we treated HEK293-SUMO3m cells with IFNα for short (0.75 h) or long periods (16 and 24 h) to determine the impact of this cytokine on global cellular SUMOylation. Ni-NTA purification of total extracts from HEK293-6xHIS-SUMO3m cells revealed that SUMO3-modified proteins increased at 0.75 h and were more abundant at 16 h following IFNα treatment (Fig. 1A). Western blot analysis of cell extracts indicated that SUMO3 expression decreased IFNα-induced STAT1 phosphorylation without affecting the abundance of phosphorylated STAT2 (Fig. 1B), consistent with a previous report (7). Indeed, phosphorylation of STAT2 in response to IFNα was similar in both in HEK293-wt and HEK293-SUMO3m cells, whereas phosphorylation of STAT1 was lower at 0.75 h in HEK293-SUMO3m cells compared to HEK293-wt cells. Also, ISG products such as STAT1 or PKR were enhanced in both cell lines after 16 h of IFNα treatment showing that IFNα response was not inhibited in SUMO3m-expressing cells.

Quantitative proteomics analyses were performed using stable isotope labelling with amino acids in cell culture (SILAC). Cells were incubated with PBS (Control cells), or IFNα for short (0.75 h) or long (16 h) time periods using cultures grown in either light, medium or heavy SILAC media, respectively (Fig. 2A). After IFNα treatment the cell pellets were pooled during the collection process prior to fractionation into cytoplasmic and nuclear fractions under hypotonic conditions. Cytoplasmic and nuclear protein extracts were divided into 3 aliquots to produce technical triplicates. Each nuclear and cytoplasmic triplicate was subjected to Ni-NTA purification to enrich SUMOylated proteins. The Ni-NTA
bound material was digested with trypsin to release the NQTGG epitope produced by the SUMO3m construct. SUMOylated peptides were further purified using the anti-K(NQTGG) antibody prior to LC-MS/MS analyses.

A summary of the identified and quantified SUMO sites is reported in Table S1. In total, we identified 558 SUMO sites with a FDR ≤ 1% and a SUMO site localization confidence ≥ 0.75 across all time points and fractions. Quantitative proteomics analyses enabled the profiling of 264 and 346 sites in cytoplasm and nuclear fractions for the two time points examined. Several SUMO sites were regulated in response to IFNα treatment (Figs 2B-2E). In contrast to the western blot analysis that depicts a substantial increase in global SUMOylation upon IFNα treatment (Fig. 1A), the proteomics analysis revealed that a similar number of SUMO substrates were up regulated and down regulated as a result of the treatment. These seemingly conflicting results can be consolidated when looking at the intensity or abundance of the SUMOylation events that are upregulated in response to IFNα (Fig. S1). The RankN plots that rank the peptide in ascending order of intensity highlight that SUMOylation sites that are upregulated during the IFNα treatments tend to be in higher abundance, leading to a global increase in a the SUMO signal observed in Fig. 1A. Of interest, we observed the increase SUMOylation of PML at Lys 490 at 16 h in both cytoplasmic and nuclear fractions (Figs 2C, 2E). We noted the increase SUMOylation of UBC9 at Lys 49 in the nuclear fraction of both 0.75 h and 16 h (Figs 2D, 2E) and in the cytoplasm at 16 h (Fig. 2C). We also observed the decrease in SUMOylation of STAT1 at Lys 703 in the cytoplasm at 0.75 h and its increase at 16 h following IFNα stimulation (Figs 2B, 2C). To distinguish changes in protein SUMOylation (Fig. 3A) from those associated with protein abundance, we analysed the cytoplasmic and nuclear extracts by western blotting (Fig. 3B). PML and STAT1 are ISG products and their protein expression increased following extended IFNα treatment in HEK293-wt and HEK293-SUMO3m cells (Fig. 3B). Therefore, the enhancement of PML and STAT1 SUMOylation at 16 h (Fig. 3A)
could be due in part to the increase of their protein levels. In contrast, IFNα treatment significantly increased UBC9 SUMOylation without altering its total protein level (Fig. 3B).

Due to the variation in the mixing of the three SILAC channels during the sample preparation stage the changes in SUMOylation site abundance in response to IFNα were obtained from the MaxQuant normalized SILAC ratios, which may have led to the loss of some regulated SUMO sites. Indeed, the normalization of the SILAC ratios may have forced SUMO sites that were weakly regulated in response to the treatment to have a greater p-value. However, we found that the error in SILAC mixing was consistent in all three technical replicates, which suggests that the workflow adopted here was reproducible (Figs. S2-S4).

**SUMOylation of endogenous PML, STAT1 and pSTAT1**—Although we did not detect an increase in PML SUMOylation at short IFN treatment by quantitative proteomics analyses (Fig. 2), we evaluated changes in endogenous protein SUMOylation in Ni-NTA-purified extracts from wt and SUMO3m cells treated with IFNα or IFNγ for 0.75 h. PML immunoblots of Ni-NTA affinity-purified extracts from HEK293-SUMO3m cells showed that PML was SUMOylated and that IFNα or IFNγ enhanced PML SUMOylation (Fig. 3C), with a further increase in PML modification when IFNα or IFNγ was combined with the proteasome inhibitor MG132.

Recently, we reported that SUMO3 expression in HeLa cells showed a decrease in STAT1 phosphorylation without affecting the transcriptional response when cells were stimulated with IFNα (7). Here, we observed a similar decrease in IFNα-induced STAT1 phosphorylation in HEK293-SUMO3m compared to HEK293-wt cells (Fig. 3D). STAT1 was found conjugated to SUMO3 in HEK293-SUMO3m cells, consistent with our proteomic analysis (Fig. 2B), and a short IFNα treatment (0.75 h) reduced slightly the level of STAT1 SUMOylation (Fig. 3D). As expected, phosphorylation of STAT1 was observed
only in response to IFNα, and immunoblots of Ni-NTA-purified extracts from HEK293-SUMO3m using anti-pSTAT1 antibodies revealed that pSTAT1 was conjugated to SUMO (Fig. 3D). To determine the stability of the corresponding proteins, we performed Ni-NTA-purification of cytoplasmic and nuclear extracts from HEK293-SUMO3m cells with and without proteasome inhibition for 4 h before adding IFNα for 0.75 h (Fig. 3E). Analysis of the inputs revealed that pSTAT1 was detected in the cytoplasm and the nucleus (Fig. 3E), though pSTAT1 migrated at a lower molecular weight in the latter fraction. This shift was associated with pSTAT1 SUMOylation since the corresponding band was only observed in the cytoplasmic fraction of the Ni-NTA purified extract of IFNα-treated cells (Fig. 3E). Furthermore, SUMOylated pSTAT1 was not stabilized with MG132, suggesting that SUMOylation did not lead to the degradation of pSTAT1. Immunoblots analyses of the inputs and Ni-NTA fractions also revealed that most SUMOylated substrates in IFNα-treated SUMO3 cells were nuclear proteins (Fig. 3E).

Previous reports indicated that IFNα induced p53-dependent apoptosis (21, 22) and that modification of p53 at K386 by SUMO1 induces p53-dependent transcription (23). Immunoblot of p53 following Ni-NTA purification of control and IFNα-treated HEK293-SUMO3m proteins revealed that p53 was SUMOylated in HEK293-SUMO3m cells, and that this modification was enhanced after 0.75 h of IFNα treatment (Fig. S5A). The increased p53 SUMOylation in SUMO3m-expressing cells was also accompanied by a positive regulation of IFNα-induced activation of p53 target gene p21 (Figs S5B, S5C) and a higher IFNα-induced apoptosis (Fig. S5D), suggesting that enhanced p53 SUMOylation in response to IFNα plays a role in this process.

*PML is required for IFN-induced global SUMOylation and UBC9 transfer to the nuclear matrix-*

PML has been reported to exert a SUMO E3 ligase activity, and may mediate the SUMOylation of many PML NB interacting proteins (24). As PML plays a key role in the IFN response (25, 26), we next examined whether the increase of protein SUMOylation in response to IFN requires PML. Accordingly,
we analyzed the changes in SUMOylation following IFN treatment in wt mouse embryonic fibroblasts (MEFs) and PML -/- MEFs untreated or treated with IFNα for a short period (0.75 or 1 h) (Fig. 4A) or with IFNα or IFNγ for a longer period (16 h) (Fig. 4B). In all treatments, IFNs enhanced SUMO2/3 conjugates in wt MEFs but not in PML -/- MEFs. Similarly, IFNα and IFNγ promoted the increase of cellular SUMO1 modification in the presence of PML, and extended IFN treatment increased the levels of PML in wt MEFs (Fig. 4B).

These results suggest that endogenous PML is required to enhance cellular SUMOylation in response to type I and II IFNs. Next, we investigated whether a specific PML isoform is implicated in the increase of SUMOylation in response to IFN. Several PML isoforms generated by alternative splicing from a single gene are designated PMLI to PMLVII (27, 28). They share the same N-terminal region, which encodes the RBCC/TRIM (RING finger, B-box, and Coiled-Coil) motif, but differ in their C-terminal region as a result of the alternative splicing. PML -/- MEFs transduced with retroviral vectors expressing each one of the seven human PML isoforms (PMLI to PMLVII) were then treated with IFNα to determine the role of the various C-terminal regions of PML. The extent of protein SUMOylation was found to vary significantly for the different PML isoforms examined (Figs 4C, 4D). IFNα was unable to stimulate global cellular SUMOylation in PML -/- cells expressing PMLI, PMLII, PMLV, PMVI or PMLVII (Fig. 4C). Also, none of these PML isoforms increased SUMO2/3 conjugates in untreated cells. In contrast, cellular SUMOylation was stimulated in untreated PML -/- cells expressing PMLIII or PMLIV with a further increase upon IFN treatment (Fig. 4D). Collectively, these results show that the enhancement of cellular SUMOylation by IFN required endogenous PML and that this process is orchestrated by PMLIII and PMLIV.

UBC9, the unique E2 SUMO conjugating enzyme, is known to interact with the RING finger domain of PML (29), a protein acting as a SUMO E3 ligase (24). The observation that UBC9 is differentially
SUMOylated upon IFNα stimulation and that this treatment enhanced protein SUMOylation via PML, prompted us to examine the interrelationship between PML and UBC9. Accordingly, we analyzed by immunofluorescence (Fig. 5A) and immunoblotting (Fig. 5B) the localization and expression of UBC9 in wt MEFs and PML-/- MEFs untreated or treated with IFNα. Analysis by confocal microscopy shows that UBC9 was found in the nucleus and the cytoplasm of untreated cells (Fig. 5A). In untreated wt MEFs expressing endogenous PML, UBC9 formed nuclear dots distinct from those of PML NBs. IFNα treatment, which enhanced PML SUMOylation at 0.75 h (Fig. 3C) and 16 h (Fig. 3A) as well as PML expression at 16 h (Figs 3C, 4B), resulted in the increase of PML NB size, where PML and UBC9 partly colocalized (Fig. 5A). In contrast, UBC9 was localized in the nucleoplasm and the cytoplasm of control and IFNα-treated PML-/- MEFs (Fig. 5A).

To determine whether IFNα treatment induced the transfer of UBC9 to the nuclear matrix, and whether PML is implicated in this process, we examined the subcellular localization of UBC9 and PML in untreated and IFNα-treated wt MEFs and PML-/- MEFs. Cells were fractionated into cytoplasmic, RIPA soluble (nucleoplasm) and RIPA-insoluble fractions (nuclear matrix and some chromatin components). In PML-/- MEFs, UBC9 was found in the cytoplasmic and nucleoplasmic fractions of control or IFNα-treated cells (Fig. 5B). Similar localization of UBC9 was observed in untreated wt MEFs, where PML was mainly localized in the nucleoplasm fraction with a small fraction in the nuclear matrix (Fig. 5B). Remarkably, IFNα treatment, which enhanced PML expression and its conjugation to SUMO, shifts UBC9 toward the nuclear matrix (Fig. 5B). To determine whether the expression of PMLIII was able to recruit UBC9, PML-/- MEFs expressing PMLIII were treated with murine IFNα. As observed in Figure 5C, PMLIII was able to recruit endogenous UBC9 within PML NBs, where both proteins colocalized in untreated and IFNα-treated MEFs. PML NBs became larger upon IFNα treatment due to PML SUMOylation and the recruitment of PMLIII partners. Taken together, our results show that IFNα increased PML expression, its conjugation to SUMO3, and the transfer of UBC9 to the nuclear matrix where UBC9 and PML colocalized within PML NBs.
DISCUSSION

IFNs play essential roles in modulating immune response against host infections via the induction of ISGs through the JAK/STAT pathway. This is achieved in part through protein modifications of key regulator of the IFN signalling transduction machinery. In particular, SUMOylation is an important modification implicated in intrinsic and innate immunity regulating IFN production, IFN signalling, the localization and the activity of many ISG products (18). Although SUMO decreases STAT1 phosphorylation in response to IFNα, it does not alter STAT2 phosphorylation, or the formation of an ISGF3-like complex responsible for binding to ISRE and inducing transcriptional and biological responses (7). Accordingly, accumulating evidences support the existence of alternative STAT2 signalling pathways that are independent of STAT1 (30, 31). We show here first that in HEK293-SUMO3m cells, STAT1 but not STAT2 activation is lower in response to IFNα compared to wt cells. Second that p53 SUMOylation increases upon IFNα treatment, correlates with higher levels of p21 protein expression, enhanced apoptosis and as previously shown induction of cellular senescence (32). Taken together these experiments confirmed the functionality of the His6-SUMO3 mutant in response to IFNα.

Importantly, we report here that in response to IFNα, cellular SUMOylation was enhanced at a short period (0.75 h) and reached a maximum 16 h post-treatment. The global increase in protein SUMOylation requires PML. Previous reports indicated that SUMO3 expression alone did not alter PML protein levels, though its proteasome-dependent degradation was noted for IFNα treatment beyond 18 h (7). This observation is consistent with the decreased protein SUMOylation observed here when cells were exposed to IFNα for 24 h (Fig.1). The identification of SUMOylated proteins in the cytoplasm and the nucleus at short and long IFNα treatment revealed that a large proportion of the SUMO proteome was regulated by IFNα (172 out of 558 SUMO sites). Among the identified proteins, some are ISG products (e.g. PML, STAT1, ADAR1, Vimentin) and others are regulators of IFN signalling or IFN production (e.g. PML, STAT1, Tif1α/TRIM24, TRIM28, TRIM33). In addition, we found that most of the
proteins from the SUMOylation machinery (e.g. UBC9, SUMO1, SUMO2, SUMO3, PIAS1, PIAS2, RanBP2, TRIM28, PML) are SUMOylated in response to IFNα.

UBC9 is SUMOylated at K14, K18, K49, K65 and K154 in vitro and its SUMOylation at K14 displayed enhanced binding to SIM-containing proteins (33, 34). However, from this list only SUMOylation at K49 was identified in vivo. We identified SUMOylation at K48 and K49 of UBC9 and have shown that IFNα enhanced UBC9 SUMOylation at K49 at an early time point in the nucleus and increased SUMOylation at K49 in both the cytoplasm and nucleus during prolonged IFNα treatment without altering its protein level. In untreated cells, UBC9 is localized in the cytoplasm and the nucleoplasm, but migrates to the nuclear matrix in response to IFNα in a PML-dependent manner. PML and PML NBs play a key role in the IFN response (25, 35-37). SUMOylation of PML is critical for the formation and function of PML NBs and it affects PML localization, stability and ability to interact with other partners. In addition, PML has SUMO E3 ligase activity, which may mediate the SUMOylation of many PML NB-associated proteins (24). It is noteworthy that PML negative cells have a defect in IFN-induced biological response (38) as well as in IFN-induced global cellular SUMOylation. Many restriction factors and key regulators of IFN pathway are SUMOylated and required this modification for their functions (18). Remarkably, introducing PMLIII or PMLIV in PML negative cells restores IFN-enhanced global cellular SUMOylation and therefore IFN functions.

The present study indicates that endogenous PML is required for increased SUMOylation in response to IFNα or IFNγ, and that this process is mediated by PMLIII and PMLIV. The mechanism by which the isoform-specific protein sequences enhance cellular SUMOylation is unclear. It is likely that they bind factors that enhance or interfere with the IFN pathway. Nuclear PML is considered to be the organizer protein of the PML NBs. All six human nuclear PML isoforms (PMLI-PMLVI) are able to form NBs when expressed in PML-negative cells (39). Although PML isoforms may have related functions due to their common functional RBCC/TRIM domain, increasing evidences suggest that the variability in the C-terminal region confers specific functions to each PML isoform (28). It has been reported that PMLIV
acts as a SUMO E3 ligase enhancing the SUMOylation of various proteins (24). We show here that PMLIII or PMLIV expression stimulated overall SUMOylation in untreated PML-/− cells and are critical for IFN increased global cellular SUMOylation. The RING domain and the B-boxes, shared by all PML isoforms, are likely required for the E3 activity of PML (24). A SUMO E3 ligase is expected to bind both UBC9 and its substrates. PML interacts with UBC9 via its RING motif, the C-terminal region specific to PMLIII and PMLIV could be implicated in the interaction with the various substrates and/or to a higher recruitment of UBC9 to PML NBs. We report here that expression of PMLIII in PML negative cells was able to recruit UBC9 to PML NBs where both proteins colocalized. Further investigations are needed to determine whether the UBC9 recruitment to PML NB is specific to PMLIII and PMLIV.

IFNs directly induce the PML gene resulting in the increase of different PML isoforms (40, 41). This effect could be partly responsible for the enhanced cellular SUMOylation observed during longer periods of IFN treatment. In addition, we show that IFNα induced the SUMOylation of PML and UBC9, and favoured the translocation of UBC9 to the nuclear matrix to promote their co-localization within PML NBs. These findings suggest that PML and UBC9 act in a cooperative manner to enhance cellular SUMOylation upon IFNα stimulation, further demonstrating that PML NBs are a hub for protein SUMOylation.

Collectively, our findings lead to the following conclusions which are illustrated in Figure 6: (i) IFN enhances cellular SUMOylation in a PML-dependent manner as early as 0.75 h post-treatment, with an increase of PML and UBC9 SUMOylation; (ii) IFN induces PML gene transcription resulting in an increase of PML isoforms 16 h post-treatment; (iii) IFN induces PML-dependent UBC9 translocation to the nuclear matrix, leading to its recruitment to PML NBs; The increase of PML expression and the recruitment of UBC9 within PML NBs promotes the enhancement of SUMOylation in response to IFN; (iv) PMLIII and PMLIV are key players of the IFN-induced increase of cellular SUMOylation. These novel findings provide further biological insights into the SUMO pathway, the contribution of PML and IFN
response.
EXPERIMENTAL PROCEDURES

**Materials and Reagents**-Recombinant human IFNα2 was from Schering (USA), human IFNγ from Roussel Uclaf (Romainville, France), recombinant murine IFNα and IFNγ from R&D Systems. Mouse monoclonal anti-PML (sc-966) antibody and rabbit polyclonal antibodies raised against PML (Sc-5621), STAT1 (sc-345), STAT1 phosphotyrosine 701 (sc-7988), PKR (sc-707), p53 (sc-126), and SUMO1 (sc-9060) as well as peroxidase-coupled secondary antibodies were from Santa-Cruz. Rabbit anti-STAT2, anti-STAT2 phosphotyrosine 689 and mouse p21 antibodies were obtained from Upstate. Mouse monoclonal anti-PML antibody recognizing murine PML (clone 36.1-104) was from Merckmillipore. Rabbit anti-UBC9 (4786) and rabbit anti-SUMO2/3 (4971) antibodies were from cell signalling, mouse anti-6His antibodies was from Clontech. Actin was detected using HRP-conjugated monoclonal anti-Actin antibody (Sigma). For immunofluorescence analyses, secondary antibodies conjugated to Alexa Fluor were purchased from Molecular Probes.

PureProteome™ Protein A/G Mix Magnetic Beads (LSK MAGAG10) was purchased from EMD Millipore (Ottawa, ON, Canada). Dimethyl pimelimidate (DMP) cross-linking reagent (21666) was obtained from ThermoFisher scientific (Burlington, ON, Canada). Modified porcine sequencing grade modified Trypsin was obtained from Promega (Madison, WI, USA). Acetonitrile (ACN) was purchased from Fisher Scientific (Whitby, ON, Canada). Ammonium bicarbonate and formic acid were obtained from EM Science (Mississauga, ON, Canada). 2-mercaptoethanol, trifluoroacetic acid (TFA), 2-chloroacetamide, protease inhibitor cocktail (4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), pepstatinA, E-64, bestatin, leupeptin, and aprotinin), phosphatase inhibitor cocktail (sodium vanadate, sodium molybdate, sodium tartrate, and imidazole) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Bradford protein reagent was obtained from Bio-Rad (Mississauga, ON, Canada). Tris base was purchased from EMD Omnipur (Lawrence, KS). Phosphate buffered saline (PBS) was obtained from HyClone (Thermo Scientific, Logan, UT). Oasis HLB cartridges (1cc, 30 mg) were purchased from Waters (Milford, MA). ECL chemiluminescence detection system was purchased from Amersham Pharmacia Biotech (Montreal, QC, Canada). Solvents for chromatographic analysis were all HPLC grade (Fisher
Scientific and in-house Milli-Q water). Capillary HPLC columns for nano-LC-MS were packed in-house using Jupiter C18 (3 μm) particles from Phenomenex (Torrance, CA), and fused silica tubing from Polymicro Technologies (Phoenix, AZ).

**Cells and lentiviral vectors**-HEK293 cells (obtained from ATCC), wild type murine embryonic fibroblasts (MEFs) and PML/- MEFs (38), immortalized by the SV40 large T antigen were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum. HEK293 cells stably expressing 6xHis-SUMO3-Q87R-Q88N (SUMO3m) were obtained as previously described (19) and maintained in DMEM supplemented with 10% fetal calf serum and neomycin (0.5 mg/ml). Lentiviral vectors encoding human PML isoforms (PMLI to PMLVII) were constructed by inserting each ORF in the HIV-1-derived pTRIP vector.

**Experimental Design and Statistical Rationale**-The quantitative analysis of changes in the SUMO proteome upon IFNα stimulation were conducted in technical triplicates. The light channel was used as an internal non-stimulated control while the medium and heavy channels were treated for 0.75 h and 16 h with IFNα, respectively. The combined SILAC samples were split into three technical replicates after the cell fractionation step. The Ni-NTA purification, tryptic digestion, peptide desalting and SUMO peptide IP experiments were performed in a parallel fashion. Each technical replicate was injected once on the LC-MS system. Technical triplicates were conducted to obtain statistically robust data that could be assessed using one-sample Student T-tests due to the normal Gaussian distribution of the log₂ of the SILAC fold-change values. Due to the amount of starting material needed per biological replicate (500 000 000 cells per condition per replicate) and the cost of the SILAC media and anti-NQTGG antibody, multiple biological replicates was not possible.

**Cytosolic and nuclear Fractionation, protein purification and anti-NQTGG Immunoprecipitation**- 500 million HEK293-SUMO3m cells were washed twice with 10 mL of ice cold PBS and fractionated into cytosolic and nuclear fraction in 15 mL of LSB buffer (10 mM Tris-HCl, 1.5
mM MgCl₂, 20 mM 2-chloroacetamide, pH 7.6 supplemented with protease and phosphatase inhibitors). Samples were incubated 30 min under gentle agitation. Nuclei were pelleted by centrifuging samples at 215 g for 15 min at 4 °C. The supernatant was recovered and constituted the cytosol. The nuclear pellet was washed with 15 mL of LSB and then lysed in 40 mL of Ni-NTA denaturing incubation buffer (6 M Guanidinium HCl, 100 mM NaH₂PO₄, 10 mM Tris-HCl, 20 mM 2-Chloroacetamide, 5 mM 2-Mercaptoethanol, pH 8) prior to sonication. Protein content was determined using micro Bradford assay according to the manufacturer’s instruction.

For each of the technical triplicates, 24 mg of cytoplasmic or nuclear protein were incubated with 960 μL of Ni-NTA beads for 16 h at 4 °C. Ni-NTA beads were washed once with 10 mL of Ni-NTA denaturing incubation buffer, 4 times with 10 mL of Ni-NTA denaturing washing buffer (8 M urea, 100 mM NaH₂PO₄, 10 mM Tris–HCl, 20 mM imidazole, 5 mM 2-Mercaptoethanol, 20 mM 2-Chloroacetamide) and finally twice with 5 mL of 100 mM ammonium bicarbonate. The protein content was determined by micro Bradford assay. On beads protein digestion was performed by adding trypsin to a ratio 1:50 (w:w) Trypsin:Protein extract for 4 h at 37 °C. Resulting Ni-NTA enriched digests were acidified by adding TFA to a final concentration of 1%, desalted on 1cc HLB cartridges as per the manufacturer’s instructions and eluted into microfuge tubes prior to their lyophilization by Speed Vac.

For each samples, 163 μL of PureProteome protein A/G magnetic beads (1 μL of slurry per 4 μg of Ni-NTA purified protein) were equilibrated with 326 μg of anti-K(NQTGG) antibody (2 μg of antibody per 1 μL of protein A/G slurry) for 1 h at 4 °C in PBS. Saturated beads were washed 3 times with 200 mM triethanolamine, pH 8.3. 1.63 mL of 5 mM DMP in 200 mM triethanolamine, pH 8.3 was added per sample and incubated 1 h at room temperature to crosslink the antibody to the beads. The reaction was quenched for 30 min by adding 81 μL of 1 M Tris-HCl, pH 8. The beads were washed 3 times with ice cold PBS and once with PBS containing 50% glycerol and stored at -20 °C until future use. The lyophilized peptides were reconstituted in 500 μL of PBS containing 50% glycerol by vortexing for 10 min at the highest speed. The anti-K(NQTGG) bound beads were added to the peptide mixture and
incubated 1 h at 4 °C. The beads were washed once with 1 mL PBS containing 50% glycerol, 6 times with 1 mL of ice cold 1X PBS, once with 1 mL of 0.1X PBS and once with water. Peptides were eluted from the beads with 3 successive elutions using 200 µL of 0.2% formic acid in water and filtered through a 0.45 µm spin tube. Eluted peptides were dried down by speed vac and stored at -80 °C for MS analysis.

**Mass Spectrometry analysis**-Peptides were reconstituted in 25 µL of water containing 0.2% formic acid and 10 µL of this mixture were injected by nanoflow-LC-MS/MS using an Orbitrap Fusion Mass spectrometer (Thermo Scientific) coupled to a Proxeon Easy-nLC 1000. Samples were separated directly on a 150 µm x 20 cm nano LC column (Jupiter C18, 3 µm, 300 A, Phenomenex) without a trapping column. The separation was performed on a linear gradient from 7% to 30% acetonitrile, 0.2% formic acid over 105 min at 600 nl/min. Full MS scans were performed on ion from m/z 350 to m/z 1500 at resolution 120 000 at m/z 200, with a target AGC of 5E5 and a maximum injection time of 200 ms. MS/MS scans were acquired in HCD mode with a normalized collision energy of 25 and resolution 30 000 using a Top 3 sec method, with a target AGC of 5E3 and a maximum injection time of 3000 ms. The MS/MS triggering threshold was set at 1E5 and the dynamic exclusion of previously acquired precursor was enabled for 20 s within a mass range of +/- 0.8 Da. Ions with charges >10 and <2 were excluded from triggering MS2 events.

**Data Processing**-MS/MS spectra were searched against Uniprot/SwissProt database including Isoforms (released on March 10, 2015, 42 084 entries) using MaxQuant (version 1.5.1.2) (42). The precursor ion tolerance was set to 20 and 4.5 ppm for the first and main searches, respectively. The MS/MS spectra search were set to a mass tolerance of 20 ppm. The maximum missed cleavage sites were set to 2 using trypsin/P as protease. Carbamidomethylation (C) was set as fixed modification and acetylation (Protein N term and K), phosphorylation (S), oxidation (M), deamination (NQ), and NQTGG (K) were set as variable modifications. Searches were conducted with the match between runs
function enabled with a 20 min alignment window and 0.7 min match time window. Identified proteins and SUMO sites were filtered with a 1% FDR using the reverse database as the decoy. MS/MS spectra for modified peptides with an Andromeda score below 40 (default values) were discarded from further analysis.

The MaxQuant output files were processed using the R-software. Possible improper SUMO site identification were filtered out by removing all “potential contaminants”, “reverse” sites from the list. Furthermore, the SUMO site list was filtered with a SUMO site probability score of 0.75 or greater, which is routinely used for PTM site identification methodologies. The reported “normalized” SILAC ratios generated by the MaxQuant software were Log₂ transformed prior to their statistical analysis. Imputations were employed for SUMO sites that were quantified in 2 of 3 triplicates using normally distributed values with a randomized 0.3 width (Log₂) and a 1.8 down shift (Log₂). Sites were deemed statistically regulated by IFNa if their p-values/fold-change combination met the permutation-based FDR of 5%, which was corrected using the significance analysis of microarrays method (S₀, correction factor) to consider the standard deviation of the data set (43). The volcano plots shown in Figure 2 panels b-e, were created by using the one-sample T-test results on the y-axis (-log₁₀(p-value)) and the Log₂FC between the IFNa treated samples compared the control samples. The corresponding dashed lines on the volcano plots depict the boundaries of statistically regulated SUMO sites based on an FDR of 5% that is adjusted using the S₀ correction factor.

**Bioinformatic analysis**-Protein networks were created using STRING database with experimentally mapped interaction with a medium confidence of 0.4 (44). Networks were generated using all identified SUMOylated proteins in this study. Cytoscape 3.2.0 was used to visualize the network (45). Gene ontology (GO) term enrichments were performed in cytoscape with Bingo 3.0.3 using a benjamini-hochberg corrected p-value below 0.01 as the cutoff for statistically significantly regulated terms (46). The following terms were analyzed versus the human proteome: Biological Processes (GOBP), Molecular Functions (GOMF), and Cellular Compartments (GOCC).
Immunoﬂuorescence analyses—Cells were grown on 12mm slides and ﬁxed with 4% paraformaldehyde for 15 min, rinsed in PBS, incubated in NH₄Cl 50 mM for 10 min and permeabilized with 0.5% BSA / 0.3% Triton X100 / 2% Normal Goat Serum for 30 min. Cells were then incubated with primary antibodies at room temperature for 30 min. Slides were rinsed in PBS 0.5% BSA and incubated at RT in the dark for 30 min with secondary antibodies and rinsed in PBS 0.5% BSA. Finally, slides were washed in PBS, counterstained with Hoechst 33342 and mounted in Fluoromount-G medium. Images were digitally acquired with a Zeiss LSM 710 confocal Microscope.

Western blot analysis of total cell extracts and Cytoplasmic, RIPA soluble and insoluble fractions—For total cell extracts, cells were washed in PBS, lysed in hot Laemmli sample buffer, and boiled for 10 min. The cytoplasmic fraction was obtained by lysing the cells in Tris-HCl 10m M pH 7.6, MgCl2 1.5 mM 0.5%, NEM 20mM, DTT 1mM 20 min at 4°C. Cells were then centrifuged at 500 g for 15 min to separate nuclei (pellet) from the cytosol (C). The RIPA soluble fraction was extracted by incubating the pellet for 20 min on ice in RIPA buffer (50 mM Tris, pH 7.5, 200 mM NaCl,1% Triton X-100/1%, deoxycholate 0.5%, SDS 0.1%, 1 mM EDTA), followed by centrifugation at 15000 g for 15 min to separate the RIPA soluble fraction (R) from the pellet (P). The RIPA insoluble fraction (P) was washed two times in RIPA buffer, suspended in PBS and boiled in Laemmli buffer. Protein extracts were separated by SDS-PAGE followed by electroblotting onto nitrocellulose membrane. After blocking of non-speciﬁc binding sites with 5% non-fat milk, the membranes were incubated with primary antibody (SUMO1, SUMO2/3, PML, PKR, pSTAT1, pSTAT2, STAT1, STAT2, p53, UBC9, p21 or Actin) followed by horseradish peroxidase-conjugated secondary antibody. The secondary antibody was detected with the ECL chemiluminescence detection system.

Real-time PCR—Total RNAs were extracted using RNeasy Mini Kit (Qiagen) following manufacturer’s instructions. RNA samples were converted to cDNA using RevertAid H Minus First
Strand cDNA Synthesis Kit (Thermo Scientific). Real-time PCR reactions were performed in duplicates using 5 μl of cDNA diluted 10 times in water using Takyon ROX SYBR MasterMix blue dTTP (Eurogentec). The following program was used on a 7900HT Fast Real-Time PCR System (Applied Biosystems): 3 min at 95°C followed by 35 cycles of 15 s at 95°C, 25 s at 60°C and 25 s at 72°C. Values for each transcript were normalized to expression levels of RPL13A (60S ribosomal protein L13a) using the 2-ΔΔCt method. Primers used for quantification of transcripts by real time quantitative PCR are as followed: 5’-AGGGATGGGGTGATAG-3’ and 5’-GGGTATATGATGGGACGTAG-3’ for p21.

Apoptosis-Cell apoptosis was assessed using Annexin V-FITC/PI Kit (BD Biosciences). Briefly, HEK293-wt and HEK293-SUMO3m were untreated or treated with 1000 U/mL of IFNα for 72 h. Cells were collected, resuspended in 100 μL of PBS and stained with Annexin V for 15 min at 4°C, followed by PI staining. Fifty thousand cells were analyzed by flow cytometry on a FACSCalibur (BD Biosciences).
AUTHOR CONTRIBUTIONS

M.A.M., G.M., F.L., F.P.M. conducted the experiments. M.K.C-A. and P.T. wrote the manuscript, developed the concept and managed the project. All authors approved the content and submission of the paper.

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The authors declare no competing financial interests. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the massIVE partner repository which can be accessed at the following location ftp://MSV000081525@massive.ucsd.edu with the following password grthibault.
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Figure legend

FIG. 1. *IFN*α enhances SUMOylation at 0.75 and 16 h post-cell treatment. A, Total cell extracts from HEK293-SUMO3m cells treated with IFN*α* at different times were purified on Ni-NTA-agarose beads. The inputs and the purified extracts were analyzed by Western blotting using anti-SUMO2/3 antibodies. B, Total cell extracts from HEK293-wt and HEK293-SUMO3m cells treated for 0.75 or 16 h with IFN*α* were analyzed by Western blot for pSTAT1, pSTAT2, STAT1, STAT2, PKR and Actin.

FIG. 2. SUMO site identification workflow and Volcano plots of IFN*α* induced changes in the SUMO proteome. A, Workflow adopted for SUMO3 site quantification. HEK293-SUMO3m cells were either mock treated with PBS (light channel), treated with 1000 Units/mL of IFN*α* for 0.75 h (medium channel) or 16 h (heavy channel). Cells were harvested, fractionated under hypotonic conditions and proteins from each channel were combined 1:1:1 based on protein content. The proteins were subjected to Ni-NTA purification, followed by trypsin digestion and subjected to immunopeptide level enrichment with an antibody recognizing the NQTGG SUMO3m remnant. The enriched peptides were analyzed on a Fusion mass spectrometer and data analyzed using MaxQuant as indicated in the methods section. Volcano plot of the SUMO site abundance changes in the cytoplasm for cells treated with IFN*α* for (B) 0.75 h and (C) 16 h. Volcano plot of the SUMO site abundance changes in the nucleus for cells treated with IFN*α* for (D) 0.75 h and (E) 16 h. See also Fig. S5 and Table S1.

FIG. 3. Increased SUMOylation of PML, STAT1 and UBC9 by IFN*α*. A, Relative changes in abundances of PML, STAT1 and UBC9 SUMOylation in response to IFN*α*, as determined by quantitative mass spectrometry. Means and standard deviations of three technical replicates are shown. B, Relative changes in PML, STAT1 and UBC9 protein expression in response to IFN*α*, as determined by Western blot. C-E, PML, STAT1 and pSTAT1 SUMOylation as determined by Ni-NTA. C, HEK293-wt and HEK293-SUMO3m cells untreated or treated with the proteasome inhibitor for 4 h, were incubated with IFN*α* or IFN*γ* for 0.75 h, immunoblots of Ni-NTA-purified extracts were performed using anti-SUMO2/3 or anti-PML antibodies. D, HEK293-wt and HEK293-SUMO3m cells were untreated or treated with IFN*α*.
for 0.75 h. Inputs and immunoblots of Ni-NTA-purified extracts were performed using anti-STAT1 or anti-pSTAT1 antibodies. E, HEK293-wt and HEK293-SUMO3m cells untreated or treated with the proteasome inhibitor for 4 h, were incubated with IFNα for 0.75 h. Inputs (upper panel) and immunoblots of cytoplasmic and nuclear Ni-NTA-purified extracts (lower panel) were analyzed by Western blot using anti-His, anti-H3 and anti-pSTAT1 antibodies. See also Fig. S6.

**FIG. 4. PML is required for IFN-enhanced SUMOylation.** A, B, wt MEFs and PML-/- MEFs were untreated or treated with murine IFNα for short (0.75 and 1 h) (A) or long (16 h) (B) periods and their extracts were analyzed by Western blot for SUMO2/3, SUMO1, PML or Actin. C, D, PML-/- MEFs were transduced with HIV-1 derived lentiviral vectors expressing each human PML isoform (C) (PMLI, PMLII, PMLV, PMLVI or PMLVII), (D) (PMLIII or PMLIV). Two days post-transduction, PML expressing cells were untreated or treated with murine IFNα for 16 h and their extracts were analyzed by Western blot for SUMO2/3, PML and Actin.

**FIG. 5. IFN enhances the recruitment of UBC9 to PML NBs.** A, wt MEFs and PML-/- MEFs untreated or treated with murine IFNα for 0.75 or 16 h were analyzed by confocal microscopy for UBC9 (green) and PML (red) staining. PML was stained with mouse monoclonal anti-PML antibody (clone 36.1-104, Merck millipore) that recognized murine PML. B, wt MEFs and PML-/- MEFs were untreated or treated with murine IFNα for 16 h. Cytoplasmic (C), RIPA soluble (R) and RIPA insoluble (P) fractions were analyzed by Western blot with anti-UBC9 and anti-PML antibodies. The Coomassie brilliant blue (CBB)-stained proteins are shown. C, Expression of human PMLIII in PML-/- MEFs is able to recruit endogenous UBC9. Two days post-transduction with PMLIII, PML-/- MEFs were untreated or treated with murine IFNα for 16 h and analyzed by confocal microscopy for UBC9 (green) and PML (red) staining. PML was stained with mouse monoclonal anti-PML antibody (sc 966, Santa Cruz) that recognized human PML.
FIG. 6. Model for enhanced cellular SUMOylation by IFN. IFN treatment at 0.75 h increases PML-dependent cellular SUMOylation, with an enhancement of PML and UBC9 SUMOylation; (ii) IFN induces PML-dependent UBC9 shift to the nuclear matrix, resulting in its recruitment to PML NBs; (iii) PMLIII and PMLIV are key players of the IFN-induced increase of cellular SUMOylation; (iv) 16 h post-treatment, IFN increases the expression of PML isoforms. Thus IFN increases UBC9 SUMOylation and its recruitment to PML NBs where UBC9 associates with PML to enhance cellular SUMOylation.
Figure 1

A

| Input       | WT | HEK293-S3m | WT | HEK293-S3m |
|-------------|----|------------|----|------------|
| IFNα (h)    |    | 0.75 16 24 |    | 0.75 16 24 |
|kDa          | 130 | 100 | 70 | 55 | 35 |

Free SUMO3

B

| Input       | WT | HEK293-S3m |
|-------------|----|------------|
| IFNα (h)    |    | 0.75 16 24 |
|             |    | 0.75 16 24 |

pSTAT2
STAT2
pSTAT1
STAT1
PKR
Actin
Figure 2

A

Combine 1:1:1

Cell Fractionation

N-NTA Purification

Tryptic Digestion

Peptide IP

LC-MS/MS

B

\[
\text{IFN 0.75 h Cytoplasm/CTRL [log2]}
\]

C

\[
\text{IFN 16 h Cytoplasm/CTRL [log2]}
\]

D

\[
\text{IFN 0.75 h Nucleus/CTRL [log2]}
\]

E

\[
\text{IFN 16 h Nucleus/CTRL [log2]}
\]
Figure 3

A

Fold Change

Cytoplasm 0.75 h IFNa 16 h IFNa Nucleus 0.75 h IFNa 16 h IFNa

B

Cyto S3 Nuc S3

STAT1

PML

UBC9

HSP90

H3

C

WT HEK293-S3m WT HEK293-S3m

IFNα - + + + - + +

IFNγ - + + + - + +

MG132 - + + + - + +

PML

SUMO3

Input Ni-NTA

D

WT HEK293-S3m

IFNα - + + +

pSTAT1

STAT1

SUMO-pSTAT1

SUMO-STAT1

* Non specific band

E

WT HEK293-S3m WT HEK293-S3m

IFNα + + + + + + +

Input Ni-NTA

pSTAT1

H3

His

100 100

Cytoplasm Nucleus
Figure 6

**Early Response**
- † SUMO on PML
- † SUMO on UBC9 at K49
- † In Global SUMOylation
- UBC9 recruitment to PML NBs

**Late Response**
- † PML protein levels
- † † UBC9 recruitment to PML NBs
- † † Global SUMOylation

![Diagram of IFNα response showing early and late responses with specific molecular interactions and time points]