

Supplementary Materials

Supplementary Materials and Methods

1. Extracellular vesicle (EV) isolation, purification and characterisation

Extracellular vesicles were purified from 7-day cell conditioned media, pre-cleared of cell debris and microvesicles by differential centrifugation followed by filtration through a 0.22 µm filter (Millipore). EVs were purified based on their density by ultracentrifugation at 100,000 x *g* on a 30% sucrose/D₂O cushion as described ¹. Purified EVs were resuspended in around 100 µl PBS, aliquoted, before storage at -80°C. Total protein was quantified by microBCA protein assay (ThermoFisher Scientific, Paisley, UK). The number, and size distribution of nano-particles was assessed by nanoparticle tracking analysis (Nanosight; Malvern Instruments, Worcestershire, UK). As a measure of EV purity, protein and nanoparticle concentrations were used to calculate a ratio of particle to protein. All preparations had a particle to protein ratio of >2x10¹⁰ particles per µg of protein, as described ². The presence of tetraspanins at the outer EV surface was determined using a plate-immobilisation of purified EVs, and indirect staining with antibodies against CD9 (R&D Systems, Abingdon, UK), CD81 or CD63 (BioRad, Hertfordshire, UK), a secondary anti-Mouse IgG-biotin conjugate (PerkinElmer) and streptavidin-Europium detection. Primary antibodies against relevant isotypes, IgG1 and IgG2b (eBioscience, ThermoFisher Scientific), were used as a control. Time resolved fluorometry was performed on a Pherastar FS instrument (BMGlabtech, Germany) as described ³. Whole cell lysates, prepared using RIPA-buffer (Santa Cruz Biotechnology) were compared to EV lysates, prepared by boiling in SDS-sample buffer containing 20 mM DTT, by western blotting running 10 µg protein per lane. After transfer to PVDF membranes (GE Healthcare), and blocking with 5% non-fat powdered milk with 0.1% Tween-20 in PBS for 1 hr, primary monoclonal antibody at a concentration of 1-4 µg/ml was added at 4°C overnight. Antibodies for expected EV proteins TSG101, Alix, LAMP1 (Santa Cruz Biotechnology), MHC Class I (eBioscience,

ThermoFisher Scientific) were used, and to assess cellular contaminants, blots were also probed for calnexin expression (Santa Cruz). After washes in 0.1% Tween-20/PBS bands were detected using an anti-mouse IgG-horseradish peroxidase conjugated antibody (Santa Cruz) and chemiluminescence substrate (PicoWest, ThermoFisher Scientific). Images of membranes were collected using the C-DiGit Chemiluminescence Blot Scanner (LI-COR Biotechnology, Cambridge, UK).

2. Extracellular vesicle analysis by Nano particle Tracking Analysis (NanoSight™)

Freshly prepared EVs, or those following fluorescent labelling were diluted in particle free water (Fresenius Kabi, Runcorn, UK) to concentrations up to 2×10^9 particles/ml, which is within the linear range of the NanoSight instrument. Analysis was performed on a NanoSight™ NS300 system configured with a temperature controlled LM14 laser module with a 488 nm laser and a high sensitivity sCMOS camera system and a syringe-pump system (Malvern Instruments, Malvern, UK). Three videos of 30-60 s were taken under controlled fluid flow with a pump speed set to 80, and temperature set to 25°C. Videos were taken in light scatter mode. On some occasions, videos were also taken following application of a long-pass fluorescence filter, so that only particles emitting light at >500 nm were visible. This required corrections for focusing, and an adjustment to the camera settings to maximise chances of visualising, and tracking fluorescent nano-particles. Videos were analysed using the batch analysis tool of NTA 2.3 software (version 2.3 build 2.3.5.0033.7-Beta7), where minimum particle size, track length and blur were set at “automatic”. The area under the histogram for each triplicate measurement was averaged and used as a particle concentration measurement.

3. Cryo-electron microscopy of purified extracellular vesicles

Extracellular vesicle preparations were adsorbed onto glow-discharging holey carbon 200-mesh copper grids (Quantifoil Micro Tools GmbH). Grids were vitrified with the aid of a Vitrobot (Maastricht Instruments BV). Vitrified samples were imaged at

liquid nitrogen temperature using a JEM-2200FS/CR transmission cryo-electron microscope (JEOL) equipped with a field emission gun and operated at an acceleration voltage of 200 kV.

4. siRNA transfection

In a 6 well plate (or 35 mm imaging dish) 100 pmols siRNA was diluted in 185 μ l Opti-MEM while in a separate container 2 μ l Oligofectamine was diluted with 13 μ l OptiMEM. The two solutions were gently mixed and incubated at room temperature for 30 min. The cells were washed with Opti-MEM and 800 μ l of Opti-MEM was added before dropwise addition of the siRNA complex mixture. Cells were returned to the incubator for 4 h before addition of 500 μ l Opti-MEM containing 30% FCS and incubated for 44 h.

5. SDS PAGE and Western blotting

5.1. Following siRNA depletion

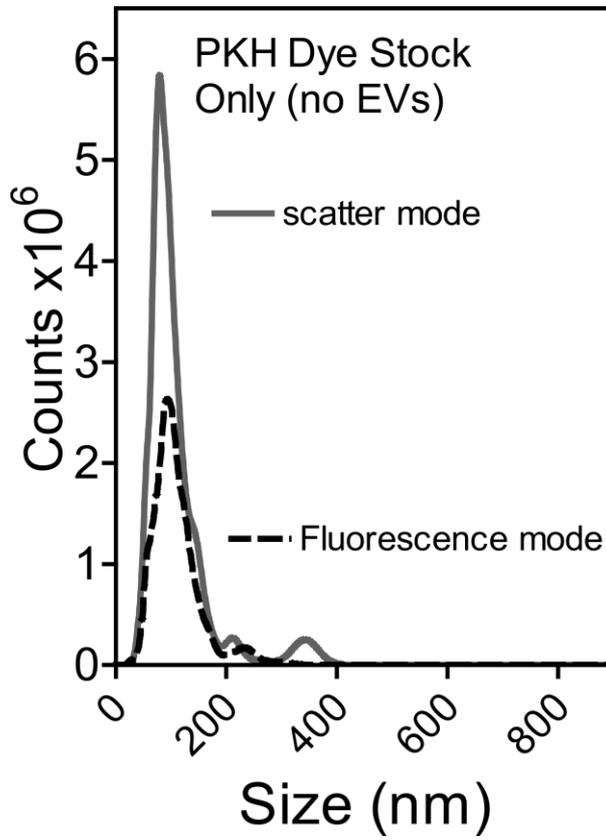
Following 48 h transfection, cells were washed with PBS followed by incubation on ice for 5 min in 100 μ l ice-cold lysis buffer - 150 mM NaCl, 50 mM Tris-base pH 8.0, 1% Triton X-100 containing protease inhibitor cocktail. Cells were scraped from the plastic surface, placed in eppendorf tubes and then centrifuged for 10 min (13000 \times g) at 4°C. The protein concentration of each sample was calculated via BCA assay and 18 μ g protein per sample was mixed with 3x SDS PAGE sample buffer, heated to 95°C and loaded on to 8%, 10% or 12% SDS-PAGE gels. Following gel electrophoresis, proteins were transferred to PVDF membranes before blocking (5% w/v dried milk in PBS 0.0025% v/v Tween 20 (PBSTM)) and incubation with primary antibodies recognising AP2 μ 2, Caveolin-1 (Cav-1), Cdc42, Flotillin-1 (Flot-1), p21-activated kinase-1 (PAK-1), or GAPDH in 2% PBSTM. Secondary antibody incubation with goat anti-rabbit HRP conjugate, goat anti-mouse HRP conjugate or HRP conjugated anti- δ -

tubulin was then performed and chemiluminescence was detected on a ChemiDoc imager using ImageLab software (Bio-Rad).

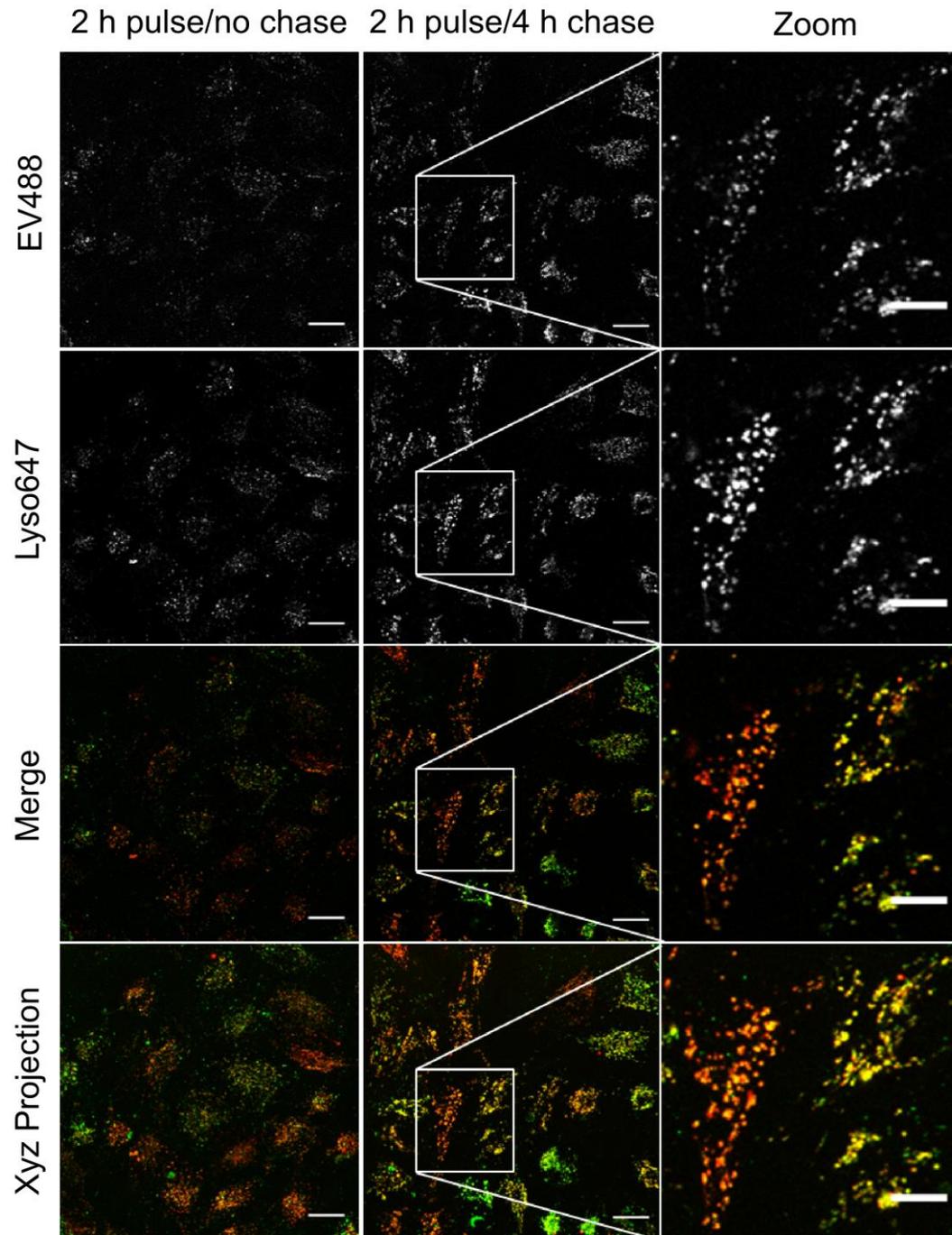
6. Optimisation and characterization of extracellular vesicle labelling

C₅-maleimide-Alexa488 (5-200 µg/ml) was added to a 30 µl EV aliquot containing 60 to 100 µg protein, and made up to a final volume of 50 µl with PBS. Incubations, with no agitation, for 60 min in the dark at room temperature (R/T), were followed by removal of unbound dye using exosome spin columns (Invitrogen) according to manufacturer's instructions. Collected labelled EVs were added to black-walled 96-well plates and the average fluorescence of triplicate wells measured on a PHERAstar FS plate reader (BMG Labtech, Ortenberg, Germany). Similarly, at a fixed dose of 200 µg dye, incubations for up to 3 h were performed before assessing intensity of labelling. In parallel, dye in the absence of EVs were included in these experiments, to assess the efficacy of free-dye capture by the exosome spin columns. For some experiments, the free sulph-hydryl bonds at the EV surface were capped by pre incubations with N-acetyl-L-cysteine (up to 1 mM) for 30 min prior to dye labelling (200 µg/ml, 1 h) and fluorescence assessment; revealing approximately 80% inhibition of labelling at maximal N-acetyl-L-cysteine dose.

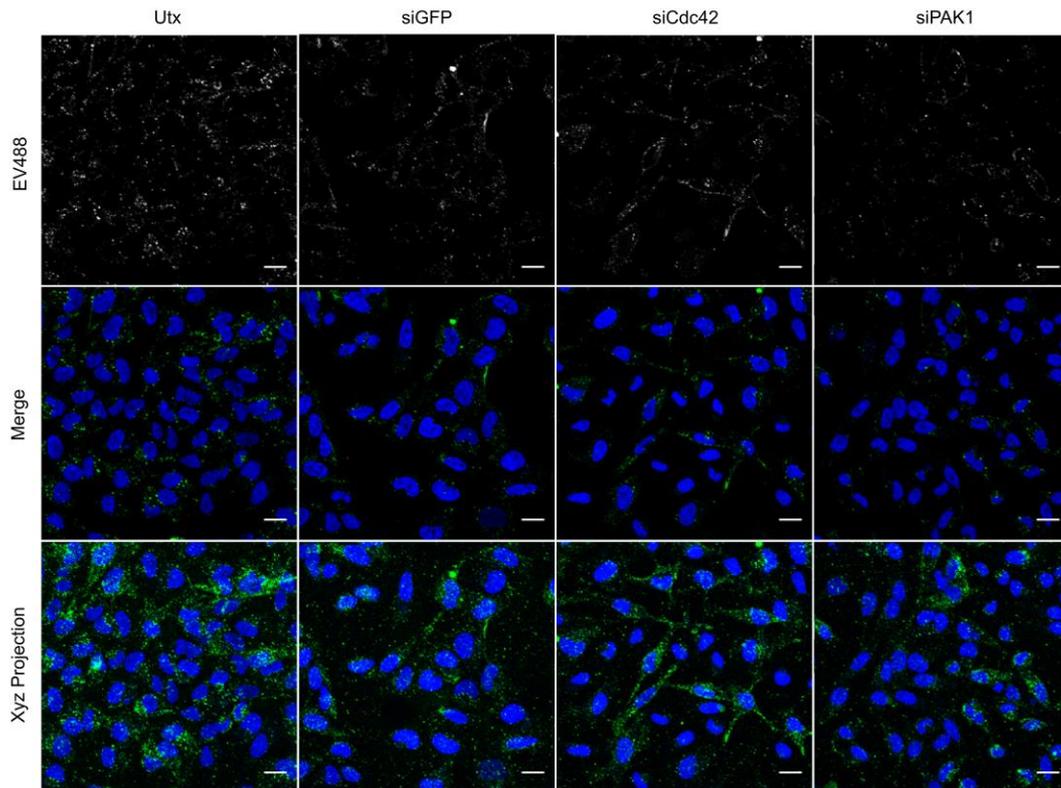
Supplementary Figures



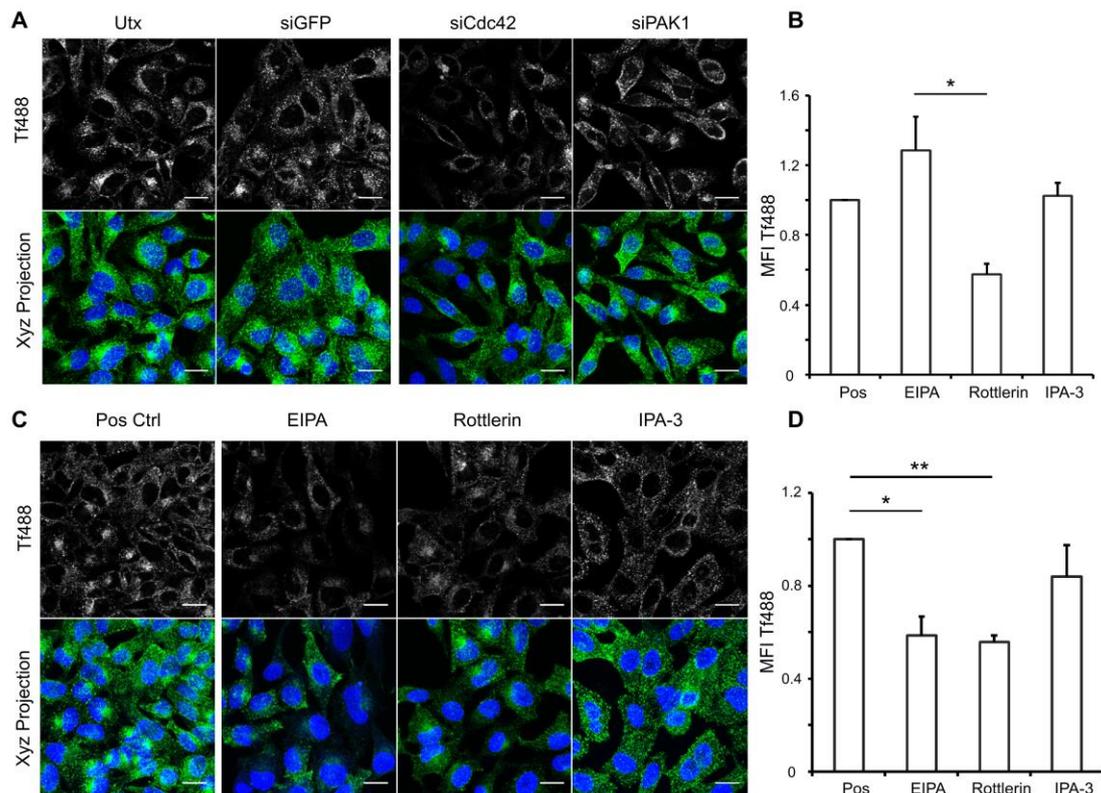
Supplementary Figure. 1. PKH26 dye was diluted 1 in 1000 in the provided dilution buffer, and analysed by NTA. The analysis was performed in light scatter mode (grey), and then following application of fluorescence filter where only fluorescing particles are visible (black, dashed line). We conclude that small particulate material spanning the size range of EVs is present in the stock solution, and a proportion (52%) of these are fluorescent.



Supplementary Figure. 2. Colocalisation of 488-labelled extracellular vesicles with dextran-loaded lysosomes in HeLa cells. Cells were incubated with Dx647 (100 $\mu\text{g}/\text{ml}$) for 2 h, washed with PBS and incubated for a further 18 h in culture media. Cells were then incubated with EV488 (60 $\mu\text{g}/\text{ml}$) for 2h with no chase or for 2 h followed by washing and a 4 hr chase. Scale bars: 20 μm and 10 μm on zoomed images. Images representative of three separate experiments.



Supplementary Figure. 3. Additional fields of view showing internalisation of extracellular vesicles in HeLa cells depleted of fluid-phase/macropinocytosis related proteins. Cells were depleted of either Cdc42 or PAK-1 via siRNA transfection for 48 h before 60 min incubation with EV488 (50 $\mu\text{g}/\text{ml}$). Cells were incubated with Hoechst for 5 min before live cell imaging. Utx: Untransfected. Scale bar: 20 μm . Images representative of three separate experiments.



Supplementary Figure. 4. Transferrin internalisation in HeLa cell models of fluid-phase/macropinocytosis inhibition. (a) Cells were depleted of either Cdc42 or PAK1 via siRNA transfection for 48 h, or (c) pre-incubated with either EIPA (25 μ M), Rottlerin (10 μ M), IPA-3 (50 μ M), or 0.05% DMSO as 'Pos Ctrl' for 30 min before 15 min incubation with Tf488 (5 μ g/ml). Cells were incubated with Hoechst for 5 min before live cell imaging. Scale bar: 20 μ m. Images representative of three separate experiments. (b) and (d) MFI quantification of the experiments presented in (a) and (c), respectively. Error bars represent Standard error. * $p < 0.05$, ** $p < 0.01$. Representative of three separate experiments.

Supplementary Video.1. Time lapse images of EV488 uptake in HeLa cells. EV488 (60 μ g/ml) were added for a period of either 120 min before confocal time lapse imaging. Hoechst and cell mask deep red were added for the final 5 min before imaging. Imaging was continuously performed for a period of 2 min.

Supplementary References

1. C. Thery, S. Amigorena, G. Raposo and A. Clayton, *Current protocols in cell biology / editorial board, Juan S. Bonifacino ... [et al.]*, 2006, **Chapter 3**, Unit 3.22.
2. J. Webber and A. Clayton, *Journal of extracellular vesicles*, 2013, **2**.
3. J. Webber, T. C. Stone, E. Katilius, B. C. Smith, B. Gordon, M. D. Mason, Z. Tabi, I. A. Brewis and A. Clayton, *Molecular & cellular proteomics : MCP*, 2014, **13**, 1050-1064.