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Title	P53-and mevalonate pathway-driven malignancies require Arf6 for metastasis and drug resistance
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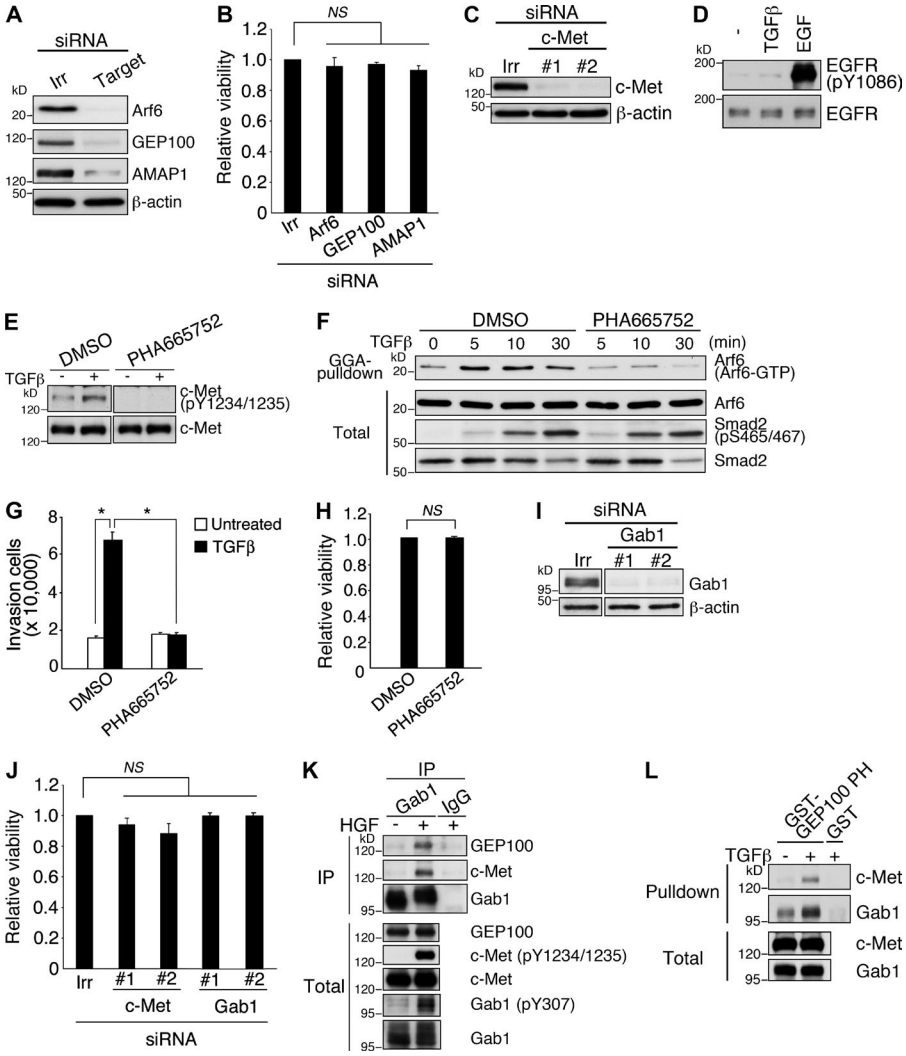


Figure S1. **TGFβ1 trans-activates c-Met to activate Arf6 in MDA-MB-231 cells.** (A) siRNA-mediated silencing of *Arf6*, *GEP100*, and *AMAP1*. Protein levels were analyzed by immunoblotting using the indicated antibodies. β-Actin immunoblotting was used as a control. The specificities of these siRNA oligonucleotides for *Arf6*, *GEP100*, and *AMAP1* have been confirmed previously, as described in the siRNA section within Materials and methods. (B) Relative viabilities of the siRNA-treated cells were measured by the MTS assay, with the viability of cells treated with siRNA with an Irr normalized to 1.0. The graph shows a representation of data from three independent experiments measuring 3 wells per experiment. (C) siRNA-mediated silencing of *c-Met*. Two different sequences (#1 and #2) were used to target *c-MET* mRNA. β-Actin immunoblotting was used as a control. (D) TGFβ1 does not notably trans-activate EGFR in MDA-MB-231 cells. Tyrosine phosphorylation of EGFR in cells treated with or without TGFβ1 or EGF for 5 min was analyzed by immunoblotting using an anti–pY1086-EGFR antibody coupled with an anti-EGFR antibody, as indicated. (E–H) PHA665752 blocks TGFβ1-mediated invasiveness. Cells were treated with 0.3 µM PHA665752 or DMSO for 16 h and then stimulated with or without TGFβ1 for 5 min in E and for the indicated times in F. Activation of c-Met was analyzed by immunoblotting using an anti-pY1234/1235–c-Met antibody and an anti–c-Met antibody (E), together with Arf6 activities (F). In F, Smad2 phosphorylation detected by an anti–pS465/467-Smad2 antibody was used as a control. Matrigel cell invasion was quantified as described in the Matrigel invasion assay section within Materials and methods (G). Cell viability was measured as in B. (n = 3; H). (I and J) siRNA-mediated silencing of *Gab1* (I) and viability of the *Gab1*-silenced cells (n = 3; J). Two different sequences (#1 and #2) were used to target *Gab1* mRNA. (K) Complex formation of Gab1 with GEP100 and c-Met upon HGF stimulation. Cells were stimulated with or without HGF (5 min), and coprecipitation of endogenous GEP100 and c-Met with anti-Gab1 immunoprecipitants was analyzed by immunoblotting as indicated. Precipitants using irrelevant IgGs were used as controls. Immunoblots using an anti–c-Met pY1234/1235 antibody and an anti–Gab1 pY307 antibody were also included as controls. (L) Binding of GST-GEP100 PH with Gab1 and c-Met upon TGFβ1 stimulation. Cells were stimulated with or without TGFβ1 for 5 min, and their cell lysates were pulled down with the GST-GEP100 PH protein, coupled with glutathione beads. Coprecipitated proteins were then analyzed by immunoblotting as indicated. Each experiment was performed at least twice using MDA-MB-231 cells. The results represent mean ± SEM. *, $P < 0.001$. Total, total cell lysate. IP, immunoprecipitation.

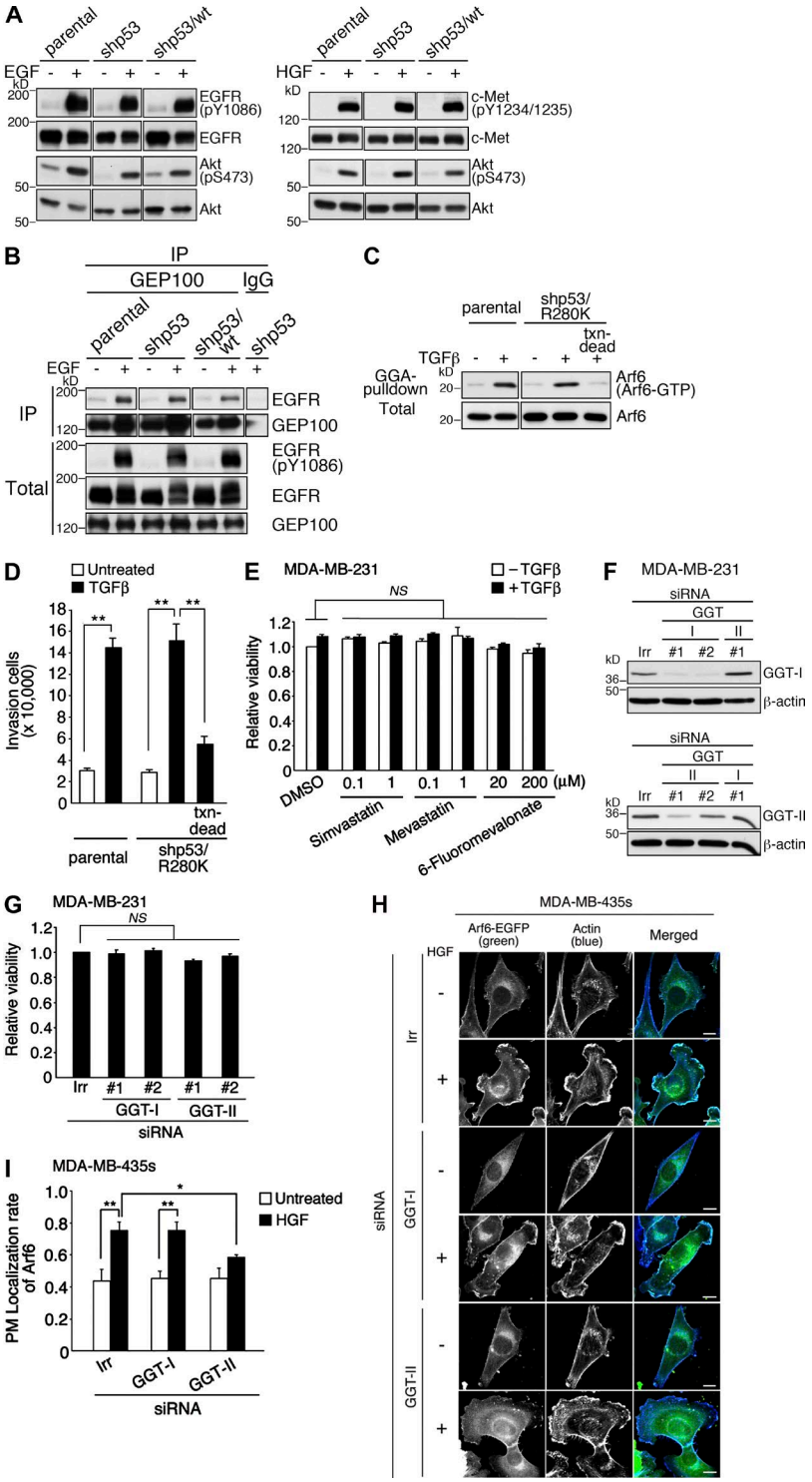


Figure S2. **Roles played by mutant p53, MVP, and GGT-II in Arf6 activation by external ligands in breast cancer cells.** (A and B) MDA-MB-231 cells and their p53 derivatives were examined regarding their activation of EGFR and c-Met by the cognate ligands. Controls included Akt activation (A) and the association of activated EGFR with GEP100 (B). IP, immunoprecipitation. (C and D) TGFβ1-induced Arf6 activation (C) and Matrigel invasion (D) in shp53 cells expressing p53 R280K or its transcriptional-dead mutant (txn-dead) were measured. In D, MDA-MB-231 cells (parental) were included as a control. Matrigel invasion activities were measured as described in the Matrigel invasion assay section within Materials and methods (n = 3). **, P < 0.001. (E) Viabilities of MDA-MB-231 cells preincubated with each inhibitor or DMSO for 16 h and then cultured with or without TGFβ1 for another 16 h in the presence of inhibitors or DMSO, as indicated. The viability of the control cells (DMSO) without TGFβ1 was normalized as 1.0. The graph shows a representation of data from three independent experiments measuring 3 wells per experiment. (F and G) siRNA-mediated silencing of *GGT-I* and *GGT-II* in MDA-MB-231 cells (F) and their viabilities as in E (n = 3; G). Two different sequences each (#1 and #2) were used to target *GGT-I* or *GGT-II*. β-Actin immunoblotting was used as a control (F). (H and I) HGF-induced (5 min) PM recruitment of Arf6-EGFP was examined using MDA-MB-435s cells treated with siRNAs for *GGT-I*, *GGT-II*, or Irr, as indicated, together with the visualization of F-actin using Alexa Fluor 647–conjugated phalloidin (H). Relative amounts of Arf6-EGFP localized at the cell periphery before and after HGF stimulation (I) were estimated from >10 cells as described in the Immunofluorescence microscopy section within Materials and methods. n = 2. Bars, 10 µm. The results represent mean ± SEM. *, P < 0.05; **, P < 0.01.

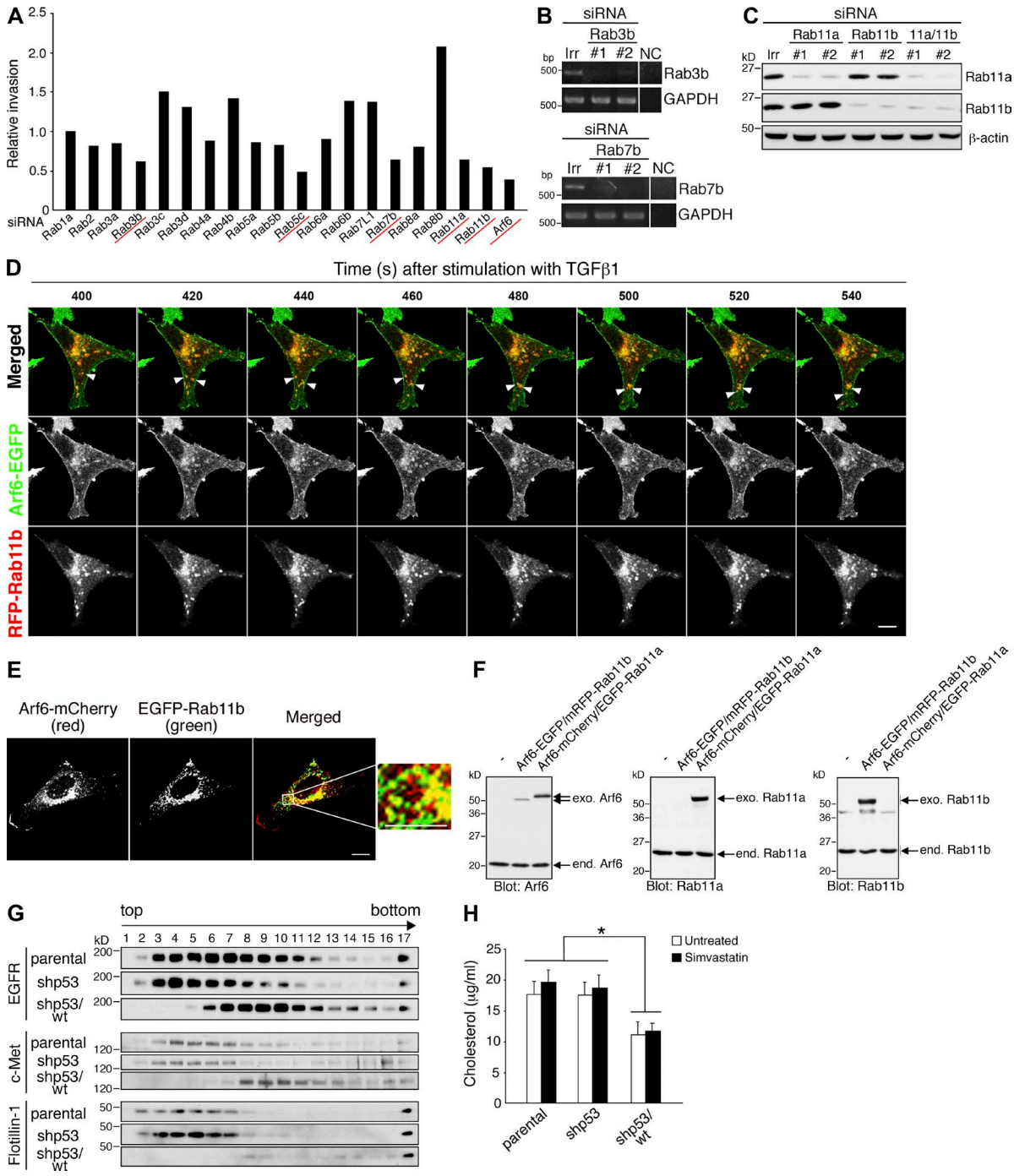


Figure S3. **Rab11b is responsible for the PM recruitment of Arf6 and the absence of any notable effects of mutant p53 in lipid rafts.** (A) Matrigel invasion activities of MDA-MB-231 cells treated with siRNAs for the indicated Rab-GTPases were measured in the presence of 10% FCS (n = 1). *Arf6* siRNA was included as a control. Candidate Rabs then found are underlined in red, and Matrigel invasion activities of MDA-MB-231 cells treated with siRNAs for these Rabs were measured in Fig. 4 A once again for reproducibility. (B and C) siRNA-mediated silencing of *Rab3b* and *Rab7b* assessed by their mRNA levels (B) and siRNA-mediated silencing of *Rab11a* and *Rab11b* assessed by their protein levels (C) in MDA-MB-231 cells. Two different sequences (#1 and #2) were used to target each of the Rabs. *GAPDH* mRNA levels (B) and β-actin protein levels (C) were included as controls. NC, no-template negative control. (D) Rab11b traffics together with Arf6 during TGFβ1-induced PM recruitment. Time-lapse confocal fluorescence images of Arf6-EGFP and RFP-Rab11b expressed in MDA-MB-231 cells were taken every 20 s after TGFβ1 stimulation, and their representative images are shown between 400 s and 540 s, as indicated. Bar, 10 μm. Also see Video 1. The arrowheads indicate representative endosomes positive for Arf6 and Rab11b, which move toward the peripheral region of the cell. (E) The intracellular colocalization of Arf6-mCherry and EGFP-Rab11b was examined in MDA-MB-231 cells. Enlarged view of the boxed area is shown on the right. Bars: (right) 5 μm; (left) 10 μm. (F) Protein levels of endogenous and exogenous Arf6, Rab11a, and Rab11b were assessed by Western blotting. -, transfection of the plasmid DNA was not done. (G and H) Mutant p53 does not affect the lipid raft localization of EGFR and c-Met (G) or total cellular cholesterol levels (H). Biochemical membrane fractionations of MDA-MB-231 cells (parental) and their p53 derivatives, as indicated, were performed to examine the lipid raft localization of RTKs, in which Flotillin-1 was used as a lipid raft marker (G). (H) Total cellular cholesterol concentrations of these cells after treatment with or without Simvastatin (1 μM for 24 h). The results represent mean ± SEM. *, $P < 0.001$. Each experiment was performed twice, except for A.

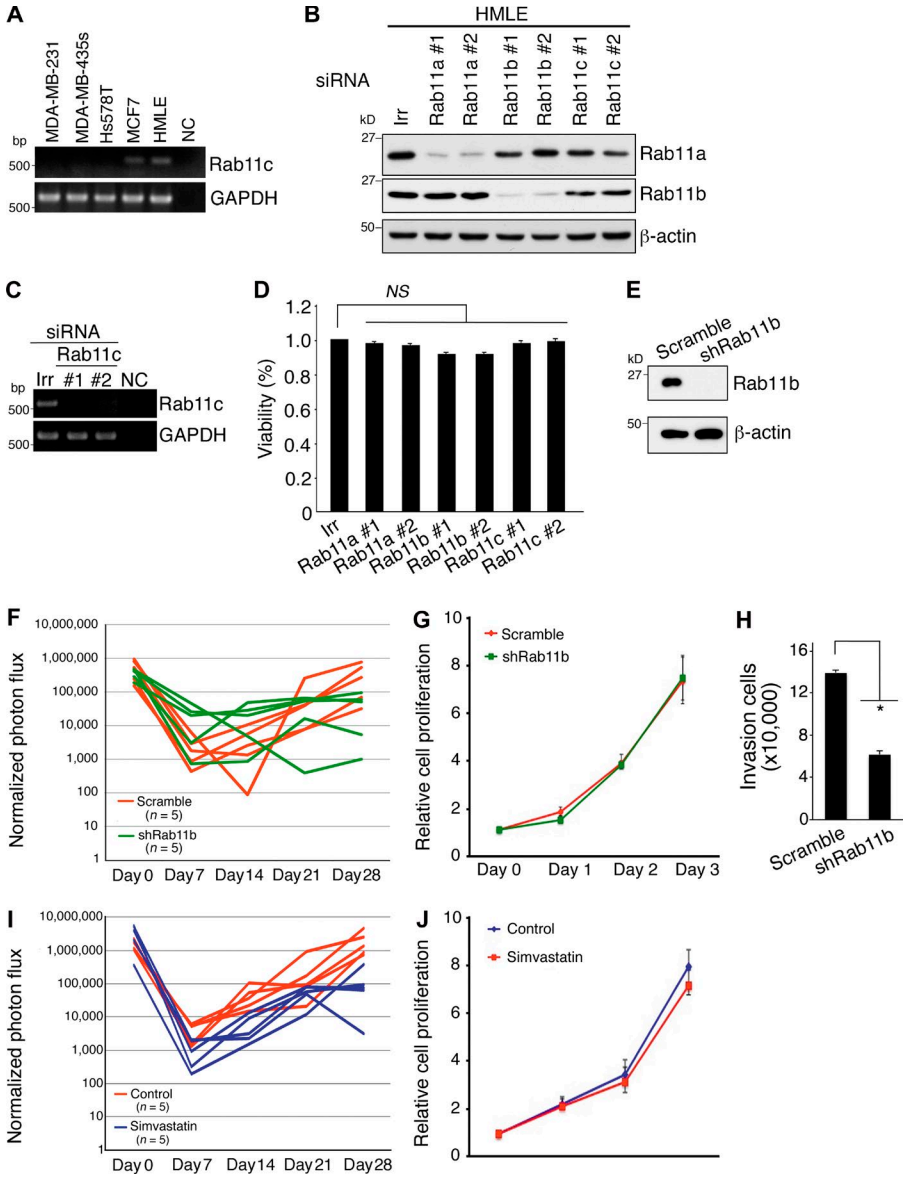


Figure S4. **Silencing of *Rab11b* in normal cells and breast cancer cells.** (A) Expression levels of Rab11c mRNA in breast cancer and HMLE cells were assessed by RT-PCR. *GAPDH* mRNA levels were included as controls. NC, no-template negative control. (B) siRNA-mediated silencing of *Rab11a*, *Rab11b*, or *Rab11c* in HMLE cells. Protein levels were analyzed by immunoblotting using the indicated antibodies. β-Actin immunoblotting was used as a control. (C) siRNA-mediated silencing of *Rab11c*, assessed by their mRNA levels in HMLE cells. Two different sequences (#1 and #2) were used to target Rab11c. *GAPDH* mRNA levels were included as controls. (D) Cell viability was measured by the MTS assay and shown as mean ± SEM, in which the viability of cells treated with Irr was normalized to 1.0. Each experiment was performed at least twice, as in Fig. S1 B. (E) shRNA-mediated silencing of *Rab11b* in MDA-MB-231 cells, which were used for the metastasis assay. Cells transfected with a control plasmid (Scramble) were used as a control. β-Actin immunoblotting was used as a loading control. (F) Time courses of bioluminescence intensities emitted from the chests of the mice shown in Fig. 6 A ($n = 5$ for each group), which were injected with *Rab11b*-silenced cells (shRab11b) or control cells (Scramble). Results were normalized as in Fig. 6 A. (G and H) In vitro proliferation (G) and Matrigel invasion (H) of the *Rab11b*-silenced MDA-MB-231 cells (shRab11b) and control cells (Scramble). Matrigel invasion was measured in the presence of TGFβ1. $n = 3$. *, $P < 0.001$. (I) Time course of bioluminescence intensities emitted from the chests of the mice shown in Fig. 6 C, which received Simvastatin or a control solution as indicated, every day after the injection ($n = 5$ for each group). (J) In vitro proliferation of the MDA-MB-231 cells used for the metastatic assay in the presence of 1 µM Simvastatin or a control solution. Relative cell growth was calculated by normalizing the values obtained from the day 0 cells as 1. In G and J, $n = 3$. Error bars represent mean ± SEM.

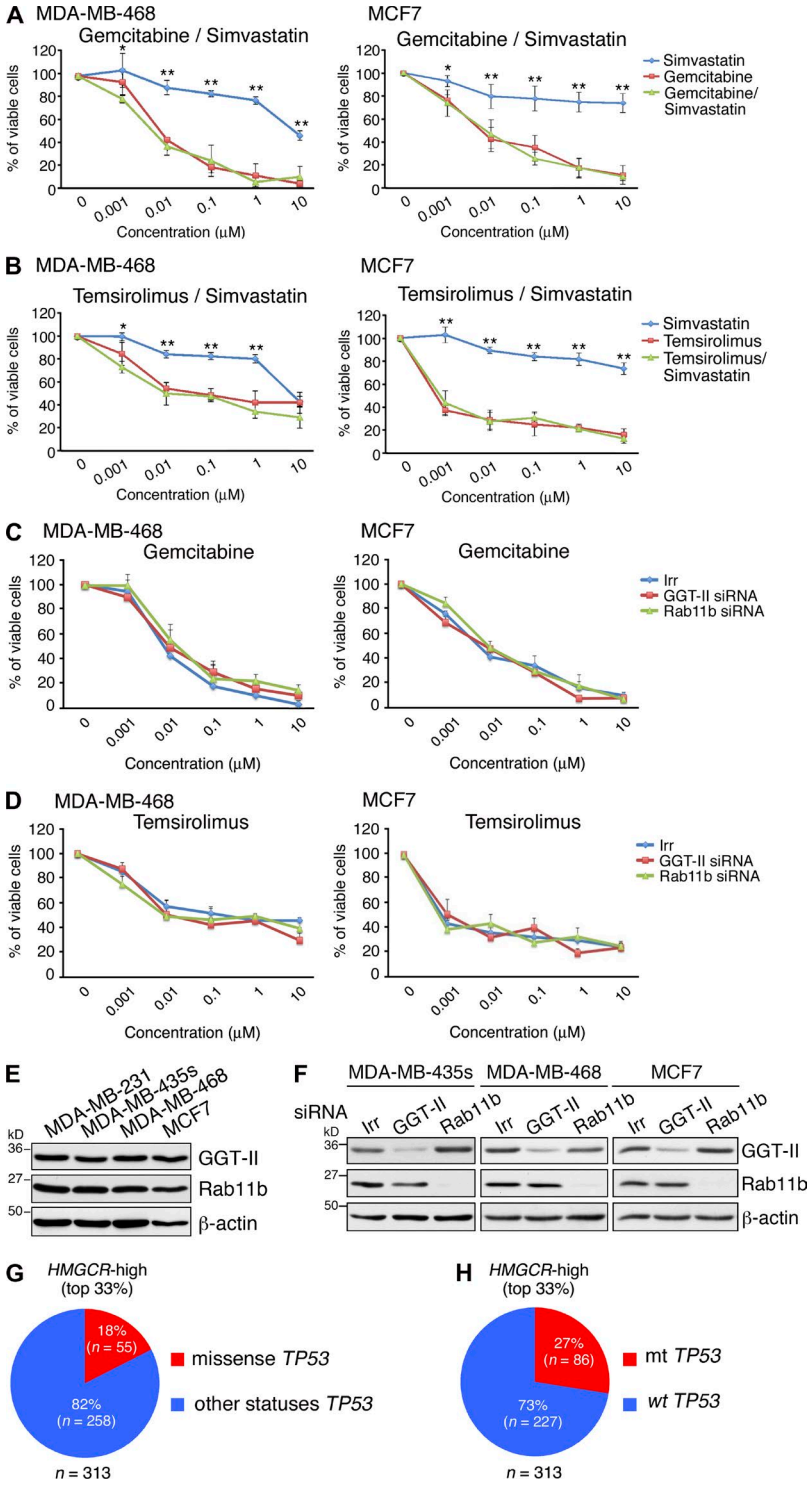
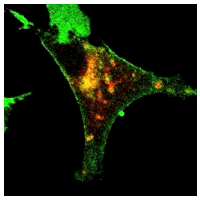


Figure S5. **Limited contribution of the *TP53* mutation to the enhanced expression of *HMGCR* mRNA in primary breast tumors.** (A and B) MDA-MB-468 or MCF7 cells were incubated with Gemcitabine (A) or Temsirolimus (B), with or without Simvastatin, as indicated. After incubation for 72 h, their viabilities were measured. At each point, concentrations of Simvastatin were equal to those of Gemcitabine or Temsirolimus. Each experiment was performed at least twice. The results represent mean ± SEM from three independent experiments measuring 3 wells per condition in each experiment. *, $P < 0.05$; **, $P < 0.01$. (C and D) MDA-MB-468 or MCF7 cells pretreated with siRNAs for *GGT-II*, *Rab11b*, or Irr were incubated with the indicated doses of Gemcitabine (C) and Temsirolimus (D) for 72 h, and their viabilities were then measured. Each experiment was performed in triplicate, and the results are shown as mean ± SEM. (E and F) Expression of GGT-II and Rab11b and siRNA-mediated silencing of *GGT-II* and *Rab11b* in breast cancer cells, as indicated. (G and H) Percentages of primary breast tumors with *TP53* missense mutations (G) or *TP53* mutations (H) among the *HMGCR*-high group. Significant populations of *TP53* missense mutations may produce oncogenic mutant p53 proteins.



Video 1. **Colocalization of Rab11b with Arf6 during the TGFβ1-induced PM recruitment of Arf6.** Time-lapse recording was performed in MDA-MB-231 cells expressing Arf6-EGFP (green) and RFP-Rab11b (red), of which representative images are shown in Fig. S3 D. Frames were taken every 20 s for 20 min in total.

Table S1. **Primers used for construction of trans-activation–deficient p53, mCherry, HA-Rab11b, or HA-Rab11b C214A/C215A and RT-PCR**

Name	Sequence (5′-3′)
Cloning primers	
hp53_22aa_L22Q_Rv	CTGGTCTGAAAATGTTTCCTGACTCAGAGG
hp53_23aa_W23S_Fw	AGCAAACTACTTCCTGAAAACAACGTTCTG
hp53_53aa_W53Q_Rv	CTGTTGTTCAATATCGTCCGGGGACAGCAT
hp53_54aa_F54S_Fw	AGCACTGAAGACCCAGGTCCAGATGAAGCT
mCherry Fw	AAATTGCGGCCGCTTTACTTGTACAGCTCGTCCATGC
mCherry Rv	CCCAAGCTTGCTGCCACCATCGGGAAGGTGCTATCCAAAATCTTC
HA-Rab11b Fw	CGCGGATCCGCCACCATGTACCCATACGATGTTCCAGATTACGCTAGCGAATTCATGGGGACCCGGGACGACGAG
HA-Rab11b Rv	CGCGGATCCTCACAGGTTCTGGCAGCACTG
HA-Rab11b C214A/C215A Fw	GCCGCCCAGAACCTGTGAGGATCCCAGTGT
HA-Rab11b C214A/C215A Rv	CTGCAGCTTGTTGGGCTTCTGTCCGTCCGT
Primers for RT-PCR	
Rab3b Fw	ATGGCTTCAGTGACAGATGGTA
Rab3b Rv	CAAGCTGCTCTGCAAGGAGC
Rab 7b Fw	ATGAATCCCCGGAAGAAGGT
Rab 7b Rv	TCAGCAGCATCTGCTCCTTGA
Rab11c Fw	CTGGTGTTTGACCTAACCAA
Rab11c Rv	GAGGTATTTGTGATAGGGCA
GAPDH Fw	ACCCAGAAGACTGTGGATGG
GAPDH Rv	TCTAGACGGCAGGTCAGGTC

Table S2. **Sequences targeted by siRNAs**

Target gene	Sequence (sense; 5′-3′)
ARF6	AAGCACCGCATTATCAATGACCG
GEP100	AAGTGAAATCACTGGCCGAG
AMAP1	AAGACCTGACAAAAGCCATTA
EPB41L5	GAGAUGGAACUGGCUAUUUUU
GAB1 #1	GAGAGUGGAUUAUGUUGUU
GAB1 #2	ACCUUUGAAAGACUGAAUA
c-MET #1	AGAUAAACCUCUCAUAAUG
c-MET #2	GUGCAGUAUCCUCUGACAG
GGT-I #1	GAGACGACUUAAGCCGAGU
GGT-I #2	GGAUAAAGAGGUGGUGUAU
GGT-II #1	GCAGAUUAUAUCGCAUCCU
GGT-II #2	GCCAACAUGAAUGUGGUGG
RAB3B #1	GCCAUGGGCUUCAUUCUGA
RAB3B #2	CUACUCAGAUCAAGACCUA
RAB7B #1	UCACCGACCCGGAGUCUUU
RAB7B #2	UACCAGAGCAUCUUAGAAA
RAB11A #1	AGAGCGAUAUCGAGCUAUA
RAB11A #2	GCAUCCAGGUUGAUGGAAA
RAB11B #1	GCAACAUCGUCAUCAUGCU
RAB11B #2	AGAACAACUUGUCCUUCAU
RAB11C #1	CCAAUCUACUCUCCCGAUU
RAB11C #2	CCGCUGCUGUCAAGGCUCA

siRNAs were synthesized with 3′ dTdT overhangs that are not indicated in the table.