Supplementary Information

Western blot analysis

Cells seeded in 100 mm culture dishes were washed three times with phosphate-buffered saline and then subjected to lysis in Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 50 mM NaF, 1x protease inhibitor mixture (Roche Biochemicals), 1 mM sodium vanadate for 15 min on ice. Total protein extracts (100 μg) were electrophoresed on 10 or 12% SDS-Polyacrylamide gel and transferred to Nitrocellulose membrane (Bio-Rad). The blots were first incubated with primary antibody and then with horseradish peroxidase-conjugated secondary antibody (Pierce, Rockfor). Immunoreactive proteins were visualized by using the ECL plus Western blotting reagent (Amersham Biosciences, Piscataway, NJ). All the experiments were carried out by culturing the cells in 10% serum until unless mentioned otherwise. Whenever needed cells were treated with ERK inhibitor U0126 (10 μM) for 2 h.

TOP-flash assay and reporter assays

β-catenin driven luciferase plasmid TOP-flash, and its mutant control, FOP-flash, were provided by Dr. M. C. Hung, MD Anderson Cancer center, Houston. In brief, cells were either transfected with 1 μg of pTOP-flash or pFOP-flash (control) reporter constructs or vector alone along with an 40 ng of β-galactosidase coding vector, which can serve as internal control for transfection. Luciferase and β-gal activities were measured 48 hours after transfection. Experiments were performed in triplicate and repeated at least 3 times. The measured luciferase activity was normalized to β-galactosidase activity levels. WISP-1 and cyclin D2 luciferase constructs were obtained from Dr. A. J. Levine and Dr. B. H. Nelson respectively and reporter assays were performed according to the

manufacturers instructions (Promega). Transcription status of Top-flash in HC11/MTA1s clones was determined by following treatment with the ERK inhibitor U0126 (10 μM) for 2 h or with the ERK specific siRNA for 48h.

RT-PCR analysis

For RT-PCR analysis, total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), and first-strand cDNA synthesis was carried out with SuperScript II reverse transcriptase (Invitrogen) using 2 μg of total RNA and poly (dT) primer. RT-PCR was performed with the gene-specific primers listed in supplementary table 1. The levels of mRNA of all the genes were normalized to that of β-actin mRNA.

siRNA transfection

siRNA against β-catenin was purchased from Santa Cruz Biotechnology (catalog #sc-29210). siRNA against MTA1s was purchased from Ambion (catalog #16708A), and ERK siRNA and GSK3-β siRNA, from Dharmacon as manufacturer's protocol.

Confocal analysis

The expression levels of MTA1s, Wnt1, GSK-3β, Phospho GSK-3β, ERK and P-ERK in HC11, T47D, ZR-75, MDA-MB-468, SKBR3 and MDA-MB-435 cells was determined by indirect immunofluorescence. The cells were grown on sterile glass cover slips, fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, and blocked in 10% normal goat serum in PBS. Cells were incubated with primary antibody, washed three times in PBS, and then incubated with appropriate secondary antibodies conjugated with 488-Alexa from Molecular Probes (Eugene, OR). The DAPI (Molecular Probes) was used as a nuclear stain. Microscopic analysis was performed using an Olympus FV300

laser-scanning confocal microscope (Olympus America Inc., Melville, NY) using sequential laser excitation to minimize fluorescence emission bleed-through.

Mammary gland whole mounts and histology

For whole-mount analysis, number 4 inguinal mammary glands were stained with carmine alum and BrdU labeling as described previously (1). Briefly, the glands were fixed with acetic acid/ethanol (1:3) for 2 h and stained with 0.5% carmine/0.2% aluminum potassium sulfate for 16 h. After being rinsed briefly with distilled water, the glands were dehydrated with the use of graded ethanol, and lipids were removed with two changes of acetone. Finally, the glands were preserved in methyl salicylate. For histological analysis, mammary gland tissue was fixed in 10% neutral buffered formaldehyde and embedded in paraffin according to standard methods. Sections (4 µm each) were stained with hematoxylin and eosin.

BrdU labeling

To detect BrdU-positive cells, a sterile solution of BrdU (20 mg/ml; Sigma-Aldrich) in phosphate-buffered saline (PBS; pH 7.4) was injected in mice intraperitoneally (50 mg/kg). Mammary glands were harvested after 3 hours, embedded in paraffin, and sectioned. BrdU incorporation was detected by immunohistochemical staining with a mouse anti-BrdU monoclonal antibody. The mean BrdU-incorporation levels were determined from the results in at least six different mice.

1. Bagheri-Yarmand R, Talukder AH, Wang RA, Vadlamudi RK, Kumar R. Metastasis-associated protein 1 deregulation causes inappropriate mammary gland development and tumorigenesis. Development 2004;131(14):3469-79.