Supplementary Materials and Methods

Cell culture

The human MDA-MB-231, MDA-MB-468 and the murine 4T1, B16, and LLC cancer cell lines were cultured in DMEM medium (Thermo Fisher Scientific) with 10% FBS (Euroclone) and 1% Penicillin Streptomycin (Thermo Fisher Scientific). Human BT549 cells were cultured in RPMI medium (Thermo Fisher Scientific) with 0.023 IU ml^{-1} insulin (Sigma Aldrich), 10% FBS (Euroclone) and 1% Penicillin Streptomycin (Thermo Fisher Scientific). Human MCF10A cells were maintained in DMEM/F12 Ham's medium 1:1, 5% horse serum (Thermo Fisher Scientific), insulin (10 µg ml^{-1}, Sigma Aldrich), hydrocortisone (0.5 µg ml^{-1}, Sigma Aldrich), epidermal growth factor (EGF 20 ng ml^{-1}, Sigma Aldrich) and 1% Penicillin Streptomycin (Thermo Fisher Scientific). T47D, HCC1937, H522, H460 and H1299 human cell lines and E0771 and CT26 murine cell lines were cultured in RPMI medium (Thermo Fisher Scientific) with 10% FBS (Euroclone) and 1% Penicillin Streptomycin (Thermo Fisher Scientific). Human CALU-1 cells were maintained in McCoy's (Thermo Fisher Scientific) with 10% FBS (Thermo Fisher Scientific) and 1% Penicillin Streptomycin (Thermo Fisher Scientific). Human BEAS-2B were cultured in LHC-9 medium (Thermo Fisher Scientific). Cells were cultured at 37°C in a humidified incubator in an atmosphere of 95% air and 5% CO_2.

Antibodies and reagents

Western blotting and immunoprecipitations were performed using the following primary homemade antibodies: mAb anti-Morgana P1/PP0 (1), mAb anti-Morgana 5B11B3 (generated in the present study), mAb anti-RFP (monoclonal antibody IgG1κ used as isotype control). The following commercial antibodies were used: Vinculin (Sigma, SAB4200080, 1:5000), CHORDC1 (Sigma, HPA041040, 1:1000), GAPDH (Merckmillipore, Mab374, 1:5000), MMP9 (Abcam, ab76003, 1:1000), Fibronectin (Santa Cruz, sc-18825, 1:1000), GST (Santa Cruz, sc-138), Laminin (Abcam, ab75344, 1:500), HSP90 (Santa Cruz, 13119, 1:1000; BD, 610418, 1:1000), HSP90 blocking antibody (2) (Enzo life sciences, ADI-SPA-830-F, 1:1000); HSP70 (Thermo Fisher Scientific, MA3-008, 1:1000), IkBα (Santa Cruz, 8404, 1:500), IKKα (Cell Signaling, 11930, 1:1000), IKKβ (Cell Signaling, 8943, 1:1000), IKKγ (Santa Cruz, 8032, 1:500), αTubulin (Sigma, T5168, 1:8000), mbp (Cell Signaling, 2396, 1:1000), ROCKI (Cell Signaling, C8F7, 1:1000), TLR2 (Blocking: Biolegend,

121802, 30 ng ml^{-1}; Western blotting: Abcam, 108998, 1:1000), TLR4 (Blocking: Abcam, 8376, 100μM; Western blotting: Santa Cruz, 293072), TLR5 (Blocking: InvivoGen, maba2-htlr5, 100μM; Western blotting: Abcam, 13876, 1:1000), Lrp1 (Abcam, ab92544, 1:1000; Thermo Fisher Scientific, MA1-27198, 1:1000), HER2 (Blocking: Selleckchem A2007, 1 μg ml^{-1}), EGFR (Blocking: BioVision, 1 μM).

The following inhibitors and reagents were used: Brefeldin A (Sigma, B7651, 10 μg ml^{-1}), Tilorone (Sigma Aldrich, 220957) 3,3'-dithiobis (sulfosuccinimidyl propionate) (DTSSP, Thermo Fisher Scientific, 21578, 1 mM), CellTracker™ Orange CMRA Dye, Green CMFDA Dye, CellTrace™ CFSE (Thermo Fisher, C34551, C7025, C34570), Puromycin (Sigma, P8833), Heparin (Sigma, H3393-100KU), Collagenase A (Roche, 10103586001), Tanespimycin/17AAG (Selleckchem, S1141, 1 μM).

Lentiviral transduction

Virus containing supernatants were collected 48 h after co-transfection of pCMV-VSV-G, pCMV Δ8.2 and the shRNA- or ORF-containing vector into HEK293T cells, and then added to the target cells. Cells were then selected with 10 μg ml^{-1} puromycin. Morgana knockdown in MDA-MB-231, BT549, E0771 and CT26 was performed by infecting cells with pGIPZ lentiviral particles expressing two different Morgana shRNAs (two against human and two against mouse Morgana), together with the TurboGFP (Open Biosystems). HSP90 and LRP1 downregulation in MDA-MB-231 was performed by infecting cells with pLKO lentiviral particles expressing HSP90 or LRP1 shRNAs (Sigma Aldrich). The following shRNAs were obtained from Open Biosystems: human CHORDC1 shRNAs V2LHS_24674 and V2LHS_24745; mouse CHORDC1 shRNAs V2THS_24746 and V2THS_24674. The following shRNAs were obtained from Sigma: human HSP90 shRNAs NM_007355.2-232s1c1.

Surface plasmon resonance analysis

Surface plasmon resonance (SPR) analyses were performed using Biacore X100 instrument (Cytiva Europe GmbH, Milan, Italy). Recombinant TLR2 was immobilized onto the surface of a single channel of a CM5 sensor chip previously coated with an anti-His antibody, by capturing the His-tag exposed on the protein (C-terminal domain), while the other channel was used as reference. TLR2 was diluted to a concentration of 10 μg/ml in PBS buffer and was injected at a flow rate of 5 μl/min

for 90 s. Taking into account the ligand (TLR2) and analytes (mbp-Morgana or mbp) molecular weights (MW) of 84 kDa, and 80 and 45 kDa, respectively, the appropriate ligand density (RL) on the chip was calculated according to the following equation: RL = (ligand MW/analyte MW) × Rmax × (1/Sm), where Rmax is the maximum binding signal and Sm corresponds to the binding stoichiometry. After TLR2 immobilization, binding experiments were performed. In the single-cycle kinetic experiment, mbp-Morgana was injected at increasing concentrations from 62.5 nM to 1000 nM over the TLR2-coated chip at a flow rate of 30 µl/min at 25°C with an association time of 120 s, and a dissociation phase of 180 s. A single regeneration step with 10 mM glycine pH 1.5 was performed following the cycle. In the binding test experiment, a single concentration (1µM) of mbp-Morgana or mbp was injected over the TLR2-coated chip at a flow rate of 30 µl/min at 25°C for 120 s. All samples were diluted in PBS buffer. The binding was detected immediately before stopping mbp-Morgana or mbp injection. The KD was evaluated using the Biacore evaluation software (Cytiva Europe GmbH, Milan, Italy) after subtraction of “zero sample concentration” and the reliability of the kinetic constants calculated by assuming a 1:1 binding model supported by the quality assessment indicators values.

Expression plasmids and recombinant protein production in ClearColi®

The recombinant full-length Morgana (aa 1-331) fused to mbp was prepared as described with minor modifications (1). Briefly, protein production was carried out in 500 mL cultures of Escherichia coli BL21 ClearColi®. Expression was induced with 1 mM IPTG at OD_{600} 0,7 and bacteria were cultured at 37 °C for 4 h. Bacterial cultures were centrifuged at 3000 rpm for 30 min at 4 °C and pellets were resuspended in Column Buffer (Tris-HCL 20 mM, NaCl 200 mM, EDTA 1 mM, $ZnCl_2$ 1 µM). Following freezing to induce partial bacteria membrane lysis, bacteria were sonicated with 8 pulses of 30 seconds at 38% of amplitude and centrifuged at 13000 rpm for 30 minutes at 4 °C. Supernatants (crude extracts) were incubated with amylose resin (New England Biolabs) overnight at 4 °C. After washing, the fusion protein was eluted with a solution of Column Buffer and 10 mM maltose. Finally, proteins were dialyzed against PBS.

Flow cytometry

Single-cell suspensions from murine tumors and lungs were prepared by mechanical and enzymatic disruption in PBS with 1 mg ml^{-1} collagenase A (Roche) for 20 minutes. Cells in suspension were filtered through a 40 μm cell strainer and centrifuged for 5 min at 120 g. After lysis of red blood cells, FcR were blocked with an anti-CD16/CD32 antibody (Becton Dickinson, 01245B) and cells were stained with the indicated fluorochrome-conjugated antibodies and Propidium Iodide (Sigma) to analyze viable cells. For lymphoid cell detection, samples were stained with anti-CD45, CD3, CD4, CD8, CD49b, B220 antibodies (Miltenyi Biotech 130-110-803, 130-119-135, 130-119-134, 130-123-865,130-123-702 and 130-123-702) and percentages of cells were calculated gating on the $CD45^+$ population. For myeloid cell analysis, samples were stained with anti-CD45, CD11b, F4/80, Ly6C, Ly6G, MHC II (Miltenyi Biotech 130-110-803, 130-110-803,130-118-320, 130-102-184, 130-102-227,130-102-139) and CD206 (BioLegend, 141706) and reported as percentage of myeloid cells on the $CD45^+$ population. The percentage of macrophages was calculated as $CD45^+$, $CD11b^+$, $F4/80^+$. The percentage of $CD8^+$ T lymphocytes was calculated as $CD45^+$, $CD3^+$, $CD8^+$. To analyze the percentage $CD8^+$ T lymphocytes/Treg $FOXP3^+$, samples were stained with anti-CD45 (Miltenyi Biotech 130-110-803), CD4, CD25 antibodies (Biolegend, 100528 and 102020) and using Foxp3 Staining Set FITC (eBioscience, 71-5775-40). For each analysis, at least 20000 $CD45^+$ living cells were analyzed. Flow cytometry analyzes were carried out on a BD FACSVerse using BD FACSSuite Software (Becton Dickinson).

Cell proliferation assay

MDA-MB-231, E0771, CT26 and 4T1 cell growth curves were generated by plating 1×10^4 cells in 96-well plates and by staining cells with 0.1% crystal violet at the indicated times. After staining, wells were destained with 20% acetic acid and the absorbance of crystal violet solution was measured at 595 nm.

Apoptotic assay

MDA-MB-231, E0771, CT26 and 4T1 cells were plated and allowed to settle for 24 hours. Cells were then treated with PBS, mAb 5B11B3 or control IgG (10 μg) for 24 h or 48 h. Percentage of apoptotic cells was assessed by annexin V and propidium iodide staining using the flow cytometer.

Sample size calculation

The sample size of each experiment is indicated in the figure legend. For sample size calculation G*Power 3.1.9.4 software was used. The effect size d was calculated on the basis of previous experiments. t test was used to determine differences between two independent means (groups). A priori computation of the required sample size, given α error probability, power and effect size was calculated by the software, with the following input parameters: Two Tails, Effect size d (obtained from previous experiments), α err prob 0,05, Power (1 – β err prob) 0,95, Allocation ratio N2/N1 1.

References

1. Ferretti R, Palumbo V, Di Savino A, Velasco S, Sbroggio M, Sportoletti P*, et al.* Morgana/chp-1, a ROCK inhibitor involved in centrosome duplication and tumorigenesis. Developmental cell **2010**;18:486-95
2. Zhang G, Liu Z, Ding H, Zhou Y, Doan HA, Sin KWT*, et al.* Tumor induces muscle wasting in mice through releasing extracellular Hsp70 and Hsp90. Nature communications **2017**;8:589