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Repression of E1AF transcriptional activity by sumoylation and PIASy

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Abstract

E1AF is a member of the Ets transcriptional factor family, and it plays a crucial role in tumor metastasis. However, the molecular mechanisms regulating its activity are not well characterized. In this study, we show that E1AF is sumoylated at four lysine residues, both *in vivo* and *in vitro*. Replacement of these lysines by arginine enhanced the transcriptional activity of E1AF, suggesting that sumoylation negatively regulates E1AF activity. We further demonstrated that PIASy enhanced sumoylation of E1AF as a specific SUMO-E3 ligase. In addition, PIASy repressed the transcriptional activity of both the wild-type and sumoylation defective mutants. However, the C342A mutant of PIASy, which abrogates SUMO-E3 ligase activity, had a significantly decreased ability to repress E1AF activity. Taken together, our results indicate that PIASy negatively regulates E1AF-mediated transcription by both E1AF sumoylation in a dependent and independent fashion.

Keywords: E1AF; PEA3; Ets; PIASy; PIAS; SUMO; SUMO-1; SUMO-E3 ligase

Post-translational modifications, such as phosphorylation, acetylation, and ubiquitination are known to regulate transcription factor function. Small ubiquitin-related modifier (SUMO) modification (sumoylation) is a post-translational modification, which has multiple functions that include involvement in protein targeting, stabilization, and transcriptional regulation [1]. Recently, many transcription factors have been reported to be sumoylated, and an important role of sumoylation in transcriptional control has been demonstrated [2,3]. Similar to ubiquitination, SUMO is conjugated to target proteins by a cascade of enzymes, including E1 (Aos1/Uba2), E2 (Ubc9) and several types of the E3 ligases, such as the protein inhibitor of activated STAT (PIAS) family members, RanBP2 or polycomb group protein Pc2 [1]. Among the SUMO-E3 ligases, PIAS family members were the first to be identified and characterized by a RING-like domain (termed SP-RING), which is similar to the RING finger structure in a subclass of ubiquitin-E3 ligases and it is required for ligase activity [4–7].

The Ets family transcription factors play a critical role in a variety of physiological processes, including cell proliferation, differentiation, development, and apoptosis [8]. They share an evolutionarily conserved DNA-binding domain, called the Ets domain [9–11]. Recently, several Ets family transcription factors have been shown to be regulated by sumoylation [12–15]. More recently, we have shown that sumoylation regulates the transcriptional activity of Ets-1, an Ets family member [16]. In addition, PIASy, which is a specific SUMO-E3 ligase, enhances Ets-1 sumoylation and represses Ets-1-mediated transcription irrespective of the sumoylation status of Ets-1 [16].

E1AF, a human homologue of mouse PEA3, is a member of the PEA3 subfamily of Ets transcription factors [17,18]. This subfamily consists of E1AF/PEA3, ER81/ETV1, and ERM/ETV5, which display a high similarity and conservation of functional domains [19]. E1AF overex-

Abbreviations: SUMO, small ubiquitin-like modifier; PIAS, protein inhibitor of activated STAT; EGFP, enhanced green fluorescent protein; GST, glutathione *S*-transferase; WT, wild-type.

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pression is associated with metastasis of breast cancer and of squamous cell carcinoma [20–23] via up-regulation of genes involved in tumor migration and invasion such as matrix metaroproteinases [24,25]. Recently, ERM has been shown to be sumoylated and its transcriptional activity is repressed by sumoylation [26]. However, the mechanisms by which sumoylation controls ERM activity remain unclear.

In this study, we present evidence that E1AF is also sumoyolated at four consensus sites both *in vitro* and *in vivo*. In addition, PIASy acts as its specific SUMO-E3 ligase, and modulates E1AF-mediated transcription both independent of E1AF sumoylation and dependent on its SUMO-E3 ligase activity. Our results demonstrate an important role for PIASy on the control of E1AF activity.

Materials and methods

Plasmid constructs. The human full-length cDNA coding E1AF was obtained through PCR of a reverse-transcribed product of mRNA from HeLa cells using SuperScript III (Invitrogen). pFLAG-E1AF and pcDNA3.1/E1AF-MycHis$_6$ were constructed by inserting full-length human E1AF cDNA into the pFLAG-CMV-2 vector (Sigma) and pcDNA3.1/Myc-His(−) vector (Invitrogen), respectively. The human SUMO-1, PIASy, and SENP1 expression plasmids have been described as previously [16]. The substitution mutants of E1AF were amplified by PCR with specific mutation primers.

For constructs encoding GST-E1AF fusion proteins, E1AF cDNA fragments were cloned in-frame into a pGEX-6P-2 vector (Amersham).

The pEBSx4-TK-Luc reporter has been described previously [16]. The pMMP7(−301/+31)-Luc was created as follows. A segment of the human *MMP7* promoter from −301 to +31 was amplified by PCR on human HeLa cell genomic DNA using the following oligonucleotides, 5′-ATATGGTACCATAATGTCCTGAATGATACC-3′ and 5′-GGGG AAGCTTCAATTGTTCTTGGACCTATGGTTG-3′. The PCR product was cloned into the KpnI/HindIII sites of the pGL3 basic vector (Promega).

Cell culture, DNA transfection, and adenovirus infection. U2OS, COS-7, and HepG2 cells were cultured in Dullbecco's modified Eagles' medium with 10% fetal bovine serum. All cells were transiently transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Recombinant adenovirus Ad/3xFLAG-SUMO-1(GG) was produced as described previously [16]. U2OS cells were infected with adenovirus at a multiplicity of infection (MOI) of 100 for 24 h.

Expression and purification of GST fusion proteins. Wild-type or mutant GST-E1AF fusion proteins were expressed in bacteria cells and purified as previously described [16].

Immunoblotting analysis. Extraction of proteins with a lysis buffer from cultured cells was followed by immunoblotting with corresponding antibodies as described previously [16].

In vitro sumoylation assays. In vitro sumoylation reactions were carried out as described previously [16]. GST-E1AF bound beads were incubated with sumoylation enzymes (human Aos1/Uba2, human Ubc9, and either GST-PIASy) and SUMO-1 in reaction buffer (50 mM Tris–HCl [pH 7.4], 5 mM $MgCl_2$, and 3 mM ATP, and 1 mM DTT).

Reporter gene assays. Luciferase reporter assays were performed as described previously [16]. Briefly, HepG2 cells were co-transfected with reporter plasmid (200 ng), 10 ng of pRL-CMV (Wako), which expresses *Renilla* luciferase as an internal control, and FLAG-E1AF (wild-type or mutant, 200 ng). In some experiments, Myc-PIASy (wild-type or C324A mutant) expression plasmids were also included in the transfection. Forty-eight hours after transfection, luciferase activities were determined in extracts made from transfected cells by using a PicaGene Dual luciferase reporter assay system (Toyo Ink) and a Lumat LB9501 luminometer (Berthold) according to the manufacturers' instructions.

Immunofluorescence microscopy. Indirect immunofluorescence analysis was performed as described previously [16]. U2OS cells grown on coverslips were fixed with paraformaldehyde (4% in PBS) and permeabilized with 0.2% Triton X-100 (0.2% in PBS). E1AF-MycHis$_6$ in the cells was detected with a monoclonal anti-Myc antibody (9E10) and an Alexa Fluor 568-conjugated goat anti-mouse secondary antibody (Molecular Probes Inc.). The stained cells were observed by a Leica TCS SP2 confocal laser scanning microscope.

Results and discussion

Sumoylation of E1AF occurs in vivo and in vitro

To examine whether E1AF is sumoylated, we first performed transient co-transfection studies using expression constructs encoding FLAG-E1AF, EGFP-SUMO-1, and EGFP-SENP1. Co-expression of E1AF with EGFP-SUMO-1 allowed for the detection of the unmodified form and three additional slower-migrating forms of FLAG-E1AF (Fig. 1A, lane 2), whereas these slower-migrating forms were markedly diminished in cells expressing SUMO specific protease SENP1 (Fig. 1A, lane 3). However, when the catalytically inactive C599A mutant was co-expressed, the intensity of slower-migrating bands was increased, and another band, which might represent a form of conjugated four EGFP-SUMO-1s, also could be detected (Fig. 1A, lane 4). To clarify the sumoylation of E1AF, we conducted *in vitro* sumoylation by using purified recombinant GST-E1AF as the substrate. In the absence of SUMO-1, E1 (Aos1/Uba2) or Ubc9, no sumoylated E1AF bands were detected (Fig. 1B, lanes 1–4). In contrast, in the presence of all components (SUMO-1, E1, and Ubc9), four higher molecular mass E1AF bands were detected (Fig. 1B, lane 5). These results suggest that E1AF is potentially sumoylated at four sites.

Mapping of the E1AF sumoylation sites

Sumoylation occurs on the lysine (K) residue within a minimal consensus sequence, ψKXE/D, in which ψ represents a large hydrophobic residue, X is any amino acid, and E/D represent an acidic residue [27]. Inspection of the amino acid sequence of human E1AF revealed five lysines, K96, K226, K260, K322, and K441 that lie within consensus sumoylation motifs. To map the E1AF sumoylation sites, we generated a series of mutants, which had these sites where lysine was substituted with arginine (*R*) and we examined their sumoylation *in vivo*. As above results, wild-type E1AF was sumoylated mainly in three sites *in vivo* (Fig. 1C, lane 2). A single site mutation at either K96, K226, or K260 abrogated the third slow-migrating band, whereas a mutation at K322 or K441 had no effect (Fig. 1C, lanes 3–7). Simultaneous mutations at K96, K226, and K260 completely abolished any detectable slow migrating bands (Fig. 1C, lane 17). To further confirm that

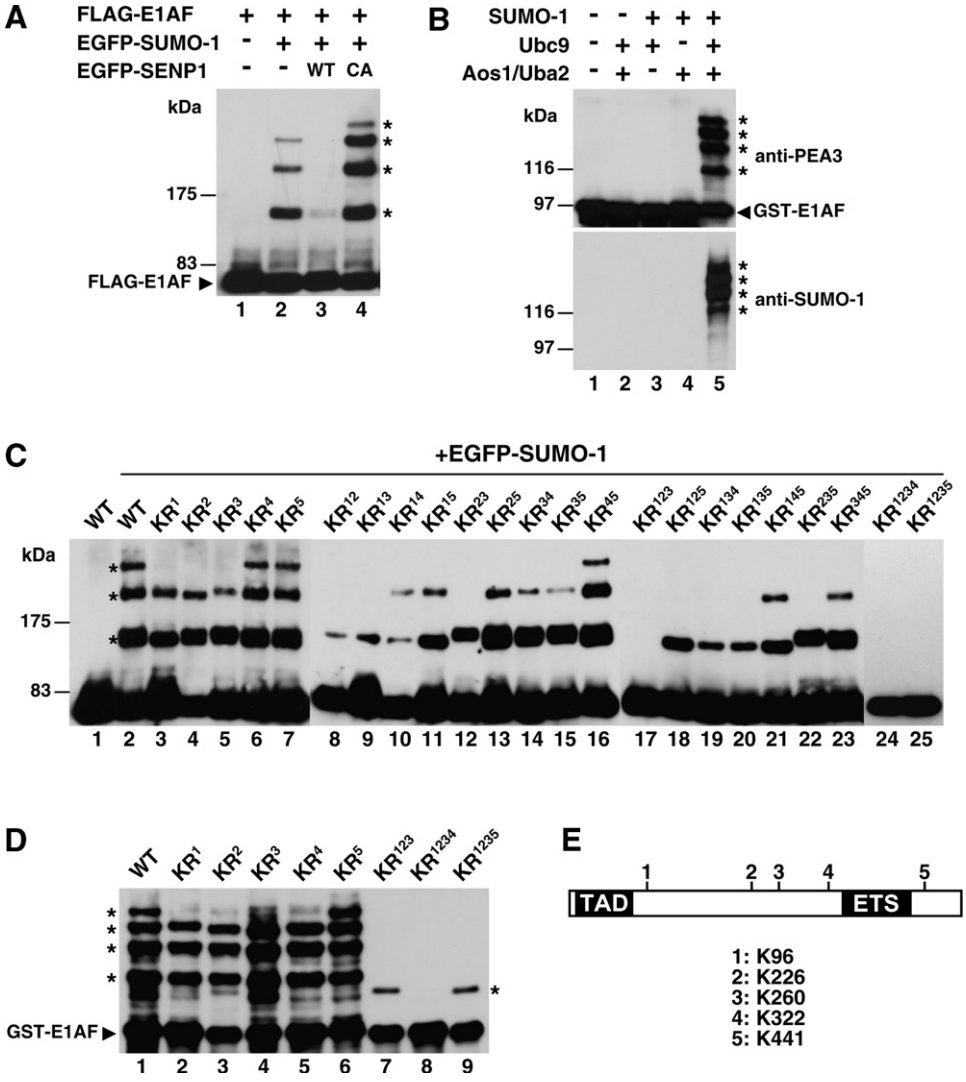

Fig. 1. E1AF is sumoylated at four sites *in vivo* and *in vitro*. (A) COS-7 cells were transiently co-transfected with expressing plasmids encoding FLAG-E1AF, EGFP-SUMO-1, and EGFP-SENP1 (either wild-type or C599A mutants). Twenty-four hours after transfection, lysates from cells were subjected to immunoblotting using anti-FLAG antibody. (B) *In vitro* sumoylation assays were performed with the indicated combination of E1 (Aos1/Uba2), E2 (Ubc9), SUMO-1 and GST-Ets-1. The reactions were immunoblotted with anti-PEA3 antibody (upper panel) or anti-SUMO-1 antibody (lower panel). (C) COS-7 cells were transiently co-transfected with expression plasmids encoding FLAG-E1AF (either wild-type or indicated mutants) and EGFP-SUMO-1. Immunoblotting was performed as in (A). (D) Wild-type or indicated mutant GST-Ets-1 proteins were used in the *in vitro* sumoylation assay. The reactions were immunoblotted with anti-PEA3 antibody. (E) Schematic representation of human E1AF. TAD, transcriptional activation domain; ETS, ETS domain. Positions of five potential sumoylation sites are indicated. Superior numbers 1, 2, 3, 4, and 5 correspond to K96, K226, K260, K322, and K441, respectively. The modified forms of E1AF are indicated by asterisks.

these lysines are sumoylated, single point mutants were tested by using an *in vitro* sumoylation assay. Wild-type E1AF was apparently sumoylated at four sites *in vitro* (Fig. 1D, lane 1). Consistent with the *in vivo* data, a single site mutation at K96, K226, or K260 abrogated the fourth slow-migrating band (Fig. 1D, lanes 2–4). In addition, a mutation at K322, but not K441, also abolished the fourth slow-migrating band (Fig. 1D, lane 5). The slow-migrating bands were completely abolished with simultaneous mutations at K96, K226, K260, and K322 (Fig. 1D, lane 8). Taken together, these data indicate that K96, K226, and K260 are major sumoylation sites both *in vivo* and *in vitro,* and K322 is an additional site *in vitro*.

PIASy enhances E1AF sumoylation as a specific SUMO-E3 ligase

We then investigated PIAS family members for their SUMO-E3 ligase activity toward E1AF. As shown in Fig. 2A, when the different PIAS family members were co-expressed with E1AF and SUMO-1 in cells, only PIASy showed enhancement of sumoylation of E1AF. To further examine the effect of PIASy, we used a purified PIASy in an *in vitro* sumoylation assay. As shown in the above experiments, E1AF was sumoylated in this assay (Fig. 2B, lane 2). When one tenth of the amount of Ubc9 that was added to lane 2 was used, bands of sumoylated forms became

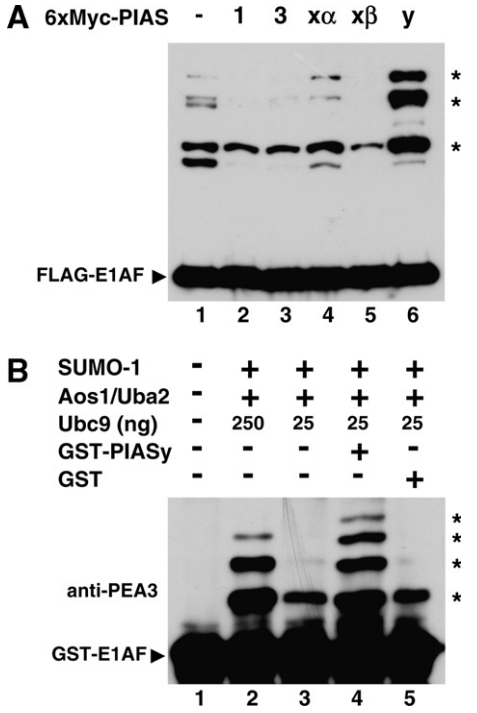

Fig. 2. PIASy enhances E1AF sumoylation *in vivo* and *in vitro*. (A) COS-7 cells were transiently co-transfected with expression plasmids encoding FLAG-E1AF, EGFP-SUMO-1, and indicated 6xMyc-PIAS. Cell lysates were subjected to immunoblotting with anti-FLAG antibody. (B) *In vitro* sumoylation assays were performed with the indicated components as in Fig. 1B. The indicated amounts of proteins were used for addition of PIASy and Ubc9. The modified forms of E1AF are indicated by asterisks.

faint (Fig. 2B, lane 3). However, the addition of a purified recombinant PIASy protein in these conditions restored the level of sumoylation (Fig. 2B, lane 4). Taken together, these results demonstrate that PIASy enhances the sumoylation of E1AF as a specific SUMO-E3 ligase.

Mutations of the sumoylation sites enhance E1AF transcriptional activity

Sumoylation has been shown to affect the activity of many transcription factors. Therefore, we investigated whether sumoylation site mutations with lysine to arginine (KR mutant) affect E1AF transcriptional activity. To test this, HepG2 cells were transiently co-transfected with expression plasmids encoding wild-type or KR mutants of FLAG-E1AF, together with EBSx4-TK-Luc containing four copies of an artificial Ets-binding site or a MMP7-Luc reporter construct. This construct bears a natural human matrilysin promoter fragment from −301 to +31 that has been shown to be upregulated by the PEA3 subfamily [28]. As shown in Fig. 3A, a single sumoylation site with mutated E1AF proteins had little effect on the activation of both reporter contexts. K96R (KR^1), K226R (KR^2), and K260R (KR^3) mutants showed slightly higher activity (~1.5-fold) compared to that of the wild-type. The K322R (KR^4) mutation caused a slight decrease in the activity. Multiply mutated E1AF K96/226/260R (KR^{123}) and K96/226/260/322R (KR^{1234}) showed comparable activities to single site mutants

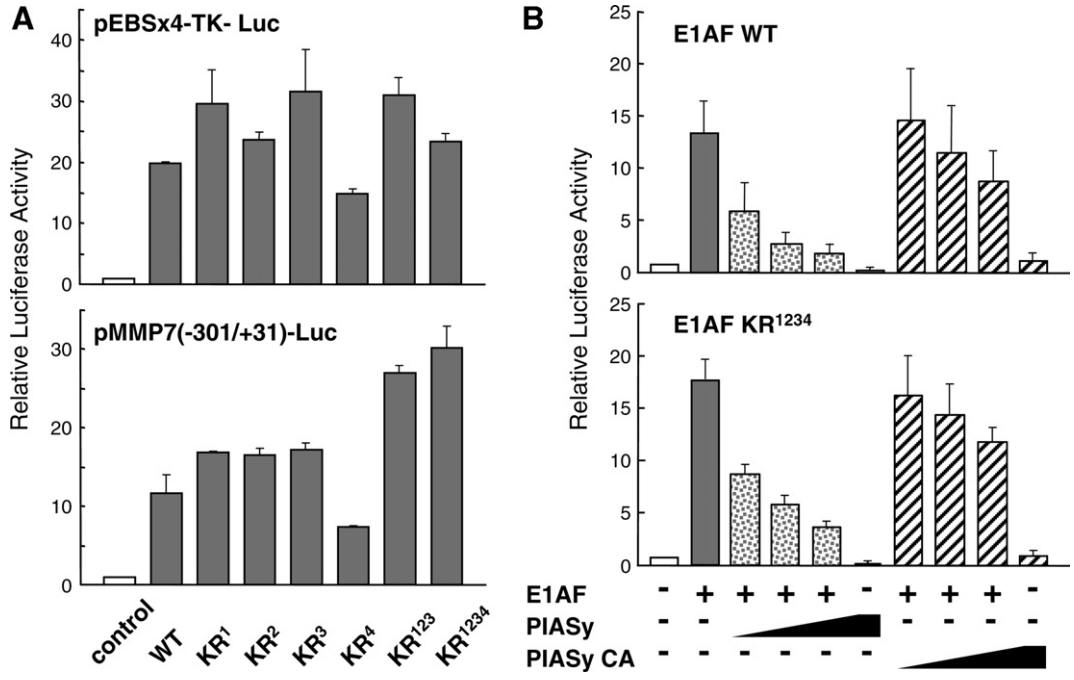

Fig. 3. SUMO acceptor site mutation and PIASy affect E1AF-dependent transcription. (A) HepG2 cells were transiently co-transfected with pFLAG-E1AF (either wild-type or indicated KR mutant) and reporter plasmid (pEBSx4-TK-Luc or pMMP7(−301/+31)-Luc). The relative activity of the luciferase construct co-transfected with the relevant empty vector (control) was set to 1. The data shown represent the averages of results from three independent experiments, and the error bars show the standard deviation. (B) HepG2 cells were transiently transfected with pFLAG-E1AF (either wild-type or KR^{1234}) and an increasing amount (50, 200, and 500 ng) of pEGFP-PIASy (either wild-type or C342A). The relative luciferase activity was determined as described above.

from the artificial promoter (Fig. 3A, upper panel). These mutants showed, however, a significantly higher activity (~3-fold) than single site mutants from the *MMP7* promoter (Fig. 3A, lower panel). These data suggest that the sumoylation of E1AF antagonizes its transcriptional activation potential depending on promoter contexts. The *MMP7* promoter region from −301 to +31 contains three candidate Ets-binding sites; one canonical AP-1 site and two consensus Tcf-binding sites [28]. Indeed, Ets transcription factors act synergistically with these factors to activate the expression of genes [28]. It is possible that there is multiple sumoylation of E1AF to modulate the synergistic control of gene expression. E1AF is a relatively unstable protein, and is degraded through the ubiquitin–proteasome pathway [29]. Sumoylation can affect ubiquitination and the following proteasome-mediated degradation in some target proteins [30,31]. However, increasing activity of E1AF sumoylation defective mutants is not due to increasing stability of mutant proteins, since both wild-type and KR^{1234} E1AF showed a similar half-life (data not shown). Consistent with this finding, mutation of the E1AF sumoylation sites did not significantly affect its polyubiquitination (data not shown).

PIASy represses Ets-1-dependent transcription

PIAS proteins can be thought of as transcriptional co-regulators whose actions can either be activating or repressive through the divergent mechanisms depending on the target gene or interacting transcriptional factor [32,33]. We examined whether overexpressing PIASy has an effect on E1AF-mediated transactivation. HepG2 cells were transiently transfected with EBSx4-TK-Luc and E1AF (either wild-type or KR^{1234}) expressing plasmids, in the presence of PIASy (either wild-type or C342A mutants) expressed constructs. PIASy decreased wild-type E1AF activity in a dose-dependent manner (Fig. 3B, upper panel). In addition, KR^{1234} activity was repressed similar to that of the wild-type in the presence of an increasing amount of PIASy (Fig. 3B, lower panel), indicating that E1AF sumoylation is not required for the repression. This effect, however, required an intact RING-like domain of PIASy, because the C342A mutant is more modest efficiently as the wild-type. The RING-like structure of PIAS proteins is essential for E3 ligase activity, but is also required for the association with substrates and/or other co-factors [34]. These data suggest that PIASy might act as an inhibitor of E1AF-mediated transcription through interaction with E1AF in a sumoylation independent manner. Alternatively, PIASy might function as a SUMO-E3 ligase for other factor(s) involved in the regulation of E1AF activity by promoting their SUMO modification.

E1AF co-localizes with PIASy in the nucleus

Sumoylation of some proteins has been shown to influence their specific subcellular localization. However, KR^{1234} E1AF is localized diffusely throughout the nucleus as well as the wild-type, and no obvious changes were observed (Fig. 4A). Co-expression of SUMO-1 also did not affect the localization of both wild-type and KR^{1234} E1AF (data not shown). These results indicate that E1AF sumoylation does not influence its nuclear localization.

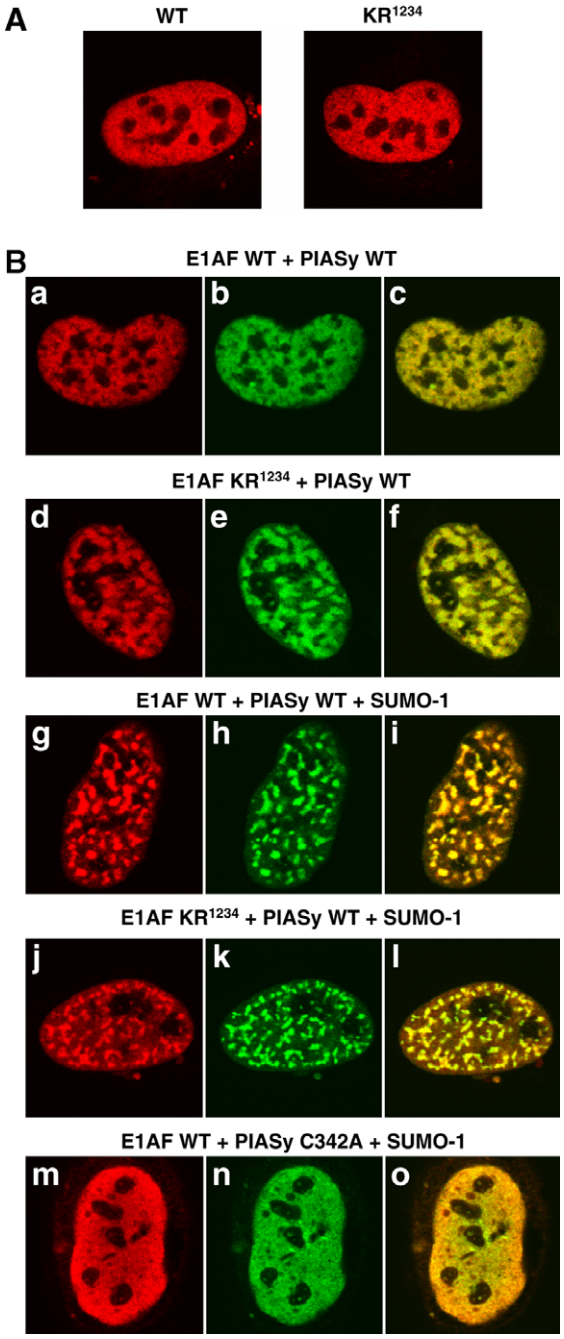

Fig. 4. E1AF co-localizes with PIASy in the cell nucleus. U2OS cells are shown to express wild-type (WT) or K96/226/260/322R mutant (KR^{1234}) E1AF-$MycHis_6$ alone (A), E1AF-$MycHis_6$ (either WT or KR^{1234}) and EGFP-PIASy WT (B, a–f), E1AF-$MycHis_6$ (either WT or KR^{1234}), and EGFP-PIASy (either WT or C342A) and FLAG-SUMO-1 (B, g–o). E1AF was detected with anti-Myc antibody (a, d, g, j, and m; red). EGFP-PIASy was visualized by autofluorescence (b, e, h, k, and n; green). FLAG-SUMO-1 was efficiently expressed in almost 100% of the infected cells (data not shown). Merged images (c, f, i, l, and o; yellow) show the co-localization of E1AF and PIASy.

One major mechanism in which PIAS proteins influence the transcriptional activity is thought to be through the relocalization of transcriptional factors to different subnuclear compartments [33]. In the case of the transcription factor LEF1, the recruitment to PML bodies by PIASy is thought to be important in repressing its activity [34]. To clarify the mechanism of PIASy-mediated repression, we investigated whether PIASy could affect E1AF nuclear localization. When co-expressed with PIASy, both wild-type and KR^{1234} E1AF showed a partly condensed pattern in the nucleus co-localizing with PIASy (Fig. 4B, a–f). Furthermore, upon co-expression with both wild-type PIASy and SUMO-1, both wild-type and KR^{1234} E1AF were co-localized with PIASy in the nuclear speckles (Fig. 4B, g–l). In contrast, the SUMO-E3 ligase defective C342A mutant PIASy was diffused throughout the nucleoplasm upon co-expression of SUMO-1, and did not induce E1AF nuclear relocalization (Fig. 4B, m–o). Collectively, E1AF co-localizes with PIASy in the nuclear bodies, independently from its sumoylation, but dependent on SUMO-E3 ligase activity of PIASy. Thus, transcriptional repression and subnuclear relocalization by PIASy are coupled. PIASy might sequestrate E1AF into the repressive complex in the subnuclear compartments, independent of its sumoylation status. Further studies will be needed to better understand the molecular mechanisms through which sumoylation and PIASy regulate E1AF-mediated transcription.

In conclusion, our findings demonstrate that E1AF is subjected to SUMO modification and is negatively regulated by this modification. Furthermore, PIASy functions as SUMO-E3 ligase for E1AF and also acts as a transcriptional inhibitor of E1AF, dependent on its SUMO-E3 ligase activity, but independent of E1AF sumoylation status. These findings provide new insight into the regulation of E1AF transcriptional activity and E1AF-induced tumor metastasis.

Acknowledgments

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