Supplemental materials and methods

Cell culture

HEK-293T cells and mouse epithelial α-TN4 cells were from ATCC, cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 4.5 g/l glucose, 10% FBS, 50 U/ml penicillin, and 50 μg/ml streptomycin, and grown in a 5% CO_2 atmosphere at 37°C. Mouse breast cancer JygMC(A) cells and 4T-1 cells were described previously (Ehata et al, 2007).

Plasmid construction and RNA interference

The mammalian expression vector p3XFLAG-CMV-10 (Sigma-Aldrich) encoding δEF1 was described previously (Shirakihara et al., 2007). Mouse CtBP1 was cloned from cDNAs of NMuMG cells by a PCR-based strategy. Amplification was performed by Platinum PCR Super Mix High Fidelity Polymerase (Invitrogen, Carlsbad, CA). Putative sites of phosphorylation by Erk kinase resided at threonine 311 and serine 422, and novel phosphorylation sites were at serine 100, 144 and 158, and threonine 176 in mouse CtBP1. These sites were mutated to alanine by mutagenesis and confirmed by sequencing. siRNAs were transfected into cells according to the protocol recommended for HiPerFect reagent (Qiagen, Valencia, CA). NMuMG cells were transiently transfected with siRNAs against mouse FGFR1IIIc (Stealth RNAi; Invitrogen). The final concentrations of the siRNAs were 5 nM.

Reagents and antibodies

Rabbit polyclonal anti-phospho-Smad2, anti-phsopho-Smad1/5/8 and anti-phospho-Erk1/2 antibodies were obtained from Cell Signaling. Mouse monoclonal anti-Smad2/3 antibody was obtained from Millipore. Mouse monoclonal anti-FLAG M2 and anti-Myc antibodies were from Sigma-Aldrich and BD Pharmingen, respectively. Rabbit polyclonal SRF antibody and mouse anti-Ki67 antibody were from Abcam and Dako, respectively. PD98059 was from Calbiochem. TGF-β type I receptor kinase inhibitor A-44-03 was used as described previously (Ehata et al., 2007), and SB431542 was from Sigma-Aldrich.

Quantitative RT-PCR analyses

The primer sequences used in the present study are shown in Supplementary Table S1, and the extraction of total RNAs and quantitative RT-PCR analyses were performed as described in materials and methods.

***In vitro* binding assays**

δEF1 and CtBP1 were separately transfected into HEK-293T cells using Fugene 6 reagent (Roche Molecular Biochemicals). After 6 h of transfection, cells were treated with or without 30 ng/ml of FGF-2 in the presence of heparin. FLAG-δEF1 or FLAG-CtBP1 immunoprecipitated with anti-FLAG M2 antibody was washed once with an alkaline phosphate buffer (50 mM Tris-HCl, pH 8.0 and 1 mM $MgCl_2$) and incubated with 2 μl of alkaline phosphate (Roche Molecular Biochemicals) at 37°C in 50 μl of alkaline phosphate buffer. One h later, the immune complexes were washed once with

the same lysis buffer and further incubated for 1 h with the cell lysates prepared from HEK-293T cells transfected with Myc-CtBP1 or Myc-δEF1. The immune complexes were washed twice with the same lysis buffer, and subjected to SDS-PAGE, followed by immunoblotting analysis.

Cell motility assay

NMuMG cells were treated with TGF-β or both TGF-β and FGF-2 for 48 h and then transfected with δEF1 siRNA. After 24 h, wound was incised and photographs were taken immediately and 12 h after incision.

Luciferase Assays

α-SMA promoter (-1.4kb/+2.7kb) was prepared from NMuMG cells by PCR-based strategy. NMuMG cells were seeded in duplicate in 24-well tissue culture plates, followed by transient transfection with α-SMA promoter-Luc, Renilla reporter (Promega, Madison QI, USA) and δEF1 expression plasmids using Lipofectamine 2000 reagent (Invitrogen) as recommended by the manufacturer's instructions. Luciferase activity was determined by a dual luciferase reporter assay system (Promega) using a luminometer (AutoLumat LB953, EG&G Berthold, Natick, MA USA). Luciferase activity was normalized to sea-pansy luciferase activity of cotransfected pRL-CMV. δEF1 mutant that does not bind to CtBP1 was generated by point mutagenesis (Furusawa et al, 1999).

Detection of miRNAs

NMuMG cells were treated with TGF-β, FGF-2 or both for 4 days. Then, total RNAs including miRNA were extracted from cells using the mirVana miRNA Isolation kit (Applied Biosystems) and subjected to reverse transcription using the TaqMan MicroRNA RT kit (Applied Biosystems) according to the manufacturer's instructions. Quantitative analysis of changes in levels of expression was performed with the ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems). Values were normalized to U6.

Co-culture of NMuMG cells with cancer cells

NMuMG cells were cultured in Cell Culture Inserts with a 0.4-μm pore size (BD Falcon) and then allowed to settled on 6-well plates pre-seeded with cancer cells. The culture medium was changed every 3 days. After 10-14 days, NMuMG cells were fixed in a 1:1 acetone-methanol solution and stained with an anti-α-SMA antibody.

Supplemental Figure Legends

Supplemental Figure S1. Effects of FGF-2 on TGF-β signaling. **(A)** Immunoblot analysis of phospho-Smad2 (p-Smad2) and -Smad1/5/8 (p-Smad1/5/8). NMuMG cells were pretreated with or without the indicated ligands for 2 days and re-stimulated without (-) or with (+) 1 ng/ml TGF-β for 1 h. The cells were harvested and examined by immunoblot analysis. **(B)** Expression profiles of Smad7 and fibronectin following treatment with TGF-β, FGF-2, or both. NMuMG cells were treated with 1 ng /ml TGF-β, 30 ng/ml FGF-2, or both for the indicated periods, and levels of Smad7 and fibronectin mRNAs were determined by quantitative RT-PCR analysis. (**C**) NMuMG cells were treated with TGF-β, FGF-2, or both for the indicated periods, and levels of E-cadherin mRNA were determined by quantitative RT-PCR analysis. Values are normalized to housekeeping TBP, indicated as fold differences compared to non-treated cells, and represent the mean ± S.D. of duplicate determinations from a representative experiment. Similar results were obtained in at least three independent experiments (**B** and **C**). (**D**) After NMuMG cells were transfected with FGFR1IIIc siRNAs (#1 and #2) and then treated with TGF-β for 24 h, the expression levels of E-cadherin were determined by quantitative RT-PCR. Each value represents the mean ± S.D. of duplicate determinations from a representative experiment. Similar results were obtained in at least three independent experiments.

Supplemental Figure S2. Prevention of EMyoT of α-TN4 cells by FGF-2. (**A**)

Suppression of spontaneous expression of α-SMA in α-TN4 cells by FGF-2. NMuMG cells (upper panels) and α-TN4 cells (lower panels) were treated with 1 ng/ml TGF-β, 30 ng/ml FGF-2, or both for 4 days, and stained with anti-E-cadherin (red), anti-α-SMA (green), and propidium iodide (blue) to detect nuclei. (**B**) Inhibition of autonomously secreted TGF-β from α-TN4 cells by TGF-β type I receptor kinase inhibitor A-44-03. α-TN4 cells were treated with 1 ng/ml TGF-β, 30 ng/ml FGF-2, and 1 μM A-44-03, as indicated, for 4 days. Levels of Smad7 (left) and α-SMA (right) were determined by quantitative RT-PCR analysis. Stimulation of α-TN4 cells by TGF-β yielded only weak induction of Smad7 and α-SMA, while treatment with A-44-03 markedly decreased basal levels of expression of Smad7 and α-SMA. Each value represents the mean ± S.D. of duplicate determinations from a representative experiment. Similar results were obtained in at least three independent experiments. (**C**) After NMuMG cells were treated with TGF-β for 3 days, immunostaining analyses were performed with anti-α-SMA (red) and phopho-Erk1/2 (p-Erk, green) antibodies. (**D**) Expression of miR-200b following treatment with TGF-β, FGF-2, or both. NMuMG cells were treated with 1 ng/ml TGF-β, 30 ng/ml FGF-2, or both for 4 days, and levels of miR-200b were determined by semi-quantitative RT-PCR analysis. Each value represents the mean ± S.D. of duplicate determinations from a representative experiment. Similar results were obtained in two independent experiments.

Supplemental Figure S3. Interaction of CtBP1 with δEF1. (**A**) After NMuMG cells were incubated in culture media containing the indicated ligands and MEK/Erk

inhibitors (U0126 and PD98059), cell lysates were examined for protein concentrations and then subjected to immunoprecipitation with normal rabbit IgG (NRS) or an anti-δEF1 antibody. Co-purified CtBP1 was detected by immunoblotting with an anti-CtBP1 antibody (top panel). The expression levels of δEF1 and CtBP1 in the same lysates were verified by immunoblotting (lower two panels). **(B)** Both threonine 311 and serine 422 in CtBP1 were converted to alanine (CtBP1mut). HEK-293T cells were transfected with combinations of plasmids for 6Myc-δEF1 and either FLAG-CtBP1 or FLAG-CtBP1 mut, and incubated with 30 ng/ml FGF-2 (F24h) or 20 μM U0126 (U24h) for 24 h. The cell lysates were immunoprecipitated with anti-FLAG M2 antibody and subsequently immunoblotted with anti-Myc antibody (top panel). **(C)** *In vitro* binding of CtBP1 to δEF1. HEK-293T cells transfected with FLAG-CtBP1 or 6Myc-δEF1 were incubated with 30 ng/ml FGF-2 for 24 h. FLAG-CtBP1 was immunoprecipitated with normal mouse serum (NMS) or anti-FLAG M2 antibody, and the immune complexes were treated with or without alkaline phosphatase (AL-P) for 1 h at 37°C. After the cell lysates prepared from 6Myc-δEF1-transfected cells were added to the immune complexes, the aliquots were taken (bottom panel) and the immune complexes were incubated for an additional 1 h. (**D**) In addition to the phosphorylation sites of 311 and 422, other phosphorylation sites (100, 144, 158 and 176) were mutated to alanine. HEK-293T cells were transfected with combinations of plasmids for 6Myc-δEF1 and either FLAG-CtBP1 or FLAG-CtBP1 mutants, and incubated with 30 ng/ml FGF-2 (F24h) or 20 μM U0126 (U24h) in the presence of FGF-2 for 24 h. The cell lysates were immunoprecipitated with anti-FLAG M2 antibody and subsequently

immunoblotted with anti-Myc antibody (top panel). (**E**) *In vitro* binding of δEF1 to CtBP1. HEK-293T cells transfected with FLAG-δEF1 or 6Myc-CtBP1 were incubated with 30 ng/ml FGF-2 for 24 h. FLAG-δEF1 was immunoprecipitated with normal mouse serum (NMS) or anti-FLAG M2 antibody, and the immune complexes were treated with or without alkaline phosphatase (AL-P) *in vitro* for 1 h at 37°C. The cell lysates prepared from 6Myc-CtBP1-transfected cells were added to the immune complexes and incubated for an additional 1 h. (**F**) The expression of SRF was determined in NMuMG cells treated with indicated ligands for 48 h. (**G**) α-SMA promoter activity by δEF1. NMuMG cells were cotransfected with the indicated plasmids and α-SMA-promoter luciferase plasmids, and 24 later, the activities of α-SMA-promoter were measured. + and ++ indicate 0.3 μg and 1.0 μg expression plasmids of δEF1 for transfection, respectively. TGF-β; cells treated with TGF-β (1 ng/ml) for 24 h (right panel), δEF1mut; δEF1 mutant that does not bind to CtBP1. Each value represents the mean ± S.D. of duplicate determinations from a representative experiment. Similar results were obtained in two independent experiments.

Supplemental Figure S4 Cell proliferation of MCF-7 cells in coculture with NMuMG cells. Immunostaining analysis with human specific anti-Ki67 antibody (green) was performed with specimens of *in vitro* three-dimension invasion assays. TOTO3 was used for detection of nuclei (blue). White dot lines, the top of collagen gel.

Supplemental Figure S5. The effects of δEF1 and CtBP1 siRNAs on EMT. (**A**) The

expression of Twist mRNA was examined in cells treated with the indicated ligands for 24 h and 48 h by quantitative RT-PCR analysis. Each value represents the mean ± S.D. of duplicate determinations from a representative experiment. Similar results were obtained in two independent experiments. (**B**) NMuMG cells were treated with TGF-β alone or TGF-β together with FGF-2 for 48 h, and then transfected with δEF1 siRNA. After 24 h, wound was incised and photographs were taken immediately and 12 h after incision (left). Bars represent the median for each category from a representative experiment (right). Similar results were obtained in two independent experiments. (**C and D**) NMuMG cells were treated with TGF-β alone or TGF-β together with FGF-2 for 48 h and then transfected with CtBP1 siRNA. After 24 h, wounding (**C**) and invasion assays (**D**) were performed. Bars represent the median for each category from a representative experiment. Similar results were obtained in two independent experiments. (**E**) NMuMG cells were transfected with CtBP1 siRNA and treated with TGF-β or both TGF-β and FGF-2 for 48 h. The conditioned media were collected and subjected to gelatin zymography analysis. (**F**) NMuMG cells were treated with TGF-β alone or TGF-β together with FGF-2 for 48 h and then transfected with CtBP1 or δEF1 siRNA. After 24 h, the cells were mixed with MCF-7 cells and invasion assay of MCF-7 was determined. Each value represents the mean ± S.D. of duplicate determinations from a representative experiment. NC, control siRNA.

Supplemental Figure S6. Differentiation of NMuMG cells by cancer cells in a co-cultivation system. (**A**) Illustration of the experimental set-up. NMuMG cells were

cultured with or without the indicated cancer cells. (**B**) NMuMG cells cultured with or without cancer cells were stained with an anti-α-SMA antibody (green) and propidium iodide to detect nuclei (red). One ng/ml TGF-β, 1 μM SB431542, and 5 μM SU5402 were added to the culture media. Scale bars indicate 100 μm. (**C**) Total RNA was extracted from JygMC(A) or 4T1 cells co-cultured with NMuMG cells, and quantitative RT-PCR analyses were performed. Each value represents the mean ± S.D. of duplicate determinations from a representative experiment. (**D**) The mRNA expression levels of MMP2 and MMP9 in crude extracts from coculture of MCF-7 cells with NMuMG cells were determined by quantitative RT-PCR using human specific primers. Cells were treated with TGF-β and FGF-2 for 72 h. NMu-β cells, NMuMG cells pretreated with TGF-β; NMu-fβ cells, NMuMG cells pretreated with TGF-β and FGF-2. Each value represents the mean ± S.D. of duplicate determinations from a representative experiment. Similar results were obtained in two independent experiments.

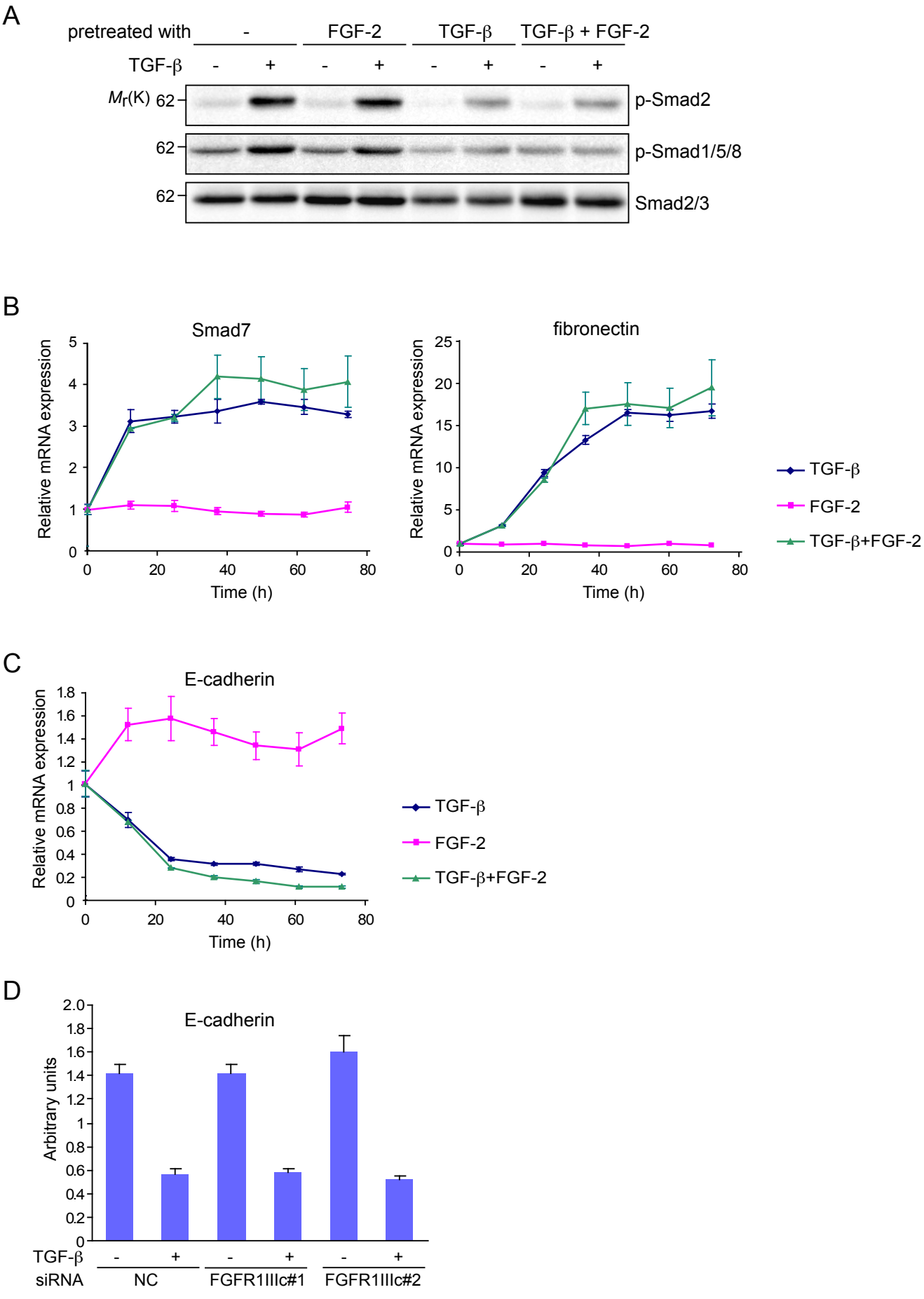
In panel (B), in the absence of cancer cells, a few NMuMG cells stained positive with an anti-α-SMA antibody. This staining disappeared with the addition of an inhibitor of TGF-β type I receptor kinases (SB431542), but increased with the addition of TGF-β (i, ii and iii). JygMC(A) cells induced α-SMA-positive NMuMG cells with a clustered morphology and this induction was inhibited by SB431542 (iv and v), indicating that TGF-β secreted from JygMC(A) cells induces epithelial cells to differentiate into α-SMA-expressing cells through EMyoT. On the other hand, co-cultures with 4T1 cells, expressing high levels of FGF-2 mRNA (Fig. S6C) failed to yield α-SMA-positive NMuMG cells (vii). With the addition of SU5402,

α-SMA-positive cells reappeared with a clustered morphology, similar to those observed by JygMC(A) cells (iv and ix).

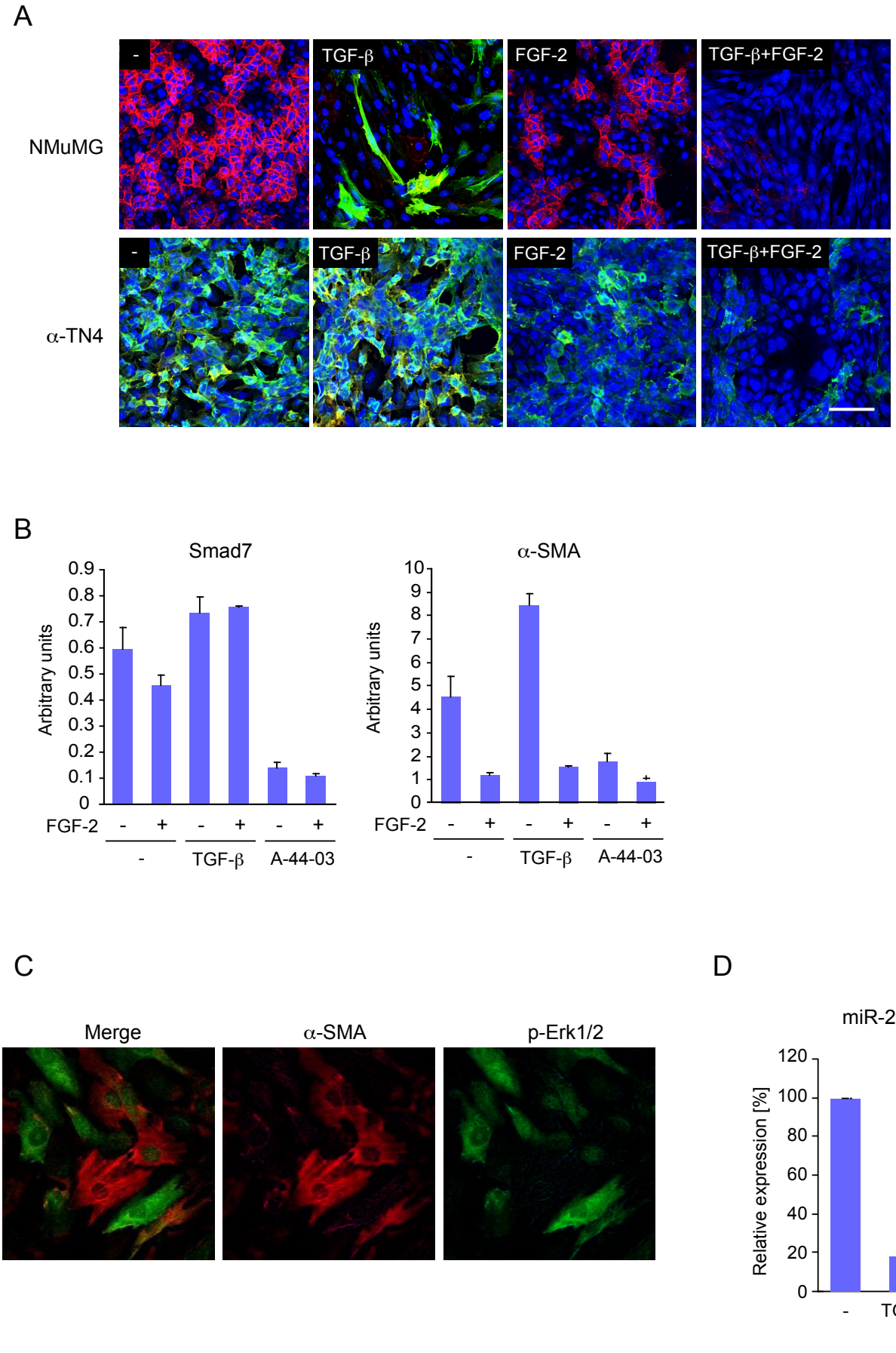
Supplemental Table S1

The primers used in the present study are shown.

Supplemental Figure S1



Supplemental Figure S2



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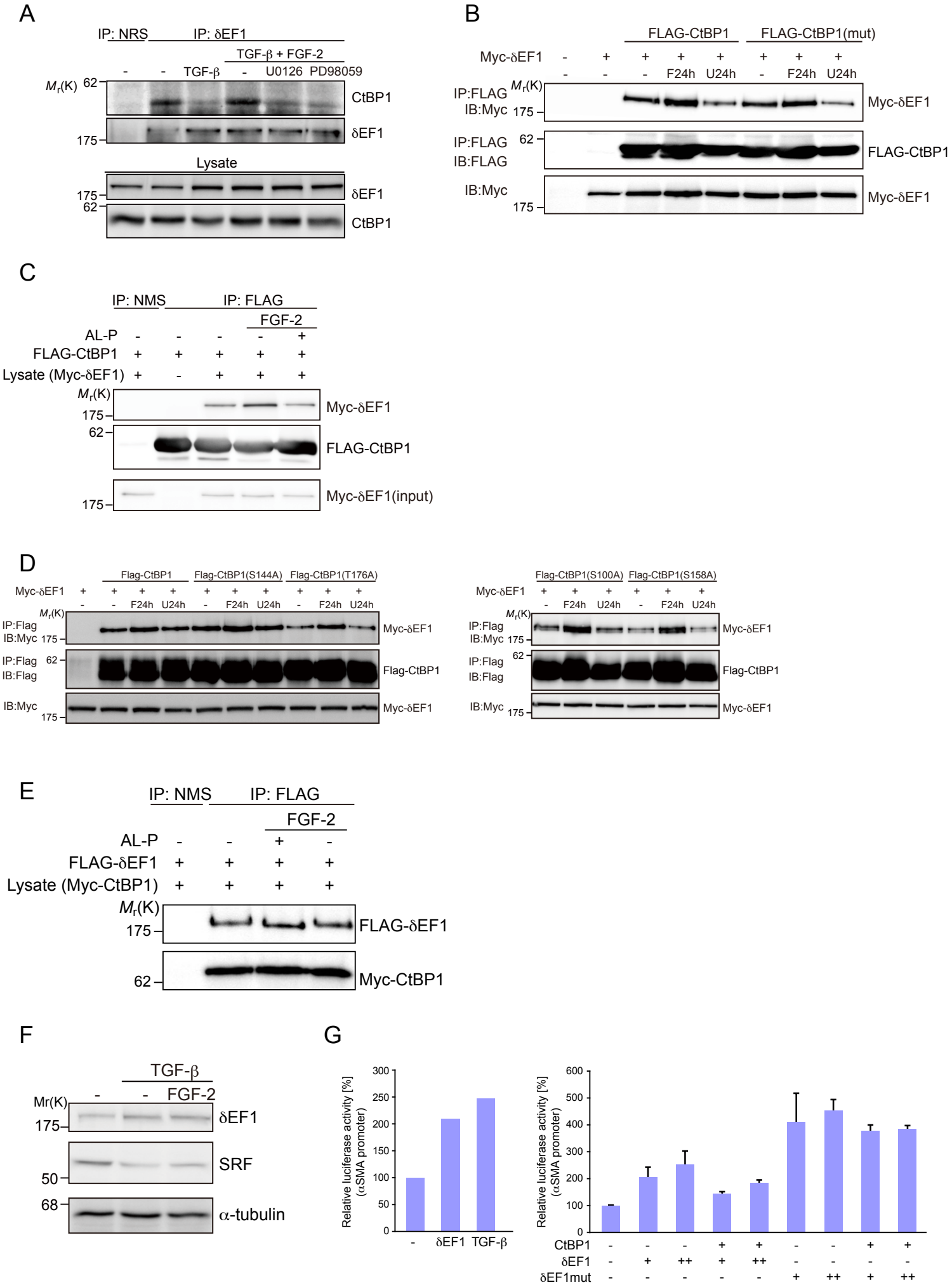

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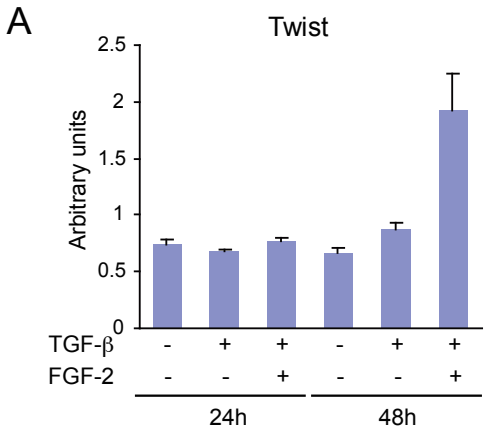
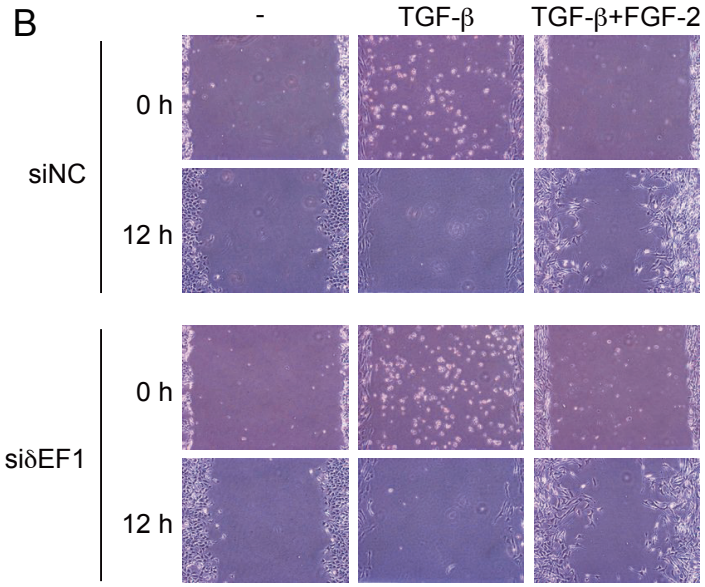
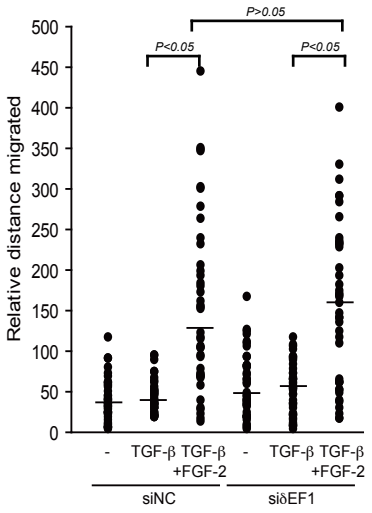
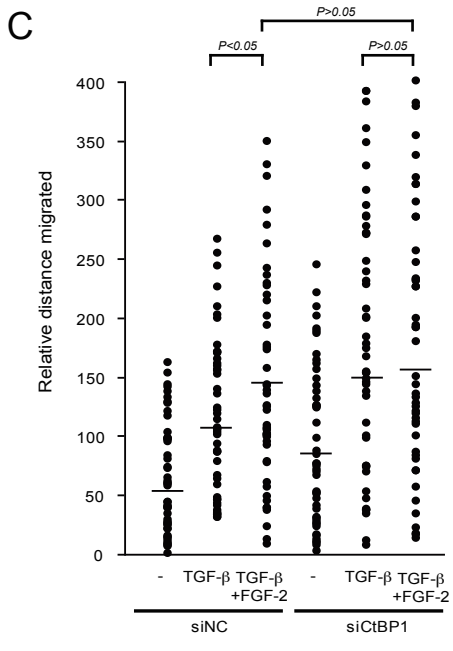
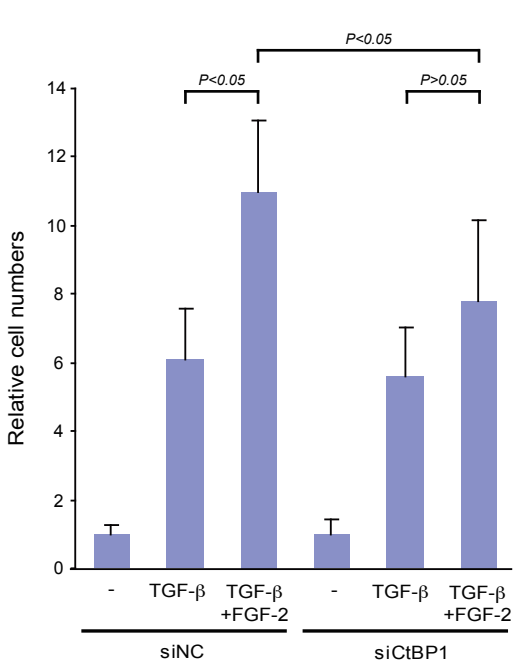
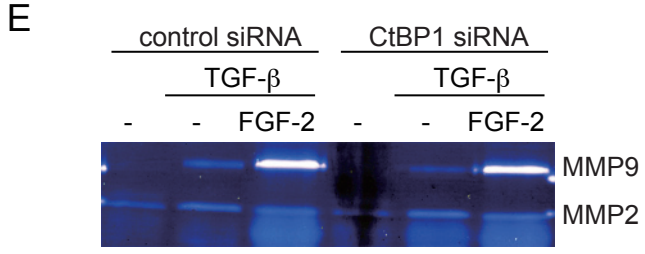


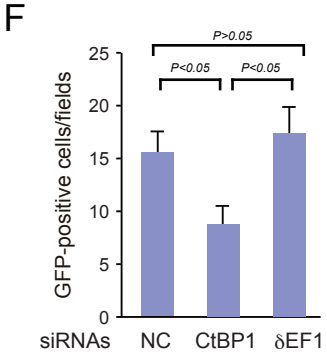




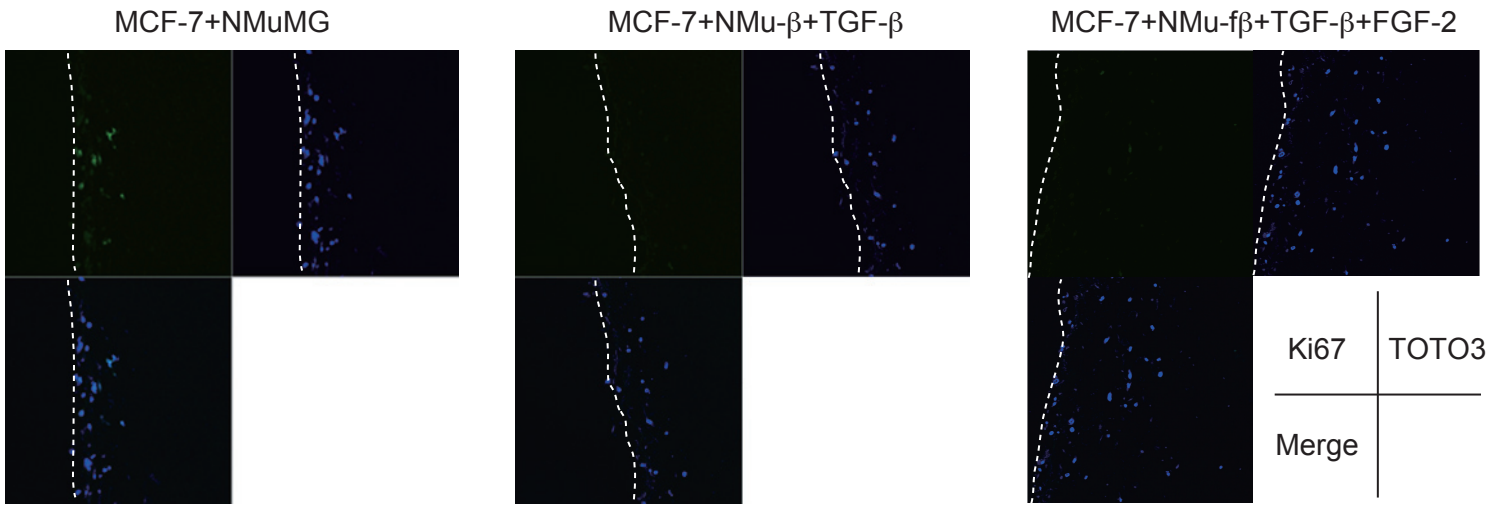




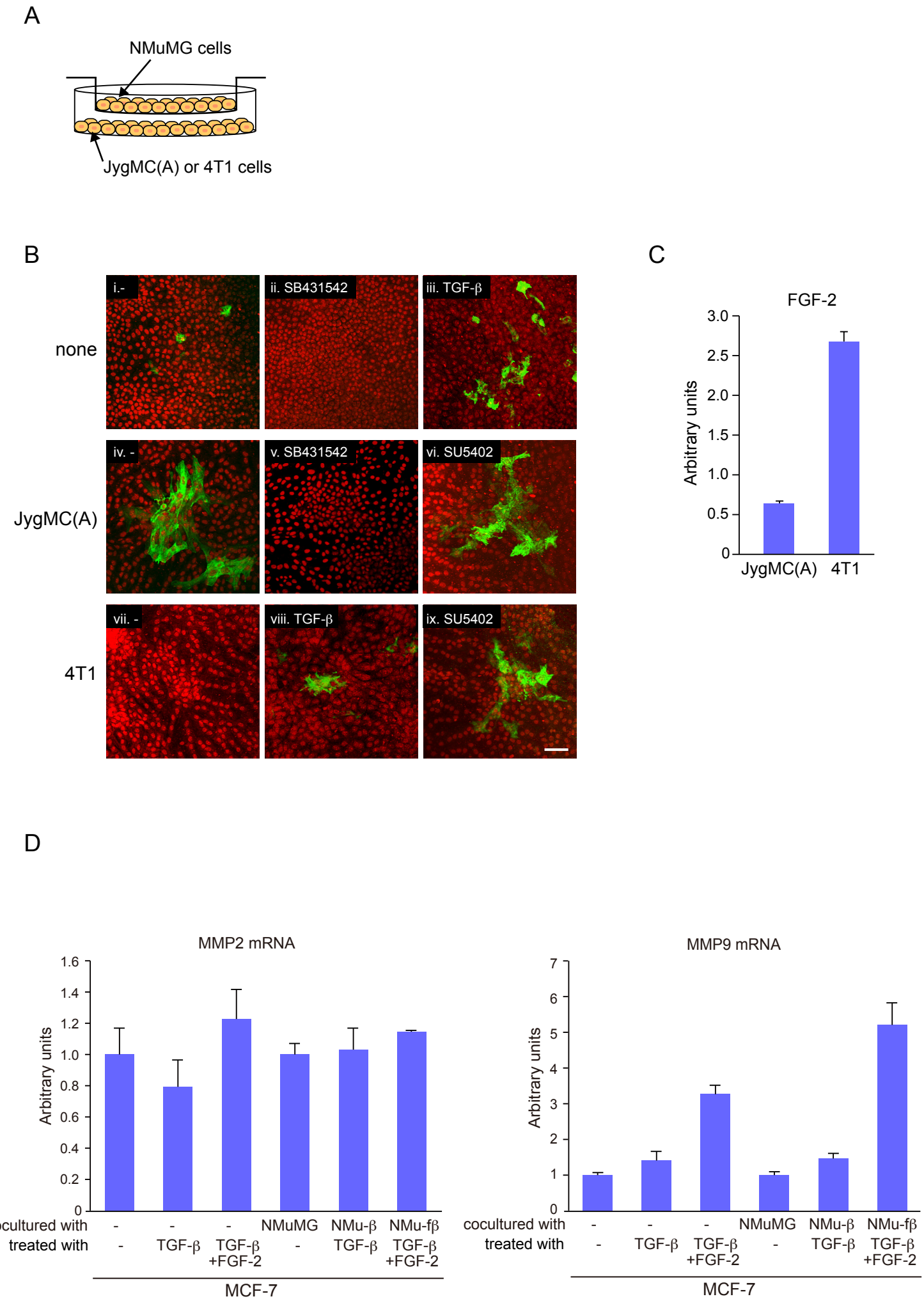




Supplemental Figure S5



Supplemental Figure S6



Supplemental Data
Table S1

Name of primer		sequences
mouse TBP	forward	5'-GCGATTTGCTGCAGTCATCA-3'
	reverse	5'-GCTGCTAGTCTGGATTGTTCTTCAC-3'
mouse α-SMA	forward	5'-CAGGGAGTAATGGTTGGAATGG-3'
	reverse	5'-GCCGTGTTCTATCGGATACTTCAG-3'
mouse calponin	forward	5'-CCAGCATGGCCAAGACAAAA-3'
	reverse	5'-CCAATGATGTTCCTGCCTTCTC-3'
mouse SIP1	forward	5'-GAAGCGACACGGCCATTATT-3'
	reverse	5'-GGCAAAAGCATCTGGAGTTCC-3'
mouse δEF1	forward	5'-ACCCCTTCAAGAACCGCTTT-3'
	reverse	5'-CAATTGGCCACCACTGCTAA-3'
mouse FGFR1	forward	5'-CACCGCTCTACCTGGAGATCATTA-3'
	reverse	5'-TTGGTGCCGCTCTTCATCTT-3'
mouse FGFR2	forward	5'-GCCTGTGAGAGAGAAGGAGATCAC-3'
	reverse	5'-AGATGACTGTCACCACCATGCA-3'
mouse GAPDH	forward	5'-TGCAGTGGCAAAGTGGAGATT-3'
	reverse	5'-TTGAAGTCGCAGGAGACAACCT-3'
mouse FGFR1IIIb	forward	5'-CGGGAATTAATAGCTCGGATGC-3'
	reverse	5'-TTGGTGCCGCTCTTCATCTT-3'
mouse FGFR1IIIc	forward	5'-GGAGTTAATACCACCGACAAG-3'
	reverse	5'-TTGGTGCCGCTCTTCATCTT-3'
mouse FGFR2IIIb	forward	5'-CACTCGGGGATAAATAGCTCC-3'
	reverse	5'-AGATGACTGTCACCACCATGCA-3'
mouse FGFR2IIIc	forward	5'-CGGTGTTAACACCACGGAC-3'
	reverse	5'-AGATGACTGTCACCACCATGCA-3'
mouse Smad7	forward	5'-TTGCCTCGGACAGCTCAATT-3'
	reverse	5'-TAAACCCACACGCCATCCA-3'
mouse fibronectin	forward	5'-GCCGTGGTCCTAACAAATCTCC-3'
	reverse	5'-CGAGACCTGTTTTCTGCCTTCC-3'
mouse FGF-2	forward	5'-CCAACCGGTACCTTGCTATGAA-3'
	reverse	5'-CAGTGCCACATACCAACTGGAGTA-3'