Supplementary information, Data S1 Materials and methods

Cell lines and cell culture

B16-F10, B16-F1, B16-F0, 67NR, 4T1, MDA-MB-231, MDA-MB-1833, MDA-MB-4175, LLC, HCT116 (Horizon Discovery), PANC-1, AsPc-1, DF (gift from Dr. Ghajar) and O97 (gift from Dr. Ghajar) cells were cultured in DMEM, and human melanoma cells (SK-MEL-, A375M and A375P), as well as Pan02, Pan02-H3, BXPC-3, HPAF-II, EO771, H292, H1975, H1650, PC-9, K-562 (DSMZ), 22RV and NB-4 (DSMZ) cells were cultured in RPMI, both medium supplemented with penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}) and 10% exosome-depleted FBS. Cell lines were obtained from American Type Culture Collection, if not otherwise mentioned. Human melanoma cell lines were obtained from Memorial Sloan-Kettering Cancer Center (MSKCC). The murine pancreatic adenocarcinoma PAN02 was purchased from the NCI cell repository. PAN02-H3, a highly liver metastatic variant of PAN02, was generated in our group by three rounds of intrasplenic injection of cells followed by collection and culturing of liver metastatic lesions

Animal models and plasma collection

C57BL/6 and NOD/SCID mice were obtained from Jackson Laboratory and maintained at the Weill Cornell Medical College (WCMC) animal facility. All procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee of WCMC and MSKCC.

To analyze circulating exoDNA from melanoma-bearing mice, NOD/SCID mice were subcutaneously implanted with 2×10^6 human melanoma SK-MEL-28 cells mixed with an equal volume of matrigel (BD Biosciences). Mice were sacrificed when the tumor reached maximum

size allowed by the IACUC protocol and peripheral blood was obtained by retro-orbital bleeding directly into anti-coagulant tubes (EDTA). Plasma (combined from 8 mice) was separated from blood cells by sequential centrifugation at $500 \times g$ for 10 min followed by $3000 \times g$ for 20 min, and subjected to exosome isolation, as described below.

Exosome preparation and exoDNA extraction

Exosomes were prepared using differential ultracentrifugation methods essentially as described in the literature [3] and resuspended in PBS for subsequent analysis. The purity of isolated exosomes was examined by EM and expression of exosomal markers such as Tsg101, Hsc70 and CD9 as described [3]. For mouse plasma samples, the plasma was filtered through a 1.2 μm membrane to remove debris and large particles, then subjected to ultracentrifugation to pellet and wash exosomes. DNA was extracted from exosomes using the QIAamp DNA mini kit (QIAGEN) following the manufacturer's protocol and eluted with 50 μl of 10 mM Tris pH 8.0. DNA quality and quantity were analyzed using Nanodrop and Agilent Bioanalyzer chips.

DNase digestion analysis of exoDNA

Purified purified exosomes resuspended in PBS were treated either with S1 nuclease, 4.5 units/μl (Fermentas) or dsDNA-specific Shrimp DNase, 0.15 units/μl (Affymetrix). Equal amounts of exosomes were used as untreated controls. The digestion was performed at 30 ^{o}C for 30 min for both S1 nuclease (Fermentas) and Shrimp dsDNase (Affymetrix). Reaction mixtures were prepared according to manufacturer's recommendations. After digestion the enzymes were heat inactivated at 70 ^{o}C for 5 min in the presence of EDTA according to manufacturer's instructions. Exosomal DNA was extracted using the QIAamp DNA mini kit (Qiagen) and the eluted DNA was distributed equally and further subjected to S1 nuclease (5 units/μl) or Shrimp dsDNase (0.20

units/ μl) treatment. All digestions were set up in 20 μl reaction and after digestion 15 μl of each sample resuspended in 1 × DNA loading dye (Fermentas) along with 1Kb DNA ladder (Fermentas) were loaded on a 1.5% agarose gel (Ultrapure agarose from Invitrogen) and run at 150 V for 45 min. The agarose gel was stained with SYBR/Gold from Invitrogen (1:5000 dilution in 1 × TAE) for 45 min and imaged with the Spectroline UV transilluminator (Kodak).

For detection of dsDNA using QuantiFluor dsDNA System (Promega), 5 μl of the digested or undigested mixes were mixed with QuantiFluor dsDNA-specific fluorescent dye and quantification of DNA was performed following manufacturer's protocol. The fluorescent intensity was measured using Spectramax M5 from Molecular devices.

Whole genome sequencing, CGH array and bioinformatic analysis

1 μg of each exoDNA and gDNA sample was submitted to the Genomic Resource Core Facility at WCMC for Illumina TruSeq library preparation and High Throughput DNA sequencing following manufacturer's instructions. Short reads were aligned to the reference mouse genome (mm9) using the BWA computer programs with default parameters. Clonal reads were collapsed using custom scripts. Aligned read densities across the entire genome were then calculated using 100 kb bins and represented using Circos plots.

For the CGH assay, ExoDNA and gDNA samples were labeled using the Genomic DNA Enzymatic Labeling Kit (Agilent Technologies) following manufacturer's instructions, and two-color hybridization was performed using SurePrint G3 and HD CGH microarrays purchased from Agilent Technologies following standard procedures. The arrays were then analyzed and copy number visualized using the Agilent Genomic Workbench software analysis tools.

Dot blot

DNA samples were denatured with 0.4 N NaOH at room temperature for 30min, then placed on ice immediately and neutralized with an equal volume of pre-cooled 0.95 M Tris (pH 6.8) buffer. A four-fold serial dilution of exoDNA starting at 200 ng was dot blotted on Nylon membrane and crosslinked in the Stratalinker. The membranes were blocked with TBST buffer containing 1% milk and then probed with anti-5'-methylcytidine (Eurogentec) and anti-DNA (American Research Products, Inc) antibodies followed by HRP-conjugated secondary antibodies and developed with SuperSignal West Femto Chemiluminescent reagent (Thermo Scientific).

Transmission electron microscopy

Exosome samples were fixed in 2% paraformaldehye and 0.2% glutaraldehyde in 0.1 M phosphate buffered saline and centrifuged to form a 1 mm thick visible pellet on the wall of a microcentrifuge tube. The pelleted exosomes were rinsed without resuspension in 0.5% sodium borohydride to block aldehyde groups and then dehydrated in a graded series of ethanol before being infiltrated in 100% LR White resin for 18 hours at 4 ^{o}C. All the processing was done in the same microcentrifuge tube and solutions were changed so as not to disturb the exosome pellet. The resin was polymerized at 60 ^{o}C overnight and the microcentrifuge tube was cut away so that the exosomes could be thin sectioned. 100 nm thick sections were collected on nickel grids. Post embedding immunogold labeling was performed for DNA labeling using the mouse monoclonal antibody AC-30-10 (EMD Millipore, Billerica, MA 01821 USA) and 10 colloidal gold conjugated to goat anti mouse IgM secondary antibodies were used to reveal the presence of DNA (BB International, Ted Pella Redding CA 96049 USA). Positive control sections consisted of sections of LR white embedded human bone marrow; negative control sections were incubated in secondary antibody without being exposed to primary antibody. Following immunogold labeling, sections were counterstained with 1% uranyl acetate and then examined in a Hitachi H7000 electron microscope at 75 kV accelerating voltage. Images were collected on Kodak 4489 film and,

after development, were scanned at 2400 DPI and the images were processed for contrast using Adobe Photoshop.

Sample preparation for AFM imaging

Exosomal DNA was extracted from exosomes as described above. Control DNA pEGFP-N1-p53 (Addgene plasmid #12091) was linearized using BamHI restriction enzyme or digested with BamHI and XhoI to release an insert of 1.2 kb. After digestion, the DNA was loaded on a 2% agarose gel and purified using QIAquick Gel Extraction Kit (QIAGEN) according to the manufacturer's instructions. DNA used for imaging was prepared at a final concentration of 1 ng/ μl and was used undiluted or diluted before imaging 1:5 or 1:10 in 1 mM Tris-HCl pH 8; 5 mM $MgCl_2$. 50 μl of this DNA solution was placed on freshly cleaved mica discs. After 3 min of incubation at room temperature the DNA was washed with 1ml of ultrapure deionized H_2O and the surface of mica containing bound DNA was dried using nitrogen.

Atomic force microscopy (AFM)

Atomic Force Microscopy imaging of the DNA samples were conducted utilizing an Asylum Research MFP-3D-BIO (Oxford Instruments). Olympus AC240 probes were first calibrated for InvOLS (nm/V) and resonant frequency (kHz) to provide stiffness (pN/nm) as guided by the software thermal tune method. All imaging was then conducted at the same set point force to ensure accurate height measurements. Whenever a probe was replaced, measurements of the control and sample were tested to verify that the height remained unchanged because of the new probe. Imaging of one sample was conducted over 40 hours to rule out changes to the sample height over time. The drive frequency was set to 5% less than the primary resonant frequency. Scan parameters were set to maintain a scan velocity of 1.95 μm/s. Set points remained constant throughout imaging, and cantilevers were retuned between every image to accommodate changes in temperature throughout the imaging period.

dsDNA was distinguished from ssDNA through two parameters. Firstly, the quintessential morphology of the DNA on mica would vary as per Adamcik *et al*., due to the ssDNA intramolecular base pairing not seen in dsDNA samples [7]. Secondly, heights of known dsDNA controls and the exosomal fractions were measured and compared. Widths were not recorded, since tip artifacts and imaging parameters would contribute heavily to this value. Images were bulk processed to apply equal masking and flattening to enable height measurements. A 400 pm mask was calculated before first-order flattening. Furthermore, due to the calculation of InvOLS, the amplitude channel (or feedback error) was added to the flattened height channel for the trace direction to convolve a more accurate image. Artifacts in the height channel due to DNA alignment in the fast-scan and slow-scan directions were clearly eliminated due to this procedure. For error substraction (Figure 1D), the amplitude was added to the height channel.

Mutational analysis of BRAF and EGFR

To detect mutations in *BRAF* and *EGFR* genes, AS-PCR assays were adopted and modified from the literature [11, 14, 15]. In brief, for both *BRAF V600E* and *EGFR T790M* mutations, standard PCR reactions containing 1.5 mM $MgCl_2$ and primer pairs either for WT or mutant alleles at 0.5 μM for *BRAF* and 2.5 μM for *EGFR* were used, respectively. The PCR programs are the following: 95 ^{o}C, 5 min; 40 cycles of (95 ^{o}C, 5 sec, 66 ^{o}C (*BRAF*)/56 ^{o}C (*EGFR*), 5 sec and 72 ^{o}C, 5 sec); 72 ^{o}C, 5 min. For detection of Exon 19 deletion in *EGFR*, PCR reactions containing 1.5 mM $MgCl_2$ and each of the four primers [11, 14, 15] at 0.25 μM were conducted. The PCR program is as follows: 95 ^{o}C, 5 min; 40 cycles of (95 ^{o}C, 30 sec, 58 ^{o}C, 30 sec and 72 ^{o}C, 30 sec); 72 ^{o}C, 5 min. A higher cycle number (80) was used when assessing the sensitivity of the assay and when circulating exoDNA was analyzed. End point PCR products were then analyzed by agarose gel (2%) electrophoresis.