Supplementary information

Supplementary Methods

*In vitro culture*

All cell lines were grown according to ATCC recommendations. Cell lines with no particular recommendations were grown in DMEM-F12 (Sigma Aldrich) with 10 % fetal bovine serum (FBS) (Gibco, Invitrogen), 1% L-glutamine (Gibco, Invitrogen), and 0.1% penicillin/streptomycin (Gibco, Invitrogen). All cells were maintained at 37°C in a 5% CO₂ incubator unless until specified. Primary cultures obtained from human colon, liver, lung and bone cancers were cultured in DMEM/F-12 medium with 10 % heat inactivated FBS, 1% L-glutamine, 100µg/ml Primocin (Invivogen) and 0.1% penicillin/streptomycin. These cell lines were designated as OST (human osteosarcoma).

CTC isolated using 84-1 antibody were cultured on fibronectin-coated plates (Thermo Fisher) in DMEM/F-12 medium with 10% heat-inactivated serum (Invitrogen) and supplementation of growth factors. CTC isolated from Osteosarcoma patients were cultured with supplementation of 10 ng/ml of Human epidermal growth factor (hEGF) (Cell Signaling) and insulin growth factor (hIGF) (R&D Systems). CTC isolated from canine and mice blood were cultured in the above-mentioned media with epidermal, insulin and fibroblast growth factors (Cell Signaling). Media was changed once every four days. All cells were maintained at 37°C in a 5% CO₂ incubator.

*Blood Collection and Processing*

For mouse samples, blood was collected from mice using sub-mandibular cheek bleeding method and at a given time 200 µl of the blood was collected in an EDTA tube (Fisher) during
the weekly follow up studies. At the end of the study, at least 800 µl of blood was collected using the left cardiac ventricle puncture method. Blood collected was then subjected to RBC Lysis using RBC Lysis buffer (eBioscience), as per manufacturer’s recommendation. Cells were then washed in PBS and used for further analysis.

Blood samples from Canine were obtained from Gulf Coast Veterinary clinic. Privately owned dogs that were referred to the Gulf Coast Veterinary clinic were selected for the study. Apart from having histologically confirmed neoplastic disease, inclusion criteria included the absence of overt heart, renal or other life-threatening illness. Dogs were staged by obtaining a thorough anamnesis, physical examination, complete blood cell count, serum biochemistry profile, urinalysis, electrocardiogram, thoracic radiographs (three-view metastasis check) and abdominal ultrasound if indicated. For confirmation of tumor type, excision biopsies from the primary tumor were obtained surgically under anesthesia and were placed in 10% neutral-buffered formalin. After being fixed, tissue was cut-in, paraffin embedded, sectioned, adhered to slides, stained with hematoxylin and eosin, cover-slipped and evaluated by boarded veterinary pathologists.

_Mutation analysis_

Whole genome amplification from small numbers of CTC cells or single cells were performed using the REPLI-g Mini Kit (QIAGEN, Valencia, CA). Briefly, cell material in 4µl PBS was lysed by adding 3µl Buffer D2 in a micro centrifuge tube and incubated for 10 min at 65°C. Whole genome amplification was carried out in the same tube following manufacture’s recommendation. Resulting DNA was tested for quality by PCR with primers on different chromosomes. Aliquot of amplified DNA was diluted 1:10 and subsequently used for PCR
analysis. Genomic DNA was also isolated from pBMC (CD45+/84-1-) with Gentra Puregene Cell Kit (Qiagen, Valencia, CA). For gene mutation detection, Q5® High-Fidelity DNA Polymerase (NEB, Ipswich, MA) was used to amplify specific gene fragments for specific mutation sites. PCR products were gel purified with Qiaquick Gel Extraction Kit (Qiagen, Valencia, CA) and Sanger sequenced by MDACC (MD Anderson Cancer Center) Sequencing and Microarray Facility. Primer sequences used here are; TP53 codon 179/213: ACAAGCAGTCACAGGCATGA and CCACTGAC AACCACCCTTAACC; Kit codon 541: CACCCCTGTCTACCTCTTTG and CAACAACTCTCCACTGTACTT.

Antibodies

Antibodies against specific markers were used for each type of cancer; CD99 (Abcam); α-SMA (Abcam); CD-31, CD11b, CD3e (eBioscience); vimentin (Cell signaling); vimentin (V9 clone) (Santacruz Biotechnology); AMF-17B, EC-40 (Developmental studies Hybridoma Bank).

Western blotting and Immunoprecipitation

Recombinant human vimentin (rhVim) (R&D Systems) was loaded in three different lanes (1, 10 and 1000 ng) and were separated using SDS gels and transferred onto polyvinylidene difluoride membranes. Detection of rhVim was by incubation of the membranes using 84-1 antibody (1:1000). The secondary goat anti-mouse IgG: HRP (horse radish peroxidase) (Promega) was used to detect 84-1 antibody. For immunoprecipitation, LM7 cells were lysed using immunoprecipitation buffer (Thermo Scientific) and vimentin was immunoprecipitated using 84-1 and 12-1 antibodies. Mouse IgG was used as control antibody. The immunoprecipitates were blotted using rabbit monoclonal anti-vimentin antibody (Cell Signaling).

Flow cytometry
5 x 10^5 cells were detached by non-enzymatic dissociation buffer, washed and stained for 20 min on ice in dark. For CSV analysis, cells were stained with 84-1 mAb (1:100) and as an isotype control (Invitrogen) for mouse primary antibody was used. Later cells were rinsed twice in PBS and labeled for secondary antibody using either AlexaFluor-405, -488 or -555 secondary antibodies (Invitrogen). Cells were then washed twice in PBS and used for data acquisition immediately using Attune Flow cytometer (Applied Biosystems). 50,000 cells were counted for the analysis. Data was later analyzed using FlowJo software (Treestar). Mean fluorescence intensity was measured using the algorithm provided by FlowJo software and was later evaluated for CSV presence on the surface. Scoring for 84-1 binding to cell lines was performed by analyzing the mean fluorescence intensity of CSV and were defined as “-“: not detectable, “+, ++, +++”: <2, <4, >4 fold expression compared to isotype control.

Alternatively, for in vivo spontaneous tumor model, the cells obtained after RBC lysis were subjected to dual staining using 84-1 antibody against CSV and CD45 antibody. Samples were compensated using antibody capture ABC beads (Invitrogen). Dot plots were plotted with CSV positive gating and CD45 negative gating to enumerate the CTCs in a given sample. Isotype controls were utilized to gate the unstained and non-specific stained cells. Entire cell population was analyzed for CTCs using Attune Flow Cytometer and analyzed using FlowJo software.

Sodium orthovanadate treatment

After 18 hr incubation, the cells were then processed for immunofluorescence staining using the above-described method for extracellular staining. For human blood specimens, mono-nucleated cells obtained from the CPT tubes were treated with SOV (100 µM) or control PBS for 15 min.
Cells were then subjected to immunofluorescence staining directly using the extracellular staining method.

*Microscopy image capture and analysis*

A total of 5,000 cells per chamber were grown on Lab-Tek 8-well permanox chamber slides (Thermo Scientific, Rochester, NY, USA) as described above. For cell surface staining, cells were fixed using 4% paraformaldehyde for 15 min, washed with PBS pH 7.4, blocked in 10% fetal calf serum (FCS) (Gibco, Invitrogen) for 1 hr and labeled for respective primary antibody (1:100) overnight at 4°C. Cells were then rinsed in PBS pH 7.4 and stained using Alexafluor-488 and -555 secondary antibodies (1:250) (Invitrogen) against respective primary antibodies. For nuclei staining, DRAQ5 (Cell Signaling) (1:500) was incorporated along with secondary antibodies for 60 min. WGA (Invitrogen) was used for staining the cell-surface, at 1:1000 for 5 minutes. The cells were then washed with PBS pH 7.4 (3 × 15 min) and mounted in Slowfade antifade (Invitrogen). For intracellular staining, cells were fixed as described above, washed with PBS pH 7.4, and permeabilized in PBS pH 7.4/0.2% NP40 (Sigma Aldrich) for 20 min. Blocking, primary and secondary antibody incubations and mounting are as described above.

For invivo labeling of cells, total cell mixture obtained after RBC lysis was washed in PBS pH 7.4 twice and labeled using 84-1 antibody for 20 min on ice, stained cells were then washed twice in PBS pH 7.4 and stained for secondary antibody Alexafluor-488 against 84-1 for 20 min and after washing twice in PBS pH 7.4, cells were plated on culture dishes and visualized for Alexafluor-488 positive cells under Jenco Epi-Fluorescence Inverted Microscope and pictures were obtained using the supplied software. Only high intensity green fluorescent cells were considered for the analysis. The sensitivity of this assay was confirmed by cell spiking assays.
For live cell imaging, laser intensities were kept to the minimum required to obtain an image to minimize photobleaching and phototoxicity. Alternatively after CD45 depletion, cells were stained for 84-1 and CD45 (AbCAM) using respective antibodies and plated on a glass slide using Cytofuge (Iris) and analyzed for 84-1+ and CD45- cells using confocal microscope. Also, for intracellular staining, 84-1+ CD45- CTC are fixed on a slide using Cytofuge and stained using the above described intracellular staining methodology.

For confocal analysis, images were acquired in 8 bits with Zeiss LSM 510 confocal microscope using LSM 5 3.2 image capture and analysis software (Zeiss). 63 x water-immersion objective (NA, 1.0) was utilized with digital zoom for image capture. All images were acquired by the same user using the same intensity and photodetector gain in order to allow quantitative comparisons of relative levels of immunoreactivity between different samples.

Animal Studies

Five six- to eight-week old C3H (NCI) mice were maintained as per NIH guidelines, and used for all animal protocols have been reviewed and approved by the Institutional Animal Care and Use Committee at the University of Texas MD Anderson Cancer Center. Mice were housed according to the institutional requirements. LM8 cancer cells were used for the in vivo inoculation and were maintained in DMEM/F-12 with 10% FBS and 0.1% penicillin/streptomycin. Confluent LM8 cells were dissociated from the culture plates and 1 x 10^5 cells were suspended in 15 µL PBS for intraosseous (i.o.) inoculations. Intraosseous inoculations were performed by inserting a 27 gauge, ½-inch needle insulin syringe (BD Bioscience) directly into the right tibia, and then suppressing the plunger on the syringe. Tumor volume was measured every week with blood collection as mentioned above. We used the sub-
mandibular bleeding method to repetitively collect blood from the mice, which is minimally invasive. Utilizing this method, we were able to collect 200 µl of blood samples for analysis of CTC. No normal epithelial or mesenchymal cells were detectable in any of these blood samples irrespective of the presence of tumor. After optimizing the assay and route of blood collection, we enumerated the CTCs in mice implanted with tumors of LM8 cells. To determine the circulating tumor cells in a mouse model that mimics Li-Fraumeni syndrome, mice heterozygote (p53515+/515A) or homozygote for the p53 missense mutation (p53515A/515A) were used. Once mice become moribund, these mice were euthanized; blood was collected and analyzed for tumor circulating cells as described above.
Supplementary Figure legends

**Supplementary Figure 1 (A)** Immunological assessment of CSV in osteosarcoma cell line LM7 using commercially available antibodies. AMF-17B, EC-40, CS and V-9 antibodies were utilized for the detection of CSV on LM7 cells using Flowcytometry, no CSV was observed using these antibodies. **(B)** Immunological assessment of CHP-TAMRA peptide to LM7 osteosarcoma cell line using flow cytometry. CHP-TAMRA peptide binds to LM7 cells, indicating the presence of CSV on these cells. **(C)** Evaluation of 84-1 binding to rhVim protein using western blotting. 10, 100 and 1000 ng of rhVim was loaded on 4-20% gradient gels and the western blotting was performed. Blots were probed with 84-1 antibody. It was observed that 84-1 has very high affinity for total vimentin. **(D)** Cell lysates from LM7 cells were prepared and immunoprecipitation was performed using 84-1 and 12-1 (different vimentin antibody clone) antibodies and western blotting was performed. Blot was probed with cell signaling antibody (rabbit origin, to exclude IgG bands). **(E)** Cell lysates from OS25, SAOS-2 and HEK-293T were evaluated for 84-1 binding. 84-1 antibody detected vimentin only in osteosarcoma cells lines OS25 and SAOS-2, while it was undetectable in HEK-293, an epithelial cell line. **(F)** Evaluation of PBMC population from human blood for CSV staining using immunofluorescence imaging. PBMC population was isolated from human blood and evaluated for different markers including CD3e, CD11b and CD45 staining along with 84-1. These cells did not show any strong immunostaining with 84-1 antibody. Scale indicates 10 μm. **(G)** Immunological assessment of CSV in cells isolated from osteosarcoma patient samples using flow cytometry. **(H)** Regression analysis of capture efficiency for different cell numbers of LM7 cells spiked in human blood. **(I)** Spiking assay using higher numbers of spiked LM7 cells.
**Supplementary Figure 2:** *(A)* Serial analysis of CTC in mice: Mice were implanted with LM8 (metastatic osteosarcoma) cells. Approximately 200 ul of blood was obtained by sub-mandibular cheek bleeding method. Samples were obtained at approximately weekly intervals until mice were euthanized because of tumor burden. Left panel indicate CTCs enumerated weekly with normal mice as control. Representative data on the right panel shows data from one mouse that represents CTCs versus tumor volume that was obtained at weekly intervals. *(B)* Micrographs of LM8 cell isolated from blood that was co-stained using antibodies against CSV (green), CD45 (red) and a nuclear stain DRAQ5 (blue). Scale indicates 10 µm. *(C)* Isolation and culture of CTCs isolated from LM8 cells that were monitored for eight days for formation of cell colonies. *(D)* Detection and isolation of CTC from spontaneous tumorigenesis mice model. CTC from spontaneously developing tumor mice were enumerated and isolated. These cells were cultured in vitro for further analysis. For fluorescence imaging, CTCs were stained for CSV (green), CD45 (red) and a nuclear stain DRAQ5 (blue) and analyzed by confocal microscopy. Scale represents 10 µm. *(E)* Detection of 84-1 positive CTCs in canine model. CTC from blood samples were isolated and analyzed by bright field and fluorescence microscopy. CTCs were isolated and cultured on 96 well plates and bright field images were acquired. For fluorescence imaging, CTCs were stained for CSV (green), CD45 (red) and a nuclear stain DRAQ5 (blue) and analyzed by confocal microscopy. Scale represents 10 µm.

**Supplementary Figure 3:** CTC isolated from GIST tumors (with and without SOV treatment) were stained for CD31, an endothelial cell marker.