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SPSB1, a Novel Negative Regulator of the Transforming Growth Factor-β Signaling Pathway Targeting the Type II Receptor*

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Background: TGF-β signaling is tightly controlled by different regulators along its signaling cascade.
Results: SPSB1 interacts and reduces the protein levels of TβRII, which results in decreasing TGF-β signaling.
Conclusion: SPSB1 acts as a new regulatory component of the TGF-β signaling pathway that targets TβRII for degradation and provides fine control of its signaling.
Significance: This is the first specific TβRII negative regulator reported.

Appropriate cellular signaling is essential to control cell proliferation, differentiation, and cell death. Aberrant signaling can have devastating consequences and lead to disease states, including cancer. The transforming growth factor-β (TGF-β) signaling pathway is a prominent signaling pathway that has been tightly regulated in normal cells, whereas its deregulation strongly correlates with the progression of human cancers. The regulation of the TGF-β signaling pathway involves a variety of physiological regulators. Many of these molecules act to alter the activity of Smad proteins. In contrast, the number of molecules known to affect the TGF-β signaling pathway at the receptor level is relatively low, and there are no known direct modulators for the TGF-β type II receptor (TβRII). Here we identify SPSB1 (a Spry domain-containing Socs box protein) as a novel regulator of the TGF-β signaling pathway. SPSB1 negatively regulates the TGF-β signaling pathway through its interaction with both endogenous and overexpressed TβRII (and not TβRI) via its Spry domain. As such, TβRII and SPSB1 co-localize on the cell membrane. SPSB1 maintains TβRII at a low level by enhancing the ubiquitination levels and degradation rates of TβRII through its Socs box. More importantly, silencing SPSB1 by siRNA results in enhanced TGF-β signaling and migration and invasion of tumor cells.

TGF-β regulates a plethora of cellular processes, including cell proliferation, differentiation, migration, organization, and death (1–5). The tight regulation of the TGF-β signaling pathway at every step is critical in homeostasis, because any perturbation of the pathway *in vivo* appears to result in the formation of cancer in mice (6, 7). For instance, deletion of one copy of the Smad4 or TGF-β1 gene resulted in gastric tumor formation (6, 7). Similarly, overexpression of a dominant negative form of TGF-β type II receptor (TβRII)[2] or the negative regulator Smad7 as a transgene in mice also resulted in tumor formation (8, 9), suggesting that complete blocking of the TGF-β signaling is not necessary for tumor formation. More recently, the threshold effect of TGF-β signaling in cancer development has been further demonstrated in $Gp130^{Y757F/+}$; $Smad3^{+/-}$ compound mice (10), with $Gp130^{Y757F/+}$ heterozygous mice desensitized to TGF-β signaling (~30%) via Stat3-mediated Smad7 expression and $Smad3^{+/-}$ mice showing ~30% reduction in TGF-β signaling (10). Interestingly, none of the single heterozygous mice develop tumors, and it is only the compound heterozygous mice, which have further suppressed TGF-β signaling, that develop gastric tumors (10), emphasizing the importance of correct levels of TGF-β signaling. In fact, the fine turning of TGF-β signaling is also critically important in normal development. The TGF-β type III (TβRIII) is an accessory, non-signaling receptor. *In vitro*, it had no effect on the TβRI- and TβRII-mediated signaling activation *per se*. However, when TβRIII was deleted by gene targeting, it resulted in a near birth, embryonic lethality with liver and heart defects (11). Careful study of the mechanism revealed that the TβRIII knockout causes desensitization of TGF-β2 signaling, highlighting the importance of appropriate TGF-β signaling in tissue homeostasis.

Biological responses to TGF-β are mediated mainly by the TβRI and TβRII transmembrane cell surface receptors, which contain cytoplasmic domains with serine/threonine kinase activity (1, 4, 12). TGF-β ligands bind TβRI and TβRII, thereby triggering phosphorylation and activation of TβRI by TβRII. The activated ligand-receptor complex then binds and phosphorylates the intracellular signaling molecules Smad2 and Smad3 through TβRI (1, 12). Once phosphorylated, these regulatory Smads (R-Smad) form complexes with Smad4 (also called Dpc4 for deleted in pancreatic carcinoma locus 4 or common Smad) and translocate into the nucleus. In the nucleus,

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[2] The abbreviations used are: TβRI, TβRII, and TβRIII, TGF-β type I, II, and III receptor, respectively; iNos, inducible nitric-oxide synthase; 293T, HEK-293T; EV, empty vector; luc, luciferase; ITR, inverted terminal repeat; EV, empty vector.

they associate with transcription factors to form transcriptionally active DNA complexes (1, 12, 13).

TGF-β signaling is tightly regulated at multiple levels both in and outside the target cells (1): both Lap and Ltbp facilitate secretion of TGF-β while retaining it in its inactive form at the basal state (14, 15); secreted molecules, such as decorin, bind directly to TGF-β ligands and neutralize their biological activity (16, 17); the transmembrane protein BAMBI sequesters ligand from binding to TβRI (18); Dapper2 promotes TβRI degradation (19); Fkbp12 blocks TβRI phosphorylation (20); TmepaI sequesters R-Smads from TβRI kinase activation (21); the E3 ubiquitin ligase Smurf1 degrades R-Smads (22) and Smad7-associated TβRI (23); and Smad7 directly competes with Smad2/3 for binding to TβRI (24). The negative regulator Smad7 is itself negatively regulated by Arkadia through ubiquitination and degradation (1). In the nucleus, transcriptional suppressors negatively regulate the transcriptional activity of the Smad complexes (25). So far, negative regulators have been discovered that target every TGF-β signaling component downstream of the receptor. However, a direct TGF-β type II receptor regulator is yet to be identified. In the present study, we discovered SPSB1 to be a novel regulator of TGF-β signaling that acts at the receptor level to specifically target TβRII.

The four members of the Spry domain-containing Socs box proteins SPSB1 to -4 were originally identified and cloned as a result of searching for Socs box-containing proteins (26–28). The Spry domain was discovered as a sequence repeat in the dual specificity kinase SplA and ryanodine receptors (29), and there are now more than 150 human proteins known to contain this domain (30, 31). Spry domains function as protein-protein interaction modules (27, 32–34) and, in the context of the SPSB proteins, act as adaptors to bring the Socs box-associated E3 ubiquitin ligase complex into close proximity with its substrate (35).

Genetic deletion of SPSB2 results in a very mild thrombocytopenia in mice (36). Recently, SPSB1 and SPSB2 have been shown to interact with inducible nitric-oxide synthase (iNos), targeting it for ubiquitination and proteasomal degradation and implicating the SPSB proteins in regulating the host response to infection (37–39). SPSB1 has also been reported to interact with c-Met to enhance the hepatocyte growth factor-induced Erk-Elk-1-Sre pathway by an unknown mechanism (27), whereas SPSB1, -2, and -4 have been reported to bind to the intracellular protein prostate apoptosis response 4 (Par4) (28). Whereas dysregulation of c-Met signaling is associated with the formation of invasive tumors, the role of the SPSB protein family in regulating cancer formation and progression remains to be determined. Interestingly, SPSB1 was identified as a TGF-β-inducible gene in a microarray screen for genes regulated during epithelial-mesenchymal transition (40). Given that some TGF-β-induced gene products, such as Smad7 and Tgif (1), feed back to regulate TGF-β signaling, the effect of SPSB1 on TGF-β signaling was examined.

Here we provide the first evidence that SPSB1 negatively regulates TGF-β signaling, interacting with TβRII via its Spry domain. Consequently, SPSB1 enhances the ubiquitination of TβRII through the Socs box-associated E3 ligase, facilitating the degradation of TβRII. Our results define SPSB1 as a new regulatory component of the TGF-β signaling pathway that restricts TβRII expression to fine tune receptor signaling.

Experimental Procedures

Antibodies and Reagents—The mouse anti-FLAG (M2) and anti-actin monoclonal antibodies were obtained from Sigma-Aldrich. The mouse monoclonal anti-HA antibody was obtained from Cell Signaling Technology, Inc. (Danvers, MA). Mouse monoclonal anti-Myc antibody was generated in house. Rabbit polyclonal anti-TβRI and anti-TβRII antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The rabbit polyclonal anti-FLAG antibody was obtained from Affinity BioReagents (Golden, CO). Rabbit polyclonal anti-phospho-Smad2 antibody was kindly provided by Prof. Peter ten Dijke (Leiden University Medical Center, Netherlands). Mouse monoclonal anti-Smad2 antibody was obtained from BD Transduction Laboratories (Rockville, MD). Goat anti-mouse IgG HRP-conjugated secondary antibody and goat anti-rabbit IgG HRP-conjugated secondary antibody were obtained from Bio-Rad (Gladesville, Australia). The anti-mouse Alexa488- and Alexa546-conjugated secondary antibodies were from Invitrogen (Mulgrave, Australia). Human recombinant TGF-β1 was obtained from R&D Systems. Doxycycline and Cycloheximide were purchased from Sigma-Aldrich, whereas MG132 was obtained from Merck (Darmstadt, Germany).

DNA Constructs and Primers—FLAG-TβRI and *HA-TβRII* were cloned into the pcDNA3 mammalian cell expression vector as described previously (41). *HA-TβRII(N236A)* and *HA-TβRII(N238A)* were generated based on *HA-TβRII* using the QuikChange® II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer's recommendations. The following primers were used in the PCR: *HA-TβRII(N236A)*, TGTGCCAACAACATCGCCCACAACACAGAGCT (forward) and AGCTCTGTGTTGTGGGCGATGTTGTTGGCACA (reverse); *HA-TβRII(N238A)*, AACAACATCAACCACGCCACAGAGCTGCTGCC (forward) and GGCAGCAGCTCTGTGGCGTGGTTGATGTTGTT (reverse). The sequences of all newly generated *HA-TβRII* mutants were confirmed by direct DNA sequencing. *FLAG/MYC-SPSB1*, *FLAG/MYC-SPSB1*Δ, *MYC-SPSB1(Y129A)*, and *MYC-SPSB1(T160A,Y161A)* were cloned into the *pEF-BOS* mammalian cell expression vector and have been described previously (28, 42).

Cell Lines, Cell Culture, and Treatments—The human embryonic kidney cell line HEK-293T (293T), the glioblastoma cell line U87MG, mouse embryonic fibroblasts, the mouse fibroblast cell line NIH3T3, and the Madin-Darby canine kidney (MDCK) cell line have all been described previously (10, 43–47). To generate the doxycycline-inducible SPSB1 NIH3T3 and MDCK cell lines, a tetracycline-inducible vector, pTRE, was utilized (48). Briefly, *pTRE-FLAG-SPSB1* and *pEFpurop-Tet-on* (48) were co-transfected into NIH3T3 and MDCK cells using FuGENE HD transfection reagent (Roche Applied Science, Basel, Switzerland) following the manufacturer's instructions and selecting with puromycin (Roche Applied Science). Positive clones were selected by Western analysis using FLAG

antibody (Sigma-Aldrich). All cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) (DKSH, Hallam, Australia), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen).

Luciferase Assays—Cells were transiently transfected with the firefly luciferase (luc) construct *pGL3-(CAGA)$_{12}$-luc* (49), together with additional DNA constructs as indicated using the FuGENE HD transfection kit for 293T cells and METAFECTENE PRO (Biontex Laboratories, San Diego, CA) for all other cells. 24 h after transfection, cells were stimulated with or without TGF-β (2 ng/ml) in medium containing 10% FCS for a further 24 h. Thereafter, cells were lysed and assessed for luciferase activity using the luciferase reporter assay kit (Promega Corp., Madison, WI) following the manufacturer's instructions.

RNA Extraction and RT-PCR—U87MG and 293T cells were transfected with control (Trilencer-27 Universal scrambled negative control siRNA from ORIGENE) or SPSB1 siRNA using FuGENE HD and METAFECTENE PRO transfection reagent, respectively (human SPSB1 siRNA, rArGrArArUrArArArCrUrCrCrUrArCrGrArArArGrCrCrCTA). 72 h after siRNA transfection, total RNA was isolated from cells using TRIzol® reagent (Invitrogen). cDNA was obtained using the SuperScript™ III CellsDirect cDNA synthesis system (Invitrogen). cDNA aliquots were subjected to PCRs using the SensiMix™ SYBR kit (Bioline) to amplify *SPSB1* and *GAPDH* with primers. Primers used were as follows: human *SPSB1*, GTATCCTGTAGTGAGTGCCGTC (forward) and CGACGGCACAAATCCATGAGCG (reverse); human *GAPDH*, AGGTCGGTGTGAACGGATTTG (forward) and TGTAGACCATGTAGTTGAGGTCA (reverse). Each cycle was carried out as follows: 10 min at 94 °C, 15 s at 94 °C, 30 s at 56 °C. Each cycle was repeated 49 times, according to the manufacturer's recommendations, using a Rotor-Gene RG-3000 thermal cycler and Rotorgene version 6.0 software (Corbett Research). On the basis of the comparative *Ct* method, gene expression levels were calculated, and *GAPDH* was used as a control gene. Control siRNA-transfected cells were set to 100%, and -fold change in expression in SPSB1- and TGF-β-treated cells is represented as the mean ± S.E.

Immunoprecipitation and Immunoblotting—After transfection, cells were lysed in lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 2 mM $MgCl_2$, 1 mM Na_3VO_4, 25 μg/ml leupeptin, and 25 μg/ml aprotinin), and cell lysates were subjected to immunoprecipitation with appropriate antibody-conjugated Sepharose-protein G beads or anti-FLAG beads (Sigma-Aldrich) for 4 h. Immunoprecipitates were washed three times with ice-cold PBS containing 0.5% Tween 20, and immunoprecipitated proteins were separated by SDS-PAGE (Invitrogen) and blotted onto nitrocellulose membrane and probed with the indicated primary antibodies. The signal was visualized using the ECL chemiluminescence detection kit (GE Healthcare, Rydelmere, Australia) following incubation with appropriate secondary antibodies.

Qualitative Analysis for Protein Half-life—The intensity of the bands in Western blot images was measured using ImageJ. Rectangular selection tool was used to select the area where the bands were located (the intensity of bands that were used to calculate the half-life of the protein was measured together in one selected area). The gaps between each band were used as relative background. The intensity of each band was measured three times by selecting three different gap intensities as the relative background (background intensity selected at low, medium, and high). Protein stability curves were generated by smoothly joining the intensity values of each set of bands in the *y* axis with their corresponding treatment times in the *x* axis using Microsoft Excel. Half-life was determined as the time at which protein band intensities were 50% of the starting level (time 0). The value of half-life shown in the results is the mean of the three estimated half-life values for each four bands. The results are shown as the mean of estimated half-life values ± S.D.

Immunofluorescence Staining and Confocal Microscopy—After transfection with appropriate DNA constructs using METAFECTENE PRO for 48 h, cells were treated with TGF-β for 1 h in medium containing 10% FCS and then washed once in preheated 37 °C PBS and fixed with 3.7% formaldehyde (Sigma) in PBS for 7 min. Following two PBS washes, cells were permeabilized with 0.2% Triton X-100 (Merck) in PBS for another 7 min. Cells were then washed three times with PBS and blocked with PBS containing 5% BSA for 1 h at room temperature. Following another three washes in PBS, cells were stained with relevant primary antibody (diluted in PBS containing 2% BSA) for 1 h at room temperature and washed in PBS three more times. Visualization was achieved with either Alexa546- or Alexa488-conjugated secondary antibody using the Nikon TE2000-E and C1 confocal microscope with a Nikon ×60 water-immerged lens. Nikon confocal EZ-C1 version 1.4 was used to collate images.

In Vitro Scratch Assay—U87MG cells were transfected with control or *SPSB1* siRNA (20 nM) and seeded onto 12-well plates and cultured until 100% confluent. 48 h post-transfection, scratches were created using a P1000 pipette tip to scratch a straight line on the culture plate. The culture medium was replaced with fresh medium to remove detached cells. Phase-contrast images were acquired at 0 and 24 h post-scratch using an inverted microscope (IX50, Olympus) equipped with a CCD camera (model 11.3, Diagnostic Instruments, Inc., Sterling Heights, MI), and SPOT advanced imaging software (version 4.0.4) was used to acquire and process images.

In Vitro Invasion and Migration Assay—U87MG cells were transfected with control or *SPSB1* siRNA (20 nM) in a 6-well plate. 48 h post-transfection, U87MG (25,000 cells/chamber) cells were resuspended in serum-free DMEM and seeded in the top chamber of a 70-μl solidified Matrigel (BD Biosciences; mixed 1:1 with DMEM)-coated, 8-μm, polycarbonate membrane transwell insert (Corning Inc.). Serum-free DMEM with or without 2 ng/ml TGF-β was added to the bottom chamber. Cells were then incubated for 24 h at 37 °C with 10% CO_2. Thereafter, cells that invaded through the coated Matrigel and migrated to the other side of the membrane of the transwell insert were fixed with 3.7% formaldehyde (Sigma) for 7 min. Cells were then washed and stained with Hoechst for 5 min. Any remaining cells in the top chamber of the transwell insert were removed by using a cotton swab. Only cells in the bottom side of the transwell insert were counted. Fluorescent images of

cell number were taken in three random fields (×20) per insert. Assays were performed in triplicate.

Generation of the TGF-β-driven tdTomato-expressing Adenoviruses—The luc reporter was cut from the *pGL3-(CAGA)$_{12}$-luc-pENTR 1A vector* (49, 50) and replaced with the tdTomato gene from *pCMV-tdTomato* vector (Clontech). attL-arrR recombination was then performed with the *pAd/PL-DEST* destination vector (Invitrogen) to generate the *pCAGA12-tdTomato* adenoviral expression plasmid. The expression plasmid was digested with PacI to expose the inverted terminal repeats (ITRs) and then transfected into the 293A cell line using Lipofectamine LTX transfection reagent (Invitrogen). Cells were harvested ~2 weeks after transfection when lysis was observed in the majority of cells. The adenovirus was amplified and used to detect TGF-β responses in cultured cell lines.

Statistics—All statistical analyses were performed using a two-tail Student's *t* test ($p < 0.05$ indicating statistical significance).

Results

TGF-β Induces SPSB1 Transcription, Which Acts in a Feedback Loop to Suppress Signaling—Many transcriptional gene targets of TGF-β signaling, such as *Smad7*, *Tgif*, *SnoN*, and *Tmepai*, feed back to regulate its signaling (1, 21, 51). *SPSB1* was identified as a TGF-β target gene in a microarray screening (40). Indeed, *SPSB1* gene expression in mouse embryonic fibroblasts is induced by TGF-β, as determined by qPCR (Fig. 1*A*). To measure TGF-β signaling activity, we first examined phospho-Smad2 levels in NIH3T3 cells, which stably express an inducible *SPSB1* construct. As shown in Fig. 1*B*, the expression of SPSB1 resulted in some reduction of the TGF-β-induced phospho-Smad2 levels. A Smad3-responsive luciferase reporter (*pCAGA-luc*) (11) was used to quantitatively measure TGF-β signaling activity in 293T cells. In the presence of increasing SPSB1 levels, *pCAGA-luc* activity decreased from ~17–18-fold to less than 10-fold (suppression of ~45%) (Fig. 1*C*), indicating a dose-dependent suppression by SPSB1. A similar inhibitory effect of SPSB1 on TGF-β signaling was also observed in human U87MG brain tumor cells (Fig. 1*D*), mouse NIH3T3 fibroblasts (Fig. 1*E*), and canine MDCK epithelial cells (Fig. 1*F*).

Next we explored which SPSB1 region is responsible for this function. Because the Socs box has been shown to be important for the function of the Socs family proteins (37–39, 52–56), its role in SPSB1 was examined in 293T cells. Expression of wild-type SPSB1 again inhibited *Smad3* reporter activity by ~50%. In contrast, expression of SPSB1 lacking the Socs box (SPSB1Δ) did not inhibit signaling and instead slightly enhanced the *pCAGA-luc* activation from ~8-fold (EV) to ~11-fold (Fig. 1*G*). A similar increase in *pCAGA-luc* activation (~35%) was observed in NIH3T3 cells (Fig. 1*H*). Furthermore, the suppression and increase of *pCAGA-luc* activity by SPSB1 and SPSB1Δ, respectively, were also evident in mouse embryonic fibroblasts isolated from transgenic mice carrying either an SPSB1 or SPSB1Δ transgene (data not shown). Collectively, these data demonstrate that the Socs box may be responsible for the negative regulatory function of SPSB1, and the Socs box deletion mutant may function as a dominant negative form of SPSB1.

Is the Spry domain also required for the negative regulatory function of SPSB1 on TGF-β signaling? The Spry domain is thought to function as a protein recognition interface (28, 37, 57), and consistent with this, mutation of Tyr-120 in the SPSB2 Spry domain disrupted SPSB2 interaction with Par4 and iNos, whereas mutation of Gln-151 together with Tyr-152 disrupted its interaction with both c-Met and Par4 (28, 37). To determine the requirement for the SPSB1 Spry domain, constructs containing the analogous SPSB1 mutations Y129A and T160A,Y161A (42), were tested for their ability to inhibit *pCAGA-luc* activity. As shown in Fig. 1*G*, mutation of Tyr-129 in *SPSB1(Y129A)* completely abolished its negative regulatory effect. In contrast, *SPSB1(T160A,Y161A)* only partially inhibited *pCAGA-luc* activity from ~8-fold (EV) to ~6-fold, a reduction of about 25%. Collectively, these results suggest that the SPRY domain is also required for the inhibitory function of SPSB1 on TGF-β signaling, and SPSB1(Y129A) and SPSB1(T160A,Y161A) may disrupt the ability of SPSB1 to interact with its targets.

SPSB1 Interacts with TβRII (and Not TβRI) through Its Spry Domain—Because SPSB1 can inhibit the transcriptional activation of Smad3 and reduce the TGF-β-induced Smad2 phosphorylation levels, SPSB1 should act at the level of the Smads or upstream in the signaling cascade. Extensive investigation failed to show any interaction between SPSB1 and any Smads (Smad2, -3, and -4; data not shown). The next logical target was therefore higher in the TGF-β signaling axis, namely the receptors, TβRI and TβRII. The tagged receptors *FLAG-TβRI* and *HA-TβRII* (41) were co-transfected together with *FLAG* or *Myc-SPSB1* in 293T cells. Western blot analysis of the anti-TβRII and TβRI immunoprecipitates showed the presence of SPSB1 (Fig. 2, *A* and *B*), indicating that SPSB1 is present in the receptor complex when both TβRII and TβRI are co-expressed. Furthermore, this complex formation was not affected by TGF-β (Fig. 2, *A* and *B*).

Can TβRI or TβRII alone interact with SPSB1? *FLAG-TβRI* or *HA-TβRII* was co-transfected with *Myc-SPSB1* in 293T cells. SPSB1 co-precipitated with TβRII but not with TβRI (Fig. 2, *C* and *D*), indicating that SPSB1 does not interact with TβRI directly but forms a complex with TβRI via TβRII (Fig. 2*B*).

Next, we explored which region of SPSB1 is responsible for the SPSB1-TβRII complex formation. As shown in Fig. 2, *D* and *E* (*lane 2*), TβRII was co-precipitated with SPSB1Δ at a similar level as with SPSB1 (*lane 1*). This result indicates that the Socs box is unlikely to be involved in the complex formation between SPSB1 and TβRII. As a result, the SPRY domain of SPSB1 is likely to be responsible for this interaction. Of note is the higher expression level of SPSB1Δ observed in the anti-FLAG blots (Fig. 2, *C–E*).

To confirm the role of the Spry domain of SPSB1 in the complex formation with TβRII, we utilized the Spry domain mutants, which interrupted the ability of SPSB1 to suppress TGF-β signaling (Fig. 1*G*). *HA-TβRII*, in combination with *Myc-SPSB1*, *Myc-SPSB1*Δ, *Myc-SPSB1(Y129A)*, or *Myc-SPSB1(T160A,Y161A)*, was co-transfected in 293T cells. TβRII was observed to co-precipitate with SPSB1 and SPSB1Δ (Fig.

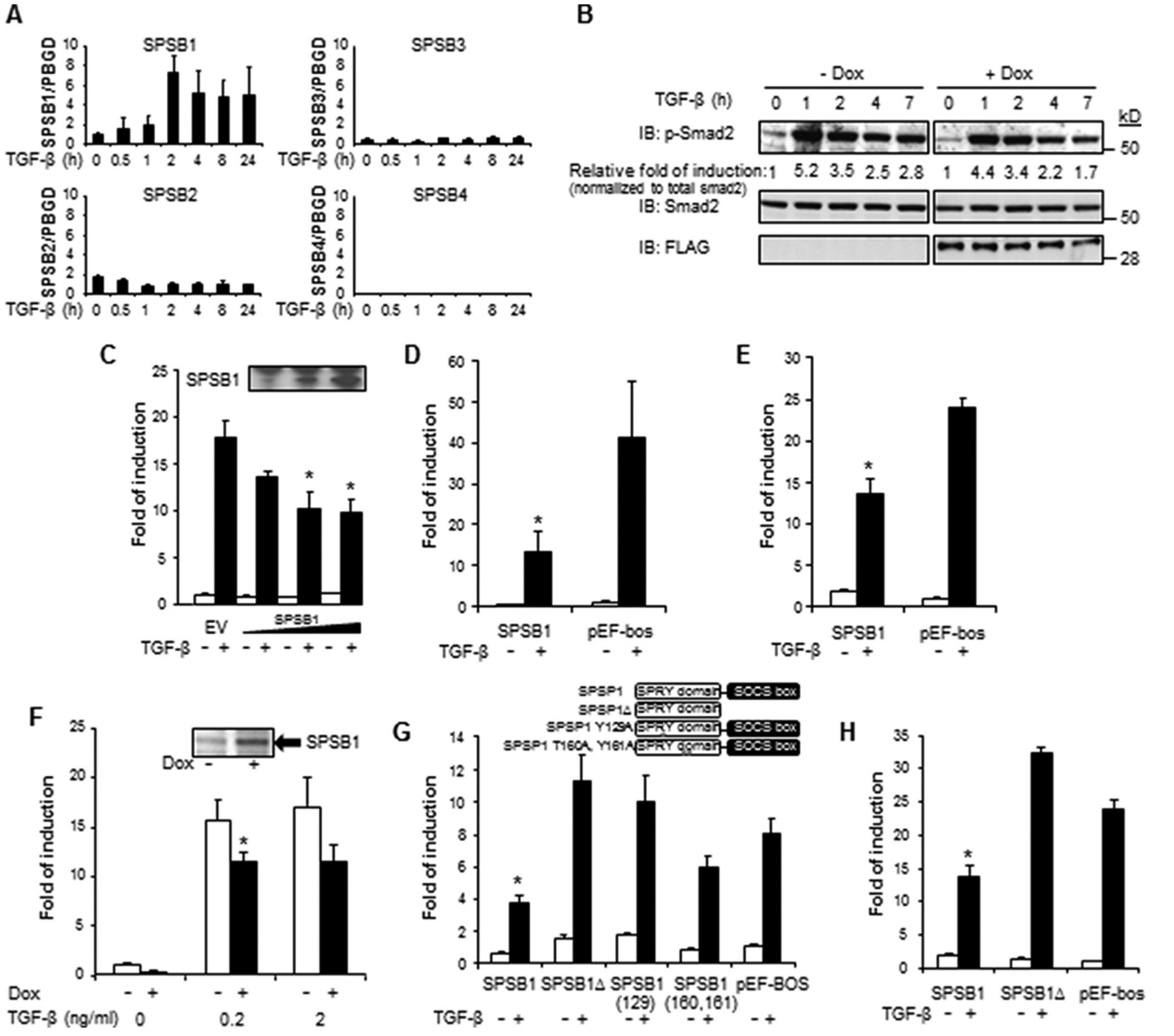


FIGURE 1. **TGF-β induces SPSB1 transcription, which feeds back to suppress its signaling.** *A*, wild-type mouse embryonic fibroblasts were treated with 2 ng/ml TGF-β, and total RNA was extracted at the indicated times post-treatment. SPSB1 to -4 mRNA levels were measured using real-time PCR and normalized to PBGD mRNA level. All results are relative to non-TGF-β-treated SPSB1 mRNA levels (time 0 h). *B*, SPSB1-inducible NIH3T3 cells were seeded in 24-well plates (~45% confluence) with or without doxycycline (2 μg/ml) for 48 h. Following the addition of TGF-β, cells were lysed at the indicated time points, and phospho-Smad2, Smad2, and FLAG-SPSB1 expression levels were examined by Western blot. 293T cells (*C* and *G*) or U87MG cells (*D*) or NIH3T3 cells (*E* and *H*) or MDCK (*F*) cells stably transfected with a doxycycline-inducible FLAG (M2)-SPSB1 construct were co-transfected with *pCAGA*$_{12}$*-luc* and SPSB1 at increasing concentrations (0, 0.025, 0.05, and 0.1 μg/well) or empty vector *pEF-BOS* (*C*) or WT or mutated/WT SPSB1 (*D*, *E*, *G*, and *H*), as indicated, for 24 h. MDCK cells (*F*) were cultured with or without doxycycline (2 μg/ml) to induce SPSB1 expression. After 24 h, cells were treated with or without TGF-β (2 ng/ml) for a further 24 h and lysed, and luciferase activity was determined. Data are expressed as relative Smad3 luciferase activity (-fold induction) by standardizing the luciferase activity of unstimulated cells transfected with EV to 1 and normalizing all other raw values accordingly. Results from a representative experiment are shown as the mean of triplicates ± S.D. (*error bars*). *, $p < 0.05$. *C* and *F*, SPSB1 expression was confirmed by Western blot of the luciferase assay cell lysates. All experiments were repeated three times, with representative results shown. A schematic illustration of the SPSB1 protein is shown in *G*.

2*E*, *lanes 1* and *2*), whereas reduced amounts of TβRII co-precipitated with the SPSB1(Y129A) and SPSB1(T160A,Y161A) mutants (Fig. 2*E*, *lanes 3* and *4*), indicating that residues Tyr-129 and Thr-160/Tyr-161 in the SPRY domain are critical for the SPSB1 interaction with TβRII. Taken together, SPSB1 interacts with TβRII through its Spry domain, supporting the previous finding that the Spry domain is involved in protein-protein interaction (28, 57).

To further investigate the TβRII-SPSB1 interaction, we created mutations in the intracellular domain of TβRII. Previous studies have shown that SPSB1 and -2 recognize a similar sequence motif in Par4 and iNos: Glu/Asp-Leu/Ile-Asn-Asn-Asn-Leu ((E/D)(L/I)NNN)). In particular, the Asn-Asn-Asn sequence was crucial for their interaction (37, 57, 58). Our sequence alignment shows that TβRII shares no overall sequence similarity with Par4 but contains a stretch of Asn^{234}-Ile-Asn-His-Asn-Thr^{239} (NINHNT) at the N terminus of the intracellular domain of the receptor. Based on the importance of the N-N-N sequence motif, we generated two mutant TβRII cDNA constructs, one containing a N236A substitution and the

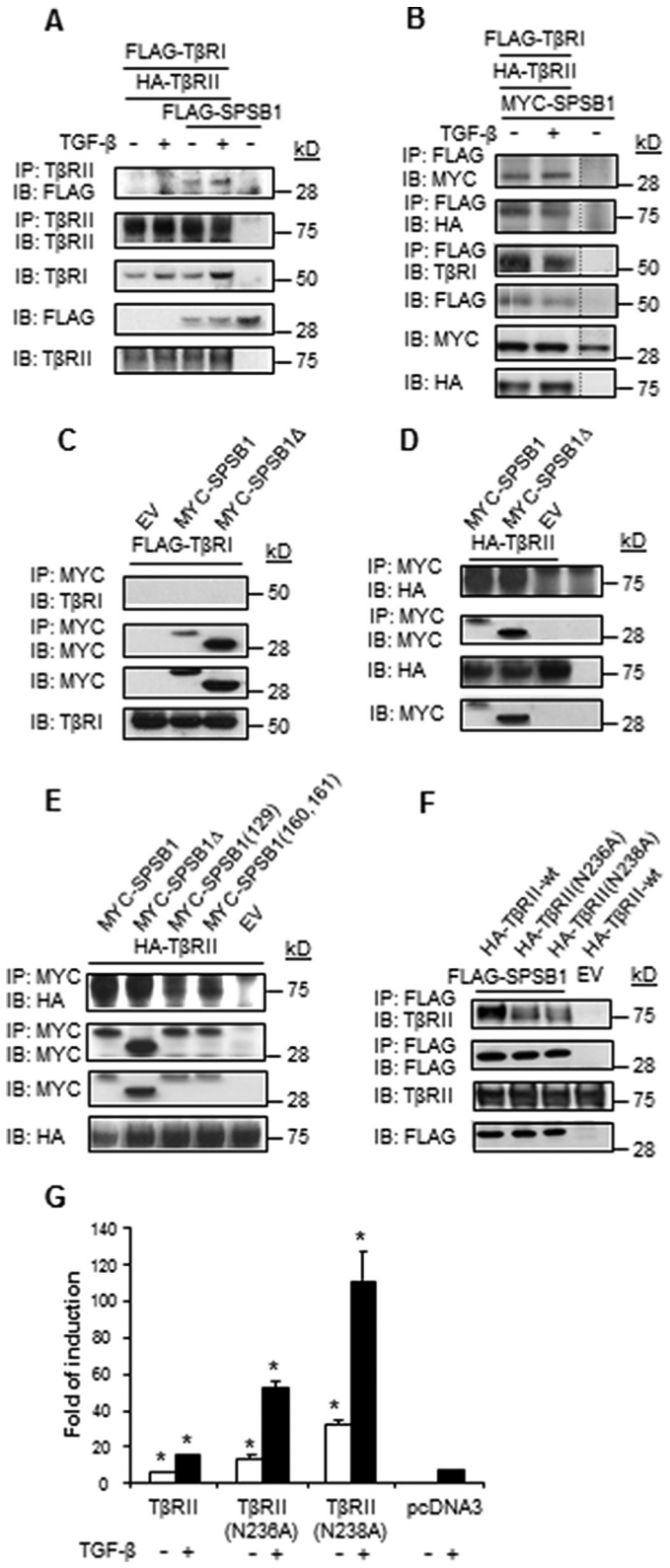


FIGURE 2. **SPSB1 interacts with TβRII but not TβRI through its SPRY domain.** 293T cells were transfected with the indicated DNA constructs (0.5 μg/well each). 48 h later, cells in *A* and *B* were treated with or without TGF-β (2 ng/ml) for a further 1 h, whereas cells in *C–F* were untreated. Cell lysates were immunoprecipitated (*IP*) with anti-TβRII antibody (*A*) or anti-Myc antibody (*C–E*) conjugated to protein G beads or anti-FLAG beads (*B* and *F*). Both whole cell lysates and immunoprecipitates were examined for the indicated proteins by immunoblotting (*IB*). The *last lane* in *B* was cut from the *right-hand side* of the same protein gel with the same exposure. 293T cells (*G*) were co-transfected with $pCAGA_{12}$-*luc* and TβRII/TβRII mutants/pcDNA3 control vector as indicated for 24 h. After 24 h, cells were treated with or without TGF-β (2 ng/ml) for a further 24 h and then lysed, and luciferase activity was determined as described in the legend to Fig. 1. *Error bars*, S.D. from representative experiments performed three times. *, $p < 0.05$. Each experiment was repeated three times, with one representative result shown.

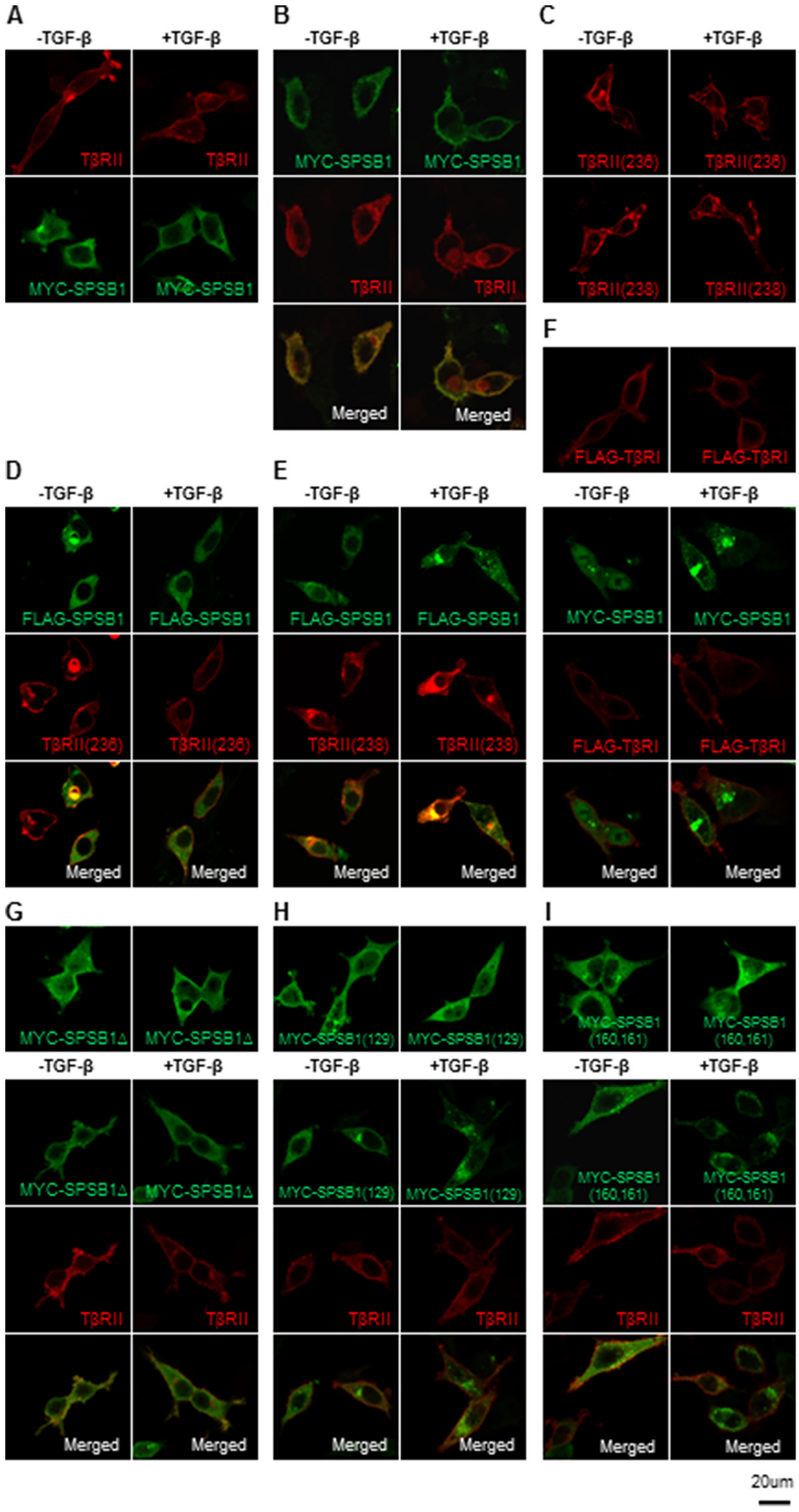
other containing a N238A substitution. Neither mutation disrupted the function of the type II receptor in a TGF-β reporter assay (Fig. 2*G*), indicating correct folding of the expressed mutants. Anti-FLAG immunoprecipitation of SPSB1 showed that TβRII was co-precipitated with SPSB1 (Fig. 2*F*). However, a reduced amount of either TβRII(N236A) or TβRII(N238A) was observed to be co-precipitated with SPSB1 (Fig. 2*F*), suggesting that Asn-236 and Asn-238 are likely to be involved in the interaction with SPSB1.

Subcellular Co-localization of SPSB1 and TβRII—To confirm that the interaction between SPSB1 and TβRII was not an artifact of cell lysis, we performed co-immunofluorescence staining. When expressed alone in 293T cells, SPSB1 was diffusely localized in the cytoplasm (Fig. 3*A*). Interestingly, in the presence of TβRII, a substantial portion of SPSB1 was found redistributed to the cell membrane (Fig. 3*B*). TβRII and SPSB1 co-localization was extensive on the cell membrane regardless of the presence or absence of TGF-β (Fig. 3*B*). This pattern of redistribution of SPSB1 and co-localization of TβRII and SPSB1 was also evident in both NIH3T3 and MDCK cells (data not shown), consistent with the co-immunoprecipitation data.

Because both TβRII(N236A) and TβRII(N238A) showed a reduced association with SPSB1, we examined their localization using immunofluorescence staining of 293T cells. Similar to the wild-type TβRII, both TβRII(N236A) and TβRII(N238A) were found predominantly localized at the cell membrane (Fig. 3*C*), regardless of whether TGF-β was present or not. Interestingly, the expression of either TβRII(N236A) or TβRII(N238A) failed to significantly redistribute SPSB1 to the cell membrane (Fig. 3, *D* and *E*); hence, the lack of co-localization with SPSB1 is consistent with our co-immunoprecipitation data, which suggests that the mutants can no longer interact with SPSB1. Expression of TβRI did not redistribute SPSB1 to the cell membrane (Fig. 3*F*), consistent with there being no detectable interaction between TβRI and SPSB1.

Because the Socs box deletion of SPSB1 did not affect its interaction with the TβRII complex (Fig. 2*D*), we next confirmed this observation by visualizing co-localization in 293T cells. As shown in Fig. 3*G*, similarly to SPSB1, SPSB1Δ was recruited to the cell surface in the presence of TβRII and extensively co-localized with TβRII in this area. This pattern of co-localization was also evident in both NIH3T3 and MDCK cells (data not shown), confirming that the Socs box domain is not required for the co-localization between SPSB1 and TβRII.

Previous Western blot (Fig. 2*E*) analysis showed that specific mutations in the Spry domain significantly altered the ability of SPSB1 to interact with TβRII. We next tested whether the same mutations in the Spry domain could affect their co-localization with TβRII. As shown in the *top panel* in Fig. 3, *H* and *I*, when expressed alone in 293T cells, SPSB1(Y129A) and SPSB1(T160A,Y161A), like SPSB1, were predominantly found across the cytoplasm and in the endosome vesicle-like struc-


A
-TGF-β
+TGF-β
TβRII
TβRII
MYC-SPSB1
MYC-SPSB1
B
-TGF-β
+TGF-β
MYC-SPSB1
MYC-SPSB1
TβRII
TβRII
Merged
Merged
C
-TGF-β
+TGF-β
TβRII(238)
TβRII(238)
TβRII(238)
TβRII(238)
F
FLAG-TβRI
FLAG-TβRI
D
-TGF-β
+TGF-β
FLAG-SPSB1
FLAG-SPSB1
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MYC-SPSB1Δ
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MYC-SPSB1(129)
-TGF-β
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MYC-SPSB1 (160,161)
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tures randomly distributed in the cytoplasm. In the presence of TβRII (Fig. 3, *H* and *I*), no obvious change in their localization was observed except that an increased amount of SPSB1 mutants seemed to be located in the endosome vesicle-like structures. In particular, no co-localization was observed between the SPSB1 mutants and TβRII on the cell membrane, even in the presence of TGF-β stimulation. Similarly, this non-association was also evident in NIH3T3 and MDCK cells (data not shown). Collectively, these results confirm that the Spry residues Tyr-129 and Thr-160/Tyr-161 are important for SPSB1 interaction with TβRII.

SPSB1 Negatively Regulates TGF-β Signaling by Destabilizing TβRII through Enhanced Ubiquitination—Given that the Socs box in Socs family proteins mediates binding to elongin C and elongin B and induces the formation of an E3 ubiquitin ligase complex (35, 37), resulting in protein degradation of the targets (59), we investigated whether SPSB1 could mediate or induce ubiquitination and degradation of its interacting proteins. Surprisingly, SPSB1 and the Socs box deletion mutation SPSB1Δ were heavily ubiquitinated when co-expressed with ubiquitin, and this occurred independently of the presence of MG132, a proteasome inhibitor (Fig. 4*A*). The observation that SPSB1Δ could be ubiquitinated at a similar level as SPSB1 suggests that the ubiquitination sites are located outside of the Socs box domain. To examine the effect of SPSB1 on TβRII ubiquitination, SPSB1 and SPSB1Δ were co-expressed with TβRII and ubiquitin in 293T cells. As shown in Fig. 4*B*, whereas TβRII was ubiquitinated when SPSB1 was not expressed, the expression of SPSB1 resulted in a clear increase in the level of ubiquitination associated with TβRII. There was a slight increase of ubiquitination associated with TβRII when SPSB1Δ was expressed; however, this is probably due to the polyubiquitinated SPSB1Δ (Fig. 4*A*) that interacts with TβRII (Fig. 2*D*). This suggests that SPSB1 can increase the ubiquitination of TβRII via its Socs box in the basal state. In contrast, there was no such increase when either SPSB1(Y129A) or SPSB1(T160A,Y161A) was co-expressed (Fig. 4*C*). In addition, SPSB1 failed to increase the ubiquitination levels of TβRII(N236A) and TβRII(N238A) (mutants disrupting the interaction of TβRII with SPSB1) (Fig. 4, *D* and *E*). These results are consistent with the ability of SPSB1 to induce ubiquitination on TβRII through the interaction of the SPSB1-SPRY domain with Asn-236 and Asn-238 of TβRII.

Normally, increased lysine 48-linked ubiquitination of a protein decreases its stability and consequently its half-life. To measure the protein half-life, a protein synthesis inhibitor, cycloheximide, was used. As shown in Fig. 5*A* (*left panels*), in the absence of SPSB1, TβRII was relatively stable during the 8-h cycloheximide treatment. However, when SPSB1 was expressed, a marked decrease in the stability of TβRII was observed (Fig. 5*A*, *middle panels*). This was not seen when SPSB1Δ was expressed, confirming the functional importance of the SPSB1 Socs box (Fig. 5*A*, *right panels*). Because both TβRII(N236A) and TβRII(N238A) showed a substantially lower level of ubiquitination than the wild-type TβRII in the presence of SPSB1, we examined their stability. As expected, in the presence of SPSB1, there was no significant increase in the

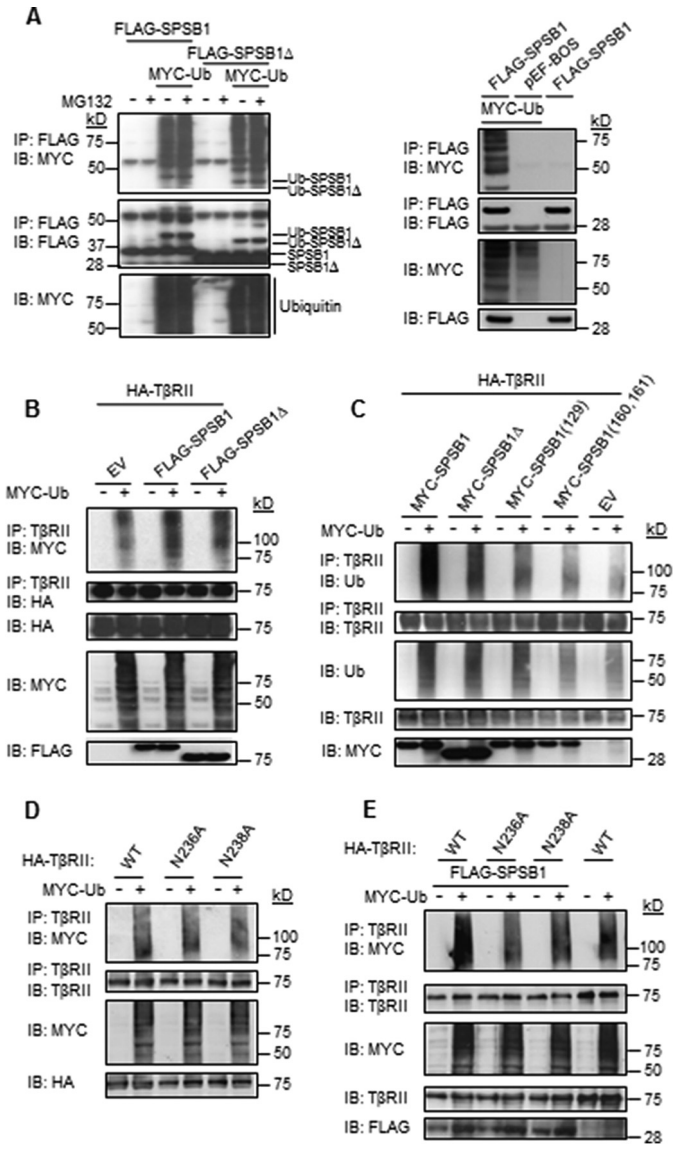


FIGURE 4. **SPSB1 enhances TβRII ubiquitination through its SOCS box.** 293T cells were transfected with the indicated FLAG/Myc-SPSB1s constructs or *pEF-BOS* (EV) and/or HA-TβRII constructs with or without Myc-ubiquitin. Cells in *A* (*left*) were treated with or without MG132 (25 μM) 42 h post-transfection for a further 6 h. In all cases, cells were lysed 48 h post-transfection. Cell lysates were immunoprecipitated (*IP*) with anti-FLAG beads (*A*) or anti-TβRII-conjugated protein G beads (*B–E*). Both whole cell lysates and immunoprecipitates were examined for the indicated proteins by immunoblotting (*IB*). Results are representative of experiments repeated at least once.

FIGURE 3. **SPSB1 co-localizes with TβRII through its SPRY domain.** 293T cells were transfected with various TβRI, TβRII, and SPSB1 constructs either alone or in combination, as indicated. 48 h later, cells were treated with or without TGF-β (2 ng/ml) for a further 1 h. Fixed cells were then immunostained with rabbit anti-TβRII, followed by Alexa546-conjugated secondary anti-rabbit IgG (*A–E* and *G–I*) and/or mouse anti-Myc followed by Alexa488-conjugated secondary anti-mouse IgG (*A*, *B*, and *F–I*) and/or rabbit anti-FLAG followed by Alexa546-conjugated secondary anti-rabbit IgG (*F*) and/or mouse anti-FLAG followed by Alexa488-conjugated secondary anti-mouse IgG (*D* and *E*), as indicated. The subcellular localization of TβRI/TβRII/TβRII mutants (*red*) and SPSB1/SPSB1Δ/SPSB1 mutants (*green*) was analyzed by confocal microscope (magnification, ×60). Co-localization of merged images appears as *yellow*. Each experiment was repeated at least once, and a representative result is shown.

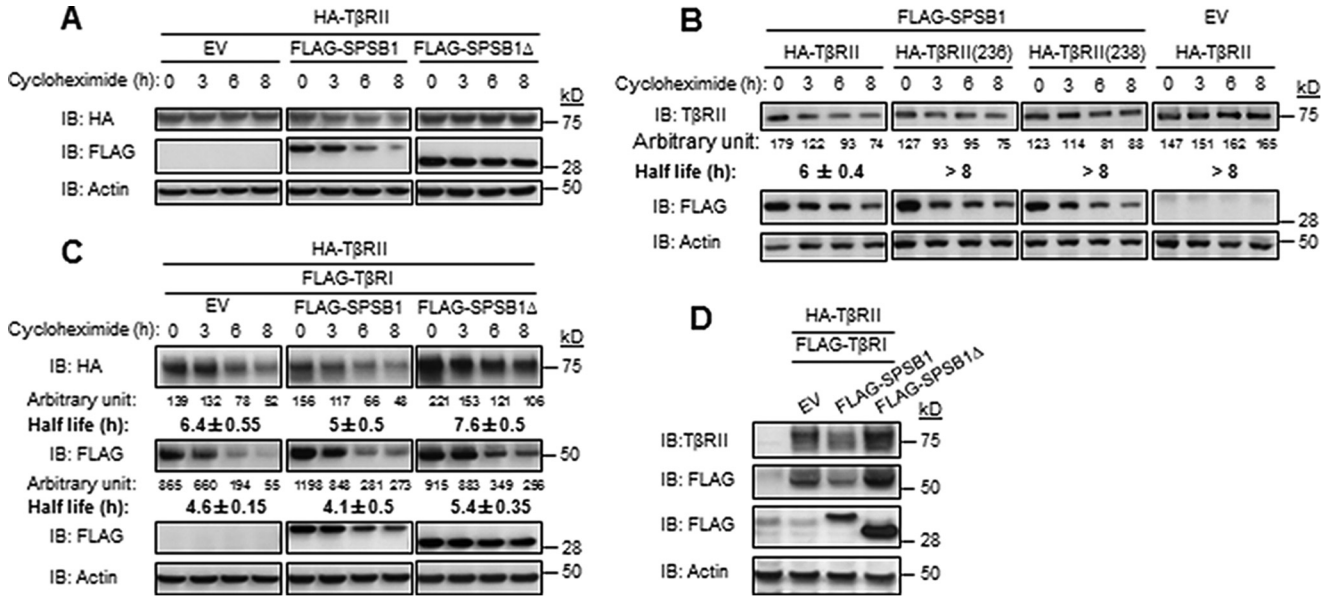


FIGURE 5. **SPSB1 negatively regulates TGF-β signaling by destabilizing TβRII.** 293T cells were co-transfected with various TβRI, TβRII, and SPSB1 constructs as indicated. 36 h later, cells were exposed to cycloheximide (20 μg/ml) for the indicated periods. Whole cell lysates were then examined for indicated proteins by immunoblotting (*IB*). Relative intensity of each TβRII and TβRI band (*B* and *C*) was qualitatively measured using ImageJ; the *number below* each *band* indicates its corresponding relative intensity (arbitrary units). The half-life ± S.D. ($n = 3$ technical replicates) for TβRI and TβRII degradation is shown *below*. Results are representative of experiments repeated at least once.

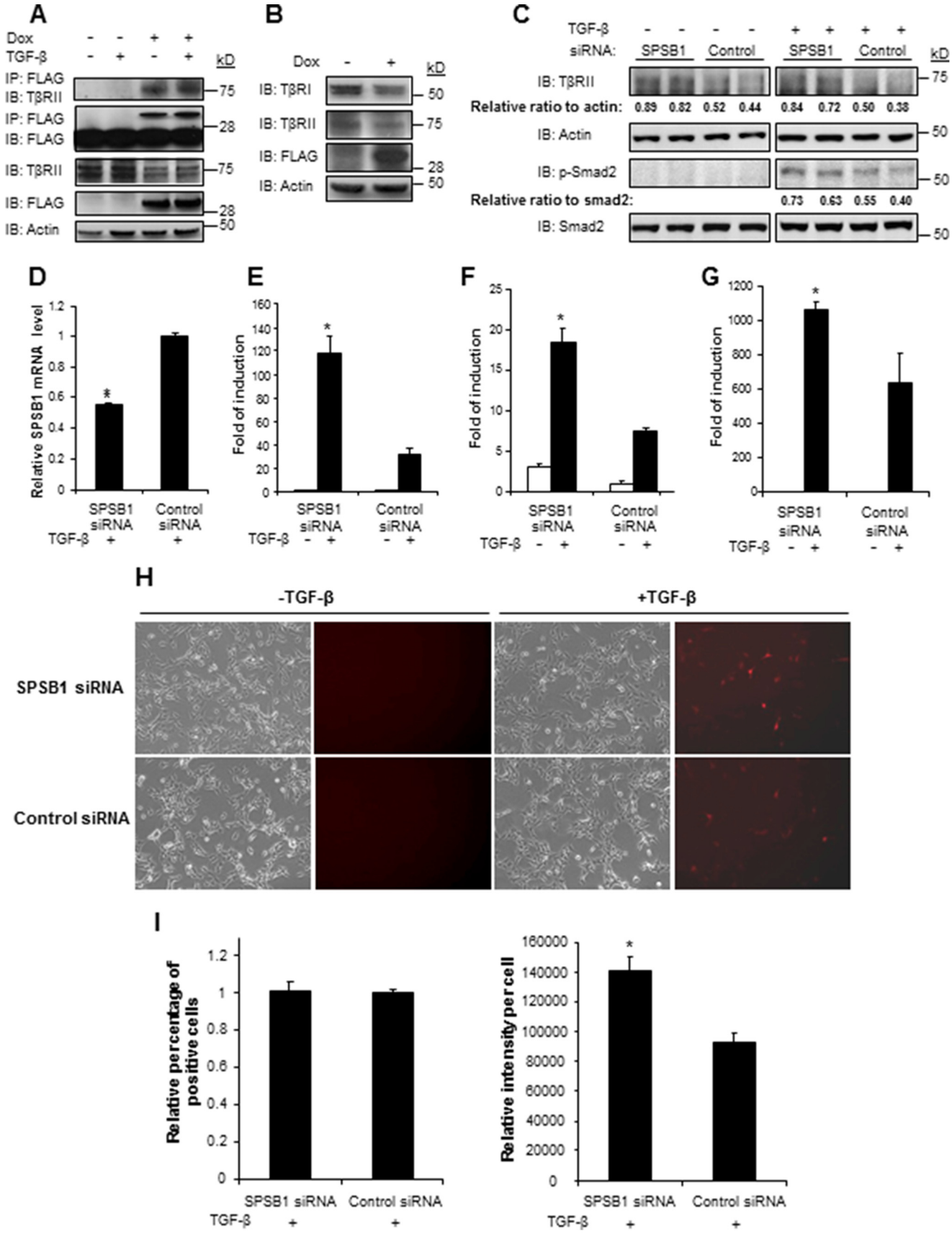
protein degradation of either TβRII(N236A) or TβRII(N238A) compared with the wild-type TβRII (Fig. 5*B*). To quantitatively measure TβRII protein stability, we measured the protein band density using ImageJ. The half-life of wild-type TβRII was estimated to be ~6 h in the presence of SPSB1. In contrast, the protein half-lives of both TβRII(N236A) and TβRII(N238A) were estimated to be more than 8 h (Fig. 5*B*). Collectively, these results suggest that SPSB1 negatively regulates TβRII protein stability by enhancing its ubiquitination.

TβRI and TβRII are expressed together in most tissues in humans (1). Therefore, the effect of SPSB1 on TβRII expression was examined in the presence of TβRI. As shown in Fig. 5*C*, when TβRI and TβRII were co-expressed, the levels of both receptors were decreased during the 8-h cycloheximide treatment, regardless of the presence or absence of SPSB1/SPSB1Δ. However, as shown in Fig. 5*C*, the half-life for TβRII reduced from ~6.4 to ~5 h in the presence of SPSB1. In contrast, the half-life for TβRII was increased to ~7.6 h in the presence of SPSB1Δ, consistent with the early observation of a dominant negative function. In addition, TβRI half-life also decreased slightly (from ~4.6 to ~4.1 h) when SPSB1 was expressed, whereas a slightly increased TβRI half-life (from ~4.6 to ~5.4 h) was observed with SPSB1Δ (Fig. 5*C*). Although TβRI is not directly targeted by SPSB1, the expression of SPSB1 seemed to have a slight but observable effect on its stability, probably because of the formation of TβRI and TβRII complexes. However, how SPSB1 exerts an effect on TβRI stability is not clear and requires further examination. Interestingly, we found that SPSB1 (but not SPSB1Δ) levels decreased in parallel with TβRII and TβRI (Fig. 5, *A* and *C*).

To examine the effect of SPSB1 expression on TβRII ubiquitination in the absence of ligand, TβRII was co-expressed with SPSB1. As expected, expression of SPSB1 decreased the levels of TβRII (Fig. 5*D*). In contrast, the expression of SPSB1Δ increased the level of TβRII (Fig. 5*D*), consistent with the observations in Fig. 5, *A* and *C*. Interestingly, the destabilizing effect of SPSB1 on TβRII was also observed for TβRI (Fig. 5*D*), indicating that in the steady-state, SPSB1 is capable of 1ulating the expression of the receptor complex.

SPSB1 Interacts with and Down-regulates Endogenous TβRII—Previous data from this study has shown that SPSB1 regulates overexpressed TβRII. To ascertain the effect of SPSB1 on endogenous TβRII, an NIH3T3 cell line was established with doxycycline-inducible FLAG-SPSB1 expression. The presence of TβRII in anti-FLAG immunoprecipitates (Fig. 6*A*) confirmed a TGF-β-independent interaction between SPSB1 and endogenous TβRII, consistent with the previous overexpression data. Also consistent was the lower level of endogenous TβRII observed when the cells were induced by doxycycline to express SPSB1 (Fig. 6*A*). Thus, SPSB1 interacts with and decreases the level of endogenous TβRII protein in NIH3T3 cells. Interestingly, endogenous TβRI levels were also slightly reduced when the cells were induced to express SPSB1, as shown by the anti-TβRI blot (Fig. 6*B*). These data support the observation that induction of SPSB1 inhibited TGF-β-mediated reporter activity and Smad2 phosphorylation (Fig. 1, *B* and *C*).

Both commercial and our own in-house SPSB1 antibodies were effective in detecting overexpressed protein; however, all of them (four in total) failed to identify endogenous SPSB1. To examine the role of endogenous SPSB1 in TβRII and TGF-β signaling, siRNAs were used to knock down endogenous SPSB1. Using a human siRNA, SPSB1 was repressed to more than 40% in U87MG cells (Fig. 6*D*). Consequently, silencing SPSB1 increased the level of endogenous TβRII (Fig. 6*C*) and enhanced *pCAGA-luc* reporter activity (Fig. 6*E*). Furthermore, a slightly higher phospho-Smad2 level was observed with TGF-β stimulation in *SPSB1* siRNA-transfected cells (Fig. 6*C*).


A
Dox
TGF-β
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IP: FLAG
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IP: FLAG
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IB: TβRII
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75
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B
Dox
kD
IB: TβRI
IB: TβRII
IB: FLAG
IB: Actin
50
75
28
50
C
TGF-β
siRNA:
SPSB1
Control
SPSB1
Control
kD
IB: TβRII
75
Relative ratio to actin:
0.89 0.82 0.52 0.44
0.84 0.72 0.50 0.38
IB: Actin
50
IB: p-Smad2
50
Relative ratio to smad2:
0.73 0.63 0.55 0.40
IB: Smad2
50
D
Relative SPSB1 mRNA level
SPSB1 siRNA
Control siRNA
TGF-β
E
Fold of induction
SPSB1 siRNA
Control siRNA
TGF-β
F
Fold of induction
SPSB1 siRNA
Control siRNA
TGF-β
G
Fold of induction
SPSB1 siRNA
Control siRNA
TGF-β
H
-TGF-β
+TGF-β
SPSB1 siRNA
Control siRNA
I
Relative percentage of positive cells
SPSB1 siRNA
Control siRNA
TGF-β
Relative intensity per cell
SPSB1 siRNA
Control siRNA
TGF-β

Similar results were also obtained in 293T and MDA231 cells (Fig. 6, *F* and *G*). These data demonstrate the involvement of endogenous SPSB1 in regulation of TGF-β signaling.

Both the *pCAGA-luc* reporter assay and the Western blotting were collectively analyzing TGF-β signaling in a pool of cells. To analyze signaling at the single cell level, we generated a new TGF-β/Smad3 reporter (*pCAGA-tdTomato*) using the much enhanced red fluorescence tdTomato gene, instead of luciferase, and subcloned it into an adenovirus expression vector (*Ad-CAGA-tdTomato*). This allowed TGF-β signaling activation to be visualized in individual live cells and quantified by the red fluorescence intensity. Interestingly, only a subpopulation of U87MG tumor cells displayed active TGF-β signaling (Fig. 6*H*) when cells were infected with *Ad-CAGA-tdTomato* virus at a multiplicity of infection of 2000, whereas the same multiplicity of infection of *eGfp* expression adenovirus delivered almost 100% infection efficiency (data not shown). Of more interest was the observation that, with *SPSB1* siRNA knockdown, the percentage of cells with active TGF-β signaling was unchanged; however, the signaling intensity increased substantially (Fig. 6*I*). Thus, silencing SPSB1 enhances TGF-β signaling in individual live cells.

Silencing SPSB1 Enhances TGF-β-mediated Cancer Cell Migration and Invasion—TGF-β signaling is known to drive tumor cell migration and invasion (60, 61). To examine whether the SPSB1 modulation of TGF-β/TβRII signaling impacts on a cellular response to TGF-β, we again silenced *SPSB1* in U87MG cells using siRNA and performed a wound healing assay. In this assay with control siRNA, the U87MG cells failed to close the wound area within 24 h, whereas TGF-β treatment promoted more cell scattering (Fig. 7*A*). In contrast, silencing *SPSB1* significantly enhanced U87MG cell migration, and TGF-β stimulation further promoted closing of the wound (Fig. 7*A*). An invasion assay using a Matrigel-coated transwell chamber was used to better quantify the TGF-β-mediated changes in cell migration. In the absence of TGF-β, very few U87MG cells invaded through the Matrigel to the other side of chamber membrane, whereas the invading cells increased ~4-fold with TGF-β treatment (Fig. 7*B*). In contrast, silencing *SPSB1* with siRNA substantially increased the number of Matrigel-invading cells, to ~14-fold in the presence of TGF-β (Fig. 7*B*). Taken together, silencing SPSB1 enhances TGF-β-mediated migration and invasion.

Discussion

The regulation of the TGF-β signaling pathway involves a variety of physiological regulators. Many of these molecules (Cdk4, Bambi, Ras, Ppm1a/Pp2ca, Smurf1, and P300 among others) act to alter the activity of Smad proteins either by regulating their stability, preventing their ability to affect transcription, or modifying their phosphorylation state (22, 62–65). In contrast, the number of molecules known to affect the TGF-β signaling pathway at the receptor level is relatively low, although the repertoire is expanding. Bambi, a TβRI-related protein lacking a cytoplasmic kinase domain, acts as a pseudoreceptor by binding endogenous TβRI and TβRII, preventing receptor activation (65, 66). TrkC, a member of the Trk family of neurotrophin receptors and Etv6-Ntrk3, a chimeric tyrosine kinase, bind to TβRII and suppress TβRII from activating TβRI (67, 68). A cholesterol-rich membrane microdomain marker caveolin-1 associates with TβRI, leading to the internalization of TβRI into caveolin-1 positive vesicles and its subsequent degradation via the proteasome pathway (69, 70). Smad7, the inhibitory Smad, can mediate the protein degradation of TβRI after association with Smurf1/2 (E3 ubiquitin regulatory factor) (71). Furthermore, Par6 mediates the TGF-β-induced tight junction dissolution during EMT (epithelial-to-mesenchymal transition) by associating with TβRII (72). However, direct regulation of TβRII has not been reported until recently, when the disintegrin and metalloproteinase Adam12 was demonstrated to stabilize the TβRII by inducing its accumulation in the early endosomal vesicles (73).

In this study, we provide the first experimental evidence that SPSB1, a Spry domain-containing Socs box protein, is a new component of the TGF-β signaling pathway, which negatively modulates TGF-β signaling by targeting TβRII. An interaction between SPSB1 and TβRII is supported by both the co-immunoprecipitation and immunofluorescence-based co-localization studies (Figs. 2*D* and 3*B*). Unlike Adam12, which stabilizes the TβRII by inducing a change in its localization from cell membrane to the early endosomes, SPSB1 is recruited from the cytoplasm to the cell membrane (Fig. 3*B*) to destabilize the type II receptor, displaying a distinct regulatory mechanism. We also determined that Asn-236 and Asn-238 in the TβRII cytoplasmic domain are required for interaction and co-localization with SPSB1 (Fig. 2*F*). Because this sequence (NINHNT) is somewhat similar to the (D/E)(I/L)NNN*X* sequence motif pres-

FIGURE 6. **SPSB1 interacts with and down-regulates endogenous TβRII.** NIH3T3 cells (*A* and *B*) containing a stably transfected doxycycline-inducible FLAG-SPSB1 construct were cultured with or without doxycycline (2 μg/ml) for 48 h to induce SPSB1 expression. After 16 h, cells were treated with or without TGF-β (2 ng/ml) as indicated. U87MG cells (*C*) were transfected with 20 nM control or SPSB1 siRNA for 72 h. After the initial 48 h, cells were treated with or without TGF-β (2 ng/ml) as indicated and lysed. *C*, Whole cell lysates were run in duplicate and examined for indicated proteins by immunoblotting (*IB*). Relative intensity was determined and presented as a ratio of TβRII to actin and phospho-Smad2 to total Smad2 expression. *D*, successful SPSB1 knockdown was confirmed in U87MG cells by analysis of SPSB1 mRNA expression. U87MG cells (*E*) or 293T cells (*F*) or MDA231 cells (*G*) were co-transfected with 20 nM control or SPSB1 siRNA and pCAGA12-luc for 48 h. Cells were treated with or without TGF-β (2 ng/ml) for a further 24 h and then lysed, and luciferase activity was determined as described in the legend to Fig. 1. Data are expressed as mean relative Smad3 luciferase activity (-fold induction), and *error bars* represent S.D. from representative experiments performed three times. *, $p < 0.05$. U87MG (*H*) cells were transfected with 20 nM control or SPSB1 siRNA and *pCAGA*$_{12}$*-luc* for 24 h and subsequently infected with adenovirus carrying TGF-β-driven tdTomato expression (*pAd-CAGA-tdTomato*) for another 24 h. Cells were then treated with or without TGF-β (2 ng/ml) for a further 24 h. Live cell images were taken using a fluorescent microscope (magnification, ×20). Fluorescence-positive cells and total number of cells per image were counted manually. Total red fluorescent intensity per image was measured by using ImageJ. Relative percentage of fluorescence-positive cells was calculated as total fluorescence-positive cells per image/total number of cells per image. Relative cell fluorescence intensity was calculated as total red fluorescent intensity per image/total fluorescent positive cells per image. The average result obtained by using two sets of representative images is shown in *I*. Similar results were obtained by using all images taken. Statistical tests incorporated comparisons with control siRNA-transfected cells. *, $p < 0.05$. All experiments were repeated, and representative results are shown.

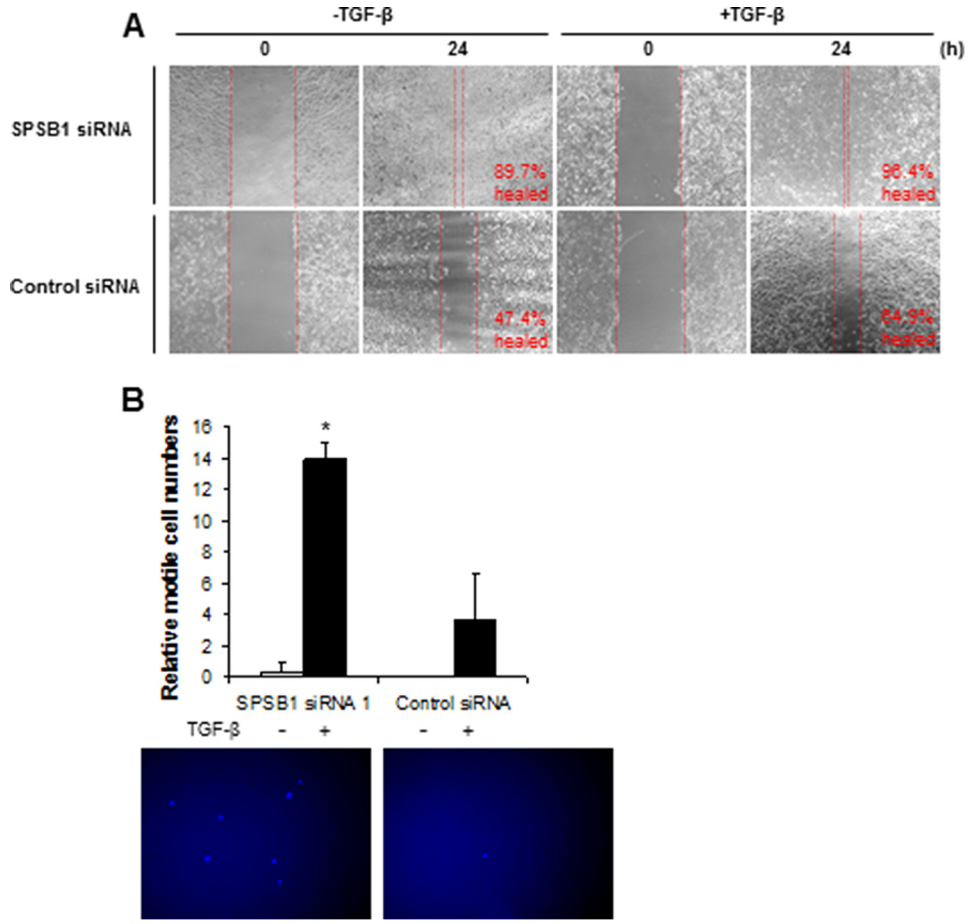

FIGURE 7. **Silencing SPSB1 in U87MG cells enhances cells migration and invasion.** U87MG cells were transfected with 20 nM control or SPSB1 siRNA for 48 h. *A*, cell monolayers were scratched as described under "Experimental Procedures" and treated with or without TGF-β (2 ng/ml), and phase-contrast images were recorded at 0 and 24 h post-scratching. Similar results were obtained in three independent experiments. *B*, following siRNA transfection, cells were collected and reseeded into the upper chamber of 8 μM pore Matrigel-coated transwell plates (Matrigel mixed 1:1 with DMEM, 70 μl/well) with or without TGF-β (2 ng/ml) as indicated for another 24 h. Cells that migrated to the bottom side of the upper chamber were then fixed and stained with Hoechst dye. Images were taken using a fluorescence microscope (magnification, ×20) in four random fields. *Bars*, means ± S.D. (*error bars*) of triplicate wells from one of three representative experiments.

ent in multiple target proteins (37, 57, 58), it is possible that this represents a direct interaction between SPSB1 and TβRII. Alternatively, another (as yet unknown) protein may act as an adaptor to bring SPSB1 to the TβRII complex. Furthermore, this interaction is TβRII-specific because TβRI alone was not associated with SPSB1 (Figs. 2*C* and 3*F*). The SPSB1-Spry domain mediates the SPSB1-TβRII interaction, because constructs that only express this domain (SPSB1Δ) retain binding to TβRII, whereas mutations in the domain (Y129A and T160A,Y161A) disrupted the SPSB1/TβRII interaction (Fig. 2*E*). Given the potential overlap in recognition of binding partners by SPSB1, SPSB2, SPSB4 and orthologues of Fsn (28, 57, 74), it is likely that additional, related SPRY domain containing proteins are capable of interacting with TβRII. On the other hand, the NINHNT sequence within the type II receptor differs from the known Spry interaction motifs ELNNNL or DINNN*X*, suggesting that the family of proteins interacting with the SPSB-Spry domain may be expanded.

The significance of this interaction is demonstrated by the observation that SPSB1 regulates TGF-β-induced Smad2 phosphorylation, Smad3 transcriptional activity, and a cellular response to TGF-β, such as cell migration (Figs. 1 (*B* and *C*) and *7*) in human (Fig. 1*D*), canine, and murine cell lines (Fig. 1, *E* and *F*). Furthermore, the inhibitory effect of SPSB1 was confirmed by depleting SPSB1 in cancer and non-cancer cell lines (Fig. 6, *E*, *F*, and *G*). This inhibition is mediated via the SPSB1-Socs box because this motif was required for the negative regulatory function of SPSB1 and the enhancement of TβRII ubiquitination levels and degradation rates (Figs. 1*G*, 4*B*, and 5*A*). Mutation of tyrosine 120 in the SPSB2 SPRY domain abolishes its interaction with Par-4 and iNos (28, 37), and the analogous SPSB1 mutant (Y129A) also abolished its interaction with TβRII, suggesting that the Spry interaction interface is the same or overlapping in each instance. Mutation of the Spry domain results in loss of the ability to enhance TβRII-associated ubiquitination, whereas TβRII mutants (TβRII(N236A) and TβRII(N238A)), which affect SPSB1 binding, maintain TβRII stability in the presence of SPSB1 (Figs. 4*C* and 5*B*). Importantly, silencing SPBS1 in U87MG cells enhances TβRII levels, indicating that SPSB1 has a physiological role in regulating TβRII signaling (Fig. 6*C*). This may explain the low levels of endogenous TβRI and TβRII in normal tissue and the rapid turnover of the TGF-β receptors by ligand-independent and ligand-dependent mechanisms (75–79). Taken together, our

results support a novel role for SPSB1 as an important negative regulator of the TGF-β signaling pathway. It regulates TGF-β signaling using a previously undiscovered mechanism by regulating TβRII ubiquitination and stability. It is particularly important to note that, unlike other TGF-β-induced genes, such as Smad7 which negatively feeds back to regulate its signaling in a dominate manner (1, 24), SPSB1 functions to fine tune TGF-β signaling.

Fine tuning TGF-β signaling can be critical physiologically, because the tight regulation of the TGF-β signaling pathway at every step is essential in homeostasis. This is strongly supported by the fact that perturbation of any of the signaling components along the cascade *in vivo* results in cancer formation in mice. For instance, deletion of one copy of the *Smad4* or *TGF-β1* gene resulted in gastric tumor formation in mice (6, 7). Similarly, overexpression of a dominant negative form of *TβRII* or the negative regulator *Smad7* as a transgene in mice also resulted in tumor formation (8, 9). This suggests that a complete blocking of the TGF-β signaling is not necessary for tumor formation. More recently, the threshold effect of TGF-β signaling in cancer development has been further demonstrated in $Gp130^{Y757F/+}$; $Smad3^{+/-}$ compound mice. It has been shown that $Gp130^{Y757F/+}$ heterozygous mice desensitize TGF-β signaling (~30%) via Stat3-mediated Smad7 expression, and $Smad3^{+/-}$ mice also result in ~30% reduction in TGF-β signaling. However, none of the single heterozygous mice develop tumors. Interestingly, the compound heterozygous mice that have further suppressed TGF-β signaling developed gastric tumors (10), demonstrating a signaling sensitivity threshold effect.

In fact, the threshold signaling sensitivity effect is also evident in the TGF-β-negative regulator-related cancers. For example, overexpression of Smurf2 can lead to a reduced level of TGF-β receptors and, maximally, a 60% decrease of TGF-β signaling activity *in vitro* (80). Transient Bambi overexpression in cells can inhibit TβRI and TβRII complex formation by up to 50% and reduce TGF-β signaling activity (18). Both overexpressed and endogenous Cdk4 can increase the phosphorylation level in the middle proline-rich region of Smad3 and reduce the Smad3-dependent TGF-β signaling activity *in vitro* (81). Although some TGF-β signaling persists, elevation of these negative regulators seems to be both crucial and sufficient for TGF-β-related cancer formation.

Despite the anti-tumor activity of TGF-β, the majority of human tumors have not suffered loss of function of TGF-β signaling components (82). During the process of carcinogenesis, tumor cells often become resistant to TGF-β-mediated growth arrest or apoptosis. Moreover, TGF-β has been shown to induce EMT in the late stage of carcinogenesis (83). It is now recognized that TGF-β actually has pro-oncogenic effects to mediate the metastasis of many different types of tumor cells in the context of advanced disease (84).

Thus, from a treatment point of view, neither up-regulation nor down-regulation of the TGF-β signaling activity drastically is desirable. As a result, modulating the threshold signaling sensitivity of TGF-β may be an effective strategy to treat cancer. SPSB1, as a novel modulator of the TGF-β signaling pathway, expands our understanding in the TGF-β threshold signaling sensitivity modulation. The ability of SPSB1 to negatively regulate (~30–50%) but not completely block TGF-β signaling via TβRII might be useful in treating tumor invasion. Whereas systemic inhibition or lack of TGF-β signaling results in acute inflammation and disruption of immune system homeostasis and other side effects, transgenic mice overexpressing SPSB1 showed no observable phenotypes (data not shown), presumably explained by the signaling threshold effect of TGF-β signaling and the modulating effect of SPSB1. In conclusion, we identified a new component of the TGF-β signaling pathway, which negatively modulates TGF-β signaling by inducing TβRII degradation. This small molecule, SPSB1, is the first direct TβRII negative regulator reported.

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