TIF1γ requires sumoylation to exert its repressive activity on TGFβ signaling

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Accepted 20 May 2013
Journal of Cell Science 126, 3713–3723

doi: 10.1242/jcs.126748

Summary

TIF1γ, a new regulator of TGFβ signaling, inhibits the Smad4-mediated TGFβ response by interaction with Smad2/3 or ubiquitylation of Smad4. We have shown that TIF1γ participates in TGFβ signaling as a negative regulator of Smad4 during the TGFβ-induced epithelial-to-mesenchymal transition (EMT) in mammary epithelial cells, and during terminal differentiation of mammary alveolar epithelial cells and lactation. We demonstrate here that TIF1γ is sumoylated and interacts with Ubc9, the only known SUMO-conjugating enzyme. Four functional sumoylation sites lie within the middle domain of TIF1γ, the Smad interaction domain. We show that a sumoylation-defective TIF1γ mutant significantly reduces TIF1γ inhibition of Smad complexes and that of the Smad-mediated TGFβ transcriptional response. Moreover, chromatin immunoprecipitation experiments indicate that TIF1γ sumoylation is required to limit Smad4 binding on the PAI-1 TGFβ target gene promoter. Ectopic expression of TIF1γ in mammary epithelial cells inhibits TGFβ-induced EMT, an effect relieved by expression of non-sumoylated TIF1γ. Taken together, our results identify a new TGFβ regulatory layer, whereby sumoylation strengthens the TIF1γ repressive action on canonical TGFβ signaling.

Key words: TIF1gamma, TRIM33, TGFbeta, Sumoylation, Smad

Introduction

Transforming growth factor β (TGFβ) exerts key functions in cell growth, differentiation, apoptosis, development and tumorigenesis. The TGFβ signaling pathway involves a small class of signaling effectors, the Smads, which act as key mediators of the cell response to TGFβ. Following ligand binding to type II/type I receptor complexes, activated type I receptors phosphorylate the receptor-activated Smads, Smad2 and Smad3. Release of activated Smad2/3 from receptors leads to the formation of a heterotrimeric complex with the common-mediator Smad (Co-Smad), Smad4. Smad complexes enter the nucleus where they interact at promoters of TGFβ target genes with other transcription factors and in cooperation with tissue-specific activators and repressors to regulate gene expression (Massagué et al., 2005). Transcriptional intermediary factor 1γ (TIF1γ; also called ectodermin, TRIM33, RFG7 or PTC7) is a member of the TIF1 family of transcriptional cofactors, characterized by an N-terminal RING-finger B-box coiled-coil (RBCC/TRIM) motif and a C-terminal bromodomain preceded by a PHD finger (Venturini et al., 1999; Yan et al., 2004). TIF1γ has been implicated in TGFβ signaling through its binding to phosphorylated Smad2/3 (He et al., 2006; Xi et al., 2011). TIF1γ could also antagonize Smad4 through its ubiquitin ligase properties (Dupont et al., 2009; Dupont et al., 2005; Levy et al., 2007; Morsut et al., 2010) and, more generally, as a repressor of TGFβ superfamily-induced transcription by restricting the residence time of activated Smad complexes on the promoters of target genes (Agricola et al., 2011). Several reports indicate that TIF1γ is an important regulator of transcription during hematopoiesis (Bai et al., 2010; He et al., 2006; Kusy et al., 2011; Ransom et al., 2004) and may also be a key actor of tumorigenesis (Aucagne et al., 2011; Herquel et al., 2011; Vincent et al., 2009). In addition, we have recently demonstrated that TIF1γ regulates the TGFβ-induced epithelial-to-mesenchymal transition (EMT) in mammary epithelial cells (Hesling et al., 2011) and during terminal differentiation of mammary alveolar epithelial cells and lactation (Hesling et al., 2013) through repression of Smad4 activity.

Sumoylation is a post-translational modification that involves the covalent addition of small ubiquitin-like modifier (SUMO) residues on a protein substrate by a mechanism similar to ubiquitylation. Sumoylation is a multi-step process initiated by the E1 SUMO-activating enzymes, followed by an E2 SUMO-conjugating enzyme (ubiquitin carrier 9, Ubc9). A third enzyme, E3 ligase, forms a complex with SUMO-conjugated Ubc9 and the protein substrate and enhances the efficiency and specificity of SUMO transfer. Conjugation to SUMO is reversible and desumoylation is catalyzed by SUMO-specific proteases (SENPs). Like other post-translational modifications, sumoylation creates an additional level of regulation and can

control diverse cellular processes, including transcription, nuclear transport and signal transduction, depending on the identity of the substrate (Gareau and Lima, 2010). The identification of TIF1α and TIF1β as SUMO substrates (Ivanov et al., 2007; Mascle et al., 2007; Seeler et al., 2001) prompted us to test whether TIF1γ might also be a substrate for this post-translational modification. In this study, we show that the middle region of TIF1γ is a specific target of SUMO-1. We further demonstrate that sumoylation of TIF1γ is required for its repressive activity on the Smad4 transcriptional response. These findings support a model whereby temporal regulation of TGFβ signaling by TIF1γ modifications implicates a blockade of Smad4 function.

Results

Modification of TIF1γ by sumoylation

We used the His6-pull down assay to determine whether TIF1γ is a substrate for post-translational modification by SUMO. Nickel affinity purification of His-tagged protein complexes from whole-cell extracts of HMEC-TR cells transfected with TIF1γ in the presence of exogenous (His-tagged) SUMO-1, yielded several immunoreactive bands. These bands were not perceptible in the absence of exogenously expressed His6–SUMO-1 (Fig. 1A). In support of the conclusion that these species correspond to sumoylated TIF1γ, they were markedly increased when Ubc9, the only known SUMO-conjugase, and the SUMO-ligase PIAS1, were expressed in the presence of SUMO-1 (Fig. 1A). A yeast two-hybrid screen confirmed that Ubc9 interacts with the TIF1γ middle region (see Materials and Methods). To corroborate this result, we next measured the endogenous interaction of TIF1γ and Ubc9 by co-immunoprecipitation in HMEC-TR cells. Co-precipitation was observed when cell lysates were immunoprecipitated with antibody against TIF1γ (Fig. 1B), confirming the interaction between TIF1γ and Ubc9. TIF1γ immunoprecipitates of TIF1γ-transfected HMEC-TR cells transfected or not with a SUMO-1 construct showed slow-migrating bands detected by a SUMO-1 antibody (Fig. 1C). SUMO modification of endogenous TIF1γ was also observed in the presence of exogenous TGFβ (Fig. 1D) and was strongly increased when both exogenous TIF1γ and SUMO-1 were expressed (Fig. 1E). No accumulation of these bands was observed after specific MG132 inhibition of proteasome-dependent degradation, consistent with a post-translational modification by sumoylation rather than ubiquitylation (Fig. 1F). Finally, expression of either SUMO-2 or SUMO-3 did not induce the TIF1γ sumoylation pattern observed after SUMO-1 transfection (Fig. 1G). Accordingly, further studies were performed with SUMO-1 only. Because desumoylation is mediated by SENP-family proteases (Yeh, 2009) and to investigate the reversibility of TIF1γ sumoylation, we expressed SENP1 together with SUMO-1. Under these conditions, TIF1γ sumoylation was abolished, whereas expression of the catalytically inactive mutant of SENP1 (R630L/K631M) (Cheng et al., 2007) restored the TIF1γ sumoylation pattern (Fig. 1H). Taken together, these results demonstrate that TIF1γ is efficiently modified by SUMO-1, a process that can be enhanced by TGFβ treatment.

Identification of functional sumoylation sites in TIF1γ

Where is TIF1γ sumoylated? We first identified 16 consensus sumoylation sites [ΨKx(D/E)] along the entire TIF1γ sequence using the SUMOplot software (Abgent, San Diego, CA, USA). We mutated each of these lysines into arginines in an attempt to

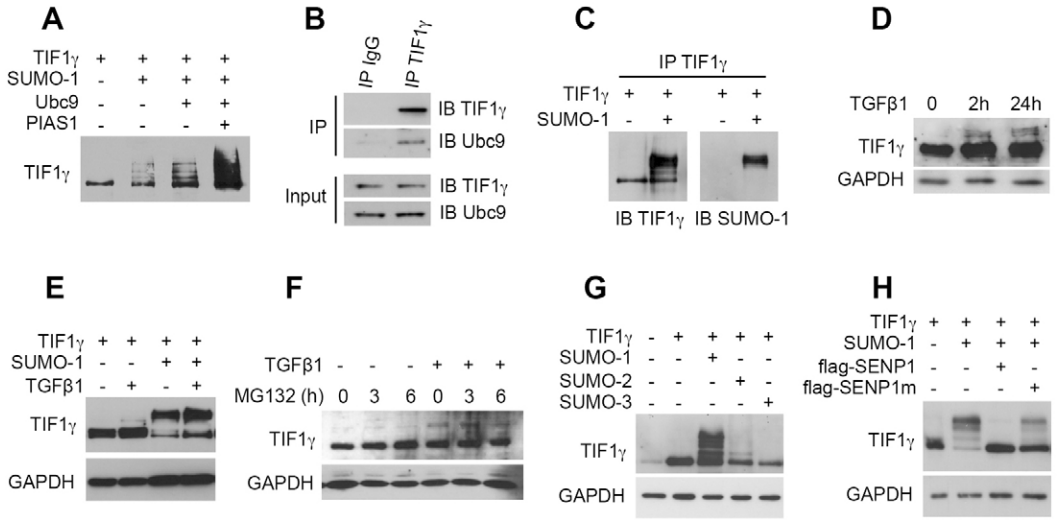

Fig. 1. SUMO modification of TIF1γ. (**A**) HMEC-TR cells were transfected with either TIF1γ expression vector alone or in combination with His_6-tagged SUMO-1, Ubc9 or PIAS1 expression vectors. Cell lysates were subjected to His_6 pull-down assays with Ni-NTA beads and immunoblotted with anti-TIF1γ antibody. (**B**) Cells lysates of HMEC-TR cells were immunoprecipitated (IP) and immunoblotted (IB) as indicated. Protein expression was monitored by immunoblot analysis of total cell extracts (Input). (**C**) Cell lysates of HMEC-TR cells transfected with TIF1γ and SUMO-1 were immunoprecipitated (IP) and immunoblotted (IB) as indicated. (**D**) HMEC-TR cells were treated or not with TGFβ for the indicated times. Total protein extracts were lysed in the presence of 10 mM N-ethylmaleimide to inhibit sumoylase activities and immunoblotted with anti-TIF1γ antibody. GAPDH was used as a loading control. (**E**) HMEC-TR cells were transfected with TIF1γ and SUMO-1 and treated with TGFβ as indicated. Total protein extracts were immunoblotted with anti-TIF1γ antibody. GAPDH loading controls are as above. (**F**) HMEC-TR cells were treated or not with TGFβ for 24 h and then with the proteasome inhibitor MG132 for the indicated time. Total protein extracts were immunoblotted with anti-TIF1γ antibody. GAPDH was used as a loading control. (**G**,**H**) Cell lysates of HMEC-TR cells transfected with the indicated expression vectors were immunoblotted with anti-TIF1γ antibody. SENP1m is a catalytically inactive mutant of SENP1 (R630L/K631M). GAPDH was used as a loading control.

disrupt TIF1γ sumoylation. Single point mutations only slightly inhibited TIF1γ sumoylation (data not shown), suggesting the existence of multiple SUMO-conjugation sites, possibly acting in concert. Accordingly, we generated a series of double, triple and quadruple lysine mutants (supplementary material Fig. S1). Only mutation of four lysines (K776R/K793R/K796R/K839R) into arginines fully abrogated sumoylation, identifying these residues as functional SUMO attachment sites (Fig. 2A). The fully sumoylation-defective quadruple mutant will be henceforth referred to as TIF1γ-Mut. Comparison of the human TIF1γ sites with those found in the same region in other organisms revealed a strong conservation of the positions of the four sumoylated lysine residues (Fig. 2B,C). Of note, these sumoylation sites were all located within the middle domain of TIF1γ (Fig. 2B), which has been identified as the interaction domain with Smad proteins (He et al., 2006; Dupont et al., 2005). We also generated a Ubc9-TIF1γ fusion protein that was efficiently sumoylated in a Ubc9-dependent manner (Jakobs et al., 2007), markedly more so in the presence of SUMO-1 (Fig. 2D).

Sumoylation has previously been shown to modulate protein stability. We asked whether such an effect could be observed for modified TIF1γ using HMEC cells transfected with either FLAG-tagged wild-type TIF1γ or TIF1γ-Mut and treated with cycloheximide. Our results conclusively show that TIF1γ sumoylation did not alter TIF1γ stability (Fig. 2E,F). Finally, to rule out the possibility that the absence of SUMO-1 modification in cells expressing TIF1γ-Mut is the consequence

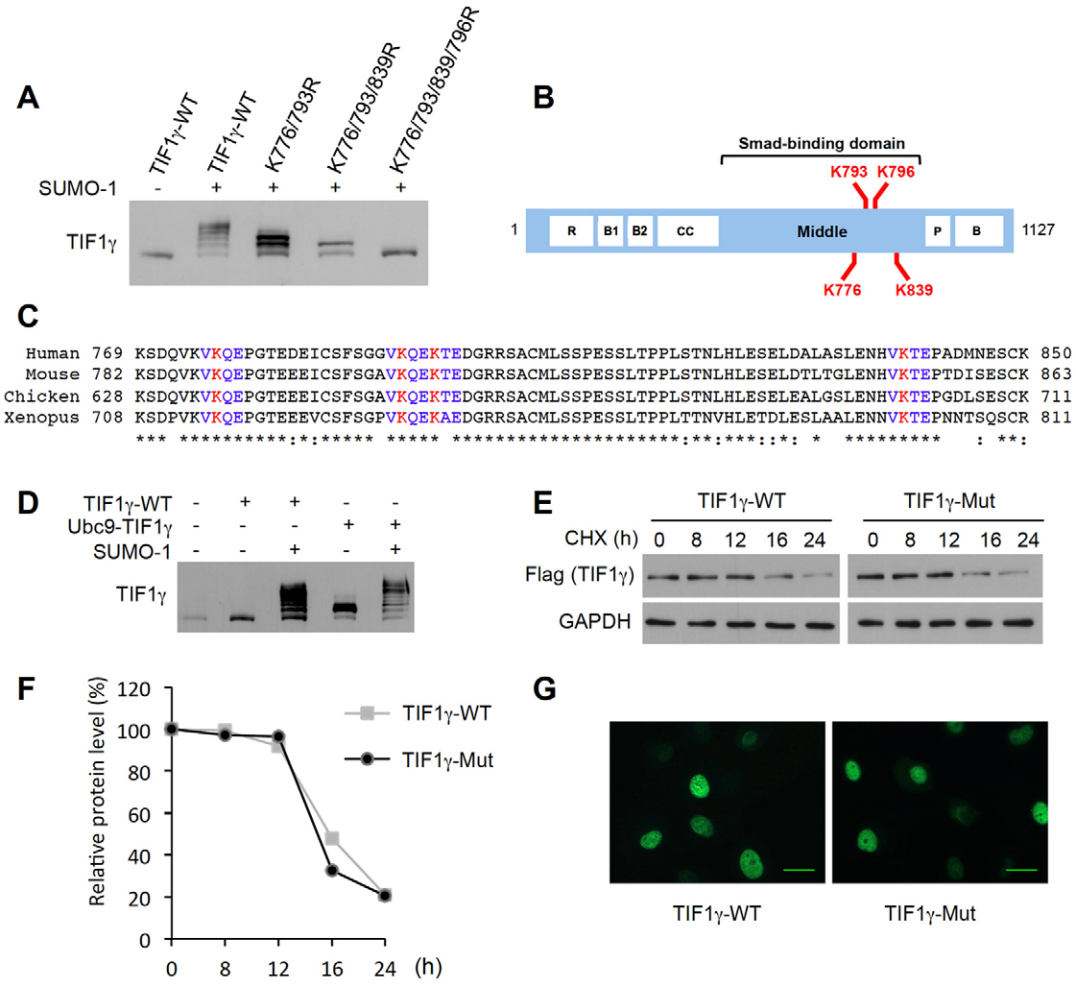

Fig. 2. Identification of TIF1γ sumoylation sites. (**A**) HMEC-TR cells were transfected with His_6-tagged SUMO-1 and TIF1γ mutants as indicated. Lysates were subjected to a His_6 pull-down using Ni-NTA beads and immunoblotted with anti-TIF1γ antibody. (**B**) Schematic representation of TIF1γ. The structure is characterized by an N-terminal motif composed of a RING finger and, surrounding the middle domain, two B-box domains (B1 and B2) and a coiled-coil (CC) domain (RBCC/TRIM), and a C-terminal PHD finger (P) and bromodomain (B). The four functional SUMO sites are shown in red. (**C**) Conservation of the ΨKx (D/E) sumoylation motifs in TIF1γ from various species. The TIF1γ sequence harboring the SUMO attachment sites was compared between the indicated species. Four conserved SUMO-targeted lysine residues (K) are shown in red. '*' and ':' denote identical or related amino acids, respectively. (**D**) Cell lysates from HMEC-TR cells transfected with the indicated constructs were processed as in A. Note that the N-terminal fusion of full-length Ubc9 to TIF1γ causes an 18-kDa shift compared with that of endogenous TIF1γ. (**E**) HMEC-TR cells were transfected with FLAG-tagged wild-type (TIF1γ-WT) or the sumoylation-defective mutant (TIF1γ-Mut) and treated with 20 μg/ml cycloheximide (CHX) for the indicated times. Protein expression was monitored by immunoblot analysis of total cell extracts using anti-FLAG antibody. GAPDH was used as a loading control. (**F**) Band intensities in E were quantified using a densitometer. Data are representative of two independent experiments. (**G**) MCF10A cells were stably infected with expression vectors encoding TIF1γ-WT or TIF1γ-Mut. TIF1γ nuclear localization was visualized by immunofluorescence using an anti-TIF1γ antibody. Scale bar: 26 μm.

of aberrant sub-cellular localization, mammary epithelial MCF10A cells stably expressing wild-type and mutant TIF1γ proteins (supplementary material Fig. S2) were analyzed by immunofluorescence. As shown in Fig. 2G, the quadruple mutant displayed the same diffuse-granular nuclear distribution as its wild-type counterpart. Taken together, our results demonstrate that TIF1γ is sumoylated on four evolutionarily conserved lysine residues (Lys-776, −793, −796 and −839) located within the middle domain of TIF1γ. These modifications, which do not alter the stability or localization of the protein, were studied further as described below.

TIF1γ is SUMO-modified in the nucleus

We first asked where in the cell TIF1γ protein is sumoylated and whether this localization is required for modification. A putative Nuclear Localization Site (NLS) was found at the C-terminus of TIF1γ (Fig. 3A). We mutated lysine 1118 into glutamic acid (K1118E mutant) using arginine or glycine mutants (K1118R and K1118G, respectively) as controls that preserve the basic charge of the sequence. As expected, wild-type TIF1γ as well as K1118R and K1118G mutants displayed nuclear staining and could be sumoylated (Fig. 3B,C). In contrast, mutation of lysine 1118 into glutamic acid (K1118E mutant) had two effects: cytoplasmic localization and loss of sumoylation (Fig. 3B,C). Most importantly, the K1118E mutant, unlike its wild-type counterpart, could not repress the TGFβ transcriptional response (Fig. 3D). These results indicate that the presumptive NLS localized at the C-terminal end of TIF1γ protein is indeed functional and that the nuclear localization of TIF1γ is required for the sumoylation of the protein and for its repressive activity.

TIF1γ sumoylation regulates Smad complex formation

We next asked how TIF1γ sumoylation might impact the TGFβ pathway. Because we localized the TIF1γ sumoylation sites within its middle Smad-protein interaction domain, we first examined whether TIF1γ sumoylation could affect Smad complex formation induced by TGFβ. As expected, TGFβ induced the formation of the Smad3/4 complex (compare lanes 1 and 2 in Fig. 4A). Note that transfection of SUMO-1 appeared to increase the interaction between Smad3 and Smad4 (compare lanes 2 and 4 in Fig. 4A). We next investigated whether TIF1γ-Mut could influence Smad complex formation. Unlike wild-type TIF1γ, which strongly decreased both Smad3/Smad4 (lane 6, Fig. 4A) and Smad2/Smad4 (lane 5, Fig. 4B) interactions, the sumoylation-defective mutant could not inhibit Smad2/Smad4 (lane 7, Fig. 4B) or Smad3/Smad4 (lane 6, Fig. 4C) complex formation. Importantly, in the presence of SUMO-1, TIF1γ-WT, but not TIF1γ-Mut, inhibited the Smad3/Smad4 interaction induced by TGFβ stimulation (compare lanes 4 and 8, Fig. 4D). These results indicate that the specific sumoylation of TIF1γ negatively regulates Smad3/Smad4 interactions and, by inference, most likely favors TIF1γ interactions with Smad2 and Smad3 instead. In contrast, the hyper-sumoylated Ubc9-TIF1γ fusion protein was more efficient than wild-type TIF1γ in inhibiting Smad interactions (see lane 8, Fig. 4C).

TIF1γ is known to interact with Smad proteins in a TGFβ-dependent manner (He et al., 2006). Accordingly, TGFβ stimulation enhanced the interaction of wild-type TIF1γ with Smad2 or Smad3 (lane 5, Fig. 4B; lane 3, Fig. 4E). Note that this interaction was favored upon SUMO-1 expression (compare lanes 2 and 3 to lanes 4 and 5, Fig. 4E). In contrast, expression of the SENP1 SUMO protease inhibited formation of the TIF1γ/Smad3 complex (lanes 6 and 7, Fig. 4E). Lack of TIF1γ sumoylation strongly inhibited the interaction between TIF1γ

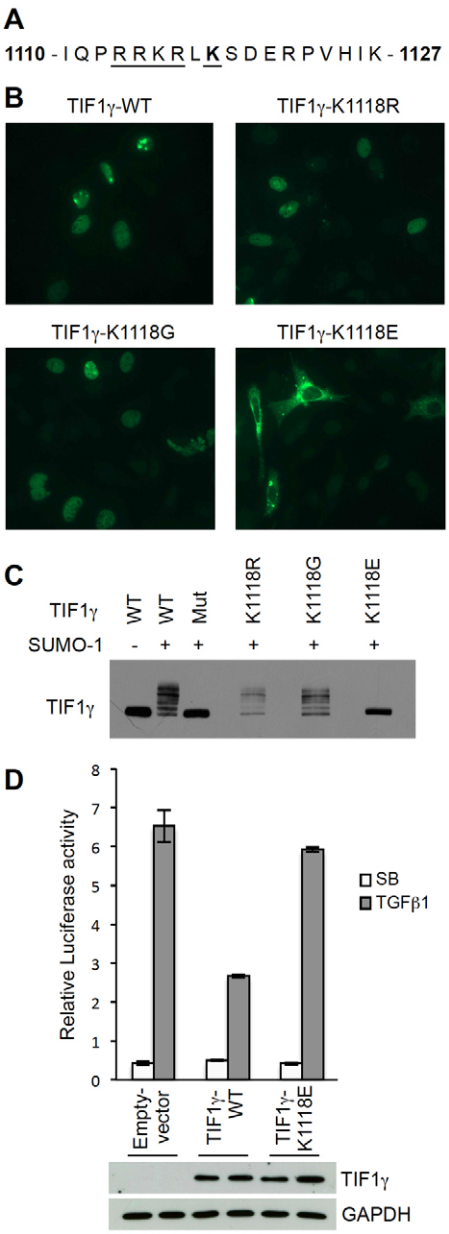

Fig. 3. TIF1γ is SUMO-modified in the nucleus. (**A**) The TIF1γ NLS: basic amino acids are underlined, the mutated lysine (K1118) is shown in bold. (**B**) TIF1γ immunofluorescence in HMEC-TR cells transfected with the indicated constructs. (**C**) Lysates from HMEC-TR cells transfected with indicated constructs were subjected to His_6 pull-down using Ni-NTA beads and immunoblotted with anti-TIF1γ antibody. (**D**) HMEC-TR cells were co-transfected with the pGL3$(CAGA)_9$-Luc reporter vector together with the pRL-SV40 internal control vector as well as with the indicated TIF1γ expression vectors. Cells were treated with the TβRI kinase inhibitor SB-431542 (SB) as control, or with TGFβ for 24 hours. SB-431542 blocks any signaling arising from low endogenous TGFβ production. Relative luciferase activity is given in arbitrary units as the mean ± s.d. of an experiment performed in triplicate, representative of three independent experiments. Protein expression (TIF1γ, GAPDH) was monitored by immunoblot analysis of total cell extracts.

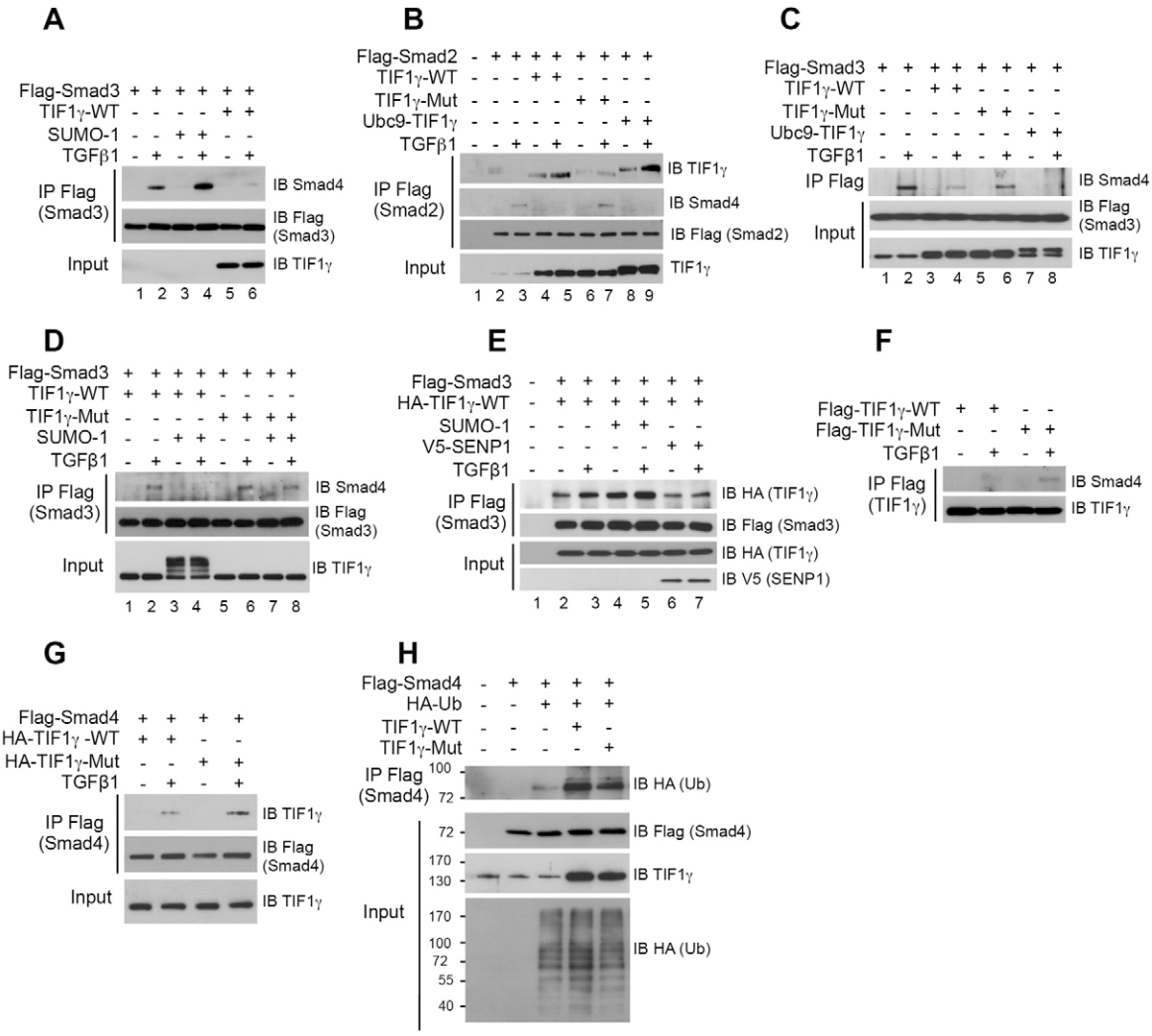

Fig. 4. Lack of sumoylation compromises the ability of TIF1γ to inhibit Smad complex formation. (**A**–**G**) HMEC-TR cells were transfected with the indicated vectors. At 48 h post-transfection, cells were treated or not with TGFβ for 2 h. Lysates were immunoprecipitated (IP) and immunoblotted (IB) as indicated. Protein expression was monitored by immunoblot analysis of total cell extracts (Input). (**H**) Sumoylation does not affect TIF1γ E3 ubiquitin ligase activity on Smad4. HMEC-TR cells were transfected with the indicated vectors. At 48 h post-transfection, cell lysates were immunoprecipitated (IP) with anti-FLAG antibody and immunoblotted (IB) with anti-HA antibody to detect Smad4 ubiquitylation. Protein expression was monitored by immunoblot analysis of total cell extracts (Input). Ub, ubiquitin.

and Smad2 (lanes 6 and 7, Fig. 4B), while TGFβ stimulation enhanced the interaction between Ubc9-TIF1γ fusion protein and Smad2 (lanes 8 and 9, Fig. 4B). Finally, we analyzed the interaction of Smad4 with TIF1γ using mutual co-immunoprecipitation in HMEC-TR cells expressing wild-type or mutant TIF1γ. We found that overexpressing sumoylation-defective TIF1γ-Mut led to a moderate but reproducible enhancement of binding to Smad4 in the presence of TGFβ (Fig. 4F,G). We next investigated whether TIF1γ sumoylation could affect the previously reported ability of TIF1γ to mono-ubiquitylate Smad4 (Dupont et al., 2009). Lack of TIF1γ sumoylation did not affect TIF1γ E3 ubiquitin ligase activity, as shown by the increased ubiquitylation pattern of Smad4 observed upon co-expression of both wild-type or mutant TIF1γ (Fig. 4H).

Taken together, these results show that lack of TIF1γ sumoylation significantly reduces the ability of TIF1γ to inhibit Smad4-Smad2/3 complex formation, suggesting that TIF1γ sumoylation is crucial for the repressive activity of TIF1γ on Smad4 function. We address this hypothesis next.

TIF1γ sumoylation restricts Smad4 residence on the PAI-1 promoter

In addition to a role in the regulation of Smad complex formation, TIF1γ has recently been shown to bind chromatin in the promoter region of TGFβ target genes (Agricola et al., 2011; Hesling et al., 2011; Xi et al., 2011). We are particularly interested in a group of genes antagonistically regulated by TIF1γ and Smad4 and involved in TGFβ-induced EMT. Our own results have shown that PAI-1 transcriptional activation by TGFβ is enhanced upon TIF1γ depletion and severely inhibited upon Smad4 depletion (Hesling et al., 2011). To determine what role, if any, TIF1γ sumoylation might play in these regulatory processes, we performed chromatin immunoprecipitation (ChIP)

assays in HMEC-TR cells known to express PAI-1 in response to TGFβ in a Smad-dependent manner. To analyze the sequence of events at the chromatin level, we followed the kinetics of Smad4 and TIF1γ recruitment at the Smad-binding region of the PAI-1 promoter (−791 to −546) after TGFβ treatment.

Consistent with its described role as a transcriptional repressor, TIF1γ was detected at the PAI-1 promoter in the absence of TGFβ treatment. TGFβ stimulation induced Smad4 enrichment onto the promoter, which was correlated with transient TIF1γ dissociation. Smad4 occupancy decreased again after 2 hours of TGFβ stimulation, with a concomitant recovery of TIF1γ occupancy of the Smad-binding region of the PAI-1 promoter beginning after 90 minutes (Fig. 5A). The optimal enrichment of TIF1γ at the promoter seen 2 hours after TGFβ stimulation suggests that TIF1γ is required for limiting Smad4 residence on the PAI-1 promoter and thus acts as a repressor of Smad4 functions. Comparable kinetics were observed upon ectopic expression of TIF1γ (Fig. 5B), although this reduced the time span of Smad4 residence on the PAI-1 promoter. Of marked interest is the observation that sumoylation-defective TIF1γ-Mut was not capable of interfering with Smad4 residence on the PAI-1 promoter (Fig. 5C), suggesting that lack of sumoylation renders TIF1γ unable to limit Smad4 association with this region, which still increased after 2 hours of TGFβ stimulation. We confirmed this requirement for sumoylated TIF1γ for termination of Smad4 signaling by performing a ChIP kinetics analysis in HMEC-TR cells upon ectopic expression of the hyper-sumoylated Ubc9-TIF1γ fusion protein. As expected if TIF1γ sumoylation serves as a signal to restrict Smad4 residence on the PAI-1 promoter, this led to strongly reduced Smad4 occupancy on the PAI-1 promoter (Fig. 5D).

TIF1γ sumoylation regulates TGFβ-induced EMT

We have shown that TIF1γ downregulation facilitates the contribution of Smad4 to TGFβ-induced EMT (Hesling et al., 2011). As demonstrated above, sumoylation of TIF1γ can regulate its interaction with Smad proteins, leading us to investigate the functional role of this post-translational modification in the regulation of TGFβ signaling. We first checked whether sumoylation of TIF1γ could affect its ability to repress TGFβ signaling. Using HMEC-TR cells expressing sumoylation-defective TIF1γ-Mut and a luciferase construct driven by the activated Smad complex, we observed that lack of TIF1γ sumoylation significantly reduced the ability of TIF1γ to repress the TGFβ transcriptional response (Fig. 6A). This result strongly suggests a correlation between TIF1γ repressive activity and its sumoylation capacity.

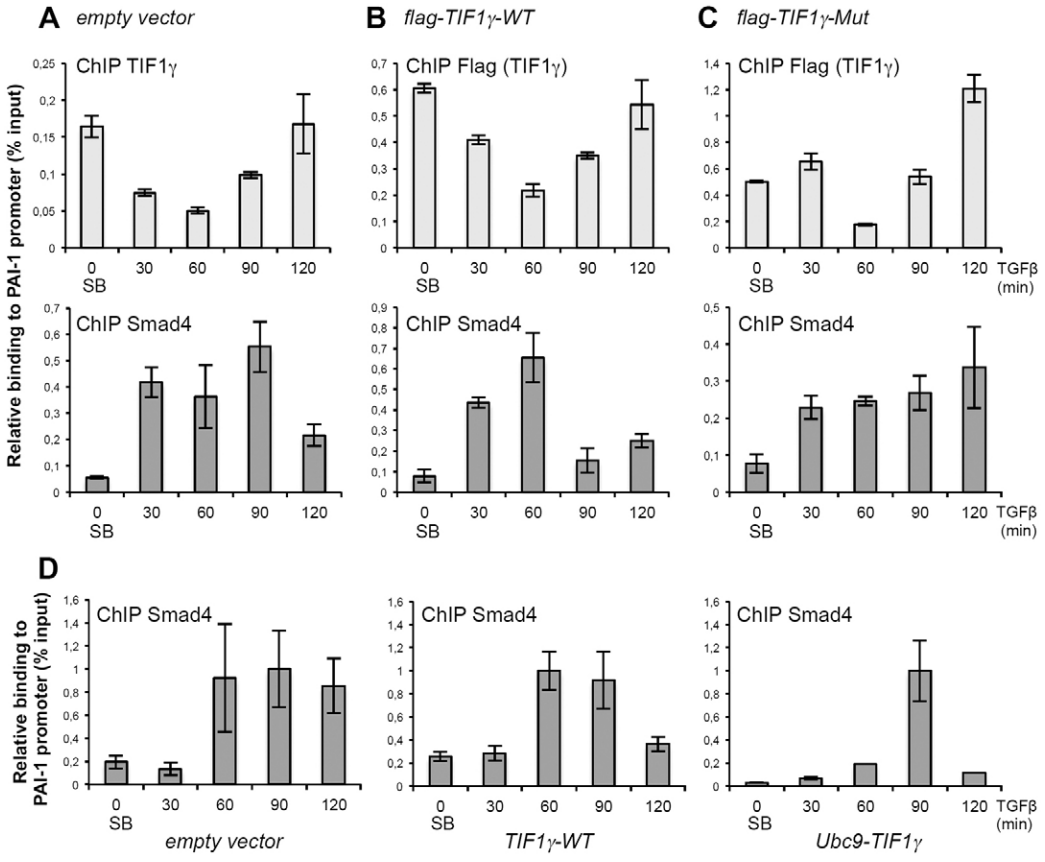

Fig. 5. TIF1γ sumoylation restricts the residence of Smad4 on the PAI-1 promoter. HMEC-TR cells were transfected with either empty vector (**A**), FLAG-TIF1γ-WT (**B**) or FLAG-TIF1γ-Mut (**C**) and treated with SB-431542 (SB) or TGFβ for the indicated time. ChIP assays were carried out using anti-Smad4, anti-TIF1γ or anti-FLAG antibodies as indicated. Precipitated genomic DNA was subjected to quantitative PCR to amplify the PAI-1 promoter region harboring the Smad-binding elements. (**D**) HMEC-TR cells were transfected with either empty vector, TIF1γ-WT or Ubc9-TIF1γ and treated with SB-431542 or TGFβ for the indicated time (minutes). ChIP assays were carried out using anti-Smad4 antibody. Precipitated genomic DNA was subjected to quantitative PCR to amplify the PAI-1 promoter region harboring the Smad-binding elements. In all panels, results are shown as the percentage of input values. Error bars represent s.d. from triplicate samples, and each panel is representative of three independent experiments.

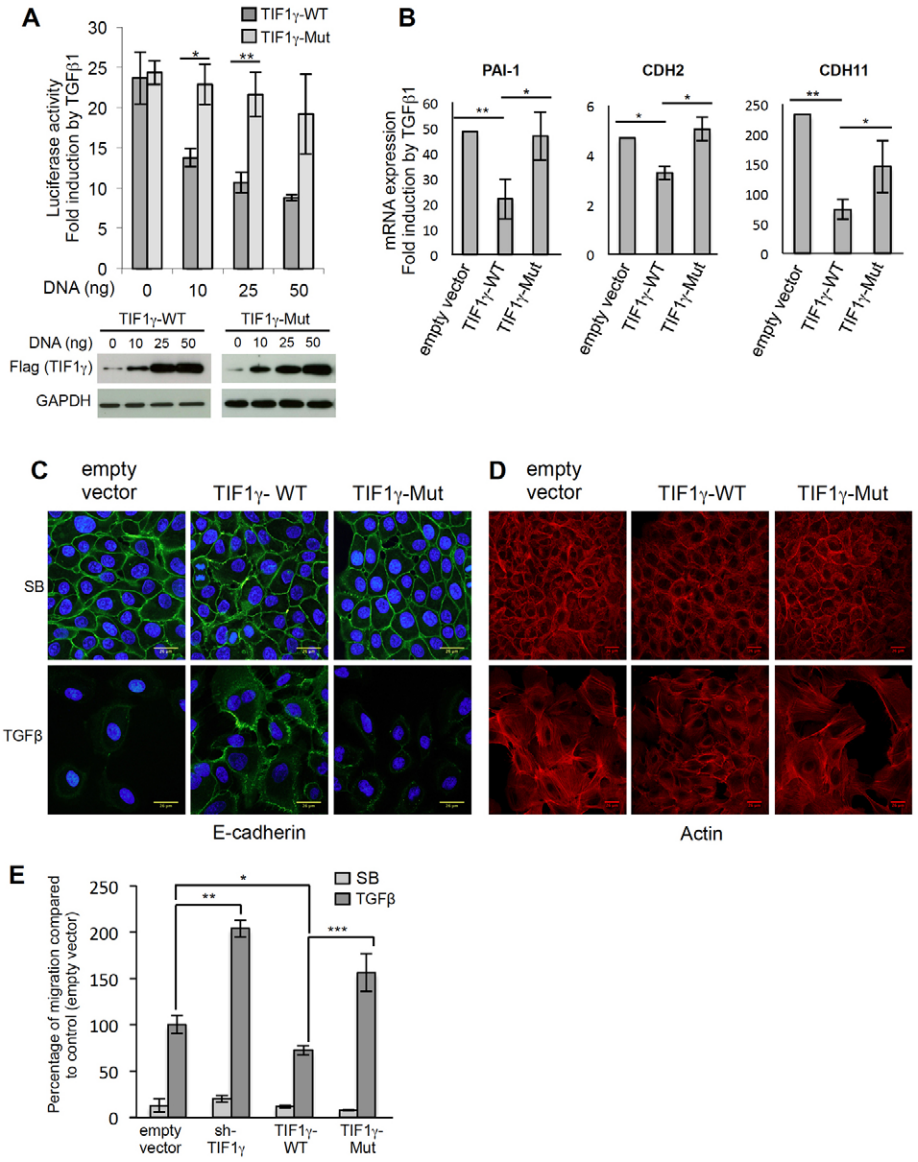

Fig. 6. TIF1γ sumoylation regulates TGFβ-induced EMT processes. (**A**) HMEC-TR cells cotransfected with the pGL3(CAGA)$_9$-Luc reporter vector together with increasing amounts of either wild-type (TIF1γ-WT) or mutated (TIF1γ-Mut) TIF1γ were treated with TGFβ for 24 h before measurement of luciferase activities. Error bars represent s.d. (n=3). Expression levels of TIF1γ and GAPDH proteins are shown. (**B–D**) Stably infected MCF10A cells expressing wild-type (TIF1γ-WT) or mutated (TIF1γ-Mut) TIF1γ were treated with SB-431542 (SB) or TGFβ for 24 h (B) or 96 h (C,D). (B) Expression of the mRNA encoding PAI-1 (serpine 1), CDH2 (N-cadherin) and CDH11 (OB-cadherin) was determined by RT-qPCR. Values were normalized to the amount of mRNA for HPRT and expressed relative to the value obtained in TGFβ-untreated controls (expressed as fold induction by TGFβ). Error bars represent s.d. (n=3). *P<0.05; **P<0.01. (C) E-cadherin expression was monitored by immunofluorescence. DAPI was used for nuclear staining. (D) The subcellular localization of actin was detected by imaging phalloidin–TRITC. Scale bars: 26 μm. (**E**) Stably transfected MCF10A cells inactivated for TIF1γ (sh-TIF1γ) or expressing WT (TIF1γ-WT) or mutated TIF1γ (TIF1γ-Mut) were treated with SB-431542 (SB) or TGFβ for 48 h prior to perform the Boyden chamber migration assay. 5% serum was used as a chemo-attractant during 22 h. Migrating cells were stained with Calcein AM and counted from random fields. Error bars represent s.d. The experiment shown is representative of three separate experiments performed in triplicate. *P<0.05; **P<0.01; ***P<0.001.

We then used MCF10A mammary epithelial cells (supplementary material Fig. S2), stably expressing either wild-type TIF1γ (TIF1γ-WT) or its sumoylation-defective mutant (TIF1γ-Mut) and analyzed by RT-qPCR the expression of the *PAI-1*, *CDH2* and *CDH11* TIF1γ-dependent genes implicated in EMT in these cells. The TGFβ-induced upregulation of *PAI-1*, *CDH2* and *CDH11* observed in control cells (empty vector) was inhibited in TIF1γ-WT cells but not in TIF1γ-Mut cells (Fig. 6B). This outcome indicates that the sumoylation-defective variant acts as a loss-of-function mutation and further establishes that sumoylation of TIF1γ is required for its repressive effect on TGFβ-dependent transcription.

Does sumoylation of TIF1γ, or lack thereof, affect the TGFβ-induced EMT process? To answer this question, we analyzed E-cadherin expression, cytoskeleton remodeling and cell migration in stably transfected MCF10A cells. Cells were treated with the TβRI kinase inhibitor SB-431542 (SB, which blocks any signaling arising from autocrine production of TGFβ) as control or with TGFβ for 96 hours. As expected, a 96 h-TGFβ treatment induced a complete loss of E-Cadherin expression in control cells (Fig. 6C, left bottom panel). In contrast to the effect of the wild-type protein (middle bottom panel), expression of TIF1γ-Mut failed to interfere with the TGFβ-induced loss of E-cadherin (compare the left and right bottom panels), establishing that TIF1γ sumoylation is also required for the repressive effects of the protein on this other aspect of TGFβ signaling.

This result was confirmed by studying actin cytoskeleton remodeling. Following stimulation with TGFβ, control cells (empty vector) underwent an EMT characterized by a transition to a spindle-like mesenchymal phenotype (Fig. 6D, compare upper and bottom left panels), whereas TIF1γ-WT expressing cells retained a much more regular epithelial morphology (middle panel). In this case as well, the sumoylation-defective TIF1γ mutant failed to inhibit the reorganization of actin fibers induced by TGFβ treatment (right panel). Finally, to correlate these effects with an additional hallmark of the EMT phenotype, cell motility, we performed a Boyden chamber migration assay using MCF10A cells stably transfected with vectors expressing an

shRNA directed against TIF1γ, TIF1γ-WT or TIF1γ-Mut (supplementary material Fig. S2). As shown in Fig. 6E, compared to empty-vector control cells, TGFβ-induced cell migration was clearly enhanced in MCF10A cells expressing the TIF1γ shRNA. As expected, cell migration was significantly reduced when wild-type TIF1γ was expressed. As a third line of evidence in support of a direct role for TIF1γ sumoylation in the repression of TGFβ-induced EMT, TIF1γ-Mut again acted as a loss-of-function mutation which did not interfere with TGFβ-dependent cell migration. Based on the results of the three independent assays illustrated in Fig. 6, we conclude that sumoylation of TIF1γ enhances its inhibitory effect on TGFβ-induced EMT. Conversely, lack of TIF1γ sumoylation abolishes this repressive function.

Discussion

Substrate modification by SUMO regulates protein–protein interactions, intracellular localization or protein functions and can therefore regulate diverse cellular processes, including transcription, replication and DNA repair (reviewed by Gareau and Lima, 2010). Post-translational modifications of TGFβ receptors and Smad proteins play a key role in the regulation of TGFβ signaling and participate in the elaboration of appropriately tuned TGFβ responses (reviewed by Xu et al., 2012). Sumoylation can play both positive and negative roles in the TGFβ pathway. In the case of TβRI, sumoylation stabilizes Smad2/3 binding to the TβRI receptor, leading to enhanced Smad activation (Kang et al., 2008). Sumoylation of Smad3 stimulates its nuclear export and inhibits Smad-dependent transcription (Imoto et al., 2008). Smad4 can also be sumoylated; this may prevent Smad4 ubiquitylation and protects it from degradation, thus enhancing TGFβ-induced Smad signaling (Lee et al., 2003; Lin et al., 2003; Ohshima and Shimotohno, 2003). However, Smad4 sumoylation was also reported to repress Smad-mediated transcription (Long et al., 2004; Miles et al., 2008). The sum total of the effects of sumoylation on TGFβ signaling are therefore quite complex (reviewed by Lönn et al., 2009).

In this study, we have extended the analysis of the effects of sumoylation of another modulator of TGFβ signaling, TIF1γ. Recently, a broad search for sumoylated proteins in brain cells identified TIF1γ as a substrate for sumoylation (Tirard et al., 2012) and, in keeping with this observation, we show here that TIF1γ is indeed covalently conjugated to SUMO-1 in the nucleus at lysines 776, 793, 796 and 839. In support of their functional significance, these four lysine residues are evolutionarily conserved and are all located within the middle domain of TIF1γ, which has been identified as the interaction domain with Smad proteins (He et al., 2006). The Ubc9 E2 enzyme and members of the E3 SUMO ligase PIAS family catalyze TIF1γ sumoylation by covalent attachment of SUMO-1, but not SUMO-2 or SUMO-3. Based on differences in their primary sequences, it appears likely that SUMO-1 and SUMO-2/3 are conjugated to distinct substrates and also differ in their ability to form SUMO chains (Gareau and Lima 2010). The biological functions of SUMO chains remain poorly characterized. SUMO chains could block the interaction of target proteins with partner proteins by masking interaction motifs, or could provide sites for interaction with SUMO chain-binding proteins such as the RING-finger 4 (RNF4) ubiquitin E3 ligase, thus facilitating SUMO chain-specific ubiquitylation (Tatham et al., 2008).

TGFβ signaling is negatively regulated by several proteins such as Smad7 or SnoN (Deheuninck and Luo, 2009; Moustakas and Heldin, 2009). It has been demonstrated that TIF1γ is also a negative regulator of TGFβ superfamily signaling. TIF1γ acts as an E3 ubiquitin ligase that targets Smad4 for ubiquitylation, thus limiting Smad4 nuclear residence on its target promoters (Agricola et al., 2011; Dupont et al., 2009). Ubiquitylation-mediated degradation of Smad4 can be antagonized by the FAM/USP9x de-ubiquitylase, thus restoring Smad4 function (Dupont et al., 2009). Results from others as well as our own have shown that TIF1γ is a potent inhibitor of Smad4 functions during TGFβ-induced EMT (Hesling et al., 2011), terminal differentiation of mammary alveolar epithelial cells and lactation (Hesling et al., 2013) and during specification of the ectoderm in *Xenopus* embryos (Dupont et al., 2005). Moreover the negative regulation of Smad4 by TIF1γ is essential to achieve proper dosage of Nodal responsiveness in mouse embryos (Morsut et al., 2010).

While TIF1γ expression inhibits TGFβ-induced transactivation, Smad4 is more available for association with Smad2/3 in TIF1γ-depleted cells, leading to an enhanced TGFβ response. Thus, varying TIF1γ/Smad4 ratios plays a critical role in the modulation of the transcriptional signal induced by TGFβ (Andrieux et al., 2012). Using promoter constructs driven by the activated Smad complex, we show that the sumoylation-defective mutant of TIF1γ significantly reduces the ability of TIF1γ to repress the TGFβ transcriptional response. This finding provides what is, to our knowledge, the first report showing that TIF1γ sumoylation is required for the inhibition of Smad4-dependent transcription. Smad4 is essential for TGFβ-induced EMT in mammary epithelial MCF10A cells and stable expression of either wild-type TIF1γ or a sumoylation-null mutant demonstrates that TIF1γ sumoylation is required to inhibit the TGFβ-induced EMT process.

It was recently proposed that TIF1γ could act as a repressor of TGFβ superfamily-induced transcription by restricting the residence time of activated Smad complexes at the promoter of target genes (Agricola et al., 2011). According to this model, TIF1γ ubiquitin ligase activity requires binding to histone H3 tails. Activated TIF1γ would then ubiquitylate chromatin-bound Smad4, leading to the disruption of Smad complexes and promoting their release from the promoter of TGFβ target genes. The major finding reported here is that TIF1γ sumoylation enhances its inhibitory effects on TGFβ signaling. In addition, we show that TIF1γ sumoylation is induced by TGFβ, by a still unknown mechanism. TGFβ stimulation triggers the nuclear accumulation of activated Smads that are targeted by the repressor TIF1γ. Induction of TIF1γ sumoylation by TGFβ could then be seen as a negative feedback signal that controls Smad4 residence on TGFβ target genes. This does not exclude that TIF1γ sumoylation might also be induced by other factors that remain to be identified.

TIF1γ has also been involved in TGFβ signaling through selective binding to phosphorylated Smad2/3 in competition with Smad4 (He et al., 2006). Massagué's laboratory recently demonstrated that TIF1γ-Smad2/3 complexes bind to H3K9me3 through recognition by PHD- and Bromo-domains, with an affinity higher than that of HP1γ (Heterochromatin Protein 1γ), the 'guardian' of the poised transcriptional state. Such a substitution of chromatin readers leads to chromatin decompaction and allows Smad4-Smad2/3 complexes to access their binding sites and switch master regulators of stem cell

differentiation from the poised to the active state (Xi et al., 2011). Accordingly, it has been proposed that, in erythroid cells, the canonical Smad4-Smad2/3 arm mediates homeostatic gene responses while the TIF1γ-Smad2/3 arm stimulates differentiation (He et al., 2006).

Our own results confirm that TIF1γ expression inhibits the formation of Smad4-Smad2/3 complexes required for Smad-dependent transcription and promotes instead the formation of TIF1γ-Smad2/3 complexes. We show that this interaction is significantly reduced by lack of TIF1γ sumoylation, as is the ability of TIF1γ to inhibit formation of Smad4-Smad2/3 complexes, enhancing instead binding of TIF1γ to Smad4. The simplest conclusion, then, is that TIF1γ sumoylation is required for inhibition of Smad4-Smad2/3 complex formation. Results of our ChIP experiments confirm that TIF1γ is required for limiting Smad4 residence on the PAI-1 promoter, probably through its ubiquitin ligase activity. Moreover, we have previously shown that TIF1γ silencing enhances Smad4 binding to DNA and that endogenous TIF1γ and Smad4 have opposite kinetics of recruitment on the promoter of PAI-1, a TGFβ target gene (Hesling et al., 2011). Interestingly, the sumoylation-defective TIF1γ mutant no longer interferes with Smad4 occupancy, suggesting that, in this case as well, TIF1γ sumoylation serves to temporally limit Smad4 binding to target promoters. In addition, TIF1γ ubiquitin ligase activity, which would not be regulated by sumoylation (Fig. 4H), has previously been shown to depend on the recognition of epigenetic modifications on the promoter of TGFβ superfamily target genes by TIF1γ PHD- and Bromo-domains (Agricola et al., 2011). We postulate that TIF1γ sumoylation might strengthen its binding to histone H3 tails in response to TGFβ treatment, therefore promoting its inhibitory effects on Smad4-dependent transcription. Taken together, our results demonstrating increased interaction of TIF1γ-Mut with Smad4 and the apparent stronger binding of TIF1γ-Mut to the PAI-1 promoter, along with Smad4, after 90 minutes of TGFβ stimulation (Fig. 5C), suggest that lack of TIF1γ sumoylation could lock Smad4 occupancy on the promoter of TGFβ target genes. Accordingly, independently of histone H3 tail recognition, promoter recruitment of TIF1γ-Mut and the subsequent absence of activation of E3 ubiquitin ligase activity would be sufficient to prevent interference with Smad4 occupancy. Future exploration of the effects of the lack of TIF1γ sumoylation in transcriptional regulation of TGFβ target genes will help prove or disprove this hypothesis.

Our results strongly suggest that the transcriptional repression mediated by TIF1γ requires its post-translational modification by SUMO, an observation also made for TIF1β (Mascle et al., 2007). TIF1β sumoylation is needed for recruitment of the SETDB1 histone methyl-transferase and the CHD3 chromatin remodeling factor and stimulates their activity, leading to gene silencing (Ivanov et al., 2007). In the same way that TIF1β sumoylation is required for KRAB domain-mediated repression, TIF1γ sumoylation would then be required for the transcriptional repression of TGFβ signaling through negative regulation of Smad complex formation and of their residence on the promoter of target genes. One could suppose that sumoylation of TIF1γ might also coordinate the recruitment of factors that regulate the chromatin state. The extremely dynamic interconnections between transcription factors, co-repressors and co-activators allow for proper and optimal transcriptional responses to a given signal. While more biochemical studies will be required to analyze in detail the interplay between different post-translational modifications in the TGFβ signaling pathway, it appears likely that the dynamics of sumoylation/desumoylation events help regulate the effect of TIF1γ on Smad4 functions.

Materials and Methods

Plasmids

Expression vectors encoding FLAG-Smad2, FLAG-Smad3 and FLAG-Smad4 were kindly provided by P. Ten Dijke (Leiden, Netherlands). For sumoylation experiments, His6-SUMO-1, SUMO-2, SUMO-3, Ubc9 and PIAS1 expression vectors were kindly provided by S. Sentis (Lyon, France). SENP1 and (R630L/K631M) catalytically inactive mutant of SENP1 expression vectors were provided by E. T. Yeh (Houston, Texas). The expression vector encoding HA–ubiquitin was generated by PCR and verified by sequencing. Sumoylation mutant plasmids were obtained by site-directed mutagenesis on the pSG5-hTIF1γ vector (a gift from R. Losson, Strasbourg, France) using the QuikChange XL Site-Directed Mutagenesis kit purchased from Stratagene. Lysines within different consensus sumoylation sites were mutated into arginines and verified by sequencing to generate the sumoylation-deficient TIF1γ-Mut mutant. N-terminus HA-tagged and FLAG-tagged TIF1γ-WT and TIF1γ-Mut expression vectors were generated using the pSG5-hTIF1γ vector. To achieve sumoylation directed by the Ubc9-TIF1γ fusion, the full-length cDNA of human Ubc9 was obtained from the pSG5-Ubc9 plasmid and the PCR product was inserted in an open-reading frame in the pSG5-TIF1γ expression vector.

Cell culture and transient transfection

Human mammary epithelial cells, immortalized by hTERT and Ras-transformed (HMEC-TR), kindly provided by R. A. Weinberg, have been previously described (Elenbaas et al., 2001). Cells were cultured in 1:1 Dulbecco's Modified Eagle's Medium (DMEM)/HAMF12 medium (Invitrogen) supplemented with 10% FBS (Cambrex), 1% penicillin-streptomycin (Cambrex), 5 ng/ml human epidermal growth factor (PromoCell), 0.5 μg/ml hydrocortisone (Sigma) and 10 μg/ml insulin (Sigma). The MCF10A mammary epithelial cell line was obtained from the American Type Culture Collection. MCF10A cells were cultured in 1:1 Dulbecco's Modified Eagle's Medium (DMEM)/HAMF12 medium (Invitrogen) supplemented with 5% horse serum (Cambrex), 1% penicillin-streptomycin (Cambrex), 10 ng/ml human epidermal growth factor (PromoCell), 0.5 μg/ml hydrocortisone (Sigma), 10 μg/ml insulin (Sigma) and 100 ng/ml cholera toxin (Sigma). Recombinant TGFβ1 (Peprotech) was used at 5 ng/ml. To block any signaling arising from autocrine production of TGFβ, experiments without TGFβ1 were performed using the TβRI kinase inhibitor SB-431542 (Sigma) at 10 μM for the indicated times. HMEC-TR cells were transiently transfected using XtremGENE transfection reagents (Roche Applied Science) according to the manufacturer's instructions.

Lentiviral infection

MCF10A cells were infected with the pLVX-based lentiviral vector (Clontech Laboratories) expressing TIF1γ-WT or TIF1γ-Mut. Stably knocked down MCF10A cells were generated by lentiviral infection with TIF1γ shRNA (Open Biosystems). Stably infected cell populations were generated following Puromycin selection (1 μg/ml) and then cultured in classical medium supplemented with 0.5 μg/ml Puromycin. TIF1γ expression levels were assessed by western blotting and RT-qPCR analysis.

Immunofluorescence

Cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized with 0.4% Triton X-100 in PBS for 5 min, blocked with 3% FBS-5% BSA in PBS for 30 min and subsequently stained with 0.25 mM tetramethyl rhodamine iso-thiocyanate-conjugated phalloidin (Sigma), anti-E-cadherin (BD Biosciences) or anti-TIF1γ (Euromedex) antibodies. Cells were incubated with AlexaFluor®488 anti-mouse antibody (Invitrogen) and mounted with media containing Hoescht nuclear stain. Fluorescence was examined by confocal laser scanning microscopy (Carl Zeiss).

His6 pull-down assay

Cells were seeded in 100 mm dishes and transfected with expression vectors encoding His6-tagged SUMO-1 and different mutants of TIF1γ. 48 hours post-transfection, cells were harvested, lysed and sonicated in buffer A (6 M guanidine-HCl; 0.1 M NaH_2PO_4; 0.01 M Tris-HCl, pH 8). His6-tagged SUMO-1 was immobilized by column chromatography using Ni-NTA beads (Qiagen). After four washes in buffer A and three washes in buffer B (8 mM urea; 0.1 M NaH_2PO_4; 0.01 M Tris-HCl, pH 6.5), elution was performed using 120 μl sample buffer. Supernatants were loaded on SDS-PAGE and immunoblotted with mouse anti-TIF1γ antibody (Euromedex).

Yeast two-hybrid screen

Automated yeast two-hybrid screens were performed at the DKFZ (German Cancer Research Center) facility as described (Albers et al., 2005). Briefly, a library of individually cloned full-length open reading frames from cDNAs of 10,070 different genes (Lamesch et al., 2007) in pGAD424 were screened to a coverage of 6.8 million clones. Yeast strains harboring the bait protein and the prey library were mated in YPDA medium containing 20% PEG 6000 before selection of positive colonies in selective medium containing 0.4 mM 3-amino triazole. Positives were identified by activation of the HIS3 and MEL1 reporters as described, and library inserts were isolated by PCR and analyzed by DNA sequence analysis. The middle domain of TIF1γ used as bait was expressed from the pGBT9 vector (Clontech). Among 65 positive clones, Ubc9 was isolated 21 times in interaction pairs with the bait.

Immunoprecipitation and Western Blotting analysis

For protein-protein interactions, cells were transfected with expression vectors encoding flag-tagged Smad2, flag-tagged Smad3, flag-tagged Smad4, HA-tagged TIF1γ-WT, HA-tagged TIF1γ-Mut, flag-tagged TIF1γ, flag-tagged TIF1γ-Mut, TIF1γ, Ubc9-TIF1γ, HA-tagged ubiquitin and/or His6-SUMO1. 48 hours post-transfection, cells were treated or not for 2 h with TGFβ1 (5 ng/ml) and lysed in RIPA lysis buffer (50 mM Tris-HCl, pH 7.5; 100 mM NaCl; 1% NP40; 0.25% NaDeoxycholate; 1 mM EDTA) supplemented with protease and phosphatase inhibitors (Roche) and 10 mM N-ethylmaleimide, which inhibits desumoylases via reductive alkylation. Total extracts (500 µg) were pre-cleared with protein A-protein G agarose beads (FastFlow, Millipore) for 10 minutes at 4℃. Pre-cleared extracts were incubated overnight at 4℃ with agitation with 2 µg of rabbit anti-flag antibody (Sigma) to form immune complexes. Immunoprecipitation was completed by incubation with protein A-protein G agarose beads during 1 hour at 4℃ under agitation, followed by three washes of 5 min at 4℃ under agitation in the same buffer. Beads were recovered with 60 µl sample buffer and supernatants were loaded on SDS-PAGE gels, followed by Western transfer and immunoblotting as described in the Figures. To analyze the endogenous interaction of TIF1γ with Ubc9, immunoprecipitation of TIF1γ was performed with 1 mg of total extracts incubated overnight at 4℃ with 3 µg of rabbit anti-TIF1γ (Bethyl laboratories) or Rabbit IgG (Sigma) used as negative control. Mouse monoclonal anti-flag antibody (EL1B11, Euromedex), mouse monoclonal anti-HA antibody (12CA5, Roche), rabbit polyclonal anti-Ubc9 antibody (#10759, SantaCruz), mouse monoclonal anti-Smad4 B8 antibody (#7966, SantaCruz), rabbit polyclonal anti-TIF1γ antibody (A301-060A, Bethyl), rabbit polyclonal anti-SUMO-1 antibody (SantaCruz), mouse monoclonal anti-TIF1γ antibody (TIF3E9, Euromedex) were used for immunoblotting. Mouse monoclonal anti-GAPDH antibody (Covalab) was used for loading controls. Peroxidase-linked anti-mouse (P0260, Dako) and anti-rabbit (P0448, Dako) secondary antibodies and ECL detection reagents (Roche) were used according to the manufacturers' instructions.

Transcription Reporter Assays

HMEC-TR cells were plated in 24-well plates and co-transfected with 150 ng of pGL3(CAGA)$_9$-Luc (Dennler et al., 1998) luciferase construct and 100 ng of the indicated expression vectors constructs. The pRL-SV40 vector (10 ng; Promega) was used as internal control for transfection efficiency. When increasing amounts of expression vectors were transfected, the amount of transfected vectors was kept constant (260 ng) by addition of an empty vector (pSG5) to the transfection mixture. After transfection, cells were cultured for an additional 24 h in complete medium with SB-431542 or TGFβ1. Transfected cells were then washed and collected. Luciferase activity was measured in equivalent amounts of each lysate using the dual luciferase kit (Promega). In all experiments, luciferase firefly activity was normalized to the *Renilla* luciferase activity of the pRL-SV40 vector. Experiments were performed in triplicate and each experiment was repeated at least three times.

Chromatin immunoprecipitation

HMEC-TR cells were transfected with 3 µg of PAI-I promoter (p800-Luc) in 100-mm culture dishes and treated with SB-431542 or TGFβ1 for the indicated times. The chromatin immunoprecipitation (ChIP) assay was carried out using the ChIP assay kit from Upstate Biotechnology. Cell lysates were immunoprecipitated with anti-Smad4 (rabbit polyclonal, SantaCruz) or anti-TIF1γ (rabbit polyclonal, Bethyl laboratories) and rabbit IgG (Abcam) was used as a negative control. Following reverse cross-linking, DNA was treated with proteinase K and purified using the Nucleospin Tissue XS kit (Macherey-Nagel). Smad4- or TIF1γ-precipitated genomic DNA was subjected to PCR. The 351-bp PAI-1 promoter region harboring the Smad-binding elements (SBE) was amplified with the forward primer 5′-AGCCAGACAAGGTTGTTG-3′ and the reverse primer 5′-GACCACCTCCAGGAAAG-3. An unrelated genomic DNA sequence (corresponding to an actin allele) was amplified with primers 5′-AGCCA-TGTACGTTGCTATCCAG-3′ and 5′-CTTCTCCTTAATGTCACGCACG-3′. In order to quantify the levels of Smad4 and TIF1γ at the PAI-1 promoter, we next carried out real-time quantitative PCR on the immunoprecipitated DNA and the inputs. Results are presented as 'percent input' values, calculated to quantify the abundance of the DNA fragment of interest added to the ChIP reaction relative to the abundance of the DNA fragment found in the final immunoprecipitate.

Boyden chamber migration assay

Cell migration assays were performed in FluoroBlok 96-Multiwell Insert plates (BD Falcon) with an 8-µm pore size PET membrane. Stably infected MCF10A cells (empty vector, shTIF1γ, TIF1γ-WT and TIF1γ-Mut) were pre-treated with SB-431542 or TGFβ1 for 48 h. Cells were cultured in serum-free medium 12 h before the cell migration assay. 1.25×10^4 cells were plated in the upper chamber in serum-free medium and medium with 5% serum was used as a chemo-attractant in the lower wells. The plates were incubated for 22 h at 37℃ and migrating cells were stained with 2 µM Calcein AM Fluorescent Dye (Interchim). Cells were counted from random fields from each well under a Zeiss inverted fluorescence microscope. Experiments were performed in triplicate and each set was repeated three times.

RNA preparation and RT-qPCR

Total RNA was extracted using TriReagent (Sigma) and precipitation by isopropanol. RNA (1 µg) was used for complementary DNA synthesis with the SuperScript II Reverse Transcriptase system (Invitrogen). mRNA levels were quantified using the SYBR Green StepOne Plus Real Time PCR system (Applied Biosystems). All values were normalized to the amount of HPRT mRNA and expressed relative to the value obtained for TGFβ1-untreated controls. Primer pairs for TGFβ1 target genes (*PAI-1*, *CDH2* and *CDH11*) were used as previously described (Hesling et al., 2011).

Acknowledgements

We thank S. Borel, R. Gadet and C. Languilaire for excellent technical assistance, Dr Emmanuel Käs (LBME, CNRS/Université Paul Sabatier, Toulouse) for help in writing this manuscript, and S. Sentis and J. Lopez for helpful discussion. We also thank the Structure Fédérative de Recherche (SFR Lyon-Est, CeCILE) for microscopic study. The authors of this work declare that they have no competing interests.

Author contributions

R.R., G.G. and C.H. conceived and designed the experiments; L.F., A.S.A., B.B., L.J., I.M., I.T. and C.H. performed the experiments; R.R., L.F., A.S.A., B.B., L.J., I.M., C.H. and G.G. analysed the data; R.R. and L.F. wrote the paper.

Funding

This work was supported by grants from Inserm; CNRS; the Ligue Nationale contre le Cancer (Savoie; grant to R.R.) and Fondation ARC (Association pour la Recherche sur le Cancer; grant to R.R.). L.F. is supported by fellowships from MESR (Ministère de l'Enseignement Supérieur et de la Recherche) and next from Fondation ARC, and C.H. from INCa (Institut National du Cancer) and from the Centre Léon Bérard.

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.126748/-/DC1

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