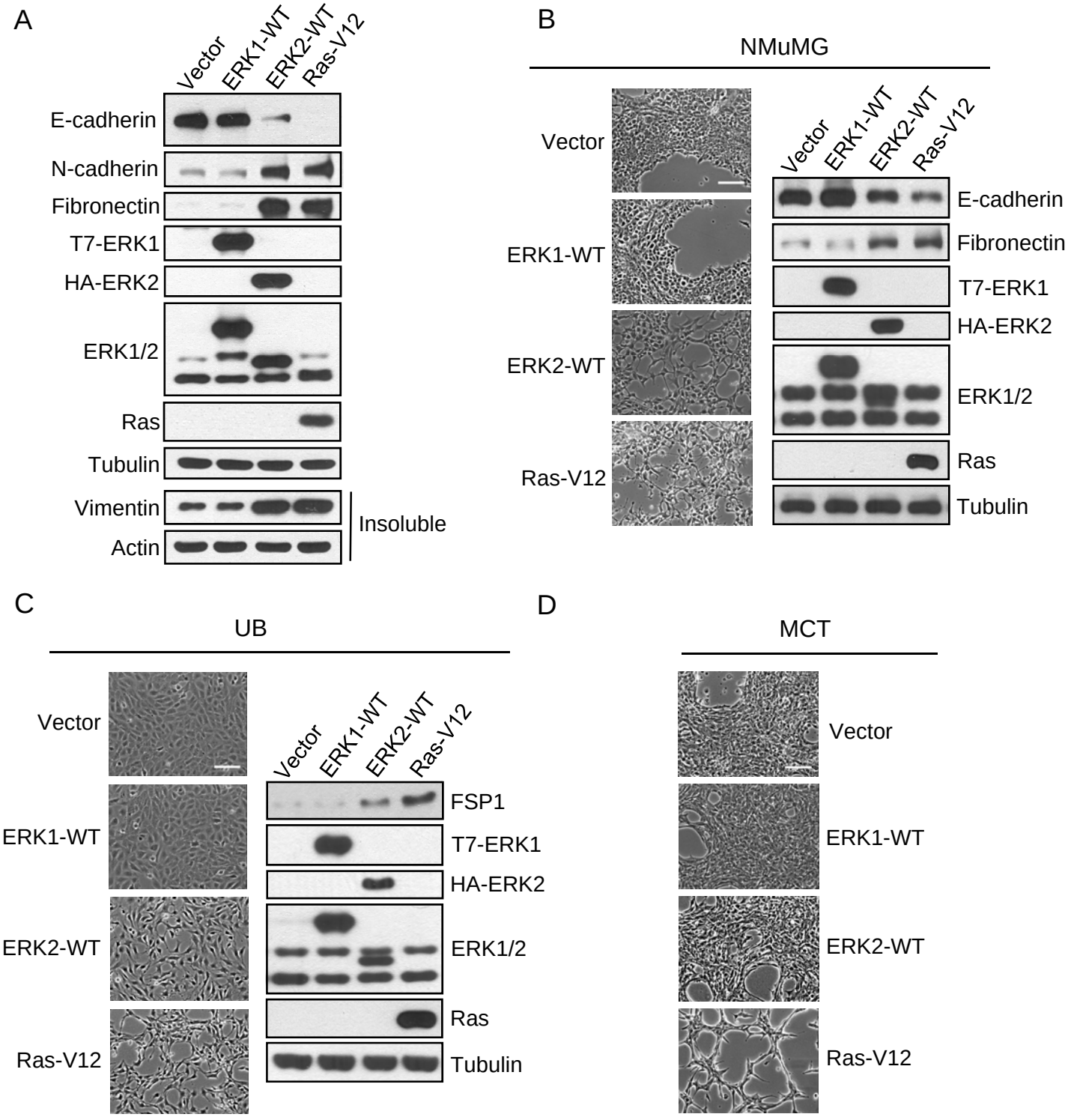


Figure S1, related to Figure 1

ERK2 specifically induces EMT. **(A)** After generating stable ERK1, ERK2 or RasV12 expressing cells by retroviral infection, cells were examined for EMT markers. MCF-10A cell lysates were prepared and subjected to immunoblot analysis. **(B-C)** Stable ERK1, ERK2 or RasV12 expressing NMuMG cells (B) and UB cells (C) were grown and fixed for phase-contrast microscopy. For the detection of proteins, cells were lysed and immunoblot analysis was performed. Bar scale, 100 μm. **(D)** Stable MCT cells expressing ERK1, ERK2, or RasV12 were grown in DMEM supplemented with 10% FBS. Media were then replaced with serum-free K1 medium (1:1 Ham's F12/DMEM with 5 μg/ml transferrin, 5 μg/ml insulin, and 50 nM hydrocortisone) containing 20 ng/ml EGF. Cell morphology was assessed by phase-contrast microscopy. Bar scale, 100 μm.

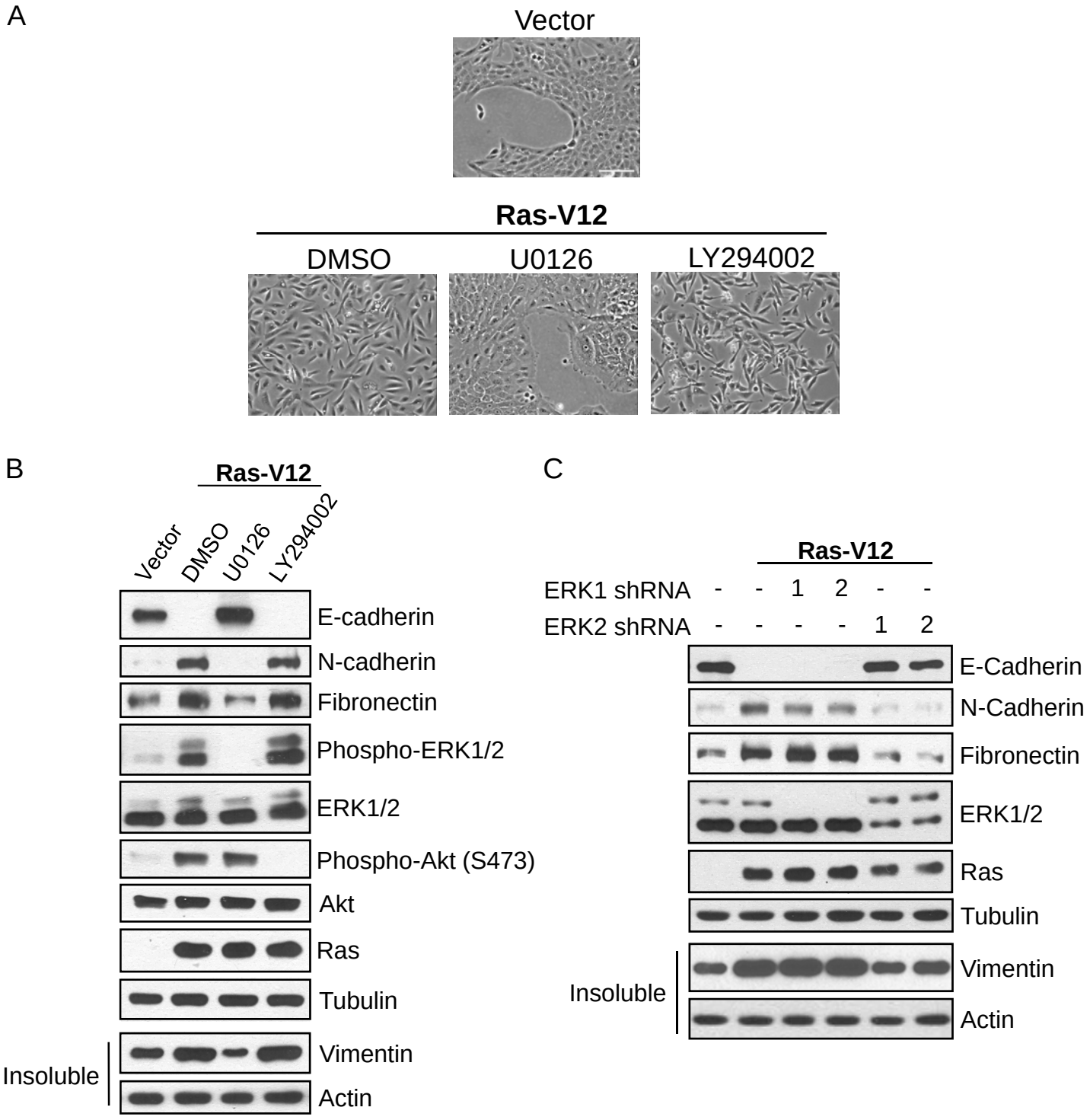
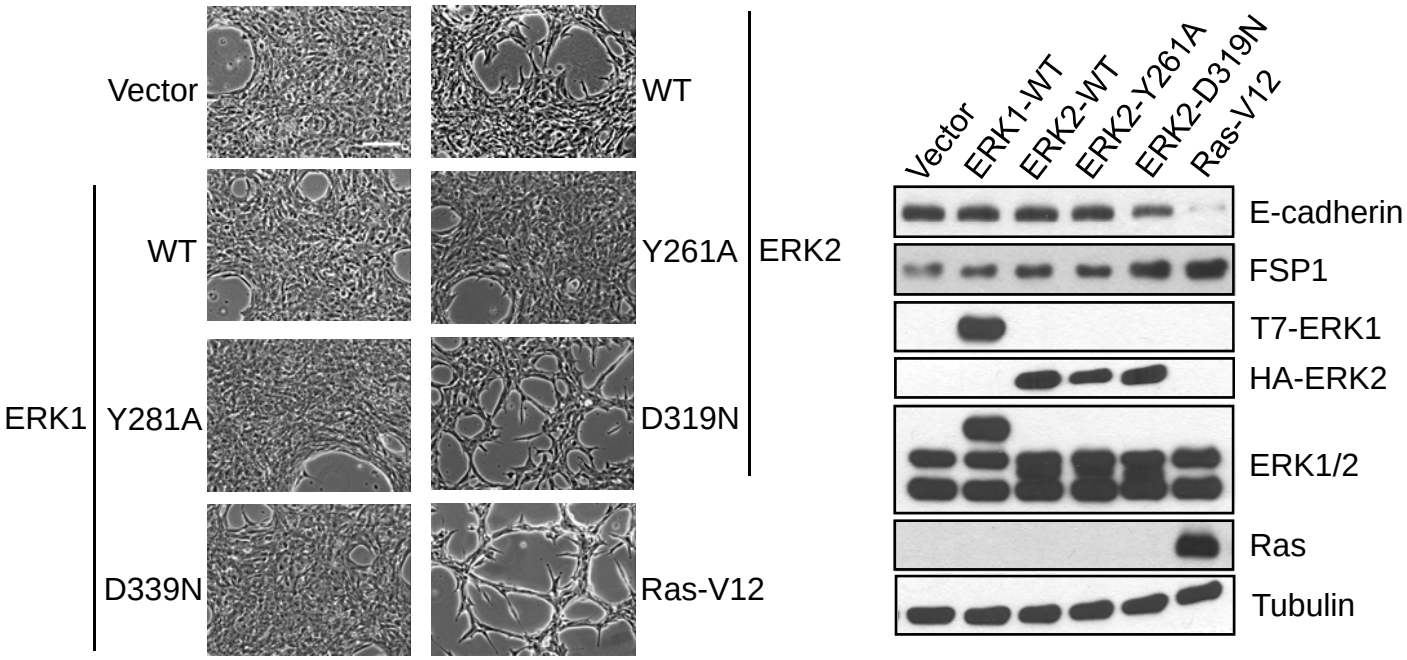


Figure S2, related to Figure 2

RasV12 induces EMT in an ERK2-dependent manner. **(A-B)** MCF-10A cells were infected with retrovirus encoding for RasV12. Prior to infection, cells were treated with either DMSO or small molecule inhibitors of the ERK-MAPK (U0126, 10 μM) or the PI-3K (LY294002, 20 μM) pathways to impair RasV12-induced signaling to these pathways. Cells were maintained in the presence of the indicated compound for one week prior to plating on glass coverslips and phase-contrast microscopy (A) or lysis and immunoblot analysis for markers of EMT (B). Bar scale, 100 μm. **(C)** MCF-10A cells were infected with lentiviral shRNA constructs targeting either endogenous ERK1 or ERK2. Two unique shRNA constructs were utilized for each target. Following knock down, cells were infected with RasV12 encoding virus and subsequently analyzed for markers of EMT by immunoblot analysis.

A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WT
Y261A
ERK2
Y281A
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D339N
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ERK2-WT
ERK2-Y261A
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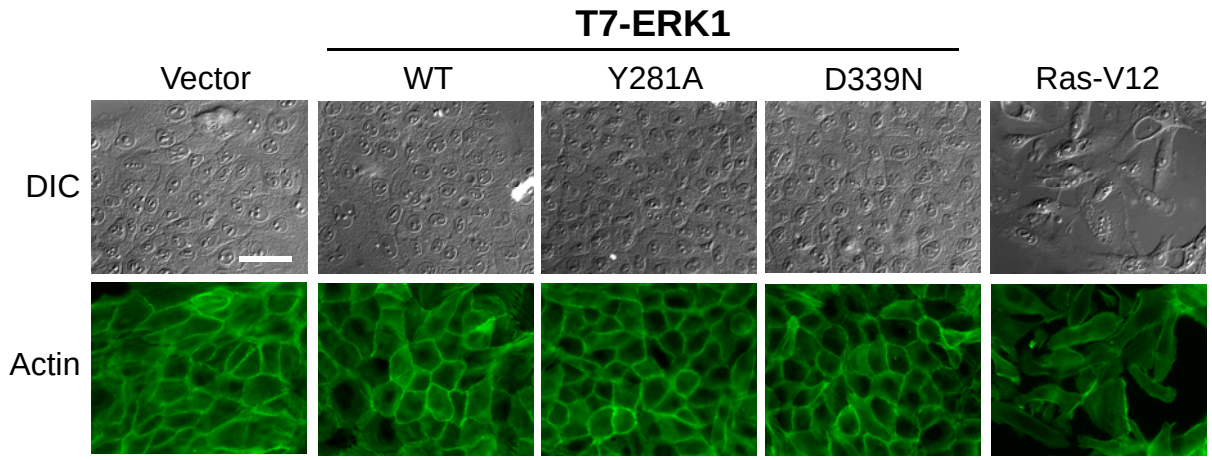
FSP1
T7-ERK1
HA-ERK2
ERK1/2
Ras
Tubulin

B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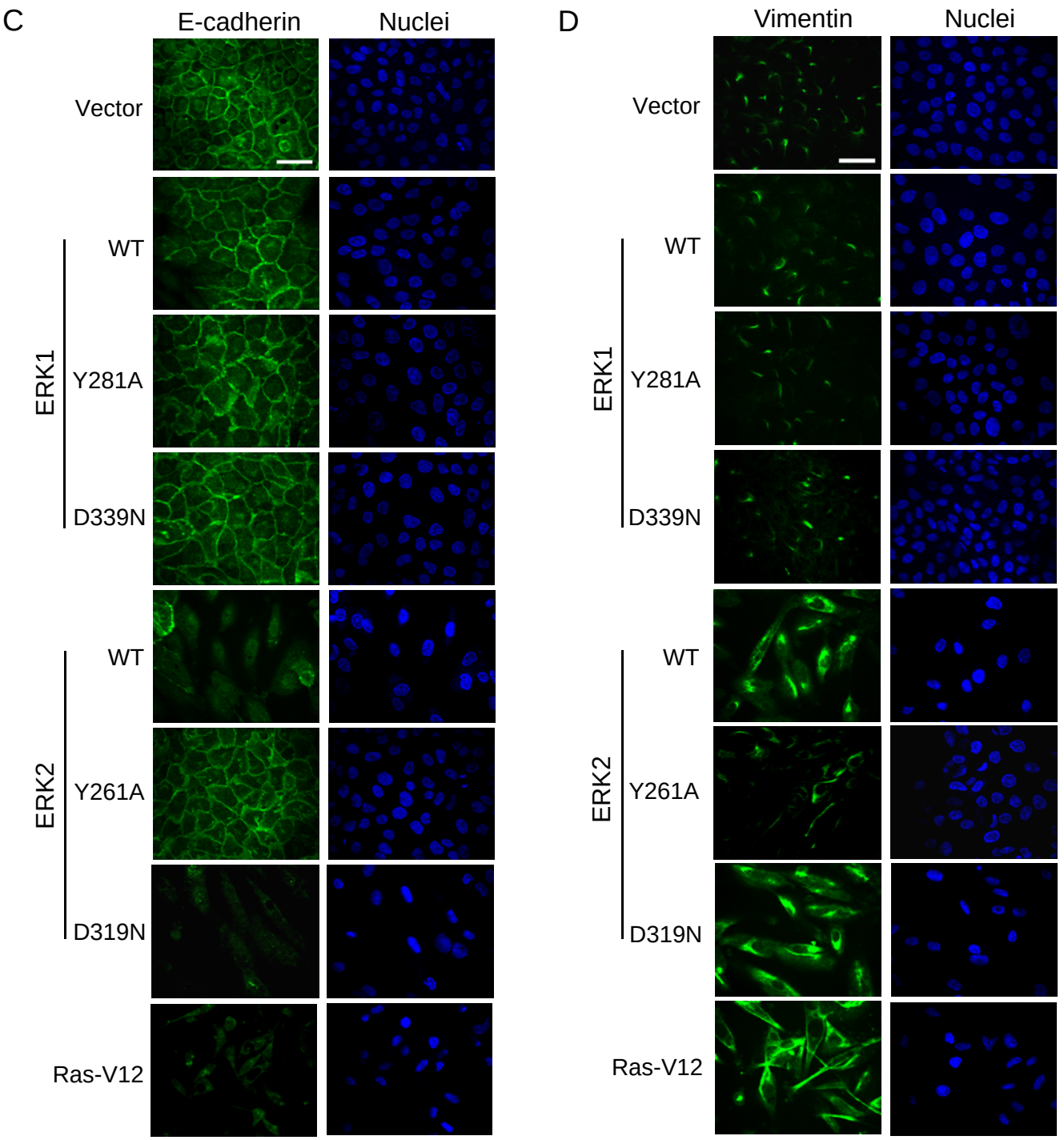
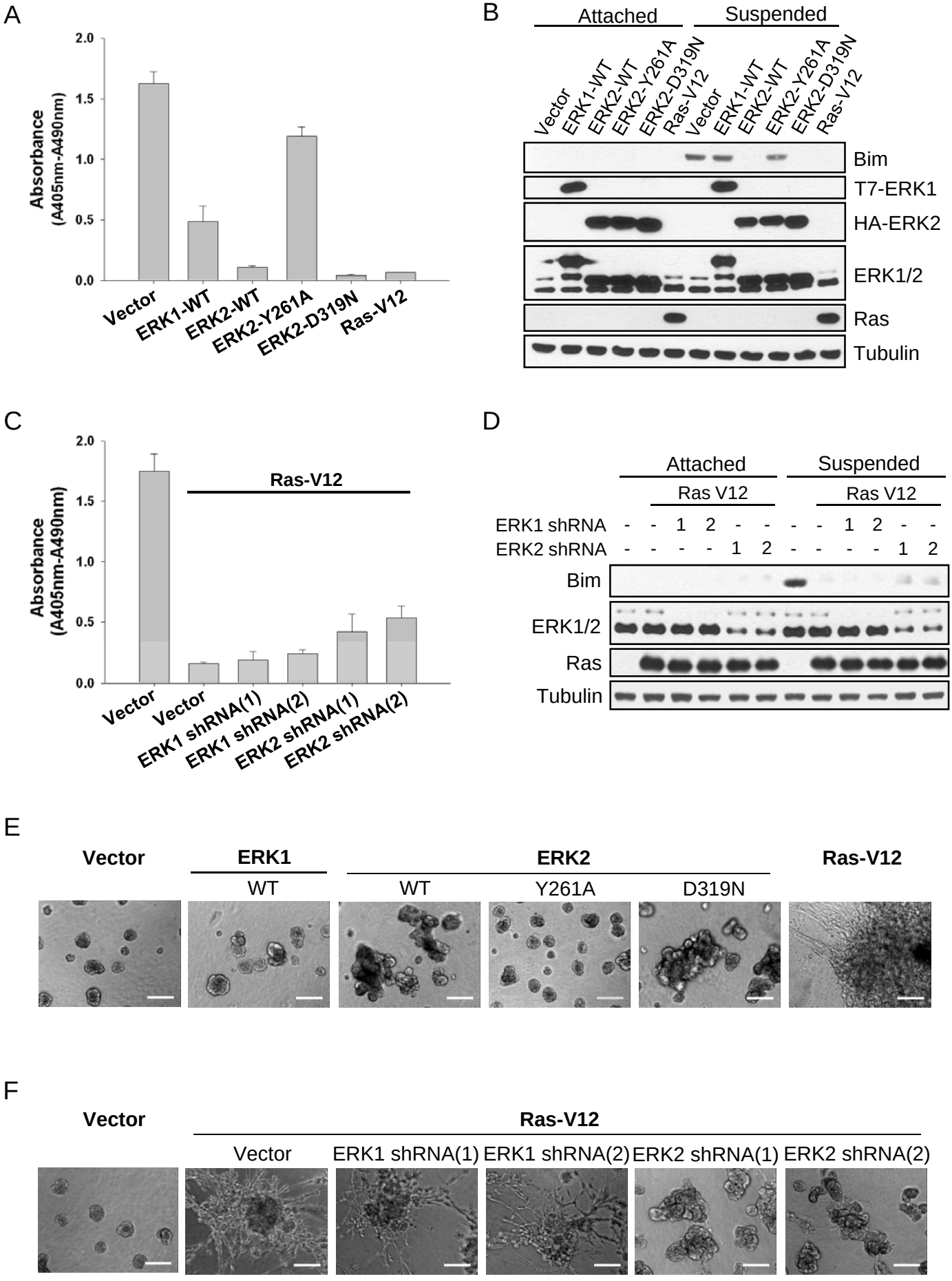


Figure S3, related to Figure 3

ERK2 specifically induces EMT in a DEF domain dependent manner. **(A)** Stable MCT cells expressing ERK1, ERK2, or RasV12 were grown in DMEM supplemented with 10% FBS and media was replace as in Fig. S1D. Cells were fixed and subject to phase-contrast microscopy. For the detection of proteins, cells were lysed and immunoblot analysis was performed. Bar scale, 100 μm. **(B)** ERK1-WT, -Y281A (a DPB mutant allele) or –D339N (a CD domain mutant allele) were overexpressed in MCF-10A cells and subsequently plated on glass coverslips, grown for 24-48 hours and fixed. Cells were processed for actin cytoskeleton and nuclear staining and then analyzed. Bar scale, 50 μm. **(C-D)** Stable MCF-10A cells were generated as previously described and assessed for E-cadherin (C) or vimentin (D) expression by immunofluorescence staining. Bar scale, 50 μm.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Ras-V12
B
Attached
Suspended
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Bim
T7-ERK1
HA-ERK2
ERK1/2
Ras
Tubulin
C
Absorbance (A405nm-A490nm)
Ras-V12
Vector
Vector
ERK1 shRNA(1)
ERK1 shRNA(2)
ERK2 shRNA(1)
ERK2 shRNA(2)
D
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Suspended
Ras V12
Ras V12
ERK1 shRNA - - 1 2 - - - - 1 2 - -
ERK2 shRNA - - - - 1 2 - - - - 1 2
Bim
ERK1/2
Ras
Tubulin
E
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ERK2
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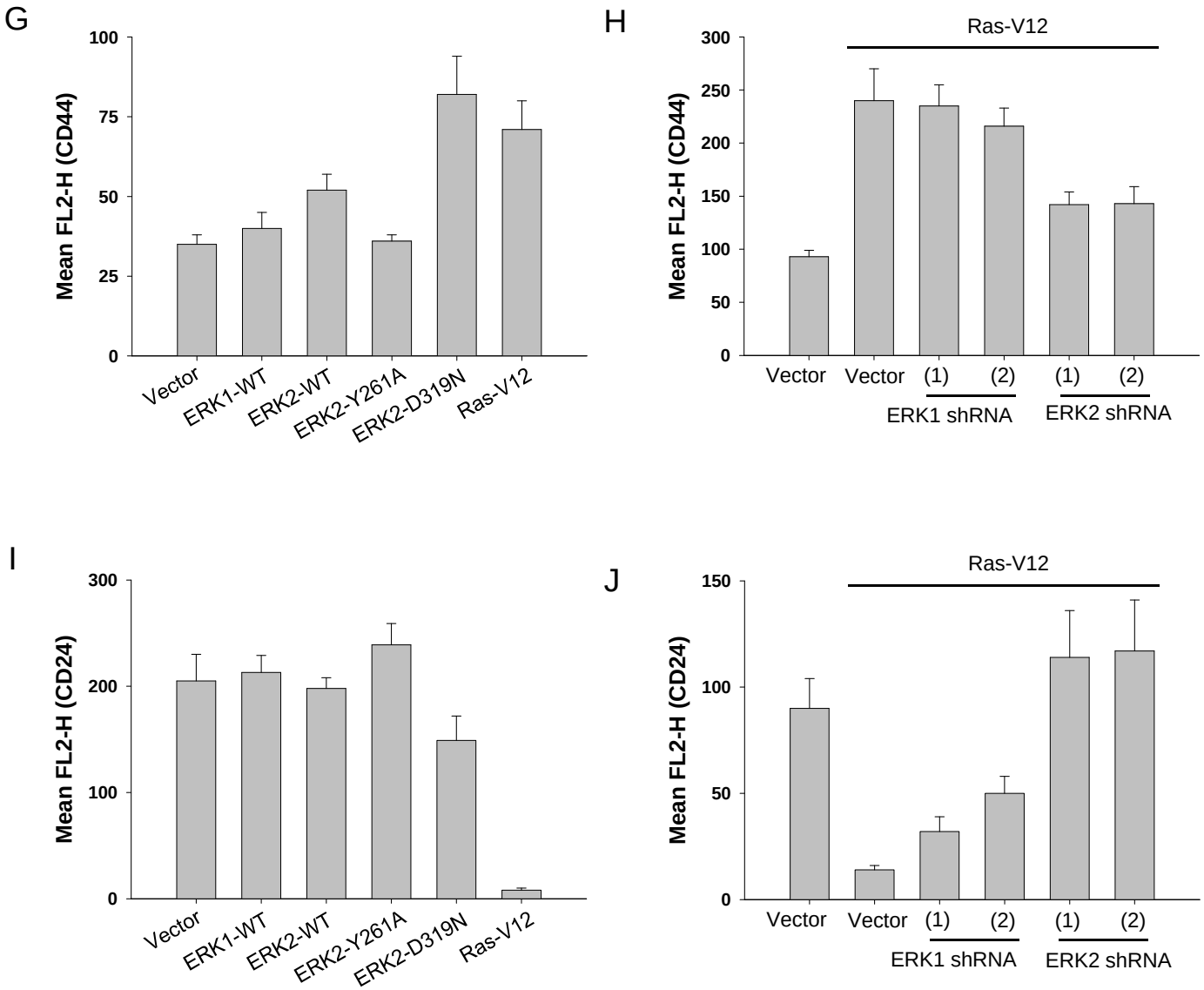
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H
Ras-V12
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200
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100
50
0
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Vector
(1)
(2)
(1)
(2)
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ERK2 shRNA
I
Mean FL2-H (CD24)
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200
100
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ERK2-WT
ERK2-Y261A
ERK2-D319N
Ras-V12
J
Ras-V12
Mean FL2-H (CD24)
150
100
50
0
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Vector
(1)
(2)
(1)
(2)
ERK1 shRNA
ERK2 shRNA
K
TGF-β1(pg/ml) / 1e5 cells
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350
300
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200
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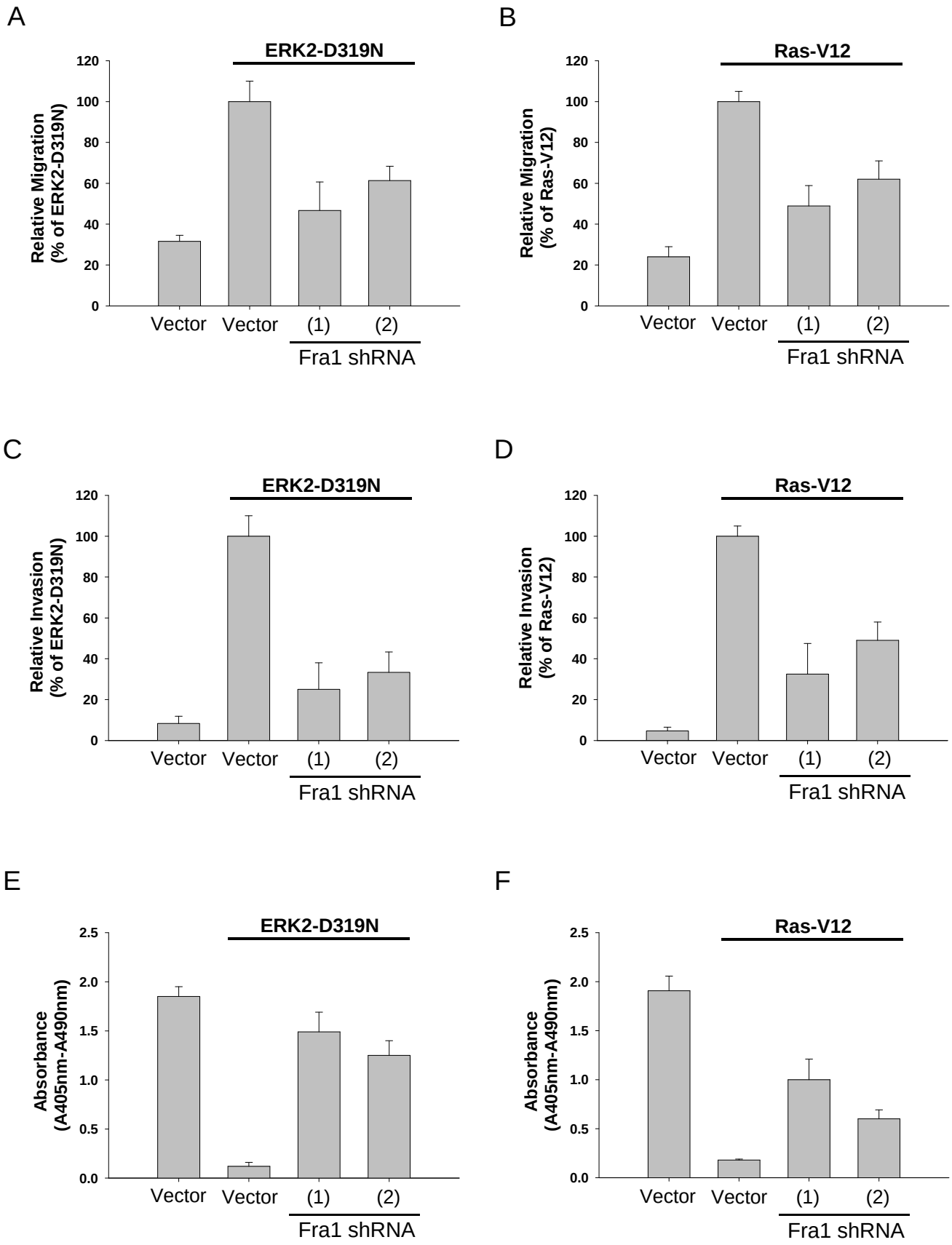
100
50
Vector
ERK1-WT
ERK2-WT
ERK2-Y261A
ERK2-D319N
Ras-V12
D1-3
D8-10

Figure S4, related to Figure 4

ERK1 and ERK2 have a differential effect on resistance to anoikis, mammary acinar morphogenesis, and cell surface expressions of CD44/CD24. **(A)** Stable MCF-10A cells were assessed for their ability to survive following plating in Poly-HEMA coated tissue culture plates. Cells were cultured for 48 hours and assayed as described in materials and methods. Increased absorbance is indicative of increased cell death. Data are the means +/- SEM of three separate experiments performed in duplicate. **(B)** Cells were treated as in Fig S4A, collected, washed and lysed as described in the materials and methods. Cellular lysates were then subjected to SDS-PAGE separation and immunoblot analysis was performed. **(C-D)** Ras V12 cells expressing vector control, ERK1 shRNA or ERK2 shRNA vector were grown in Poly-HEMA coated plates for 48 hours and their anoikis sensitivity was measured (C). Data are the means +/- SEM of three separate experiments performed in duplicate. Cell lysates collected and proteins were analyzed by immunoblotting with the indicated antibodies (D). **(E)** Stable ERK1, ERK2, or Ras V12 expressing cells were grown in 3D Matrigel cultures for 15-16 days and subjected to phase-contrast microscopy. Bar scale, 100 μm. **(F)** Stable ERK1 or ERK2 knockdown cells were infected with Ras V12, and then cultured in 3D Matrigel for 8 days. Similar results were obtained when monitored out to 16 days. Bar scale, 100 μm. **(G)** ERK1, various ERK2 alleles, or Ras V12 expressing cells were prepared for FACS analysis as described in the materials & methods, and CD44 cell surface expression was measured. Data are the means +/- SEM of three separate experiments performed in duplicate. **(H)** ERK1 or ERK2 knockdown cells were infected with RasV12 encoding virus and subsequently analyzed for cell surface CD44 levels by FACS. Data are the means +/- SEM of three separate experiments performed in duplicate. **(I)** ERK1, various ERK2 alleles, or Ras V12 expressing cells were prepared and CD24 cell surface expression was measured. Data are the means +/- SEM of three separate experiments performed in duplicate. **(J)** ERK1 or ERK2 knockdown cells were infected with RasV12 encoding virus and subjected to FACS analysis for detection CD24 surface levels. Data are the means +/- SEM of three separate experiments performed in duplicate. **(K)** Cells were plated at 1 day and 8 days post-retroviral infection and grown for 48 hours in TGF-β assay media as described in materials and methods. Cell culture supernatant was collected and analyzed using a TGF-β1 ELISA kit. Data show are the means +/- SEM of three separate experiments preformed in duplicate.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Vector
(1)
(2)
Fra1 shRNA
B
Ras-V12
Relative Migration (% of Ras-V12)
C
ERK2-D319N
Relative Invasion (% of ERK2-D319N)
D
Ras-V12
Relative Invasion (% of Ras-V12)
E
ERK2-D319N
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1.0
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F
Ras-V12

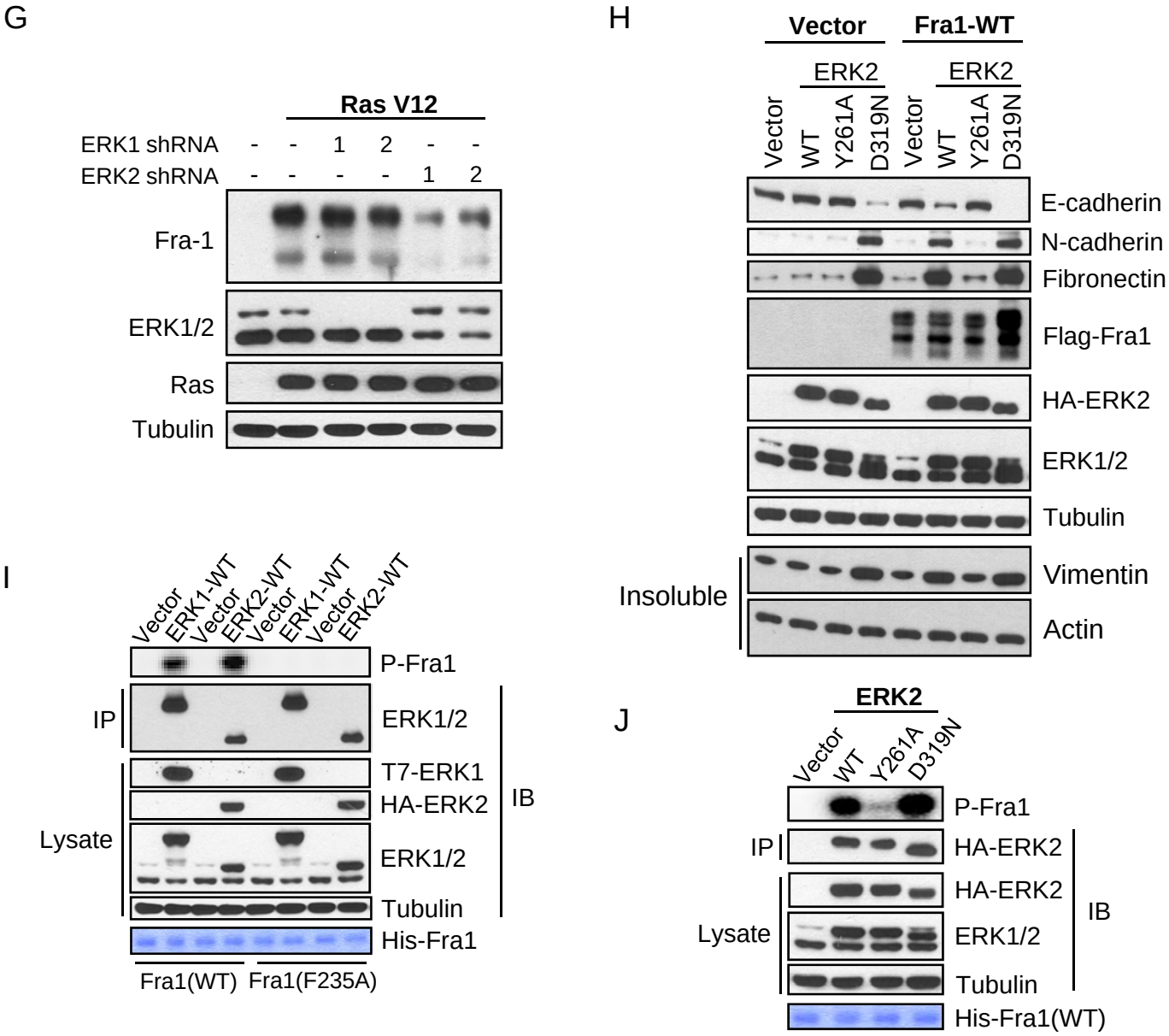


Figure S5, related to Figure 5

Fra1 is regulated by Ras/ERK2 and is necessary for ERK2 or Ras-induced migration, invasion, resistance to anoikis. **(A-B)** Fra1 knockdown cells were infected with ERK2-D319N (A) or RasV12 (B) encoding retrovirus and migration assays were performed. Data are the means +/- SEM of three separate experiments performed in duplicate. **(C-D)** Invasion assays were performed with the stable cells described in A, B. Data are the means +/- SEM of three separate experiments performed in duplicate. **(E-F)** The cells described in A, B were placed in suspension and assayed for detachment-induced cell death. Data are the means +/- SEM of three separate experiments performed in duplicate. **(G)** Endogenous ERK1 or ERK2 were downregulated using two unique lentiviral shRNA constructs prior to infection with RasV12. After stable cells were selected, expression of Fra1 was assessed by immunoblot analysis. **(H)** Following infection with Fra1-WT, cells were infected with various HA-ERK2 encoding retrovirus. Cell lysates were analyzed by immunoblot analysis with the indicated antibodies. **(I)** Stable cells expressing ERK1 and ERK2 were lysed, and immunoprecipitation was performed. Phosphotransferase assays were performed using purified His-Fra1-WT and His-Fra1-F235A as substrates. Band intensity was quantitated using Image J software. Phosphorylation of Fra1 by ERK2 was ~1.5 times greater than that by ERK1 in this experiment, and the amount of immunoprecipitated of ERK1 protein was ~3 times greater than that of ERK2. Therefore, ERK2 exhibits significantly greater phosphotransferase activity towards Fra1 than ERK1 in vitro. **(J)** Stable cells expressing various ERK2 alleles were lysed, and immunoprecipitation was performed. Phosphotransferase assays were performed using purified His-WT-Fra1 as a substrate.

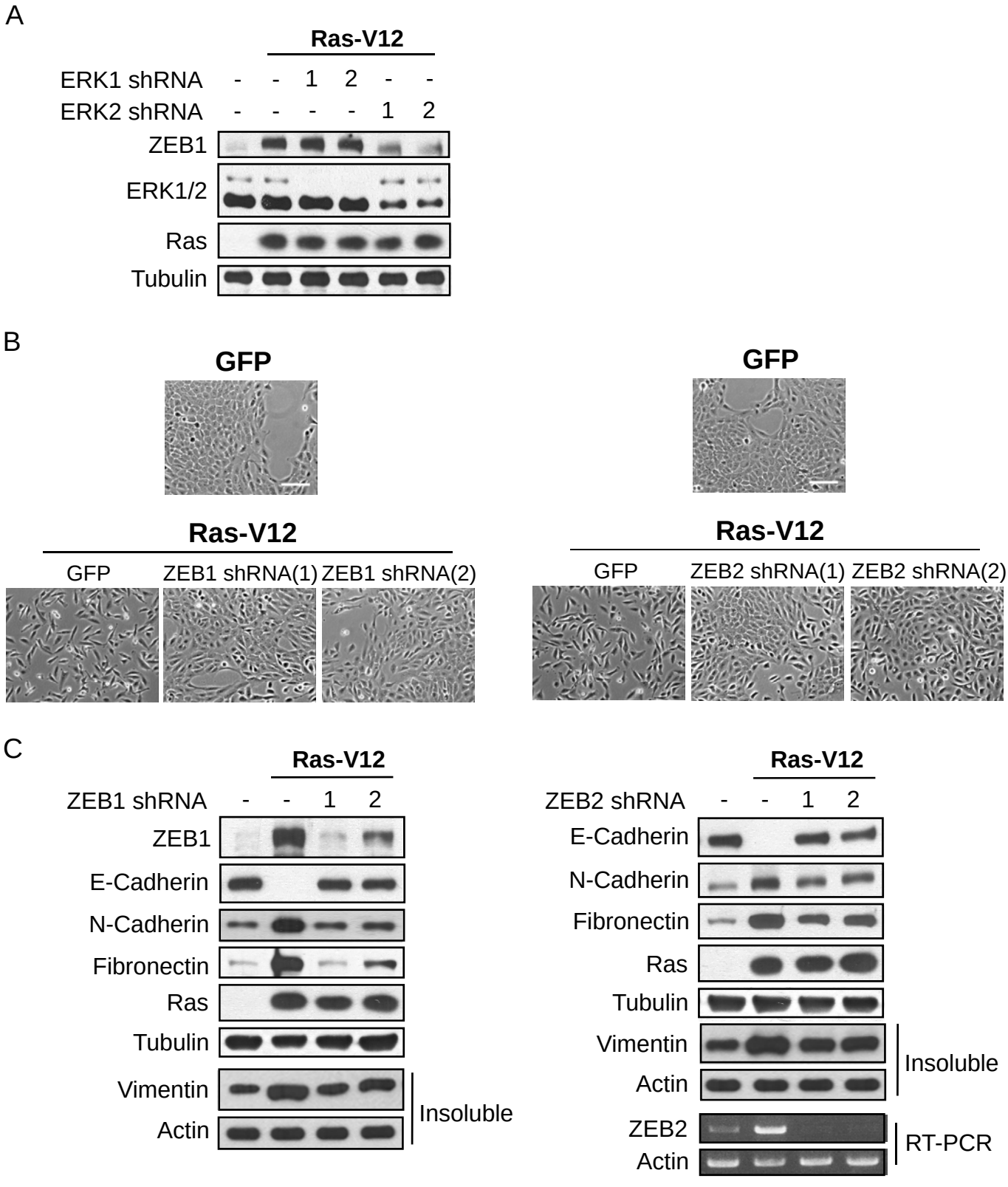
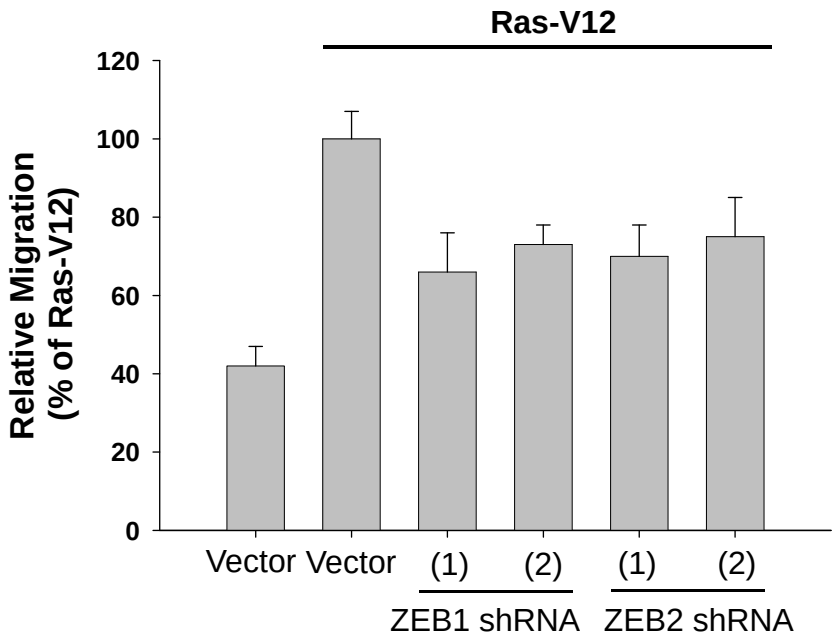


Figure S6, related to Figure 6

ZEB1/2 are downstream targets of Ras/ERK2/Fra1. **(A)** ERK1 or ERK2 knockdown cells were infected with RasV12 and lysed for immunoblot analysis. **(B-C)** Stable ZEB1 or ZEB2 knockdown cells were infected with Ras V12 encoding retrovirus. Cell morphology (B) and protein expression (C) were assessed by phase-contrast microscopy and immunoblot analysis, respectively. Bar sacle, 100 μm.

A



B

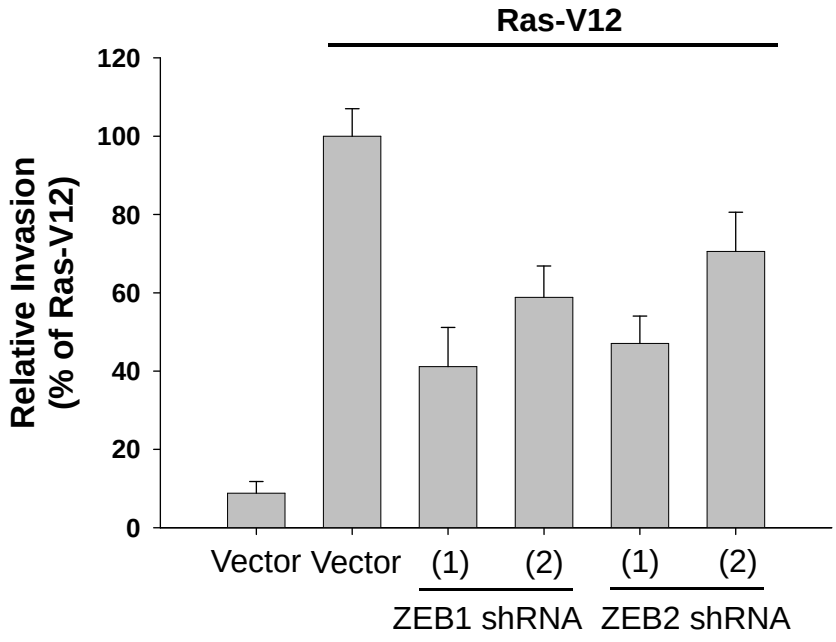


Figure S7, related to Figure 7

ZEB1/2 are necessary for Ras-induced migration and invasion. ZEB1 or ZEB2 down-regulated cells were infected with RasV12, and migration (A) and invasion (B) assays were performed.

Experimental Procedures

Reagents

The Gal4-Elk1 luciferase construct and the Gal4-Fra1 luciferase reporter were kindly provided by Dr. Kun-Liang Guan (University of Michigan, MI) and Dr. Nancy Colburn (National Cancer Institute, MD), respectively. pRK7-Flag-HA-RSK1 was kindly provided by Dr. Yuki Abe (Harvard Medical School, MA). All lentiviral shRNA constructs were kindly provided by Dr. William C. Hahn (Dana-Farber Institute, MA).

EGF was purchased from Peprotech, and U0126 and LY294002 were from Biomol. Anti-T7 antibody and anti-FSP1 antibody were from Novagen and Abcam, respectively. Anti-HA (clone 12CA5) antibody was prepared from ascites and anti-ERK1/2 antibody was previously described (Chen et al., 1992). Anti-phospho-ERK1/2 (pTEpY), anti-ERK5 (BMK), anti-tubulin and anti-Flag (M2) antibodies were from Sigma. Anti-phospho-RSK (T573) antibody was from R&D Systems and anti-Bim antibody was from Calbiochem. Anti-actin and anti-Fra1 antibodies were from Santa Cruz Biotechnology. Anti-Ras, anti-phospho-Akt (S473) and anti-Akt antibodies were purchased from Cell Signaling Technology. Anti-E-cadherin, anti-N-cadherin, anti-fibronectin, anti-vimentin, anti-CD44, and anti-CD24 antibodies were from BD Biosciences. Anti-mouse, anti-rabbit, and anti-goat HRP-conjugated antibodies were purchased from Chemicon, Amersham and Santa Cruz Biotechnology, respectively.

Indirect Immunofluorescence Microscopy

Cells were plated on glass coverslips for 24-48 hours in growth media. Cells were washed once with ice cold phosphate-buffered saline (PBS), fixed in 3.7% formaldehyde/PBS for 15 minutes at room temperature, and permeabilized with 0.1% Triton X-100/PBS for 5 minutes. Coverslips were blocked for 20 minutes in blocking solution (1% BSA/PBS) and then incubated with specific antibodies in blocking solution for 90-120 min. The cells were rinsed four times and incubated with a secondary antibody conjugated to a flurochrome (Jackson Immuno Research) in blocking solution for 1h. Cells were rinsed with PBS four times, mounted with mounting buffer (1% propylgallate in a mixture of glycerol and PBS (8:2)) and examined using a Nikon TE2000 w/PerkinElmer Spinning Disk Confocal microscope. DNA was visualized by TOPRO-3 (Molecular Probes) staining.

For staining of the actin cytoskeleton, Oregon Green 488 conjugated phalloidin (Molecular Probes) was used and nuclei were stained with DAPI. DIC/phalloidin images and phase-contrast images were obtained using a Nikon 80i upright microscope and a Nikon Eclipse TE300 microscope, respectively.

Cell Culture and Generation of Stable Cell Lines

MCF-10A mammary epithelial cells and NMuMG mammary epithelial cells were obtained from the American Type Culture Collection and were cultured as previously described (Debnath et al., 2003, Xie et al, 2004). UB ureteric bud cells and MCT mouse cortical tubule epithelial cells were kindly provided from Dr. Roy Zent (Vanderbilt University) and Dr. Raghu Kalluri (Harvard Medical School), respectively, and cultured as previously described (Pozzi et al., 2006; Okada et al., 1997). To generate retroviruses, 293 GPG packaging cells (Ory et al., 1996) were transfected with plasmids using Lipofectamine2000 (Invitrogen). Media was changed 24 hours following transfection. Cell supernatant was collected on days 4 through 7 after plating, filtered through 0.45 μm pore filters and aliquots were frozen at -80˚C. Stable pools of cells overexpressing individual proteins were generated by retroviral infection in the presence of growth media supplemented with 8 μg/ml polybrene. Following infection, the cells were selected using puromycin or neomycin as described (Debnath et al., 2003). For the co-expression of Fra1 and ERK2, cells were infected with Fra1 viral supernatants and selected with neomycine first, and then infected with ERK2 viral supernatants and selected with puromycin. Cells were used immediately following selection and for up to three weeks after selection. Fresh stable cell lines were made before each group of experiments and experiments were performed following at least two separate infections.

Generation of Stable Knockdown Cells

To generate lentiviruses, shRNA plasmids or control shRNA plasmids were transfected into 293T cells with the packaging (Δ8.9) and envelope (VSVG) expression plasmids. The following day, transfection media was removed and cells were incubated in DMEM/10% FBS for two additional days. Three days after transfection, viral supernatants were harvested, filtered through 0.45 μm pore filters, and aliquots were frozen at -80˚C. Cells were infected in the presence of growth media supplemented with 8 μg/ml polybrene. Following infection for 24 hours, cells were treated with 2.0 μg/ml puromycin and cells that stably expressed targeting shRNA vectors or control shRNA

vectors were selected. The decreased level of individual targets was confirmed by immunoblot analysis or RT-PCR.

Immunoblot Analysis and Immunoprecipitation

To examine protein expression, cells were plated for 24-48 hours before cell lysis, unless otherwise indicated. For lysate preparation, cells were washed once with ice cold PBS prior to lysis on ice with cold lysis buffer (10 mM KPO_4 (pH 7.2), 1 mM EDTA, 10 mM $MgCl_2$, 5 mM EGTA, 50 mM β-glycerophosphate, 0.5% NP-40, 0.1% Brij-35, 1mM Na_3VO_4, 1mM NaF, 1mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1μg/ml pepstatin). Lysates were cleared by centrifugation at 16,000 g for 10 minutes at 4°C. In order to examine the NP-40 insoluble material, the cell pellet was resuspended in SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 8.3% glycerol, 1.5% SDS, 1.5% 2-mercaptoethanol, 0.005% bromophenol blue) using a 25 gauge syringe needle. The protein concentration of the soluble fraction was determined by Bradford analysis; equal amounts of protein were loaded on SDS-PAGE gels and transferred to nitrocellulose. The membranes were blocked with TBST (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat dried milk, and probed overnight at 4°C with primary antibodies. The following day, membranes were incubated with secondary antibodies and developed using enhanced chemiluminescence.

To examine ERK1/2 phosphorylation of RSK1, pRK7-Flag-HA-RSK1 was transfected into stable ERK1-WT, various ERK2 alleles expressing MCF-10A cells using Fugene6 (Roche) transfection reagent. Following overnight transfection, growth media was removed and cells were starved overnight in MCF-10A assay media (Debnath et al., 2003). The next day, cells were stimulated with 50 ng/ml of EGF for 5 minutes and then lysed as described above. Following cell lysis and protein determination, equal concentrations of lysate were subjected to immunoprecipitation with anti-Flag (M2) antibody for 2 hours at 4˚C and precipitated with protein A sepharose beads. Beads were eluted in 2x SDS-PAGE sample buffer and immunoblot analysis was performed.

Protein purification

Human Fra1 cDNA was cloned into pQE30 (Qiagen) vector. The F235A point mutation was introduced using the Quickchange kit (Stratagene) and sequences were verified. The plasmids were transformed into *Escherichia coli* strain M15, and purified to

homogeneity from crude lysates using Ni-NTA-Agarose (Qiagen) according to the manufacture's protocol. Briefly, the protein expression was induced by 1 mM IPTG and bacterial lysates were prepared by sonication in buffer A (20 mM Tris-HCl, pH 7.5, and 100 mM NaCl). After centrifugation at 14,000 rpm for 15 min, the supernatant was applied to Ni-NTA-Agarose column, washed with buffer A containing 30 mM imidazole, and eluted with buffer A containing 200 mM imidazole. Eluted proteins were dialyzed extensively with PBS containing 1 mM DTT and stored at -80 ^{o}C.

Protein Phosphotransferase Assays

ERK1-WT, ERK2-WT, or ERK2-mutants expressing cells were lysed and immunoprecipitation was performed using either anti-T7 or anti-HA antibodies. The immunoprecipitates were washed two times with lysis buffer and twice with kinase buffer (25 mM Tris, pH 7.4, 2 mM DTT, 10 mM $MgCl_2$, 5 mM β-glycerophosphate), and kinase assays were performed as described previously (Roux et al, 2007) with recombinant His-Fra1 protein as a substrate. The reaction products were subjected to SDS-PAGE, and subsequent autoradiography.

Flow Cytometric Analysis

Cells were trypsinized and washed twice with ice-cold PBS containing 0.2% BSA. Aliquots of cells were incubated for 1 hr at 4°C with either anti-CD44, anti-CD24, or isotype-matched control IgG antibody in the PBS/BSA solution. The cells were washed three times with PBS/BSA and then incubated with secondary antibodies coupled to R-phycoerythrin for 1 hr at 4°C. After washing three times with PBS/BSA, the cells were resuspended in PBS and analyzed using a FACScan (Becton Dickinson).

RT-PCR analysis

Total cellular RNA was purified from cultured cells using the RNeasy mini kit (Qiagen) following the protocol of the manufacturer. After a 50°C/50-min reverse transcription step, each mRNA was PCR amplified in 25 cycles for 1 min at each of the following temperatures; 94°C, 55°C, and 72°C. PCR products were analyzed on 1% agarose gels. The sequences of amplification primers were as follows: Snail forward, TTTACCTTCCAGCAGCCCTA; Snail reverse, CCAGGCTGAGGTATTCCTTG; Slug forward, GAGCATACAGCCCCATCACT; Slug reverse, CTCCCCCGTGTGAGTTCT-

AA ; Twist forward, GTCCGCAGTCTTACGAGGAG ; Twist reverse, CTAGTGGGACG-CGGACAT ; ZEB1 forward, GCACCTGAAGAGGACCAGAG ; ZEB1 reverse, TGGTG-ATGCTGAAAGAGACG ; ZEB2 forward, AATGCACAGAGTGTGGCAAG ; ZEB2 reverse, CGACAGGCGGAATATTAGGA ; β-actin forward, AAATCTGGCACCACACC-TTC ; and β-actin reverse, AGCACTGTGTTGGCGTACAG .

Luciferase Assay

Pools of stable MCF-10A cells were plated in 12-well tissue culture plates for 24 hours and transfected with Gal4-firefly luciferase and Gal4-Elk-1 or Gal4-Fra1 using Fugene6 (Roche) transfection reagent. pRL-tk renilla luciferase (Promega) was also transfected and served as an internal control. After six hours, fresh growth media was added to the transfection reaction and the cells were incubated overnight. Cells were harvested and luciferase activity was measured using the Dual Luciferase Reporter assay system (Promega).

Transwell Migration and Invasion Assays

Cells were trypsinized and collected as previously described (Debnath et al., 2003). Resuspension media was aspirated and cells were resuspended in assay media. For migration assays, assay media supplemented with EGF (5 ng/ml) was added to the bottom chamber of the cell culture inserts. Cells (5×10^4 / 100 μl) were then added to the top chamber of cell culture inserts (BD Biosciences, 8 μm pore size) in a 24-well companion plate. After overnight incubation, the cells that had invaded the lower surface of the membrane were fixed with methanol and stained with 0.2% crystal violet in 2% ethanol. The number of cells that had migrated was quantified by counting 10 random distinct fields using a light microscope. For cell invasion assays, BD BioCoat invasion chambers coated with Matrigel were used. Invasion chambers were prepared according to manufacturers specifications and assays were performed as described for migration assays, except that 20 ng/ml of EGF was added to MCF-10A assay media to serve as the chemo-attractant.

Anoikis Assays

Six-well tissue culture plates were coated with 6 mg/ml Poly-HEMA in 95% ethanol and incubated at 37˚C until dry. MCF-10A cells were plated at 2 x 10^5 cells/ml in growth media for 48 hours. Cells were collected, washed with PBS, and cell death was measured using a cell death ELISA kit (Roche Diagnostics) according to the manufacturer's specifications. Each stable cell line was assayed in duplicate and duplicates were averaged to obtain the mean value for cell death.

Morphogenesis Assay

The three-dimensional culture of MCF-10A cells in Matrigel was carried out as previously described (Debnath et al., 2002).

TGF-β1 ELISA Assay

Stable cells were plated one day or eight days following retroviral infection at 3 x 10^5 cells per 6-well plate in TGF-β assay media (MCF-10A assay media supplemented with 20 ng/ml EGF) and allowed to grow for 48 hours. Cell culture supernatant was then collected, snap-frozen in liquid nitrogen, and stored at -80˚C until assayed. Activated TGF-β1 levels were determined using the Human TGF-β1 Quantikine ELISA (R&D Systems) according to manufacturer's directions. Autocrine production of TGF-β1 was normalized to cell number. Cell number was determined by washing the cells with PBS, trypsinizing at 37˚C, and collecting cells with resuspension buffer following the collection of cell culture supernatant. An aliquot of the cells was taken and counted using a hemacytometer to determine cell number.

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