SUPPLEMENTAL DATA

Biomechanical remodeling of the microenvironment by stromal caveolin-1 favors tumor invasion and metastasis

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SUPPLEMENTAL FIGURES LEGENDS

Figure S1, related to Figure 1:

(A) Example FDM labeled for FN (green) (autofluorescence in blue). Cav1WT and Cav1KO MEFs were plated for 4 h on NIH-3T3-derived FDMs (3D) or on soluble FN (5µg/ml) (2D). The charts show the evolution of EF (B) and cell-occupied area (C) after plating. (D) Cav1WT and Cav1KO MEFs were plated for 4 h on FN (5µg/ml). (a) Cells were labeled for Rac1-activated phospho-S141PAK (pPAK, green) and counterstained with Hoechst (blue); pPAK concentrates at the edge of Cav1KO MEFs (asterisks). (b) Pixel intensity for pPAK was measured from at the cell edge. (c) Immunoblot showing pPAK levels. Tubulin was used as loading control. (E) Effect of cell number on collagen gel contraction by Cav1WT and Cav1KO MEFs.

Figure S2, related to Figure 2:

(A) SMA expression by Cav1WT and KO MEFs, both immortalized and primary (pMEFs), cultured under the indicated conditions. (B) Representative images of tubulin staining (red) and calculated EFs for Cav1KO MEFs rescued with either WT or Y14F Cav1 and plated for 6 h on the indicated FDMs. (C) Quantification of total and average numbers of protrusions per cell in samples depicted in B. (D) Cav1WT and Cav1KO MEFs were grown on soluble FN (2D) or on Cav1WT- or Cav1KO-derived FDMs (3D). Fixed cells were stained for active integrins (9EG7, green), pY397FAK (red), and nuclei (Hoechst; blue). Boxed areas in the top row are shown in magnified view in the lower panels. (E) Cav1WT and KO MEFs plated on the indicated FDMs were fixed and labeled

for FN (red) and active integrins (9EG7, green). Numbers indicate the average length of integrin-dependent adhesions.

Figure S3, related to Figure 3:

(A) Representative force curves from Cav1WT FDMs and Cav1KO FDMs. Curves overlap when the tip is at a distance from the fiber, but once the tip establishes mechanical contact with the fiber the curves diverge. To achieve the same force with both FDMs, the tip needs to push the Cav1KO FDMs harder, and consequently the gradient is shallower. (B) Matrix thickness and cell density in un-extracted FDMs generated by Cav1WT and KO MEFs. Representative images show cultures stained for FN (red) and nuclei (blue). (C) Identification and quantification of ITRAQ-labeled ECM proteins by liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS). (a) Experimental design. (b) Design of iTRAQ reagents and FDM labeling by methods A and B (see Supplemental Experimental Procedures). (c) Identified proteins and relative ratios. (D) Skin sections of 3-week old WT and Cav1KO mice stained with Masson's trichrome and picrosirius red (PR). Polarized (orthogonal) light highlights fibrillar collagen. (E) Multiphoton excitation microscopy of intact fixed mammary glands from Cav1WT and KO mice. Images show second harmonic generation (SHG) and autofluorescence signals of surrounding stroma. Red and yellow arrows mark curly and straight collagen fibers devoid of SHG signal.

Figure S4, related to Figure 4:

(A) Staining for FN (red) and Hoeschst (blue) in cultures of Cav1 WT and KO MEFs grown on FN-gels (3D cultures) for the indicated times. Remodeling of soluble FN is visible as the formation of bright FN fibrils, leaving clear areas depleted of FN stain. The chart tracks FN fibrillogenesis (AU) over time. (B) FN expression in 2D cultures of Cav1 WT and KO MEFs grown without serum. The immunoblot shows total expression. Tubulin was used as loading control. (C) FN fibrillogenesis of Cav1KO MEFs stably expressing WT or Y14F Cav1. The chart shows quantification (AU) and the panels display representative images. (D) Effect of stable depletion of p190RhoGAP in Cav1KO MEFs. (a) Quantification of FN fibrillogenesis in Cav1KO MEFs stably expressing

p190RhoGAP shRNA sequence#1. (b) Rac1 membrane distribution. Representative images highlight the loss of strong Rac intensity at the plasma membrane of p190RhoGAP-silenced cells (asterisks). Graph shows Rac1 pixel intensity from the cell edge to the nucleus. (E) Immunoblot showing the effectiveness of three independent p190RhoGAP shRNA sequences on p190RhoGAP expression in Cav1KO MEFs. Cav1 and tubulin blots are shown as internal controls. (F) Effect of the three p190RhoGAP shRNAs on collagen gel contraction.

Figure S5, related to Figure 5:

(A) Paired paraffin-embedded sections of normal kidney and renal tumor tissue from a representative patient. Sections are stained for Cav1, α-SMA and Collagen deposition (Masson's trichrome) as indicated. (B) Scoring of stromal activation and Cav1 expression in normal kidney and renal tumor tissue from 5 patients. (C) Fibroblasts from renal tumor tissue (CAFs) were isolated and infected with lentivirus encoding scrambled or Cav1-siRNA. Immunoblot shows Cav1 silencing efficiency. Tubulin shows equal loading. Collagen gel contraction assay confirms impaired contraction by Cav1-silenced CAFs. (D) Hmb45 (white) and Cav1 (red) staining in distant tumor-associated stroma of metastases from melanoma samples. (Note that in Patient #97 tumor cells are positive for Cav1). (E) Staining for Cav1, Hmb45, CD90, Collagen (COL1), FN and nuclei (Hoechst) in distant tumor-associated stroma of a melanoma metastasis. S= stroma, T=tumor.

Figure S6, related to Figure 6:

(A) A 1:1 mix of calcein-pre-labeled ATCC-231 cells (green) and WT or Cav1KO MEFs was embedded in collagen gels, fixed, and stained for actin (red) and nuclei (Hoechst, blue). EFs of ATCC-231 cells are indicated. (B) Higher magnification of invading areas from Fig.6C, shown to highlight polarization and directionality of migration of ATCC-231 tumor cells and Cav1WTMEFs. (C) Invasion index of the experiment shown in Fig.6D calculated for tumor cells (PC3) and MEFs. (D) Quantification of MEF proliferation rate in the Matrigel invasion assay. (E) Tumor cells and MEFs were spatially separated during Matrigel invasion to mimic physiological topography. Start and

end points are shown. The chart represents the invasion index for tumor cells (PC3) and MEFs.

Figure S7, related to Figure 7:

(A) Orthotopic mammary gland allografts of E0771 tumor cells in WT or CavKO mice. (a) Primary tumor photon flux. (b) Representative pictures of organs extracted 26 days after allograft. Quantification of the total number of metastatic foci and their organ distribution detected by bioluminescence *ex vivo*. (c) Quantification of SHG collagen content. (B) Orthotopic mammary gland xenografts of LM-4175 cells in WT or CavKO mice. (a) Multiphoton excitation microscopy of intact fixed tumors extracted from Cav1WT and KO mice 8 days after injection. Images show second harmonic generation (SHG) and GFP signal of LM-4175 cells. The same tumors were processed and stained with Masson's trichrome and picrosirius red (PR). Polarized light highlights fibrillar collagen. Arrows indicate cells with elongated pro-invasive morphology in WT. S= stroma, T=tumor. (b) Quantification of the angle of collagen fibers angle to the tumor boundary (for SHG and PR). TACS = tumor-associated collagen signature. (c) Primary tumor photon flux in nude mice 102 days after xenografting. (d) Quantification of primary tumors stained for FN, SMA and nuclei. The angles of single thresholded FN-labeled fibers were calculated and the percent of fibers within ±20° compared. EF and angle distribution of SMA positive cells is shown. (C) Subcutaneous injection of Matrigel containing LM-4175 tumor cells plus the indicated pMEFs. (a) In vivo primary tumor photon flux. (b) Representative images of primary tumors stained for FN, SMA and nuclei. (c) Quantification as described for Bd. (D) Experimental metastasis assay. (a) Experimental scheme. (b) Representative images of metastatic growth over time. (c) Plot of metastatic growth over time. Insets show representative images of photon flux in lungs at 20 days after allograft.

SUPPLEMENTAL VIDEOS

Supplemental video 1, **related to Figure 1:** Confocal sections (z-stacks) of FN staining in NIH-3T3 FDMs

Supplemental video 2, **related to Figure 1:** 3D reconstruction of confocal sections of Cav1WT MEFs embedded in collagen I gels and stained using phalloidin

Supplemental video 3, related to Figure 1: 3D reconstruction of confocal sections of Cav1KO MEFs embedded in collagen I gels and stained using phalloidin

Supplemental video 4, **related to Figure 6:** ATCC-231 cells were plated for 6 hours and imaged for 12h within Cav1WT FDMs (5min intervals, 145 frames, 15msec/frame)

Supplemental video 5, **related to Figure 6:** ATCC-231 cells were plated for 6 hours and imaged for 12h inside Cav1KO FDMs (5min intervals, 145 frames, 15msec/frame)

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Extracellular matrices produced by immortalized or primary fibroblasts (FDMs)

3D matrices reminiscent of *in vivo* ECM were prepared as published (Beacham et al., 2007; Castelló-Cros and Cukierman, 2009). Briefly, 2.5×10^5 cells/ml were plated on chemically cross-linked gelatin on tissue culture dishes or coverslips and maintained in confluence for 6-8 days. Cells were supplemented every 48 h with 50 μg/ml fresh L-ascorbic acid (AA) to stabilize ECM components thus facilitate collagen production and polymerization. The resulting 3D cultures were checked for quality by indirect immunofluorescence (Castelló-Cros and Cukierman, 2009) or cells were removed from the matrix by alkaline detergent extraction (Beacham et al., 2007; Castelló-Cros and Cukierman, 2009), yielding cell-free 3D matrices for further analysis.

Collagen gel contraction assay

The assay was performed as previously described (Orimo et al., 2005). Briefly, 1.2×10^5 fibroblasts (alternatively, 6×10^4 CAFs/NFs) were mixed with NaOH-titrated collagen I (PureCol^{TM}, INAMED) to a final collagen I concentration of 1mg/ml. In some experiments, 4×10^5, 1×10^6 or 1.5×10^6 fibroblasts were used (Fig.S1). The mixture was immediately transferred to a 24-well plate and lattices were allowed to solidify for 20 min at room temperature. Serum-containing medium was added to each well and gels were manually detached by circular movements using a sterile pipette tip. Gels were placed at 37ºC and contraction was documented. Four or five fibroblast-containing gels were assayed for each condition. Gel contraction index was calculated with Metamorph software from the gel surface area measured on acquired images, and reported as the percentage of contraction of the initial surface area.

Spheroid invasion assay

The protocol is based on (Gaggioli et al., 2007). ATCC-231 tumor cells were labeled (30 min) with calcein and mixed 1:1 with MEFs. The mixes were embedded in serum-free collagen I gels (15 μl, 1 mg/ml) in the well centers of an 8-well IBIDI chamber. After polymerization, the gel plug containing the cells was embedded in a second serum-containing collagen gel (150 μl, 1 mg/ml) and covered with serum-containing medium supplemented with SDF-1α (50 ng/ml). After 6 days, gels were fixed, labeled with

fluorescently-conjugated phalloidin, imaged and analyzed. Invasiveness was quantified as the area invaded by tumor cells.

Matrigel invasion assay

Equal numbers of GFP-expressing PC-3 cells and fibroblasts were mixed, loaded into the well centers of Matrigel-coated IBIDI angiogenesis wells, and covered with a 1:1 mix of Matrigel and medium (11 μl). After polymerization, chambers were filled with medium containing 2% FBS. Alternatively, PC-3 cells and fibroblasts were separated by a thin Matrigel layer. For this, PC-3 cells were first seeded into Matrigel and a thin Matrigel layer was overlaid three hours later and left to polymerize before seeding MEFs. During the 6 day invasion period, 5 μl medium containing 10% serum was added to the chambers every second day. The cells were fixed and labeled with phalloidin and Hoechst, and imaged by confocal microscopy. Invasiveness of each cell type was quantified as the area invaded. Total numbers of MEFs at the end point were quantified by automated counting of nuclei in all the acquired planes (ImageJ) and subtraction of GFP-positive (PC3) cells.

Orthotopic xenografts, subcutaneous tumorigenicity assay, experimental metastasis assay and bioluminescent imaging

In the first orthotopic xenograft protocol (Fig 7A), E0771 C57BL/6 mammary adenocarcinoma cells (kindly provided by Adam Schrum) were lentivirally infected a vector encoding GFP and luciferase (a kind gift from A. Alfranca). GFP-positive cells ($4x10^6$ in 200 μl PBS:Matrigel 1:1) were injected into mammary glands of WT and Cav1KO mice (strain B6.Cg-CAV1^{tm1mls}/J). The same cells ($1x10^6$ in 100 μl PBS) were injected into tail veins of WT and Cav1KO mice (strain B6.Cg-CAV1^{tm1mls}/J) in an experimental metastasis assay. Successful injection was verified by immediate bioluminescent imaging. In the second orthotopic xenograft experiment (Fig.7D), LM-4175 cells prepared as before were injected into mammary glands of lethally-irradiated WT and Cav1KO mice (strain STOCK Cav1^{tm1Mls}/J). After 7 days, tumors and surrounding stroma were extracted and subcutaneously transplanted to Nude mice. Alternatively, tumor architecture was analyzed using SHG imaging. In the subcutaneous tumorigenicity assay, 10^6 LM-4175 tumor cells (Minn et al., 2005) were unmixed or mixed 1:1 with Cav1WT or Cav1KO pMEFs, or with Cav1KO pMEFs infected with p190RhoGAPshRNA lentiviral vector (+p190shKO MEFs). Cells were resuspended in a

1:1 mix of PBS and Matrigel (200 μl), and injected subcutaneously into anaesthetized nude mice using a 25-gauge needle (Elenbaas et al., 2001; Karnoub et al., 2007). For bioluminescent imaging, mice were injected with luciferin (17.5 mg/ml), and after 20 min were placed in the IVIS™ Imaging System and ventral views captured. Tumor growth and metastasis formation were monitored at regular intervals. To facilitate metastasis detection in axillary/brachial lymph nodes, front limbs were secured with tape and the lower portion of the animal was shielded to block bioluminescence from the primary tumors. Exposure time for photon flux quantification was 0.2 s, but ranged from 0.2 s to 2min for metastasis detection. At the end of the *in vivo* analysis, luciferin-injected mice were killed and the organs extracted and reimaged *ex vivo* to detect metastatic foci. Images acquired from multiple exposure times were used to manually quantify every visible metastatic focus. Small metastatic foci could be detected by adjusting the scale of photon flux in Living Image 3.2 software. Extracted primary tumors were frozen in tissue-Tec and prepared for histology.

ITRAQ labeling, identification and quantification of ECM proteins by liquid chromatography coupled to tandem mass spectrometry

FDMs from WT and KO MEFS were extracted as described above (Method #A). Alternatively, FDMs were extracted with three successive treatments with hypotonic PBS (diluted 1:5) containing 0.5% w/v sodium deoxycholate (DOC) (Method #B). Extracted FDMs were extensively washed with PBS, scraped off the plate and proteolyzed with 4 μg trypsin (30 mM Tris, pH8) overnight at 37°C. The resulting soluble protein extracts were quantified by Bradford assay. Each sample was then denatured and digested as described in the iTRAQ™ reagents protocol. For protein digestion, modified porcine trypsin (Promega) was added at a final trypsin:protein ratio of 1:50. After digestion overnight at 37ºC, samples were labeled with iTRAQ tags as follows: proteins obtained by "Method A" were labeled with WT-iTRAQ 115 and KO-iTRAQ 114; proteins prepared by "Method B" were labeled with WT-iTRAQ 116 and KO-iTRAQ 117. After labeling, the samples were vacuum dried and dissolved in buffer A (0.5% acetic acid) for desalting and removal of excess iTRAQ reagent in reverse-phase (RP) C-18 cartridges.

The resulting tryptic peptide mixtures were separated by nano-liquid chromatography coupled to mass spectrometry for protein identification. Peptides were injected onto a C-

18 RP nano-column (100 μm I.D. and 12 cm; Mediterranea sea, Teknokroma) and analyzed on a continuous acetonitrile gradient consisting of 0-43% B for 140 min, 50-90% B for 1 min (B=95% acetonitrile, 0.5% acetic acid). Peptides were eluted from the RP nano-column at a flow rate of 300 nl/min into an emitter nanospray needle for real-time ionization and peptide fragmentation on an LTQ-Orbitrap mass spectrometer (Thermo Fisher, San José, CA, USA). An enhanced FT-resolution spectrum (resolution=60000) followed by the MS/MS spectra of the three most intense parent ions were analyzed during the chromatographic run (180 min). Each parent ion was fragmented by two dissociation methods: CID for peptide sequence analysis and HCD for quantification of the iTRAQ reporter low mass signals. Dynamic exclusion was set at 0.5 min.

To identify proteins, tandem mass spectra were extracted and the charge state deconvoluted with Proteome Discoverer 1.0 (Thermo Fisher). All MS/MS samples were analyzed with SEQUEST™ (Thermo Scientific, version 1.0.43.2), MASCOT™ (MatrixScience, version 2.2.01), and X! Tandem (The GPM, thegpm.org; version 2007.01.01.1). Two mixed cleavages were allowed, and an error of 15 ppm or 0.8 Da was set for full MS and MS/MS spectra searches, respectively. Phosphorylation on Ser, Thr, or Tyr was specified as a variable modification. Scaffold (version _3_00_03, Proteome Software Inc., Portland, OR) was used to validate MS/MS peptide and protein identifications. Protein probabilities were assigned by the Protein Prophet algorithm. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Nanomechanical and imaging measurements by atomic force microscopy

Experiments were performed with a multimode atomic force microscope fitted with a Nanoscope V controller (Veeco Digital Instruments). Imaging and force spectroscopy were carried out in PBS (0.01 M, pH=7.4). Images and force curves were obtained using cantilevers with a force constant $k \approx 0.01$ N/m (Olympus BL-RC150VB-C1). The force constant was calibrated by the thermal tuning method (Butt and Jascke, 1995). The sensitivity of the photodiode was calibrated from a deflection versus distance curve performed on a mica substrate (36.14 nm/V). Prior to measurements, the ECM was rinsed with deionized water and dried in gaseous N_2. Once the sample was mounted into the

fluid cell of the microscope, 100 µl PBS was introduced into the liquid cell and the experiment proceeded. To acquire the force curves, we first recorded a 30 µm x 30µm image of a representative area of the sample. We then selected points on the fiber force spectroscopy measurements. The force curves were acquired by approaching and retracting the tip toward the sample by 2 µm at a rate of 1 Hz. Each curve has 512 x 512 points. To prevent damage to fibers, tip excursion was stopped when the force reached 2 nN. The Young modulus was derived by the Hertz model,

$$F = 4/3\ \sqrt{R}\ E/(1-(\dagger 2)\ (\dagger(3/2)$$ (Equation 1)

where F is the applied load, R the tip radius (20 nm), E the effective Young modulus of the tip-sample interface, ν the Poisson ratio (0.5 for soft materials) and δ the indentation. The ECM stiffness is several orders of magnitude smaller than that of the AFM probe, so that $E \approx E_{ECM}$. Equation 1 requires transformation of the cantilever deflection dependence on sample displacement into force versus indentation curves. This is accomplished by determining the indentation from

$$(= (S_{\downarrow}P - S_{\downarrow}0) - (z - z_{\downarrow}0)$$ (Equation 2)

where S_p is the piezo movement, S_0 the contact point, z the deflection recorded by the photodiode and z_0 the deflection offset. The measurements were performed at the point of maximum indentation, which in turn was limited by the maximum force applied.

FRAP

MEFs transfected with vinculin-GFP were plated overnight on the assorted FDMs. Two pre-bleach events were acquired before bleaching by stimulation with the SP5 scanner at 488 nm. Fluorescence recovery was monitored at 4s intervals until the intensity reached a plateau. Fluorescence during recovery was normalized to the pre-bleach intensity. Relative recovery of vinculin-GFP at 3D-matrix adhesions and FAs was evaluated by comparing the half-times of fluorescence recovery toward the asymptote. Mobile and immobile fractions were calculated from comparison of intensity ratios in the bleached area before bleaching and after recovery. Graphs are representative of a minimum of 3 independent experiments in which between 6 and 15 adhesion structures were bleached. The procedure based on (Goetz et al., 2008).

Multiphoton microscopy and second harmonic generation

For MPE and SHG imaging of fixed, intact (non sectioned), non-stained mammary glands and mammary tumors, we used an optical workstation built around a Nikon Eclipse TE300 (Provenzano et al., 2006). A Ti:sapphire laser excitation source (Spectra-Physics-Millennium/Tsunami, Mountain View CA), producing around 100 fs pulse widths and tuned to 890–900 nm, was used to generate both multiphoton excitation (cellular autofluorescence from FAD) and SHG. The beam was focused onto the sample with either a Nikon 40× Plan Fluor oil-immersion lens (N.A. = 1.4) or a Nikon 60× Plan Apo water-immersion lens (N.A. = 1.2). All SHG imaging was detected from the back-scattered SHG signal. Additionally, due to the fundamental differences between MPE (autofluorescence) and SHG signals, filtering can separate the emission signals. Using a 470 nm (cut-on) long pass (LP) filter, autofluorescence was discriminated from the total emission, while a 445 nm narrow band pass filter (±20 nm) was used to separate SHG (filters from TFI Technologies, Greenfield, MA). For mammary tumors, a 520/535nm filter was used to detect GFP signals. All power settings were held constant during acquisition to allow comparison of intensities. Interestingly, some collagen fibers were visible using the LP filter. Acquisition was performed with WiscScan, a software acquisition package developed at LOCI (http://www.loci.wisc.edu/wiscscan/). Image analysis for combined MPE-SHG was performed with ImageJ software, which was also used to quantify differences between SHG signals. Fluorescence intensity of collagen SHG in mammary tumors was measured in 1000 μm^2 regions of interest drawn in the stroma adjacent to tumor cell islands; multiple regions were examined for each tumor. The angle of collagen fibers to the tumor boundary was determined for regions in which such a boundary was evident. Alignment was analyzed using Curvelet-Based Alignment Analysis software (www.loci.wisc.edu/software/curvelet-based-alignment-analysis), in which a line was drawn at the tumor/stroma boundary and collagen angles determined relative to that line. Quantitative data were graphed and statistically analyzed with GraphPad software.

Isolation of primary fibroblasts associated with normal and tumor breast, and kidney samples

To protect patient privacy, samples were decoded according to approved IRB procedures, while relevant clinical information was made available to the researchers upon request.

Fresh surgical specimens (paired normal kidney and renal tumor tissue or non patient matched normal and tumor breast tissues) were minced and dissociated by incubation overnight at 37°C in 0.2% collagenase with agitation at 200 rpm. Fibroblasts were isolated by 10 minute centrifugation at 200 g. Pellets were re-suspended and serially filtered through 500 μm nylon mesh and 100 μm and 40 μm cell strainers. Isolated fibroblasts were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM; Mediatech, Inc.) supplemented with 15% fetal bovine serum (FBS; Hyclone), 100 units/ml penicillin, and 100 μg/ml streptomycin (Mediatech, Inc.). Non-adherent material was removed after 8 hours. After characterization (Castelló-Cros and Cukierman, 2009), kidney fibroblasts were immortalized using a combination of hTERT and bmi-1, while breast fibroblasts were used as primary fibroblasts. Both immortalized (at early passages) and primary cells were forced to produce their own matrix and subsequently grown in this 3D matrix (as opposed to a 2D plastic substrate) to allow them to reproduce their *in vivo* features (Amatangelo et al., 2005; Quiros et al., 2008).

Immunohistochemistry

Paraffin-embedded sections were deparaffinized in xylene, rehydrated through a graded series of ethanol and water and boiled in 10 mM sodium citrate-buffer for antigen retrieval. Sections were incubated with assorted antibodies overnight at 4°C. HRP-secondary antibodies were detected with the Liquid DAB Substrate Pack (Biogenex, San Ramon, CA) according to the manufacturer's instructions. Sections were counterstained with Harris modified hematoxylin (Thermo Fisher Scientific). Negative controls included omission of primary antibody and its substitution with normal rabbit or mouse IgG (Sigma-Aldrich). Collagen deposition and organization was visualized by standard Masson's trichrome staining or picrosirius red staining. To highlight fibrous structures, picrosirius red labeled samples were visualized with a Leica DM 2500 microscope fitted with an orthogonally oriented polarizer.

Immunofluorescence and confocal microscopy of melanoma tissues.

Thin sections (4 μm) of cryopreserved tissue were first blocked for 10 min with 1% human immunoglobulins and then incubated for 1 h with a mixture of primary antibodies from different species (for example, rabbit polyclonal antiserum against Cav-1, and HMB-45 monoclonal antibodies, or isotype-matched control antibodies). Primary

antibodies were used at 1-5 µg/mL, followed by incubation with Cy5-labeled antimouse and Cy3-labeled antirabbit secondary antibodies. After blocking with 10% mouse immunoglobulins, samples were incubated with FITC-labeled anti-CD90 or anti-CD45, and with DAPI to visualize nuclei. Samples were imaged using an AOBS/SP2 inverted scanning confocal microscope (Leica Microsystems) fitted with a 63x PL-APO NA 1.3 immersion objective. Image processing and colocalization analyses were assessed with LCS-15.37 Leica Confocal Software.

Plasma membrane purification

The protocol was as described in (Smart et al., 1995). Briefly, cells were washed twice with buffer A (250 mM sucrose, 1mM EDTA, 20 mM Tricine, pH 7.8) and collected and harvested in 3 ml buffer A. After centrifugation, cells were resuspended in 1 ml of buffer A and hand homogenized with 20 strokes. Homogenates were centrifuged at 1000g for 10 minutes. The postnuclear supernatant (PNS) was layered (4 mg protein) on top of 23 ml of 30% Percoll in buffer A, and centrifuged at 84,000g for 30 min. The plasma membrane fraction appeared as a visible band approximately 5.7 cm from the bottom of the tube. The membrane fraction was collected, diluted in buffer A and centrifuged at 105,000g for 1 h to remove Percoll. Purified plasma membranes in the resulting pellets were analyzed by immunoblot.

Purification of detergent-resistant membrane fractions

The protocol was as described in (Navarro-Lerida et al., 2002). Briefly, MEFs were scraped off the plate and resuspended at 4°C in 2 ml Mes buffered saline (MBS) (25 mM MES, pH 6.5, 0.15 M NaCl, 1 mM PMSF plus 1% Triton X-100). Cells were homogenized on ice by a minimum of 10 strokes through a syringe (0.5x16 mm). The homogenate was adjusted to 40% sucrose by the addition of 2 ml 80% sucrose in MBS (4 ml in total), and was transferred to a Beckman SW40 13 ml Ultraclear tube. 30% and 5% sucrose in MBS (4 ml each) were successively overlaid to form a 40-30-5% discontinuous sucrose gradient. Homogenates were separated by centrifugation at 200,000 ***g*** for 18 h a SW40 rotor (Beckman) at 4°C. Most Cav1 was contained in a light, scattered band confined to the 30-5% sucrose interface, which excluded most cell proteins. Twelve 1 ml fractions were collected from the bottom of the tube. Proteins in each fraction were precipitated by addition of 1 ml of cold acetone and incubation

overnight at 4°C. Samples were centrifuged at 16,000 ***g*** in a microcentrifuge, and the protein pellets were dried for 2 hours to eliminate all traces of acetone. Precipitated proteins were analyzed by SDS-PAGE and immunoblot.

Immunoprecipitation of p190RhoGAP

MEF plasma membranes were resuspended in lysis buffer (5mM Tris-HCl pH 7.5, 100 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40 containing 0.5 mM PMSF, 1mM Na3VO4, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 10 mM NaF) and incubated overnight with 4 µg anti-p190RhoGAP antibody. 50 µl protein G-agarose beads were added and samples incubated for 3 h at 4ºC. After washing and boiling in sample buffer, eluted proteins were analyzed by 7.5% SDS-PAGE and immunoblot.

RNA interference–mediated knockdown of p190RhoGAP and Cav1

Sequences targeting mouse p190RhoGAP were cloned into short hairpin RNA (shRNA) vector pSuper.Retro.Neo+GFP (Oligoengine). The sequences, obtained from GenBank/EMBL/DDBJ under accession no. NM_172739, were as follows: sequence#1, nucleotides 2935–2953, 5′-GTTATGGACGCAACATTAA-3′; sequence#2, nucleotides 1225–1243, 5′-ACAGGAACTTCGATGATCA-3′; and sequence#3, nucleotides 1955-1963, 5′-GACACCAACCTTCCAACCC-3′. The nontargeting control (scrambled) sequence was 5′-GCGCGCTTTGTAGGATTCG-3′. Retroviral supernatants were generated by transfecting 293T/17 cells with each shRNA and pSVψ2 vector using the Fugene 6 transfection reagent (Roche). Cav1KO MEFs were infected with retroviral supernatants and high GFP-expressing cells were sorted (~15% of the cell population). To silence p190RhoGAP in primary Cav1KO MEFs for the subcutaneous tumorigenicity assay, sequence#1 was cloned into pLVX-shRNA2, which contains a ZsGreen1 reporter (Clontech), and primary KO MEFs were infected with supernatants derived from HEK-293T cells. Infection efficiency was monitored by ZsGreen1 expression. A representative immunoblot confirming silencing efficiency is presented in Fig7.Ca. An alternative Cav1 siRNA was designed (sequence #2, Fig.S5C: 5'-GACGTGGTCAAGATTGACTTT-3') corresponding to bases 254-277 of the human Cav1. This sequence was also cloned into pLVX-shRNA2. Normal and tumor-associated fibroblasts were infected with supernatants derived from HEK-293T cells. Infection efficiency was monitored by ZsGreen1 expression.

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