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Daxx Mediates the Small Ubiquitin-like Modifier-dependent Transcriptional Repression of Smad4*[S]

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Che-Chang Chang‡§, Ding-Yen Lin§, Hsin-I Fang§, Ruey-Hwa Chen¶, and Hsiu-Ming Shih‡§‖

From the ‡Graduate Institute of Life Sciences, National Defense Medical Center, the §Division of Molecular and Genomic Medicine, National Health Research Institutes, and the ¶Institute of Molecular Medicine, College of Medicine, National Taiwan University, Taipei, 11529 Taiwan, Republic of China

Daxx has been shown to function as an apoptosis regulator and transcriptional repressor via its interaction with various cytoplasmic and nuclear proteins. Here, we showed that Daxx interacts with Smad4 and represses its transcriptional activity via the C-terminal domain of Daxx. *In vitro* and *in vivo* interaction studies indicated that the binding of Smad4 to Daxx depends on Smad4 sumoylation. Substitution of Smad4 SUMO conjugation residue lysine 159, but not 113, to arginine not only disrupted Smad4-Daxx interaction but also relieved Daxx-elicited repression of Smad4 transcriptional activity. Furthermore, chromatin immunoprecipitation analyses revealed the recruitment of Daxx to an endogenous, Smad4-targeted promoter in a Lys^{159} sumoylation-dependent manner. Finally, down-regulation of Daxx expression by RNA interference enhanced transforming growth factor β-induced transcription of reporter and endogenous genes through a Smad4-dependent, but not K159R-Smad4-dependent, manner. Together, these results indicate that Daxx suppresses Smad4-mediated transcriptional activity by direct interaction with the sumoylated Smad4 and identify a novel role of Daxx in regulating transforming growth factor β signaling.

Sumoylation, the covalent attachment of ubiquitin-like SUMO[1] to lysine residues, is an important post-translational modification that regulates the functions of proteins involving in many cellular processes (1–3). With an increasing number of sumoylated proteins being identified, it has been proposed that SUMO conjugation affects target protein function by two major mechanisms (1, 4–6). First, sumoylation alters the molecular interaction properties and/or the subcompartmentalization of its targets. For instance, sumoylation of the transcriptional factor Elk-1 not only regulates the nucleo-cytoplasmic shuttling of this protein (7) but also results in the recruitment of histone deacetylase HDAC2 to Elk-1-regulated promoters, thereby repressing their transcription (8). Second, sumoylation antagonizes other post-translational modifications, such as ubiquitination and acetylation, by targeting a common acceptor lysine residue. The prototypical example is that SUMO conjugation of IκBα at lysine 21 stabilizes this protein by blocking ubiquitination at the same site (9). Because many transcriptional regulatory proteins are subjected to SUMO modification (1–3), sumoylation has emerged as an important mechanism in controlling gene expression.

Transforming growth factor (TGF) β regulates a wide array of biological activities (for reviews, see Refs. 10 and 11). The cellular effects of TGF-β are mediated by both the type I and type II receptor serine/threonine kinases. Upon ligand binding, the type II receptor phosphorylates the type I receptor, which subsequently phosphorylates Smad2 and Smad3 (receptor-regulated Smads, R-Smad). The activated R-Smads then form complexes with the common-mediator Smad4 (Co-Smad) and translocate into the nucleus to regulate the transcription of target genes that mediate TGF-β-induced cellular processes (12–16). Recently, several groups have demonstrated that Smad4 could be covalently conjugated by SUMO-1 at lysine 113 and 159, and mutation of both sumoylation residues significantly increases Smad4 transcriptional activity, suggesting a negatively regulatory mode of sumoylation on Smad4 activity (17–19). Currently, the underlying mechanism as to how the sumoylation modulates Smad4 transcriptional activity has not been completely unraveled. Mutation of Smad4 sumoylation sites does not alter the ability of Smad4 to form a complex with its interacting partners on promoter (17) but rather modestly increases the stability of Smad4 (18, 20). In addition, sumoylation was found to inhibit Smad4 intrinsic transcriptional activity *per se* (19), implicating a mechanism involving the recruitment of specific transcriptional factors to Smad4-regulated promoters via SUMO-modified Smad4.

Daxx is initially identified as a cytoplasmic signaling molecule linking Fas receptor to Jun N-terminal kinase signaling (21). Daxx has also been reported to associate directly with the cytoplasmic domain of the type II TGF-β receptor, thereby mediating TGF-β-induced apoptosis and Jun N-terminal kinase activation (22). Besides functioning as a signal transducer in the cytoplasm, Daxx also acts as a transcriptional corepressor in the nuclear compartments. Daxx was found to suppress several transcription factor-responsive reporter activities, including reporters of CRE, E2F1, Sp1, and NF-κB (23). Furthermore, through direct protein-protein interactions, Daxx can inhibit the transcriptional potential of several transcription factors, such as ETS1 (24), Pax3 (25, 26), glucocorticoid receptor (27, 28), p53 family proteins (29), and mineralocorticoid receptor (30). Whether Daxx is involved in TGF-β-induced Smad4 transcriptional regulation has not been explored.

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‖ To whom correspondence should be addressed. Present address: Institute of Biomedical Sciences, Academia Sinica., 128, Sec. 2, Academia RD, Taipei 11529, Taiwan. Tel.: 886-2-2652-3520; Fax: 886-2-2785-8594; E-mail: hmshih@ibms.sinica.edu.tw.

[1] The abbreviations used are: SUMO, small ubiquitin-like modifier; TGF, transforming growth factor; PML, promyelocytic leukemia protein; HDAC, histone deacetylase; PAI-1, plasminogen activator inhibitor-1; NEM, *N*-ethylmaleimide; GST, glutathione *S*-transferase; HA, hemagglutinin; β-Gal, β-galactosidase; SBE, Smad-binding element.

In the present study, we showed that Daxx is capable of repressing the transcriptional activity of Smad4 through its interaction with SUMO-modified Smad4. Mutation of Smad4 sumoylation residue Lys^{159} but not Lys^{113} disrupted its association with Daxx, thereby abolishing the inhibitory effect of Daxx on Smad4 transactivation. Furthermore, chromatin immunoprecipitation experiments showed that Daxx forms complexes with the wild-type Smad4 but not with K159R mutant on the promoter of plasminogen activator inhibitor-1 (PAI-1), suggesting that Daxx controls Smad4 transcriptional activation via Lys^{159} sumoylation. Accordingly, down-regulation of Daxx expression by RNA interference increased Smad4 transactivation and PAI-1 expression regulated by TGF-β. Our results identify an important role for Daxx in mediating sumoylation-dependent modulation of Smad4 transcriptional potential.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen and β-Galactosidase (β-Gal) Assays—Yeast two-hybrid array screen using LexA-Smad4 as bait against preys consisting of 600 human full-length cDNA clones fused to the VP16 transactivation domain. Briefly, AMR70 yeast strain expressing LexA-Smad4 was mated with L40 yeast expressing different preys in 96-well plates. After overnight incubation, the resulting yeast cells were cultured in medium lacking tryptophan and leucine for selection of diploid cells. Diploid cells were further transferred to medium lacking tryptophan, leucine, and histidine for scoring protein-protein interactions. Positive clones were selected and subsequently verified by one-on-one transformation assays. β-Gal liquid assays were performed with the Galacto-light Plus kit (Tropix Inc., Bedford, MA), and three separate liquid cultures for each yeast transformant were assayed.

Plasmid Construction—The cDNA fragments for various Smads and Smad4 subdomains were amplified by PCR and subcloned into the pBTM116 in-frame with the LexA to generate pBTM116-Smad1,-Smad2, -Smad3, -Smad4, -MH1, -Linker, and -MH2. The cDNA fragments of Smad1, Ubc9, Daxx, and its deletion mutants were inserted into the pACT2 vector to produce pGalAD-Smad1, -Ubc9, -$Daxx_{FL}$, -$Daxx_{1-625}$, -$Daxx_{1-501}$, and -$Daxx_{570-740}$. HA-Daxx and its mutant constructs were described (27, 28). pCMV-Myc-Smad4, pCMV-FLAG-Smad4, and pRK5-HA-Smad4 were constructed by inserting full-length cDNA of Smad4 into the pcDNA3.1-Myc vector (Invitrogen), pCMV-Tag 2 (Stratagene), and pRK5-HA vector, respectively. pEGFP-Daxx and p2XFLAG-Daxx were generated by subcloning full-length cDNA of Daxx into the pEGFP-C1 vector (Clontech) and pCAG-2XFLAG vector. The mammalian and yeast constructs expressing sumoylation site mutants K113R, K159R, and K113/159R were created using a QuikChange site-directed mutagenesis kit (Stratagene) with pCMV-Myc-Smad4 and pBTM116-Smad4 as templates, respectively. For constructing pGST-Smad4-Linker, cDNA fragment coding the linker domain was subcloned into the pGEX-4T-1 vector (Amersham Biosciences). To generate p6XHis-$Daxx_{570-740}$, the cDNA fragment of Daxx was subcloned into the gateway pENTR3C vector (Invitrogen) followed by a switch to the pDEST17 vector (Invitrogen). pSUPER-Daxx was engineered by inserting an oligonucleotide corresponding to Daxx sequence ^{46}GAT GAA GCA GCT GCT CAG C^{64} into the pSUPER vector (a generous gift from Dr. Reuven Agami). SBE4-Luc is a gift from Dr. Bert Vogelstein. The sequences of all constructs were confirmed by DNA sequencing analysis.

Cell Culture, Transient Transfection, and Luciferase Reporter Assay—COS-1 and MDA-MB-468 cell lines were obtained from the American Type Culture Collection (Manassas, VA). Mv1Lu mink lung cell line is a gift from Dr. Neng-Yao Shih (National Health Research Institutes). All of the cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. To generate stable transfectants of Smad4 and its mutants, MDA-MB-468 cells were transfected with pCMV-Myc-Smad4, pCMV-Myc-$Smad4_{K113R}$, pCMV-Myc-$Smad4_{K159R}$, or pCMV-Myc-$Smad4_{K113/159R}$ and then selected in medium containing 1 mg/ml G418. Transient transfections of COS-1 and Mv1Lu mink lung cells were carried out using the Lipofectamine transfection kit (Invitrogen). For coimmunoprecipitation and Western blot analyses, the cells were harvested at 48 h after transfection. For the reporter gene assay, 2×10^5 cells of COS-1, Mv1Lu, MDA-MB-468, or its derivatives were seeded on 6-well plates 24 h prior to transfection. Various expression constructs, together with the β-Gal expression vector (as an indicator for normalization of transfection efficiency), were introduced into these cells by FuGENE 6 (Roche Applied Science). The transfectants were starved for 12 h followed by TGF-β treatment for additional 18 h. The cells were lysed and assayed for relative luciferase activity (firefly luciferase for the reporter and β-Gal activity for the indicator) as manufacture instructed (Packard, Meriden, CT). For reporter gene experiments involving pSUPER-Daxx, transfection and reporter assays were performed as described above, except the cells were harvested at 60 h after transfection.

Immunoprecipitation and Western Blot Analysis—The cells were lysed in lysis buffer containing 50 mM Tris (pH 7.8), 0.15 M NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.5% Nonidet P-40, and 0.1% sodium deoxycholate and protease inhibitor mixture (Complete; Roche Applied Science). For assaying Daxx-Smad4 interaction, the lysis buffer was supplemented with 20 mM *N*-ethylmaleimide (NEM). Lysates containing equal amounts of proteins were subjected to immunoprecipitation and immunoblot analyses as described previously (28). Antibodies to Smad4 (B-8, monoclonal) (G-20, polyclonal), Smad3 (I-20), and SUMO-1 (D-11) were purchased from Santa Cruz. Another anti-SUMO-1 antibody (GMP-1) was purchased from Zymed Laboratories Inc.. Antibodies to Daxx and FLAG were purchased from Sigma. Anti-Myc and anti-HA antibodies were from Covance. Anti-actin antibody was from Chemicon.

Immunofluorescence—MDA-MB-468 cells were plated onto coverslips the day before transfection. The expression vectors for EGFP-Daxx and/or HA-Smad4 were transiently transfected into MDA-MB-468 cells. Twenty-four hours after transfection, the cells were incubated in Dulbecco's modified Eagle's medium containing 0.2% fetal bovine serum together with or without 200 pM TGF-β for 2 h. The cells were then fixed in 4% paraformaldehyde in phosphate-buffered saline, permeabilized with 0.4% Triton X-100, and then incubated with the anti-HA antibody for 1 h at room temperature. Following this incubation, the cells were washed three times for 10 min with phosphate-buffered saline at room temperature and the incubated with the Texas Red-conjugated anti-mouse IgG (DAKO) for 1 h at 20 °C. The nuclei were revealed by 4′,6′-diamidino-2-phenylindole staining (10 μg/ml). The coverslips were inverted and mounted on slides. The images were visualized by fluorescent microscopy.

In Vitro Sumoylation Assays and Binding Assays for Sumoylated Proteins—The FLAG-tagged Smad4 proteins were purified from transiently transfected COS-1 cells using immunoprecipitation as described above. The precipitated Smad4 proteins were divided equally into two portions for sumoylation reactions in the presence or absence of SUMO-1 proteins. *In vitro* sumoylation was performed in 20 μl of reaction mixture containing 2 mM ATP, 20 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 15 ng of SUMO E1 recombinant proteins (LAE Biotechnology), 200 ng of Ubc9, 100 ng of SUMO-1, and FLAG-Smad4 proteins bound on beads. The reactions were carried out at 37 °C for 1 h and then washed extensively with phosphate-buffered saline. Half of the resulting sample was examined for sumoylation by Western blot analysis with anti-FLAG antibody. The other half of the sample was further incubated with lysates of COS-1 cells overexpressing HA-Daxx or its mutants at 4 °C for overnight. Beads of samples were collected by centrifugation and washed with phosphate-buffered saline, and bound proteins were analyzed by Western blot with anti-HA antibody.

GST Pull-down Assay—The expression and purification of GST-Smad4-Linker fusion protein were performed as described (27, 28). 2 μg of purified GST fusion proteins bound on glutathione agarose beads were subjected to *in vitro* sumoylation assay. A fraction of the reaction mixture was analyzed by immunoblotting to indicate the amount of input and SUMO-1-modified GST fusion proteins. The resulting samples were washed, blocked with BSA, and then incubated for 2 h with 0.3 ml of binding mixture containing 10 mM HEPES (pH 7.5), 50 mM NaCl, 0.1% Nonidet P-40, 0.5 mM dithiothreitol, and 0.5 mM EDTA, together with recombinant $Daxx_{570-740}$ proteins generated from BL21 codon plus strain transformed with p6XHis-$Daxx_{570-740}$. The bound samples were washed four times and analyzed by immunoblotting with anti-Daxx antibody.

Chromatin Immunoprecipitation Analysis—The chromatin immunoprecipitation experiments were performed essentially as described (31). Briefly, $\sim 8 \times 10^6$ cells of each MDA-MB-468 Smad4 stable clone were seeded on four plates (10 cm), cultured for 36 h, and then starved in Dulbecco's modified Eagle's medium with 0.2% fetal bovine serum for additional 12 h. The cells were further stimulated with TGF-β (200 pmol) for 2 h and then subjected to fixation with 1% formaldehyde for 20 min at room temperature. Glycine was added to a final concentration of 125 mM to stop cross-linking. After chromatin extraction, shearing, and preclearing steps, the samples were split for immunoprecipitation with 5 μg of anti-Daxx (Sigma), anti-Smad4 (B-8, Santa Cruz), control rabbit IgG (SC-2027, Santa Cruz) antibodies, or no antibody (as input chromatin control). Bound DNA-protein complexes were washed and then eluted as described (31), and the cross-links were removed by incubat-

ing in 250 mM NaCl at 65 °C for 4 h. The resulting samples were precipitated and resuspended for proteinase K digestion and followed by DNA purification with a PCR purification kit (Qiagen). 5 μl of purified DNA (one-ninth of each sample) was used for PCR amplification (42 cycles). Primers flanking Smad-binding elements (SBEs) in the PAI-1 promoter were 5′-GACAAGGTTGTTGACACAAGAG-3′ (forward) and 5′-GATAACCTCCATCAAAACGTGG-3′ (reverse), which are corresponding to −894/−873 and −614/−593 from the initiation ATG, respectively. PCR products were run on a 2% agarose gel and analyzed by ethidium bromide staining.

Quantification of PAI-1 Expression Level in pSUPER-Daxx-transfected MDA-MB-468 Smad4 Stable Cells—MDA-MB-468 Smad4 wild-type and mutant stable cells were cotransfected with pSUPER or pSUPER-Daxx along with pEGFP-C1 vector (in a 10:1 ratio). Transfected cells were cultured for 48 h, and the GFP-positive cells were isolated by FACS Vantage flow cytometer (BD Biosciences). The resulting cells were cultured for additional 24 h and subsequently treated with or without TGF-β for 4 h. Total cellular RNAs from these cells were extracted using the TRIzol reagent (Invitrogen). Five microgram of RNA of each sample was then reverse transcribed using ThermoScript reverse transcription-PCR system (Invitrogen) in 20 μl of reaction mix. A 1-μl aliquot of the reverse transcription reaction product was used for semiquantitative PCR analysis with specific PAI-1 primers (forward primer: 5′-CAGACCAAGAGCCTCTCCAC-3′ and reverse primer: 5′-ATCACTTGGCCCATGAAAAG-3′) for an initial denaturation step at 95 °C for 5 min; 35 cycles of 15 s at 95 °C, 15 s at 52 °C, and 30 s at 72 °C; and a final elongation step at 72 °C for 10 min. As an internal control, an aliquot of each sample was analyzed for the level of glyceraldehyde-3-phosphate dehydrogenase RNA by semiquantitative and real time PCR with the forward primer (5′-TGGTATCGTGGAAGGACTCA-3′) and reverse primer (5′-AGTGGGTGTCGCTGTTGAAG-3′). The PCR products were then subjected to electrophoresis on 1% agarose gel containing ethidium bromide. The real time quantitative PCR were performed on the Applied Biosystem PRISM 7700 sequence detector with SYBR Green dye (Applied Biosystems) for detection as described in the manufacturer's guidelines. For each sample, the average threshold (C_t) value was resulted from quadruplicate assays, and the ΔC_t value was determined by subtracting the average glyceraldehyde-3-phosphate dehydrogenase C_t value from the average PAI-1 C_t value. Three independent experiments were performed for measuring PAI-1 levels of pSUPER-Daxx-transfected MDA-MB-468 Smad4 stable cells.

RESULTS

Daxx Interacts with Smad4 and Suppresses Its Transcriptional Activity—In a search for potential partner of the Smad4, we carried out a yeast two-hybrid array screen using a fusion protein comprised of the full-length human Smad4 and the LexA DNA-binding domain (LexA-Smad4) as bait. Daxx and Ubc9 were recovered from this screen as positive clones. The specificity of the interactions of Daxx, Ubc9, and Smad4 was verified by one-on-one transformation (Fig. 1*A*, *left panel*). Introduction of both LexA-Smad4 and GalAD-Daxx or GalAD-Ubc9 (Daxx or Ubc9 fused with Gal4 activation domain, respectively) constructs conferred onto transformants the ability to grow in medium lacking histidine. By contrast, yeast transformed with the control bait, LexA-MST3, or LexA-Smad1, along with GalAD-Daxx or GalAD-Ubc9 failed to do so, indicating that the interactions of Smad4 with Daxx and Ubc9 are specific. We further tested the possibility of Daxx associating with other Smad proteins and demonstrated that neither Smad2 nor Smad3 interacted with Daxx in yeast two-hybrid assays (Fig. 1*A*, *right panel*).

Because Daxx can act as a transcriptional coregulator, identification of Daxx interacting with Smad4 prompted us to examine whether Daxx is involved in regulating Smad4 transcriptional activity. To this end, expression constructs of Smad4 and Smad3 were cotransfected with increasing amount of HA-Daxx into COS-1 cells along with the 3TP-Lux reporter, which contains TGF-β-responsive elements from the PAI-1 and collagenase promoters (32). Consistent with previous reports (33, 34), overexpression of both Smad3 and Smad4 induced a TGF-β-independent 3TP-Lux reporter activity (Fig. 1*B*). Introduction of Daxx, however, suppressed the transcriptional activity of Smad3/4 in a dose-dependent manner. To further substantiate the Daxx transcriptional repression on Smad4, MDA-MB-468 breast cancer cells lacking endogenous Smad4 were transfected with FLAG-Smad4 and HA-Daxx along with the reporter 3TP-Lux or SBE4-Luc, with the latter containing four copies of the Smad-binding element CAGA (35), followed by TGF-β treatment. As expected, Daxx also inhibited the TGF-β-induced reporter activities of 3TP-Lux (Fig. 1*C*) and SBE4-Luc (Fig. 1*D*) in MDA-MB-468 cells carrying exogenous Smad4. Furthermore, the repressive effect of Daxx on Smad4-mediated reporter activities of 3TP-Lux (Fig. 1*E*) and SBE4-Luc (Fig. 1*F*) was also observed in Mv1Lu mink lung cells, another TGF-β-responsive cell line. Together, these results indicate that Daxx could suppress Smad4-mediated transcriptional activity, and this suppression is not a cell type-specific event.

To further establish the link between the interaction and regulation of Smad4 by Daxx, we delineated the domain(s) of Daxx required for interacting with Smad4. Several Daxx deletion mutants were generated and then characterized for their interplays with Smad4 by yeast two-hybrid assays and Smad4 reporter experiments. In yeast two-hybrid assays, a truncated mutant of Daxx expressing amino acid residues 570–740 ($Daxx_{570–740}$) was still able to interact with LexA-Smad4 (Fig. 2*A*). By contrast, two N-terminal fragments, $Daxx_{1–501}$ and $Daxx_{1–625}$, failed to do so. Therefore, the C-terminal domain of Daxx is sufficient for binding Smad4. Consistent with the results from yeast two-hybrid assays, $Daxx_{570–740}$, instead of $Daxx_{1–625}$, inhibited the 3TP-Lux reporter gene activity in COS-1 cells cotransfected with a fusion construct consisting of both TGF-β type I and II receptor cytoplasmic domains, R(II-I)C (Fig. 2*B*) that has been shown to activate TGF-β responses in a ligand-independent manner (36). Likewise, the repressive effect of Daxx and $Daxx_{570–740}$, but not $Daxx_{1–625}$, on Smad4 was also observed in the Smad4-transfected MDA-MB-468 cells (data not shown). Thus, the interaction capabilities of these Daxx mutants are well correlated with their transcriptional repression abilities toward Smad4.

Daxx Does Not Alter the Nuclear Translocation of Smad4—Because Daxx was reported to associate with the TGF-β type II receptor in the cytoplasm (22), we next examined whether the repressive effect of Daxx on Smad4 is due to an inhibition of Smad4 nuclear translocation. To test this possibility, MDA-MB-468 cells were transiently transfected with HA-Smad4 and/or EGFP-Daxx followed by TGF-β treatment. Immunofluorescence analysis revealed the translocation of Smad4 from the cytoplasmic to the nuclear compartment upon TGF-β stimulation (Fig. 3, *panel a versus panel c*). Daxx, however, was mainly distributed in the nucleus, and its localization was not altered by TGF-β treatment (*panel e* and *panel g*). Notably, when both Smad4 and Daxx were coexpressed in the same cells, Daxx did not block Smad4 nuclear translocation upon TGF-β-treatment (*panel m*). In fact, Smad4 subcellular distribution was not affected by overexpression of Daxx (*panel i* and *panel m*). Therefore, the effect of Daxx on repressing Smad4 transcriptional activity could not be attributed to the sequestration of Smad4 in the cytoplasm and is likely due to an inhibition of Smad4 nuclear function.

SUMO-1 Modification of Smad4 Mediates Daxx Interaction—To further study the interplay between Daxx and Smad4, we determined their interactions *in vivo*. COS-1 cells were cotransfected with expression constructs for HA-tagged Smad4 and double FLAG (2XFLAG)-tagged Daxx. Western blotting of anti-HA immunoprecipitates from lysates of transfected cells revealed the coprecipitation of a small amount of FLAG-tagged Daxx with Smad4 (Fig. 4*A*, *top panel*, *lane 4*). Interestingly, a

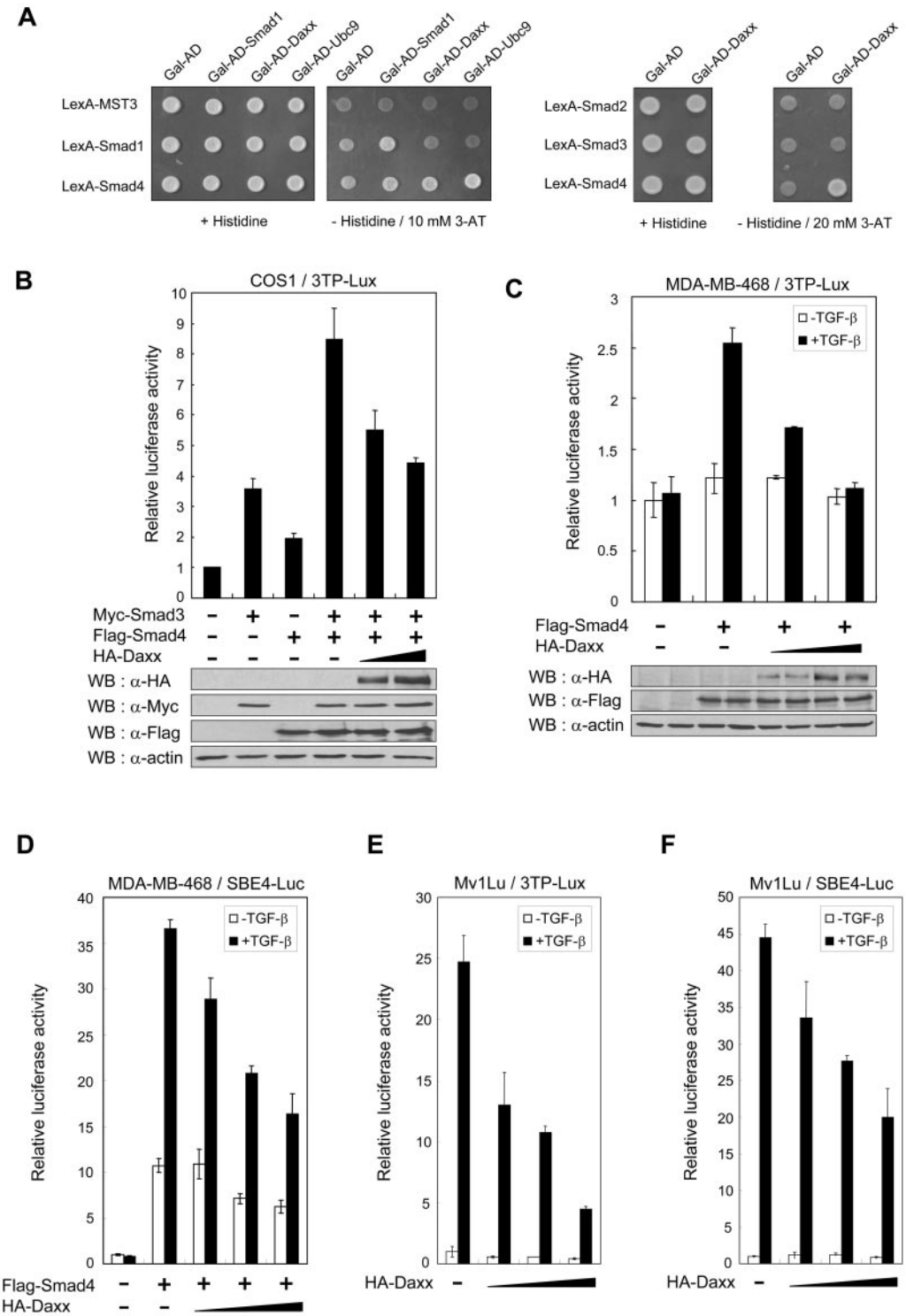

FIG. 1. **Daxx interacts with Smad4 and suppresses its transactivation potential.** *A*, identification of Daxx as a Smad4-interacting protein. L40 yeast strain cotransformed with a bait (LexA-based fusion construct) and a prey plasmids (GalAD-based fusion construct) was plated on the medium plates in the presence or absence of histidine and various concentrations of 3-aminotriazole (*3-AT*) as indicated. 3-Aminotriazole was used to inhibit basal activity conferred by the bait plasmid. The colonies grown in *His*$^-$ plate indicate the interaction between proteins encoded by the two plasmids. *B*, the effect of Daxx on Smad3/4-mediated transcription. COS-1 cells were transfected with 600 ng of 3TP-Lux, 100 ng of pCMV-β-Gal, together with or without 300 ng of Smad3, 300 ng of Smad4, and increasing amounts of HA-Daxx (500 and 1000 ng) as indicated. Total amount of plasmid in each transfection was kept constant by the addition of the empty pcDNA3 vector as needed. Transfected cells were incubated in Dulbecco's modified Eagle's medium with 10% fetal bovine serum for 48 h. Relative luciferase activity is represented as the means ± S.D. from at least three independent experiments. The expression levels of transfected Smad3, Smad4, and Daxx proteins and endogenous actin in whole cell extracts were analyzed by Western blot with antibodies as indicated (*bottom panel*). *C* and *D*, suppression of TGF-β-induced reporter activity by Daxx. MDA-MB-468 cells were transfected with 3TP-Lux (*C*) or SBE4-Luc (*D*) reporter construct along with FLAG-Smad4 and increasing amount of HA-Daxx plasmids as indicated. After transfection, the cells were starved for 12 h followed by TGF-β treatment for 18 h. The cells were harvested and subjected to reporter assays. Relative luciferase activity is represented as the means ± S.D. from three independent experiments. *E* and *F*, Daxx represses TGF-β-induced reporter activity in Mv1Lu mink lung cells. Mv1Lu mink lung cells were transfected with 3TP-Lux (*E*) or SBE4-Luc (*F*) reporter construct with increasing amount of HA-Daxx construct followed by TGF-β treatment. The relative reporter activity was determined as described above.

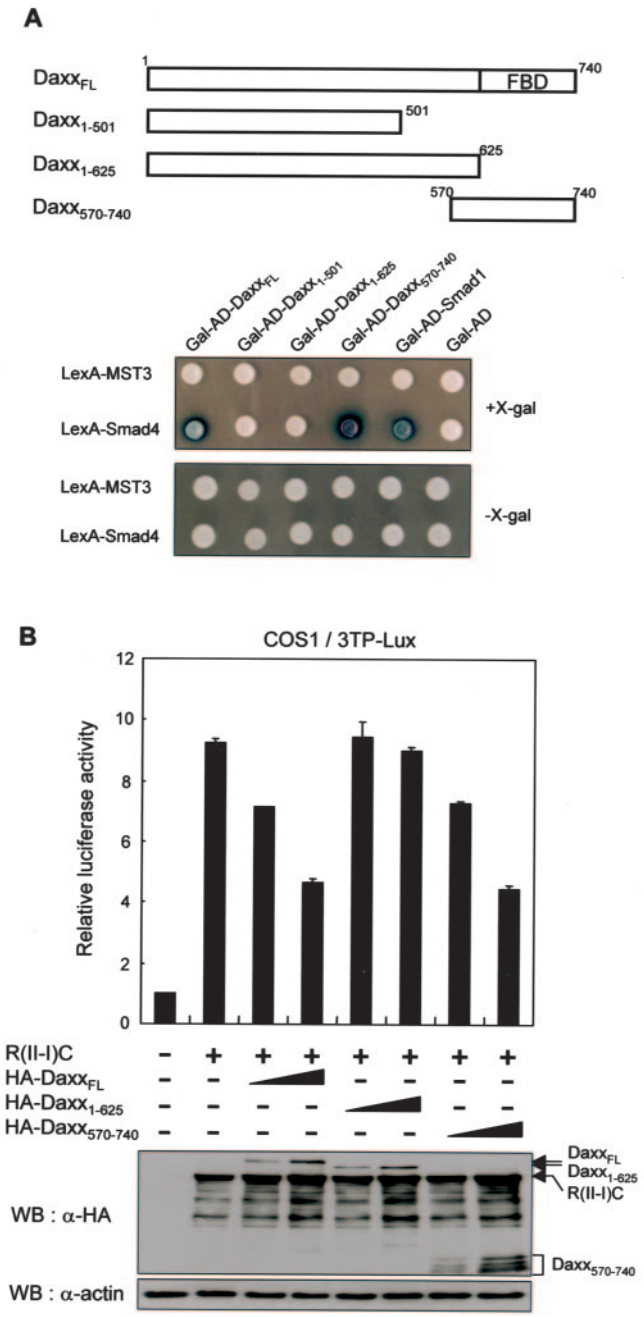

FIG. 2. **The C-terminal domain of Daxx mediates Smad4 interaction and transrepression.** *A*, interaction of Smad4 with Daxx deletion mutants. *Top panel*, schematic representation of Daxx deletion mutants tested in yeast two-hybrid and mammalian transfection assays. *Bottom panel*, yeast stain L40 was transformed with various Gal-AD-based constructs as indicated. The resulting transformants were mated with different AMR70 bait strains carrying LexA-MST3 (as a negative control) or LexA-Smad4 and further cultured on His$^+$ plates in the presence or absence of X-gal. *B*, the effect of Daxx mutants on TGF-β-stimulated reporter activity in COS-1 cells. The cells were co-transfected with 3TP-Lux reporter (600 ng) and pCMV-β-Gal (100 ng), together with or without the expression vector for active TGF-β receptor R(II-I)C (900 ng) and increasing amounts of expression plasmids for Daxx or its mutants (250 and 500 ng) as indicated. Luciferase activity was assayed as in Fig. 1. The data presented are the means ± S.D. from at least three independent experiments. Western blot analysis shows the expression of different transfected Daxx mutants as well as the active receptor R(II-I)C protein in whole cell extracts (*bottom panel*).

significantly higher level of Daxx could be detected in the Smad4 immunoprecipitates when cells were lysed in the presence of NEM (*top panel, lane 8*), a cysteine protease inhibitor usually used to preserve the sumoylation of cellular proteins. As seen in Fig. 4*A*, NEM treatment resulted in the detection of two slowly migrating bands of HA-Smad4 (indicated by *asterisks*), which corresponds to Smad4 SUMO modification (see below). In the reciprocal immunoprecipitation experiments, the upper slowly migrating band and the unmodified band of Samd4 were detected in the Daxx immunoprecipitates from cells lysed with NEM (Fig. 4*B*, *top panel*, *lane 2*), whereas a small amount of unmodified Smad4 was precipitated by Daxx from cells lysed without NEM (*lane 1*), suggesting that SUMO modification enhances Daxx-Smad4 association. These results, in conjunction with the findings that the C-terminal domain of Daxx is able to interact with SUMO-1 (37)[2] and that Daxx binds to sumoylated androgen receptor (38), raised a hypothesis that the association between Daxx and Smad4 is sumoylation-dependent. To test this possibility, we performed an *in vitro* binding assay of Daxx with sumoylated Smad4. Sumoylated Smad4 was generated by *in vitro* sumoylation reaction of Smad4 proteins immunoprecipitated from Smad4-transfected COS-1 cells and was subsequently subjected to a binding assay with Daxx. In line with the previous reports (17–19), two more slowly migrating forms of Smad4 were exclusively observed from the sample with sumoylation reaction, although the yield of the lower sumoylated band appeared less as compared with the upper sumoylated band (Fig. 4*C*). Notably, Daxx was only pulled down by SUMO-conjugated Smad4 but not by unmodified Smad4 protein (Fig. 4*C*), thus demonstrating a critical role of Smad4 sumoylation in its interaction with Daxx. Consistent with the results of Daxx domain mapping studies, Daxx$_{570-740}$ but not Daxx$_{1-625}$ proteins could be precipitated by sumoylated Smad4 (Fig. 4*C*). Together, these results indicate that Daxx interacts with Smad4 via a SUMO-dependent manner.

Sumoylation of Smad4 at Lys^{159} Is Critical for Daxx Interaction—If sumoylation of Smad4 indeed accounts for the recruitment of Daxx and subsequent repression of Smad4 transcriptional activity, interfering with Smad4 sumoylation should abrogate these effects of Daxx. To specifically block Smad4 sumoylation, we defined its sumoylation site(s). During the progress of our studies, three groups reported that the Lys^{113} and Lys^{159} residues within the Smad4 MH1 and linker domains, respectively, can be sumoylated (17–19). Our analyses of Smad4 sumoylation were consistent with these findings (supplemental data). We therefore examined the interaction of Daxx with Smad4 sumoylation site mutants in yeast two-hybrid assays. Interestingly, the K113R mutant retained a capacity similar to that of the wild-type protein to associate with Daxx. By contrast, the K159R mutant completely lost its interaction with Daxx, although it exhibited a higher basal activity in the β-Gal reporter assay (Fig. 5*A*). Furthermore, the K113/159R mutant, as expected, also failed to interact with Daxx. Together, these results indicate that the sumoylation site of Lys^{159} within the linker region is responsible for the binding of Daxx to Smad4. In agreement with these findings, Daxx is able to bind the linker segment rather than the MH1 domain of Smad4 in yeast two-hybrid assays (Fig. 5*B*). To further demonstrate a direct interaction between Daxx and sumoylated Smad4, the recombinant GST-Smad4 linker segment purified from bacteria was subjected to an *in vitro* sumoylation reaction and then used to pull down the bacterially expressed, purified Daxx$_{570-740}$ fragment. As shown in Fig. 5*C*, the Daxx fragment was only precipitated with the sumoylated Smad4 linker segment but not with the unmodified one, thus providing a definitive evidence for a direct and sumo-dependent interaction between Smad4 and Daxx.

[2] D.-Y. Lin and H.-M. Shih, unpublished data.

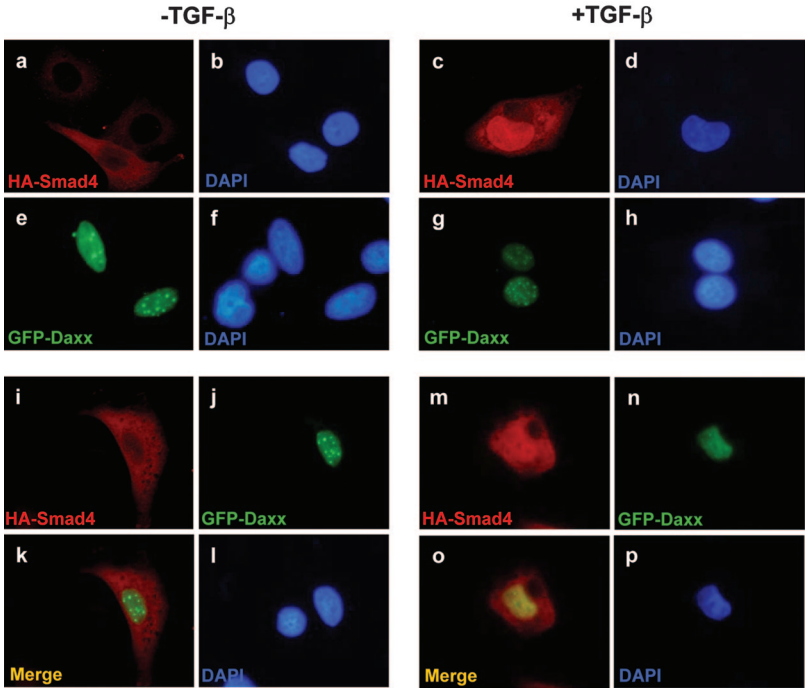

FIG. 3. **Daxx does not inhibit TGF-β-stimulated Smad4 nuclear translocation.** MDA-MB-468 cells were transfected with plasmid expressing HA-Smad4 (*a–d*), EGFP-Daxx (*e–h*), or both (*i–p*). Transfectants were treated with or without 200 pM TGF-β for 2 h and then immunostained with anti-HA antibody (*red*) followed by 4′,6′-diamidino-2-phenylindole staining (*blue*). The cells were examined by immunofluorescent microscopy. The overlay of the *green* (GFP) and *red* images is shown in *k* and *o* (merge).

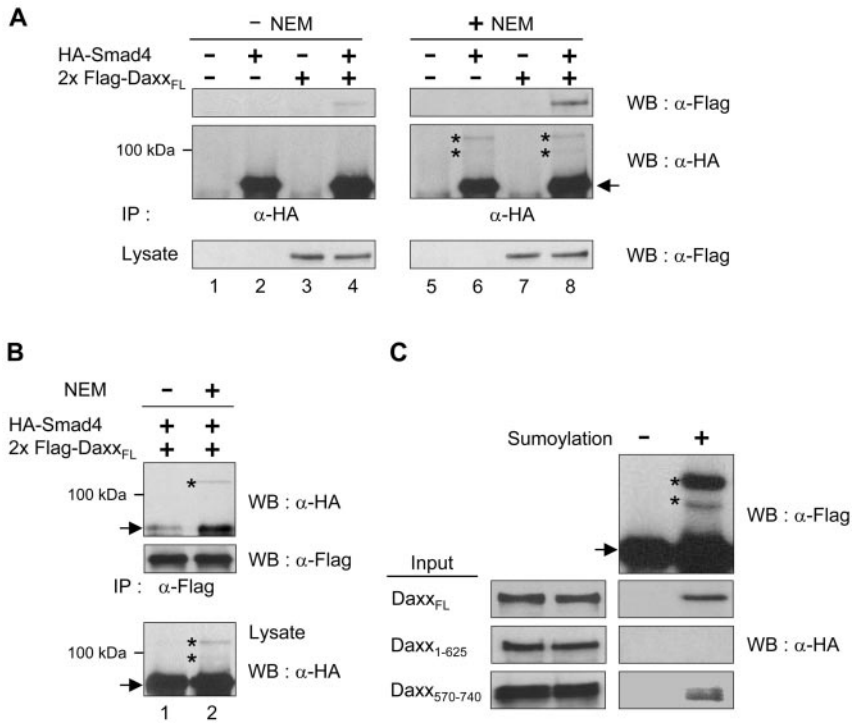

FIG. 4. **Sumoylation of Smad4 mediates Daxx interaction.** *A* and *B*, coimmunoprecipitation of Daxx and Smad4. COS-1 cells were transiently transfected with expressing constructs for HA-Smad4 and/or 2XFLAG-Daxx. The cells were then lysed in the radioimmune precipitation assay buffer in the absence or presence of 20 mM NEM as indicated. Equal amounts of lysates were immunoprecipitated (*IP*) with anti-HA (*A*) or anti-FLAG (*B*) antibody followed by immunoblotting with anti-FLAG or anti-HA antibody, respectively. The *asterisks* and *arrow* indicate the SUMO-1 modified and unmodified Smad4, respectively. *C*, Daxx binds sumoylated Smad4 *in vitro*. FLAG-tagged Smad4 proteins were subjected to *in vitro* sumoylation assays as described under "Experimental Procedures." Half of the SUMO-1 modified and unmodified Smad4 proteins were analyzed by immunoblotting with anti-FLAG antibody (*top panel*). The other half of modified and unmodified proteins bound on agarose beads were incubated with cell lysate expressing HA-Daxx, HA-Daxx$_{1–625}$, or HA-Daxx$_{570–740}$. After extensive washing, the bound proteins were analyzed by Western blotting (*WB*) with anti-HA antibody. The *arrow* and *asterisks* indicate the SUMO-1 unmodified and modified Smad4, respectively. *Input* represents the 5% amount of various Daxx proteins subjected to this binding assay.

Lys^{159} Sumoylation Is Critical for Daxx-induced Transcriptional Repression of Smad4—We next established the cause-effect relationship between the SUMO-dependent Smad4-Daxx interaction and transcriptional repression of Smad4 by Daxx. MDA-MB-468 breast cancer cells were utilized to establish cell lines expressing Myc-tagged wild-type and sumoylation-defective mutants of Smad4. Pooled stable clones of each Smad4 wild-type and mutants were selected, and the expression levels of these Smad4 proteins were analyzed by Western blot with anti-Myc antibody. As shown in Fig. 6*A*, the expression levels of Smad4 wild-type and SUMO mutant proteins in the stable clones were comparable. These cells were transiently transfected with 3TP-Lux reporter along with or without Daxx expression construct followed by TGF-β stimulation. Luciferase assays showed that expression of various Smad4 proteins in MDA-MB-468 cells conferred TGF-β responsiveness, indicating that these Smad4 mutants are functional (Fig. 6*B*). Notably, in the absence of ectopically expressed Daxx, the K159R and K113/159R mutants displayed higher levels of TGF-β-induced reporter activities than the wild-type or K113R mutant, which is consistent with previous reports (17–19) and might reflect a relief of the repressive effect by endogenous Daxx (see "Discussion"). As expected, introduction of Daxx into cells expressing the wild-type Smad4 resulted in the transcriptional repression

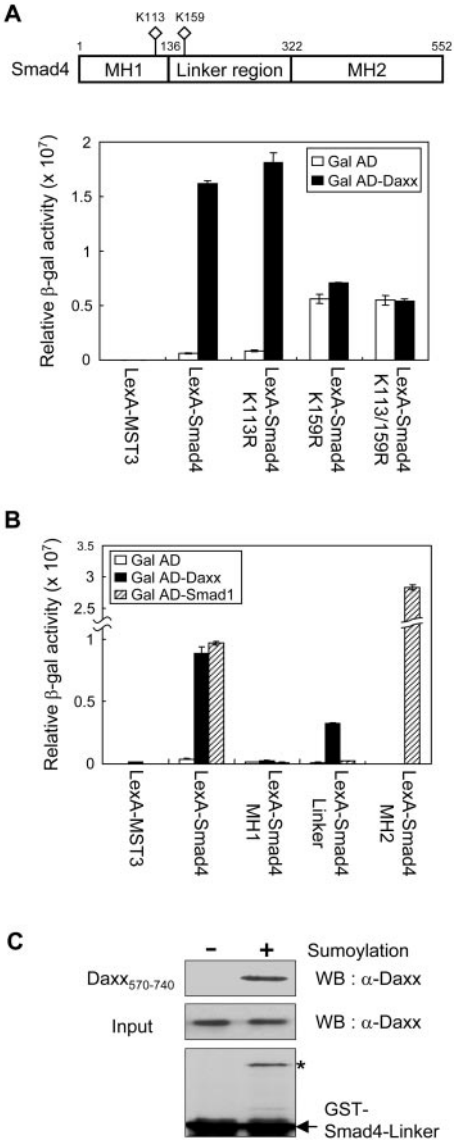

FIG. 5. **Sumoylation of Smad4 Lys^{159} is critical for mediating Daxx interaction.** *A*, mutation of Smad4 Lys^{159} but not Lys^{113} abolishes Daxx interaction in yeast two-hybrid assays. *Top panel*, schematic representation of the domain structure of human Smad4. The sumoylation sites Lys^{113} and Lys^{159} of Smad4 are indicated. *Bottom panel*, the L40 yeast strain was transformed with the plasmid constructs as indicated. The relative strength of protein interactions was determined by measuring β-galactosidase activities using the Galacto-light Plus kit (Tropix Inc., Bedford, MA) and normalized by cell density (A_{600}). The data shown are the means ± S.D. from three independent experiments performed in triplicate. *B*, Daxx binds to Smad4 linker region in yeast two-hybrid assays. Liquid β-Gal assays were performed with L40 transformants as indicated. The data represent the means ± S.D. of three independent experiments. *C*, Daxx interacts directly with sumoylated Smad4 linker region *in vitro*. 2 μg of recombinant GST-Linker fusion protein was subjected to *in vitro* sumoylation reaction, and the resulting products were incubated with 1 μg of recombinant Daxx$_{570-740}$ proteins for 2 h. The samples were further washed and analyzed by SDS-PAGE and autoradiography. *Input* represents 5% amount of recombinant Daxx$_{570-740}$ subjected to the GST pull-down assay. Western blot (*WB*) analysis of SUMO-1 modified (*asterisk*) and unmodified (*arrow*) GST-Linker proteins used in this pull-down assay is shown in the *bottom panel*.

of Smad4 activity. Daxx, however, could no longer suppress the transcriptional activity of the K159R- and K113/159R-Smad4, whereas the K113R-Smad4 showed a similar repression by Daxx as the wild-type protein. Together, these results strongly suggest that the sumoylation of Lys^{159} is crucial for Daxx recruitment, leading to Smad4 transcriptional repression.

Next, we sought to demonstrate the Lys^{159} sumoylation-dependent recruitment of Daxx at endogenous gene promoter. MDA-MB-468 cells stably expressing the wild-type or sumoylation-defective Smad4 were treated with TGF-β and then subjected to chromatin immunoprecipitation analysis. After formaldehyde cross-linking and precipitation of the chromatin with anti-Smad4, anti-Daxx, or a control antibody, the precipitated DNA was PCR-amplified with a set of specific primers flanking the Smad-binding sites in the PAI-1 promoter. Upon TGF-β stimulation, the wild-type and sumoylation-defective Smad4 interacted with the endogenous PAI-1 promoter to a very similar extent (Fig. 6*C*), consistent with the recent report that sumoylation does not alter the DNA binding activity of Smad4 *in vitro* (17). Recruitment of Daxx to the PAI-1 promoter, however, was detected in the Smad4 wild-type and K113R stable cells but not in K159R and K159/113R stable cells (Fig. 6*C*). These findings demonstrate a specific loading of Daxx to the endogenous, Smad4-regulated promoter in a Lys^{159} sumoylation-dependent manner.

Knockdown of Daxx Expression Increases Smad4 Transactivation and PAI-1 Expression Induced by TGF-β—To further demonstrate the role of endogenous Daxx in regulating Smad4 transactivation, we used a RNA interference approach to knockdown endogenous Daxx protein expressed in MDA-MB-468 cells. A Daxx-specific oligonucleotide was engineered into the pSUPER vector for generating small interfering RNA (pSUPER-Daxx). pSUPER-Daxx or pSUPER control vector was transfected with 3TP-Lux reporter and the expression construct of the active TGF-β receptor R(II-I)C into MDA-MB-468 Smad4 stable cells. Western blot analysis revealed that introduction of pSUPER-Daxx into MDA-MB-468 stable cells resulted in a dose-dependent decrease of endogenous Daxx without affecting the protein levels of actin, Smad3, and Smad4 (Fig. 7, *A* and *B*). Under such condition, the receptor-induced 3TP-Lux reporter gene activity was further elevated by pSUPER-Daxx, correlating with the repressive effect of Daxx on Smad4-mediated transcriptional activation (Fig. 7*A*). Notably, when pSUPER-Daxx was transfected into the K159R-Smad4 stable cells, no significant increase in the 3TP-Lux reporter activity was observed, although the endogenous protein level of Daxx was down-regulated (Fig. 7*B*). Together, these results further support our conclusion that Daxx suppresses the Smad4 transactivation through a Lys^{159} sumoylation-dependent mechanism.

Finally, we sought to investigate the effect of Daxx knockdown on TGF-β-induced expression of endogenous genes. MDA-MB-468 cells stably expressing wild-type or K159R Smad4 were transfected with pSUPER-Daxx or control vector followed by TGF-β stimulation. The level of PAI-1 transcript in these transfected cells was measured by both semiquantitative PCR (Fig. 7*C*, *top panel*) and real time PCR (Fig. 7*C*, *bottom panel*) analyses. Transfection of pSUPER-Daxx plasmid into cells carrying wild-type Smad4 resulted in a significant increment of TGF-β-induced PAI-1 transcript, compared with cells receiving pSUPER control vector (*top* and *bottom panels*, *lane 2 versus lane 4*). However, this enhancement of TGF-β-induced PAI-1 expression by down-regulating Daxx was not observed in cells expressing K159R-Smad4, which already displayed a higher PAI-1 induction level in the absence of Daxx small interfering RNA (*lane 6 versus lane 8*). Together, these results indicate a physiological role for Daxx in repressing TGF-β-induced expression of endogenous genes and highlight a critical function for Smad4 Lys^{159} sumoylation in mediating this effect of Daxx.

DISCUSSION

In the present study, we have identified a novel function of Daxx in regulating Smad4-mediated TGF-β signaling. We

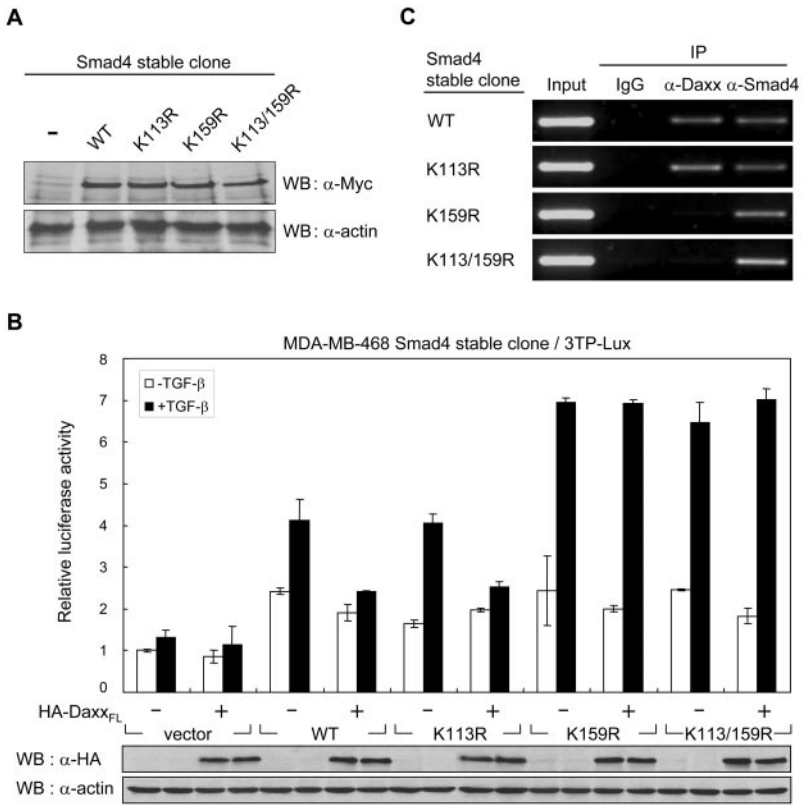

FIG. 6. **Mutation of Lys^{159} abrogates Daxx-dependent repression of Smad4 transcriptional activity.** *A*, establishment of MDA-MB-468 stable clones. 20 μg of cell lysates from each pooled stable clone expressing Myc-Smad4 or its mutants were subjected to Western blot (*WB*) analysis with anti-Myc and anti-actin antibodies. *B*, Daxx suppresses Smad4-mediated transcription through a Lys^{159} sumoylation-dependent manner. Stable clones described in *A* were transiently transfected with HA-Daxx construct and 3TP-Lux reporter. The cells were starved, stimulated with or without TGF-β, and harvested for luciferase assay. The relative luciferase activity was determined as described in the legend to Fig. 1. The data presented are the means ± S.D. of at least three independent experiments. Western blot indicates the expression of transfected Daxx (*bottom panel*). *C*, Daxx recruitment to the PAI-1 promoter requires Lys^{159} sumoylation. Stable clones of MDA-MD-468 cells expressing wild-type (*WT*) and mutant Smad4 as indicated were treated with TGF-β for 2 h and then subjected to chromatin immunoprecipitation analysis as described under "Experimental Procedures." One-ninth of the immunoprecipitated and purified DNA were subjected to PCR amplification for 42 cycles with primers flanking Smad-binding elements in the PAI-1 promoter. *Input* represents 2.5% of the chromatin used for the immunoprecipitation.

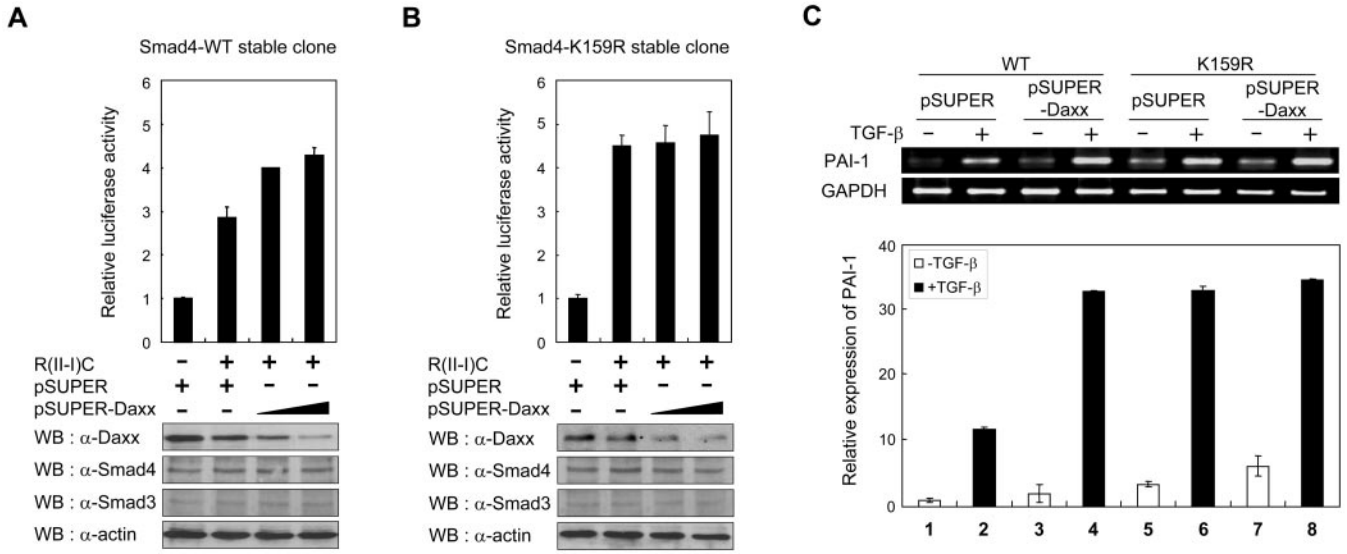

FIG. 7. **Knockdown of Daxx expression potentiates Smad4-mediated transactivation and TGF-β-induced gene expression.** *A* and *B*, down-regulation of Daxx enhances TGF-β-induced transactivation in cells expressing wild-type (*WT*) Smad4 but not Smad4 K159R. MDA-MB-468 cells stably expressing wild-type Smad4 (*A*) or Smad4 K159R (*B*) were transfected with pSUPER-Daxx (300 and 500 ng) or pSUPER (500 ng) together with R(II-I)C plasmid (900 ng) and 3TP-Lux reporter construct (600 ng). The transfected cells were harvested for luciferase assay as described in the legend to Fig. 1. The data shown are the means ± S.D. of three independent experiments. Expression levels of Daxx, Smad4, Smad3, and actin in each transfectant were determined by immunoblotting with corresponding antibodies (*bottom panel*). *C*, down-regulation of Daxx potentiates TGF-β-induced PAI-1 transcripts in MDA-MB-468 stable cells expressing Smad4 wild-type but not K159R mutant. MDA-MB-468 Smad4 stable cells as indicated were cotransfected with pSUPER-Daxx or pSUPER empty vector along with pEGFP-C1 vector. After 48 h, the transfected cells were sorted for GFP positive cells followed by TGF-β stimulation for 4 h. Total RNA from these cells was subjected to reverse transcription followed by semiquantitative PCR (*top panel*) and real time PCR (*bottom panel*) analyses of PAI-1 expression as described under "Experimental Procedures." Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) serves as a control in both semiquantitative PCR and real time PCR analyses. The *bottom panel* indicates the relative expression of PAI-1 in three independent experiments. *WB*, Western blot.

showed that Daxx interacts with Smad4 and represses its transcriptional potential through the C-terminal domain of Daxx. Importantly, these effects of Daxx are dependent on Smad4 sumoylation at Lys^{159}. Additionally, Daxx is recruited to an endogenous, Smad4-targeted promoter *in vivo* through a Lys^{159} sumoylation-dependent manner. Finally, knockdown of the endogenous Daxx protein increases the transactivation potential of Smad4 and TGF-β-induced expression of endogenous PAI-1. Together, these findings not only identify Daxx as a negative Smad4 cofactor but also provide an important clue of sumoylation in regulating Smad4 transcriptional activity.

Daxx was previously reported to mediate the TGF-β-induced

Jun N-terminal kinase activation and apoptosis by associating with the cytoplasmic domain of the TGF-β type II receptor (22), which delineates a cytoplasmic role of Daxx in TGF-β signaling pathway. Our study, however, defines a nuclear role for Daxx in this pathway, because Daxx is found to be recruited to Smad4-regulated promoter to repress the transactivation of Smad4. Furthermore, our immunofluorescence analysis indicated that the distribution of Daxx is mainly in the nuclear compartment of MDA-MB-468 cells under both TGF-β-stimulated and unstimulated conditions. This nuclear localization of Daxx is consistent with a number of reports (25, 27, 39–43). However, Daxx is found in the cytoplasmic fraction in AML12 cells (22) and can be shuttled from the nucleus to the cytoplasmic compartment under circumstances (42–44). Although the regulation of Daxx subcellular localization requires further investigation, several lines of evidence indicate that repressive effect of Daxx on Smad4-mediated TGF-β signaling observed in our study is unlikely because of its modulation of the type II receptor. First, Daxx suppressed the overexpressed Smad4/Smad3-induced reporter activity (Fig. 1*B*), which is independent of TGF-β receptors. Second, overexpression of Daxx did not affect TGF-β-induced Smad4 nuclear translocation, an event lying downstream of the type II receptor. Third, overexpression or depletion of Daxx protein did not affect the transcriptional potential of K159R-Smad4 induced by TGF-β (Figs. 6*B* and 7*B*). Fourth, down-regulation of Daxx in cells stably expressing K159R-Smad4 did not alter the expression of PAI-1 in response to TGF-β (Fig. 7*C*). Therefore, at least in cell systems used in our study, Daxx acts at the level of Smads rather than the type II receptor in the TGF-β-induced signaling pathways.

A growing list of transcription factors has been shown to be positively or negatively regulated by SUMO modification (1, 45). Likewise, Smad4 was found by our and other groups (17–19) to undergo SUMO modification on lysine 113 and 159, and mutation of both residues or lysine 159 markedly enhances Smad4 transcriptional activity. However, the study by Ohshima and Shimotohno (46) identified only Lys^{159} as the SUMO acceptor site, and the K159R mutant conferred a lower transcriptional activity than wild-type Smad4 in HepG2 cells. The discrepancy in K159R-mediated transcriptional activity might result from the usage of different cell systems. Very little is known about the underlying mechanism of this effect of K159R mutation. Two possible scenarios of sumoylation in regulating Smad4 transcriptional activity have been encountered: (i) sumoylation alters Smad4 protein stability and (ii) sumoylation modulates Smad4 protein-protein interactions. Because the K113/159R-Smad4 displays a modestly higher stability than the wild-type protein (18, 20), a model for the potential competition between sumoylation and ubiquitination at the same lysine residues was proposed to explain the increased stability of this mutant (18, 20). A similar competition mechanism was reported to account for the stability of IκBα by sumoylation (9). However, controversial results were reported for the ubiquitination capacities of sumoylation-defective mutants of Smad4 (18, 20), implying the existence of a competition-independent mechanism. Furthermore, because the MDA-MB-468 stable cell lines used in our study contained a very similar, if not identical, steady-state expression level of the wild-type and mutant Smad4 proteins (Fig. 6*A*), the stability issue may be insufficient to account for the significant difference in the transcriptional activities of these proteins (Fig. 6*B*). On the other hand, we demonstrated that sumoylated Smad4 is capable of interacting with Daxx. Although *in vivo* interaction assay revealed the coprecipitation of Daxx with both sumoylated and unmodified Smad4, yeast two-hybrid analysis (Fig. 5*A*) and *in vitro* binding assay using purified proteins (Fig. 5*C*) clearly indicate that Smad4-Daxx interaction is strictly dependent on the presence of the Lys^{159} SUMO acceptor residue and the sumoylation event *per se*, respectively. Therefore, the observed coprecipitation of Daxx with unsumoylated Smad4 may result from the oligomerization of sumoylated Smad4 with unmodified one and from the rapid desumoylation after coimmunoprecipitation, and the latter is further supported by the significant reduction of Daxx-Smad4 coprecipitation in the absence of sumoylation-stabilizing agent NEM. Given that a strong correlation was observed between Daxx-Smad4 interaction and Daxx-induced repression of Smad4, the loss of Daxx transrepression on K159R-Smad4 most likely results from the lack of interaction with Daxx rather than a change in protein conformation triggered by this mutation. Thus, our results obtained from the Daxx overexpression and knockdown systems strongly suggest that Daxx is the factor that mediates Lys^{159} sumoylation-dependent protein-protein interaction and transcriptional regulation of Smad4. Collectively, our findings provide the mechanistic insight underlying SUMO-dependent transcriptional repression of the Smad4.

Recent reports showed that overexpression of Ubc9 and/or SUMO-1 enhances the Smad4-mediated transcription in both mammalian cells (17, 18) and *Xenopus* animal caps (18). These findings, however, need to be interpreted with caution, and it cannot be simply concluded that sumoylation increases the intrinsic Smad4 transcriptional potential. Because several Smad-associated coactivators (*e.g.* p300 (47–49) and PIAS3 (50)) and corepressors (*e.g.* HDAC1 (51), PIASy (52, 53), and Daxx (54)) could also be regulated by sumoylation, such global overexpression of Ubc9 and/or SUMO-1 may indirectly regulate Smad4-mediated transcription via sumoylation of these cofactors (54–58). Given the highly context-dependent nature of TGF-β and Smad signaling effects, it is conceivable that Long *et al.* (19) recently reported an inhibitory role of Ubc9 and/or SUMO-1 overexpression in TGF-β-induced reporter activity, contrary to previous reports. Importantly, using SUMO-1 fusion assay to covalently attach SUMO-1 to various Smad4 sumoylation-defective mutants, this group demonstrated a great reduction of Smad4 intrinsic transcriptional activity by SUMO conjugation (19), which supports our notion that sumoylation negatively regulates intrinsic Smad4 transcriptional activity.

The C-terminal domain of Daxx interacts not only with sumoylated Smad4, but also with several transcription factors as well as PML (24–29, 41, 59). Interestingly, the binding of Daxx to PML is also dependent on the sumoylation of PML (28, 39, 41, 60). In view of Daxx binding to sumoylated Smad4, one can envision that sumoylated PML may compete with sumoylated Smad4 for Daxx interaction, leading to a promotion of Smad4 transactivation. Indeed, PML but not PML sumoylation-defective mutant could enhance TGF-β-induced Smad4 transactivation.[3] Daxx has recently been shown to bind SUMO-1 (37).[2] Whether a SUMO-binding motif within Daxx mediates the SUMO-dependent protein-protein interaction is under investigation. In addition, it is currently unclear how Daxx only binds to sumoylated Lys^{159} but not sumoylated Lys^{113}. Whether the flanking region of sumoylated Lys^{159} provides a conformational preference for interaction requires further investigation as well.

Although Daxx functions as a transcriptional corepressor, the molecular mechanism by which it suppresses the transcriptional activation remains largely unclear. A previous report demonstrated that the deacetylase inhibitor trichostatin A can efficiently reverse the repressive effect of Daxx (41), suggesting

[3] C.-C. Chang and H.-M. Shih, unpublished data.

an involvement of histone deacetylation. In support of this model, Daxx was reported to interact with HDAC1 both *in vitro* and *in vivo* (41) and to associate with multiple proteins that are critical for transcriptional repression, such as HDAC2, components of chromatin such as core histone H2A, H2B, H3, and H4, and a chromatin-associated protein Dek (61). In addition, Daxx has been shown to associate with ATRX, a protein binding to heterochromatin protein HP1 and functioning as part of a chromatin-remodeling complex (62–64). Also reported is its association with condensed chromatin in the cells lacking PML (39), indicating a role for Daxx in association with a transcriptionally silenced chromatin structure. Thus, it is likely that the repressive effect of Daxx on sumoylated Smad4 is mediated by the recruitment of HDACs and chromatin silencing factors. Alternatively, the binding of Daxx to sumoylated Lys^{159} of Smad4 may affect the function of the Smad4 activation domain in the linker region (65), which is capable of recruiting SMIF1 and CBP/p300 to enhance Smad4-mediated transactivation (66, 67). The binding of Daxx to sumoylated Lys^{159} in the linker region may exclude the recruitment of these coactivators, leading to transcriptional repression. Further studies will be required to define the repressive effect of Daxx on sumoylated Smad4.

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