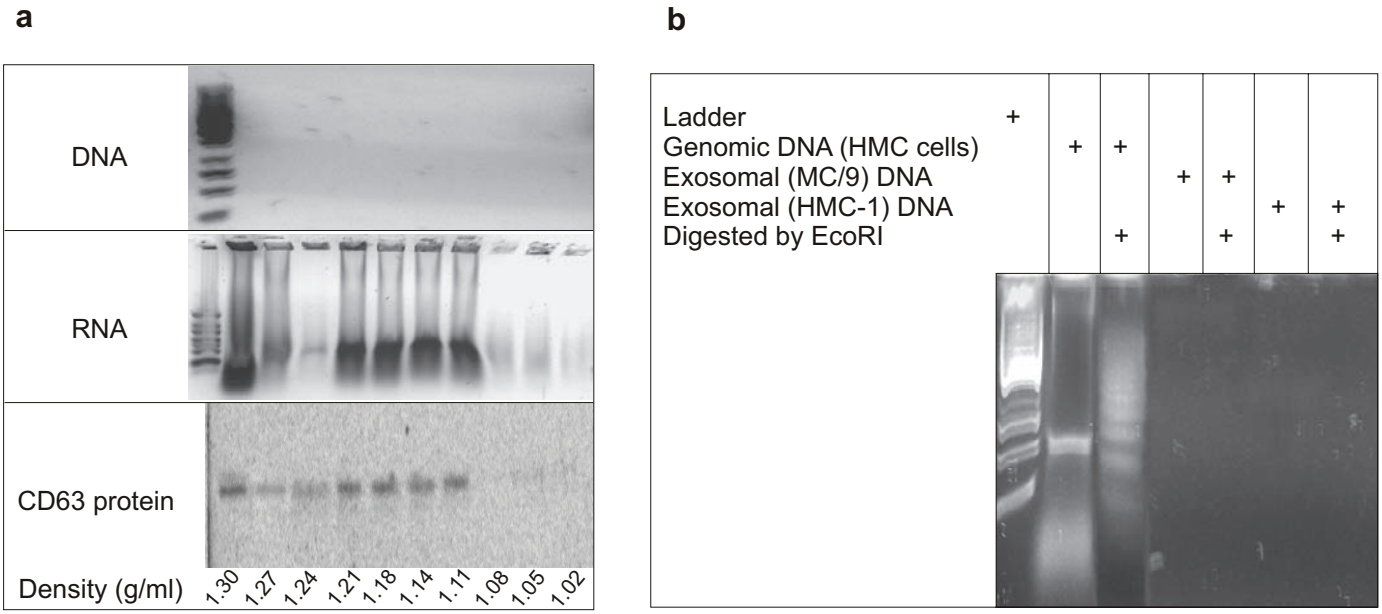
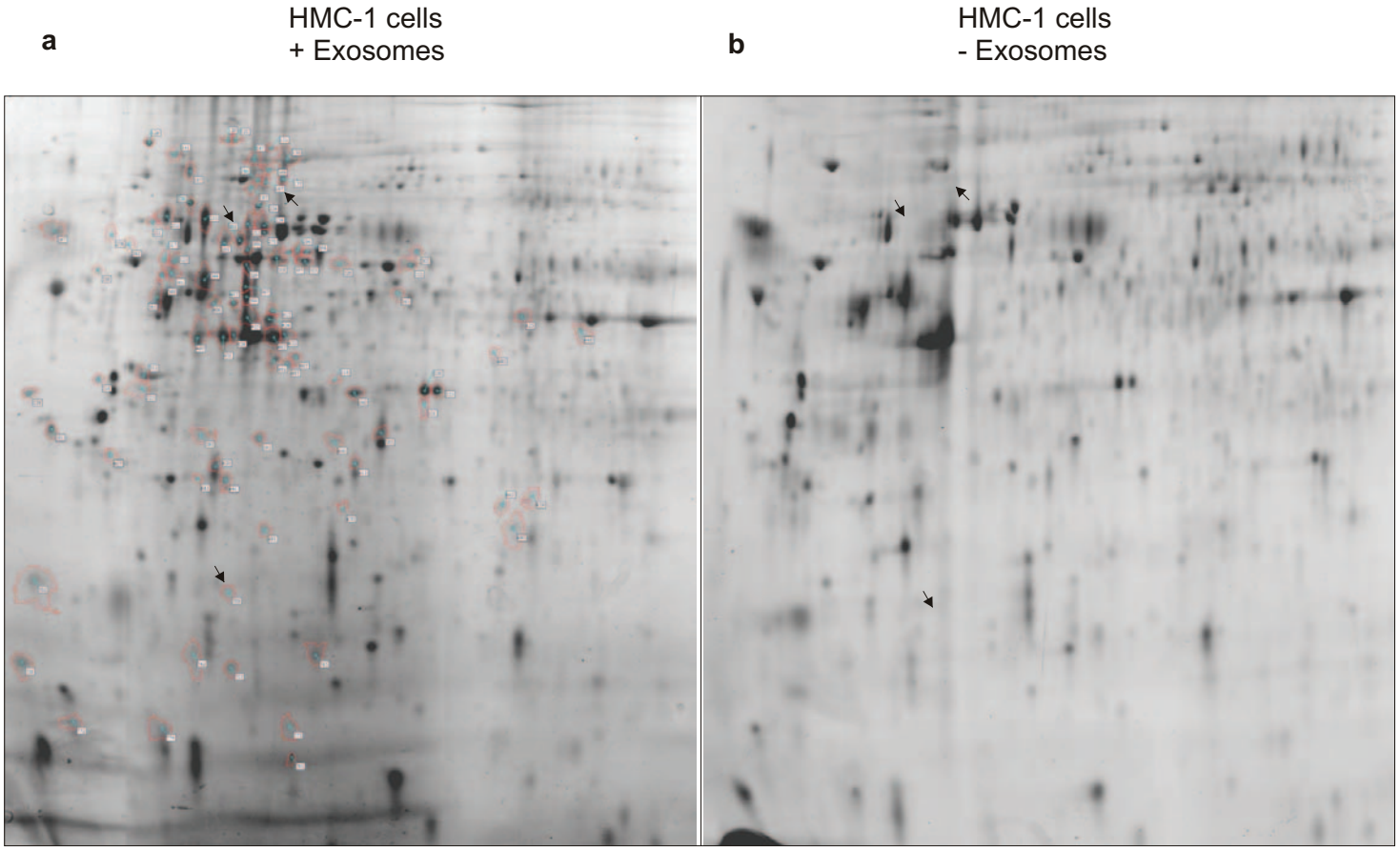
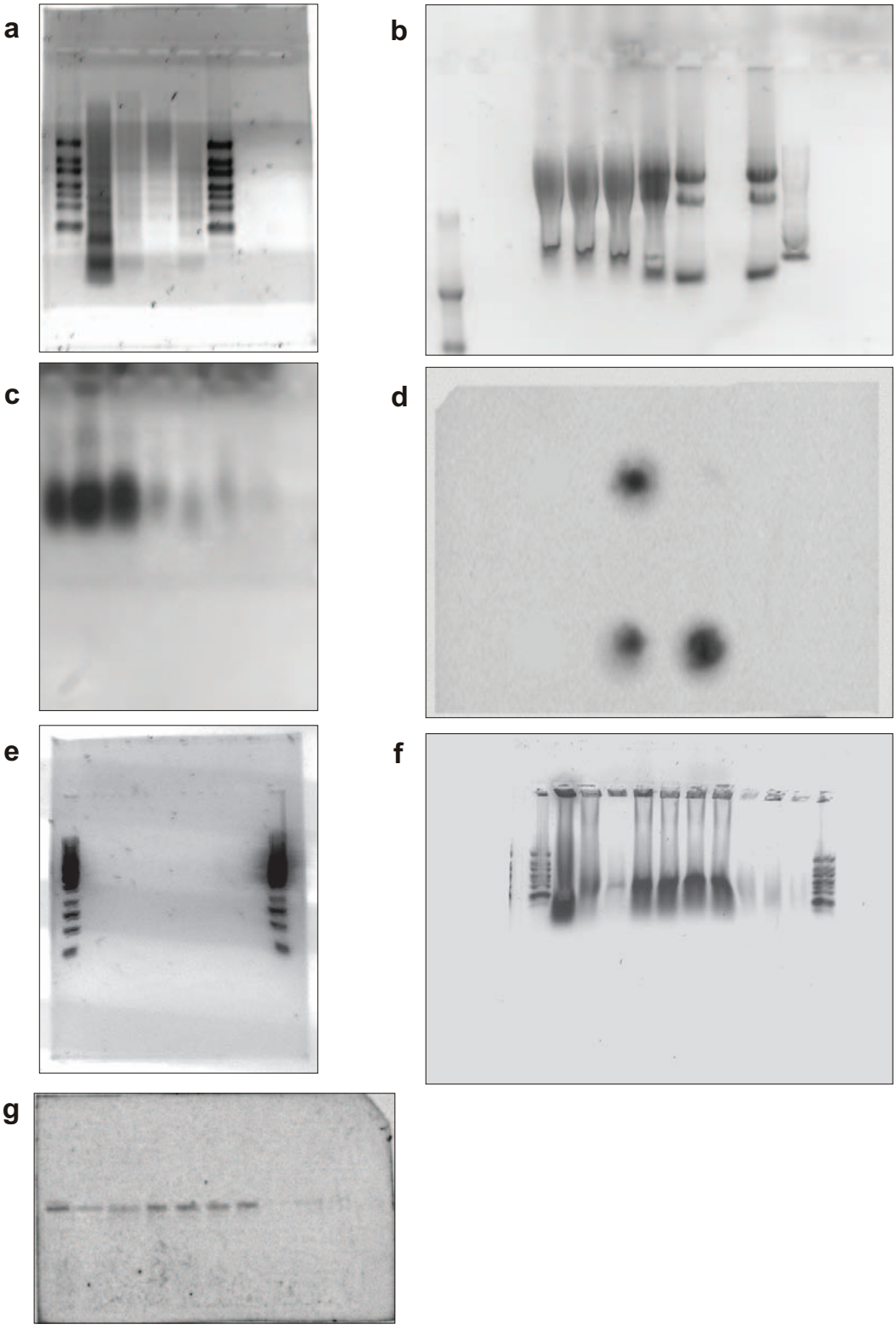
nature cell biology



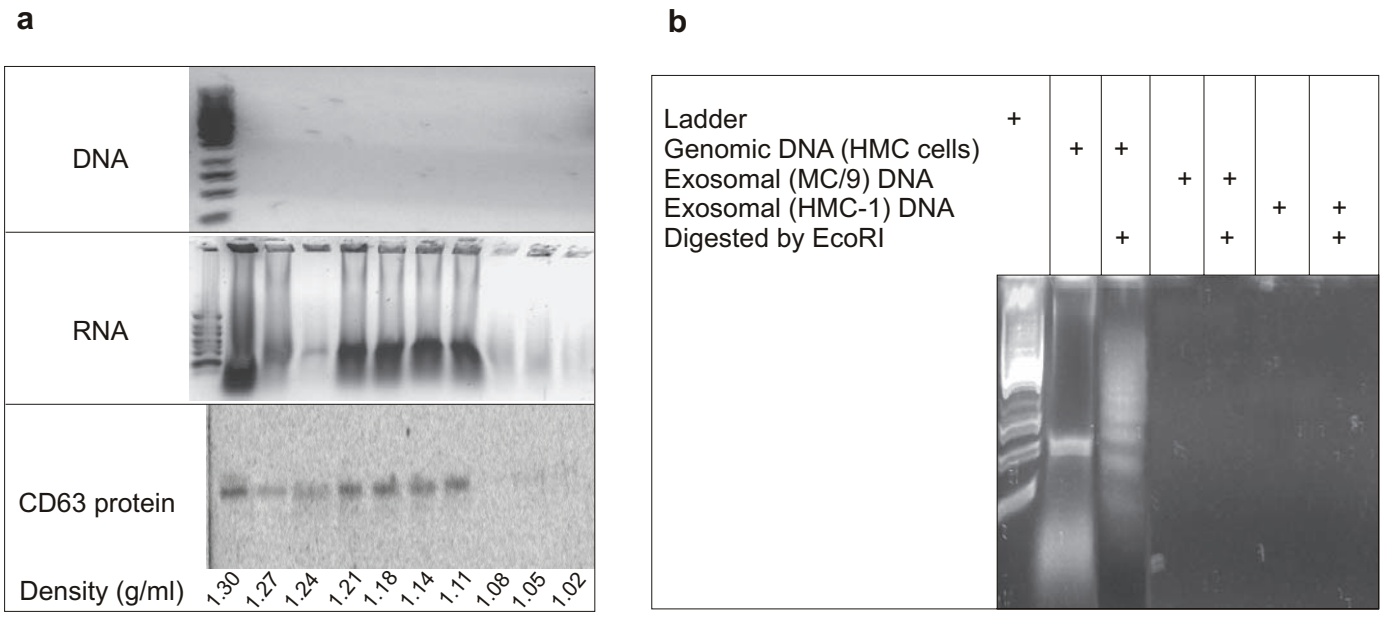
Supplementary Information, Figure 1.
Detection of DNA, RNA, and protein of MC/9 exosomes.
Exosomes were isolated as previously described in methods. (**a**) To further examine the purity of the exosomes, the exosome pellet harvested after the 120 000 g centrifugation was floated in a sucrose gradient (0.25-2M sucrose, 20 mM Hepes/NaOH, pH 7.2). The exosomes were dissolved in 2.5 M sucrose and the gradient was layered on top of the exosomes. The gradient was centrifuged at 100 000 g for 15 h according to previously published methodology6. Gradient fractions (10 x 3.8 ml) were collected from the bottom of the tube, and was diluted with 10 ml PBS and ultracentrifuged for 2h at 150 000 g (Beckman Ti70.1 rotor), and the pellets were dissolved in Trizol. The DNA, RNA and proteins from each fraction was extracted. No DNA could be detected in any fraction of the sucrose gradient. The RNA and CD63 protein was found at the characteristic density of exosomes (1.11-1.21 g/ml). RNA and CD63 protein could also be detected in fractions 1 and 2 (density 1.27-1.30 g/ml), probably because of aggregated exosomes. (**b**) To confirm absence of DNA in exosomes, DNA was extracted (Qiagen DNA easy tissue kit Cat No 69506) from HMC-1 cells and from exosomes from both MC/9 and HMC-1. Five microgram of the DNA from the cells and 50% of the sample volume from the exosomes were digested by the restriction enzyme EcoRI. The samples were separated in a 1% agarose gel. The results show that HMC-1 cells contain DNA, but the exosomes from both HMC-1 and MC/9 cells do not contain any DNA. Same results have been obtained using Trizol extraction method. For full scan see supplementary information figure 3.



Supplementary Information, Figure 2
Proteomic results from transfer of MC/9 exosomes to HMC-1 cells.
Human mast cells HMC-1 were incubated with the mouse MC/9 exosomes (**a**) and without (**b**) for 24 hours. Proteins between the two gels were matched and 96 newly produced proteins were identified by MALDI-tof. Spots labelled with arrows are good candidates as mouse protein produced from exosomal mRNA. For further information see supplementary information, Table S5.



Supplementary Information, Figure 3.
Full scans of all key gel and western data.
Gels **a-d** are original scans from figure 2 in the manuscript, gels **e-g** are the original scans for supplementary figure 2.



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Supplementary Methods

Cells

MC/9 cells (ATCC) were cultured according to manufacturer's recommendations. To eliminate exosomes present in serum, Rat T-Stim and FBS were ultracentrifuged at 120 000 g for 90 min using a Ti70 rotor (Beckman optima LE-80k Ultracentrifuge). The human mast cell line HMC-1 (Dr Joseph Butterfield, Mayo Clinic, USA), was cultured in IMDM containing 10% FBS, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 2 mM L-glutamine and 1.2 mM alph-thioglycerol. For release of exosome, the HMC-1 cells were cultured in the presence of 1 µM calcium ionophore for 30 min. Bone marrow mast cells (BMMC) were prepared by culturing bone marrow cells from femurs of 7-10 wk old male BALB/c in the presence of IL-3 (R&D systems) as described previously[24]. After 4 weeks of culture, the cells were harvested and consisted of 96% pure MCs as analysed by morphology. During the last 48 h, BMMC were cultured at $3x10^6$ cells ml^{-1} in complete medium with ultracentrifuged FBS supplemented with 10 ng ml^{-1} IL-4 (R&D-systems), and in some experiments in the presence of 1 µl ml^{-1} ^{3}H-Uracil (Amersham Biosciences). For culture of $CD4^+$ T cells, mouse spleens were collected and passed through a 70µm followed by 30 µm filter. $CD4^+$ T cells were purified by negative selection using the Spincep® mouse $CD4^+$ T cells enrichment cocktail (Stemcell Technologies) according to the manufactures instructions. The purity of the $CD4^+$ T cells ranged from 89 to 91%, as analysed by flow cytometry. The cells were cultured in RPMI 1640 containing 10% FBS, 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin at $1x10^6$ cells per ml in flat bottom 48 well plates.

Exosome purification

Exosomes were prepared from the supernatant of MC/9, BMMC and HMC-1 cells by differential centrifugations as previously described[4] with some modifications. Briefly, cells were harvested, centrifuged at 500 g for 10 min to eliminate cells and at 16 500 g for 20 min, followed by filtration through 0.22 µm filter to remove cell debris. Exosomes were pelleted by ultracentrifugation (Beckman Ti70 rotor) at 120 000 g for 70 min. For mass spectrometry, the exosome pellet was washed once in PBS. Exosomes were

measured for their protein content using BCA™ Protein Assay Kit (Pierce). For the density gradient experiment the 120 000 g exosome pellet was floated in a sucrose gradient (0.25-2 M sucrose, 20 mM Hepes/NaOH, pH 7.2). The exosomes were dissolved in 2.5 M sucrose and the gradient was layered on top of the exosome suspension. The gradient was centrifuged at 100 000 g for 15 h according to[4] with some modifications. Gradient fractions (10 x 3.8 ml) were collected from the bottom of the tube, diluted with 10 ml PBS and ultracentrifuged for 2 h at 150 000 g (Beckman Ti70.1 rotor), and the pellets were extracted by Trizol. For exosome yields, see Supplementary Information Table S1.

Flow cytometry

For FACS analysis, exosomes from MC/9 and BMMC cells were adsorbed onto 4-μm aldehyde/sulphate latex beads (Interfacial Dynamics) for 15 min in 30 μl PBS followed by 3 h with agitation in 200 μl PBS at RT (for MC/9; 25 μg exosomes per $1.5x10^5$ beads and for BMMC; exosomes from 10^7 cells per $1.5x10^5$ beads). The reaction was stopped by incubation in 100 mM glycine for 30 min. Exosome-coated beads were washed three times, incubated with CD63 antibody (Santa Cruz), washed twice, incubated with PE-conjugated secondary antibody (Santa Cruz), washed twice and analysed on a FACSScan (Becton Dickinson, San Diego CA). For immunoisolation, 4-μm-diameter aldehyde/sulphate latex beads were incubated with purified anti-CD63 mAb (BD) or mouse IgG1 (Sigma-Aldrich) under gentle agitation at RT overnight according to manufactures recommendation (Interfacial Dynamics). For FACS analysis, 30 μg of HMC-1 exosomes were incubated with 1.5×10^5 anti-CD63 or mouse IgG1 beads in 30 μl PBS at RT for 15 min, the volume was made up to 400 μl and the beads were incubated at 4 °C overnight under gentle agitation. The reaction was stopped by incubation in 100 mM glycine for 30 min. Exosome-coated beads were washed twice, incubated in 1% human serum at 4 °C for 15 min, washed twice and incubated with PE-conjugated CD63 antibody (BD), washed and analysed on a FACSScan (Becton Dickinson, San Diego CA).

Electron microscopy

The exosome pellets from MC/9 cells and HMC-1 cells resuspended in PBS were loaded onto formwar carbon coated grids (Ted Pella Inc, Redding, CA, USA cat no 01800N-F). Exosomes were fixed in 2% PF, washed and the HMC-1 exosomes were immunolabelled with anti-CD63 (BD) antibody followed by 10 nm gold labelled secondary antibody (Sigma Aldrich). The exosomes were post-fixed in 2.5% glutaraldehyde, washed, contrasted in 2% uranyl acetate, embedded in a mixture of uranyl acetate (0.8%) and methyl cellulose (0.13%), and examined in a LEO 912AB Omega electron microscope (Carl Zeiss NTS, Germany).

Protein analysis by LC-MS/MS

Exosome proteins were extracted and collected in the stacking part of a 10% SDS gel. Total proteins were cut from the gel, trypsinated and analysed using the LC-MS/MS by Core facilities (http://www.proteomics.cf.gu.se/). Briefly for the liquid chromatography an Agilent 1100 binary pump was used, together with a reversed phase column, 200 x 0.075 mm, packed in-house with 3 μm particles Reprosilpur C_{18}-AQ. To separate the peptides, the flow through the column was reduced by a split followed by a 50 min gradient of 0-50% CH_3CN. The nano-flow LC-MS/MS were done on a 7-Tesla LTQ-FT mass spectrometer (Thermo Electron) equipped with a nanospray source modified in-house. The spectrometer was operated in a data-dependent mode, automatically switching to MS/MS mode. MS-spectra were acquired in the FTICR, while MS/MS-spectra were acquired in the LTQ-trap. For each scan of FTICR, the three most intense, doubly or triply charged, ions were sequentially fragmented in the linear trap by collision induced dissociation. All the tandem mass spectra were searched by MASCOT (Matrix Science, London, UK) program to identify proteins. The analysis was repeated three times.

Autonomous translation assay

MC/9 exosomes (740 μg protein weight), cells ($3x10^6$) or extracted proteins (310 μg) were kept for 4 h in complete medium containing 10% of the standard concentration of methionine, and incubated in the presence of L-[^{35}S]-methionine (Amersham Biosciences), 10 mCi ml^{-1}, for 18 h to label newly produced proteins. Proteins were

extracted, washed three times using a 10 kDa ultra filter (Millipore) and radioactivity was measured by scintillation.

Isolation of RNA, DNA and proteins

RNA, DNA and proteins were isolated using Trizol® (Invitrogen) or RNeasy® mini kit (Qiagen) according to the manufactures protocol. For co-purification of microRNA and total RNA, the RNA was extracted using Trizol, followed by the RNeasy® mini kit. Cells and exosomes were disrupted and homogenized in Buffer RLT (Qiagen) and 3.5 volumes of 100% ethanol were added to the samples prior use of the RNeasy mini spin column. The rest of the procedure was performed according to the manufactures protocol.

RNA detection

Detection of RNA was performed using agarose gel followed by EtBr staining, by QC-RNA (www.exiqon.com), and by using Agilent 2100 Bioanalyzer® (www.chem.agilent.com). For detection of RNA in BMMC exosomes, the cells were cultured in the presence of ^{3}H-Uracil for 48h, exosomes were isolated, and washed with a 10 kDa cut-off column to eliminate unbound nucleotides. The exosomal RNA was isolated and the signal was detected by scintillation. For detection of mRNA in both BMMC and MC/9 exosomes, cDNA was synthesised using reverse transcriptase (Fermentas) in the presence of radioactive [α-^{32}P]-CTP, 10 mCi ml^{-1} (Amersham) according to manufactures recommendations. Total cDNA was run on a 0.8% agarose gel, dried over night, and visualized using phosphoimager. As control, samples were RNase treated before cDNA synthesis. To confirm that the RNA is confined inside the exosomes, MC/9 exosomes were treated with 0.4 µg µl^{-1} RNase (Fermentas) for 10 min at 37°C. As a control, 5µg cellular RNA was added to the exosomes before the RNase treatment. To elute any macromolecules bound to the exosomes, MC/9 exosomes were treated with 0.25% trypsin for 10 min at 37 ºC. Anti-CD63 (Santa Cruz) was added to the exosomes, before the trypsin treatment to determine whether external proteins were removed by the treatment. Exosomes were isolated by ultracentrifugation. RNA and proteins were isolated using Trizol. RNA was run on an agarose gel and the anti-CD63 antibody was detected by horseradish peroxidase-coupled secondary antibody (Harlan

Sera-lab) followed by enhanced chemiluminescence (Amersham Biosciences, Inc.) on a nitrocellulose membrane.

Western blot

Total proteins from the 10 fractions were extracted and run on polyacrylamide gels, before transfer to nitrocellulose membranes (BioRad). Membranes were blocked in TBS containing 0.5% skim milk, incubated with the CD63 antibody (Santa Cruz) followed by the horseradish peroxidase-coupled secondary antibody (Harlan Sera-lab), and subjected to enhanced chemiluminescence (Amersham Biosciences, Inc.).

Microarray

The microarray experiments were performed by SweGene (www.swegene.org/) according to Affymetrix microarray DNA chip analysis. The expression level Signals were scaled in GCOS 1.2 to give a median array intensity of 100. This was done to enable different arrays to be compared. The program Spotfire DecisionSite 8.2 (www.spotfire.com) was used for gene-profiling analysis. For the network analysis to identify biological mechanisms, the program Ingenuity was used (www.ingenuity.com).

MicroRNA array and profiling

Methods for purification of small RNA were described in the section of RNA isolation. Identification of microRNA was performed by the Exiqon company (www.exiqon.com). Briefly, the quality of the total RNA was verified by an Agilent 2100 Bioanalyzer profile. Two μg total RNA from the exosome and the donor mast cell (MC/9) samples were labelled with Hy3™ and Hy5™ fluorescent stain, respectively, using the miRCURY™ LNA Array labelling kit, following the procedure described by the manufacturer. The Hy3™-labeled exosome samples and a Hy5™-labeled mast cells were mixed pair-wise and hybridised to the miRCURY™ LNA array version 8.0, which contains capture probes targeting all human, mouse and rat miRNA listed in the miRBASE version 8.0. The hybridisation was performed according to the miRCURY™ LNA array manual using a Tecan HS4800 hybridisation station (Tecan Systems, Inc. San Jose, CA). The miRCURY™ LNA array microarray slides were scanned by a ScanArray 4000 XL

scanner (Packard Biochip Technologies, Billerica, MA ,USA) and the image analysis was carried out using the ImaGene 6.1.0 software (BioDiscovery, Inc,El Segundo, CA USA). The quantified signals were normalised using the global Lowess (LOcally WEighted Scatterplot Smoothing) regression algorithm.

Transfer experiments

To label MC/9 exosome RNA, cells were cultured in complete medium supplemented with 1 µl ml^{-1} ^{3}H-Uracil 72 h before exosome isolation. Exosomes were isolated according to the isolation protocol and washed by ultra-filtration (10 kDa, Millipore) to remove free nucleotides. The exosomes were added to MC/9, $CD4^{+}$, and HMC-1 cells in the ratio of 8:1 between donor cells and recipients at the starting point of labelling. At 0 h and 24 h, cells were harvested and washed twice. RNA was isolated by RNeasy® mini kit and the signal of radioactive RNA was measured using scintillation. Medium supplemented with 1 µl ml^{-1} ^{3}H-Uracil absent from donor cells was treated equally and used as negative control.

In vitro translation

Total exosomal RNA was purified using RNeasy® mini kit and 0.5 µg was used for the translation. The *in vitro* rabbit lysate translation kit (Promega Corporation) was used according to manufactures recommendation to translate exosomal mRNA to proteins. A sample without exosomal RNA was treated equally and used as negative control. After the translation procedure was accomplished, total proteins were precipitated using acetone and determined using RC DC protein assay (BioRad). The protein content of the samples (presence and absence of the exosomal RNA) was compared using 2D-PAGE, BioRad instruments (Mini-protean®3cell) and recommendation. The 2D-gels were visualised using SyproRuby (BioRad) and digitalised using phosphoimager. Protein spots of the samples were compared and a selection of the newly produced proteins was cut, trypsinated, and identified using LC-MS/MS followed by MASCOT program search. The newly produced proteins of mouse origin were compared to the genes identified from the DNA microarray analysis.

In vivo translation

MC/9 exosomes (1000 μg) were added to HMC-1 cells (8 x 10^6) in three different time points (0, 3, 6 h) and the cells were incubated for approximately 24 h. The HMC-1 cells were harvested, washed, and the total proteins of the cells were separated by 2D-PAGE according to Core facility (www.proteomics.cf.gu.se). A sample without exosomes was treated equally and used as negative control. The newly produced proteins were detected using PDQUEST and 96 spots were cut and identified using MALDI-tof followed by MASCOT program search, according to Core facility (www.proteomics.cf.gu.se).

Statistics

Kruskal Wallis test followed by Mann-Whitney was used for the statistical analysis when required and a p-value <0.05 was considered significant.

Accession numbers

Details about the microarray deposition can be found at http://www.ncbi.nlm.nih.gov/projects/geo/ (the GEO accession number is: GSE7275).