Sumoylation of the Transcriptional Intermediary Factor 1β (TIF1β), the Co-repressor of the KRAB Multifinger Proteins, Is Required for Its Transcriptional Activity and Is Modulated by the KRAB Domain

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Small ubiquitin-related modifier (SUMO) has emerged as a key post-translational modifier of protein functions. Here we show that TIF1β, a developmental regulator proposed to act as a universal co-repressor for the large family of KRAB domain-containing zinc finger proteins, is a heavily SUMO-modified substrate. A combined analysis of deletion and punctual mutants identified TIF1β as a multitype acceptor for SUMO which specifically targets six lysine residues (Lys554, Lys575, Lys676, Lys750, Lys779, and Lys804) within the TIF1β C-terminal repressive region. Reporter gene assays indicate that TIF1β requires SUMO-modification for its repressive activity. Indeed, sumoylation-less mutants failed to recapitulate TIF1β-dependent repression. TIF1β homodimerization properties and interaction with the KRAB domain are preserved in the mutants with lysine to arginine substitutions as confirmed by in vivo bioluminescence resonance energy transfer (BRET). Using histone deacetylase (HDAC) inhibitors, we also demonstrate that TIF1β sumoylation is a prerequisite for the recruitment of HDAC and that TIF1β SUMO-dependent repressive activity involves both HDAC-dependent and HDAC-independent components. Finally, we report that, in addition to relying on the integrity of its PHD finger and on its self-oligomerization, TIF1β sumoylation is positively regulated by its interaction with KRAB domain-containing proteins. Altogether, our results provide new mechanistic insights into TIF1β transcriptional repression and suggest that KRAB multigene proteins not only recruit TIF1β co-repressor to target genes but also increase its repressive activity through enhancement of its sumoylation.

The transcriptional intermediary factor 1β (TIF1β, KAP-1, TRIM 28) is an essential developmental regulator belonging to the TIF1 family and is believed to act as the universal transcriptional co-repressor for the large family of vertebrate-specific Krüppel-associated box (KRAB) domain-containing zinc finger transcription factors (1–7). All TIF1 family members (TIF1α, β, γ, δ, and bonus) are characterized by an N-terminal tri-partite motif (named TRIM or RBCC) composed of a RING (really interesting new genes) domain followed by a coiled-coil domain as well as a C-terminal bi-partite motif encompassing a PHD (Plant HomeoDomain) finger and a bromo domain (2, 8–11). The RBCC motif of TIF1β is known to homodimerize and to specifically interact with the KRAB domain of KRAB multifinger proteins (12–14). The TIF1β PHD finger and bromo domain were reported to cooperate in transcriptional repression via recruitment of repressive enzymatic activities such as histone deacetylase complexes (NurD/Mi2-α) and a methyltransferase protein (SETDB1) (15, 16). Moreover, TIF1β (like all TIF1 family members) displays the canonical amino acid motif, PXVXL, interacting with HP1 heterochromatin proteins (HP1α, β, and γ) involved in gene silencing (17–19). Recently, the TIF1β/HP1 interaction was shown to be essential for histone modifications associated with transcriptional repression and progression through cellular differentiation (20–23).

In attempts to unveil other determinants involved in the strong repressive activity mediated by TIF1β, we found that this co-repressor is a SUMO target protein. Recent studies have demonstrated that post-translational modification of transcription factors and their co-regulators by the small ubiquitin-related modifier protein, SUMO, can modulate their transcriptional activity (24–28). The four mammalian SUMO, SUMO1, -2, -3, and -4, all resemble ubiquitin in terms of tertiary structure and their mechanism of conjugation to protein substrate. However, unlike ubiquitination, the main known functional consequences of SUMO modification appear not to be degradation (29–33). Several reports illustrate the direct involvement of SUMO modification in gene regulation and in particular in gene repression (34–44). To explain the transcriptional repression induced by SUMO-modified proteins, some studies have suggested that SUMO proteins display intrinsic repressive activity, whereas others have proposed that SUMO-modified transcription factors can recruit HDAC proteins or HDAC-containing complexes (35, 40, 42, 45–50).

SUMO proteins form an isopeptide bond between their C-terminal glycine and an ε-NH2-amino group of a lysine residue...
on their target proteins (51). The target lysine to which SUMO is conjugated is usually contained within a short consensus sequence, ψKKXE (E/D) (where ψ represents a large hydrophobic residue) (52). The SUMO conjugation process, so called sumoylation, involves SUMO-activating E1 enzyme (SAE1/SAE2 heterodimer) and a SUMO-conjugating E2 enzyme (UBC9) (53). Furthermore, SUMO-specific E3 ligases that enhance SUMO conjugation have also been described. The known E3 ligases belong to three structurally independent protein classes represented by RanBP2, Pc2, and PIAS family members (protein inhibitor of activated STAT) (54–57). SUMO E3 ligases display limited substrate specificity and are reported to stabilize the interaction between the E2-conjugating enzyme, UBC9, and the target substrates to be sumoylated (53, 58–60). Like ubiquitination, sumoylation is a dynamic and reversible process because of the existence of several SUMO-specific proteases, referred to as members of the SENP (sentrin proteases) family. These proteases are involved both in the maturation of SUMO as C-terminal hydroxylases and in the removal of SUMO from its target protein as isopeptidases (58).

In this study, we report that TIF1β is a strong substrate for SUMO modification exhibiting several sumoylation sites clustered in its C-terminal repressive region. Prevention of TIF1β sumoylation by using sumoylation-deficient mutants abrogates its repressive activity both as a protein artificially targeted to DNA or indirectly recruited to DNA via a KRAB domain-containing protein. Furthermore, we demonstrate that TIF1β SUMO modification is required for recruitment of HDAC-containing complexes. Finally, this study indicates that TIF1β sumoylation can be positively regulated by its homodimerization and its interaction with KRAB domain-containing multifinger proteins which are recruiting this co-repressor to target genes.

**EXPERIMENTAL PROCEDURES**

**Expression Vectors**

**TIF1β Constructs**—Full-length TIF1β (GenBank™ accession number NM_011588; aa1–834) was derived from the pMFI-Gal4-TIF1β construct (generous gift from Dr. J. V. Bonventre) (3, 61). It was subcloned as an EcoRV-NotI fragment into the blunt BamHI site of pCGN (62) (HA-TIF1β), as an EcoRI-NotI fragment into pBluescript SK+ (SK+ TIF1β), and as an EcoRI-NotI-blunted fragment into the EcoRI-Smal sites of the yeast pGBT9 vector (encoding the first 1–147 amino acids of the yeast transcription factor Gal4, that corresponds to the DNA binding domain) (Clontech) (Gal4DBD-TIF1β). To generate Myc-tagged TIF1β, a Clal-NotI-blunted fragment was transferred from SK+ TIF1β into the blunt XbaI site of pcDNA3.1-Myc (pcDNA3.1-Myc-TIF1β). Flag-HA-TIF1β was obtained by subcloning an EcoRV-NotI fragment from SK+ TIF1β into pcDNA3-Flag-HA (pcDNA3-Flag-HA-TIF1β). pcDNA3.1-Gal4DBD-TIF1β construct was obtained by subcloning Gal4DBD-TIF1β as an HindIII-Sall fragment from pGBT9 vector (Clontech) into the HindIII-Xhol sites of pcDNA3.1 (Invitrogen). C-terminal deletion mutants Gal4DBD-TIF1β-(1–735) (Δ1) or Gal4DBD-TIF1β-(1–675) (Δ2) were generated from pcDNA3.1-Gal4DBD-TIF1β by removing the KpnI-Xbal or EcoNI-Xbal C-terminal fragments, respectively. Gal4DBD-TIF1β-(1–563) (Δ3) or Gal4DBD-TIF1β-(1–482) (Δ4) were obtained by subcloning a HindIII-Sacl or HindIII-AflI fragments from pGBT9-TIF1β into the HindIII-EcoRV sites of pcDNA3.1, respectively. TIF1β devoid of the N-terminal homodimerization RBCC motif and encoding the HP1 binding domain, the PHD finger and the bromo domain (ΔN-TIF1β; from nucleotides 1806 to the stop codon of TIF1β at position 3063) was amplified by PCR and cloned in-phase as a BamHI fragment into the BamHI site of pCGN and as a BamHI-Sall fragment into pGBT9 to generate pGBT9 ΔN-TIF1β. A Gal4DBD-ΔN-TIF1β construct was obtained by subcloning the HindIII-Sall fragment from pCBT9 ΔN-TIF1β into the HindIII-Xhol sites of pcDNA3.1. TIF1β point mutations were generated using the QuikChange site-directed mutagenesis kit (Stratagene) starting with fragments of the wild-type cDNA cloned in pBluescript SK+. Only the sense oligonucleotides are indicated here for L271P (5′-gggaggaacctgcaccacactctgaaaac-3′ where a silent Spfl restriction enzyme site is incorporated), L307P (5′-ctcaagaggaattggcttca-3′), K554R (5′-cctggtgatgttctggagcaagag-3′ where a silent Mun1 restriction enzyme site is incorporated), K757R (5′-ccgggtactcgtctctctgacc-3′ where a silent BsmI restriction enzyme site is incorporated), K676R (5′-ctggctgcgctgcattggaggaagtcgag-3′ where a silent Pfcl restriction enzyme site is incorporated), K795R (5′-cgtgctgctcctgagaacg-3′ where a silent BsrGI restriction enzyme site is incorporated), K804R (5′-ggtcagcgacgctcgctgctgctg-3′ where a PvuII restriction enzyme site is incorporated), H,C648,651A,AA (5′-atgagctgctgctcctgtcagacgacg-3′) mutations. All mutants were verified by DNA sequencing, and mutated fragments were subcloned in replacement of the wild-type fragment in appropriate vectors containing the full-length or truncated TIF1β cDNA.

**KRAB Domain-containing Protein Constructs**—The 75-amino acid repressive KRAB domain from ZNF74-II isoform was used as a prototype for the conserved KRAB domain (63). A 43 amino acid truncated and non-repressive domain (KRAB tr) derived from another ZNF74 isoform (ZNF74-1) (63) was used as a control, which does not interact with TIF1β (14). KRAB domains in fusion with Gal4DBD (1–147) (which encodes a nuclear localization signal) were cloned as XbaI fragments in pRSV (63) or as a BsrGI-BamHI fragment in pcDNA3.1-Gal4DBD. The Myc-tagged ZNF74 constructs were obtained by cloning the cDNAs encoding for ZNF74-1 (GenBank™ accession number X71623; aa1–572) (64) or ZNF74-11 (GenBank™ accession number X92715; aa1–643) (63) as an XbaI fragment into the Xbal site if the Myc-tagged version of pcDNA3.1 vector.

**BRET2 Constructs**—Rluc-TIF1β, GFP-TIF1β and GFP-NLS, which target GFP to the nucleus via a nuclear localization signal (NLS), were previously described in Ref. 14. TIF1β 4KR, TIF1β L271P, and TIF1β L307P were subcloned as an EcoRI-Pmel fragment from pcDNA3.1-Gal4DBD into the EcoRI-EcoRV sites of phRluc and pGFP10 (PerkinElmer Life Sciences). The GFP-KRAB-NLS construct, targeted to the nucleus by a NLS,
was prepared by subcloning a SacI-Apal fragment from pBlue-script SK + in-phase into the SacI-Apal sites of pGFP10.

Other Constructs—The FLAG epitope-tagged PIASy is described in Ref. 65 (generous gift from Dr. R. Grosschedl). The FLAG epitope-tagged wild-type SENP1 and catalytically inactive SENP1C603S (generous gift from Dr. D. Bailey) are described in Ref. 66. SUMO1 (GenBank accession number U67122) cloned in pcDNA3.1-myc (myc-SUMO1) (generous gift from Dr. F-M. Boisvert) was subcloned as an XhoI fragment in pGFP10 (PerkinElmer Life Sciences). SUMO2 was subcloned as a BstYI-NotI fragment from the pGEX4T-1 vector containing EGFP-SUMO2 (generous gift from Dr. M. Dasso) into the BgIII-NotI site of pGFP10.

Antibodies
The following mouse monoclonal antibodies 12CA5 anti-HA (67), 9E10 anti-Myc (68), M2 anti-FLAG (Sigma), anti-GFP (Roche Applied Science), anti-Gal4 (Santa Cruz Biotechnology), and polyclonal antibody anti-TIF1β (amino acids 381–564) were used (14).

Cell Culture and Transfection
Human embryonic kidney 293T cells (HEK 293T) maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Wisent), 100 µg/ml penicillin and streptomycin, 1 mM L-glutamine were seeded at a density of 3 × 10⁵ cells per 60-mm dish for cell extracts and immunoprecipitation experiments, 1 × 10⁶ cells per 100-mm dish for BRET experiments, and 2 × 10⁵ in 6-well plates for transcriptional assays. Transient transfections of plasmids were performed on the following day using the calcium phosphate precipitation method except for transcription assays where FuGENE transfection reagent (3 µl/1 µg DNA) (Roche Applied Science) was used. The total amount of transfected DNA was kept constant (10 µg for 100-mm dishes, 6 µg for 60-mm dishes, and 1–1.3 µg for 6-well plates).

Cells Extracts and Immunoprecipitations
For preparing whole cell extracts to be separated by SDS-polyacrylamide gel electrophoresis, cells were lysed in denaturing Laemmli buffer (400–600 µl/dish) containing 20 mM NEM (N-ethylmaleimide), a cysteine protease inhibitor usually used to preserve the sumoylation of cellular proteins. DNA was immediately sheared using a 1-ml syringe with a 26-gauge ½ needle or sonicated to reduce sample viscosity. For immunoprecipitation, cells were lysed in SDS-solubilizing buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 0.8% SDS, 20 mM NEM) (1.5 ml/dish) completed with a mixed proteases inhibitors. The following steps were done at 4 °C. Extracts were passed through a 26-gauge ½ needle to reduce viscosity and centrifuged at 15,000 × g for 2 min. Extracts were then precleared with protein A-Sepharose (1 ml/10–20 µl stacked beads) (Sigma) for 1 h. Immunoprecipitations of the precleared extracts (1 ml) were then carried out for 16 h using 10 µl of the appropriate antibody and 40–50 µl of protein A-Sepharose. The protein A-Sepharose beads were washed three times with 1 ml of washing buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 0.4% SDS, 2% Triton, 20 mM NEM). Washed immunoprecipitates were resuspended in Laemmli buffer. Whole cell extracts and immunoprecipitates were separated on SDS-polyacrylamide gel electrophoresis and electrotransferred for Western blotting. Following probing of the nitrocellulose membranes with the relevant antibodies, a chemiluminescence reagent was used (Renaissance kit, PerkinElmer Life Sciences).

BRET² Experiments
The BRET assays were conducted as we previously described in Ref. 14. In brief, transiently transfected cells were resuspended and distributed in 96-well microplates (white Optiplate from Packard). Upon addition of the cell permeant luciferase substrate (coelenterazine deep blue, PerkinElmer Life Sciences), the bioluminescence signal resulting from its degradation was detected using a 370–450-nm band pass filter (donor emission peak 400 nm). The energy transferred resulted in a fluorescence signal emitted by the GFP10 acceptor (excitation peak 400 nm and emission peak 510 nm) that was detected using a 500–530-nm band pass filter. The BRET signal (BRET ratio) was quantified by calculating the acceptor fluorescence/donor bioluminescence ratio as previously reported. Expression level of each construct was determined by direct measurements of total fluorescence and luminescence on aliquots of transfected cell samples. The GFP10 total fluorescence was measured using a FluoroCount (PerkinElmer Life Sciences) with an excitation filter at 400 nm, an emission filter at 510 nm. The total luminescence was measured using the same cells incubated with coelenterazine H for 10 min (Molecular Probes) (emission peak 485 nm). The BRET ratios were plotted as a function of the GFP/LUC fusion protein expression ratio, both fusion proteins expression being assessed with the same cells as described above, to take into account the potential variations in the expression of individual constructs from transfection to transfection. As a negative control, the Rluc-TIF1β/GFP-NLS BRET pair evaluating random collision was used as in Ref. 14.

Transcriptional Assays
Transient transfections were done as described above. The firefly luciferase reporter plasmid pGL3–5xGal4 corresponds to pGL3 vector under the control of the SV40 promoter (Promega) and a multimerized Gal4 DNA binding sequence (AGGGTATATAATG) ×5. The Renilla luciferase vector pRLuc-C1 (PerkinElmer Life Sciences) (20 ng) was co-transfected to normalize for transfection efficiency. The effector plasmid corresponds to pcDNA3.1 (Invitrogen) in which Gal4DBD–(1–147) was cloned and fused to TIF1β, the KRAB domain or their mutants (see description above). The total amount of transfected DNA was kept constant by addition of the pcDNA3.1 empty vector. Histone deacetylase inhibitor treatments were done 24-h post-transfection either with 5 mM sodium butyrate (NaBut) (from a 3 M stock solution resuspended in PBS pH 7.5) or with 200 nM trichostatin A (from a 300 mM stock solution resuspended in ethanol). Cell lysates were prepared 48 h after transfection and split into two samples for determination of the luciferase activity and assessment of the level of protein expression by Western blotting. The equivalent of 35 µg of cell lysates was processed for the luciferase activity using the Dual GloTM luciferase assay kit (Promega).
RESULTS

SUMO Modification of TIF1β in Vivo—Considering that sumoylation is often associated with a change in the activity of transcriptional regulators, we wondered if TIF1β co-repressor could be a target of SUMO protein as previously shown for TIF1α, another member of the TIF1 family (69). To determine whether TIF1β could be covalently modified by SUMO in mammalian cells, human 293T cells were transfected with HA-tagged TIF1β in the presence or absence of GFP-SUMO1, considering that free SUMO1 is limited in the cells (70). Western blot using anti-TIF1β revealed that the addition of GFP-SUMO1 results in the appearance of several bands migrating above the unmodified TIF1β (Fig. 1A, lanes 3 and 4). These bands of reduced mobility are predicted to correspond to TIF1β species covalently linked to GFP-SUMO1. Accordingly, these SUMO-modified forms were lost in cells co-transfected with a SUMO-specific isopeptidase, SENP1 that cleaves SUMO from its protein substrate, but not in cells expressing its catalytically inactive form, SENP1C603S (Fig. 1B, lanes 3 and 4) (66). Further confirmation was obtained in immunoprecipitation experiments where five slow migrating Flag-HA-tagged TIF1β species revealed by an anti-FLAG antibody were also detected by an anti-GFP antibody recognizing the GFP-SUMO1-modified TIF1β (Fig. 1D, lane 6 in left and right panels).

We then obtained evidence that endogenous TIF1β was a substrate for SUMO. Endogenous TIF1β sumoylation was detected with a specific anti-TIF1β antibody only when we overexpressed either Myc-SUMO1 or GFP-SUMO1, or when endogenous SUMO-modification of TIF1β was enhanced by using the E3 ligase, PIASy (Fig. 1E, lanes 2, 4, and 6). This suggests that only a small percentage of TIF1β is normally covalently linked by SUMO at the steady state as also reported for other sumoylated proteins (71). Altogether, these results clearly demonstrate that TIF1β is strongly sumoylated in mammalian cells overexpressing SUMO1 and that co-expression of
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components of the SUMO pathway can regulate TIF1β SUMO modification.

Identification of TIF1β SUMO Modification Sites—Depending mostly on the exposure times, four to five sumoylated species were easily detected in cells co-transfected with TIF1β and either GFP-SUMO1 or GFP-SUMO2 (Fig. 1C). Thus, at least four main sumoylated sites are predicted to be present on TIF1β. SUMO1 is less likely to give rise to polysumoylation chain formation in vivo than SUMO2 because its sequence does not include a bona fide SUMO acceptor lysine involved in SUMO chain formation (30). Accordingly, even if the sumoylation patterns obtained with SUMO1 and SUMO2 were similar (Fig. 1C), our studies to identify TIF1β sumoylation sites were done with SUMO1. TIF1β primary sequence encodes 42 lysine residues with only two being part of a classical sumoylation consensus (Lys554 and Lys676). In an attempt to delineate the TIF1β region(s) subjected to sumoylation, C-terminal deletion mutants were generated and subjected to sumoylation in vivo either in cells co-transfected with GFP-SUMO1 or not (Fig. 2, A and B). To ensure that all deletion mutants are efficiently targeted to the nucleus, deletion constructs were all fused to Gal4 DNA binding domain (Gal4DBD), which includes a nuclear localization signal. The sumoylation pattern of deletion mutants was compared with that of wild-type TIF1β, also fused to Gal4DBD. Analysis of the number and intensity of sumoylated species observed for each deletion mutant suggest that sumoylation sites are progressively lost from Δ1 to Δ4 deletion mutants (Fig. 2, A and B). Only the unmodified TIF1β was detected with the Δ4 mutant suggesting that all sumoylation sites are located in the C-terminal part of TIF1β between amino acids 482 and 834. This region of around 350 amino acids contains 13 lysine residues, including Lys554 and Lys676 (Fig. 2, B and C).

To determine which of the 13 C-terminal lysine residues of TIF1β serve as a substrate for sumoylation, each of them were mutated to arginine residue (R) individually or in combination. A detailed analysis of the sumoylation profile of these mutants suggested that the first two bands migrating above the unmodified TIF1β correspond to monosumoylated forms exhibiting different apparent molecular weight as also reported for other sumoylated proteins (38, 72). Accordingly, as seen in Fig. 2D (compare lanes 1 and 2), the second band above the unmodified TIF1β was strongly reduced by mutating K554 and the faint remaining signal was completely abolished in the double K554R/K575R mutant (Fig. 2D, lane 3). This suggests that two distinct populations of monosumoylated species comigrate, one being monosumoylated on Lys554 and one on Lys676. Considering the relative abundance of these monosumoylated species, Lys554 appears as a major sumoylation site compared with Lys676. Two additional major sumoylation sites were evidenced with the use of the K779R and double K779R/K804R mutants (compare lane 4 with lanes 5 and 6). These mutants led to significant reduction in the first sumoylation band migrating just above the unmodified TIF1β. Only a faint doublet of bands closely migrating with the species monosumoylated on Lys779 or Lys804 were seen with the K779R/K804R mutant (lane 6). These remaining bands (less than 5% of the sumoylation detected with wild-type TIF1β) are likely to correspond, respectively, to the monosumoylation of Lys750 (lower band) and Lys676 (higher band) as demonstrated with mutants containing five (5KR mutant) and six (6KR mutant) mutated lysines (Fig. 2D, lanes 7–9). The above results demonstrate that TIF1β includes six SUMO acceptor sites. Our deletion and punctual mutant results also suggest that the bands migrating above the monosumoylated species correspond to multisumoylated forms of TIF1β with potentially two to six SUMO moieties linked to any of the different acceptor lysines (scheme in Fig. 2D). The TIF1β species expected to contain five SUMO moieties was seen on longer exposure of some gels. However, we have never observed the one predicted to contain six SUMO moieties presumably because this modified species represents a very minor product not detectable at the level of sensitivity of our assay or because the sumoylation of some lysine residues is mutually exclusive. Consistent with the multisumoylation of TIF1β, the removal of any sumoylation site by mutation of one target lysine leads to a decrease in the intensity or in the disappearance of the highest migrating band. Altogether, our results show that among the six potential sumoylation sites, three of them are major sumoylation sites (Lys554, Lys779, Lys804) and three are minor sumoylation sites (Lys575, Lys676, and Lys750).

Regulation of TIF1β Transcriptional Repression by Sumoylation—Knowing that TIF1β is a strong transcriptional co-repressor involved in gene silencing, we assessed the potential impact of TIF1β sumoylation on its transcriptional repressive properties. We used a gene reporter assay to compare the transcriptional repressive activity of wild-type TIF1β with that of various sumoylation-deficient mutants containing target lysines mutated to arginines. Because TIF1β needs to be recruited to DNA to repress transcription (2–4), both wild-type and SUMO-deficient mutant TIF1β effectors were fused to Gal4DBD. This fusion protein recognizes the Gal4 response element (5×Gal4UAS) placed in front of the SV40 strong promoter that drives the expression of the luciferase reporter gene (Fig. 3A). As seen in Fig. 3C, the repressive activity of wild-type TIF1β is dose-dependent, reaching a maximum of ~20-fold. At comparable maximal doses, TIF1β 4KR (K554R/K575R/K779R/K804R), that includes mutations of the three major sumoylation sites, represses transcription 4–5-fold less than wild-type TIF1β. At doses of transfected TIF1β varying in the 10-fold range, a change limited to ~1.5-fold was observed in the repressive activity elicited by TIF1β 4KR in contrast to ~4-fold increase obtained with the wild type. This suggests that the TIF1β sumoylation-deficient mutant is unable to fully repress the luciferase reporter in our assay. A comparison of the transcriptional repression activity of various mutants that impair sumoylation was also carried out (Fig. 3B). Interestingly, TIF1β repressive activity was progressively lost by increasing the number of mutated sumoylation sites as observed in conditions where all sumoylation deficient mutants are expressed at comparable levels. These above results strongly suggest a correlation between TIF1β repressive activity and its sumoylation capacity.

While controversial, some studies suggested that the repressive activity of TIF1β was partially sensitive to histone deacetylase (HDAC) inhibitors (15, 17, 73). Our results using wild-type TIF1β or a deletion mutant (ΔN-TIF1β), containing the previ-
**FIGURE 2.** Identification of TIF1β sumoylation sites. A, sumoylation profiles of Gal4DBD-TIF1β and C-terminal deletion mutants (Δ1–Δ4) (3 μg) co-transfected (left) or not (right) with GFP-SUMO1 (3 μg) revealed by immunoblot of 293T whole cell extracts; the faster migrating dark band corresponds to the unmodified TIF1β. For the GFP sumoylation, a longer exposure is shown for Δ1 and Δ2 mutants (last two lanes). For the wild-type protein and C-terminal deletion mutants (lanes 1–5, right panel), stars identify TIF1β species modified by endogenous SUMO. B, schematic representation of the full-length 834 amino acids TIF1β and mutants. The characterized domains represented are the RING finger, B-box 1, B-box 2, coiled-coil domain, HP1BD (HP1 binding domain), PHD (Plant-Homeo Domain) finger, and bromo domain. Mutated residues used in this study are indicated. C, illustration of the amino acid context surrounding TIF1β sumoylation target lysines. Target lysine residues are in bold and those that are part of a classical KXXE consensus sequence are underlined. D, sumoylation profiles of lysine to arginine mutants of Gal4DBD-TIF1β (2–3 μg) in the presence of GFP-SUMO1 (2 μg) revealed by immunoblot of 293T whole cell extracts. Arrowhead points to the unmodified TIF1β. Arrows point to the bands containing monosumoylated species, and the modified lysine is indicated. A schematization of the sumoylation profile is provided on the right. Brackets identify mono- and multisumoylated species, and bands in parentheses correspond to TIF1β species containing potentially five (observed on some blots) or six SUMOs (never detected).
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FIGURE 3. Regulation of TIF1β transcriptional repression by sumoylation. A, schematic drawing of luciferase reporter and Gal4-effector plasmids. The luciferase reporter is under the control of the strong SV40 promoter, five

ous characterized repressive region of TIF1β including the six SUMO-targeted lysines (scheme in Fig. 2B), confirmed that treatment with sodium butyrate (Fig. 3D) and another inhibitor for class I and II HDAC, Trichostatin A (data not shown), reduces the repressive activity of TIF1β by ~30–50% (HDAC-dependent repression) (Fig. 3D). To investigate if there is a potential functional link between HDAC recruitment and TIF1β sumoylation, we compared the sodium butyrate sensitivity of ΔN-TIF1β and its 6KR derivative that is refractory to sumoylation. Clearly, the residual 1.5-fold repressive activity of the ΔN-TIF1β 6KR mutant was not modulated by the HDAC inhibitor suggesting that the recruitment of HDAC is SUMO-dependent (Fig. 3D, left panel). Furthermore, comparative analysis of the repression of ΔN-TIF1β with that of the ΔN-TIF1β 6KR sumoylation-less mutant in the presence of HDAC inhibitor revealed that the SUMO-related repression (see arrow on Fig. 3D) involves both HDAC-dependent and HDAC-independent components (see dotted arrows on Fig. 3D).

Because TIF1β acts as a co-repressor indirectly recruited to DNA via DNA-binding KRAB multifinger proteins (2, 23), we also compared the co-repressive activity of wild-type TIF1β and its sumoylation-deficient mutant following its indirect recruitment to DNA using a Gal4-KRAB fusion protein (Fig. 4A). Interestingly, in the presence of Gal4-KRAB, the co-repressive activity elicited by the endogenous TIF1β was enhanced by up to 30% by adding increasing amounts of wild-type TIF1β (Fig. 4A). In contrast, the co-repressive activity was decreased by 10% in the presence of comparable amounts of 4KR mutant suggesting that this sumoylation-deficient mutant exerts a dominant negative effect over the repression induced by endogenous TIF1β proteins (Fig. 4A). Thus, TIF1β co-repressor activity is clearly impaired by mutation of its major sumoylation sites. This loss of co-repressive activity of the 4KR mutant is not caused by a change in its subcellular localization because TIF1β sumoylation-deficient mutants display the same nuclear localization patterns as the wild-type TIF1β (observed

consensus Gal4 UAS (5xGal4 UAS) and the SV40 enhancer sequence (SV40 E). Effectors proteins correspond to wild-type TIF1β or its lysine to arginine mutants. B and C, transcriptional activity of TIF1β wild-type and sumoylation-less mutants directly recruited to the luciferase reporter. All transient transfections were done in 293T cells using 200 ng of reporter plasmid and a fixed amount of Gal4DBD-TIF1β effectors as indicated in B or increasing amounts of either Gal4DBD-TIF1β WT or K554R/K575R/K779R/K804R (4KR) mutant (from 25 to 300 ng). (C). Fold repression represents the ratio of luciferase activity measured for the reporter alone to the activity measured in the presence of the indicated effector proteins after normalization for transfection efficiency. Error bars represent the S.D. for at least three independent experiments performed in duplicate or triplicate. D, effect of sodium butyrate, a class I and II HDAC inhibitor, on the transcriptional activity of TIF1β wild-type and its sumoylation-less mutant. Transfections were done using 200 ng of reporter with 500 ng of the Gal4DBD-ΔN-TIF1β or 100 ng of the Gal4DBD-TIF1β constructs for untreated cells. Considering the observed ~5-fold increase in the expression of TIF1β and the normalizing Renilla luciferase in sodium butyrate-treated cells, the amount of Gal4DBD-effectors (Gal4DBD-ΔN-TIF1β 100 ng or Gal4DBD wild type 200 ng) was adjusted to obtain similar protein expression in treated and untreated cells. Cells were treated with sodium butyrate 24 h post-transfection and recovered 24 h later for transcriptional assays. Error bars represent the S.D. for at least three independent experiments performed in duplicate. SUMO-related repression (arrow) is composed of HDAC-dependent and HDAC-independent components (dotted arrows). For all assays (B–D), the expression level of TIF1β was assessed by immunoblots of 293T whole cell extracts from pooled duplicate or triplicate samples derived from one independent experiment.

FIGURE 3. Regulation of TIF1β transcriptional repression by sumoylation.
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BRET saturation curves were obtained when a fixed amount of Rluc-TIF1β or -TIF1β 4KR was co-expressed with increasing amount of GFP-KRAB-NLS (Fig. 4B). As typically seen when specific interactions occur, these curves showed an increase in the energy transfer (BRET\(^2\)) ratio between the RLuc fusion donor and GFP fusion acceptor until a plateau is reached when the amount of acceptor is no longer limiting compared with the donor. Noticeably, the BRET\(^2\) ratio of Rluc-TIF1β or -TIF1β 4KR at the maximal dose of GFP-KRAB-NLS is 4-fold higher than the one obtained with the negative control BRET pair Rluc-TIF1βL307P/GFP-KRAB-NLS. The TIF1β L307P mutant was previously shown to have an abrogated interaction with a KRAB domain in an in vitro experiment using the RBCC fragment of TIF1β (12). We also ruled out that the differential co-repressive activity elicited by TIF1β and its 4KR mutant was caused by an impaired homodimerization because they exhibit similar BRET saturation curves (Fig. 6A).

Noticeably, the increased repression observed when overexpressing wild-type TIF1β in the presence of Gal4-KRAB correlated with the appearance of a higher molecular weight band characteristic of TIF1β sumoylated by endogenous SUMO (Fig. 4A). This result suggests that the Gal4-KRAB fusion favors TIF1β sumoylation as explored in detail below (Fig. 5). In contrast, the endogenous sumoylation of TIF1β transfected alone remains undetectable in our transcriptional assays (Fig. 3).

**Regulation of TIF1β Sumoylation by the KRAB Domain of Multifinger Proteins**—Based on observations mentioned above, we investigated the possibility that the KRAB domain could modulate the sumoylation of TIF1β. Strikingly, co-transfection of wild-type TIF1β together with a prototypical KRAB domain (derived from ZNF74 zinc finger protein) significantly boosts the sumoylation level of TIF1β in condition in which GFP-SUMO was limiting (Fig. 5A, left panel compare lanes 1 and 2). Such enhancement was neither observed using a non-repressive truncated version of this KRAB domain (KRAB tr), which does not interact in vitro with TIF1β (14, 63) nor with the TIF1β L307P mutant, which failed to bind the KRAB domain as shown in vitro (12) and confirmed here in living cells by BRET (Figs. 4B and 5A, left and middle panels). Importantly also, in the presence of Gal4–KRAB, a higher molecular weight band characteristic of TIF1β sumoylated by endogenous SUMO appeared above the endogenous TIF1β (Fig. 5B). These results suggest that the sumoylation of TIF1β co-repressor is favored by its interaction with the KRAB domain. To confirm that such a domain can regulate sumoylation of TIF1β in the context of a full-length KRAB multifinger protein, we used two isoforms of a prototypical KRAB multifinger protein, ZNF74, that include the full-length repressive KRAB domain (ZNF74-II isoform) and the non-repressive-truncated KRAB domain (ZNF74-I isoform) used above (63). Only ZNF74-II interacts with TIF1β as previously shown in living cells by BRET (14). Clearly also, only ZNF74-II repressive isoform increases TIF1β sumoylation (Fig. 5A, right panel). Taken together, our results strongly suggest that the interaction of TIF1β with KRAB domain-containing zinc finger proteins positively regulates its SUMO modification and consequently its repressive activity.

**FIGURE 4. Regulation of TIF1β transcriptional co-repression by sumoylation.** A, co-repressor activity of TIF1β wild-type and sumoylation-less mutant recruited to the luciferase reporter by a KRAB domain. Transfections were done under conditions where the endogenous TIF1β becomes limiting using 600 ng of luciferase reporter, 200 ng of Gal4DBD-KRAB, and increasing amounts of wild type or 4KR Flag-HA-TIF1β (up to 480 ng). The percentage of change in repression because of the transfected TIF1β and KRAB domain constructs was evaluated relative to the observed 10-fold repression of the reporter luciferase activity elicited by the KRAB domain construct alone. A typical experiment out of three performed in duplicate is presented. The duplicate are within 5.5%. The expression level of TIF1β or KRAB domain proteins was assessed by immunoblots of 293T whole cell extracts from pooled duplicate samples derived from one independent experiment. Stars identify TIF1β species modified by endogenous SUMO. B, BRET\(^2\) titration curves showing interaction in living mammalian cells between the KRAB domain and full-length wild-type TIF1β and mutants. The BRET\(^2\) pairs tested were Rluc-TIF1β (○), Rluc-TIF1β 4KR (△), or Rluc-TIF1β L307P (□) used in a fixed amount (in the range of 1–2 μg) with increasing amounts of GFP-KRAB-NLS (from 0.2 to 8 μg). Rluc-TIF1β L307P/GFP-KRAB-NLS BRET\(^2\) pair (△) was used as negative control. For each pair, data from three independent experiments performed in duplicate were pooled. The data were represented as described under “Experimental Procedures.”

by immunolocalization with Flag-HA- or GFP-tagged constructs, data not shown) nor to an impaired interaction with the KRAB domain as shown by a BRET interaction assay in living cells (Fig. 4B) (14, 74). This is evidenced by the fact that similar
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Requirement RBCC Oligomerization Motif and PHD Finger Integrity for Efficient TIF1β Sumoylation—TIF1β and other members of the TRIM family are suggested to function as oligomers homodimerizing via their RBCC motif (13, 14, 75–77). Interestingly, we noticed that the sumoylation of TIF1β RBCC punctual mutants, L271P and L307P, was significantly reduced compared with that of the wild-type TIF1β (Fig. 6A). These mutants were previously found to be deficient for homodimerization in vitro and to exhibit a reduced homodimerization in cell extracts while only L307P was found to be deficient for interaction with the KRAB domain as shown by Peng et al. (12).

Because our BRET studies also indicate altered homodimerization properties of the RBCC mutants compared with the wild-type TIF1β (or the 4KR mutant) (Fig. 6A), we suspected that efficient sumoylation of TIF1β may require its oligomerization. In agreement with this, a HA-tagged deletion mutant lacking the RBCC dimerization motif, ΔN-TIF1β, did not show any significant sumoylation despite the presence of the six possible SUMO-acceptor sites (Fig. 6B, left panel). In contrast, a version of ΔN-TIF1β fused to the heterologous Gal4 dimerization domain exhibits a ladder of sumoylated bands (Fig. 6B, lane 1 in middle panel) as typically observed for the wild-type protein. Considering these results and the fact that HA-ΔN-TIF1β retains a nuclear localization (data not shown), this suggests that homodimerization of TIF1β is required for its sumoylation.

As demonstrated here, the RBCC motif of TIF1β appears dispensable for its sumoylation when replaced by a heterologous dimerization domain. Recently however, sumoylation of another RBCC motif-containing protein, PML, was found to depend on the integrity of part of its RBCC motif, the RING finger (78, 79). Interestingly, Capili et al. (80) suggested structure similarity between PML RING finger and TIF1β PHD finger which are both cysteine-rich, zinc-binding domains. To assess the potential involvement of the PHD finger in the sumoylation of TIF1β, we generated mutants where the PHD finger structure was disrupted by mutating two amino acids responsible for zinc-binding (H648A, C651A) (80). Interestingly, the intensity of sumoylated species was strongly reduced when the PHD finger mutations were introduced either in the Gal4-ΔN-TIF1β or in the full-length TIF1β (Fig. 6B, middle and right panels, lanes 1 and 2). The above results indicate that the oligomerization of TIF1β via its RBCC motif and the structural integrity of its PHD finger domain are essential for TIF1β-efficient sumoylation.

DISCUSSION

This study identified TIF1β, the universal co-repressor of KRAB multifinger proteins as a sumoylation substrate, which can be either mono-sumoylated on one of its six targeted lysines or multisumoylated on a combination of these lysine residues. Our results indicate that the sumoylation of TIF1β is an essential determinant for the repressive activity of this co-repressor of transcription and is a prerequisite for recruitment of histone deacetylase complexes. Interestingly, TIF1β sumoylation is shown to be dependent on its ability to homodimerize. Furthermore, it can be positively modulated by its interaction with KRAB domain-containing proteins. These results lead us to suggest that KRAB multifinger proteins not only recruit TIF1β co-repressor to target genes but also increase its repressive activity through enhancement of its sumoylation.

TIF1β, a Substrate Rich in Lysine Residues Targeted by SUMO—Using TIF1β lysine-acceptor and C-terminal deletion mutants, we provided evidence that TIF1β possesses six SUMO-acceptor sites involved in multisumoylation events. Noticeably, in the conditions of our assays using SUMO1, we
ruled out that TIF1β is polysumoylated through the formation of SUMO chains on individual lysine residues. While the vast majority of SUMO target proteins were described to contain one or two SUMO modification sites, TIF1β appears as a multi-acceptor protein for SUMO such as PML, Daxx, and N-CoR (44, 81, 82). Interestingly, all TIF1β acceptor sites, characterized as two major (Lys^{779}, Lys^{804}) and two minor (Lys^{575}, Lys^{756}) sumoylation targets. During the completion of this manuscript, three of the six sumoylation sites characterized here were also identified by others (86).

Analysis of sumoylation profiles revealed that the position of the targeted lysine within TIF1β primary sequence strongly influences the migration of each TIF1β mono-sumoylated species on denaturing polyacrylamide gel. Noticeably, the closer the target lysine is to the terminal end of the protein, the smaller is the apparent molecular weight of the monosumoylated species (Fig. 2D, scheme).

TIF1β appears as a substrate heavily sumoylated under conditions where it is overexpressed in the presence of SUMO. As for most sumoylated proteins, it was difficult to obtain evidence of the sumoylation of endogenous TIF1β by endogenous SUMO. However, we revealed such endogenous modification in cells transfected with either a SUMO E3 ligase, PIASy, or a KRAB domain reported here to enhance TIF1β sumoylation.

**TIF1β Sumoylation, a Prerequisite for Transcriptional Repression—** Our functional assays strongly suggest that the transcriptional repression mediated by TIF1β requires its post-translational modification by SUMO, an observation also made for other transcriptional regulators such as Sp3, Elk-1, and BKLF transcription factors (38, 46, 49) or co-regulators such as p300, CtbP, and N-CoR (34, 44, 87). Reducing the number of TIF1β SUMO acceptor sites by mutating single, double, and multiple sumoylation target lysine residues progressively decreased TIF1β-dependent transcriptional repression to near a basal repressive level. Remarkably, a TIF1β sumoylation-deficient mutant in which all the target lysine residues were mutated to arginine residues prevents almost completely the TIF1β-dependent repression activity. This is suggesting that the TIF1β repressive ability is mostly dependent on its capacity to be covalently modified by SUMO proteins.

Because no obvious difference has been observed in the subcellular localization of wild-type TIF1β and its various sumoylation-deficient mutants (TIF1β 4KR and -6KR) (data not shown), it is unlikely that a variation in their subcellular distribution contributes to their differential transcriptional repres-

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**FIGURE 6.** TIF1β sumoylation status depends on the integrity of its homodimerization domain and of its PHD finger domain. A. TIF1β optimal sumoylation requires an intact RBCC motif. Sumoylation profiles of wild-type HA-tagged-TIF1β (1.5 μg) and coiled-coil mutants (L271P or L307P, 2.5 μg) in the presence of GFP-SUMO1 (1 μg) revealed by immunoblot of 293T whole cell extracts. BRET\(^{+}\) titration analysis showing homodimeric interaction of TIF1β wild type, its coiled-coil domain mutants, or its sumoylation-less mutant in living 293T cells. The BRET\(^{+}\) pairs tested were Rluc-TIF1β/GFP-TIF1β (○), Rluc-TIF1β L271P/GFP-TIF1β L271P (○), Rluc-TIF1β L307P/GFP-TIF1β L307P (○), Rluc-TIF1β 4KR/GFP-TIF1β 4KR (○), and the negative control pair Rluc-TIF1β/GFP-NLS (*). Briefly, 293T cells were transfected with fixed amount of Rluc fusion protein (in the range of 1–2 μg) and increasing amounts of GFP fusion protein (from 0.05 to 6 μg). For each pair, data of at least three independent experiments performed in duplicate were pooled. The data were represented as described under “Experimental Procedures.” B. TIF1β sumoylation depends on its homodimerization and on the integrity of the PHD finger domain. Comparison of the sumoylation profiles obtained in 293T cell extracts between wild-type HA-tagged-TIF1β (2 μg) and an HA-tagged-ΔN-TIF1β mutant devoid of the RBCC homodimerization motif (2 μg) (left panel), Gal4-ΔN-TIF1β (10 μg) and its derivative mutated in the PHD finger (middle panel) as well as Gal4-TIF1β and its derivative mutated in the PHD finger as shown in the right panel. GFP-SUMO1 (1 μg in right panel or 2 μg) was co-transfected with TIF1β constructs. For each experiment, the total GFP fluorescence was measured to confirm equivalent GFP-SUMO1 expression level. Arrowheads point to the unmodified TIF1β.
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tive activity. Previous studies trying to decipher the mechanisms involved in the repressive activity of sumoylated proteins have suggested that SUMO displays an intrinsic repressive activity when directly targeted to DNA as a Gal4-SUMO fusion (49, 50). Furthermore, it was shown that the covalent attachment of SUMO promotes recruitment of HDAC complexes and thus inhibition of transcription both in conditions where SUMO is fused with transcriptional regulators to generate a chimera (35, 49) or is conjugated to the e-NH2 group of a lysine residue from target proteins (34, 40, 46). Importantly, we found that the SUMO-dependent repressive activity of TIF1β is accounted both by HDAC-dependent and HDAC-independent mechanisms. This is consistent with the recent results of Lee et al. (86) showing that TIF1β sumoylation correlates with both an attenuation of histone H3 acetylation (H3-K9, -K14) and with an enhancement of histone H3 di-methylation (H3-K9, -K14) at a specific TIF1β-targeted promoter. Because TIF1β is known to interact with Mi2-α, a zinc finger helicase, which is part of NuRD histone deacetylase complexes, as well as with the SETDB1 methyltransferase (15, 16), it is tempting to speculate that the recruitment of these repressive enzymatic activities is modulated by TIF1β sumoylation status. This may occur as a result of a differential interaction of the sumoylated and unsumoylated forms of TIF1β with these enzymatic complexes. Interestingly, a recent article identified SETDB1 as a protein, which directly interacts with SUMO2 (47). Because the interaction of TIF1β and SETDB1 has been previously characterized by yeast-two hybrid and co-immunoprecipitation experiments (16), it remains to be determined whether this interaction is direct or indirect and if it requires TIF1β sumoylation.

**TIF1β Sumoylation Is Regulated by Its Interaction with KRAB-containing Proteins and Requires TIF1β Oligomerization**—TIF1β, a ubiquitous and abundant protein, is believed to function as the obligate co-repressor for KRAB multifinger proteins, which are mostly expressed in stage-specific and tissue-specific manner during vertebrate development (3). In the present study, we demonstrate that KRAB domain-containing proteins positively modulate TIF1β sumoylation, because of their direct interaction with TIF1β RBCC motif. Thus, the recruitment of TIF1β to gene promoters regulated by KRAB multifinger proteins and its subsequent KRAB-induced sumoylation may represent a specific and unique way to enhance the repressive activity of TIF1β at selected promoters. This could explain why only a small proportion of endogenous TIF1β is SUMO modified at a steady state. It must be acknowledged that most of our work was done with transient transfections and Gal4-based systems. Thus, further studies should confirm the importance of TIF1β sumoylation in gene repression and its regulation by KRAB-containing proteins at promoters in chromatinized and natural chromatin contexts.

Whereas it is generally recognized that TIF1β can repress gene transcription when targeted to DNA through KRAB domain interaction, a few studies have found that TIF1β could also behave as a transcriptional co-activator (88, 89). More specifically, TIF1β has been characterized as a co-activator when recruited by transcriptional regulators such as C/EBPβ, the glucocorticoid nuclear hormone receptor and the TRIP-Br family members (88, 89). Thus, one could propose that specific protein interactors such as KRAB multifinger proteins may regulate TIF1β repressive activity by stimulating its sumoylation, a prerequisite for efficient recruitment of repressive enzymatic complexes. In contrast, other protein complexes, such those described above, may prevent TIF1β sumoylation and switch this co-regulator into a co-activator with the capacity to recruit activating enzymatic complexes. In such case, sumoylation may represent a critical determinant by which TIF1β is regulated.

At least two hypotheses, not mutually exclusive, can be proposed to explain how the KRAB domain increases TIF1β sumoylation. First, it could induce a TIF1β conformational change favoring its interaction with the sumoylation machinery and/or a better accessibility of TIF1β sumoylation sites. We ruled out however the possibility that the KRAB domain itself recruits UBC9, the E2 conjugating enzyme for SUMO (data not shown). Second, because the present study demonstrates that efficient sumoylation of TIF1β requires its oligomerization, an attractive possibility, is that KRAB domains may enhance TIF1β sumoylation by reinforcing its oligomerization. Consistent with this hypothesis, another group suggested that KRAB domain can stabilize TIF1β homo-oligomers formation in vitro (12). Our previous results also demonstrated that a KRAB domain-containing protein, ZNF74, can bind to TIF1β oligomers in living cells suggesting that oligomers represent the transcriptionally active species (14). Interestingly, a dimerization-dependent sumoylation has also been reported for at least two other proteins, namely HDAC4 and PML (90, 91). In the case of PML, it was recently suggested that its dimerization favors its auto-sumoylation, a process requiring the presumed intrinsic SUMO E3 ligase activity of the PML RING finger (78, 79). Since we found here that TIF1β efficient sumoylation also requires the integrity of its PHD finger, a domain reported to be structurally related to the RING finger of PML (80), further studies will determine if the TIF1β PHD finger may exhibit a SUMO E3 ligase activity allowing the auto-sumoylation of this strong co-repressor of transcription.

Whereas the number of SUMO target proteins is still growing, the physiological determinants regulating their specific sumoylation remain unknown. We report here that members of a family of transcription factors recruiting specifically the co-repressor TIF1β can modulate its sumoylation and consequently its repressive activity. Further studies will determine if sumoylation may antagonize other post-translational modifications of TIF1β co-regulator.

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