

Supplementary Material

Materials and Methods

Proteomics analysis

Protein extraction and digestion. Chemicals used for protein extraction and digestion were of analytical grade, and Milli-Q water was employed in buffers and solutions. After purification, about 150 µg of exosomes in 200 µL of 50 % TFE in PBS were vigorously vortexed and sonicated for 5 minutes in an ice bath and finally incubated with constant shaking for 2h at 60°C. Proteins were reduced with 5 mM DTT (Ultrapure-grade, Sigma-Aldrich, Oakville, ON, Canada) for 30' at 60°C and alkylated with 25mM IAA (Ultrapure-grade, Sigma-Aldrich, Oakville, ON, Canada) for 30' in the dark at room temperature. Before adding mass spectrometry-grade trypsin (Pierce), the samples were diluted 5-fold with 100 mM ammonium bicarbonate pH 8.0. Protein samples were digested by adding trypsin at a ratio of 1:50 (w/w) for 18 hours at 37°C with constant shaking, in presence of 2mM CaCl₂. To stop digestion 100 µl of 2.5% TFA (puriss. P.a. for HPLC, Sigma-Aldrich, Oakville, ON, Canada) were added to the samples. Digested samples were then centrifuged at 14,000g for 10 min at 4°C. The resulting supernatant, containing the peptide mixture, was extracted using the 100 µL Bond Elute OMIX C18 pipette tips (Agilent Technologies), according to the manufacturer's instructions. Eluted peptide mixtures were vacuum dried and reconstituted in 5% acetonitrile 0.1% formic acid for mass spectrometry analyses.

Generation of the reference spectral library. Approximately 4µg of tryptic peptides from each of the two samples (Exo and IL3L Exo) were run for Data-Dependent Acquisition (DDA) analysis. The resulting list of protein/peptides was used for construction of the SWATH reference spectral library. The sample was analyzed via reverse-phase high-pressure liquid chromatography electrospray ionization tandem mass spectrometry (RP-HPLC-ESI-MS/MS) using a TripleTOF® 5600 mass spectrometer (AB SCIEX; Framingham, US). The mass spectrometer was coupled to a nanoLC Eksigent 425 system (AB SCIEX; Framingham, US). RP-HPLC was performed with a trap and elution configuration using an Acclaim PepMap100 Nano Trap Column 100µm x 2cm, C18, 5µm, 100Å and an Acclaim PepMap® RSLC Nano Column 75µm x 250mm, C18, 2µm, 100Å. The reverse-phase LC solvents were: solvent A (0.1% formic acid in water) and solvent B (2% water and 0.1% formic acid in acetonitrile). The samples were loaded in the trap column at a flow rate of 5 µl/min for 10 min using a solvent, from loading pump, containing 2% acetonitrile and 0.1% v/v TFA in water and eluted at a flow rate of 300µl/min using a gradient method according to which solvent B is linearly increased from 2% to 10% within 10 min, from 10% to 30%

within 110 min and then to 60% within 15 min; afterwards, phase B is further increased to 95% within 2 min. Phase B is maintained at 95% for 10 min to rinse the column. Finally, B is lowered to 2% over 2 min and the column reequilibrated for 21 min (170 min total run time). The eluting peptides were on-line sprayed in the Triple TOF 5600 Plus mass spectrometer, that it is controlled by Analyst TF 1.7 software (AB SCIEX, Toronto, Canada).

Each of the two samples used to generate the SWATH-MS spectral library was subjected to four DDA runs. For these experiments, the mass range for MS scan was set to m/z 400–1250 and the MS/MS scan mass range was set to m/z 230–1,500. Using the mass spectrometer, a 0.25 s survey scan (MS) was performed, and the top 50 ions were selected for subsequent MS/MS experiments employing an accumulation time of 0.065 s per MS/MS experiment for a total cycle time of 3.5485 s. Precursor ions were selected in high resolution mode ($>30,000$), tandem mass spectra were recorded in high sensitivity mode (resolution $>15,000$). The selection criteria for parent ions included an intensity of greater than 500 cps and a charge state ranging from + 2 to + 5. A 15 s dynamic exclusion was used. The ions were fragmented in the collision cell using rolling collision energy, and CES was set to 5.

Eight DDA MS raw files were combined and subjected to database searches in unison using ProteinPilot™ 4.5 software (AB SCIEX; Framingham, US) with the Paragon algorithm. The samples were input as unlabeled samples with the following parameters: iodoacetamide cysteine alkylation, digestion by trypsin and no special factors. The searches were conducted through identification efforts in a UniProt Swiss-Prot database (downloaded in July 2014, with 137216 protein sequence entries) containing whole *Homo sapiens* proteins. A false discovery rate analysis was also performed.

SWATH-MS analysis and targeted data extraction. Two samples (2 μ g each) were subjected to the cyclic data independent acquisition (DIA) of mass spectra. Data were acquired by repeatedly cycling through 40 consecutive 15-Da precursor isolation windows (swaths). For these experiments, the mass spectrometer was operated using a 0.05 s survey scan (MS). The subsequent MS/MS experiments were performed across the mass range of 100 to 1600 m/z on all precursors in a cyclic manner using an accumulation time of 0.03 s per SWATH window for a total cycle time of 1.2990 s. Ions were fragmented for each MS/MS experiment in the collision cell using rolling collision energy, and CES was set to 15. The spectral alignment and targeted data extraction of DIA samples were performed using PeakView v.2.2 (AB SCIEX; Framingham, US) with the reference spectral library. All DIA files were loaded and exported in .txt format in unison using an extraction window of 15 min and the following parameters: three hundred peptides/protein, seven transitions/peptide, peptide confidence level of 90%, excluded shared and modifies peptides, and XIC width set at 75 ppm. This export procedure generated three distinct files

containing the quantitative output for (1) the peak area under the intensity curve for individual ions, (2) the summed intensity of individual ions for a given peptide, and (3) the summed intensity of peptides for a given protein. For each protein, seven individual ion intensities summed as peptide intensity, ten peptides intensities summed as protein intensity. Mean of all technical replicates were used to compare proteins of the two exosome populations.

Viability assay (MTT assay)

Cell viability was assessed with Methyl-thiazoltetrazolium (MTT) assay as previously described²¹. Briefly, CML cells were seeded at a density of 1×10^5 in a 96-well plate and exposed to 0.1, 0.5, 1 and 10 $\mu\text{g/ml}$ of Imatinib-loaded exosomes for 24 and 48 hours, and with 0.5 μM of Imatinib as positive control. Similarly, Imatinib sensitive and resistant CML cells were exposed to 1 $\mu\text{g/ml}$ of siRNA-loaded exosomes for 24, 48 and 72 hours, and with 0.5 μM of Imatinib. The absorbance was measured at 540 nm.

Dinamic light scatter (DLS)

Exosome size distribution was determined by dynamic light scattering (DLS) experiments. Collected nanovesicle samples were diluted to avoid inter-particle interaction and placed at 20°C in a thermostatic cell compartment of a Brookhaven Instruments BI200-SM goniometer, equipped with a solid-state laser tuned at 532 nm. Scattered intensity autocorrelation functions were measured by using a Brookhaven BI-9000 correlator and analyzed in order to determine the size distribution. The size at the maximum of the distribution (moda) is reported as a significant average size.

RNA extraction and Real-Time PCR

Imatinib sensitive and resistant CML cells were treated for 24 and 48 hours with 1 $\mu\text{g/ml}$ of exosomes derived from transfected HEK293T cells, containing BCR-ABL specific siRNA or scrambled siRNA (negative control). Tumor biopsies soon after removal were stored in RNAlater solution (Applied Biosystems, Foster City, California, USA). Each sample was lysed in a tissue homogenizer. RNA was extracted using the commercially available Illustra RNAspin Mini Isolation Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK), according to manufacturer's instructions. Total RNA was reverse-transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem). RT-QPCR was performed in 48-well plates using the Step-One Real-Time PCR system (Applied Biosystem). For quantitative SYBR®Green realtime PCR, the following primers were used: BCR-ABL

(5' TCCGCTGACCATCAAYAAGGA 3', 5' CACTCAGACCCTGAGGCTCAA 3') and GAPDH (5'ATGGGGAAGGTGAAGGTCG3', 5'GGGTCATTGATGGCAACAATAT3'), all obtained from Invitrogen (Foster City, CA, USA). Real-time PCR was performed in triplicates for each data point. Relative changes in gene expression between control and treated samples were determined with the $\Delta\Delta C_t$ method. Levels of the target transcript were normalized to GAPDH endogenous control, constantly expressed in all samples (ΔC_t). For $\Delta\Delta C_t$ values, additional subtractions were made between treated samples and control ΔC_t values. Final values were expressed as fold of induction.

Western Blotting

Imatinib sensitive CML cells were treated for 24 hours with 0.5 μ M of Imatinib (as positive control), with 1 or 10 μ g/ml of exosomes derived from HEK293T transfected or not and treated with Imatinib. Imatinib sensitive or resistant CML cells were treated for 72 hours with 0.5 μ M of Imatinib or with 1 μ g/ml exosomes derived from HEK293T expressing IL3-Lamp2b, and transfected with BCR-ABL specific siRNA or scrambled siRNA (negative control).

Total protein cell lysates or exosome lysates were obtained and analyzed by SDS-PAGE followed by Western blotting. Antibodies used in the experiments were: anti-6xHIS, anti-GAPDH, anti-CD81, anti-Tsg101 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Alix, anti-IL3-Ra, anti-c-Abl, anti-phospho-Abl (Cell Signaling Technology, MA, USA).

Figure legends

Figure S1. Quantitative proteomic analysis of exosomes and IL3L exosomes. (A) Graphical representation of the ratio among the number of proteins of the whole dataset (blue), the number of proteins differentially represented in Exo and IL3L Exo (green) and the number of ribosomal proteins significantly enriched in Exo (yellow). (B) GO enrichment analysis of proteins differentially represented in Exo and IL3L Exo performed by FunRich. The graph reports data of molecular function terms.

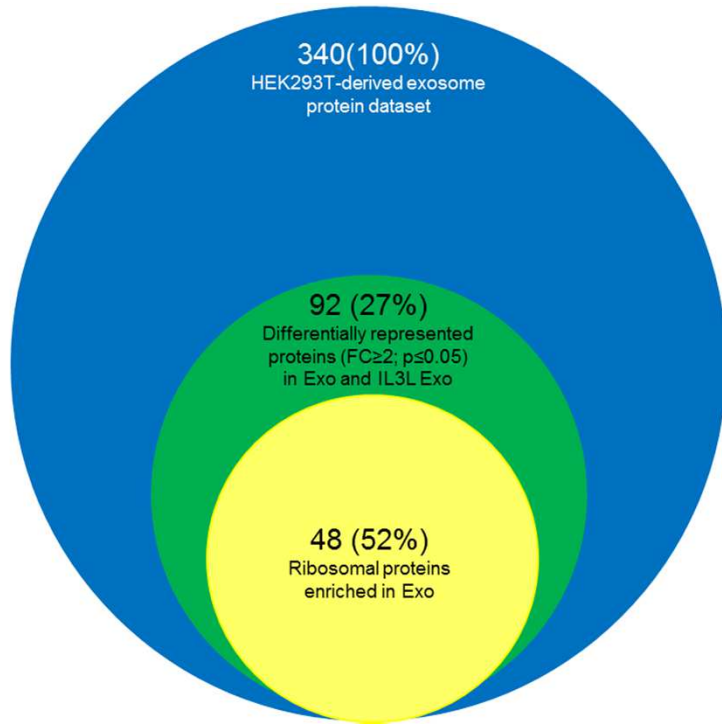
Figure S2. (A) Histograms show fluorescence intensity expressed as ratio between a.u. and number of LAMA84 and K562R cells treated for 3h and 6h with 10 µg/ml of HEK293T-derived exosomes (Exo) and IL3L-HEK293T-derived exosomes (IL3L Exo). Significant higher values of fluorescence intensity were observed for IL3L Exo treated cells in comparison to cell treated with Exo control (*, $p < 0.05$; ***, $p < 0.0005$). As well as significant higher values of fluorescence intensity were found for cells treated for 6h in comparison to 3h, independently by the type of exosome (°, $p < 0.005$; °°, $p < 0.0005$); (B) Analysis at confocal microscopy of LAMA84 (upper panel) or K562R (lower panel) cells treated for 6h at 4°C with 10µg/ml of HEK293T-derived exosomes (Exo) and IL3L-HEK293T-derived exosomes (IL3L Exo). Nuclei were stained with Hoechst (blue), exosomes were labelled with PKH26 (red); (C) LAMA84 (left panel) and K562 (right panel) growth was measured by MTT assay after 24, 48 h of treatment with 1 µg/ml of IL3L Exo-Imatinib with different amounts of empty IL3L Exo (specific competition, SC) or with different amount of empty Exo (non-specific competition, AC). The values were plotted as % of growth vs Ctrl (untreated cells – dot line). Each point represents the mean ± SD of three independent experiments.

Figure S3. (A) Histograms of tumor weight (mg) of Imatinib-sensitive CML xenograft for each type of treatment: ^a, versus Ctrl ($p < 0.0005$); ^b, versus Ctrl ($p < 0.05$); ^c, versus Imatinib ($p < 0.005$); ^d, versus Imatinib ($p < 0.0005$); ^e, versus Imatinib ($p < 0.05$); ^f, versus Exo-Ctrl ($p < 0.05$); ^g, versus IL3L-Exo ($p < 0.0005$); ^h, versus Exo-Imatinib ($p < 0.005$); (B) Histograms of tumor weight (mg) of Imatinib-resistant CML xenograft for each type of treatment: ^a, versus Ctrl ($p < 0.05$); ^b, versus IL3L Exo-scrambled siRNA ($p < 0.005$).

Figure S4. (A) mRNA levels BCR-ABL were evaluated in samples from mice xenografts. The values were plotted as fold change compared to xenograft control; (B) Mice organs were excised and imaged after 24 h. A scale of the radiance efficiency is presented to the right.

Supplementary table S1. List of identified and quantified proteins by SWATH-MS.

A



B

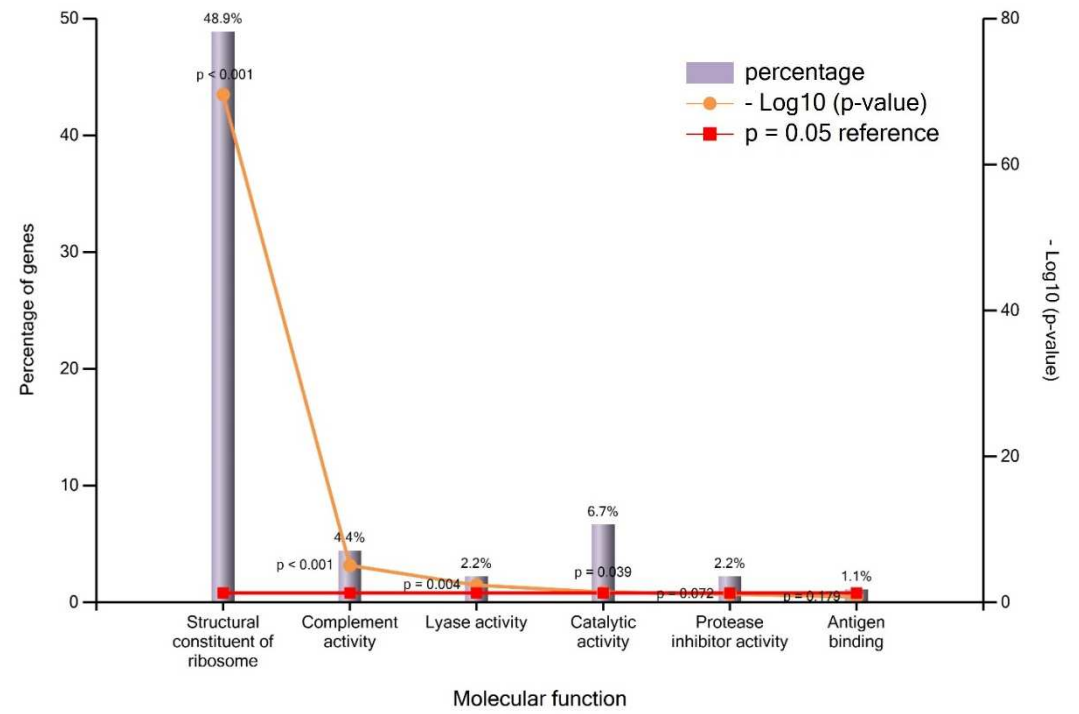


Figure S1

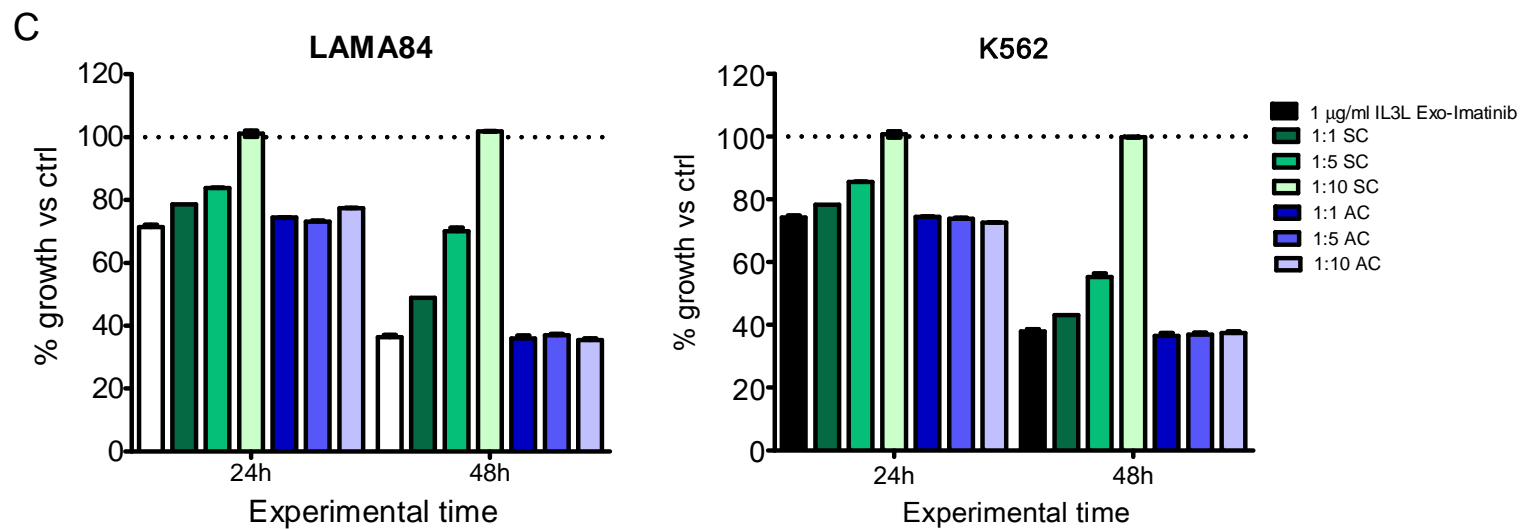
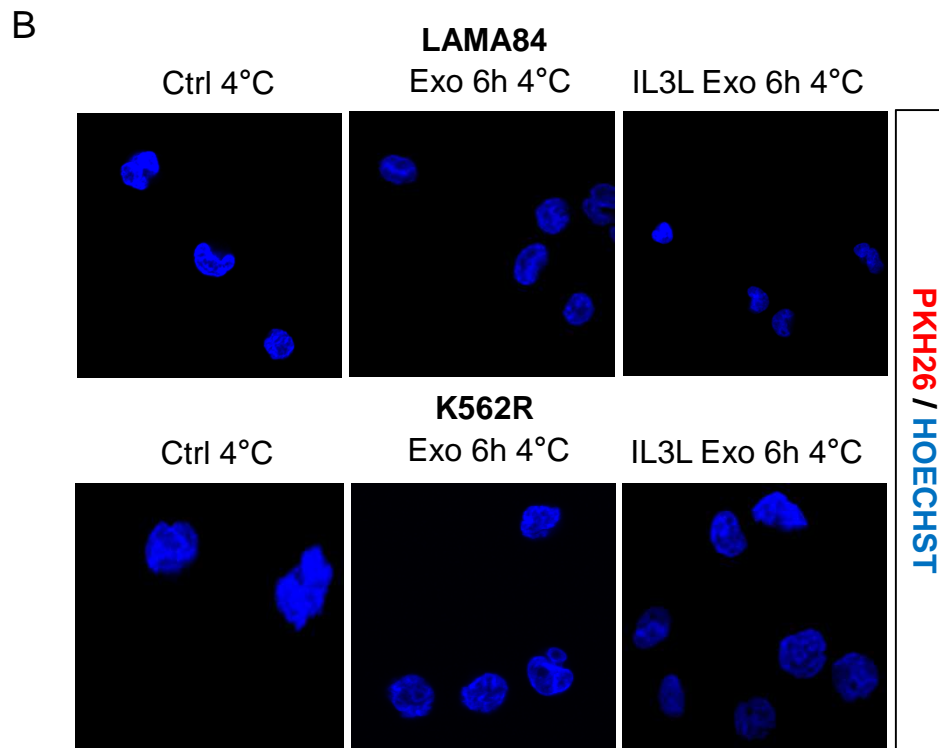
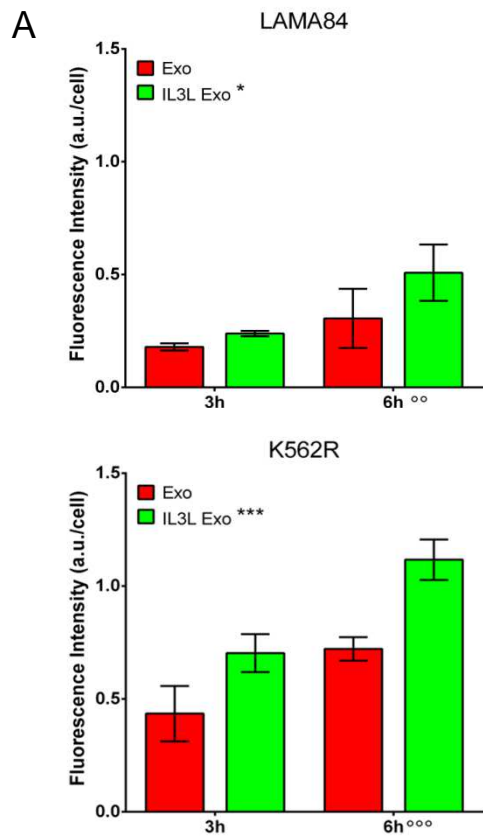


Figure S2

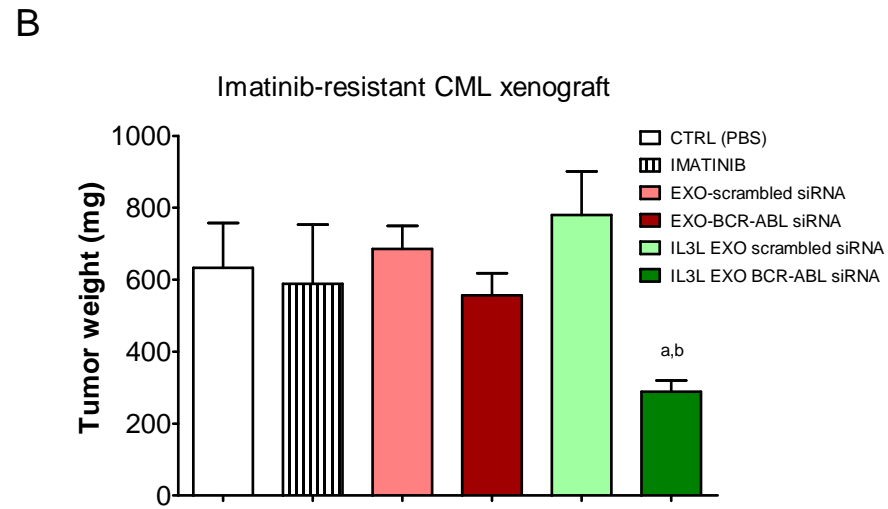
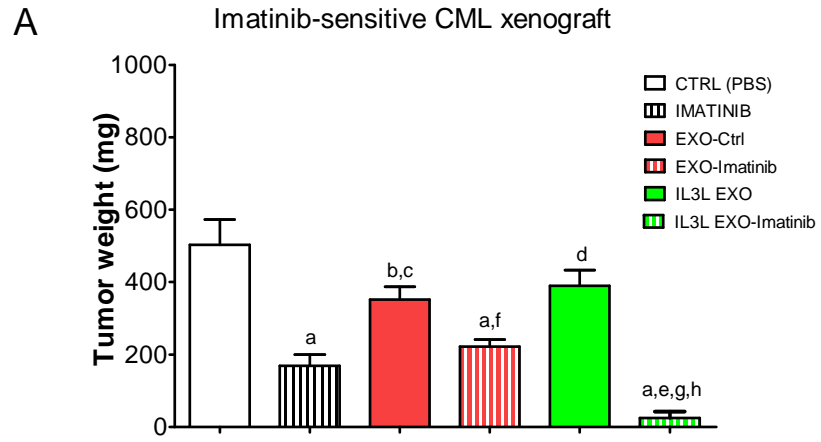
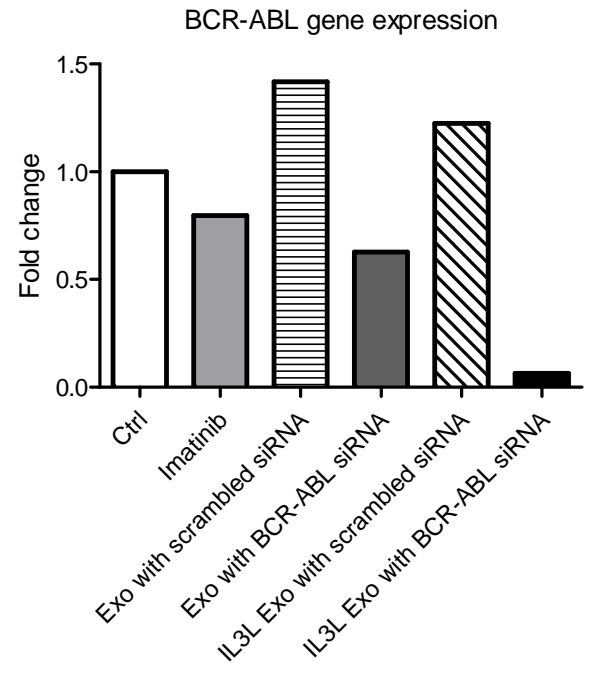


Figure S3

A



B

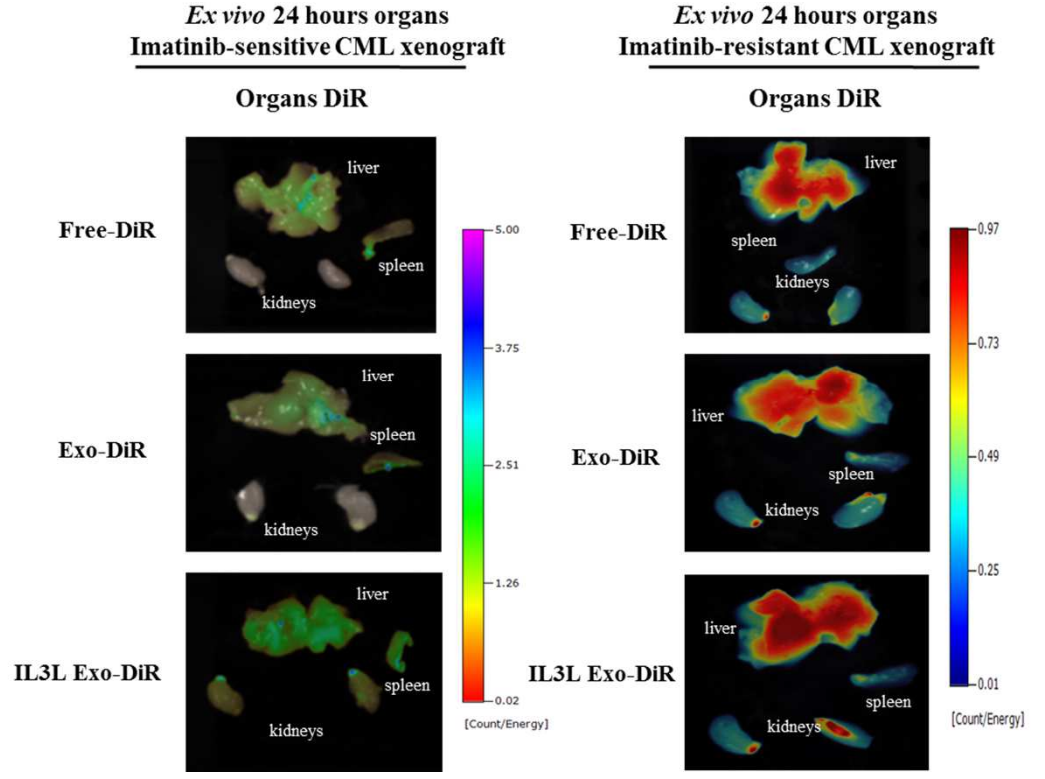


Figure S4