Supplemental Data

B-myb Positively Regulates Serine-Threonine Kinase Receptor-associated Protein (STRAP) Activity through Direct Interaction

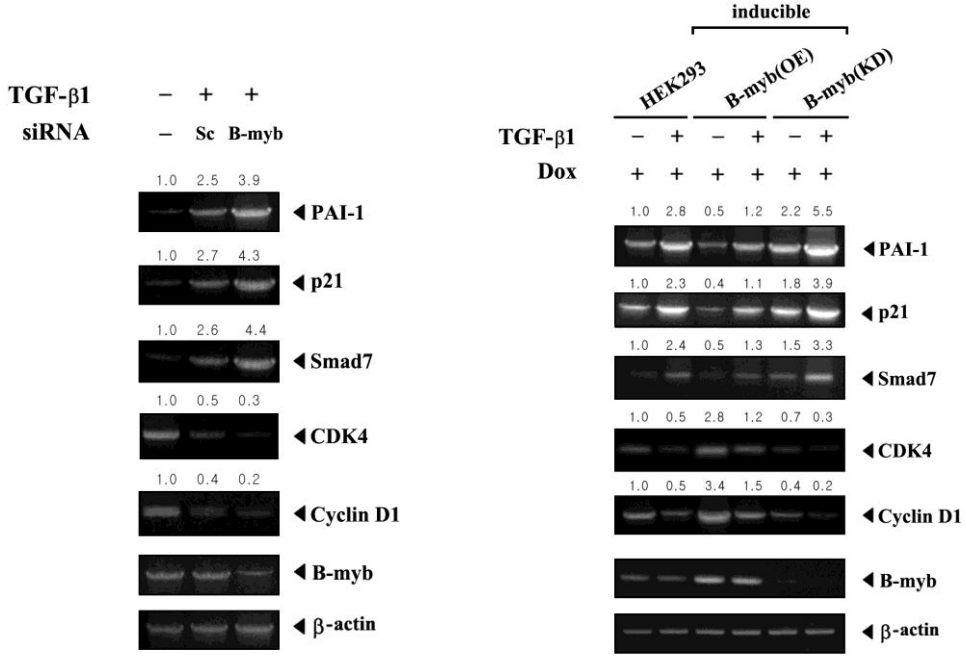
Hyun-A Seong, Ravi Manoharan, and Hyunjung Ha

Materials and Methods

Generation of Inducible STRAP(KD) and Smad7(KD) Cell Lines—An inducible STRAP shRNA HEK293 cell line [STRAP(KD)] has been described previously (5). An inducible Smad7 shRNA HepG2 cell line [Smad7(KD)] was generated using the following oligonucleotides: forward, 5'-TCGAGGGCTCAATTCGGACAACAAGTTCAAGAGACTTGTTGTCCGAATTGAGCCTTTTTTA-3'; and reverse, 5'-AGCTTAAAAAAGGCTCAATTCGGACAACAAGTCTCTTGAACTTGTTGTCCGAATTGAGCCC-3', as described previously (5). The Smad7 sequence was underlined.

RT-PCR—HepG2 or MCF7 cells were transiently transfected with control scrambled siRNA or B-myb-specific siRNA #1 as indicated, and then treated with TGF-β1 (100 pM, 20 h) or 5FU (0.38 mM, 30 h). Total RNA was isolated from transfected cells using easy-BLUE™ reagent according to the manufacturer's instructions (iNtRON Inc., Seongnam, Korea). 1-2 μg total RNA was reverse transcribed with RevertAid™ reverse transcriptase (Fermentas Inc., Glen Burnie, Maryland). The reaction was conducted at 42°C for 1 h. 2-5 μl cDNA mixture diluted 5-fold in DEPC-treated water was subjected to PCR. The reaction parameters were 5 min at 94°C, 1 cycle; 30 s at 94°C, 1 min at 50°C, 1.5 min at 72°C, 20-30 cycles; 5 min at 72°C, 1 cycle. The primers used for PCR were as follows: for p21, forward 5'-GTCCGTCAGAACCCATGC-3', reverse 5'-TTAGGGCTTCCTCTTGGAGA-3'; for PAI-1, forward 5'-CAACTTGCTTGGGAAAGGAG-3', reverse 5'-GTGGAGAGGCTCTTGGTCTG-3'; for Smad7, forward 5'-CCAACTGCAGACTGTCCAGA-3', reverse 5'-TGCTGCATAAACTCGTGGTC-3'; for CDK4, forward 5'-GGCCCTCAAGAGTGTGAGAG-3', reverse 5'-CCAACACTCCACATGTCCAC-3'; for Cyclin D1, forward 5'-CGTGGCCTCTAAGATGAAGG-3', reverse 5'-TCCTCCTCTTCCTCCTCCTC-3'; for p53, forward 5'-TCCCAAGCAATGGATGATTT-3', reverse 5'-ACACGCAAATTTCCTTCCAC-3'; for Mdm2, forward 5'-AGATTCCAGCTTCGGAACAA-3', reverse 5'-GTGGCGTTTTCTTTGTCGTT-3'; for Bax, forward 5'-GCCGTGGACACAGACTCC-3', reverse 5'-AACCACCCTGGTCTTGGAT-3'; for β-actin , forward 5'-GGCATCCTCACCCTGAAGTA-3', reverse 5'-CCATCTCTTGCTCGAAGTCC-3'; for B-myb, forward 5'-CCCTTCAAACTCTTCCAGCC-3', reverse 5'-ATGAGAATGGGCTCGTGACA-3'.

A


TGF-β1 − + +
siRNA − Sc B-myb
1.0 2.5 3.9
PAI-1
1.0 2.7 4.3
p21
1.0 2.6 4.4
Smad7
1.0 0.5 0.3
CDK4
1.0 0.4 0.2
Cyclin D1
B-myb
β-actin
inducible
HEK293
B-myb(OE)
B-myb(KD)
TGF-β1 − + − + − +
Dox + + + + + +
1.0 2.8 0.5 1.2 2.2 5.5
PAI-1
1.0 2.3 0.4 1.1 1.8 3.9
p21
1.0 2.4 0.5 1.3 1.5 3.3
Smad7
1.0 0.5 2.8 1.2 0.7 0.3
CDK4
1.0 0.5 3.4 1.5 0.4 0.2
Cyclin D1
B-myb
β-actin

B

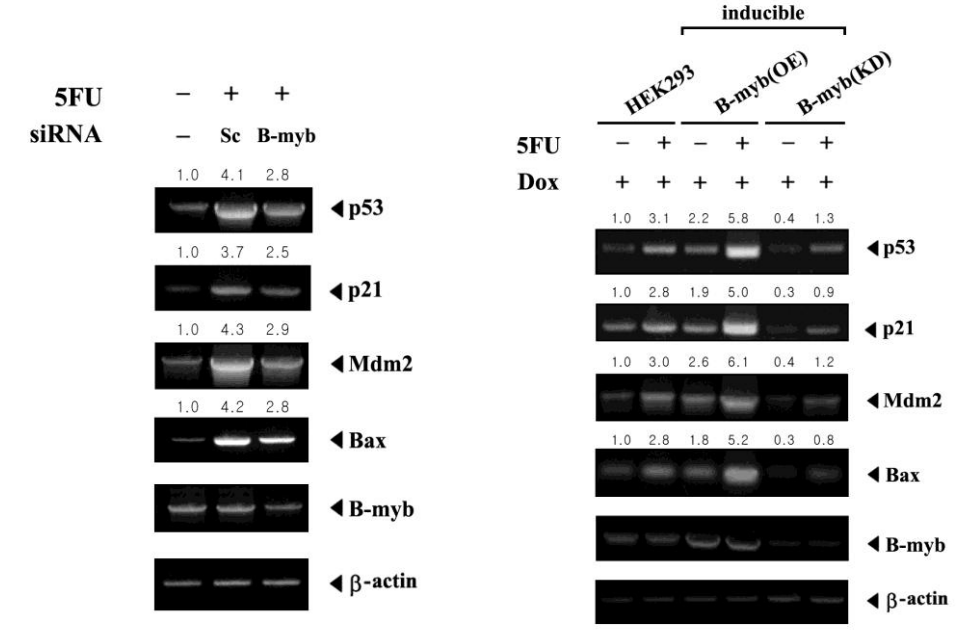
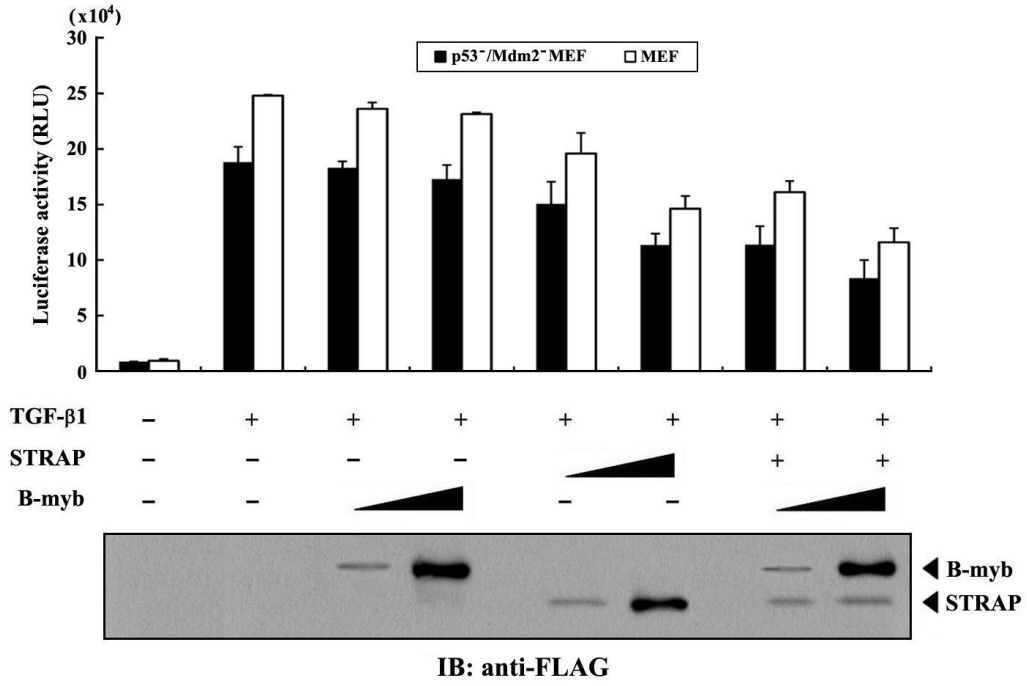

5FU − + +
siRNA − Sc B-myb
1.0 4.1 2.8
p53
1.0 3.7 2.5
p21
1.0 4.3 2.9
Mdm2
1.0 4.2 2.8
Bax
B-myb
β-actin
inducible
HEK293
B-myb(OE)
B-myb(KD)
5FU − + − + − +
Dox + + + + + +
1.0 3.1 2.2 5.8 0.4 1.3
p53
1.0 2.8 1.9 5.0 0.3 0.9
p21
1.0 3.0 2.6 6.1 0.4 1.2
Mdm2
1.0 2.8 1.8 5.2 0.3 0.8
Bax
B-myb
β-actin

FIG. S1. **Effect of B-myb on the mRNA expression of TGF-β and p53 targets.** HepG2 (*A*) or MCF7 (*B*) cells were transiently transfected with control scrambled siRNA (*Sc*) or B-myb-specific siRNA #1 (*B-myb*) as indicated, and then treated with TGF-β1 (100 pM, 20 h) or 5FU (0.38 mM, 30 h) (*left panels*). HEK293 cells harboring stably integrated pcDNA4™/TO/myc-HisA vector containing wild-type B-myb [*inducible B-myb(OE)*] or pSingle-tTS-shRNA vector containing B-myb-specific shRNA [*inducible B-myb(KD)*] treated with TGF-β1 (100 pM, 20 h) or 5FU (0.38 mM, 30 h) were cultured in the presence or absence of 1 μg/ml doxycycline (*Dox*) for 72 h (*right panels*). The mRNA expression of TGF-β (*A*) or p53 (*B*) targets was analyzed by RT-PCR as described in "Materials and Methods". The amount of β-actin mRNA was used as a loading control.

A


(x10⁴)
30
25
20
15
10
5
0
Luciferase activity (RLU)
■ p53⁻/Mdm2⁻ MEF
□ MEF
TGF-β1 – + + + + + + +
STRAP – – – – + +
B-myb – – – – –
B-myb
STRAP
IB: anti-FLAG

B

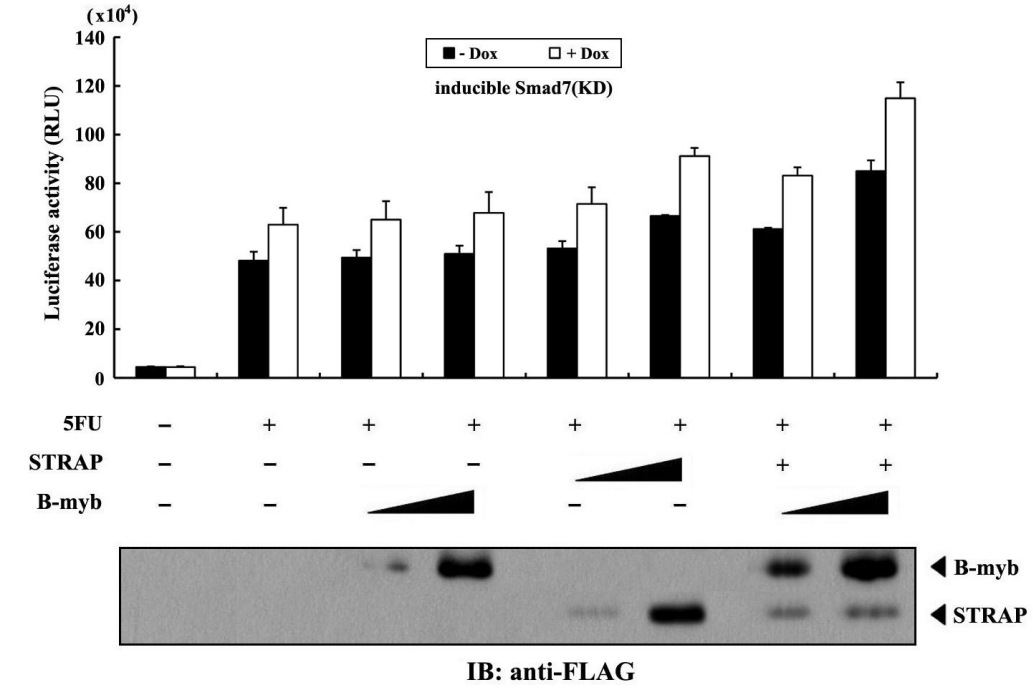
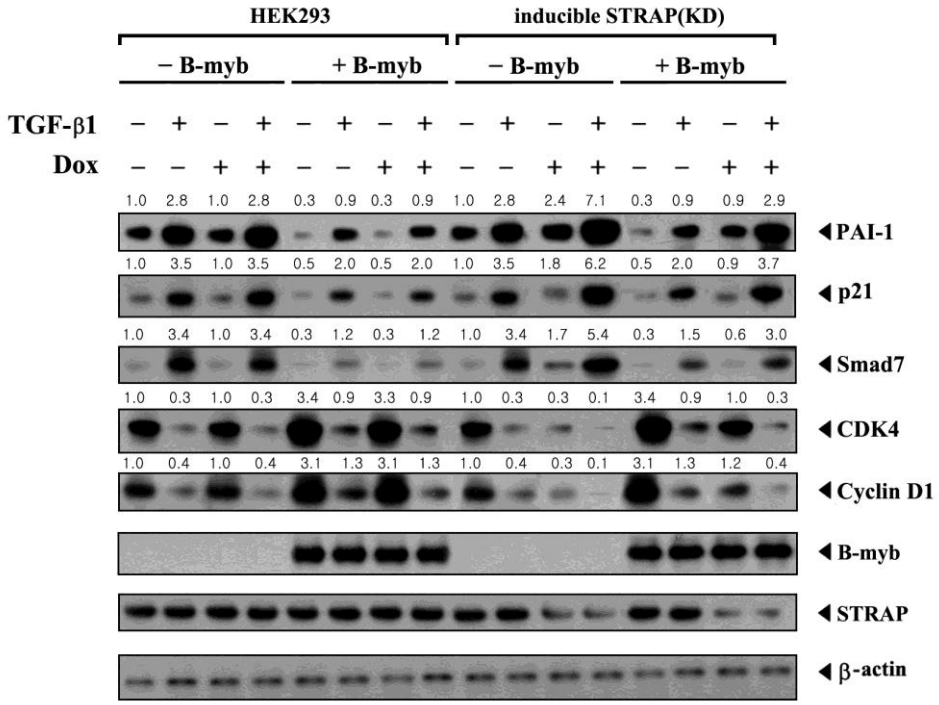

(x10⁴)
140
120
100
80
60
40
20
0
Luciferase activity (RLU)
■ - Dox
□ + Dox
inducible Smad7(KD)
5FU – + + + + + + +
STRAP – – – – + +
B-myb – – – – –
B-myb
STRAP
IB: anti-FLAG

FIG. S2. **B-myb-STRAP complex functions as an intermediate of cross-talk between the TGF-β and p53 signaling pathways.** *A*, effect of B-myb on STRAP-mediated inhibition of TGF-β-induced transcription in p53-null system. Wild-type MEF or p53/Mdm2 double null MEF cells (6) were transiently transfected with 0.3 μg p3TP-Lux reporter, 0.1 μg expression plasmid for β-galactosidase as an internal control, and increasing amounts (0.3 and 0.6 μg) of FLAG-tagged B-myb or STRAP, and subsequently stimulated by TGF-β1 (100 pM, 20 h) and 5FU (0.38 mM, 30 h). *B*, effect of B-myb on STRAP-mediated stimulation of p53 transactivation in Smad7-knockdown system. HepG2 cells harboring stably integrated pSingle-tTS-shRNA vector containing Smad7-specific shRNA [*inducible Smad7(KD)*] were transfected with increasing amounts (0.3 and 0.6 μg) of FLAG-tagged B-myb or STRAP as indicated, together with 0.2 μg p53-Luc reporter plasmid and 0.1 μg β-galactosidase internal control, in the presence of TGF-β1 (100 pM, 20 h) and 5FU (0.38 mM, 30 h), and cultured in the presence or absence of 1 μg/ml doxycycline (*Dox*) for 72 h. Luciferase activity was measured 48 h after transfection and normalized to β-galactosidase activity. The expression level of FLAG-tagged B-myb and STRAP was analyzed by anti-FLAG antibody immunoblot.

A


HEK293
inducible STRAP(KD)
− B-myb
+ B-myb
− B-myb
+ B-myb
TGF-β1 − + − + − + − + − + − + − + − +
Dox − − + + − − + + − − + + − − + +
1.0 2.8 1.0 2.8 0.3 0.9 0.3 0.9 1.0 2.8 2.4 7.1 0.3 0.9 0.9 2.9
PAI-1
1.0 3.5 1.0 3.5 0.5 2.0 0.5 2.0 1.0 3.5 1.8 6.2 0.5 2.0 0.9 3.7
p21
1.0 3.4 1.0 3.4 0.3 1.2 0.3 1.2 1.0 3.4 1.7 5.4 0.3 1.5 0.6 3.0
Smad7
1.0 0.3 1.0 0.3 3.4 0.9 3.3 0.9 1.0 0.3 0.3 0.1 3.4 0.9 1.0 0.3
CDK4
1.0 0.4 1.0 0.4 3.1 1.3 3.1 1.3 1.0 0.4 0.3 0.1 3.1 1.3 1.2 0.4
Cyclin D1
B-myb
STRAP
β-actin

B

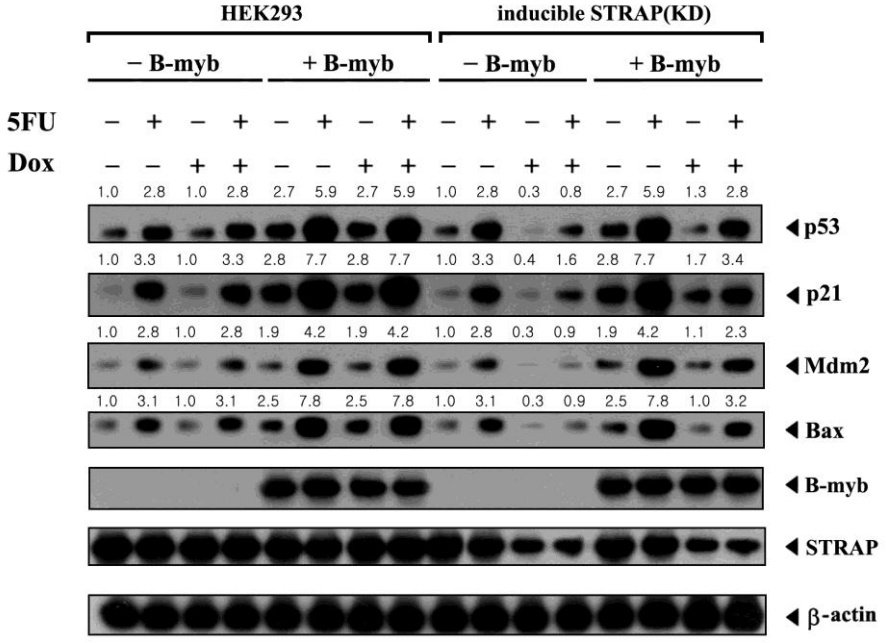

HEK293
inducible STRAP(KD)
− B-myb
+ B-myb
− B-myb
+ B-myb
5FU − + − + − + − + − + − + − + − +
Dox − − + + − − + + − − + + − − + +
1.0 2.8 1.0 2.8 2.7 5.9 2.7 5.9 1.0 2.8 0.3 0.8 2.7 5.9 1.3 2.8
p53
1.0 3.3 1.0 3.3 2.8 7.7 2.8 7.7 1.0 3.3 0.4 1.6 2.8 7.7 1.7 3.4
p21
1.0 2.8 1.0 2.8 1.9 4.2 1.9 4.2 1.0 2.8 0.3 0.9 1.9 4.2 1.1 2.3
Mdm2
1.0 3.1 1.0 3.1 2.5 7.8 2.5 7.8 1.0 3.1 0.3 0.9 2.5 7.8 1.0 3.2
Bax
B-myb
STRAP
β-actin

FIG. S3. **STRAP is required for B-myb-mediated regulation of TGF-β and p53 signaling.** *A*, effect of STRAP on the expression of TGF-β target genes. Parental HEK293 or HEK293 cells harboring stably integrated pSingle-tTS-shRNA vector containing STRAP-specific shRNA [*inducible STRAP(KD)*] cells were transfected with FLAG-B-myb as indicated, and then treated with 100 pM TGF-β1 for 20 h and cultured in the presence or absence of 1 μg/ml doxycycline (*Dox*) for 72 h. Cell lysates were subjected to immunoblot analysis using anti-PAI-1, anti-p21, anti-Smad7, anti-CDK4, anti-cyclin D1, anti-FLAG, anti-STRAP, and anti-β-actin antibodies. *B*, effect of STRAP on the expression of p53 target genes. Parental HEK293 or inducible STRAP(KD) cells were transfected with or without FLAG-B-myb, and then treated with 0.38 mM 5FU for 30 h and cultured in the presence or absence of 1 μg/ml doxycycline (*Dox*) for 72 h. Cell lysates were subjected to immunoblot analysis using anti-p53, anti-p21, anti-Mdm2, anti-Bax, anti-FLAG, anti-STRAP, and anti-β-actin antibodies. The relative level of the expression of TGF-β and p53 target genes was quantified by densitometric analysis. The fold increase relative to untreated HEK293 cells in the absence of B-myb was calculated.

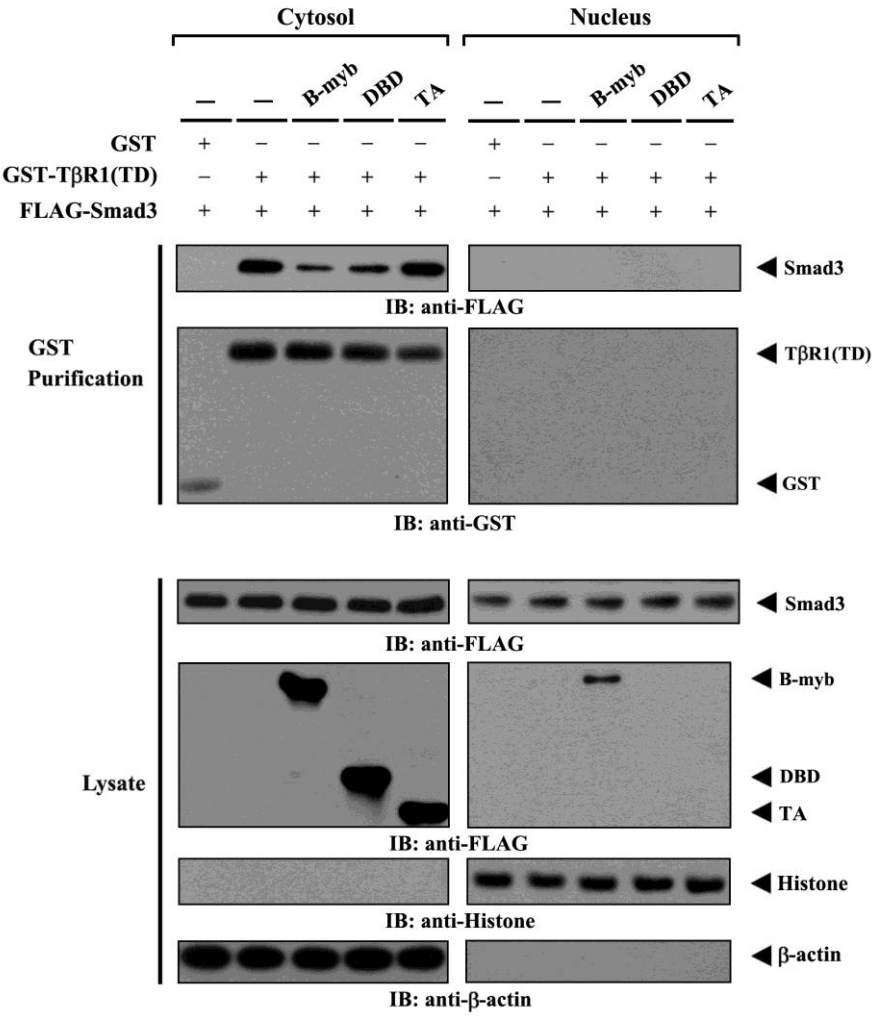


FIG. S4. **B-myb decreases the activated type I TGF-β receptor-Smad3 complex formation in the cytoplasm.** Cytoplasmic and nuclear fractions from the extracts of HEK293 cells transfected with the indicated combinations of plasmid vectors expressing GST-TβR1(TD), FLAG-Smad3, and FLAG-tagged wild-type B-myb or its deletion mutants DBD and TA were separated from each other, and the each fraction was subjected to precipitation with glutathione-Sepharose beads (*GST purification*). Complex formation between TβR1(TD) and Smad3 was analyzed by anti-FLAG antibody immunoblot (*top panels*). *Cytosol* and *Nucleus* indicate the cytoplasmic and nuclear fractions of cell extracts, respectively.