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PIAS3 induces SUMO-1 modification and transcriptional repression of IRF-1

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Abstract Interferon regulatory factor-1 (IRF-1) is a transcriptional activator that is involved in interferon response, regulation of cell growth and oncogenesis. To try to identify the molecules that regulate the function of IRF-1, we performed yeast two-hybrid screening and isolated protein inhibitor of activated STAT3 (PIAS3) as an IRF-1-binding protein. This protein was also found to bind with small ubiquitin-related modifier-1 (SUMO-1) and ubiquitin-conjugating enzyme 9, an E2 in the SUMO-1-conjugating system. Co-expression of PIAS3 induced SUMO-1 modification of IRF-1 in a RING finger domain-dependent manner and also repressed transcriptional activity of IRF-1. Thus, PIAS3 functions as a SUMO-1 ligase for IRF-1 and also as a repressor of IRF-1 transcriptional activity.

Key words: Interferon regulatory factor-1; Protein inhibitor of activated STAT3; Small ubiquitin-related modifier-1 ligase; Small ubiquitin-related modifier-1 modification; Transcriptional repression

1. Introduction

Interferon regulatory factor-1 (IRF-1), which has been identified as a transcriptional activator of interferon-β [1], regulates the expression of various genes involved in anti-viral and anti-bacterial immune responses, is involved in DNA damage-induced cell cycle arrest in collaboration with the tumor suppressor p53 through induction of CDK inhibitor $p21^{Cip1/Waf1}$, and functions as a negative regulator of cell growth [2]. In addition, analyses of IRF-1/p53 double null mice and patients with acute myeloid leukemia and myelodysplasia suggest that IRF-1 has a tumor-suppressing activity [2].

Small ubiquitin-related modifier-1 (SUMO-1) is a protein that shares 18% identity with ubiquitin and is covalently conjugated to target proteins by a system analogous to the ubiquitin conjugation system: SUMO-1 is activated by E1 enzyme (Aos1/Uba2 heterodimer) in an ATP-dependent manner, is transferred to E2 enzyme (ubiquitin-conjugating enzyme 9, Ubc9), and then attaches to ε-amino groups of specific lysine residues of target proteins [3–5]. Recently, four members of the mammalian PIAS (protein inhibitor of activated STAT) family, PIAS1, PIASxα, PIASxβ and PIASy, and a yeast PIAS homologue, Siz1, have been reported to have SUMO-1 ligase activities toward various target proteins, including p53, androgen receptor, LEF1 and septin [6–12]. In addition, it has been shown that PIAS-mediated SUMO-1 modification of various transcription factors causes up- or down-regulation of their transcriptional activity [8,11,12].

We previously reported that the stability of IRF-1 is governed by the ubiquitin–proteasome system [13]. In a search for proteins that regulate the function and the stability of IRF-1, we identified PIAS3 and Ubc9 as IRF-1-binding proteins. In the present study, we found that PIAS3 mediates SUMO-1 modification of IRF-1 and suppresses IRF-1 transcriptional activity in a RING finger domain-dependent manner. To the best of our knowledge, this is the first report on PIAS3 as a SUMO-1 ligase for the specific target protein. We propose that the function of IRF-1 is regulated by PIAS3 through its SUMO-1 ligase activity.

2. Materials and methods

2.1. Cell culture and transfection

Human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (Gibco) and antibiotics at 37°C under 5% CO_2 atmosphere. Transfection was performed using FuGENE6 (Roche) according to the manufacturer's protocol.

2.2. Plasmid construction

Mouse IRF-1 cDNA [13] was subcloned into the pCI-neo-FLAG plasmid [13], and a fragment containing FLAG-tagged IRF-1 from pCI-neo-FLAG-IRF-1 was subcloned into the pEF-BOS vector (a gift from Dr. S. Nagata of Osaka University) [14] to construct a mammalian expression plasmid of IRF-1, pEF-FLAG-IRF-1. The open reading frames of PIAS3 and UBC9 were amplified by polymerase chain reaction (PCR) with the respective cDNAs obtained from positive clones in yeast two-hybrid screening as templates. The resulting PCR fragment of PIAS3 was subcloned into the pCI-neo-6Myc vector that had been generated by inserting a fragment containing six copies of the Myc epitope into the pCI-neo mammalian expression vector (Promega). Deletion mutants of PIAS3 were generated by PCR and subcloned into the pAD-Gal4 vector (Stratagene). The PIAS3 (C334S) mutant was generated using a QuickChange Site-Directed Mutagenesis kit (Stratagene) and subcloned into the pCI-neo-6Myc vector. pGLex, the LexA-derived bait vector for a yeast two-hybrid assay, and pCGT-T7-SUMO-1, a SUMO-1 mammalian expression plasmid, were gifts from Dr. H. Ariga of Hokkaido University. UBC9 and SUMO-1 cDNAs were subcloned into the pGLex vector for yeast two-hybrid analysis.

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Abbreviations: IRF-1, interferon regulatory factor-1; PIAS3, protein inhibitor of activated STAT3; SUMO-1, small ubiquitin-related modifier-1; Ubc9, ubiquitin-conjugating enzyme 9; PCR, polymerase chain reaction; ISRE, interferon-stimulated response element

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2.3. Yeast two-hybrid screening

pGLex-IRF-1 (1–190) was constructed by fusing the IRF-1 (1–190) coding sequence in-frame to the LexA DNA-binding domain in the pGLex vector. *Saccharomyces cerevisiae* L40 cells transformed with pGLex-IRF-1 (1–190) and a mouse embryonic fibroblast MATCHMAKER cDNA library (Clontech) were plated onto media that lacked tryptophan, leucine and histidine and had been supplemented with 5 mM 3-amino-1,2,4-triazole (Sigma). Approximately 1.5×10^6 colonies were screened for growth in the absence of histidine and for LacZ expression. The activity of β-galactosidase was determined using X-gal as a substrate. Plasmid DNAs derived from double-positive clones were extracted from yeasts, and the nucleotide sequences were determined using an ABI Prism 377 DNA sequencer (Perkin-Elmer).

2.4. Immunoprecipitation and immunoblotting

Human 293T cells were transiently transfected with the indicated plasmids using FuGENE6, and at 36 h after transfection, the cells were treated with 10 μM MG132 (Peptide Institute, Osaka, Japan) for 4 h to inhibit proteasomal degradation of IRF-1. After the cells had been washed with ice-cold phosphate-buffered saline, they were lysed with a buffer containing 50 mM Tris–HCl, pH 7.5, 0.3 M NaCl, 0.5% Triton X-100, complete protease inhibitor cocktail (Roche), 10 mM *N*-ethylmaleimide (Sigma) and 50 μM MG132. The cell lysate was sonicated for 10 s and the debris was removed by centrifugation at $13\,000\times g$ for 20 min. The resulting supernatant was incubated with anti-FLAG M2 affinity gel (Sigma) for 2 h at 4°C, and the immunocomplex produced was washed five times with lysis buffer. For Western blotting, the whole cell lysate and immunoprecipitates were separated by SDS–PAGE and transferred onto nitrocellulose membranes (Bio-Rad). The membranes were immunoblotted with anti-T7 (Novagen), anti-Myc (9E10, Santa Cruz) and anti-IRF-1 (Santa Cruz) antibodies and incubated with horseradish peroxidase-conjugated antibodies against mouse or rabbit immunoglobulin (Amersham), followed by detection with ECL Western blotting detection reagents (Amersham).

2.5. Reporter gene assay

Human 293T cells seeded onto 24-well dishes were transfected with the pISRE-Luc reporter plasmid (Clontech) and with combinations of IRF-1 and wild-type PIAS3 or PIAS3 C334S mutant expression plasmids, together with phRG-TK (Promega) for normalizing transfection efficiency. The total amounts of plasmids were adjusted with empty vectors pCI-neo and pEF-BOS. Twenty-four hours after transfection, luciferase activity was measured by using a Dual-Luciferase Reporter Assay System (Promega) and an AB-2000 luminescencer-PSN (Atto, Tokyo, Japan). The same experiments were repeated three times.

3. Results and discussion

3.1. PIAS3 is an IRF-1-binding protein

To identify proteins that regulate the function of IRF-1, we

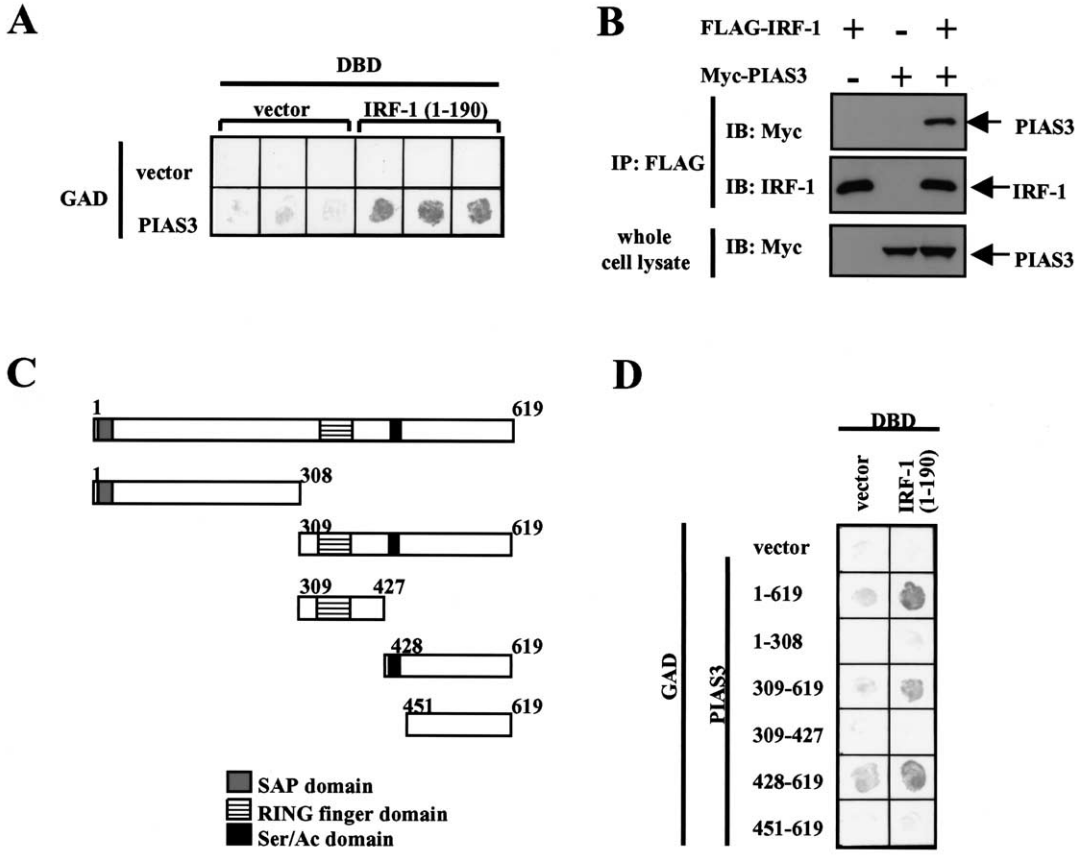

Fig. 1. PIAS3 is capable of interacting with IRF-1. A: Protein interaction in a yeast two-hybrid system. Yeast L40 cells were transformed with the indicated expression vectors containing IRF-1 (1–190) fused to the LexA DNA-binding domain (DBD) and PIAS3 fused to the GAL4 activation domain (GAD). β-Galactosidase activity of each transformant was measured. B: Interaction of IRF-1 and PIAS3 in mammalian cells. 293T cells were transiently transfected with expression vectors containing FLAG-tagged IRF-1 and Myc-tagged PIAS3 as indicated. Cell lysate was subjected to immunoprecipitation (IP) with anti-FLAG antibody, and the resulting precipitates were subjected to immunoblotting (IB) with anti-Myc and anti-IRF-1 antibodies. A portion of the cell lysate was directly subjected to IB with anti-Myc antibody in order to verify the expression level of PIAS3 protein. C: Schematic representation of various PIAS3 constructs. PIAS3 contains a putative chromatin-binding SAP (SAF-A/B, Acinus and PIAS) domain, a RING finger domain and a Ser/Ac (serine- and acidic amino acid-rich) domain. D: Interaction of IRF-1 with PIAS3 deletion mutants in yeast. L40 cells were transformed with the indicated expression vectors containing IRF-1 (1–190) fused to LexA DBD and various deletion constructs of PIAS3 fused to GAD. β-Galactosidase activity of each transformant was measured.

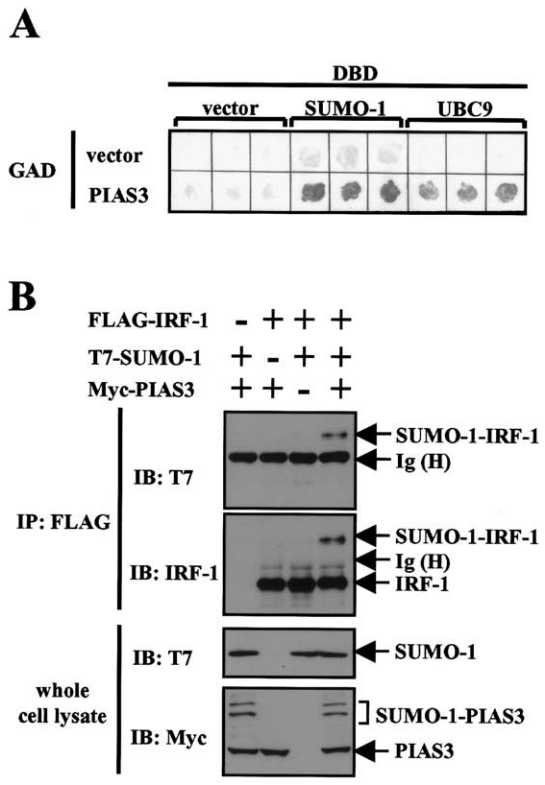

Fig. 2. PIAS3 mediates SUMO-1 modification of IRF-1. A: Interaction of PIAS3 with Ubc9 and SUMO-1. A yeast two-hybrid assay was performed as described in Fig. 1A. B: SUMO-1 modification of IRF-1 by PIAS3 in vivo. 293T cells were transiently transfected with expression vectors containing FLAG-tagged IRF-1, T7-tagged SUMO-1 and Myc-tagged PIAS3 as indicated. Cell lysate was subjected to immunoprecipitation (IP) and then to immunoblotting (IB) as described in Fig. 1B. A portion of the cell lysate was directly subjected to IB with anti-T7 and anti-Myc antibodies in order to verify the expression levels of SUMO-1 and PIAS3 proteins, respectively. Ig (H), heavy chain of immunoglobulin.

performed yeast two-hybrid screening with a mouse embryonic fibroblast cDNA library using the N-terminal region (amino acids 1–190) of IRF-1 as bait (Fig. 1A). A screen of approximately 1.5×10^6 yeast transformants yielded 20 colonies that grew in selective media and expressed LacZ. DNA sequencing analysis revealed that four of these clones were identical to a long form of PIAS3 [15] that contains a 105-bp (35-amino acid) insertion in the N-terminal region (GenBank accession number BC023128).

We next carried out an experiment to determine whether IRF-1 is capable of associating with PIAS3 in mammalian cells. FLAG-tagged IRF-1 and Myc-tagged PIAS3 were transiently expressed in 293T cells and the cell extract was prepared. The extract was subjected to immunoprecipitation using anti-FLAG antibody, and the co-immunoprecipitated PIAS3 was detected by immunoblotting with anti-Myc antibody. As shown in Fig. 1B, PIAS3 was co-immunoprecipitated with IRF-1. These results indicate that IRF-1 and PIAS3 are capable of forming a complex in mammalian cells.

To determine the domain of PIAS3 required for its interaction with IRF-1, we constructed various deletion mutants of PIAS3 (Fig. 1C) and fused them to the Gal4 activation domain. Yeast two-hybrid analysis using IRF-1 (1–190) as bait revealed that the serine- and acidic amino acid-rich domain (Ser/Ac domain) in the C-terminal region of PIAS3 is responsible for the association with IRF-1 (Fig. 1D).

3.2. PIAS3 mediates SUMO-1 modification of IRF-1

In the yeast two-hybrid screening described above, Ubc9 was also identified as a binding partner of IRF-1 (data not shown). Ubc9 functions as an E2 in the SUMO-1-conjugating system [3–5]. In addition, it has recently been reported that PIAS3 interacts with Ubc9 and SUMO-1 in vitro [11]. Our results of yeast two-hybrid analysis also indicated that PIAS3 interacts with Ubc9 and SUMO-1 in yeast (Fig. 2A). From these results, we speculated that PIAS3 has SUMO-1 ligase activity toward IRF-1. To confirm this possibility, we transiently expressed FLAG-tagged IRF-1, T7-tagged SUMO-1 and Myc-tagged PIAS3 in 293T cells. The cell extract was subjected to immunoprecipitation with anti-FLAG antibody, and the immunoprecipitates produced were probed by Western blotting with anti-T7 or anti-IRF-1 antibody (Fig. 2B). One band with slower mobility than that of intact IRF-1 was detected by anti-IRF-1 antibody only in the case of co-transfection with FLAG-IRF-1, T7-SUMO-1 and Myc-PIAS3 expression plasmids, and this band was also detected with anti-T7 antibody. These results suggest that IRF-1 is conjugated with SUMO-1, the conjugation being mediated by PIAS3. Furthermore, in immunoblotting of cell lysate with anti-Myc antibody, two bands with slower mobilities than that of intact PIAS3 were detected only in the presence of SUMO-1, suggesting that PIAS3 is also conjugated with SUMO-1, the conjugation being mediated by PIAS3 itself. This is consistent with the previous reports on SUMO-1 modification of other PIAS family proteins by themselves [11,12]. Thus, PIAS3 mediates SUMO-1 modification of IRF-1 and of itself.

3.3. PIAS3 functions as a SUMO-1 ligase

PIAS family proteins contain a RING finger domain called

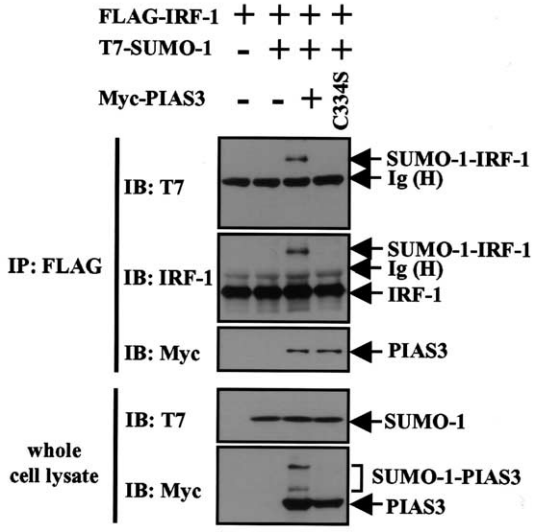

Fig. 3. The RING finger domain of PIAS3 is required for SUMO-1 modification of IRF-1. 293T cells were transiently transfected with expression vectors containing FLAG-tagged IRF-1, T7-tagged SUMO-1 and Myc-tagged wild-type or C334S mutant of PIAS3 as indicated. Cell lysate was subjected to immunoprecipitation (IP) and then to immunoblotting (IB) as described in Fig. 1B. A portion of the cell lysate was directly subjected to IB as described in Fig. 2B. Ig (H), heavy chain of immunoglobulin.

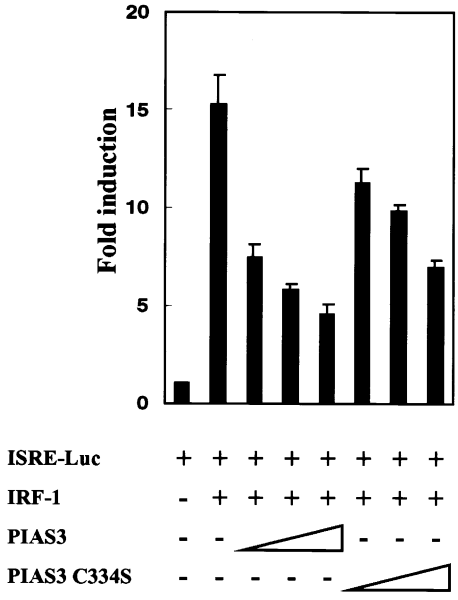

Fig. 4. PIAS3 represses transcriptional activity of IRF-1. 293T cells were transiently transfected with expression vectors containing FLAG-tagged IRF-1 (100 ng) and increasing amounts of Myc-tagged wild-type or C334S mutant of PIAS3 (50, 100 and 200 ng), together with the pISRE-Luc reporter vector containing multimerized ISRE and the phRG-TK vector that expresses *Renilla* luciferase under the HSV-TK promoter for normalizing transfection efficiency. Twenty-four hours after transfection, cells were harvested and luciferase activity was assayed. The level was normalized on the basis of the level of activity of control *Renilla* luciferase. Results are expressed as fold induction in luciferase activity relative to control cells that had been transfected with an empty vector. Triplicate experiments were carried out, and the error bars represent standard deviations.

the SP-RING (Siz/PIAS RING) domain [16], which is essential for binding of PIAS to Ubc9 and for SUMO-1 ligase activity [6–12]. To determine the requirement of the RING finger domain of PIAS3 for SUMO-1 modification of IRF-1, we constructed a point mutant, PIAS3 (C334S), in which the conserved cysteine residue in the RING finger domain had been changed to serine [7,17], and we performed co-transfection experiments followed by immunoprecipitation (Fig. 3). Although the C334S mutant was co-precipitated with IRF-1, co-expression of PIAS3 (C334S) and SUMO-1 in 293T cells did not enhance SUMO-1 conjugation of IRF-1, in contrast to the case with wild-type PIAS3. In addition, immunoblotting of cell lysate with anti-Myc antibody showed that PIAS3 (C334S) also failed to enhance SUMO-1 modification of the mutant itself. Thus, the RING finger domain of PIAS3 is essential for SUMO-1 modification of IRF-1 and PIAS3, suggesting that PIAS3 functions as a SUMO-1 ligase.

It has been demonstrated that SUMO-1 is conjugated to the specific lysine residue in the consensus sequence (ΨKXE; Ψ, hydrophobic amino acid) in many, but not all, target proteins [3–5]. On the other hand, recent studies have indicated that this sequence is a binding site for Ubc9 in the case of RanGAP1 in vitro [18,19]. IRF-1 lacks the above consensus sequence, although this protein is conjugated with SUMO-1. Studies to identify the SUMO-1 conjugation sites in IRF-1 are now in progress in our laboratory.

3.4. PIAS3 represses transcriptional activity of IRF-1

Next, we carried out an experiment to determine whether PIAS3 modulates transcriptional activity of IRF-1. 293T cells were co-transfected with IRF-1 and wild-type PIAS3 or PIAS3 (C334S) mutant expression plasmids together with a reporter gene containing ISRE (interferon-stimulated response element), which is known to be a binding sequence for the IRF family [2], and luciferase activity was measured (Fig. 4). Wild-type PIAS3 suppressed the transcriptional activity of IRF-1 in a dose-dependent manner, while the PIAS3 (C334S) mutant exhibited weaker suppressive activity than did the wild-type. Similar results were obtained when human HepG2 cells were used in place of 293T cells (data not shown). These results suggest that the suppressive action of PIAS3 on IRF-1 transcriptional activity is partially dependent on its SUMO-1 ligase activity.

PIAS3 was originally identified as a transcriptional repressor of activated STAT3 [15] and subsequently as a binding protein of other transcriptional regulators such as androgen receptor [20–22], Gfi-1 [23], HMG-1C [24] and MITF [25]. Co-expression of PIAS3 up- or down-regulates the activities of these transcription factors, although it is not known how PIAS3 regulates these transcriptional activities. It has recently been reported that PIASy represses the activity of the transcription factor LEF1 by targeting LEF1 into nuclear bodies in a RING finger domain-dependent manner [8]. In connection with this, it should be noted that SUMO-1 modification is required for the nuclear body localization of various nuclear proteins [3–5]. PIAS3 has been reported to be located in nuclear bodies [11,23] and we found in the present study that SUMO-1 ligase activity of PIAS3 is partly responsible for its repressive action on IRF-1 activity. On the other hand, PIAS3, as well as PIAS1, has been reported to function as a direct inhibitor of DNA binding of STAT in vitro [15,17]. Taken together, the results suggest that PIAS3 may negatively regulate the activity of IRF-1 by sequestering IRF-1 to nuclear bodies through PIAS3-mediated SUMO-1 modification, while PIAS3 may also regulate IRF-1 activity through a SUMO-1-independent mechanism, such as direct inhibition of DNA binding of IRF-1. Further detailed analysis is needed to elucidate the mechanism by which PIAS3 regulates transcriptional activity of IRF-1.

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