Supplemental Information

FoxM1 sustains Smad3/4 complex and controls TGF-β-signaling and cancer metastasis

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Inventory of Supplemental Information

1. Supplemental Data

2. Supplemental Experimental Procedures

1. Supplemental Data

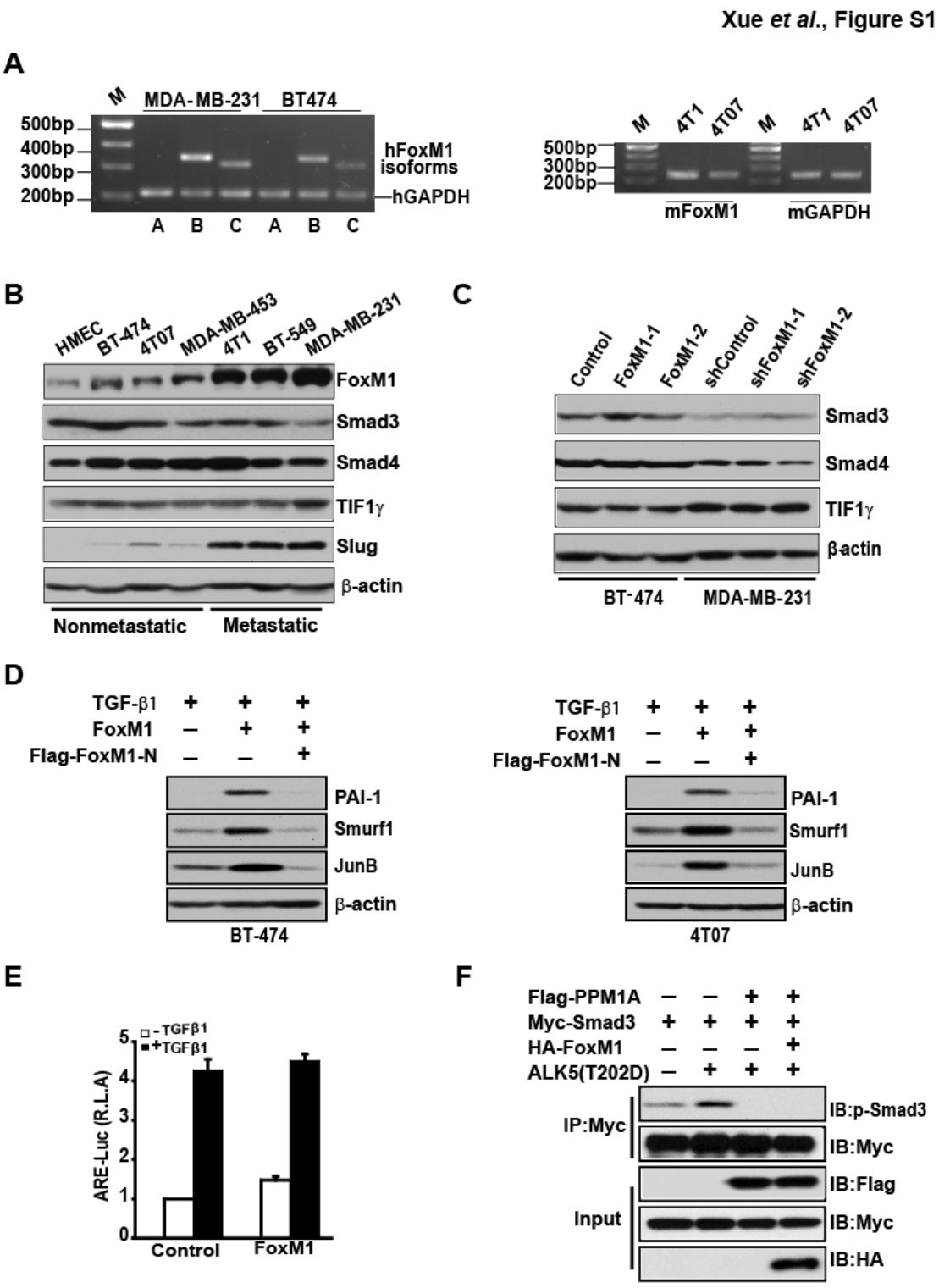


Figure S1

FoxM1 expression in human breast cancer cell lines, *Related to Figure 1*. (**A**) Left

panel: *FoxM1A*, *FoxM1B,* or *FoxM1C* mRNA expression in human breast cancer MDA-MB-231 and BT474 cells was analyzed by semiquantitative RT-PCR using the specific primers for human FoxM1A, FoxM1B, or FoxM1C. Right panel: Mouse *FoxM1* (mFoxM1) expression in mouse breast cancer 4T1 and 4T07 cells was analyzed by semiquantitative RT-PCR using the specific primers for mouse FoxM1. (Note that the *FoxM1A* mRNA was not detected in the human cell lines and that the levels of *FoxM1B* were higher than the levels of *FoxM1C*. 4T1 and 4T07 cells expressed mouse *FoxM1*). (**B**) Western blotting of total Slug, FoxM1, TIF1γ, Smad3, and Smad4 proteins in 7 cell lines, including human mammary epithelial cell line HMEC, 3 nonmetastatic breast cancer cell lines, and 3 metastatic breast cancer cell lines. (**C**) Cell lysates were extracted from BT-474 control and FoxM1 overexpression (FoxM1-1, and -2) cells, MDA-MB-231 shControl, and shFoxM1 (shFoxM1-1 or -2) cells. The indicated protein expression in these cells was analyzed by immunoblotting. (**D**) 4T07 or BT474 control (-) and FoxM1 overexpression (+) cells were transfected with Flag-FoxM1 *N*-terminal mutant. After 48 h, the cells were incubated with or without TGF-β1 (5 ng/ml) for 2 h. Whole-cell extracts were prepared, and the expression levels of the indicated genes were analyzed by western blotting. (**E**) HaCaT cells were transfected with the ARE-Luc construct, a Smad2-specific luciferase reporter, pRL-TK reporter, and FoxH1 constructs together with control vector or the Flag-FoxM1 vector. After 28 h of transfection, the cells were treated with TGF-β1 (5 ng/ml) for another 20 h. Luciferase activity was then measured. Luciferase activity is shown as relative luciferase activity (RLA) to that in cells transfected with the control vector and without TGF-β1 treatment. Values are mean ± SD for triplicate samples from a representative experiment. (**F**) 293T cells were transfected with plasmids encoding PPM1A, Smad3, FoxM1, or ALK5-T202D. The phosphorylation of Smad3 was analyzed by immunoblotting using an anti-phospho-Smad3 antibody. The level of Smad3 in the immunoprecipitates was determined using an immunoblot with an anti-Myc antibody.

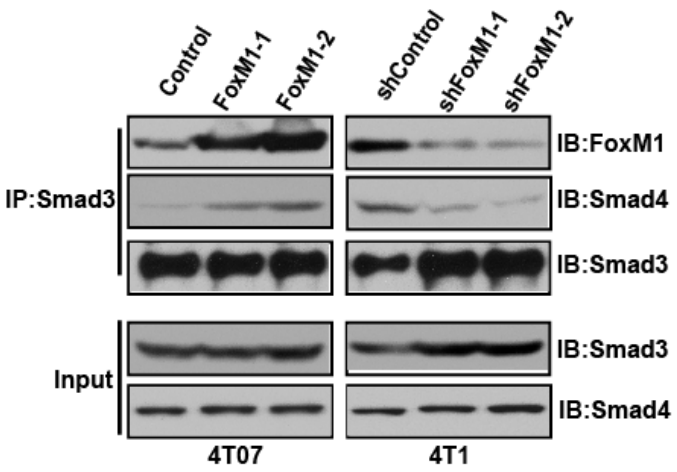


Figure S2

FoxM1 interacts with Smad3 in 4T07 and 4T1 cells, *Related to Figure 2*. Nuclear extracts were collected from 4T07 cells stably transfected with a control pcDNA3.1 vector or FoxM1 vector (FoxM1-1 or -2) and from 4T1 shControl or shFoxM1 stable cells. The nuclear extracts were then immunoprecipitated with anti-Smad3 antibodies. The resulting immunoprecipitated complexes were analyzed for expression of FoxM1, Smad3 or Smad4 by immunoblotting.

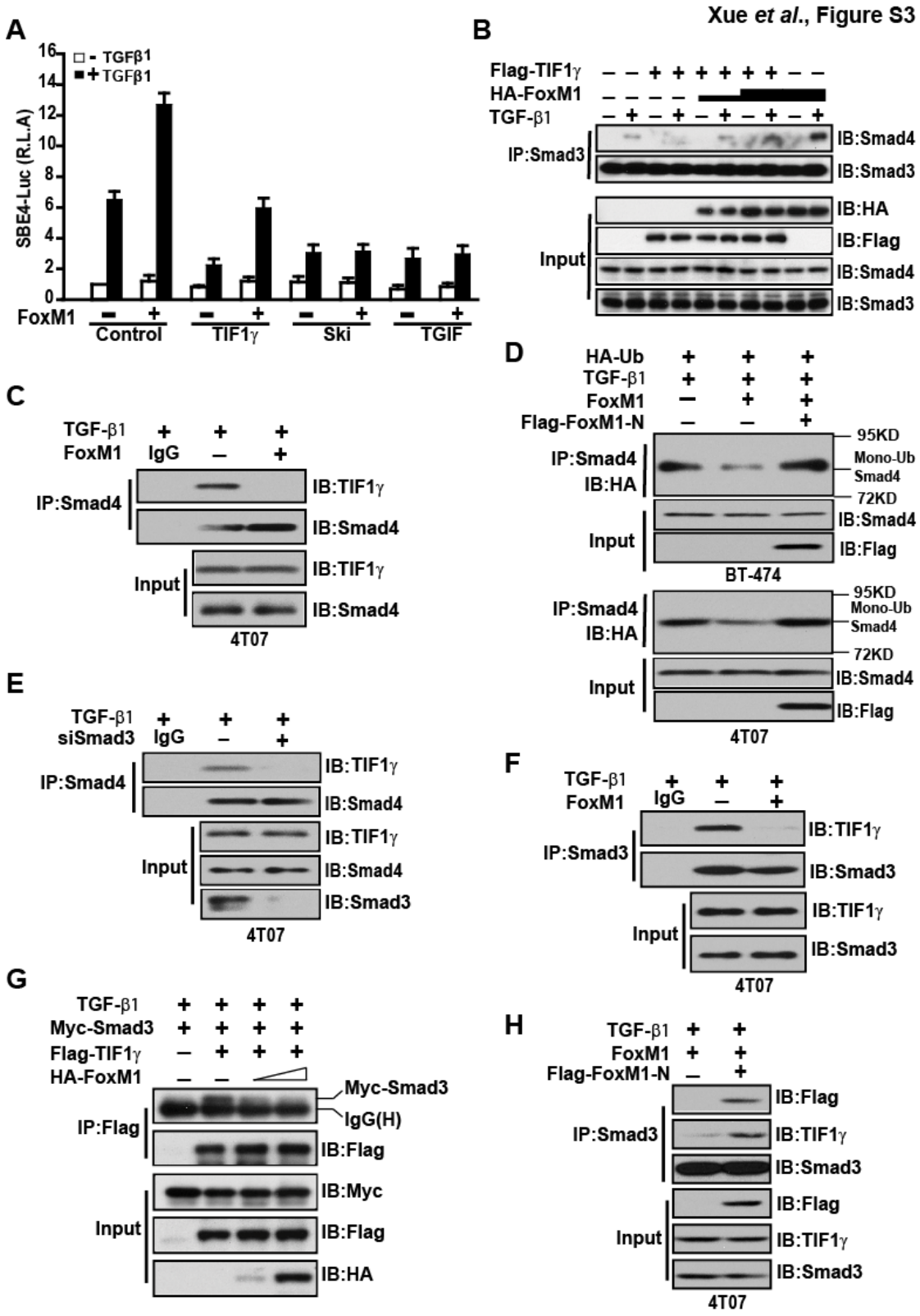


Figure S3

FoxM1 attenuates the inhibitory effects of TIF1γ on TGF-β signaling, *Related to*

Figure 6. (**A**) HaCaT cells were transfected with plasmids encoding FoxM1, TIF1γ, Ski, or TGIF, along with the SBE4-Luc and pRL-TK reporter constructs, and then treated with TGF-β1 (5 ng/ml) for 20 h before being harvested for luciferase assay. (**B**) HaCaT cells were transfected with plasmids encoding FoxM1 and TIF1γ expression constructs. The cells were harvested 48 h after treatment with TGF-β1 (5 ng/ml) for 2 h. (**C**) FoxM1 impaired the endogenous association of Smad4 with TIF1γ. 4T07 control (-) and FoxM1 overexpression (+) cells were harvested 48 h after treatment with TGF-β1 (5 ng/ml) for 2 h. (**D**) 4T07 or BT474 control (-) and FoxM1 overexpression (+) cells were transfected with Flag-FoxM1 *N*-terminal mutant. (**E**) 4T07 cells were transfected with control or Smad3-siRNA. The cells were harvested 48 h after treatment with TGF-β1 (5 ng/ml) for 2 h. (**F**) 4T07 control (-) and FoxM1 overexpression (+) cells were harvested 48 h after treatment with TGF-β1 (5 ng/ml) for 2 h. (**G**) Overexpression of FoxM1 prevented the association of Smad3 with TIF1γ. 293T cells were transfected with the indicated plasmids. (**H**) The N-terminal region of FoxM1, which can bind to Smad3, could not prevent TIF1γ from binding to Smad3. 4T07 FoxM1 overexpression cells were transfected with Flag-FoxM1 *N*-terminal mutant. Cells were harvested 48 h after treatment with TGF-β1 (5 ng/ml) for 2 h.

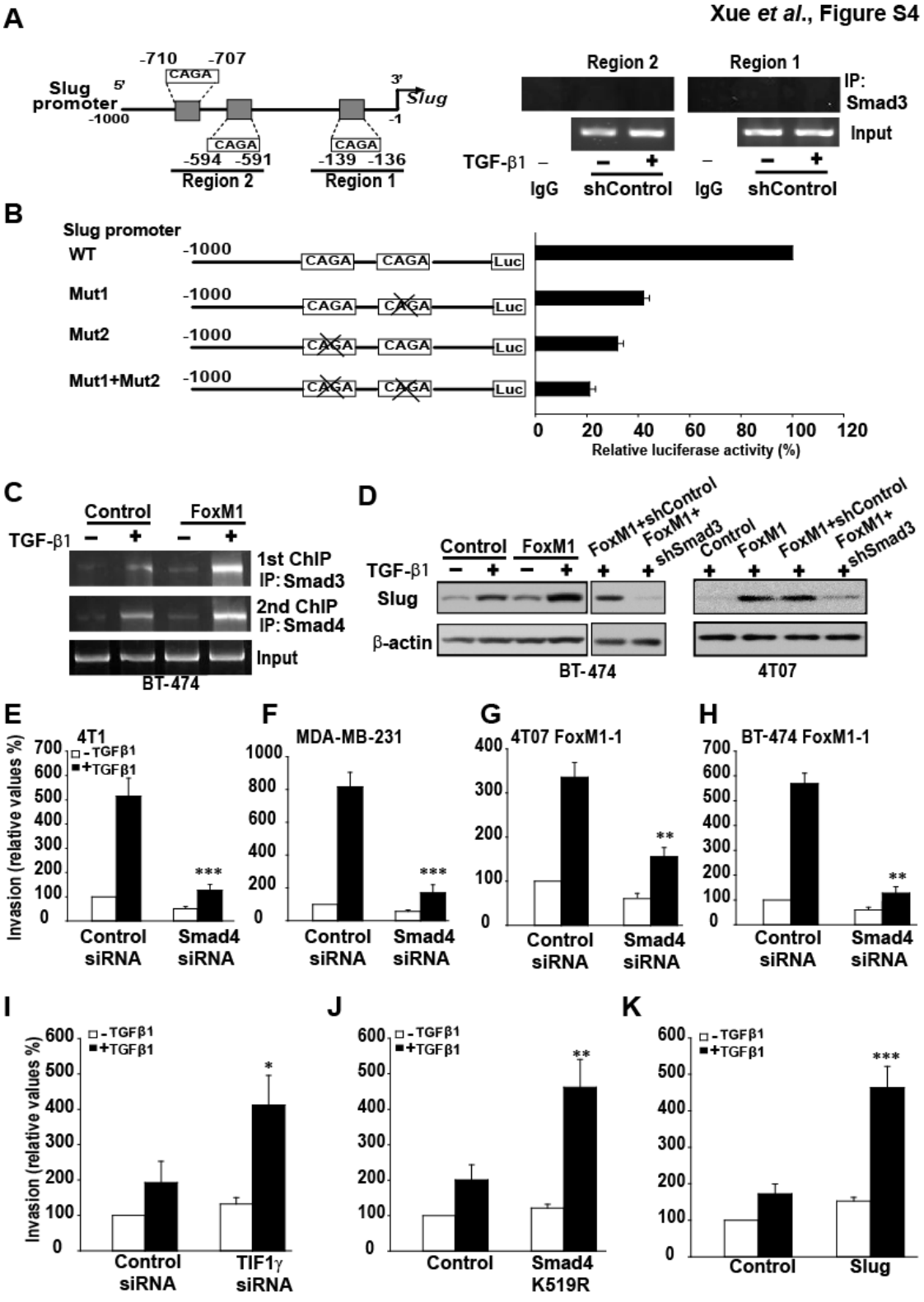
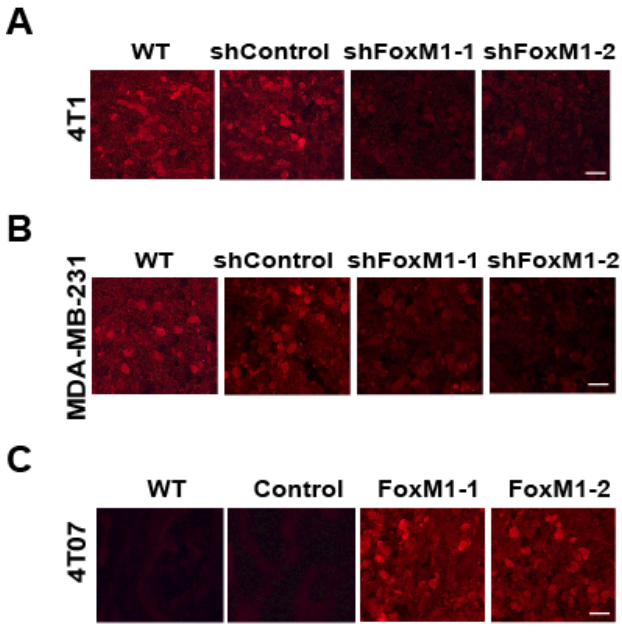


Figure S4

Overexpression of FoxM1 increases the binding of Smad3/Smad4 to the *Slug* promoter, and FoxM1 regulates cell invasion by affecting

TIF1γ–mediated inhibition of TGF-β signaling and depends on Smad4, *Related to Figure 8*. (**A**) Upper panel: diagram showing the putative Smad3-binding elements in the 1,000-bp genomic DNA sequence of the *Slug* promoter. Sequences of Smad3-binding element sites are shown. Lower panel: The binding of Smad3 to the *Slug* promoter could not be detected within a 200-bp region of the *Slug* promoter in the two regions as shown. ChIP assays were performed in MDA-MB-231 shControl cells that were incubated with or without TGF-β1 (5 ng/ml) for 24 h. (**B**) CAGA-box mutation reduces promoter activity. A luciferase assay was carried out as described for panel (E) in Figure 1 using a reporter gene construct with either wild-type promoter or the promoter with the mutated CAGA (TACA-mut)-box. MDA-MB-231 shControl cells were transfected with the indicated mutant luciferase reporters. Luciferase activity was measured 48 h after incubation of cells with TGF-β1 (5 ng/ml) for 20 h. (**C**) ChIP and re-ChIP assays were performed in BT-474 control and FoxM1 cells that were incubated with or without TGF-β1 (5 ng/ml) for 24 h. (**D**) Overexpression of FoxM1 promoted expression of *Slug* induced by TGF-β1. BT-474 or 4T07 control and FoxM1 cells or FoxM1 shControl and shSmad3 cells were incubated with or without TGF-β1 (5 ng/ml) for 24 h. Whole-cell extracts were prepared and Slug levels were analyzed by immunoblotting. (**E**) 4T1 cells were transfected with control siRNA or Smad4 siRNA and then were subjected to *in vitro* invasion assay with or without TGF-β1 treatment. ***$P < 0.001$. (**F**) MDA-MB-231 cells were transfected with control siRNA or Smad4 siRNA and then were subjected to *in vitro* invasion assay with or without TGF-β1 treatment. ***$P < 0.001$. (**G**) 4T07-FoxM1-1 cells were transfected with control siRNA or Smad4 siRNA and then were subjected to *in vitro* invasion assay with or without TGF-β1 treatment. **$P < 0.01$. (**H**) BT-474-FoxM1-1 cells were transfected with control siRNA or Smad4 siRNA and then were subjected to *in vitro* invasion assay with or without TGF-β1 treatment. **$P < 0.01$. (**I**) 4T1 shFoxM1 cells were transfected with control or siTIF1γ. The cells were treated with TGF-β1 and then subjected to invasion assay. *$P<0.05$. (**J**) 4T1 shFoxM1 cells were transfected with control or Smad4 K519R mutant plasmids. The cells were treated with

TGF-β1 and then subjected to invasion assay. ***P*<0.01. (**K**) 4T1 shFoxM1 cells were transfected with control or *Slug* expression vector. The cells were treated with TGF-β1 and then subjected to invasion assay. ****P*<0.001.



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Liver metastases		
Cell line	Incidence	Mean (range)
4T07–WT	0/10	0
4T07–Control	0/10	0
4T07–FoxM1-1	4/10	4.8 (0-10)*
4T07–FoxM1-2	2/10	15.6(0-23)**

Figure S5

FoxM1-Smad3 pathway promotes breast cancer metastasis, *Related to Tables 1 and 3.* (**A-C**) Sections of lung metastatic nodule tissue from mice injected with the indicated breast cancer cells were IF-stained with p-Smad3 antibody (*red*), followed by confocal microscopic analysis. Bar=20μm. (**D**) Overexpression of FoxM1 promoted liver metastasis. The mice were killed when they were moribund or on day 25 after mammary fat pad injection. Their livers were harvested, and the number of metastatic tumor nodules on each liver was counted and the mean number of nodules for each group was calculated. *$P < 0.05$ and **$P < 0.01$ compared with the parental (WT) group. Results are shown for one representative experiment of two.

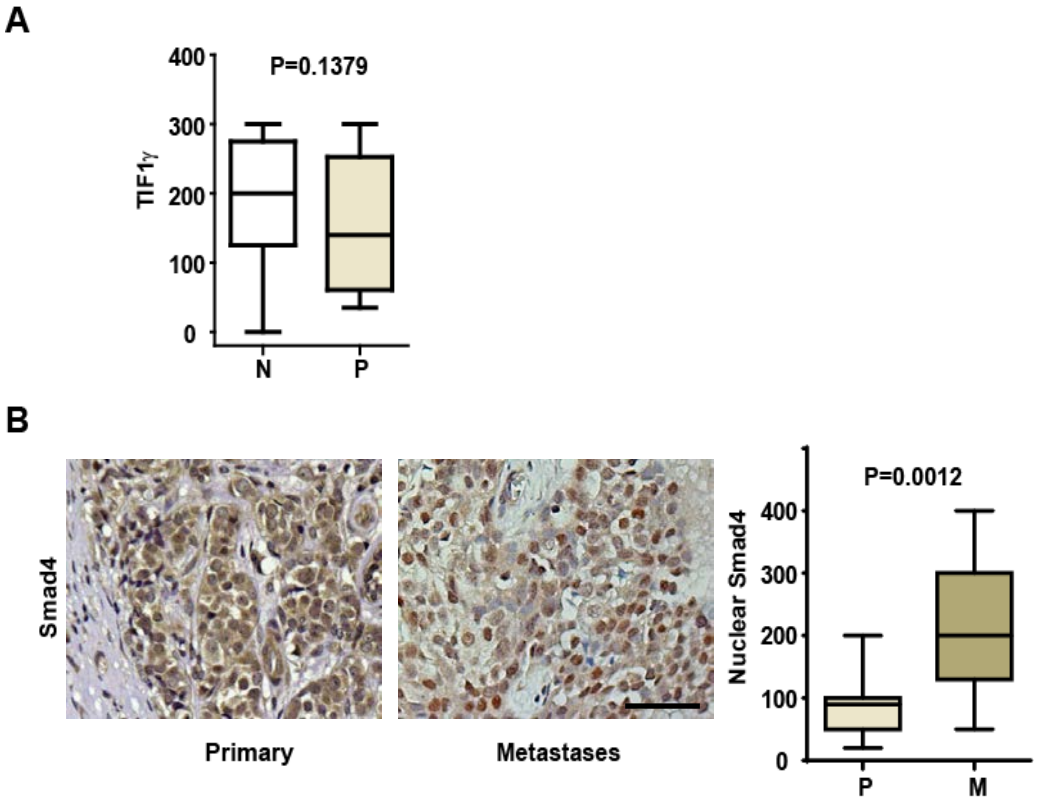

Figure S6

The expression of Smad4 and TIF1γ in human breast tissues, *Related to Figure 9.* (**A**) TIF1γ expression was slightly lower in human breast cancer tissues than in normal breast tissues. Expression of TIF1γ was examined via immunohistochemical staining in 12 human normal breast tissues (N) and 12 primary breast tumors (P). Boxes indicate interquartile range. Bars from each box extend to the largest and smallest observations. Levels of TIF1γ expression were slightly lower in primary breast cancer tissues than in normal breast tissues (P = 0.1379) according the chi-square test. (**B**) The expression of Smad4 in representative cases of primary breast tumors (P) and lymph node metastasis tissue specimens (M). Original magnification, ×400. Scale bars=200μm. Levels of nuclear Smad4 expression were significantly higher in lymph node metastasis tissues than in primary breast cancer tissues (P = 0.0012) according the chi-square test.

2. Supplemental Experimental Procedures

Plasmids, siRNAs and reagents. Human FoxM1, FoxM1 mutants, sh-RNA-resistant FoxM1 (FoxM1-shR), or sh-RNA-resistant R286A/H287A (R286A/H287A-shR) lentivirus vectors were described previously (20). Smad3 mutant, Smad4 mutant, and PPM1A were generated by polymerase chain reaction (PCR). The expression vectors encoding Flag-Smad1, Flag-Smad6, and Flag-TGIF were obtained from Dr. Joan Massagué (Memorial Sloan-Kettering Cancer Center, New York, NY, USA). The plasmids encoding Flag-Smad2, Flag-Smad3, Flag-Smad4, Smad3N-Flag, Smad3NL-Flag, Smad3C-Flag, Smad4-Myc, and T202D-Flag were obtained from Dr. Rik Derynck (University of California-San Francisco, San Francisco, CA, USA). The Flag-Smad5 and ARE-Luc expression vectors were obtained from Dr. Jeffrey L. Wrana (University of Toronto, Toronto, Ontario, Canada). The HA-Ski and HA-SnoN expression vectors were obtained from Dr. Robert A. Weinberg (Massachusetts Institute of Technology, Boston, MA, USA). The SBE4-Luc and FoxH1 plasmids were obtained from Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD, USA), and Flag-TIF1γ was obtained from Dr. Stefano Piccolo (University of Padua, Padua, Italy). SMARTpool siRNA duplexes specific for human or mouse Smad3, Smad4, TIF1γ, and a nontargeting siRNA (siControl) were purchased from Dharmacon. Recombinant human TGF-β1 was purchased from R&D Systems.

Antibodies. The following antibodies were from Sigma: anti-HA-tag (H3663; 1:1,000 IB, 2 μg immunoprecipitation), anti-Flag-tag (F1804; 1:1,000 IB, 2 μg IP), anti-Myc-tag (M4439; 1:1,000 IB, 2 μg IP). The following antibodies were from Cell

Signaling Technology: Smad3 (#9523; 1:1,000 IB), p-Smad3 (#9520; 1:1,000 IB), TGF-β receptor 1 (#3712; 1:1,000 IB), TGF-β receptor 2 (#3713; 1:1,000 IB), TIF1γ (#8972; 1:1,000 IB), Smurf1 (#2174; 1:1,000 IB), JunB (#3746; 1:1,000 IB), and Slug (#9585; 1:1,000 IB, 1:100 immunohistochemistry). The following antibodies were from Santa Cruz Biotechnology: FoxM1 (sc-500; 1:1,000 IB, 1:100 immunofluorescence), GAPDH (sc-137179; 1:1,000 IB), Lamin B (sc-6216; 1:1,000 IB), normal rabbit IgG (sc-2345; 2 μg IP), normal mouse IgG (sc-2025; 2 μg IP), β-actin (sc-47778; 1:5,000 IB), and Smad4 (sc-7966; 1:1,000 IB, 2 μg IP, 1:100 immunohistochemistry). The following antibodies were from BD Transduction Laboratories: Smad2/3 (610842; 1:100 immunofluorescence), PAI-1 (612024; 1:1,000 IB). FoxM1 (ab83097, Abcam; 1:100 immunohistochemistry), p-Smad3 (ab52903, Abcam; 1:100 immunohistochemistry), and TIF1γ (IHC-00216, Bethyl Laboratories; 1:100 immunohistochemistry).

Establishment of Stable Cell Lines. The human FoxM1 shRNA lentivirus vectors were described previously (20). The mouse FoxM1 shRNA lentivirus vectors were generated by ligation of vector PRNAT-U6.2 (GenScript) with oligonucleotides (5'-gatccccGGACCACTTCCCTTACTTTttcaagagaAAAGTAAGGGAAGTGGTCCtttttggaac-3') or (5'-gatccccGACGGAGGCTGCCCCTTCCttcaagagaGGAAGGGGCAGCCTCCGTCtttttggaac-3'). To establish shFoxM1 stable cell lines, metastatic breast cancer cells were transduced by using shFoxM1 lentiviruses with polybrene (6 μg/ml, Sigma). After 72 h of transduction, cells were selected with 500 μg/ml neomycin or

hygromycin (150 μg/mL) for 14 days. To establish FoxM1 overexpression stable cell lines, nonmetastatic breast cancer and HaCaT cells were transfected with pcDNA3.1-FoxM1B or control plasmids. To establish 4T07 FoxM1 or BT-474 FoxM1 shSmad3 stable cell lines, cells were transduced by using mouse or human shSmad3 lentiviruses (MISSION shRNA Lentiviral Particles, Sigma). To avoid clonal variation, stable cell lines were established from the mixed population of multiple clones.