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Tumor Suppressor Bromodomain-containing Protein 7 Cooperates with Smads to Promote Transforming Growth Factor-β Responses

Ting Liu[#1,2,*], **Meiling Zhao**[#1], **Jinquan Liu**[1], **Zhou He**[1], **Ye Zhang**[1], **Han You**[3], **Jun Huang**[1], **Xia Lin**[4], and **Xin-Hua Feng**[1,4,*]

[1] Life Sciences Institute and Innovation Center for Cell Signaling Network, Zhejiang University, Hangzhou, Zhejiang 310058, China

[2] Department of Cell Biology and Program in Molecular Cell Biology, Zhejiang University School of Medicine, Hangzhou, Zhejiang 310058, China

[3] State Key Laboratory of Cellular Stress Biology and Innovation Center for Cell Signaling Network, College of Life Sciences, Xiamen, Fujian 361102, China

[4] Michael E. DeBakey Department of Surgery and Department of Molecular & Cellular Biology, Baylor College of Medicine, Houston, TX 77030, USA.

[#] These authors contributed equally to this work.

Abstract

Smad proteins are central mediators in the canonical transforming growth factor-β (TGF-β) signaling pathway in mammalian cells. We report here that bromodomain-containing protein 7 (BRD7) functions as a novel transcription coactivator for Smads in TGF-β signaling. BRD7 forms a TGF-β inducible complex with Smad3/4 through its N-terminal Smad-binding domain. BRD7 simultaneously binds to acetylated histones to promote Smad-chromatin association, and associates with histone acetyltransferase p300 to enhance Smad transcriptional activity. Ectopic expression of BRD7, but not its mutants defective in Smad binding, enhances TGF-β transcriptional, tumor suppressing and epithelial-mesenchymal transition (EMT) responses. Conversely, depletion of BRD7 inhibits TGF-β responses. Thus, our study provides compelling evidence for a new function of BRD7 in fine-tuning TGF-β physiological responses.

Keywords

BRD7; Transcription coactivator; Transforming growth factor beta; Smad

[*]Corresponding authors: liuting518@zju.edu.cn; xfeng@bcm.edu.

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INTRODUCTION

Transforming growth factor-β (TGF-β) is a ubiquitously expressed cytokine that controls a plethora of cellular responses from cell proliferation, differentiation, and apoptosis to embryonic development in metazoan (1-3). Numerous studies have established a model for the transduction of signals from TGF-β receptors to the nucleus in mammalian cells. In the canonical TGF-β signaling pathway, Smad proteins are the central mediators and are classified into three groups, including receptor associated Smads (R-Smads), common Smad (Smad4) and inhibitory Smads (I-Smads) (4). Upon TGF-β stimulation, heteromeric receptors phosphorylate R-Smads on their SXS motifs (5). Subsequently, R-Smads form a heter-oligomeric complex with Smad4 and then translocate into the nucleus, where they cooperate with transcriptional co-activators and co-repressors to regulate responses of target genes (6,7).

Smad proteins share two highly conserved domains at their N and C termini, known as the Mad homology 1 (MH1) and Mad homology 2 (MH2) domains, respectively. The MH1 domain is responsible for the binding with Smad-binding element (SBE), whereas the MH2 domain contributes to the Smad transcriptional activity (8,9). In fact, Smad transcriptional complexes target specific genes by recruitment of transcription co-factors to the target promoters (9). The most well-characterized Smad co-activator is the general co-transcriptional factor CBP/p300, which facilitates transcription by decreasing chromosome condensation through its intrinsic histone acetyltransferase (HAT) activity and by increasing the accessibility of transcription factors to the basal transcription machinery (10). By directly binding with Smads, CBP/p300 positively regulates Smad-mediated transcriptional activation in the TGF-β signaling pathway (11). Increasing numbers of transcription (co)factors, including P/CAF, SIP1, TGIF, and SMIF, have been identified, which further help to understand the context-dependent regulation of TGF-β transcriptional responses (8,12-15).

BRD7 is an evolutionarily conserved protein that contains a single bromodomain. Previous studies showed that BRD7 exerts multiple cellular functions through regulation of cell cycle progression. BRD7 affects PI3K signaling, mediates chromatin remodeling and regulates p53-dependent replicative senescence (16-20). As a component of chromatin remodeling SWI/SNF complexes, BRD7 function as a transcriptional co-activator or co-repressor. Indeed, BRD7 serves as a transcription co-activator for tumor suppressors BRCA1 and p53 to regulate target gene transcription (18-20). Together with the previous report that a low level of BRD7 protein is found in various tumor types (21-24), BRD7 could be an important tumor suppressor that deserves more attention.

Here we demonstrate a novel function of BRD7 in TGF-β signaling. BRD7 interacts with the Smad tumor suppressor complex, enhances both DNA binding ability and transcriptional activity of Smads. BRD7 functions in growth inhibition and tumor suppression partly as a transcriptional co-activator of Smads.

RESULTS

BRD7 interacts with Smad3 and Smad4 under physiological conditions

In search for Smad regulatory proteins, we identified BRD7 as a Smad-interacting protein in protein interaction screens (unpublished observations). To validate this interaction with physiological significance, we examined the ability of BRD7 protein to bind to Smads in mammalian cells. First, HEK293T cells were co-transfected with HA-tagged BRD7 and FLAG-tagged Smads (Smad1 to 4), and co-immunoprecipitation (co-IP) of cell lysates was carried out and followed by immunoblotting. Results indicate that BRD7 strongly interacted with Smad1 and Smad3, and weakly with Smad2 and Smad4 in HEK293T cells (Fig. 1A). Similar results were obtained by using *in vitro* protein binding assays (Fig 1B). We were further interested in the Smad-BRD7 interaction under physiological conditions by performing co-IP experiments with both proteins at endogenous levels. In the absence of good BRD7 antibodies, we first established A549 cells stably expressing HA-BRD7. Co-IP analysis revealed that TGF-β induced the association between BRD7 and Smad3 (Fig 1C, lane 4). Consistent with the fact that TGF-β-induces the R-Smads-Smad4 protein complex formation, BRD7 also interacted with Smad4 at the endogenous levels, and this interaction was further enhanced by TGF-β treatment (Fig 1D, lane 3). Furthermore, we carried out sequential co-IP to explore if BRD7 forms a ternary complex with both Smad3 and Smad4. The result showed that BRD7 could associate with activated Smad protein complex upon TGF-β treatment (Fig 1E), implicating a potential role of BRD7 in the TGF-β signaling pathway. Interestingly, Smad3 depletion has no obviously effect on the interaction between BRD7 and Smad4 (Fig 1F, lane3), indicating that the interactions between BRD7 and Smad3 or Smad4 are independent.

The N terminus of BRD7 contains a Smad-binding domain

Next, we generated a series of deletion mutants to map the regions within Smad3/4 and BRD7 that mediate their interactions. We first determined the domains of Smads for BRD7 interaction. Smad proteins contain the highly conserved MH1 and MH2 domains, linked with the less conserved linker region (Fig 1G). Results of co-IP analysis demonstrate that the MH1 and MH2 domains of Smad3 sufficed to confer BRD7 binding as deletion of the linker region had no effects on BRD7 binding (Fig 1H). In accordance, Smad3 with deletion of either the MH1 domain or the MH2 domain failed to interact with BRD7 (Fig 1H). Similar results were obtained for Smad4 binding to BRD7 (Supplemental Fig. 1A and 1B).

BRD7 is an under-characterized protein with 651 amino acids. It has a conserved Bromodomain (aa 135-232) and uncharacterized yet conserved domain (aa 286-534) (Fig 1I). Deletion analysis of individual regions of BRD7 demonstrates that the very N terminus before bromodomain on BRD7 (BRD7N, aa 1-129) is required for BRD7 binding to Smad3 (Fig 1I and 1J) and Smad4 (Supplemental Fig 1C and 1D). Moreover, BRD7N alone sufficed to mediate BRD7 interaction with both Smad3 (Fig 1I and K) and Smad4 (Supplemental Fig 1E). GST pull-down assays confirmed the direct interaction of BRD7N with Smad3 (Fig 1L) or Smad4 (Supplemental Fig 1F).

BRD7 potentiates TGF-β-induced transcriptional responses

In order to reveal the function of BRD7 in TGF-β signaling pathway, we determined the effects of BRD7 on TGF-β-induced Smad-dependent transcriptional responses. First, we evaluated the effect of overexpressed BRD7 on TGF-β responses by using Smad-binding element (SBE)-luc, a synthetic TGF-β-responsive reporter gene dependent on Smad activation, in both HaCaT and A549 cells. Overexpressed BRD7 caused a significant increase in TGF-β-induced transcriptional activation from SBE-luc reporter in both HaCaT (Fig 2A) and A549 cells (Supplemental Fig 2A). Since TGF-β up-regulates transcription of the extracellular matrix component plasminogen activator inhibitor-1 (PAI-1), we also examined the effect of BRD7 on PAI-1-luc reporter expression. Similarly to SBE-luc expression, PAI-luc expression was profoundly increased by BRD7 (Fig 2B; Supplemental Fig 2B).

We further investigated whether BRD7 enhances TGF-β-induced expression of endogenous genes. As reported, TGF-β inhibits epithelial cell growth partially via induction of the cell cycle inhibitor p21 (WAF/Cip1) (25). Consistent with the increased TGF-β-induced reporter transcriptional activation (Fig 2A and 2B), increased expression of BRD7 significantly enhanced the TGF-β-induced expression of endogenous p21, as demonstrated by qRT-PCR (Fig 2C and 2D).

We then took a loss-of-expression approach to address the effect of BRD7 on TGF-β transcriptional responses. An approximately 90% knockdown efficiencies of BRD7 could be achieved at both mRNA and protein levels (Fig 3E). When endogenous BRD7 was knocked down in A549 cells, we noted a dramatic reduction of TGF-β-induced transcriptional activation of the SBE-luc and PAI-1-luc reporter genes (Supplemental Fig 2C and 2D). Importantly, depletion of BRD7 decreased the TGF-β-induced expression of p21 mRNA in both HaCaT and A549 cells (Fig 2F and 2G). In accordance, knockdown of BRD7 reduced the levels of p21 proteins in response to TGF-β (Fig 2H and 2I).

BRD7 bridges the Smad-chromatin interaction via its bromodomain

To elucidate the mechanism of how BRD7 enhances TGF-β-induced cell signaling, we examined the possibility that BRD7 is directly involved in TGF-β-induced transcriptional regulation. Smads, as transcription factors, possess both DNA-binding and transactivation properties. Since both Smad3 and Smad4 can directly bind to DNA, we first tested whether BRD7 could promote Smad3/Smad4 DNA-binding ability by using SBE oligo pull-down assay. The DNA pull-down assay revealed that BRD7 dramatically increased binding of Smad3/Smad4 to the SBE oligonucleotide (Fig 3A). To further address the effect of BRD7 on Smad DNA-binding in the chromatin environment, we carried out ChIP assays on the p21 promoter in A549 cells. The result demonstrated that BRD7 increased Smad4 binding to the p21 promoter in the presence of TGF-β (Fig 3B).

BRD7 contains a highly conserved bromodomain which is present in many chromatin associated transcription factors and some nuclear histone acetyltransferases (HATs) (16). Considering the facts that BRD7 interacts with acetylated histone H3 through its bromodomain to exhibit transcriptional regulation activity (26), we examined whether BRD7

stabilizes the Smad4-DNA complex through its association with acetylated histone on chromatin. Notably, BRD7 bound to acH3K9 more strongly upon TGF-β treatment (Fig 3C), and it significantly enhanced Smad4-acH3K9 interaction (Fig 3D, lane 4) and Smad3-acH3K9 association (Fig 3E). In sharp contrast, BRD7Δ2, the BRD7 mutant lacking the bromodomain failed to exhibit such enhancing effect (Fig 3D, lane 6). Active TGF-β type I receptor could have some stimulatory effects on the Smad4-acH3K9 or Smad3-acH3K9 association (Fig 3D, 3E). Moreover, knockdown of endogenous BRD7 significantly reduces TGF-β-induced Smad4-acH3K9 binding (Fig 3F). Taken all the results together, BRD7 promotes the Smad3/4-chromatin association via its bromodomain.

BRD7 cooperates with p300 to enhance Smad4 transcriptional activity

The MH2 domain of Smads displays transcriptional activity when tethered to DNA via a heterogonous GAL4 DNA-binding domain (27), partly through the function of coactivators CBP/p300 (11). To examine if BRD7 enhances Smad4 transcriptional activity, we performed a heterologous reporter gene assay using the GAL4 fusion system. Results demonstrate that BRD7 enhanced the transcription activity of Smad4C (Smad4MH2) (Fig 4A). Since CBP/p300 binds to Smads (11) and BRD7 (19), we reason that BRD7 and CBP/p300 cooperate to mediate Smad-dependent transcription. There was indeed an interation between BRD7 and CBP/p300, and furthermore, TGF-β significantly increased the BRD7-p300 association (Fig 4B). We further mapped the interaction domain in BRD7 that is responsible for p300 binding, and located it in the very C terminus of BRD7, designated BRD7C (Fig 4C). As a histone acetyltransferase, CBP/p300 also interacts with acH3K9 (Fig 4D, lane 2). Interestingly, the p300-acH3K9 association was enhanced by constitutively active type I TGF-β receptor ALK5T202D (Fig 4D, lane 3), and this interaction is abolished when BRD7 was depleted (Fig 4D, lane 4 and 5). Taken together, we believe that BRD7 could facilitate Smad4 transcriptional activity by cooperating with p300. These results indicate that BRD7 is a physiological co-activator in TGF-β signaling through its interactions with both Smads and CBP/p300.

After having determined the roles of various domains in BRD7, we further analyzed whether deletions of identified domains could affect the function of BRD7 in TGF-β transcriptional responses. As shown in Fig 4E, deletions of any functional domains weakened its ability to potentiate TGF-β-induced SBE and PAI-1 reporter gene activation. We also examined the functions of individual domains by rescue experiments. We introduced back deletion mutants into otherwise BRD7 depleted A549 cells, and found that BRDΔ1, BRDΔ2 and BRDΔ5 all failed to fully rescue TGF-β-induced responses (Fig 4F). These results support the notion that the Smad-binding, p300-binding and Bromo domains are critical for BRD7-dependent co-activation in TGF-β signaling.

BRD7 enhances TGF-β-induced cytostatic and tumor suppressive responses

As a multifunctional cytokine involved in many important cellular processes, TGF-β controls numerous cellular processes, including cell proliferation, apoptosis and epithelial-mesenchymal transition (EMT) (28). Since we already established the role of BRD7 in Smad-DNA binding and Smad-mediated transcriptional activity, we next sought to examine whether BRD7 could control the strength of TGF-β physiological responses. For this

purpose, we first performed BrdU staining in HaCaT cells to dissect the role of BRD7 in TGF-β-induced inhibition on cell proliferation. As shown in Fig 5A, BRD7 knockdown attenuated the phenotype of TGF-β-induced anti-proliferation. Consistently, TGF-β-induced G1-S cell cycle arrest was also inhibited in BRD7 knockdown cells, compared with control cells (Fig 5B). This suggests that BRD7 functions in TGF-β-induced Smad-dependent cell growth inhibition.

As a Smad co-activator in the TGF-β antiproliferative response, it is reasonable to speculate that BRD7 could inhibit tumorigenesis *in vivo*. To test this, we evaluate the ability of BRD7 to suppress tumor formation. Because BRD7 was reported to function in oncogenic-induced p53-dependent senescence (19), we attempted to exclude possible involvement of p53 in TGF-β tumor suppressive actions. We stably expressed BRD7 wild-type (WT) and deletion mutants (Δ1, Δ2, Δ5) in H1299 cells. H1299 cells are human non-small cell lung carcinoma with partial deletion of the TP53 gene, resulting in the lack of p53 expression. As shown in Fig 5C-E, when H1299 tumor cells were injected into nude mice, BRD7 wild-type displayed an apparently suppressive effect on tumor formation, as H1299-BRD7WT cells produced fewer and smaller tumors than H1299-GFP control cells. Meanwhile, three mutants that lack the Smad-binding domain (BRD7Δ1), bromodomain (BRD7Δ2) and p300-binding domain (BRD7Δ5) exhibited weak or no tumor suppressive functions in comparison to wild-type BRD7. Moreover, we found that the p21 protein level is dramatically increased in tumors expressing BRD7, but not BRD7Δ1 and BRD7Δ2 mutants (Fig 5F). These results indicate that BRD7 enhances TGF-β signaling in the tumors dependent of its binding to Smad, acetyl group and p300.

BRD7 potentiates TGF-β-mediated EMT responses

TGF-β has a paradoxical effect on tumorigenesis. Whilst it inhibits cell proliferation and tumor formation, TGF-β is a potent inducer of EMT, cell motility and cell invasiveness. Thus, we also tested if BRD7 is involved in TGF-β-induced EMT. To this end, we used immortalized mammary epithelial cell line MCF10A, which undergoes EMT in response to TGF-β (29). To examine the effect of BRD7 on EMT, we established MCF10A cells that stably express HA-BRD7. Interestingly, expression of BRD7 enabled MCF10A cells to produce slightly loose cell-cell adhesion toward an EMT-like morphological change in comparison with MCF10A-GFP cells (Fig 6A). TGF-β stimulation further profoundly promoted the EMT, while treatment with TGF-β type I receptor kinase inhibitor SB431542 reverted the cells to epithelial phenotype (Fig 6A). The morphological transition was further confirmed by examination of specific protein markers characteristic of epithelial and mesenchymal cells, e.g. E-cadherin, Vimentin and Actin. First, the immunofluorescence experiment found that MCF10A-BRD7 cells exhibited decreased staining of E-cadherin at cell adhesion and increased staining of Vimentin in cells. These cells responded to TGF-β more potently to induce the corresponding changes (Fig 6B). Second, Western blotting and qRT-PCR analyses demonstrated that the decrease in protein and mRNA levels of E-cadherin, was more obviously observed in MCF10A-BRD7 cells than control cells (Fig 6C and 6D). Likewise, more profound increases in Vimentin protein and Fibronectin mRNA were found in MCF10A-BRD7 (Fig 6D and 6E). Lastly, as a result of EMT, expression of BRD7 conferred MCF10A cells much higher motility than control MCF10A-GFP cells in

wound healing assays, which could also be blocked by SB431542 (Fig 6F). These results suggest that BRD7 enabled or sensitized cells to undergo EMT.

DISSCUSSION

Emergent evidence suggests that BRD7 functions in regulating cell proliferation, oncogene-induced senescence and other cellular processes (30-32). BRD7 acts as a tumor suppressor in a number of cancer types (17-19,24,30-33). Consistently, its promoter was hypermethylated in tumors (24). BRD7 has been reported to inhibit cell proliferation and promote cell cycle arrest through various mechanisms (19,31). BRD7 can act through the p53 and the PI3K pathways (20,26,34), yet there are conflicting results about the absolute role of these pathways in mediating physiological functions of BRD7. Through the interaction with p53, BRD7 is recruited to the promoters of p53 target genes to mediate p53-dependent oncogenic-induced senescence. On the contrary, BRD7 can confer tumor suppression in tumors lacking p53 (19), suggesting that p53 is not essential in mediating BRD7 tumor suppressing functions. Moreover, BRD7 binds to p85α subunit of PI3K and sequesters p85 from the catalytic subunit p110, thereby resulting in attenuation of the growth-promoting PI3K signaling (17). Opposite data have found that BRD7 is able to induce cell cycle inhibitor p21 in cells with p85α and/or p85β deficiency, indicating that PI3K is dispensable for the growth inhibitory function of BRD7 (17). These conflicting data suggest that there are alternative mechanisms underlying the growth inhibitory actions of BRD7. Our current results provide convincing evidence that BRD7 achieve its functions perhaps partly through the TGF-β growth inhibitory pathway. Indeed, BRD7 profoundly promotes TGF-β-induced cell growth inhibition and inhibits tumorigenesis even in the p53 null background. Knockdown of BRD7 dampens the ability of TGF-β in inducing growth inhibition and G1 arrest. Thus, our study assigns a new function to BRD7 in cell growth regulation through the TGF-β pathway.

Our results have found that BRD7 has no effects on receptor-mediated phosphorylation and activation processes (unpublished observations). Notably, BRD7 interacts with the Smad3/4 complex in a TGF-β-dependent manner. Furthermore, BRD7 mediates a stronger Smad4-DNA complex formation and induces Smad transactivation activity through its binding with p300 upon TGF-β stimulation. Functional analysis demonstrates that BRD7 strongly potentiates TGF-β-induced transcriptional responses in various cell lines. Our studies highlight BRD7 as a transcriptional co-activator of Smads in TGF-β-induced signaling (Fig 6G). Interestingly, a direct interaction between BRD7 and Smad1, a BMP-specific Smad, has also been observed in our experiments. Similarly, BRD7 can enhance BMP2-induced transcriptional response (Supplemental Fig 3), which indicates that BRD7 may also be involved in BMP-regulated cell functions.

BRD7 interacts with Smad3/4 through its N terminus, which also binds to p53 (19). Our attempts to further distinguish the Smad-binding and p53-binding have failed, as any destruction on the BRD7N domain results in the failure in Smad4 binding, indicating that the structural integrity of BRD7N is critical for Smad4 binding (unpublished observations). In addition to the Smad binding, BRD7 binds to acetyl-histones. As previously reported, BRD7 is a component of SWI/SNF complexes to regulate transcription (26,35). Notably,

BRD7 enhances the DNA-binding activity of Smad proteins in both *in vitro* and *in vivo* assays (Fig 3A and 3B). Considering the fact that the bromodomain of BRD7 directly associates with acetylated histone (26), we hypothesize that BRD7 may stabilize the Smad-DNA complex through its interaction with acetylated histone. Indeed, TGF-β enhances BRD7-acetyl-histone interaction. Furthermore, the association between Smad3/4 and acetyl-histone is dependent on the presence of BRD7 (Fig 3D and 3E), but not bromodomain-defective mutant BRD7Δ2 (Fig 3D). In consistence, BRD7 depletion dramatically decreases TGF-β-induced Smad4 association with acetyl-histone. Taken together, BRD7 promotes Smad-chromatin association through its bromodomain.

In the Smad nucleoprotein complex, BRD7 has a dual role in augmenting transcriptional activity. In addition to its bridging role to connect Smad with acetyl histones, it further promotes engagement of p300/CBP, another transcriptional co-activator. It has previously been reported that CBP/p300 cooperates with the Smads complex to facilitate the transcriptional regulation function of Smad proteins (11,19). We found that TGF-β enhances the association between p300 and BRD7 as well as that between p300 and acetyl-histone, while this enhancement is absent when BRD7 is depleted in cells. Thus, TGF-β induces the formation of a more stable nucleoprotein complex consisting of Smad4, BRD7, p300/CBP and chromatin (DNA and acetyl-histone).

In summary, our study has elucidated an important function of BRD7 in the TGF-β signaling pathway. As the TGF-β/BMP signaling pathways also play important roles in tissue differentiation and embryogenesis, BRD7 may be a pivotal regulator in development. Genetic ablation of the *Brd7* gene has resulted in impaired cognitive behavior in mice (36). Given the involvement of BRD7 in multiple pathways, BRD7 may converge these signaling pathways to function in cell growth control, development and tumorigenesis. Whether the *Brd7* knockout phenotype is attributed to the alterations in TGF-β/BMP signaling in development awaits further investigation.

MATERIALS AND METHODS

Plasmids and Antibodies

Expression plasmids for FLAG-tagged Smads and mutants, FLAG-ALK5T202D (a constitutively active mutant of rat TGF-β type I receptor with the Thr-to-Asp substitution at the amino acid residue 202), HA-p300, and reporter plasmids SBE-Luc and, PAI-1-Luc were described previously (37,38). HA-BRD7 was subcloned into pRK3HA vector. BRD7 deletion mutants and HA-ALK5T204D (a constitutively active mutant of human TGF-β type I receptor with the Thr-to-Asp substitution at the amino acid residue 204; an equivalent of rat ALK5T202D) were generated by PCR-directed site mutagenesis. Antibodies were obtained as follows: anti-FLAG (F3165), mouse IgG (I5381) and anti-β-actin (A5441) from Sigma; anti-Myc (sc-40), and anti-Smad4 (sc-7966) antibodies from Santa Cruz; anti-HA (3724), anti-p21 (2947), anti-acetylated-H3K9 (9649s), anti-Vimentin (5741), anti-E-cadherin (3195s) from Cell Signaling Technology; peroxidase-conjugated goat anti-rabbit (111-035-045) and rabbit anti-mouse (315-035-048) from Jackson ImmunoResearch.

Cell culture and transfection

HEK293T, A549 and H1299 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), HaCaT cells were maintained in MEM supplemented with 10% FBS. HaCaT and A549 cells were transfected with X-tremeGENE (Roche) and 293T cells with PEI (Polyscience). A549-BRD7 stable cells, HaCaT-BRD7 stable cells, MCF10A-BRD7 stable cells and H1299-BRD7 stable cells were obtained by using lentiviral infection. Briefly, full-length or mutated BRD7 cDNA was subcloned into pWPI-puro vector and transfected into 293T cells together with packaging plasmid psPAX2 and envelope plasmid pMD2.G. Media containing viruses were collected after 48 h and then used to infect host cells. Stable cells were then selected with 2 μg/ml puromycin (Sigma).

Transcriptional reporter assay

HaCaT cells were transfected with reporter plasmids and expression plasmids for BRD7 and/or Smads as indicated in the figures. 24 h after transfection, cells were treated with TGF-β (2 ng/mL) for 12 h. Cells were then harvested and analyzed with the Dual Luciferase Reporter Assay system (Promega) (39). All assays were repeated for three times. The values are the average of triplicates ± SD.

Immunoprecipitation and Western blotting analysis

Cells were transfected with expression plasmids for BRD7 and/or Smads as indicated in the figures. 24 h after transfection, cell lysates were harvested by NEDT lysis buffer (150 mM NaCl, 150 mM Tris-HCl (pH7.5), 2 mM EDTA, 0.5% NP40) and incubated together with protein A Sepharose CL-4B (GE Healthcare) and appropriate antibodies for 4 h at 4°C. After extensive washes, immunoprecipitated proteins were eluted in SDS sample loading buffer, separated by SDS-PAGE, transferred onto PVDF membranes (Millipore), and detected by Western blotting analysis.

GST pull-down assay

GST fusion proteins were expressed in *E. coli* BL21 (DE3) strain and purified by glutathione-Sepharose 4B beads (GE Healthcare) according to manufacturer's instructions. *In vitro* transcription/translation was performed with the TNT@ quick system (Promega). GST pull-down assays were carried out as described previously (38).

DNA pull-down assay

Cells transfected with expression plasmids were lysated in DNAP-lysis buffer (50 mM NaCl, 25 mM Tris-HCl (pH7.4), 0.5% Triton X-100) and then were incubated with 1 μmol biotinylated SBE oligonucleotides (5'- GTACATTGTCAGTCTAGACATACT-3') in DNAP binding buffer (50 mM KCl, 10 mM Tris-HCl [pH7.4], 5% glycerol) at room temperature for 30 min. DNA-protein complexes were collected by incubating with streptavidin beads (GE Healthcare) for 15 min, and identified by Western blotting after extensive washes.

Real-time RT-PCR (qRT-PCR)

Total RNAs were prepared by TriZol reagent (Invitrogen) from A549 or HaCaT cells. RNAs were reverse-transcribed to complementary DNAs using PrimeScript ® RT reagent Kit

(TaKaRa). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed by the SYBR green method (ABI). All targets were measured in triplicates. Primers used for each gene are listed as follows:

Human p21 - Forward: 5'-ACCATGTGGACCTGTCACTGT-3'

Human p21 - Reverse: 5'-TTAGGGCTTCCTCTTGGAGAA-3'

Human β-actin - Forward: 5'-CAAAGTTCACAATGTGGCCGAGGA-3'

Human β-actin - Reverse: 5'-GGGACTTCCTGTAACAACGCATCT-3'

Human BRD7 - Forward: 5'-GCTGTTGCACTCAGGAATGA-3'

Human BRD7 - Reverse: 5'-ACTCTTGAAGGCGTGTGCTT-3'

Human Fibronectin - Forward: 5'-TCCACCAACAAACTTGCATCTGGAG-3'

Human Fibronectin - Reverse: 5'-CTCAGGTTATCAAAAGTGCAGG-3'

Human E-cadherin - Forward: 5'-CGGGAATGCAGTTGAGGATC-3'

Human E-cadherin - Reverse: 5'-AGGATGGTGTAAGCGATGGC-3'

RNA interference

Small interfering RNAs (siRNAs) targeting human BRD7 were purchased from Dharmacon, (si#1 target sequence: GUGCCAAGAUUAUCCGUAU; si#2 target sequence: GUACUAAUGCCAUGAUUUA). siRNA were transfected into A549 or HaCaT cells for 48 h using Lipofectamine ® RNAiMAX Reagent (Invitrogen).

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed using a kit from Upstate Biotechnology and following the manufacturer's instructions. The immunoprecipitated nucleoprotein complexes were eluted by incubation twice for 15 min at 25°C with 200 μl of elution buffer (1% SDS, 100 mM $NaHCO_3$), and crosslinks were reversed at 65°C for 4 h. DNAs were extracted with phenol/chloroform and precipitated with ethanol. The p21 promoter DNA was amplified in a PCR by using forward primer (5'-TGCTGGAACTCGGCCAGGCT-3') and reverse primer (5'-AGCGCGGCCCTGATATACAAC-3').

Cell proliferation assay

Cell proliferation was measured by using bromodeoxyuridine (BrdU) ELISA kit (Roche) according to the manufacturer's instructions. Cells were seeded in 96-well culture plates

(4000 cells per well) and treated for 4 h with BrdU labeling solution. After fixation and denaturation, cells were detected by anti-BrdU antibody and results were read in a luminometer.

FACS analysis

Cell cycle was measured by using cell cycle staining kit (MultiSciences Biotech) according to the manufacturer's instructions and then analyzed immediately by flow cytometry using FACScan (Becton Dickinson).

In vivo tumor formation assay

A total of 1×10^6 H1299 cells stably expressing GFP (control) or wild-type or mutated BRD7 were suspended in 100 μl RPMI 1640 cell culture media and then injected subcutaneously into the hind legs of 6-week old nude mice. Four groups of mice (n=4) were tested. Mice were sacrificed at 45 days post-injection and tumors were excised and measured.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

acH3K9	acetyl histone H3 Lys9
BMP	Bone Morphogenetic Protein
BRD7	bromodomain-containing protein 7
EMT	epithelial-mesenchymal transition
GST	glutathione S-transferase
HAT	histone acetyltransferase
P/CAF	p300/CBP-associated factor
qRT-PCR	quantitative real-time PCR
SIP1	Smad-interacting protein 1
TGF-β	Transforming Growth Factor beta
TGIF	TG-interacting factor
SMIF	Smad4-interacting protein

SXS motif Ser-X-Ser motif

WT wild-type

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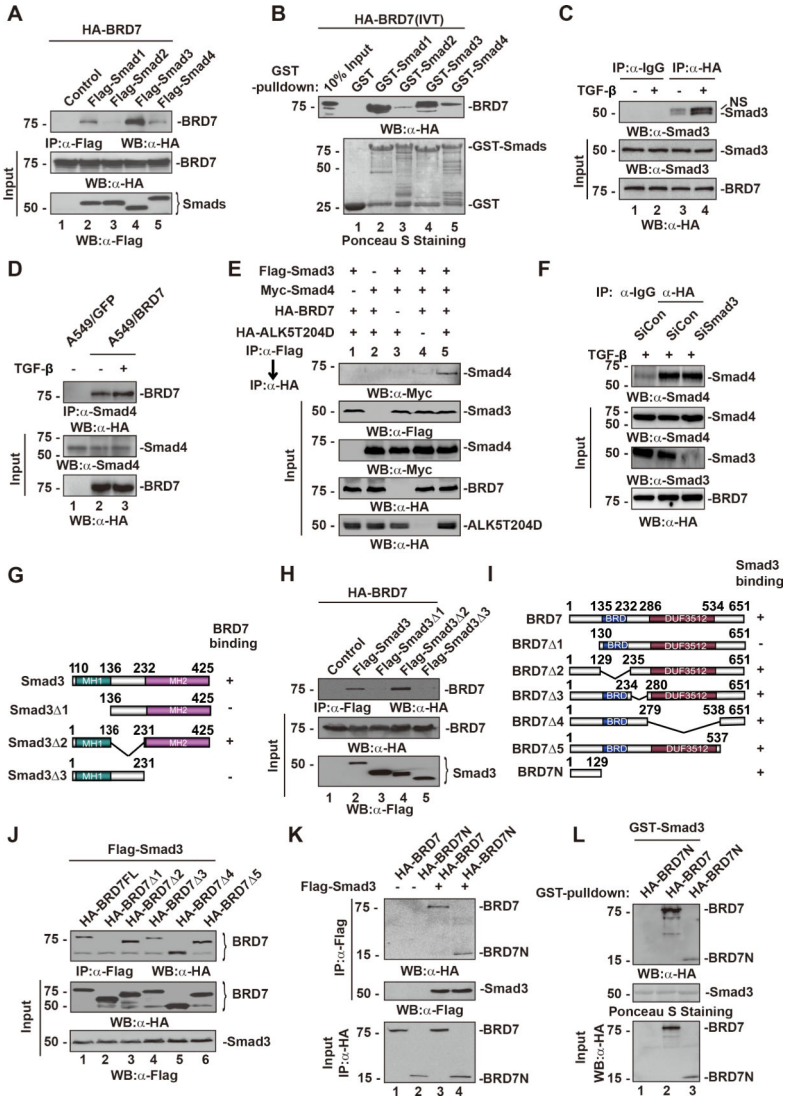

FIGURE 1. BRD7 interacts with Smad3 and Smad4 under physiological conditions

A. BRD7 interacts with Smad proteins *in vivo*. HA-BRD7 and Flag-Smad1/2/3/4 were co-transfected into HEK293T cells. Cells lysates were harvested after 24 h and then immuoprecipitated (IP) with anti-FLAG antibody (α-FLAG). After extensive wash, the immunoprecipitates were then separated on SDS-PAGE and analyzed by Western blotting (WB) using appropriate antibodies.

B. BRD7 interacts with Smad proteins *in vitro*. GST-Smad1/2/3/4 proteins were purified from *E. coli* strain BL21 (DE3). HA-BRD7 proteins, generated by using *in vitro* transcription and translation kit, were incubated with GST-Smads or GST only for 3 h at 4°C, and then detected by Western blotting with anti-HA (α-HA) antibody. Inputs of GST fusion proteins were stained by Ponceau S dye on membrane.

C. BRD7 binds to Smad3 under physiological conditions. A549 cells stably expressing BRD7 were harvested and immunoprecipitated with anti-Smad3 antibody (α-Smad3) after 2 h TGF-β treatment. Anti-IgG antibody (α-IgG) was used as a control. Immunoprecipitated proteins and inputs were analyzed as described in Fig. 1A.

E. BRD7 forms a ternary complex with Smad3 and Smad4. Expression plasmids for FLAG-Smad3, Myc-Smad4, HA-BRD7 and/or HA-ALK5204D were co-transfected into HEK293T cell. Cell lysates were harvested after 24h and then IP with anti-FLAG (α-FLAG) antibody. After extensive wash, the binding proteins were eluted by incubation with FLAG peptide, followed by second IP with anti-HA (α-HA) antibody. The final immunoprecipitates were then separated on SDS-PAGE and analyzed by WB using appropriate antibodies.

F. Depletion of Smad3 has little effect on the BRD7-Smad4 association. Control or Smad3-targeting siRNA was transfected into A549-BRD7 stable cells and the cells were treated with TGF-β for 2h. Cell lysates were harvested and IP with anti-HA antibody (α-HA). Anti-IgG (α-IgG) was used as control. The immunoprecipitates were then separated on SDS-PAGE and analyzed by WB using appropriate antibodies.

G. Diagram of Smad3 deletion mutants used in domain mapping experiments. The start and end amino acid residues for each fragment are indicated. “+” marks detectable interaction, whereas “−” marks lack of detectable interaction.

H. Smad3 interacts with BRD7 through its MH1 and MH2 domains. FLAG-Smad3 mutants and HA-BRD7 were co-transfected into HEK293T cells. IP-Western blotting was done as described in Fig. 1A.

I. Diagram of BRD7 deletion mutants. The start and end amino acid residues for each fragment are indicated. “+” marks detectable interaction, whereas “−” marks lack of detectable interaction.

J. BRD7N (aa 1-129) is essential for Smad3 binding. HEK293T cells were transfected with HA-BRD7 mutants and FLAG-Smad3 plasmids. IP-Western blotting was done as described in Fig. 1A..

K. BRD7N is sufficient for Smad3 binding *in vivo*. BRD7N and BRD7WT were co-transfected into HEK293T cells with or without Smad3. IP-Western blotting was done as described in Fig. 1A.

L. BRD7N is sufficient for Smad3 binding *in vitro*. GST-Smad3 was purified from *E. coli* strain BL21 (DE3), BRD7 and BRD7N proteins were generated by *in vitro* transcription and translation kit. GST pulldown was done as described in Fig. 1B.

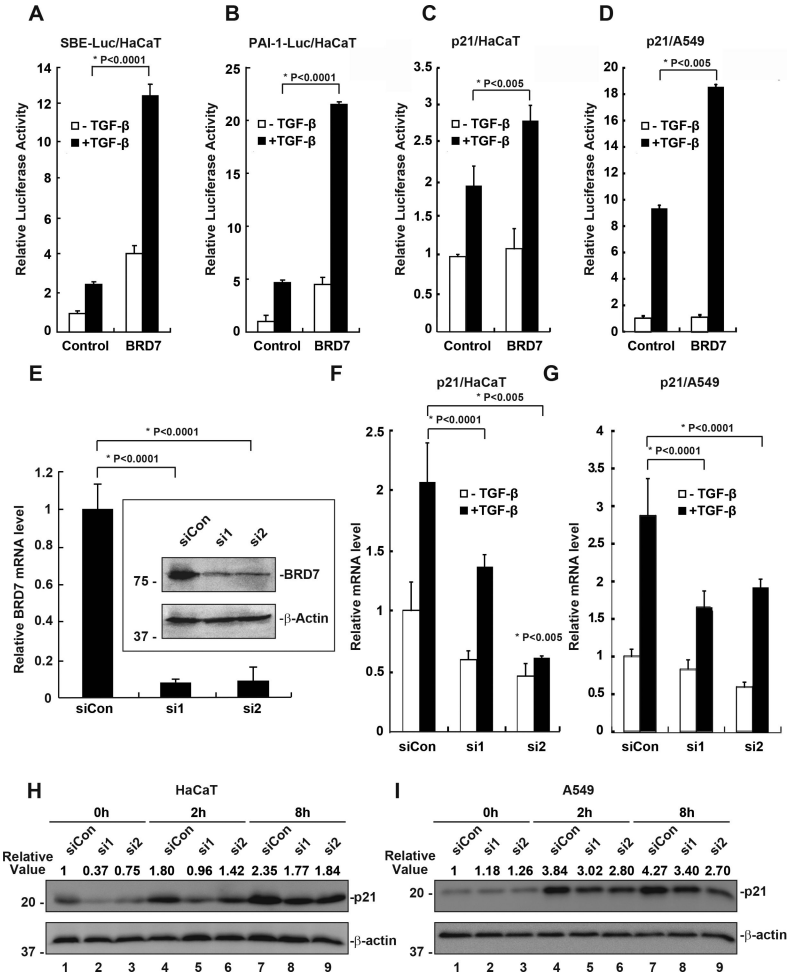

FIGURE 2. BRD7 promotes TGF-β-induced transcriptional responses

A. BRD7 enhances TGF-β-induced SBE-luc reporter gene expression in HaCaT cells. BRD7 and SBE-luc were co-transfected into HaCaT cells. Relative luciferase activities were measured after 8 h TGF-β treatment (2 ng/mL). Values are the means (±s.e.) of three separate experiments performed in triplicates and normalized for transfection efficiency against Renilla luciferase activities.

B. BRD7 enhances TGF-β-induced transactivation of the PAI-1 promoter. HaCaT cell transfection, reporter assays and data analysis were done essentially as described in Fig. 2A.

C. Ectopic BRD7 enhances TGF-β-induced p21 gene transcription. HaCaT cells were transfected with GFP or BRD7 plasmids and stimulated with 2 ng/mL of TGF-β for 8 h. Total mRNAs were then isolated and analyzed by qRT-PCR.

D. BRD7 enhances TGF-β-induced p21 gene transcription in A549 cells.

E. BRD7 expression is effectively reduced by siRNA-mediated knockdown in HaCaT cells. About 90% knockdown of BRD7 mRNA (with β-actin as an internal control) and protein levels was achieved, as shown in qRT-PCR (bar graphs) and Western blotting (insert) analyses.

F. Knockdown of BRD7 inhibits TGF-β-induced p21 mRNA level. Control siRNA or siBRD7 were transfected into HaCaT cells. TGF-β treatment, mRNA isolation and qRT-PCR analysis were done as in Fig. 2C.

G. Knockdown of BRD7 inhibits TGF-β-induced p21 expression in A549 cells.

H. Knockdown of BRD7 decreases TGF-β-induced p21 protein levels. Control siRNA or BRD7 siRNA were transfected into HaCaT cells and then stimulated with 2 ng/mL of TGF-β for 0, 2, and 8 h. Cell lysates were examined by Western blotting with anti-p21 antibody (α-p21) and β-Actin. The value of band intensity of the p21 protein relative to that of β-actin was measured and shown above the panel.

I. Knockdown of BRD7 decreases TGF-β-induced p21 protein levels in A549 cells.

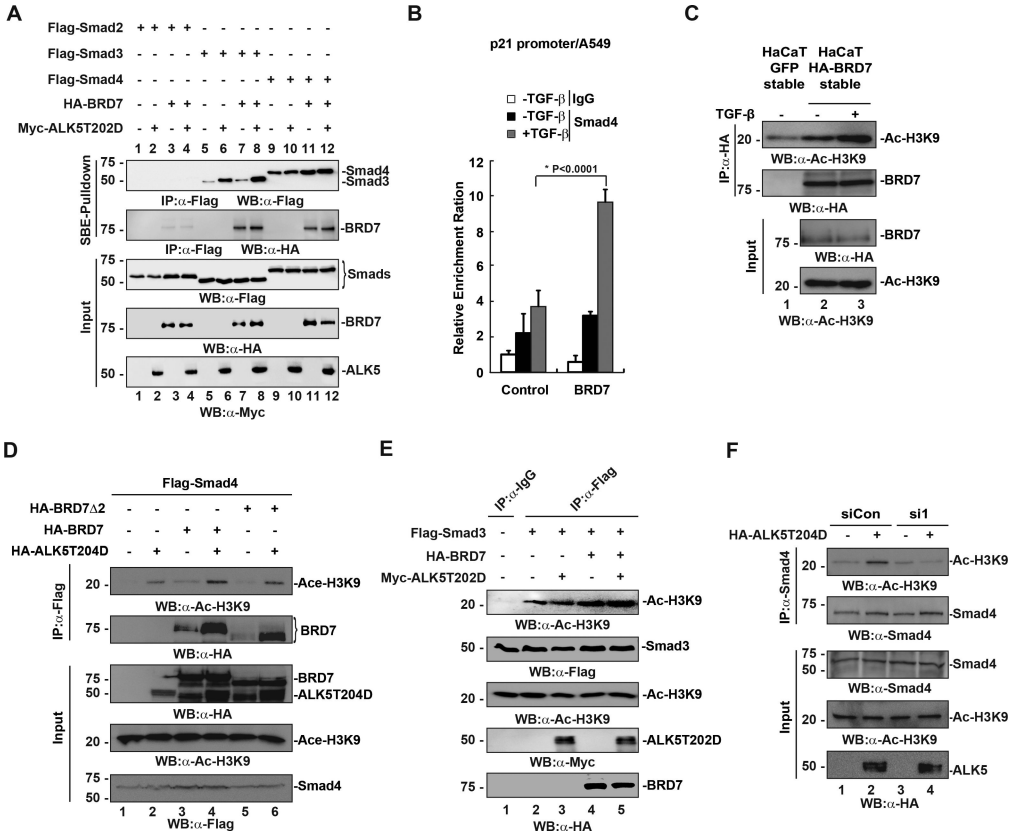

FIGURE 3. BRD7 potentiates Smad-chromatin association via its bromodomain

A. BRD7 increases Smad3/4-DNA binding. HA-BRD7 was transfected into HEK293T cells with FLAG-Smad2, FLAG-Smad3 or FLAG-Smad4. Cell lysates were harvested and incubated with biotinylated SBE oligonuleotides. The DNA-Smad complex was affinity-purified using streptavidin beads and then examined by WB with anti-FLAG antibody (α-FLAG).

B. BRD7 promotes the occupancy of Smad4 on the p21 promoter. A549-BRD7 stable cells or A549-GFP control cells were treated with TGF-β (2 ng/mL) for 2 h. Isolated chromatins were immunoprecipitated with anti-Smad4 (α-Smad4). Smad4-bound DNA was analyzed by amplifying a 106 bp fragment of the p21 promoter in qPCR. Values are the means (±s.e.) of three separate experiments performed in triplicates.

C. TGF-β enhances BRD7-acH3K9 interaction. HaCaT cells stably expressing HA-BRD7 were treated with 2 ng/mL of TGF-β for 2 h. Cell lysates were harvested and immunoprecipitated with anti-IgG (α-IgG) or anti-HA antibody (α-HA). BRD7-bound acetylated histones were detected with anti-acH3K9 antibody (α-acH3K9).

D. BRD7 promotes Smad4-acH3K9 interaction. FLAG-Smad4 and HA-BRD7 or HA-BRD7Δ2 were co-transfected into HEK293T cells. Constitutively active TGF-β type I receptor HA-ALK5T204D was used to activate TGF-β signaling. IP-Western blotting using indicated antibodies was done as described in Fig. 1A.

E. The association between Smad3 and Ac-H3K9 is enhanced by BRD7 and ALK5T202D. FLAG-Smad3 and HA-BRD7 and/or Myc-ALK5T202D were co-transfected into HEK293T cells. Cell lysates were immunoprecipitated with anti-FLAG (α-FLAG). Anti-IgG (α-IgG)

were used as control. IP-Western blotting was done using indicated antibodies as described in Fig. 1A.

F. BRD7 is essential for Smad4-acH3K9 binding. Control siRNA or siRNA against BRD7 were transfected into HEK293T cells for 24 h, HA-ALK5T204D was introduced to activate TGF-β signaling. IP-Western blotting using indicated antibodies was done as described in Fig. 1A.

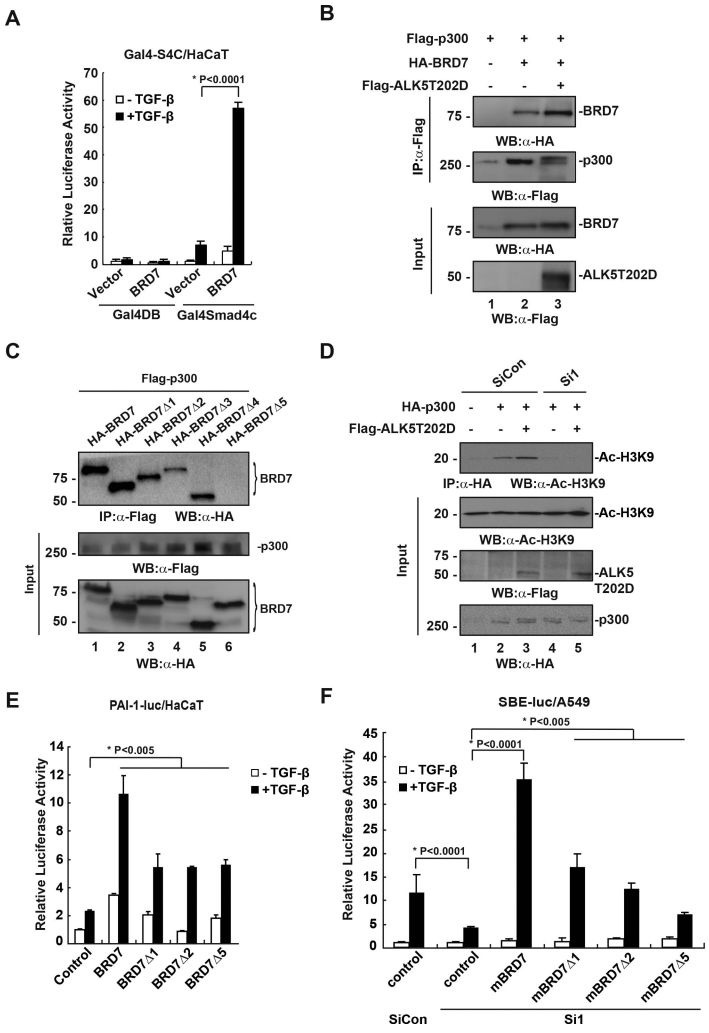

FIGURE 4. BRD7 enhances Smad4 transcriptional activity

A. BRD7 stimulates the transactivation activity of Gal4-Smad4C. HaCaT cells were transfected with reporter plasmid pFR-luc and indicated combinations of Gal4 empty vector (Gal4DB), Gal4-Smad4C, and BRD7. Relative luciferase activities were measured after 8 h TGF-β treatment (2 ng/mL). Values are the means (±s.e.) of three separate experiments performed in triplicates.

B. TGF-β enhances the BRD7-p300 interaction. HEK293T cells were transfected with HA-BRD7 and FLAG-p300. IP-Western blotting was done using indicated antibodies as described in Fig. 1A.

C. BRD7 interacts with p300 through its C terminus. HA tagged BRD7 or its deletion mutants were transfected into HEK293T cells together with FLAG-p300. Domains responsible for p300 binding were examined by IP-Western blotting as described in Fig. 1A.

D. BRD7 is essential for the p300-acH3K9 binding. Control siRNA or siRNA against BRD7, HA-ALK5T204D and HA-p300 were transfected into HEK293T cells for 24 h. IP-Western blotting using indicated antibodies was done as described in Fig. 1A.

E. Bromodomain, Smad-binding and p300-binding domains are required for TGF-β-induced signaling. BRD7, BRD7 mutants and PAI-1-luc were co-transfected into HaCaT cells. Cell transfection, reporter assays and data analysis were done essentially as described in Fig. 2A.
F. Full-length BRD7 rescues TGF-β-induced responses in BRD7-depleted cells. Mouse derived full-length BRD7, BRD7Δ1, BRD7Δ2 or BRD7Δ5 were stably expressed in A549 cells. Control siRNA or siBRD7 were transfected into these cell lines by using RNAiMax. Reporter assays and data analysis were done essentially as described in Fig. 2A.

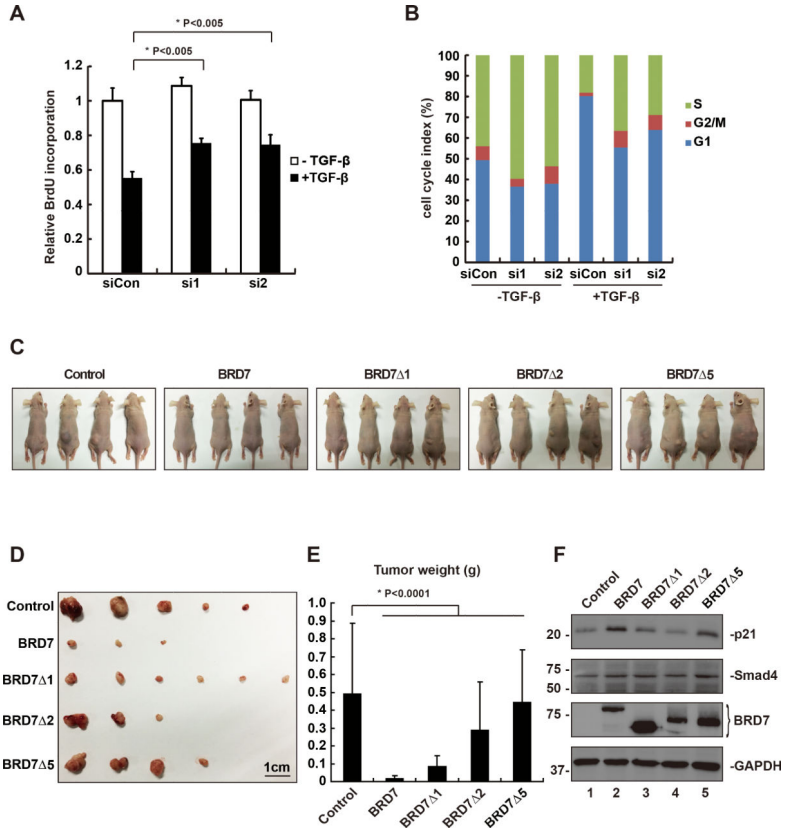

FIGURE 5. BRD7 enhances TGF-β-induced cytostatic and tumor suppressive responses

A. BRD7 knockdown attenuates TGF-β-induced anti-proliferation. HaCaT cells were transfected with control siRNA or siBRD7 and treated with TGF-β (2 ng/mL) for 48 h. Cell proliferation rate was examined by using BrdU staining method.

B. BRD7 depletion attenuates TGF-β-induced G1 arrest. Control siRNA or siBRD7 were transfected into HaCaT cells. After 48 h, cells were treated with TGF-β (2 ng/mL) for 24 h and then analyzed by flow cytometry.

C-E. BRD7 suppresses tumorigenesis in mouse models. H1299 cells that stably express GFP, BRD7 or a BRD7 mutant were subcutaneously injected into nude mice. 45 days after cell implantation, mice were scarified and photographed (C), and tumors were photographed (D) and weighed (E).

F. Expression levels of BRD7 (WT and mutants), Smad4 and p21 protein in tumors were examined by Western blotting analysis.

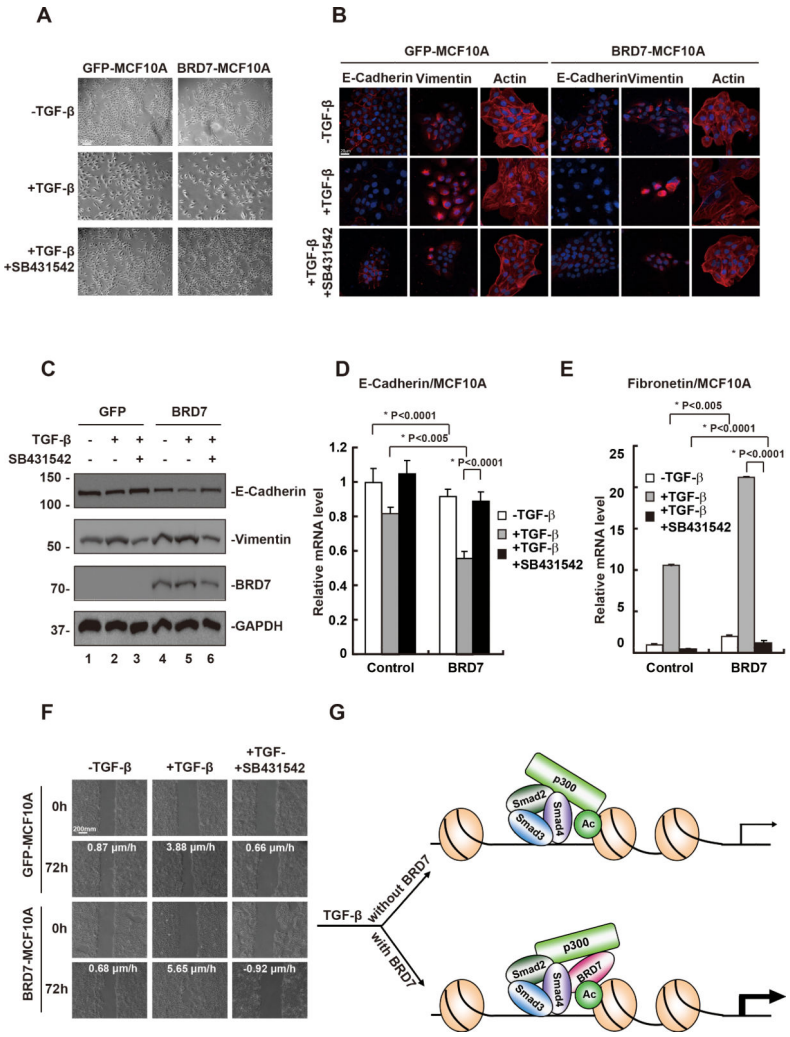

FIGURE 6. BRD7 potentiates TGF-β-mediated EMT responses

A. BRD7 overexpression induces EMT-like phenotype in MCF10A cells. MCF10A cells that stably express BRD7 or GFP were established by using lentiviral infection method. Cells were treated with TGF-β (2 ng/mL) and/or type I receptor inhibitor SB431542 for 48 h. Morphology of cells were recorded in bright fields under microscope.

B. BRD7 enhances TGF-β-induced EMT. Immunofluorescent images for indicated EMT markers (E-Cadherin, Vimentin and Actin) were captured in MCF10A-BRD7 and control GFP cells. 4',6-Diamidino-2-phenylindole (DAPI; Blue) staining was used to detect nuclei in all panels. Representative results from 3 independent experiments are shown.

C. BRD7 enhances TGF-β-induced EMT marker gene responses. Expression of epithelial cell marker (E-cadherin) and mesenchymal cell marker (Vimentin) in MCF10A-BRD7 stable cells or control MCF10A-GFP cells were examined by Western blotting analysis.

D. BRD7 enhances TGF-β-mediated E-Cadherin mRNA down-regulation. E-Cadherin mRNA levels in MCF10A-BRD7 stable cells or control MCF10A-GFP cells were analyzed by using qRT-PCR.

E. BRD7 enhances TGF-β-induced fibronectin mRNA up-regulation. Fibronectin mRNA levels in MCF10A-BRD7 stable cells or control MCF10A-GFP cells were analyzed by using qRT-PCR..

F. BRD7 enhances TGF-β-induced cell migration. Confluent MCF10A-BRD7 or control MCF10A-GFP cells were wounded and photographed at 0 or 72 h after wounding. Numbers in the 72h panels indicate the migration rate.

G. A working model for the function of BRD7 in TGF-β signaling. BRD7 binds to acetylated chromatin upon TGF-β treatment to promote Smad4-DNA binding ability and cooperates with p300 to enhance Smad transcriptional activity.