Supporting Information

Wang et al. 10.1073/pnas.1319190110

SI Materials and Methods

Cell Culture. HEK 293T cells were grown in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin in 5% CO_2 at 37 °C. MDA-MB-231 cells were maintained in Leibovitz L-15 Medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin without CO_2 at 37 °C. 4T1 cells were grown in RPMI-1640 supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin in 5% CO_2 at 37 °C. Transfection of plasmids was performed with Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. Hydrogel assay were performed as previously described (1).

Chemicals. The following compounds used in this study were purchased from Sigma-Aldrich: simvastatin (S6196), DL-mevalonolactone (M4667), GGTI-2133 (G5294), cytochalasin D (C8273), and geranylgeranyl pyrophosphate (G6025). Rho inhibitor C3 transferase was from Cytoskeleton.

Plasmids. Human Yes-associated protein (YAP) expression plasmid V5-YAP was provided by Makiko Fujii (Aichi Cancer Center Research Institute, Nagoya, Japan) (2). Human MST2 expression plasmids Flag-MST2 was generously provided by Kunliang Guan (University of California, La Jolla, CA). To generate expression plasmids for human RhoA, cDNAs were PCR-amplified and cloned into a pFlag-CMV2 vector, and then constitutively active RhoA (Q63L) expression plasmid was constructed from WT RhoA by using site-directed mutagenesis. Then, constitutively active RhoA (Q63L) was subcloned into a pBoBi lentivirus expression plasmid. To produce the lentivirus, the recombinant pBoBi-RhoA (Q63L) vector was cotransfected with pMDLg/pRRE, pRSV-Rev, pVSV-G into HEK 293T cells, and culture supernatants containing the virus were collected 48 h and 72 h after transfection. Receptor for hyaluronan-mediated motility (RHAMM) promoter constructs were amplified from human genomic DNA and cloned as XhoI/NcoI fragments into pGL3 enhancer luciferase reporter vector (Promega). The promoter construct −1830/1 was cloned as previously described (3). The promoter construct −1830/1 (ΔTB1), −1830/1 (ΔTB2) and −1830/1 (ΔTB1/2) were constructed with TEAD-binding site 1 (−291 to −285), TEAD-binding site 2 (−103 to −97), and TEAD-binding sites 1/2 (both −291 to −285 and −103 to −97) deletion, respectively.

Cell Migration and Invasion Assays. Twenty-four transwell chambers were used to monitor tumor cell migration and invasion as previously described (4). Briefly, cells were treated with DMSO or 5 μM simvastatin for 24 h and then trypsinized and transferred to a transwell chamber with 8-μm pore size membrane in the medium without FBS. The growth medium containing 20% FBS was placed in the lower chamber of the transwell chamber. After 6 h incubation, cells on the upper side of the membrane were removed by wiping with a cotton swab and stained with crystal violet, and then the migrated cells were photomicrographed. For invasion assay, the transwell chambers with 8-μm pore size membrane were coated with Matrigel. The rest of the assays were performed as described earlier except incubating cells in the transwell chamber for 24 h. For quantification, the migrated (i.e., invaded) stained cells on the other side of the membrane were extracted with 33% acetic acid. The absorbance of the eluted stain was determined at 570 nm.

Real-Time RT-PCR Analysis. Total RNA was extracted using RNAiso Plus (TaKaRa) and was then reverse-transcribed into cDNA by Primescript RT Reagent kit (TaKaRa) according to the manufacturer's instructions. Prepared cDNA was then subjected to quantitative PCR analysis using FastStart Universal SYBR Green Master (ROX; Roche).The primer pairs used for quantitative PCR amplification were as follows: RHAMM (human), sense, 5′-AGAACCAACTCAAGCAACAGG-3′; antisense, 5′-AGGAGACGCCACTTGTTAATTTC-3′; RHAMM (mouse), sense, 5′-CCTTGCTTGCTTCGGCTAAAA-3′; antisense, 5′-CTGCTGCATTGAGCTTTGCT-3′; CTGF (human), sense, 5′-GTTTGGCCCAGACCCAACT-3′; antisense, 5′-GGAACAGGCGCTCCACTCT-3′; GAPDH (human), sense, 5′-CCAGAACATCATCCCTGCCTCTACT-3′; anti-sense, 5′-GGTTTTTCTAGACGGCAGGTCAGGT-3′; and GAPDH (mouse), sense, 5′- TGGATTTGGACGCATTGGTC-3′; antisense, 5′-TTTGCACTGGTACGTGTTGAT-3′.

Western Blot Analysis. Cells or tumor tissues were harvested, sonicated in lysis buffer (20 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 2 μg/mL leupeptin, and 1 mM PMSF). Equal amounts of proteins were separated by SDS/PAGE and analyzed by immunoblotting. Western blot were prepared by standard procedures by using anti-RHAMM (provided by Volker Assmann, University Medical Center Hamburg-Eppendorf, Hamburg, Germany), YAP1 (Abnova and Proteintech), phospho-YAP (Ser127; Cell Signaling), LATS1 (Cell Signaling), phopho-LATS1 (Ser909; Cell Signaling), TEAD4 (Abnova), Flag (Sigma), V5 (GeneTex), phopho-MST1 (Thr183)/MST2 (Thr180; GeneTex), RhoA GTPase (Epitomics), Flag (Abmart), EGFP (Abmart), Tubulin (Sigma), Lamin B (Santa Cruz), GAPDH (Santa Cruz), and β-actin (Santa Cruz) antibodies. The protein bands were scanned and the density of the bands was quantified as a ratio to the loading control by using Quantity One software (Bio-Rad).

Nuclear and Cytoplasmic Protein Extraction. To separate nuclear and cytoplasmic extract, high salt isolation of nuclear was carried out as previously described (5). Briefly, cells were extracted in a buffer containing 10 mM Pipes, pH 6.8, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.5% Triton X-100 supplemented with 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 2 μg/mL leupeptin, and 1 mM PMSF. After 3 min at 4 °C, nuclei were pelleted by centrifugation at 5,000 × *g* for 3 min, and both fractions were subjected to Western blot analysis.

Immunofluorescence Staining. After treatment, MDA-MB-231 or 4T1 cells were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.4% Triton X-100. Following blocking, cells were incubated with primary antibodies for 2 h at room temperature. FITC- or Cy3-conjugated goat anti-rabbit IgG antibodies were used as the secondary antibody. Primary antibody used were anti-YAP1 antibody (Abnova or Proteintech). Phalloidin-FITC was used to stain actin cytoskeleton. For visualization of cell nucleus, DAPI was used. Sections were observed by using an Olympus laser scanning confocal microscope.

ChIP Assay. The ChIP assay was performed by using ChIP-IT Express Enzymatic Chromatin Immunoprecipitation kit (Active Motif) according to the manufacturer's instructions. Then, the eluted DNA was amplified by PCR with RHAMM promoter-specific primers as

follows: sense, 5′- TATTGGATGGCAGCCTAAAC-3′; and antisense, 5′- CACAGGAACACGAAGAAAGAA-3′. This was followed by analyzing by agarose gel electrophoresis. The antibodies used were anti-YAP (H-125; Santa Cruz) and TEAD4 (Abnova).

Luciferase Reporter Gene Assay. Cells were transfected with the indicated expression vectors and a luciferase reporter driven by RHAMM promoter by using Lipofectamine 2000 according to the manufacturer's instructions. Cell lysates were used for luciferase assay by using the luciferase assay kit (Promega).

Lentiviral shRNA Cloning, Production, and Infection. Constructs expressing shRNA against YAP, TEAD1/3/4, and RHAMM were generated by subcloning the following oligonucleotides into pSilencer 2.1-U6 hygro vector with the BamHI/HindIII site, and then shuttled into FG12 vector as previously described (6). The target sequences of YAP shRNA (7), TEAD1/3/4 shRNA (7), LATS1 shRNA (1), and LATS2 shRNA (1) were reported previously. The sequences of RHAMM oligonucleotides are as follows: RHAMM1 sense, 5′-GATCCGCTAGATATTGCCCAGTTATTCAAGAGATAACTGGGCAATATCTAGCTTTTTTGGAAA-3′, RHAMM1 antisense, 5′-AGCTTTTCCAAAAAAGCTAGATATTGCCCAGTTATCTCTTGAATAACTGGGCAATATCTAGCG-3′; and RHAMM2 sense, 5′-GATCCGGACCAGTATCCTTTCAGATTCAAGAGATCTGAAAGGATACTGGTCCTTTTTTGGAAA-3′, RHAMM2 antisense, 5′-AGCTTTTCCAAAAAAGGACCAGTATCCTTTCAGATCTCTTGAATCTGAAAGGATACTGGTCCG-3′. To produce the shRNA lentivirus, the recombinant FG12 vector was cotransfected with pMDLg/pRRE, pRSV-Rev, pVSV-G into HEK 293T cells, and culture supernatants containing the virus were collected 48 h and 72 h after transfection. For infection with lentivirus, MDA-MB-231 cells were cultured with lentiviral solution for 24 h in the presence of 1 μg/mL Polybrene (Sigma).

Breast Tumor Animal Model Study. Female BALB/c nude mice were obtained from the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Science. All animal studies were carried out according to the protocols approved by the Administrative Committee on Animal Research of the Graduate School at Shenzhen, Tsinghua University. According to previously described protocols (4), mice were injected with 5 mg/kg body weight of simvastatin every day for 7 d. Control mice received PBS solution. Then, MDA-MB-231 cells were injected s.c. into the right flank of BALB/c nude mice at a dosage of 5×10^6 cells per mouse. After inoculation, simvastatin was administrated until the mice were killed at 3 wk.

Immunohistochemistry of Tissues. Formalin-fixed, paraffin-embedded tissues from patients with breast carcinoma were selected for immunohistochemical examination by using an indirect immunoperoxidase method. The antibodies used for immunohistochemical staining were YAP (H-125; Santa Cruz) and RHAMM (provided by Volker Assmann).

Statistical Analysis. Significant difference of data were determined using two-way ANOVA (Prism 4.00; GraphPad). To analyze the significance of data, a two-tailed Student *t* test (unpaired) was used. Results are presented as mean ± SD.

1. Dupont S, et al. (2011) Role of YAP/TAZ in mechanotransduction. *Nature* 474(7350): 179–183.
2. Fujii M, et al. (2012) TGF-β synergizes with defects in the Hippo pathway to stimulate human malignant mesothelioma growth. *J Exp Med* 209(3):479–494.
3. Sohr S, Engeland K (2008) RHAMM is differentially expressed in the cell cycle and downregulated by the tumor suppressor p53. *Cell Cycle* 7(21):3448–3460.
4. Mandal CC, Ghosh-Choudhury N, Yoneda T, Choudhury GG, Ghosh-Choudhury N (2011) Simvastatin prevents skeletal metastasis of breast cancer by an antagonistic interplay between p53 and CD44. *J Biol Chem* 286(13):11314–11327.
5. Reyes JC, Muchardt C, Yaniv M (1997) Components of the human SWI/SNF complex are enriched in active chromatin and are associated with the nuclear matrix. *J Cell Biol* 137(2):263–274.
6. Qin XF, An DS, Chen IS, Baltimore D (2003) Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. *Proc Natl Acad Sci USA* 100(1):183–188.
7. Zhao B, et al. (2008) TEAD mediates YAP-dependent gene induction and growth control. *Genes Dev* 22(14):1962–1971.

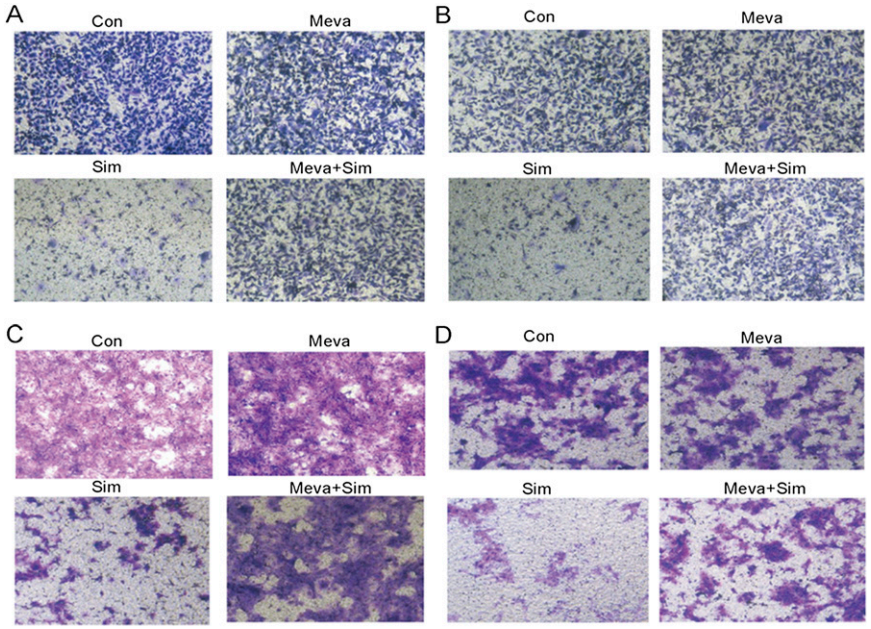

Fig. S1. Simvastatin inhibits migration and invasion of breast cancer cells through mevalonate pathway. (*A* and *B*) Simvastatin blocked migration and invasion of MDA-MB-231 breast cancer cells via mevalonate pathway. MDA-MB-231 cells grown in medium containing 10% FBS were incubated with or without 250 μM mevalonate (Meva) for 6 h before treatment with DMSO or 5 μM simvastatin for 24 h and used for migration (*A*) and invasion (*B*) assays. (*A*) The cells were then trypsinized and transferred to a transwell chamber in the same medium for 6 h. The cells were fixed and stained with crystal violet, and then the stains were eluted and the migrated cells were photomicrographed. (*B*) The cells were then trypsinized and transferred to a transwell chamber coated with matrigel for 24 h. The cells were fixed and stained with crystal violet, and then the stains were eluted and the migrated cells were photomicrographed. (*C* and *D*) As in *A* and *B* but with 4T1 cells.

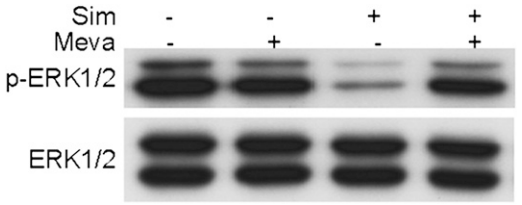

Fig. S2. Simvastatin significantly inhibits ERK activity, whereas adding mevalonate abolishes the inhibitory effect. 4T1 cells were incubated with or without 250 μM mevalonate (Meva) for 6 h before treatment with DMSO or 5 μM simvastatin for 24 h. Western blot was performed with the indicated antibodies.

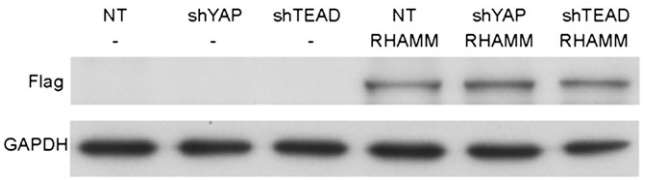

Fig. S3. Reexpression of Flag-RHAMM in lentivirus-infected MDA-MB-231 cells. The expression of Flag-RHAMM was confirmed by Western blot.

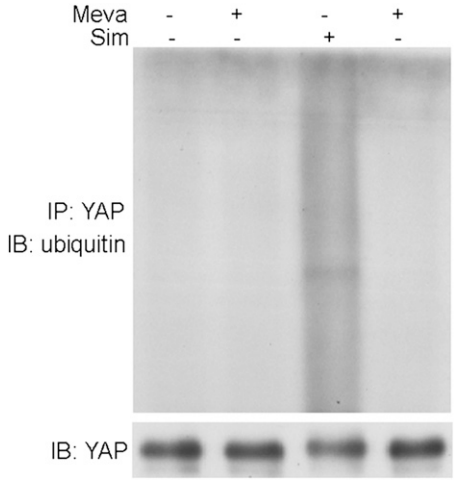

Fig. S4. YAP is ubiquitinated after simvastatin treatment, and the addition of mevalonate abolishes the ubiquitination. MDA-MB-231 cells were incubated with the indicated reagents for 24 h and then treated with 25 μM MG132 for 5 h before harvest. Endogenous YAP was immunoprecipitated with anti-YAP antibody and Western blot was done with anti-YAP or anti-ubiquitin antibodies.

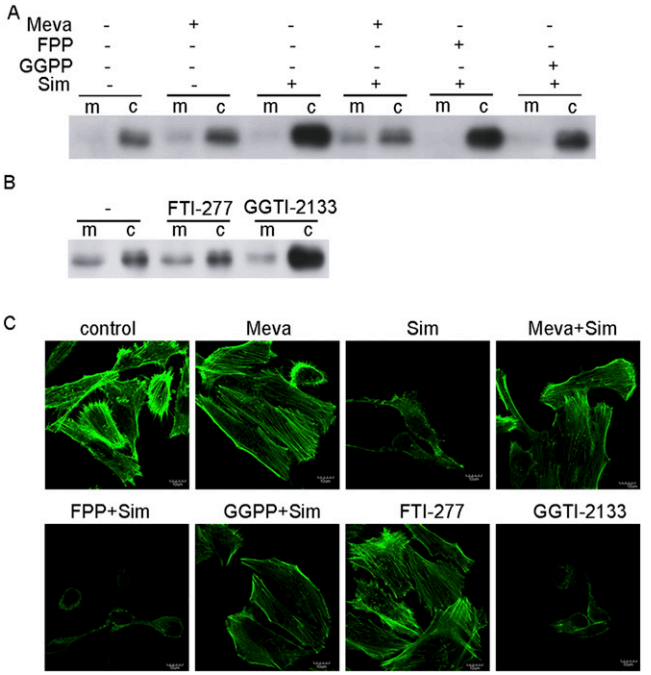

Fig. S5. The mevalonate pathway inhibitor simvastatin or GGTI-2133 significantly inhibits RhoA GTPase activity and disrupts actin cytoskeleton, and the addition of mevalonate or geranylgeranyl pyrophosphate (GGPP) abolishes the effect. (*A*) Simvastatin inhibited RhoA GTPase activity, and the addition of mevalonate or GGPP abolished the inhibitory effect. MDA-MB-231 cells were treated with the indicated reagents for 24 h, and the membrane and cytosol were isolated and subjected to Western blot by using RhoA GTPase antibody. (*B*) GGTI-2133 inhibited RhoA GTPase activity, and the addition of mevalonate or GGPP abolished the inhibitory effect. MDA-MB-231 cells were treated with GGTI-2133 for 24 h, and the membrane and cytosol were isolated and subjected to Western blot by using RhoA GTPase antibody. (*C*) Simvastatin or GGTI-2133 disrupted actin cytoskeleton, and the addition of mevalonate or GGPP abolished the inhibitory effect. MDA-MB-231 cells were treated with the indicated reagents for 24 h, and then the cells were fixed and stained with phalloidin (green).