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The SUMO E3-ligase PIAS1 regulates the tumor suppressor PML and its oncogenic counterpart PML-RARA

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Abstract

The ubiquitin-like SUMO proteins covalently modify protein substrates and regulate their functional properties. In a broad spectrum of cancers, the tumor suppressor PML undergoes ubiquitin-mediated degradation primed by CK2 phosphorylation. Here we report that the SUMO E3-ligase inhibitor PIAS1 regulates oncogenic signaling through its ability to sumoylate PML and the PML-RARA oncoprotein of acute promyelocytic leukemia (APL). PIAS1-mediated SUMOylation of PML promoted CK2 interaction and ubiquitin/proteasome-mediated degradation of PML, attenuating its tumor suppressor functions. In addition, PIAS1-mediated SUMOylation of PML-RARA was essential for induction of its degradation by arsenic trioxide, an effective APL treatment. Moreover, PIAS1 suppression abrogated the ability of arsenic trioxide to trigger apoptosis in APL cells. Lastly, PIAS1 was also essential for PML degradation in non-small cell lung cancer cells, and PML and PIAS1 were inversely correlated in NSCLC cell lines and primary specimens. Together, our findings reveal novel roles for PIAS1 and the SUMOylation machinery in regulating oncogenic networks and the response to leukemia therapy.

Introduction

SUMOylation regulates fundamental cellular processes such as apoptosis, response to cellular stress and cellular proliferation. SUMOylation occurs though an enzymatic cascade, which involves E1, E2 and E3-ligase enzymes, that leads to covalent conjugation of SUMO

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proteins (SUMO1 and SUMO2/3) to target substrates creating an interface for protein-protein interactions. Typically, only a small fraction of a given protein is SUMOylated (1).

The promyelocytic tumor suppressor (PML), initially identified as a component of the PML-RARA oncoprotein of acute promyelocytic leukemia (APL), critically regulates multiple tumor suppressive pathways (2–4). Indeed, *Pml* inactivation in mice leads to cancer susceptibility (2, 5, 6). Moreover, PML deficiency occurs commonly in a broad spectrum of human cancers through a mechanism that involves aberrant ubiquitin/proteasomal degradation (6–8).

PML encodes a RING finger, 2 beta boxes and a coiled-coil domain (RBCC) followed by a variable C-terminal region that gives rise to several isoforms (9). PML concentrates in the cell in nuclear bodies (PML-nuclear bodies, PML-NBs thereafter), which have been implicated in tumor suppression (4). PML-RARA promotes APL through several mechanisms that include the disruption of PML dependent functions and the disruption of PML-NBs into PML microspeckles (10–12).

SUMOylation has been implicated in the regulation of both PML and of PML-RARA (13–15). For example, arsenic trioxide (As_2O_3), a drug of first choice for the treatment of APL, induces degradation of both PML and PML-RARA through their SUMOylation. However, the SUMO E3-ligases that mediate these events have remained elusive.

To elucidate the role of SUMOylation in the control of oncogenic networks, we have addressed the mechanisms that regulate the SUMOylation of PML and of its oncogenic counterpart PML-RARA. We have determined that PIAS1 is a PML SUMO E3-ligase that plays a critical role in the regulation of both PML and PML-RARA. Our findings unveil a novel role for the SUMOylation machinery in the regulation of oncogenic networks and in response to targeted leukemia therapy.

Material and Methods

Reagents, cell lines, antibodies and plasmid constructs

We used the Matchmaker yeast two-hybrid system (Clontech, Mountain view, CA). HEK293T cells and BJ fibroblasts were obtained from the ATCC. NSCLC cell lines A549, H157, H322, H358, H460, H1299, H1650, HCC1171, PC9 were obtained from Dr. John Minna (UT Southwestern Medical Center, Dallas, USA). NSCLC cell lines have been DNA fingerprinted for provenance and confirmed to be the same as the DNA fingerprint library maintained by ATCC. Dr. Pier Paolo Pandolfi (Beth Israel Deaconess Medical Center, Boston, USA) provided the NB4 cells. All cell lines were used withing 6 months of thawing. Cycloheximide, Arsenic trioxide, MG-132 and all other chemicals were purchased from Sigma-Aldrich. A complete list of antibodies and plasmids is available in the Supplemental Information Section.

Immunoprecipitation, immunoblotting, immunofluorescence, histidine-purification and SUMOylation assays

Immunoprecipitations, Western Blots and immunofluorescences staining were performed according to standard procedures (6). Histidine-purifications and SUMOylation assays were performed as described (14–16).

Immunohistochemistry

Tumor tissue microarray of paraffin-fixed tumor specimens were constructed and analyzed by immunohistochemistry as described previously (7). PML and PIAS1

immunohistochemicals staining were scored blindly by 2 pathologists. Discrepancies were resolved by re-examination of the samples. PML complete loss was defined as undetectable levels of PML and partial loss was defined by two or fewer PML nuclear bodies per cell (7). PIAS1 staining was scored as 0 = negative; 1 = weak less than 50% of tumor cells; 2 = moderate to strong, greater than 50% of tumor cells.

RNA interference

We used pGIPZ retroviral vectors encoding 2 non-overlapping specific shRNAs sequences or non-silencing control shRNA. On-target siRNA pools against PML or non-targeting siGENOME control pools were purchased from Dharmacon (Thermo Scientific).

Apoptosis and growth curve assays

Apoptosis was evaluated by FACS analysis using propidium iodide staining (17). For growth curves, cells were plated in triplicate at 2.5×10^5 per well in 6-well plates or 1.0×10^5 per well in 12-well plates (6).

Single nucleotide polymorphism and mRNA expression microarrays

Whole genome single nucleotide polymorphism (SNP) array profiling was done with the Illumina Human1M-Duo DNA Analysis BeadChip (Illumina, Inc.). Processing was done with Illumina BeadStudio and DNA copy number was derived from the “Log R Ratio”, which measures the relative probe intensity compared with normal diploid controls. Copy number for chromosomal positions mapping to *SKOR1* or *PIAS1* were averaged for analysis. For mRNA expression analysis the same NSCLC cell lines were profiled with Illumina HumanWG-6 V3 (GEO submission: GSE32036). Data processing involved background correction (18), quantile normalization and log transformation.

Statistical analysis

Chi-square test was used for TMA immunohistochemical stain analysis. Expression and copy number array data for PIAS1 were compared using Pearson correlation.

Results

PIAS1 and PIASxα interact with PML promoting its SUMOylation

To identify novel PML interacting proteins, we performed yeast two-hybrid screening using as prey a rat lung cDNA library and the PML RBCC motif, which is present in all PML isoforms, as bait. We discovered that the protein inhibitor of activated STAT1 (PIAS1) and PIASxα interact with the PML RBCC domain (Fig. S1A and data not shown).

To perform functional studies, we focused on PML IV, the PML isoform most intensely studied (4, 9). We found that PML interacts with PIAS1 and PIASxα also in mammalian cells, including NSCLC cells, both when ectopically or endogenously expressed (Fig. 1A–D, S1B and S1C). Moreover, PIAS1 and PIASxα co-localize with SUMO1 and SUMO2, which are PML-NB resident proteins (Fig. S1D). Finally, we determined that the Box2-CC domain of PML is sufficient to mediate the interaction with PIAS1 (Fig. S2A and S2B). These experiments indicate that PIAS1 and PIASxα interact with PML in PML-NBs, and that the Box2-CC domain is sufficient to mediate this interaction.

By performing *in vitro* SUMOylation assays, we discovered that both PIAS1 and PIASxα SUMOylate PML. PIAS1 led to the appearance of three discrete high molecular weight bands, while PIASxα induced a discrete high molecular band in addition to several slower migrating bands (Fig. 2A and S3A). In this setting, the PIAS1 E3 SUMO ligase activity

toward PML is comparable to its ability to SUMOylate a *bona fide* substrate such as the p53 tumor suppressor (Fig. 2A, lane 4) (19).

With immunoprecipitation (IP) assays in transfected HEK293T cells, we detected PML-reactive high-molecular weight bands indicating that in cells expressing PIAS1 and PIASxα together with SUMO1 or SUMO2 a fraction of PML undergoes SUMOylation (Fig. 2B). Histidine-purification experiments performed under denaturing conditions to detect SUMO covalently bound to PML confirmed that PIAS1 and PIASxα promote the SUMOylation of PML (Fig. 2C). During these experiments we also observed that PIAS1 downregulates PML, while PIASxα does not (Fig. 2C, compare lane 4 with lanes 3 and 5).

Next, we determined with histidine-purification assays whether PIAS1 or PIASxα affect the SUMOylation of the three major PML SUMOylation sites (i.e. K65, K160 and K442 in the PML isoform we used here) (20). Since PIAS1 appeared to promote PML degradation, we performed this experiments in the presence of the proteasome inhibitor MG132. In the absence of ectopic expression of PIAS1 or PIASxα, we detected 2 major SUMOylated PML protein species (Fig. 2D, lane1). The faster migrating PML band was absent in cells transfected with PML 3KR, a mutant carrying a lysine (K) to arginine (R) substitution ablating the three major PML SUMOylation sites (Fig. 2D, lane 2). This observation suggests that: 1. PML contains at least one additional SUMOylation site that is used in *vivo*; 2. one of the major SUMOylation sites is not efficiently SUMOylated in basal conditions, at least in HEK293T cells.

Ectopic expression of PIAS1 or PIASxα with wild type PML led to the appearance of three SUMOylated PML species (Fig. 2D, lane 3 and 5). Moreover, PIAS1 appeared to SUMOylate PML at a higher efficiency than PIASxα (Fig. 2D and Fig. S3B). Transfection of PML 3KR with either PIAS1 or PIASxα, led to an overall decrease in PML SUMOylation and to the appearance of a novel pattern of SUMOylated PML species (Fig. 2D and Fig. S3B). Thus, both PIAS1 and PIASxα SUMOylate the three major PML SUMOylation sites. We also conclude that deletion of the three major PML SUMOylation sites unmask cryptic SUMOylation sites that appear to be differentially used by either PIAS1 or PIASxα. This observation is consistent with the report that PML encodes several minor SUMOylation sites (21). Finally, PIAS1 has a higher affinity for both major and minor PML SUMO acceptor sites.

PIAS1 promotes ubiquitin-mediated degradation of PML

We tested the ability of PIAS1 and PIASxα to affect PML degradation in HEK293T cells exposed to cycloheximide, an inhibitor of protein translation. We determined that expression of PIAS1, but not PIASxα decreases the half-life of PML from 6 to about 2 hours (Fig. 3A, left panels). However, PIAS1 and PIASxα do not affect the half-life of PML 3KR (Fig. 3A, right panels). Furthermore, PIAS1, but not PIASxα, dramatically upregulates PML ubiquitination (Fig. 3B, lane 3). Neither PIAS1 nor PIASxα affects the baseline ubiquitination of PML 3KR. Finally, point mutations that abrogate each of the major SUMOylation sites do not affect the ability of PIAS1 to promote PML ubiquitination (Fig. S3C).

We also determined the effect of PIAS1 knockdown in NSCLC cells where PML undergoes aberrant ubiquitin-mediated degradation (6). We discovered that PIAS1 silencing up-regulates PML in all cell lines tested (Fig. 3C and S4A). These observations indicate that PIAS1, by SUMOylating PML, promotes its ubiquitin-mediated degradation.

As_2O_3 induces degradation of both PML and PML-RARA through a mechanism that requires direct SUMOylation of PML or of the PML moiety of PML-RARA (13–15). Thus,

is notable that we found that PIAS1 knockdown significantly impairs the ability of As_2O_3 to degrade PML (Fig. 3D and S4B). These data led to the conclusion that PIAS1 plays a critical role in the degradation of PML not only in cancer cells but also in cells treated with As_2O_3.

PIAS1 promotes interaction between PML and CK2

In cancer cells, CK2 triggers PML ubiquitin-mediated degradation by direct phosphorylation of PML serine 517 (S517) (6). Furthermore, CK2 and PIAS1 physically interact *in vitro* (22). Thus, we tested whether PIAS1 promotes the interaction between PML and CK2. With IP assays, we determined that PIAS1 promotes the interaction of endogenous CK2 with PML and that this interaction is enhanced by transfection of SUMO1-GFP or SUMO2-GFP (Fig. 4A). These data suggest that SUMOylation promotes PML-CK2 interaction, which in turn promotes PML phosphorylation and degradation.

To test this hypothesis we determined whether phosphorylation of PML S517 affects PML ubiquitination. We found that a serine to alanine substitution at PML S517 significantly impairs PML ubiquitination (Fig. 4B). Next, we assessed whether PML S517 is required for PIAS1 mediated-PML SUMOylation with histidine-purification assays. Transfection of PIAS1 with either SUMO1-GFP or SUMO2-GFP induces additional SUMOylation events in both His-PML or His-PML S517A, as demonstrated by the appearance of high-molecular weight bands that are recognized by an anti-GFP antibody (Fig. 4C, right panel and Fig. S4C). Mobility of SUMO-GFP fusion proteins is slower than wild type SUMO1/2, thus we reason that the fuzzy bands of Fig. 4C, lanes 4 and 5, represent PML species concomitantly SUMOylated by endogenous SUMO and SUMO-GFP. In this qualitative assay, cells transfected with SUMO1-GFP display reduced intensity of the bands corresponding to SUMOylated PML, most likely due to preferential degradation (Fig. 4C, 4^{th} lane). Transfection with SUMO2-GFP led to the robust appearance of SUMOylated PML and PML S517A (Fig. 4C, lane 6 and 7). Consistently with our previous publication, PML S517A, which is resistant to ubiquitin-mediated degradation, appears to be more abundant than wild type PML (6).

We determined that SUMOylated PML is phosphorylated by CK2 using an antibody specific for PML phosphorylated at S517 (Fig. 4D) (6). Taken together, this data indicate that PML SUMOylation does not require phosphorylation of PML at S517 supporting a model whereby PIAS1-mediated PML SUMOylation provides an interface for the recruitment of CK2, which in turn phosphorylates PML at S517 triggering its ubiquitination (Fig. 4E).

PIAS1 promotes SUMOylation of PML-RARA and its As_2O_3-dependent degradation

Our data led to the conclusion that PIAS1 mediates PML degradation in cancer cells, suggesting that both oncogenic stress and As_2O_3 utilize PIAS1 to induce PML SUMOylation. Therefore, we tested whether PIAS1 promotes SUMOylation of PML-RARA.

Transfection of PIAS1 with PML-RARA in HEK293T cells leads to the appearance of high molecular bands that correspond to SUMOylated PML-RARA as previously defined by others (23). SUMOylated PML-RARA was dramatically upregulated when we co-expressed PIAS1 with SUMO1-GFP or SUMO2-GFP. Furthermore, PIAS1 and PML-RARA readily co-IP and that SUMO1 or SUMO2 promote their interaction (Fig. 5A, Fig. S5A). Finally, PIAS1 leads to PML-RARA upregulation, which is further increased by the presence of SUMO1 or SUMO2 (Fig. 5A and S5A).

Next, we determined that mutations that ablate K65 and/or K160 significantly reduce PIAS1 dependent PML-RARA SUMOylation. The PML-RARA SUMOylation pattern suggests that the slowest-migrating high molecular weight band corresponds to SUMOylated at K65 and the faster-migrating high-molecular weight band corresponds to SUMOylation at K160 in addition to, yet to be identified, SUMO acceptor site(s) (Fig. 5B and S5B). In this regards, it is noteworthy that others have reported that PML undergoes SUMOylation also at K380 and K 400 and 497, thus it is possible that PIAS1 mediates SUMOylation also at any of these sites (21).

The therapeutic effect of As_2O_3 in APL is due to its ability to degrade the PML-RARA oncoprotein through a process that involves SUMOylation of its PML moiety. This event promotes the recruitment of the RNF4 ubiquitin E3-ligase to SUMOylated PML-RARA, leading to its ubiquitin mediated proteasomal degradation (12–15). Thus, we hypothesized that PIAS1 would prime the degradation of PML-RARA induced by As_2O_3.

Ectopic expression of PIAS1 strikingly increases the ability of As_2O_3 to down-regulate PML-RARA (Fig. 5C). Moreover, knockdown of PIAS1 in NB4 cells, an APL cell line that recapitulates the effects elicited by As_2O_3 in primary APL cells (12, 24) (Fig. S5C and S5D), significantly reduces the ability of As_2O_3 to degrade PML-RARA, resulting in persistence of PML microspeckles (Fig. 5D, 5E and S5E). As_2O_3 also upregulates PIAS1 in NB4 cells (Fig. S5F). Finally, PIAS1 silencing in NB4 cells leads to a significant resistance to apoptosis induced by As_2O_3 (Fig. 5F).

We conclude that PIAS1 is required for As_2O_3-dependent PML-RARA degradation and induction of apoptosis in APL cells.

PIAS1 promotes tumorigenesis in NSCLC

Our findings demonstrate that PIAS1 targets PML to ubiquitin-mediated degradation. Therefore, we assessed whether PIAS1-dependent PML degradation is biologically significant.

PIAS1 is elevated in H322, A549 and H1299 cells as compared to H157, HCC1171, H1650 and PC9 cells. PML is barely detectable in H322, A549 and H1299 cells (Fig. 6A and S6A). We previously demonstrated that PML is aberrantly ubiquitinated in H322, A549 and H1299 (6).

We found that the PIAS1 locus is frequently amplified in NSCLC cells, where we also found a direct correlation between amplification of the PIAS1 locus and its expression (Fig. 6B and Supplemental Table 1). Finally, we observed that PML and PIAS1 proteins are also inversely correlated in primary NSCLC specimens (Fig. 6C and 6D).

Taken together these data indicate that an inverse correlation exists between PIAS1 and PML in NSCLC cells suggesting that PIAS1 promotes tumorigenesis in virtue of its ability to promote PML SUMOylation and its subsequent degradation. Consistent with this notion, we discovered that stable knockdown of PIAS1 leads to significant anti-proliferative effects and enhancement of UV-induced apoptosis in H1299 and A549 cells, which are representative of NSCLC cells that aberrantly degrade PML (Fig. 6E, S6B and S6C) (6). Western blot analysis confirmed that PIAS1 knockdown was effective and correlated with PML upregulation (Fig. S6B and S6C). Notably, co-silencing of PML and PIAS1 abrogates the anti-proliferative effect elicited by PIAS1 knockdown (Fig. 6F and S6D). This data indicate that PIAS1 knockdown exerts PML-dependent anti-proliferative effects. This finding implies that PIAS1 has a novel oncogenic activity in virtue of its ability to promote PML degradation.

Discussion

SUMOylation has been involved in the regulation of several fundamental processes such as cellular trafficking and localization, transcriptional regulation and protein turnover (1). However, the role of SUMOylation in the regulation of oncogenic networks is not well understood.

The identity of the SUMO E3-ligase that mediates the SUMOylation of PML and PML-RARA has remained elusive. To the best of our knowledge, our manuscript reports the first identification of PML and PML-RARA SUMO E3-ligase enzymes.

PIAS1 has been mainly implicated in the regulation of innate immunity through epigenetic mechanisms (25, 26). However, it has been suggested that PIAS1 may regulate oncogenic networks through its ability to inhibit the p53 tumor suppressor, STAT proteins or BRCA1 (16, 27, 28).

Our discovery that PIAS1-mediated SUMOylation is essential for PML degradation in cancer cells and for As_2O_3-mediated degradation of PML-RARA unveils a novel function of the SUMOylation machinery in the regulation of oncogenic networks and targeted anti-leukemic therapy. We propose that PIAS1 has an oncogenic role when it SUMOylates PML in NSCLC cells and in other cancer cell types where PML is aberrantly degraded by ubiquitination (Figure 7A, left panel). In contrast, by degrading PML-RARA, PIAS1 exerts a critical therapeutic effect in APL cells treated with As_2O_3 (Figure 7A, right panel).

PIAS1 recognizes both PML and the PML moiety of the PML-RARA oncoprotein, therefore, the biological activity of PIAS1 depends on the status of PML. In cancer cells, PIAS1 leads to PML SUMOylation that, in turn, recruits CK2 to PML promoting the phosphorylation of PML S517 (Figure 7A, left panel). This event triggers PML ubiquitin-mediated degradation with consequent loss of PML-tumor suppressive functions and promotion of tumorigenesis (6). In this regard, it is noteworthy that CK2 positively regulates PIAS1 (22). Moreover, CK2 undergoes SUMOylation, thus it is likely that PIAS1 and CK2 are part of an oncogenic cellular network responsible for aberrant PML degradation in cancer cells (29). We speculate that CK2 mediated phosphorylation of PML promotes the recruitment of, a yet to be identified, PML ubiquitin E3-ligase.

As_2O_3 is a major therapeutic agent in APL due to the ability to physically interact with the PML RING domain of PML and of the PML-RARA oncoprotein. This event causes a conformational change that triggers the recruitment of the SUMO E2-ligase Ubc9 to PML-RARA, promoting its SUMOylation (12). Therefore it is likely that by recruiting Ubc9 As_2O_3 stimulates the activity of PIAS1 toward PML and PML-RARA.

Our model also provides a framework to reconcile reports implicating SUMOylation of PML and of PML-RARA in contradictory biological outcomes, such as engagement of PML tumor suppressive activities and PML-RARA induced leukemogenesis, but also PML and PML-RARA degradation (15, 23, 30–32). Our observation that PIAS1-mediated SUMOylation upregulates PML-RARA and is not sufficient to trigger its degradation is consistent with the report that SUMOylation of PML-RARA at K160 is required for leukemogenesis (23) (Figure 7A, right panel). We propose that PIAS1-mediated SUMOylation not only activates the biological functions of PML and PML-RARA, but also provides a signal to promote their degradation (Figure 7A, right and left panels). This mechanism would allow the downregulation of PML tumor suppressive functions when they are no longer needed or in cancer cells where CK2 is upregulated (6). In this regard, is noteworthy that preliminary experiments performed in primary mouse embryonic

fibroblasts, a cell type where CK2 is not upregulated, indicate that PIAS1 induces growth arrest and senescence in a PML dependent way (data not shown).

Our proposed model is also consistent with recent reports indicating that the ubiquitin E3-ligase RNF4 is essential for As_2O_3-induced PML-RARA degradation because of its ability to recognize poly-SUMOylated PML and PML-RARA (14, 15). It is also tempting to speculate that RNF4 may mediate the degradation of PML also in cancer cells in the absence of As_2O_3, however it is not yet know whether RNF4 mediates PML degradation in cancer cells not exposed to As_2O_3. Future studies should investigate whether PIAS1, CK2 and RNF4 are part of an integrated cellular network that leads to aberrant degradation of PML in cancer cells. It will be also of future interest to determine whether RNF4 is regulated by As_2O_3 or by oncogenic signaling pathways (Figure 7).

We also speculate that the amount of SUMOylation may determine the likelihood of PML or of PML-RARA to undergo degradation. In this respect, we have not yet investigated the functional consequences of PIASxα mediated PML SUMOylation, but it is possible that the failure of PIASxα to mediate PML degradation is due to a its decreased affinity for PML. Alternatively, it is possible that PIASxα may synthesize SUMO chains qualitatively different than PIAS1.

PIAS1, which is located on chromosome 15q23, has not been implicated in recurrent chromosomal aberrations in human cancer or tumor-associated mutations. To the best of our knowledge this is the first report that the PIAS1 locus is amplified in significant proportion of NSCLC cells and inversely correlates with PML in primary NSCLC specimens. This finding strongly suggest that amplification/overexpression of PIAS1 may confer a selective advantage to NSCLC. Future experiments will be needed to further define the mechanism(s) regulating PIAS1 protein levels and function in cancer cells.

Our data reveal a novel and unexpected function of PIAS1 and the SUMOylation machinery in the regulation of oncogenic networks and in regulating the response to targeted APL therapy. Remarkably, the biological output of PIAS1 depends on PML status. PIAS1 promotes tumorigenesis in cancer cells expressing PML, but exerts a therapeutic activity in APL by degrading PML-RARA. These data provide the rationale for the development of pharmacologic inhibitors of PIAS1 to prevent aberrant degradation of PML in common cancer types such as NSCLC or prostate cancer, where PML is aberrantly degraded. Conversely, strategies that stimulate PIAS1 activity may have a beneficial role in the therapy of APL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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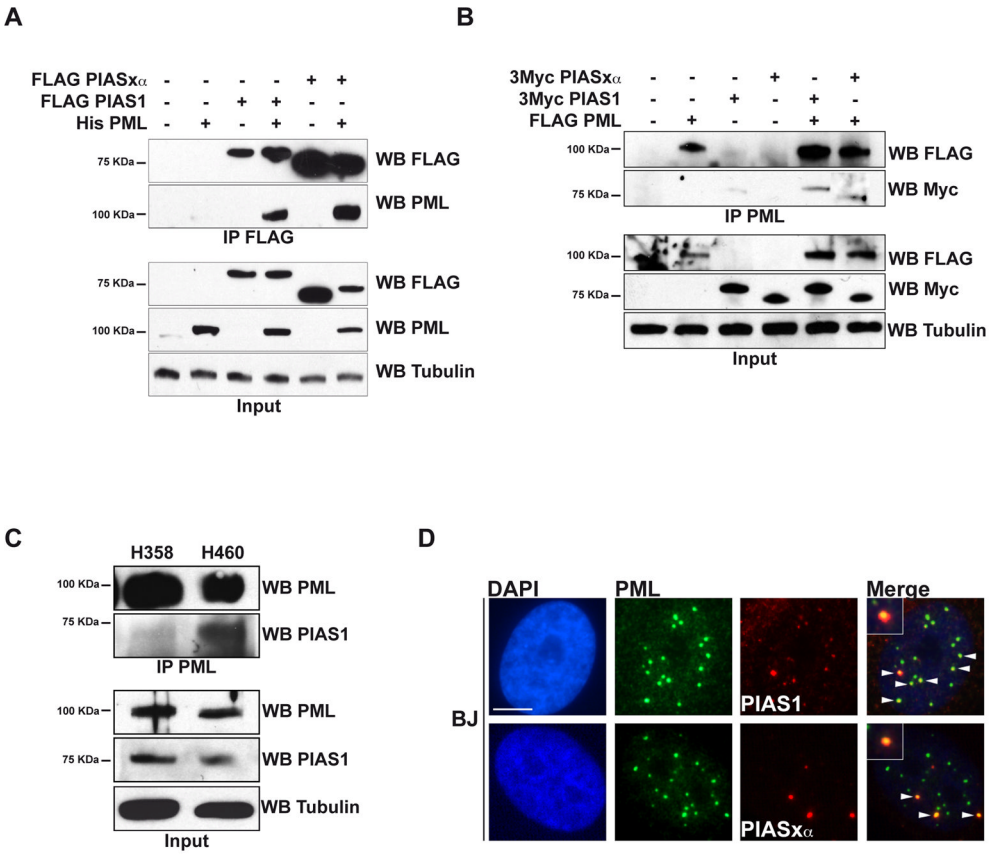

Fig. 1.

PIAS1 and PIASxα interact with PML. (A–C) Cell lysates of HEK293T (A–B) or H358 and H460 (C) cells were analyzed by IP followed by WB as indicated. (D) BJ fibroblasts were analyzed by IF. PML (green), PIAS1 or PIASxα (red), co-localization of PML and PIAS1 or PIASxα (yellow). Bar represents 5 μm.

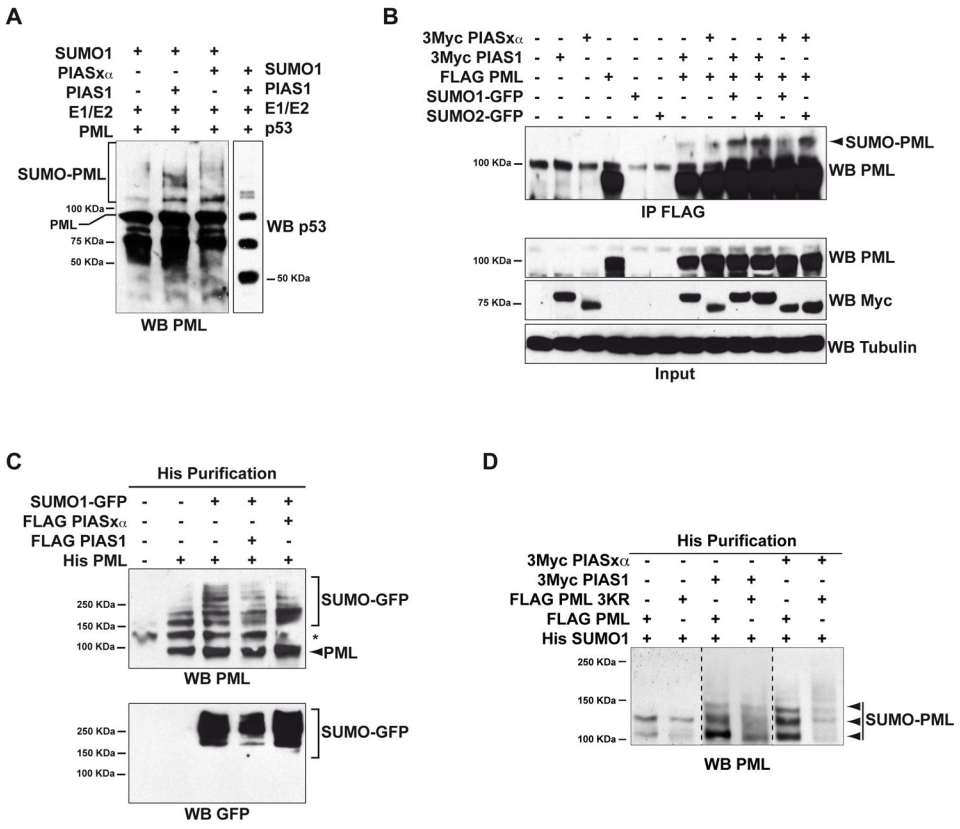

Fig. 2.

PIAS1 and PIASxα promote PML SUMOylation. (A) *In vitro* SUMOylation reactions were analyzed by WB. (B) HEK293T cells were transfected with the indicated expression vectors and analyzed by IP as indicated. (C–D) HEK293T cells were transfected as indicated and lysates were analyzed by histidine-purification followed by WB. Asterisk indicates an aspecific band.

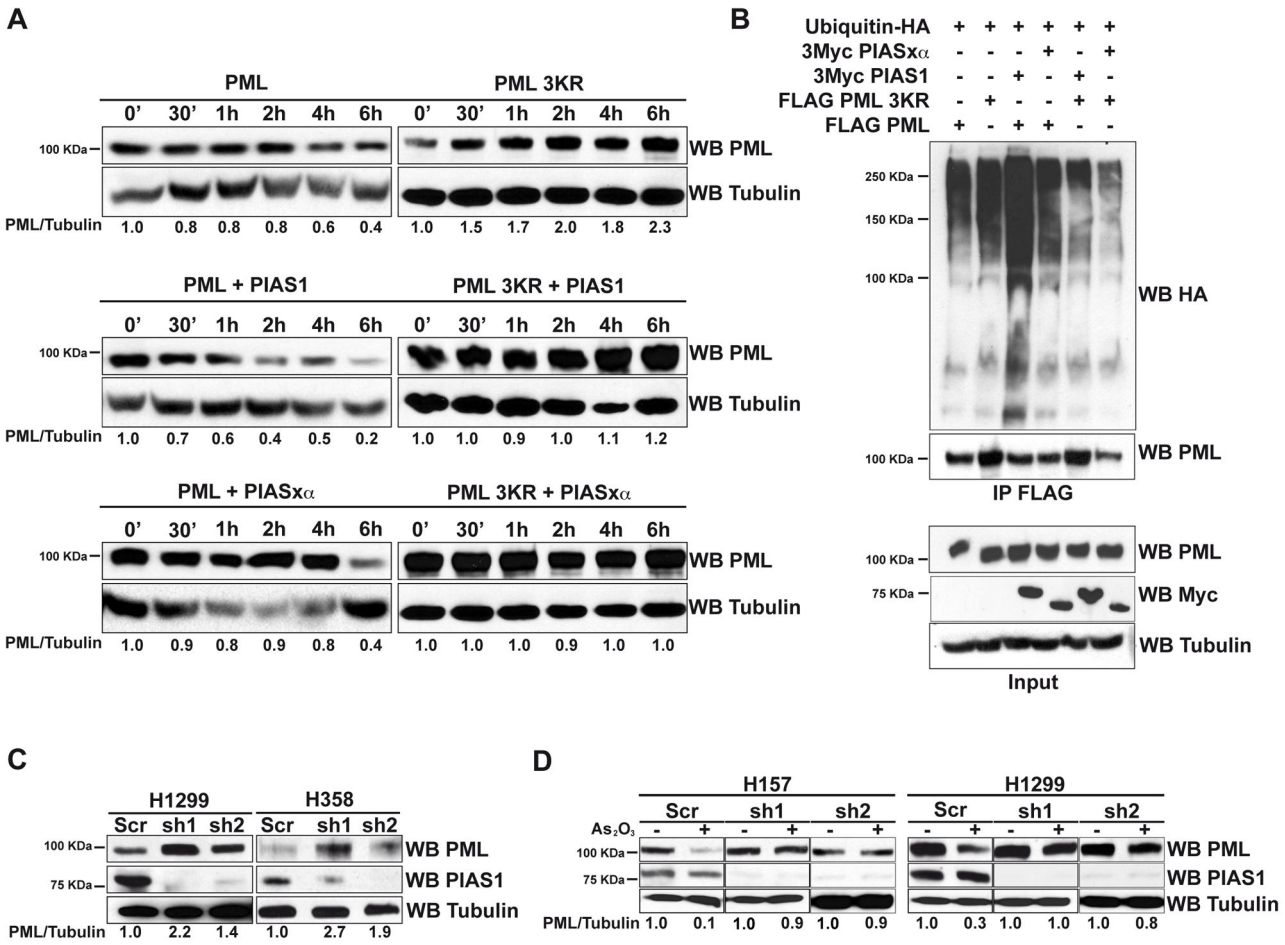

Fig. 3.

PIAS1 promotes ubiquitin-mediated degradation of PML. (A) Transfected HEK293T cells were treated with cycloheximide and analyzed by WB. (B) Transfected HEK293T cells were treated with MG132. Ubiquitinated FLAG-PML was detected by IP followed by WB. (C) NSCLC cell lines were transduced with lentiviruses expressing PIAS1 shRNAs (sh1 and sh2, respectively) or scrambled control (Scr) and analyzed by WB. (D) NSCLC cell lines were transduced with the indicated lentiviruses, treated with 1 μM As_2O_3 for 24 hours and analyzed by WB.

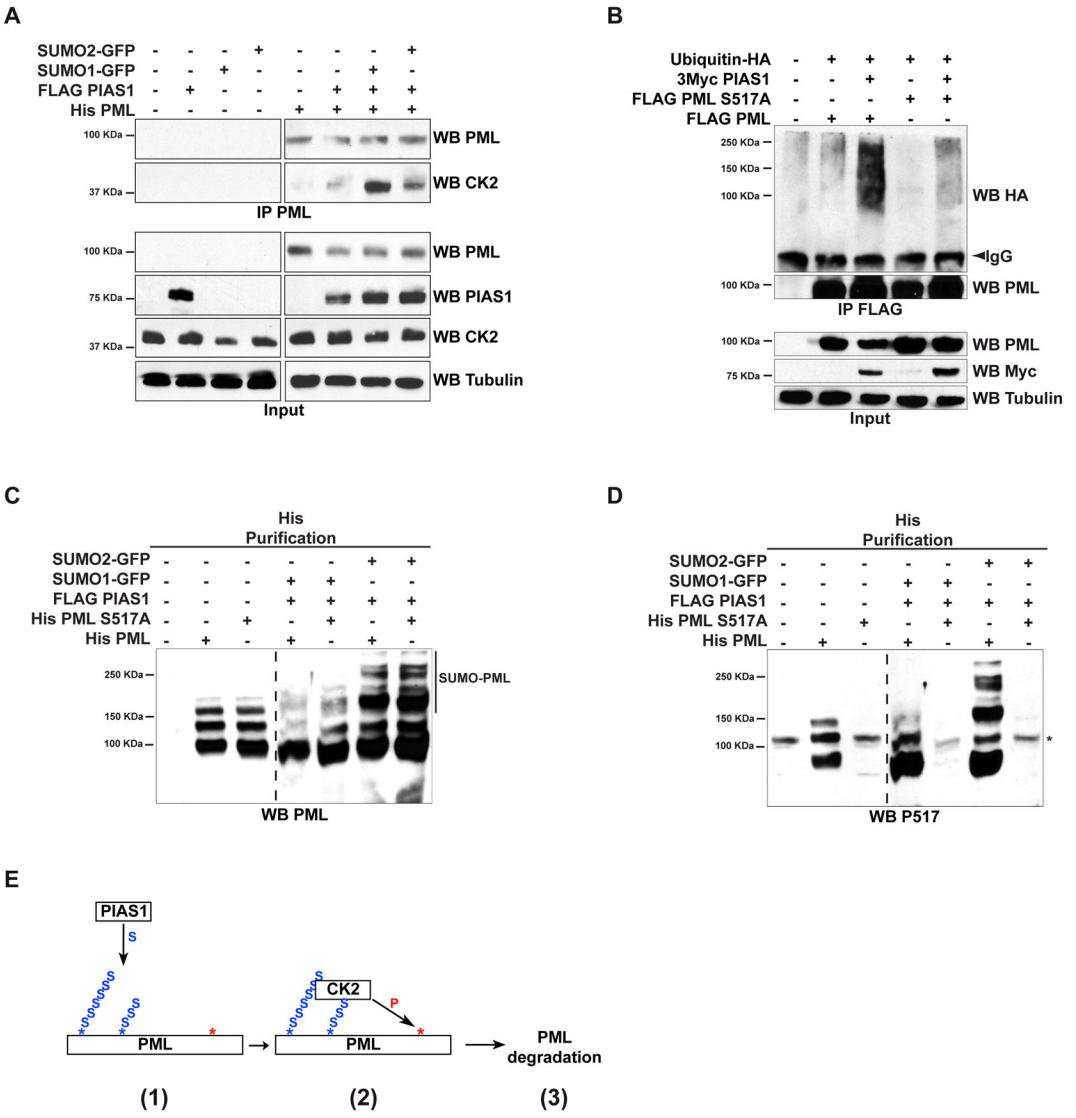

Fig. 4.

PIAS1-dependent PML SUMOylation promotes interaction with CK2 and phosphorylation of PML at residue S517. (A) Transfected HEK293T cells were analyzed by IP followed by WB. (B) HEK293T cells were transfected as indicated and treated with MG132. Ubiquitinated FLAG-PML or FLAG-PML S517 was detected by IP followed by WB. (C–D) Transfected HEK293T cells were analyzed by histidine-purification. WB was first probed with antibody anti-PML (C) and after stripping with anti-PML phospho-serine 517 (D). The asterisk indicates an aspecific band. (E) Model of SUMOylation-dependent PML degradation: PIAS1 promotes PML SUMOylation [1], which in turn recruits CK2 with consequent phosphorylation of PML S517 [2]. Phosphorylation of PML at S517 triggers PML degradation [3]. S = SUMO, P = phosphorylation.

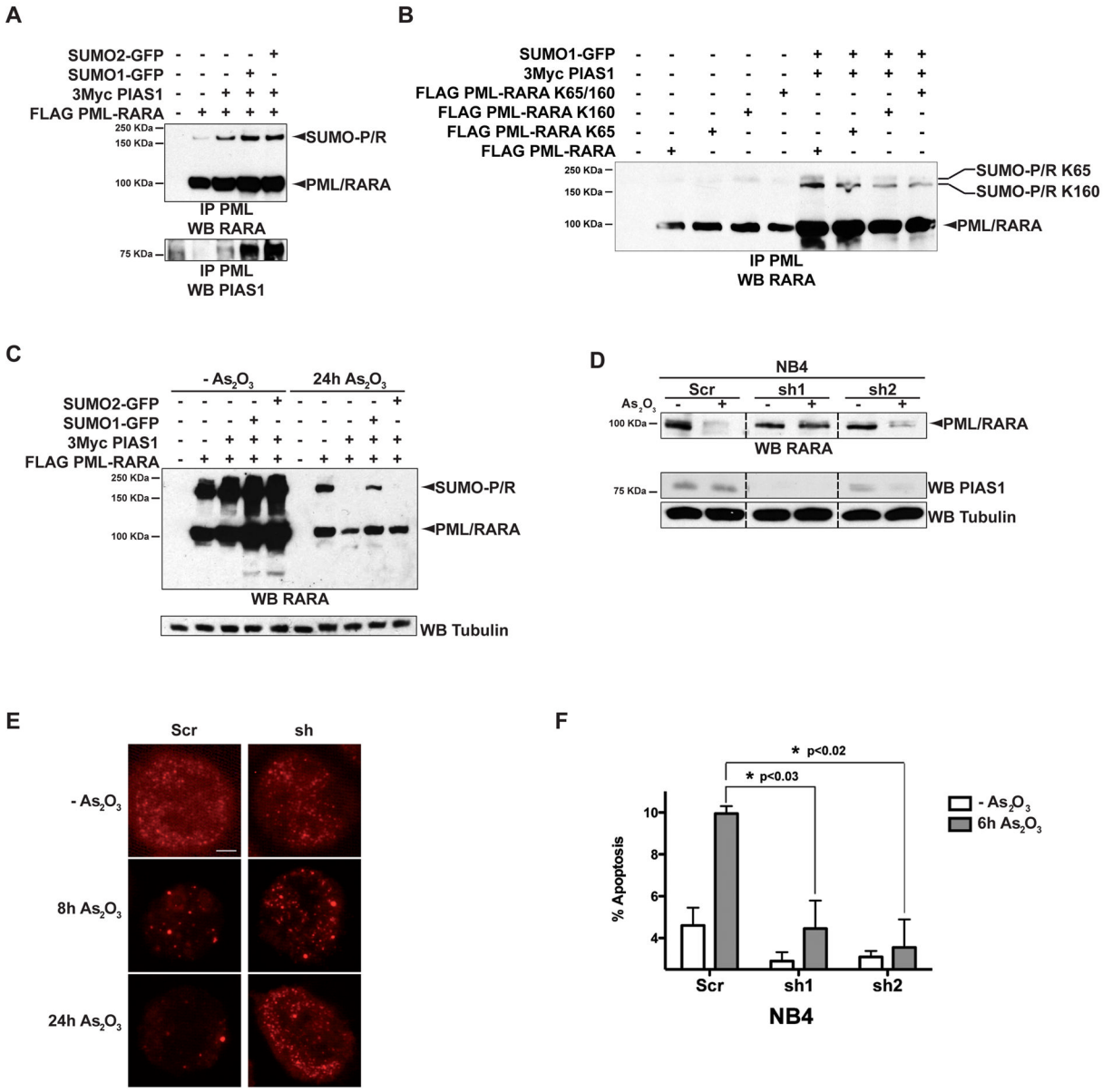

Fig. 5.

PIAS1 promotes SUMOylation of PML-RARA and its As_2O_3-dependent degradation. (A–B) HEK293T cells were transfected as indicated and analyzed by IP followed by WB. P/R = PML-RARA. Bands corresponding to P/R SUMOylation at K65 or K160 are indicated. (C) Transfected HEK293T cells were treated with 1 μM As_2O_3 for 24 hours and analyzed by WB. (D) NB4 cells expressing a PIAS1 shRNAs (sh1 and sh2) or a scrambled shRNA (Scr) were treated with As_2O_3 for 24 hours and analyzed by WB. (E) NB4 cells expressing a PIAS1 specific (sh) or control (Scr) shRNA were treated with 1 μM As_2O_3 for 8 or 24 hours and analyzed by IF with an anti-PML antibody. Bar represents 5 μm. (F) Histogram shows the percentage of apoptosis induced by As_2O_3 in NB4 cells expressing the indicated shRNAs. Asterisks indicate statistical significance.

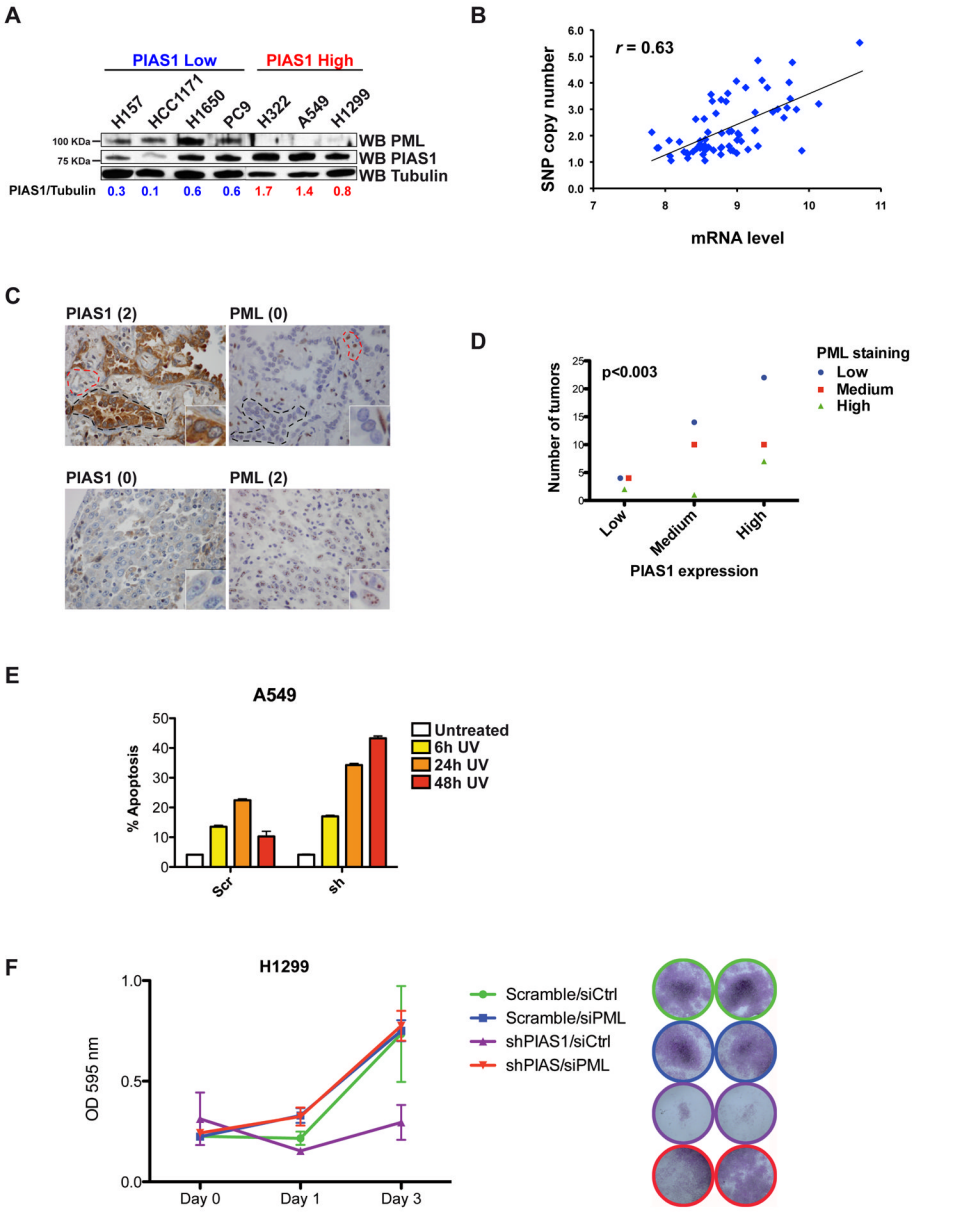

Fig. 6.

PIAS1 promotes tumorigenesis in NSCLC. (A) PML and PIAS1 were detected by WB in the indicated cell lines. (B) Scatter plot showing a high Pearson correlation coefficient between mRNA expression (Illumina WG6-V3; log scale) and copy number (inferred from Illumina Human1M-Duov3 SNP profiling) of PIAS1 in NSCLC cell lines. (C) Histopathological analysis of PML and PIAS1 in representative primary NSCLC specimens (200X magnification). Upper row: (left panel) an example of a NSCLC specimen with intense nuclear and cytoplasmic PIAS1 staining in cancer cells, but not in stromal cells; (right panel) a section of the same NSCLC shows that cancer cells are negative for PML, while stromal cells are positive. Lower row: (left panel) NSCLC sample displaying absence of PIAS1 in cancer cells; (right panel) a section of the same NSCLC shows that in this specimen PML is readily detected. Inserts display higher magnifications. Black dotted line: representative area of cancer cells. Red dotted line: representative area of stromal cells. (D) Histogram shows distribution of PML and PIAS1 staining in NSCLC primary samples. Y axis: number of

tumors; X axis: PIAS1 and PML intensity of staining. (E) The histogram shows the percentage of apoptosis in cells treated with UV at the indicated time and transduced with either a lentivirus expressing a control shRNA (Scr) or a PIAS1 shRNA (sh). (F) Proliferation of H1299 cells stably expressing a scrambled (Scr) or PIAS1 shRNA (shPIAS1) transfected with either siRNA against PML (siPML) or control non-targeting siRNA (siCtrl). Representative tissue culture wells stained with crystal violet are presented on the right.

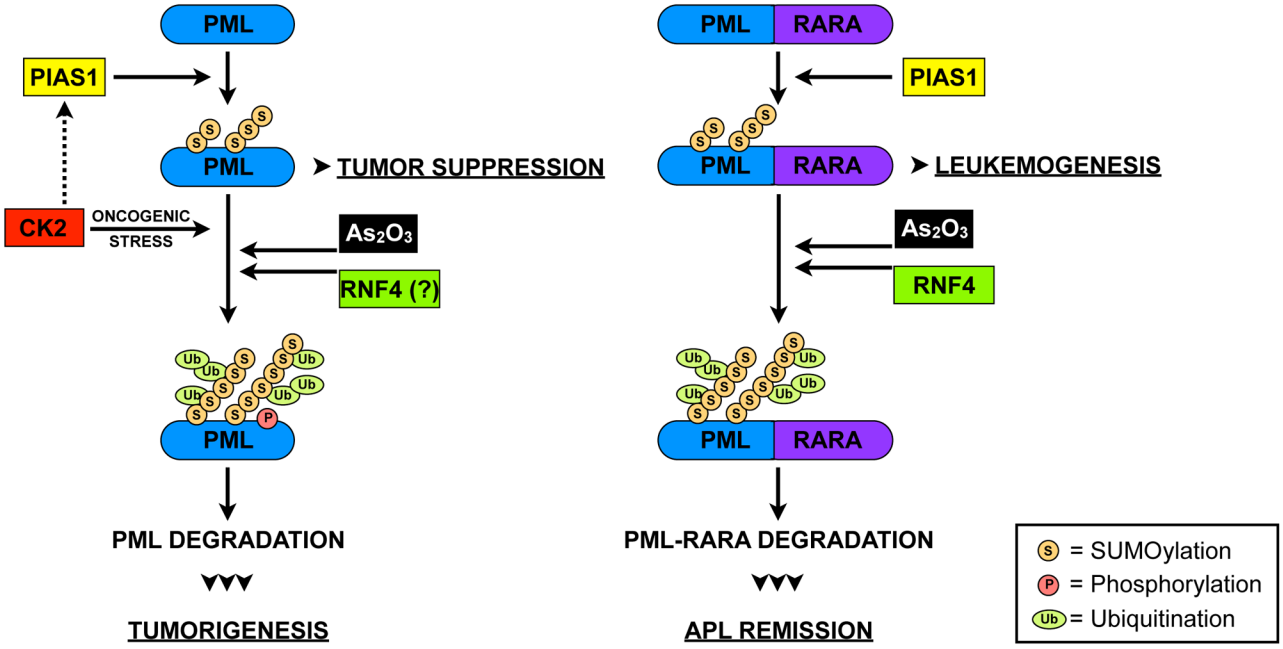

Fig. 7.

PIAS1-mediated SUMOylation of PML and PML-RARA promotes their degradation. In cancer cells (left panel), PIAS1 leads to PML SUMOylation that, in turn, recruits CK2 to PML promoting the phosphorylation of the PML degron. This event triggers PML ubiquitin mediated degradation with consequent loss of PML-tumor suppressive functions and promotion of tumorigenesis. CK2 and PIAS1 interact (dashed arrow), thus it is possible that a tertiary PML-CK2-PIAS1 protein complex exists. As_2O_3 facilitates PIAS1-mediated SUMOylation of PML and recruitment to SUMOylated PML of the RNF4 ubiquitin E3-ligase. It is unknown whether RNF4 mediates the ubiquitination of PML in cancer cells not exposed to As_2O_3. In APL (right panel), PIAS1 promotes SUMOylation of PML-RARA, which in the absence of As_2O_3 is not sufficient to trigger ubiquitination of PML-RARA. As_2O_3 facilitates PIAS1-mediated SUMOylation of PML-RARA causing the recruitment to SUMOylated PML-RARA of the RNF4 ubiquitin E3-ligase. The mechanism that regulates the recruitment of RNF4 to SUMOylated PML or PML-RARA is presently not known.