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The small GTPase Rab27a can promote tumor progression by modifying the tumor microenvironment via both exosome-dependent and –independent mechanisms.

Online Supplemental Material
4 supplemental figures, plus detailed informations for the Materials and Methods are available with this article.
Suppl Figure S1: Most shRNA to Rab27a or Rab27b affect the expression level of the other gene.
Suppl Figure S2: Metastasis formation by 4T1 after i.v. injection is decreased (A), whereas growth of 4T1 tumors in vitro is not affected (B) by Rab27a inhibition.
Suppl Figure S3: Involvement of the immune system in differential growth of TS/A, 4T1, and their sh27a-expressing counterparts.
Suppl Figure S4: Complete list of secreted proteins analyzed by antibody microarrays.

Supplementary informations for Materials and Methods
Mice
For suppl figure S3: C57Bl/6 $Rag2^{-/-}$ mice were bred in our animal facility (Curie Institute, Paris, France).
Cells
Cells were cultured in medium supplemented with 10% fetal calf serum (Abcys), 2mM glutamine, penicillin/streptomycin (100 U/mL and 100 µg/mL respectively): RPMI + 1 mM pyruvate (Invitrogen) for 4T1, DMEM with 4.5 g/L glucose for TS/A, MB49 and B16F10.
Reagents
Antibodies for FACS analysis of the immune infiltrate: antibodies to CD16/CD32 (to block Fc receptors), CD8-Pacific Blue, CD8-FITC, TCRß-FITC, CD45.2-PerC-Cy5.5, CD4-PE-Cy7, CD19-APC-Cy7, $I\text{-}A^{d}$-FITC, Ly-6G-PE, CD11c-PE-Cy7, CD11b-APC-Cy7 (from BD Biosciences), NKp46-PE, Ly-6C-APC (eBiosciences) and FoxP3-APC (Anti-mouse/rat FoxP3 staining set APC kit from eBiosciences). Intracellular FoxP3 was stained according to manufacturer's instructions. Viability was assessed by DAPI staining (Molecular Probes) after the stainings or using the Live/Dead fixable violet dead cell stain kit from Invitrogen before the stainings, according to manufacturer's instructions.
Antibodies against mouse proteins for Western blotting: Hsc70 (Stressgen Biotech, SPA815), Tsg101 (Genetex, clone 4A10), CD63 (MBL, clone R5G2), Alix/AIP1 (BD Bioscience, 49/AIP1), Actin (Millipore, C4). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Jackson Immunoresearch. Mouse Anti-Rab27a was generated in M. Seabra's lab (Tolmachova et al, Mol Biol Cell. 2004;15:332).
Depleting antibodies to CD4+ T lymphocytes (clone GK1.5) and Ly6G+ neutrophils (clone 1A8) and corresponding isotype controls were purchased from BioXCell.
pLKO.1puro plasmids allowing expression of shRNA specific for mouse Rab27a or Rab27b, or a scrambled sequence of shRNA to GFP as control (Scr), and a puromycin resistance gene were obtained by L. F. Moita from the library described in (Moffat et al, Cell 2006; 124: 1283). Lentiviruses were produced as previously described and used to infect subconfluent cells in 96-well plates.
sequence of shRNA Rab27a2:
CCGG**CGAAACTGGATAAGCCAGCTA**CTCGAG**TAGCTGGCTTATCCAGTTTCG**T TTTTG
Sequence of shRNA Rab27b1 :
CCGG**CCTGAGACAATGTCAAACCAT**CTCGAG**ATGGTTTGACATTGTCTCAGG**T TTTTG

Exosome purification and characterization

Cells plated in 145 mm^2 plates were incubated 48 hours in complete medium depleted from serum-derived exosomes and microvesicles by overnight ultracentrifugation at 100,000 g. Supernatants were collected and exosomes were purified by successive centrifugations: 300 g for 10 min; 1,000 g for 20 min; 10,000 g for 30 min and 100,000 g for 90 min. The 10,000 g and 100,000 g pellets were washed in large volumes of PBS, and resuspended in 80 µl PBS. Producing cells were counted, cell lysatesm (obtained by lysing cells in 50mM Tris, pH 7.5, 0.3 M NaCl, 0.5% Triton X-100, 0.1% sodium azide, with a cocktail of antiproteases (Roche)) were prepared from 4.10^6 cells. Proteins in pellets and lysates were quantified by Micro-BCA (Thermo Scientific) in the presence of 2% SDS. Exosomes secreted by 10.10^6 to 15.10^6 cells (i.e around 3 µg) or 30 µg of lysates (corresponding to around 2.10^5 cells) were loaded on NuPAGE 4-12% BisTris gels (Invitrogen) and separated under non-reducing conditions. For Rab27 detection by Western blotting, 150 µg of lysates were analyzed in reducing conditions. RNAs were extracted from 10^6 cells using Macherey Nagel Nucleospin RNA II kit. 200 ng of RNA (measured by Nanodrop (Thermo Scientific)) were reverse transcribed with SuperScript II (Invitrogen). $1/5^{th}$ of the obtained cDNA was used for quantitative PCR, performed in triplicate.

In vivo experiments

For lung metastasis evaluation, lungs were fixed in AFA and nodules were manually counted. In some experiments, black ink was injected in the trachea at sacrifice before lung harvesting, to allow better contrasting of tumor metastases.

For analysis of immune cells in blood and organs, single cell suspensions were generated from spleens, or from tumors pre-digested with collagenase and DNAse (1 mg/ml and 100 µg/ml respectively, Roche), by straining on 0.2 µm filter. Red blood cells were lysed before incubation with fluorescent antibodies. To analyse cell morphology, Ly6C+/G+ and Ly6C+/G- cells were sorted on a FACSAria, centrifuged by cytospin, and stained with Giemsa/May-Grünewald staining.

Mouse Cytokine antibody arrays

Medium conditioned by 10^6 Scr or sh27a 4T1 and TS/A cells, or unconditioned medium were hybridized on antibody microarray membranes (Mouse Cytokine Array C2000, Raybiotech), and quantified according to manufacturer's instructions. Two independent experiments were performed for 4T1, one for TS/A, and four for unconditioned medium. Intensity of signal in each spot (two spots/protein/membrane) was quantified by ImageJ and rapported to the maximum signal obtained in the positive control spots of the same membrane.

Bone marrow differentiation in vitro

Bone marrow cells obtained from femurs of Balb/c mice and depleted from red blood cells were plated in 100 µL RPMI-10% FCS (Biowest), 500 nM 2-β-mercaptoethanol (Invitrogen), in non culture-treated 24-well plates. Tumor cell supernatants and enough complete medium to reach a final volume of 500 µL medium (containing a final concentration of 1mM pyruvate, 1X nonessential amino acids, 2mM glutamine) were added. Cells were cultured for seven days, fresh medium was added every two days. On day 7, floating and adherent cells were collected by trypsinization and stained with the following antibodies: I-Ad-FITC, Ly6G-PE, CD45.2-PerCP-Cy5.5, CD11c-PE-Cy7, Ly6C-APC, CD11b-APC-Cy7, and analyzed by FACS in the presence of DAPI (50 nM) to assess viability. Acquisitions were performed on MacsQuant (Miltenyi Biotech), allowing to analyze the different cell populations and to count the absolute number of cells in each well.

For these experiments, tumor cell-conditioned supernatants were collected from 24 hours cultures in depleted medium, and centrifuged at 300 g and 1,000 g as above, or further ultracentrifuged at 10,000 g and 100,000 g to deplete exosomes and other particulate materials. After one wash, the pellet from the 100,000 g centrifugation was resuspended in the

initial volume of complete medium and used alone or mixed with exosome-depleted conditioned supernatant (for the reconstitution experiments). Volumes of conditioned medium or exosomes secreted by 3.10^5 or 1.10^5 cells were added in duplicate to wells containing $3.7x10^5$ bone marrow cells.

Legends for supplementary Figures

Suppl Figure S1: Most shRNA to *Rab27a* or *Rab27b* affect the expression level of the other gene. Expression of *Rab27a* and *Rab27b* in TS/A and 4T1 cells expressing 5 different shRNA to either *Rab27a* (sh27a1 to sh27a5) or *Rab27b* (sh27b1 to sh27b5) as compared to control shRNA (Scr) was quantified by quantitative RT-PCR. Results are expressed as % of expression level in control cells, and graphs show mean + s.d. calculated from 1 (TS/A sh27a5) to 5 experiments for all shRNA except sh27a2 and sh27b1 (see legend of figure 1C).

Suppl Figure S2: Metastasis formation after i.v. injection and growth of 4T1 tumors in vitro upon Rab27a inhibition. A- Number of metastasis foci counted in lungs of individual mice (left), lung weight (right), and representative images at 20 days after i.v. injection of Scr or sh27a-4T1. Ctl = mice without i.v. injection of 4T1. **B-** Number of Control (Scr), or sh27a-expressing 4T1 cells present in culture wells at different time points after plating was measured by cell titer glow assays (mean + s.d. of triplicates in one representative experiment of 3).

Suppl Figure S3: Involvement of the immune system in differential growth of TS/A, 4T1, and their sh27a-expressing counterparts. A- Representative examples of CD11b, CD11c, Ly6C and Ly6G stainings obtained in viable CD45-positive (i.e. hematopoietic) cells invading control (Scr) or sh27a-expressing TS/A and 4T1 tumors. Inset shows typical aspect by cytospin and Giemsa/May-Grünewald staining of the major cell populations in the Ly6C+/G+ and Ly6C+/G- quadrants. Scale bar : 10 µm. **B-** Control (Scr) and sh27a-expressing 4T1 tumors were injected subcutaneously in C57Bl/6-Rag2$^{-/-}$ mice devoid of T and B lymphocytes. Tumor growth over 4 weeks (left panels), and tumor size at d15 (right panels) are represented for individual mice (18 mice pooled from 3 experiments). Growth of Rab27a-impaired tumors is slowed down in Rag2$^{-/-}$ hosts (** = $p<0.01$, student's T-test), showing that the adaptive immune system is not required for the decreased growth rate. **C–**Growth of Control (Scr) and sh27a-expressing 4T1 tumors in Balb/c mice depleted from CD4+ T cells by injection of anti-CD4, as compared to mice injected with isotype control. Tumor size at d15 (left panel) and efficacy of CD4 depletion are shown in blood at d17 of tumor growth (right panel). Individual mice in one experiment.

Suppl Figure S4: Complete list of secreted proteins analyzed by antibody microarrays. A- Mouse cytokine antibody microarrays (Raybiotech) were used to compare the amount of 144 proteins in the supernatant of equal numbers of control (Scr), or sh27a-expressing 4T1 or TS/A cells. Medium that had not been incubated with cells was also used to determine the background signal of each antibody pair (Medium). Results are expressed as arbitrary units of signal for each protein rapported to the positive control signal of each membrane. Left panel: proteins displaying a.u. above 30; Right panel: proteins with a.u. below 30. Proteins for which signal was twice higher than Medium + s.e.m in at least one cell type are shown. Mean values + s.e.m of 4 (Medium) or two independent experiments (4T1), or of duplicates from one experiment (TS/A). **B-D-** Organization scheme of the 4 membranes contained in the C2000 antibody microarray. Pos = positive control spots, Neg = negative control spots, Blank = empty spots. Proteins for which medium conditioned by either 4T1 or TS/A gave a signal stronger than unconditioned medium + s.e.m are highlighted. All the other proteins were not significantly detected over background in 1 ml of medium conditioned by 10^6 TS/A or 4T1.