Specificities of exosome versus small ectosome secretion revealed by live intracellular tracking of CD63 and CD9

Mathilde Mathieu, Nathalie Névo, Mabel Jouve, José Ignacio Valenzuela, Mathieu Maurin, Frederik J. Verweij, Roberta Palmulli, Danielle Lankar, Florent Dingli, DAMARYS LOEW, Eric Rubinstein, Gaëlle Boncompain, Franck Perez & Clotilde Théry

Despite their roles in intercellular communications, the different populations of extracellular vesicles (EVs) and their secretion mechanisms are not fully characterized: how and to what extent EVs form as intraluminal vesicles of endocytic compartments (exosomes), or at the plasma membrane (PM) (ectosomes) remains unclear. Here we follow intracellular trafficking of the EV markers CD9 and CD63 from the endoplasmic reticulum to their residency compartment, respectively PM and late endosomes. We observe transient co-localization at both places, before they finally segregate. CD9 and a mutant CD63 stabilized at the PM are more abundantly released in EVs than CD63. Thus, in HeLa cells, ectosomes are more prominent than exosomes. By comparative proteomic analysis and differential response to neutralization of endosomal pH, we identify a few surface proteins likely specific of either exosomes (LAMP1) or ectosomes (BSG, SLC3A2). Our work sets the path for molecular and functional discrimination of exosomes and small ectosomes in any cell type.

https://doi.org/10.1038/s41467-021-24384-2
ll cells release membrane-enclosed vesicles, collectively called Extracellular Vesicles (EVs), in their environment. These EVs contain a selected set of lipids, nucleic acids, and proteins from their cell of origin and thus can transfer a complex array of information to surrounding or distant cells. EVs can form by direct outward budding from the plasma membrane (PM) of prokaryotic and eukaryotic cells. In eukaryotic cells, EVs can also form first as intraluminal vesicles of internal multivesicular compartments of the endocytic pathway (MVBs), and are then secreted upon fusion of these compartments with the PM. To clarify the nomenclature, it has been recently recommended to use the term “exosomes” specifically for the MVB-derived EVs, rather than for all small EVs. The PM-derived EVs, on the other hand, are called various names, such as microvesicles, microparticles, or ectosomes: we will use here the latter term which is exclusively used for PM-derived EVs, whereas the others are also used for any type of EVs.

Since they form at different subcellular sites, exosomes and ectosomes will likely contain different sets of specific cargoes, and thus different functions. However, this hypothesis has not been conclusively confirmed so far, given the difficulty to separate exosomes from ectosomes of the same size present in biofluids or cells’ conditioned medium, the lack of specific protein markers to distinguish exosomes from ectosomes, and of molecular machineries and tools with demonstrated full specificity for one or the other.

Several tetraspanins, especially CD63, CD81, and CD9 have been used as markers of exosomes for the last two decades, due to their accumulation in small EVs as compared to whole cell lysates, and to the steady-state accumulation of CD63 in MVBs. More recently, however, their presence in other EVs has been observed. By capture of EVs bearing specifically either CD63 or CD9 or CD81, followed by analysis of their protein composition and enrichment in endosomal markers, we proposed that EVs bearing only CD9 or CD81 but not CD63 probably did not form in endosomes (and were thus ectosomes), whereas those bearing CD63 together with one or the two other tetraspanins may correspond to endosome-derived exosomes. This observation was made using EVs released by primary human immune dendritic cells, which complicated a direct validation of the model by carrying out cell biology analyses.

This work aims to determine the actual exosomal or ectosomal nature of EVs containing the different tetraspanins. We use HeLa cells, a cellular model amenable to experimental manipulations necessary to address cell biology questions. Our reasoning is that, to identify the subcellular origin of the EVs released by these cells, we need to follow in a time-controlled manner intracellular trafficking of tetraspanins from their initial synthesis in the endoplasmic reticulum (ER) until their secretion in EVs. We have adapted CD63 and CD9 to the Retention Using Selective Hook (RUSH) system, which has been used to follow and control trafficking of numerous transmembrane and secreted proteins, and thus identify atypical pathways of secretion. This allows us also to synchronize the release of CD9- or CD63-containing EVs, to determine the composition of a more homogenous mixture of newly synthesized EVs. Our results demonstrate that both CD63 and CD9 can be released in small ectosomes formed at the plasma membrane, and that in HeLa cells, ectosomes represent a minor subpopulation of small EVs (sEVs) that bear CD63 together with other late endosomal molecules such as LAMP1/2. Their secretion is specifically susceptible to neutralization of the endosomal pH. Secretion of ectosomes, for which we identify specific markers in HeLa cells as BSG and SLC3A2, is conversely insensitive to endosomal pH neutralization. Interestingly, CD81, another tetraspanin used commonly as exosome and/or small EV marker, behaves like CD9 rather than like CD63. The markers and molecular mechanisms specific of exosomes versus ectosomes identified here will pave the way for further studies to decipher their respective functions.

Results

CD63 and CD9 are present on two distinct and one common populations of EVs. In dendritic cells, CD63 and CD9 are secreted abundantly in small EVs, where an EV subpopulation bearing CD63 with CD9 and/or CD81 seemed to correspond to endosome-derived exosomes, but both are also detected in larger EVs. We first analyzed their distribution in EVs released by HeLa cells. CD81, CD9, and CD63 can be found in material recovered from HeLa conditioned medium (CM) after 2 h centrifugation at 200Kxg (200 K pellet) (Fig. 1a). By contrast, hardly any signal was detected in the pellets recovered at 2Kxg (2 K) and 10Kxg (10 K), which, in dendritic cell CM, contained large and medium EVs. The total protein content was very low in the 2 K and 10 K pellets, suggesting that HeLa release mainly small and/or light EVs requiring high g-force for efficient pelleting. The 200 K pellets also contained the cytosolic protein syntaxin, confirming (as recommended by the MISEV2018 guidelines) the presence of membrane-enclosed vesicles. The endoplasmic reticulum (ER) transmembrane protein calnexin was not detected, showing that the HeLa EVs do not originate from the ER. Finally, acetylcholine esterase (AChE), which we recently showed not to be an exosome nor EV marker, but rather a co-isolated serum-derived non-EV component was detected in the 200 K pellets. Analyzed by Transmission Electron Microscopy (TEM), the 200 K pellet contained a majority of vesicles of around 50 nm in diameter, a less abundant population of about 120 nm, and a minor population of about 200 nm (Fig. 1b, right panel). The smallest (50 nm) particles were not detected by Nanoparticle Tracking Analysis (NTA), whose detection limit for biological particles is around 70 nm. By NTA, the 200 K pellets contained a major population of 120 nm, less abundant EVs of 200–300 nm, and few of 300–350 nm in diameter (Fig. 1b, left panel). These results thus confirm that a majority of EVs released by HeLa are smaller than 250 nm, and we will call them here small EVs (sEVs).

To determine whether CD9 and CD63 are on the same or on different sEVs, we immuno-isolated these EVs with antibodies specific for one or the other tetraspanin (Fig. 1c). The HeLa CM was filter-concentrated, rather than ultracentrifuged, to concentrate EVs while avoiding aggregation which can be induced by ultracentrifugation of some biofluids like plasma. CM was obtained in the absence of fetal bovine serum to avoid clogging of the filter concentrator by excess of serum-derived proteins. Side-by-side analysis of the isolated EVs (pull-down: PD) and the EVs that had not bound the antibody (Flow-Through: FT) shows that anti-CD63 co-precipitates around 50% of CD9, and that conversely anti-CD9 co-precipitates around 50% of CD63 (Fig. 1c). Immunoprecipitation of CD63 and CD9 in a mixed conditioned medium from CD63-KO and CD9-KO cell lines shows much lower co-isolation of the other tetraspanin by either antibody (Supplemental Fig. 1a, b). Therefore, the observed co-precipitation of CD63 and CD9 from WT CM is not due to aggregation of single positive EVs but rather due to simultaneous presence of the two tetraspanins on the same EV. This suggests that HeLa cells secrete at least three EV populations defined by these markers: one population with both CD63 and CD9, one with CD63 only and one with CD9 only. Indeed, analysis of the 200 K pellets by immuno-EM after double-labeling for CD9 and CD63 confirmed the existence of these three populations (Fig. 1d). We next questioned the sub-cellular origin of the CD63 +/CD9 + EV population released by HeLa cells. By immunofluorescence, we observed that CD63 is located mainly in intracellular
Fig. 1 CD63 and CD9 are found on two different and one common EV populations. 

a Western blot showing transmembrane proteins (CD9, CD63, and CD81), a cytosolic protein (syntenin-1) and two “negative” controls (AChE and calnexin) in cell lysates (CL) and the pellets obtained from HeLa 24 h conditioned medium after differential ultracentrifugation (2 K, 10 K, 200 K). The loaded material comes from 20 × 10⁶ cells for the centrifugation pellets, and from 0.2 × 10⁶ cells for the cell lysate. Representative of 3 independent experiments.

b NTA and EM analysis of 200 K pellets obtained from HeLa 24 h conditioned medium. The quantifications represent the mean of concentration or frequency of EVs of different diameters, error bars show the standard deviation (SD). N = 3 independent experiments. Scale bar of the zooms: 0.1 μm.

c Principle of immunoprecipitation of CD63 and CD9 EVs in HeLa concentrated conditioned medium and representative Western blot of the pull-down (PD) and flow-through (FT) of the immunoprecipitation, with quantification of the relative CD63 and CD9 bands intensity of three independent experiments (mean ± SD is represented). Immuno-EM analysis of the 200 K pellet labeled with anti-CD9 (5 nm gold particles) and anti-CD63 (10 nm gold particles) antibodies. This experiment was performed once. Scale bar of the zooms: 0.1 μm.

d Confocal imaging of immunofluorescence staining of CD63 and CD9 in HeLa cells. Pink arrows show CD9 localized in intracellular compartments. Representative picture of two independent experiments. Scale bar: 5 μm.
compartments. In contrast, CD9 is mainly found at the plasma membrane but also in rare dim intracellular compartments. No clear co-localization of the two proteins was observed at steady-state (Fig. 1e). Thus, following the dynamics of CD63 and CD9 localization in the cell during their transport is required to understand biogenesis of the double-positive EVs.

**CD63 and CD9 traffic transiently through late endosomes and the PM.** The RUSH system was used to follow CD9 and CD63 synchronously by live imaging from the ER to their residency compartment (Fig. 2a). The principle of this assay is to reversibly retain a protein of interest in a donor compartment, like the ER, and to monitor its release by live imaging. To this end, the protein of interest is fused to a fluorescent moiety and to the streptavidin-binding peptide (SBP), and co-expressed with another protein localized in the donor compartment, fused to streptavidin and used as a hook (here: Streptavidin-KDEL for retention in the ER). Upon synthesis, the SBP-fluorescent protein is retained in the ER by its interaction with streptavidin-KDEL. Biotin is then added to release the protein of interest. Biotin binds to streptavidin and induces the release of the SBP-fused protein that can then follow its normal trafficking route. The fluorescent protein and the SBP were inserted in the small extracellular loop of CD63 and CD9 (Fig. 2a), following a strategy previously used to follow trafficking of multivesicular bodies (MVBs) and their fusion with the PM. Indeed, the CD63-pHluorin construct used in this previous study behaved similarly to the endogenous CD63 in terms of steady-state intracellular localization and release in EVs, thus showing that this particular site of insertion of the fluorescent protein did not create artefacts for further study of its trafficking.

The trafficking of the two tetraspanins was quantified and compared in HeLa transiently co-transfected with the RUSH plasmids coding for CD63-mCherry and CD9-eGFP (Fig. 2b). Between 15 and 40 min after leaving the ER, both enter and leave the Golgi with similar kinetics. Then they separate (evidenced by a decrease of their Pearson’s co-localization coefficient) and CD63 eventually accumulates in intracellular compartments while CD9 reaches the cell periphery. However, between 30 min and 1 h after biotin addition, after leaving the Golgi, a fraction of both proteins reaches the cell periphery. A decrease of their Pearson’s coefficient was co-localization of the two proteins was observed at steady-state, contrary to CD63-WT, CD63-YA was located mainly at the cell periphery and in a few small intracellular compartments (Fig. 3b). Immunolabeling with antibodies against EEA1, Rab5, Rab7, and Lamp1, which label endosomes of the different stages of the endocytosis pathway (Supplementary Fig. 3a), showed a similar trend of colocalization of CD63-YA and CD63-WT with all markers, but to a lower overall level for CD63-YA, due to its detection at regions of the PM. In addition, CD63-YA was clearly less co-localized than CD63-WT with Lamp1 (−62%), and with Rab5 (−72%), whereas colocalization with EEA1 and Rab7 was less decreased (−35% and −38%, respectively). This observation suggests that, when inside the cell, and among the markers we tested, CD63-YA is more localized in EEA1- and in Rab7-positive early and late endosomes than other compartments (Supplementary Fig. 3a). CD63-YA arrival and exit from the Golgi showed similar kinetics as those of CD63-WT and CD9 (Supplementary Fig. 3b). CD63-YA then appeared in small intracellular compartments, rather than large ones like CD63 (Supplementary Fig. 3b). When NH$_4$Cl was added 1 h after biotin addition, only a slight increase of total fluorescence intensity was observed (Supplementary Fig. 3c), similar to the increase observed for CD9-eGFP. This confirms that CD63-YA does not go to acidic compartments as much as CD63-WT. Finally, co-transfecting HeLa with CD63-YA and CD63-WT or CD9 RUSH plasmids showed a similar trafficking for CD63-YA and CD9, with mainly peripheral localization 2 h after biotin addition (Fig. 3c).

**CD9 and CD63-YA have similar kinetics of PM localization and internalization, which differ from those of CD63.** To confirm that the different tetraspanins use the PM as transport intermediate, and quantify more precisely the extent and kinetics of such transport, we quantified PM exposure carrying out anti-GFP staining of non-permeabilized HeLa transfected with CD63-WT-eGFP, CD63-YA-eGFP, or CD9-eGFP RUSH plasmids. The relative expression of GFP at the surface (anti-GFP-AF647 signal normalized to total GFP expression in fixed cells) was then quantified by flow cytometry at different times after biotin addition and at steady state corresponding to continuous presence of biotin from the time of transfection (Fig. 4a, Supplementary Fig. 4). CD9 and CD63-YA reached a similar surface (AF647/total (GFP) fluorescence intensity ratio of 1 around 1 h after biotin addition, which increased further to around 2 at steady state. For CD63, by contrast, the maximum surface/total ratio increased but remained below 1 until 2 h post biotin addition, and decreased between 2 h and the steady-state (Fig. 4a). This kinetic quantitative analysis thus confirms the microscopy observations: a portion of CD63 is transiently localized at the plasma membrane, whereas CD63-YA and CD9 behave similarly.
Fig. 2 CD63 and CD9 transiently co-localize in multivesicular bodies and at the plasma membrane. a Principle of the RUSH system used to follow CD63 and CD9 intracellular trafficking. SBP streptavidin binding peptide, strept streptavidin, ER endoplasmic reticulum. b Micrographs and quantifications of live imaging of HeLa cells co-transfected with the CD63-mCherry and CD9-eGFP RUSH plasmids. Biotin at 40 μM was added at $T = 0$. White arrows show peripheral compartments where CD63 and CD9 co-localize. Z-projection of 11 planes. Scale bar: 5 μm. Quantification upon time of three independent experiments showing the mean ± SD eGFP and mCherry fluorescence intensity in the Golgi and in large compartments, the mean ± SD number of eGFP- or mCherry-positive small compartments and the median and range of the Pearson’s co-localization coefficient between eGFP and mCherry where automatically quantified. $N = 3$ independent experiments. 5 fields per experiments where imaged, for a total of at least 10 individual cells to analyze per experiment. c Representative electron microscopy images of HeLa cells co-transfected with RUSH constructs of CD63-mCherry and CD9-eGFP, 1h or 2 h after incubation with biotin, or at steady-state, labeled with anti-eGFP gold 10 nm (red arrows) and anti-mCherry gold 15 nm (blue arrows). Relative labeling index (RLI) in each compartment quantified from 7 different fields per replicate is represented as mean ($n = 2$ independent biological replicates). d Confocal microscopy pictures of HeLa cells (z-projection) co-transfected with CD63-mCherry- and CD9-eGFP-RUSH plasmids, and stained with anti-Rab7 after 1 h of incubation with biotin. Scale bar: 10 μm. Mander’s coefficients representing the % of CD9+ /CD63-, CD9-/CD63+, and CD9+ /CD63+ intracellular compartments also positive for the Rab7 signal in each cell are shown. Results from two independent experiments are shown, each dot represents one cell (23 cells from replicate 1, and 24 cells from replicate 2) and the median is represented. Ordinary one-way ANOVA with a Tukey’s multiple comparisons test was performed to compare the different categories of intracellular compartments shown on the graph.
and accumulate at the plasma membrane. These different behaviors could be due to different rates of internalization from the plasma membrane. To quantify this internalization, we performed an antibody uptake assay: 2 h after biotin addition, cells were incubated with anti-GFP-AF647 antibodies to label RUSH-tetraspanins at the surface, and incubated at 37 °C for 1 h. Cells were then treated by trypsin to remove antibodies still present at the cell surface (=stripping, Fig. 4b) before quantitative analysis by flow cytometry. This treatment efficiently removed antibodies remaining at the cell surface, since the signal was reduced by 77% ± 2.8 for CD63-eGFP, 87% ± 0.4 for CD63-YA-eGFP and 94.9% ± 0.8 for CD9 in cells incubated at 4 °C. While around 80% of CD63 is internalized (i.e., the AF647 signal is reduced by 20% in the stripped condition), only 30% of CD9 and 40% of CD63-YA are internalized (Fig. 4b). Collectively, the live tracking and cell surface arrival experiments show that CD63-YA behaves mostly like CD9: it accumulates at the cell surface, but a minor portion also undergoes re-internalization, whereas the WT CD63 is quickly and massively re-internalized, as previously shown13.

EVs released by HeLa contain more CD9 and CD63-YA than CD63. We then asked whether altering subcellular localization of the same molecule, CD63, affects its release in EVs. After 24 h of treatment with biotin, the protein synthesized from the transfected RUSH CD63-YA-mCherry was efficiently released in small EVs (200 K pellet, Fig. 5a). Interestingly, more mCherry signal was detected in EVs from CD63-YA than CD63-WT-transfected cells. This difference was even more striking when EVs released during 24 h with biotin were recovered by anti-GFP

---

**Fig. 3** The mutant CD63-YA displays different trafficking from CD63-WT and similar trafficking as CD9. **a** Scheme of the structure and C-terminal sequences of CD63-WT and the mutant CD63-YA. **b** Immunofluorescence of HeLa cells transfected with the RUSH CD63-eGFP or CD63-YA-eGFP plasmids at steady state. Scale bar 10 μm. This experiment was performed once. **c** Micrographs of HeLa cells co-transfected with CD63-YA-eGFP and CD63-WT-mCherry or CD9-eGFP and CD63-YA-mCherry, and median ± range of the Pearson’s co-localization coefficient over time between eGFP and mCherry. Biotin was added at T = 0. Z-stacks of 11 planes. Scale bar 5 μm. CD63/CD63-YA: 3 independent experiments n = 51 cells, CD9/CD63-YA: 2 independent experiments n = 33 cells. 5 fields per experiment where imaged, for a total of at least 10 individual cells to analyze per experiment.
Immuno-precipitation from CM of HeLa cells transfected with CD9-, CD63-WT-, or CD63-YA-eGFP RUSH constructs (Fig. 5b). While similar percent of cells expressed GFP upon transfection with the 3 RUSH constructs (Supplementary Fig. 5a), and similar numbers of EVs of similar sizes were released by the RUSH constructs-transfected cells (Supplementary Fig. 5b, c), between 2 and 4 times more GFP signal was detected per particle for both CD63-YA and CD9 than for CD63-WT (Fig. 5b).

BafilomycinA1 (BafA1) is a vacuolar ATPase inhibitor which inhibits the acidification of late endosomes and has been
Fig. 5 CD63-WT is less efficiently released in EVs than CD9 and CD63-YA. a Western blot of the cell lysate (CL) and of the different EV pellets obtained by differential ultracentrifugation of CCM from HeLa cells transfected with the RUSH plasmids CD63-WT-eGFP or CD63-YA-mCherry (24 h release with biotin). EVs from 20 × 10⁶ cells and CL from 0.2 × 10⁶ cells were loaded. The intensity of the band corresponding to the mCherry fusion proteins was quantified and normalized by the intensity of the CD9 band in 3 independent experiments, the mean ± SD is represented. Two-tailed paired t test.

b Representative Western blot of the pull-down (PD) and flow-through (FT) of the immunoprecipitation of EVs from HeLa cells transfected with the RUSH plasmids CD63-WT-eGFP, CD63-YA-eGFP, or CD9-eGFP, recovered 24 h after biotin addition. 60 × 10⁸ total particles quantified by NTA were used for each IP. Percent of GFP + cells quantified by flow cytometry were similar in the three conditions (Supplementary Fig. 5a). The GFP bands intensity in the PD normalized to endogenous CD9 in the corresponding PD are represented as mean ± SD for 3 independent experiments. Ordinary one-way ANOVA, Tukey’s multiple comparisons test.

c Representative Western blot of EVs (200 K pellets) from HeLa cells transfected with the CD63-WT, CD63-YA, or CD9-eGFP RUSH plasmids treated with DMSO or BafA1 100 nM during 16 h. The same number of EVs between the DMSO and the BafA1 conditions were loaded on the gel (around 100 × 10⁸ particles). The fold change between DMSO and BafA1 treatment for each construct is represented as mean ± SD for 3 independent experiments. Two-tailed one sample t test to compare each condition with a theoretical mean of 1.

d Proportion of cellular endogenous CD9 and CD63 released in EVs, as semi-quantified on Western blots. The signal for CD9 and CD63 in 200 K pellets released by 20 × 10⁶ HeLa cells was divided by the signal for the same molecule in the total lysate of 0.2 × 10⁶ cells, run on the same blot. 1 representative Western blot and quantification (mean ± SD) of 3 independent experiments. Two-tailed paired t test.
described to stimulate the secretion of CD63 + EVs and we confirmed that CD63-eGFP release in EVs was strongly increased by BafA1 (Fig. 5c). In contrast, only minor increases in CD63-YA-eGFP or CD9-eGFP content of EVs were observed upon BafA1 treatment (Fig. 5c), while the total amount of released EVs was increased for all cells (Supplementary Fig. 5d). These results show that CD63 can be efficiently released in small EVs when its location is stabilized at the plasma membrane through mutation of its endocytosis signal, and confirm that different mechanisms are responsible for the release of CD63 in EVs depending on its localization in endosomes or at the PM. They also suggest more abundant release of CD9 and the PM-localized mutant CD63 in EVs than the MVB-enriched WT CD63. Consistent with this hypothesis, in the non-transfected HeLa cells, although both CD9 and CD63 are detected in EV pellets, the ratio of total signal in pellets versus whole cell lysates was always lower for CD63 than for CD9, also suggesting a lower efficiency of release of CD63-bearing EVs (Fig. 5d). Collectively, these results suggest that in HeLa cells, sEVs are more likely to be secreted from the PM than from MVB under steady-state conditions.

Quantitative mass spectrometry analysis of synchronously secreted CD63 or CD9 EVs reveals signatures of plasma membrane- and endosome-derived EVs. To better determine the origin of CD63 + and CD9 + EVs and to identify specific markers of EVs released from PM or from endosomes, we performed a quantitative mass spectrometry analysis of GFP + EVs recovered by immunoprecipitation from HeLa transfected with the CD63-WT-eGFP or CD9-eGFP RUSH plasmids, incubated during 3 h or 24 h with biotin. The 3 h time point was chosen to analyze freshly released EVs, while allowing sufficient recovery of EV proteins for proteomic analysis. The 24 h time point matched a classical timing used to prepare conditioned medium for EV isolation. At 3 h after biotin addition, CD63-mCherry and CD9-eGFP displayed similar low colocalization level, quantified on fixed cells, as at 2 h, and slightly higher than at 24 h (Supplementary Fig. 5e).

The RUSH system had several unique advantages in this approach. First, the analysis of EVs after short-term trafficking of the RUSH constructs enabled to study a majority of freshly secreted EVs avoiding too many cycles of re-uptake and recycling. Second, use of the same anti-eGFP antibody to isolate both RUSH EVs secreted EVs avoiding too many cycles of re-uptake and recycling. The RUSH constructs enabled to study a majority of freshly released EVs, while allowing sufficient recovery of EV proteins for proteomic analysis. The 24 h time point matched a classical timing used to prepare conditioned medium for EV isolation. At 3 h after biotin addition, CD63-mCherry and CD9-eGFP displayed similar low colocalization level, quantified on fixed cells, as at 2 h, and slightly higher than at 24 h (Supplementary Fig. 5e).

A total of 333 and 397 proteins specifically isolated by the anti-GFP IP were quantitatively compared between CD63-eGFP and/or CD9-eGFP EVs at, respectively, 3 h and 24 h (Supplementary dataset 1, Supplementary dataset 2). They were categorized as enriched (over two-fold) or unique in CD63- or CD9-bearing EVs, versus common to CD63- and CD9-bearing EVs (Fig. 6a, see Materials and methods for detailed criteria).

To try to determine the subcellular origin of these EVs, we compared our protein lists with a reference database of >5000 proteins specifically assigned to HeLa subcellular compartments using the FunRich software. For each category of proteins (enriched in CD63-EVs, enriched in CD9-EVs or common to CD63- and CD9-EVs), we calculated the percent assigned to the different intracellular organelles and whether this percent was significantly different from that of the reference database of intracellular proteome. At 3 h (Fig. 6b, left), the CD63/CD9 common category was significantly enriched for PM proteins (14.8% versus 9.7% in the total proteome), whereas this enrichment was not observed for either CD63- or CD9-EV-specific proteins (7 and 5%, respectively). A significant enrichment was observed for lysosomal proteins in both CD63-EVs and in the CD63/CD9 common category (7 and 5.7% versus 1.7% in the total proteome). Interestingly, transmembrane lysosomal proteins (LAMP1, LAMP2, PLD3) were only found in CD63-EVs, whereas only soluble lysosomal enzymes (CTSV/D/C and TPP1) were enriched in the CD63/CD9 common category (Table 1). This suggests that a specific sorting of transmembrane lysosomal cargoes into EVs by CD63 may occur, without involvement of CD9, whereas the CD63- and/or CD9-EVs trafficking into lysosomes would capture on their surface some luminal proteins. At 24 h (Fig. 6b, right), we observed in CD9-EVs a much stronger and significant enrichment of PM components (28.3%), than in the common CD9/CD63 category or the CD63-EVs (respectively, 10% and 2%). Lysosomal protein enrichment decreased slightly but was still significantly higher than that in the total proteome, in both the CD63-EVs and the CD63/CD9-common category, and LAMP1 was enriched in the latter (Fig. 6c, Table 1). Thus in 24 h conditioned media, a time routinely used to recover EVs, CD9-bear EVs come from the PM, whereas CD63/CD9-EVs come from both PM and lysosomes.

We then searched for transmembrane (TM) proteins, using both the GO term analysis and manual annotation, which could associate specifically to one or the other EV type and could be used to label or isolate them by antibodies recognizing their extracellular domains. Only 6–14 TM proteins were present in the CD9-EVs (respectively 3–24 h), 8–8 in the common CD63/CD9 category, and 18–16 in the CD63-EVs (red text in Supplementary dataset 2). Among these, proteins assigned to lysosomes, endosomes (considering all stages of the endocytic pathway: early, recycling, and late endosomes including MVs) or the PM are shown in Fig. 6c, Table 1. Only PM proteins were enriched in CD9-EVs, whereas both endosome/lysosome and PM transmembrane proteins were enriched in CD63-EVs.

We then searched for transmembrane (TM) proteins, using the GO term analysis and manual annotation, which could associate specifically to one or the other EV type and could be used to label or isolate them by antibodies recognizing their extracellular domains. Only 6–14 TM proteins were present in the CD9-EVs (respectively 3–24 h), 8–8 in the common CD63/CD9 category, and 18–16 in the CD63-EVs (red text in Supplementary dataset 2). Among these, proteins assigned to lysosomes, endosomes (considering all stages of the endocytic pathway: early, recycling, and late endosomes including MVs) or the PM are shown in Fig. 6c, Table 1. Only PM proteins were enriched in CD9-EVs, whereas both endosome/lysosome and PM transmembrane proteins were enriched in CD63-EVs.

BafA1 and GW4869 drugs affect differently secretion of CD63 and LAMP1, versus CD9, BSG, and SLC3A2. We selected for further analysis: LAMP1 (or CD107a) for its known lysosomal steady-state localization and its enrichment in CD63-EVs and/or in the common CD63/CD9 category, basigin (BSG, a plasma membrane protein also called EMPRINN or CD147) for its different association to CD63 at 3 h and CD9 at 24 h, and the PM 4F2 cell surface antigen heavy chain (SLC3A2 or CD98), for its common presence in CD63- and CD9-EVs at 3 h and its enrichment in CD9-EVs at 24 h (Fig. 6a).

We first asked how these markers behaved, in terms of enrichment in the 200 K pellets versus cell lysates of non-transfected HeLa cells (Fig. 7a). As observed before (Fig. 4d), CD63 was less enriched than CD9 in the 200 K pellets recovered from 24 h CM. LAMP1 was even less enriched than CD63, whereas both BSG and SLC3A2 were as enriched as CD9, in accordance with their preferential association with CD9-EVs in the proteomic analysis.

We then studied the effects of two drugs used in the literature to disturb exosome secretion (BafA1 and the inhibitor of neutral sphingomyelinase GW4869 (30)) on the secretion of our selected proteins, other sEV markers (syntenin-1/SDCBP and CD81), and negative controls (AChE and Calnexin). A 16 h treatment of cells during the medium conditioning period was chosen, as it did not induce loss of cell viability (Fig. 7b). GW4869 treatment decreased in a variable manner the total number of released particles in the 200 K pellet, while it increased the

NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-021-24384-2
ARTICLE

(2021)12:4389 | https://doi.org/10.1038/s41467-021-24384-2 | www.nature.com/naturecommunications
**Fig. 6** Quantitative proteomic analysis of CD63-eGFP-RUSH and CD9-eGFP-RUSH EVs. EVs were isolated by anti-GFP immuno-isolation from either non-transfected HeLa cells, or HeLa transfected with CD63-eGFP-RUSH or CD9-eGFP-RUSH, either 3 h or 24 h after biotin addition, and their composition was analyzed by mass-spectrometry. **a** Volcano plots representing quantified proteins with at least 2 peptides in 2 replicates in at least one condition. Shown are the fold changes of peptide abundancy between CD63- and CD9-eGFP expressing EV samples and the $p$-value of this quantification, for EVs recovered 3 h (left) or 24 h (right) after biotin addition. Position of the membrane-associated proteins selected for further analysis is indicated. **b** Results of the FunRich gene enrichment analysis among the proteins either enriched in the CD63- (blue), or CD9-eGFP (red) samples, or common between the CD63 and CD9-eGFP samples (purple) at 3 h or 24 h after biotin addition. For each subcellular compartment protein list, % of proteins of this category in the list of CD63-, CD9-, or common CD63/CD9 proteins is indicated, and the $p$-value of this percentage being different to its counterpart in the whole HeLa cell database is calculated. $P$-value (hypergeometric uncorrected). **c** Schematic representation of the transmembrane proteins identified in the CD63-, CD9-, or CD63/CD9-eGFP EVs at 3 h and 24 h.
Table 1: Names of proteins identified in EVs and used for the endosome, lysosome, and PM (plasma membrane) categories are listed.

| 3 h | 24 h |
|-----|-----|
| **CD63 enriched** | **CD63 enriched** |
| **CD9 enriched** | **CD9 enriched** | **CD63 enriched** | **CD9 enriched** |
| LAMP1* | GBA | CTSV | DNASE2 | HEXB |
| RNASET2* | CTSD | RNASET2 | TPPI |
| PLD3* | TPI* | CD63* | CTSC |
| CTSD | CTSC | SCPEPI | CTSD |
| DNASE2 | HEXB | LAMP2* | LAMP1* |
| LAMP2* | | | |
| **Endosomes** | **Endosomes** |
| CHMPS4B | IST1 | BLMH | CHMP1B | IST1 | CPNE8 |
| ATP6V1D | TOLLIP | VPS26A | EHD4 | BLMH |
| ATP6V1G | CPNE8 | ATP6V1D | ATP6V1G | TOLIP | CHMPS4B |
| | | | | | PRSS23 |
| | | | | | GM2A |
| | | | | | EHD1 |
| | | | | | COL5A1 |
| **Plasma membrane** | **Plasma membrane** |
| IGFBP7 | DDAH1 | JUP | TSPAN4* | BASPI | JUP |
| BSG* | DAG1* | S100A14 | PLP2* | CD9* | SLC7A5* |
| TSPAN4* | ALDOA | GNG2 | IGFBP7 |
| CD44* | BASPI | B2M | ANXA2 |
| LMNB1 | ANXA2 | BS* | BANF1 |
| TLN1 | VCP | SLC3A2* | EZR |
| | LMNA | SLC3A9I0* | S100A10 |
| | BANF1 | DDAH1 | ALDOA |
| | B2M | GNAI3 | MSN |
| CD9* | CALMI | DAG1* | LMNA |
| | SLC3A9I0* | GPC1* | VCP |
| | SLC3A2* | L1CAM* | CALMI |

*Membrane associated proteins.

Discussion

In this work, we provide evidence that sEVs bearing tetraspanins, especially CD9 and CD63 with little CD63, bud mainly from the plasma membrane, whereas others bearing CD63 with little CD9 but containing some late endosome proteins form in internal compartments and qualify as exosomes. We also identified a small set of additional transmembrane proteins that can be used to distinguish the small ectosomes from bona fide exosomes. To obtain these evidences, we followed in live cells the intracellular trafficking of the tetraspanins and identified their colocalization and segregation over time (Figs. 2–4), we quantified the release in EVs of an endocytosis-defective mutant form of CD63 which traffics like CD9 (Figs. 3–5), we performed quantitative proteomic analysis to identify proteins that are specifically released in EVs with CD9 or CD63 (Fig. 6, Table 1), and we quantified the effect on EV secretion of a drug known to increase the pH of late endosomes, BafA1, to evidence distinct behavior of endosomal versus PM EV markers (Fig. 7).

Incidentally, our study revealed that a drug commonly used as an exosome inhibitor, GW4869, which inhibits neutral sphingomyelinase and thus prevents ceramide accumulation, should be used with caution. The low or variable effect of GW4869 on release of our EV markers questions its specificity for exosomes. This drug might affect a specific subpopulation of EVs containing syntenin-1 and LAMP1, but not CD63 in HeLa. In addition, the requirement of ceramide for EV secretion largely depends as well on the cell type. For instance, in the breast carcinoma cell line SKBR3, GW4869 increased release of exosomes while it decreased the amount of small EVs.

Comparison of small EVs (containing a mixture of exosomes and small ectosomes), and larger EVs/ectosomes showed differences in their respective protein composition and oncogenic activities, but these studies did not explore the diversity within small EVs. More recently, further approaches to separate subtypes of small EVs and compare their cargos have been published. Importantly, the two most recent studies demonstrated the presence of non-EV components, called extracellular nanoparticles, and small lipidic structures called exonemes of unclear vesicular nature within the bulk of small EV preparations. Subtypes of small EVs of different sizes within the 50–150 nm range, or slightly different densities in gradients, were also reported. However, the subcellular origin of these different small EVs was not specifically evaluated, and an exosomal nature could only be speculated, based on enriched presence of molecules known to associate with endosomes in some of the recovered small EVs. Our study has the advantage of combining the use of several markers from endosomes and lysosomes or from the PM with a search for mechanisms involved...
Fig. 7 Different effect of BafA1 and GW4869 on secretion of CD63, CD9, and the novel EV markers. a Western blot showing CD9, CD63, and CD81, and the new markers LAMP1, BSG, and SLC3A2 in cell lysates (CL) and the pellets obtained from HeLa conditioned media after differential ultracentrifugation (2K, 10K, and 200K). The loaded material comes from 20 × 10^6 cells for the centrifugation pellets, and from 0.2 × 10^6 cells for the cell lysate. One representative image. For each marker, mean ± SD of the quantification is shown for 3 independent experiments. Ordinary one-way ANOVA, Tukey’s multiple comparisons test. b Viability of HeLa cells at the end of the 16 h medium conditioning period in the presence of DMSO (control) or BafA1 (100 nM) or GW4869 (10 μM) drugs, measured by trypan blue in 6 independent experiments, mean ± SD is represented. No significant difference observed with an ordinary one-way ANOVA, Tukey’s multiple comparisons test. c Nanoparticle tracking analysis (NTA) of EVs obtained by differential ultracentrifugation from equal numbers of HeLa cells treated with DMSO (control), BafA1 or GW4869 during 16 h. The particles concentration according to their size and the fold change of the total particle concentration between treated and control conditions are represented as mean ± SD of 5 (200 K) or 3 (10 K) independent experiments. Two-tailed one sample t test to compare each condition with a theoretical mean of 0. d TEM analysis (1 representative image) and size measurement (in 3 independent experiments) of EVs in 200 K pellets divided by the signal in the total lysate, run on the same blot, is shown for 3 independent experiments. Two-tailed one sample t test to compare each condition with a theoretical mean of 0.

| Concentration (particle / mL) | DMSO | BafA1 | GW4869 | GW4869 |
|-----------------------------|------|-------|--------|--------|
| 10^10                        |      |       |        |        |
| 10^9                         |      |       |        |        |
| 5×10^8                      |      |       |        |        |
| 1×10^9                      |      |       |        |        |
| 2×10^9                      |      |       |        |        |
| 3×10^9                      |      |       |        |        |
| 4×10^9                      |      |       |        |        |

The mean fold change ± SD between DMSO and BafA1 or GW4869 treatment of the bands intensity in the 200 K and 10 K pellets divided by the cell lysate is represented for 6 independent experiments. Two-tailed one sample t test to compare each condition with a theoretical mean of 0.

Fig. 7 Different effect of BafA1 and GW4869 on secretion of CD63, CD9, and the novel EV markers. a Western blot showing CD9, CD63, and CD81, and the new markers LAMP1, BSG, and SLC3A2 in cell lysates (CL) and the pellets obtained from HeLa conditioned media after differential ultracentrifugation (2K, 10K, and 200K). The loaded material comes from 20 × 10^6 cells for the centrifugation pellets, and from 0.2 × 10^6 cells for the cell lysate. One representative image. For each marker, mean ± SD of the quantification is shown for 3 independent experiments. Ordinary one-way ANOVA, Tukey’s multiple comparisons test. b Viability of HeLa cells at the end of the 16 h medium conditioning period in the presence of DMSO (control) or BafA1 (100 nM) or GW4869 (10 μM) drugs, measured by trypan blue in 6 independent experiments, mean ± SD is represented. No significant difference observed with an ordinary one-way ANOVA, Tukey’s multiple comparisons test. c Nanoparticle tracking analysis (NTA) of EVs obtained by differential ultracentrifugation from equal numbers of HeLa cells treated with DMSO (control), BafA1 or GW4869 during 16 h. The particles concentration according to their size and the fold change of the total particle concentration between treated and control conditions are represented as mean ± SD of 5 (200 K) or 3 (10 K) independent experiments. Two-tailed one sample t test to compare each condition with a theoretical mean of 0. d TEM analysis (1 representative image) and size measurement (in 3 independent experiments) of EVs in 200 K pellets divided by the signal in the total lysate, run on the same blot, is shown for 3 independent experiments. Two-tailed one sample t test to compare each condition with a theoretical mean of 0.

| Concentration (particle / mL) | DMSO | BafA1 | GW4869 | GW4869 |
|-----------------------------|------|-------|--------|--------|
| 10^10                        |      |       |        |        |
| 10^9                         |      |       |        |        |
| 5×10^8                      |      |       |        |        |
| 1×10^9                      |      |       |        |        |
| 2×10^9                      |      |       |        |        |
| 3×10^9                      |      |       |        |        |
| 4×10^9                      |      |       |        |        |

The mean fold change ± SD between DMSO and BafA1 or GW4869 treatment of the bands intensity in the 200 K and 10 K pellets divided by the cell lysate is represented for 6 independent experiments. Two-tailed one sample t test to compare each condition with a theoretical mean of 0.
in their secretion by chemical inhibition, to demonstrate the existence of different subpopulations of small EVs, to characterize their protein content and their subcellular origin.

Our results demonstrate that HeLa cells release more small ectosomes than bona fide exosomes. An interesting tool that allowed us to demonstrate this proposal is the mutant CD63 molecule devoid of its lysosome-addressing signal, CD63-YA. CD63-YA accumulates at the PM, like CD9, and is more efficiently released in EVs by HeLa cells than the WT CD63 (Fig. 5). The eGFP-RUSH constructs of CD63 and CD63-YA could now be used as tools to quantify relative secretion of exosomes versus ectosomes in any cell, by transient transfection followed by quantification of the GFP signal recovered from each construct in the conditioned medium. It would be interesting to analyze this way cells secreting at steady-state more CD63 in EVs than HeLa, and determine whether they indeed display a high ability to secrete bona fide exosomes, or whether instead their CD63-EVs mainly form at the PM.

Our results also led us to propose to use a restricted combination of molecules (CD63, LAMP1, syntenin-1, CD9, CD81, BSG, and SLC3A2) to determine the proportion of ectosomes versus exosomes in a given small EV preparation. Concerning LAMP1, which behaves like CD63 in response to endosomal pH neutralization, its presence on EVs suggests that exosomes can be released from secretory lysosomes as well as from late endosomes. Concerning the PM-EV markers: SLC3A2 is the heavy chain of various heterodimeric amino-acid transporters, and one of its possible partners, Large amino-acid transporter LAT-1 (SLC7A5 also named 4F2 light chain)\textsuperscript{26}, was also identified in the proteomic analysis, but was found as a common protein between

Fig. 8 Trafficking of RUSH-CD81 compared to RUSH-CD63 and -CD9. Micrographs of live imaging of HeLa cells co-transfected with either CD63-mCherry or CD9-mCherry and CD81-eGFP RUSH plasmids. Biotin was added at $T = 0$. The median ± range of the Pearson’s co-localization coefficient between eGFP and mCherry is represented over time after biotin addition. Scale bar: 10 μm. 2 independent experiments. 5 fields per experiment where imaged, for a total of at least 10 cells to analyze per experiment.
CD63 and CD9 EVs at 24 h. BSG and SLC3A2 directly interact together27, and these two proteins are part of a protein complex interacting with integrins28. BSG also interacts with CD4429. Interestingly, BSG and CD44 were specifically present in CD63-bearing EVs at an early time point of secretion, whereas BSG and SLC3A2 were specific to CD9-EVs at a later time point (Fig. 6c). Thus, BSG is probably released with different types of EVs, depending on its interacting partner. Of note, SLC3A2 is highly expressed in several cancers30, and BSG has already been described as a cancer cell exosome marker21,31, which makes these two proteins good candidates as tumor cell EVs biomarkers.

In addition, a few other markers identified by the proteomic analysis could be in the future also used as either exosome or exosome-specific markers (Fig. 6). This includes for instance LAMP2, PLD3, PLP2 or the tetratraspan TSPAN4 as exosome markers, and LICAM, DAG1, CD99, or DSG3 as exosome markers. Another interesting exosome marker could be PTGFRN, a known major partner of CD9 and CD81, which has been shown to remain associated with these tetratraspans on EVs secreted by K562 cells.32 It was identified by 18 antibodies with CD9-EVs at 24 h (Supplementary dataset 2), but could not be reliably compared to CD63-EVs because a single common peptide was present in the two samples. BaFa1 treatment of cells in combination with quantitative assessment of the resulting EV level of these EV-associated proteins would have to be performed to validate their proposed specificity. Of course, although the action of BaFa1 on EVs seems exosome-specific compared to ectosomes in HeLa according to our observations, this should be further demonstrated in other cell types, by similarly assessing an array of EV-associated proteins. In particular, BaFa1 can affect also Golgi trafficking33 if prominent over the late endosome-specific effect in some cells, this action of BaFa1 would be expected to affect more generally secretion and trafficking of most membrane proteins, hence the global composition of EVs. Finally, it would be interesting to use other drugs, recently tested in a small screen specifically designed to quantify CD63-EV release15, to quantify their effect on EV release of the exosome and exosome markers, and of the mutant CD63-YA reporter.

Our findings and our methodology to discriminate different sEVs subpopulations will be a basis for further studies on the heterogeneity of EVs and their specific functional properties.

**Methods**

**Cell culture and transfections.** HeLa cells (obtained from Dr. B. Goud in 1995, and in-house validated by short tandem repeat [STR] analysis in 2018) were cultured in Dulbecco’s modified Eagle’s medium (DMEM-Glutamax, Gibco), with 10% fetal calf serum (FCS, Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco).

HeLa KO for CD63 or CD9 were generated by CRISPR/Cas9 using sgRNA (sequences provided in Supplementary Table 1) selected using the CRISPR design tool available at the Broad Institute (https://portals.broadinstitute.org/ggpp/public/analysis-tools/sgrna-design). The corresponding guide DNA sequences were cloned into the lentiviral CRISPRv2 plasmid (Addgene #52861) according to the instructions of the Zhang laboratory. (https://www.addgene.org/25986). The plasmids were transfected using Fugene HD according to the manufacturer’s instructions, and cells were treated after 36–48 h with 5 μg/mL puromycin for 36–48 h. Cells were stained for CD63 or CD9 and negative cells for the antigen of interest were sorted using a FACS Aria cell sorter (Becton Dickinson). CD63 KO cells were subcloned after two sorting rounds and one clone was kept for experiments. CD9 KO cells were subcloned after two sorting rounds and a pool of 5 clones was used for experiments. CD9 KO cells generated by E. Rubinstein and the CD9 KO clone isolated by R. Pamluli are available upon request to, respectively, C. Théry and G. van Niel (guillaume.van-niel@inserm.fr).

Transient plasmids transfections were performed on 80% confluent cells using the calcium phosphate method: 2.5 μg/mL of plasmids were mixed 1/20 vol/vol with 1 mM Tris pH 8 and 2.5 M CaCl2, and incubated 5 min at room temperature (RT) before being mixed with an equal volume of HEBS (160 mM NaCl, 1.5 mM Na2HPO4, 50 mM Hepes, pH 7.03). This mix was added at 1/10 vol/vol into culture medium and cells were incubated during 8 h before washing with PBS and replacing with fresh culture media for an additional 16 h before performing experiments.

**Antibodies and reagents**

**Primary antibodies for Western blot.** Primary antibodies for Western blot were mouse anti-human CD63 (BD Bioscience, clone H565, 1/250), mouse anti-human CD9 (Millipore, clone MM2/57, 1/50), -GFP (Invitrogen, clone GF28R, 1/1000), -human CD147/BSG (Proteintech, clone 1G12B5, 1/1000), -human CD98/SLC3A2 (Proteintech, clone 2B10F5, 1/500), -human actin (Millipore, clone C4, 1/500), -human CD81 (Diaclone, clone TS81, 1/1800), rabbit anti-human LAMP1 (Biovision, clone EPR4204, 1/2000), -mCherry (Biovision, polyclonal, 1/1000), -human calnexin (Abcam, recombinant clone EPR3633(2), 1/1000), goat-anti human acetylcholinesterase (Abcam, polyclonal, ab 31276, 1/500), monoclonal rabbit anti-human syntaxin-1 (1/1000) was a gift from P. Zimmermann (pascale.zimmermann@kuleuven.be).

**Secondary antibodies.** Secondary antibodies HRP-conjugated goat anti-rabbit IgG (H + L) and HRP conjugated goat anti-mouse IgG (H + L) were purchased from Jackson Immuno-Research and used 1/10000.

**Antibodies for immunoprecipitation.** Antibodies for immunoprecipitation were mouse anti-human CD63 (clone TS63, 1/100, available upon request to E. Rubinstein: eric.rubinstein@inserm.fr) and mouse IgG1 anti-human CD9 (clone TS90 1/1000) (commercially available at Diaclone or Abcam), mouse IgG1 anti-human EEA1 (BD Transduction Laboratories, clone 14/EEA1, 1/1000), mouse IgG2a anti-human Rab5B (BD Transduction Laboratories, clone 1/Rab5B, 1/100), rabbit anti-human Rab87 (Cell Signaling, D9F2, 1/100), mouse IgG1 anti-human LAMP1 (Developmental Studies Hybridoma Bank, HH43, 1/400), goat anti-mouse IgG2b Alexafluor 647 (Invitrogen, 1/300), goat anti-mouse IgG Alexafluor 488 (Invitrogen 1/300), goat anti-mouse IgG Alexafluor 568 (Invitrogen, 1/200), goat anti-rabbit IgG Alexafluor 647 (Invitrogen, 1/200).

**Antibodies for immunofluorescence.** Antibodies for immunofluorescence were mouse IgG2b anti-human CD63 (clone TS63, 1/100, available upon request to E. Rubinstein: eric.rubinstein@inserm.fr) and mouse IgG1 anti-human CD9 (clone TS90 1/1000) (commercially available at Diaclone or Abcam), mouse IgG1 anti-human EEA1 (BD Transduction Laboratories, clone 14/EEA1, 1/1000), mouse IgG2a anti-human Rab5B (BD Transduction Laboratories, clone 1/Rab5B, 1/100), rabbit anti-human Rab87 (Cell Signaling, D9F2, 1/100), mouse IgG1 anti-human LAMP1 (Developmental Studies Hybridoma Bank, HH43, 1/400), goat anti-mouse IgG2b Alexafluor 647 (Invitrogen, 1/300), goat anti-mouse IgG Alexafluor 488 (Invitrogen 1/300), goat anti-mouse IgG Alexafluor 568 (Invitrogen, 1/200), goat anti-rabbit IgG Alexafluor 647 (Invitrogen, 1/200).

**Plasmids.** A RUSH vector encoding for the KDEL-Streptavidin Hook (available upon request to F. Perez: franck.perez@curie.fr) was used for all CD63, CD9, and CD81 reporters. A CD63-SBP-pHluorin RUSH plasmid was initially generated by inserting the SBP sequence upstream of the pHluorin fragment in the CD63-pHluorin sequence described in ref. 12 (generated by F. J. Verweij and G. Boncompain and available upon request to G. Van Niel: guillaume.van-niel@inserm.fr). The CD63-SBP-pHluorin fragment was synthesized and ligated in a Str-KDEL Neomycin vector after Ascl/PacI digest, thereby replacing the neomycin cassette with CD63-SBP-pHluorin. The pHluorin sequence was then replaced by the eGFP or the mCherry (fragments taken from plasmids described in ref. 8) sequence (synthesized by Integrated DNA Technologies) to generate RUSH plasmids allowing the expression of human CD63 with the SBP and eGFP or mCherry in the small luminal loop. The RUSH CD9-eGFP and RUSH CD81-eGFP plasmids were obtained from this plasmid, replacing the CD63 sequence by a synthetic sequence of human CD9 or human CD81 containing restriction sites allowing the later cloning of the SBP-eGFP or mCherry sequence from the RUSH-CD63 plasmids and a Myr-Palm terminal sequence provided in Supplementary Table 1, restriction sites in bold, linker in italics) (gBlocks Gene Fragments purchased from Integrated DNA Technologies). The RUSH CD63-YA-mCherry plasmid was obtained by performing directed mutagenesis on the RUSH CD63-mCherry plasmid, using CD63-YA forward and reverse primers (Supplementary Table 1). The parental plasmid was digested with the DpnI enzyme (NEB). The mCherry was then replaced by eGFP to generate the RUSH CD63-YA-eGFP plasmid. The Myr-Palm-mCherry plasmid (available upon request to F. Perez) was generated by fusing the myristoylation–palmitoylation sequence and a linker in N-term of the mCherry sequence. Primers and original sequences used for cloning are indicated in Supplementary Table 1. RUSH plasmids encoding WT and mutant CD9, CD63, CD81 are available upon request to C. Théry.

**EV isolation by differential ultracentrifugation.** FCS-EV depleted medium was first prepared by centrifuging DMEM with 20% FCS at 200K×g overnight with a
45Ti rotor (Beckman Coulter). Supernatant was then recovered by pipetting without taking the last 5 mL to avoid disturbing the pellet, and filtered through a 0.22 μm filter (Millipore). Culture medium (less than 80%) 150 cm dishes was changed, after a PBS wash, for FCS-DEP deleted media diluted with DMEM to 10% FCS. Twenty-four or 16 h later, medium was collected for EV isolation and cells were trypsinned and counted. (generally around 25 × 106 cells/dish). Three consecutive centrifugations of 20 min at 300 g at 4 °C were performed to remove any floating cells. The supernatant of the supernatant at 2Kg, 10Kg, and 200Kg at 4 °C were centrifuged. 2Kg centrifugation was performed during 20 ml, the washings with PBS and re-centrifuged for 20 min at 2Kg in an Eppendorf Centrifuge 5810. 10Kg and 200Kg were performed in a Beckman Coulter centrifuge for 40 min and 2 h, respectively. Pellets were resuspended in 6 mL PBS for a wash and centrifuged again at the same speed in a MLA80 rotor (Beckman Coulter) during 20 min and 1 h, respectively. The pellets were resuspended in 1 μL PBS. 1 × 10^6 secreting cells.

**Immunofluorescence on cells.** Cells were seeded on 12 mm diameter coverslips. One day before confluence (untransfected cells, Fig. 1e), 24 h after transfection with the RUSH-CD63-mCherry and -CD9-EGFP and 1 h of incubation with biotin (Fig. 2d), or 24 h after transfection with the RUSH-CD63-WT-EGFP and -CD63-YA-EGFP constructs in the presence of biotin (Supplementary Fig. 3a), they were fixed with 4% paraformaldehyde (PFA) (EMS) during 15 min at RT. Primary and secondary antibodies were successively incubated during 1 h each at RT in PBS containing 0.05% saponin and 0.2% BSA, except for anti-Rab7: cells were permeabilized in 0.3% Triton X-100 + 5% BSA (in PBS) for 15 min at RT, blocked in 5% BSA (in PBS) for 30 min at RT, primary antibody was incubated overnight at 4 °C and secondary antibody 1 h at RT, both in blocking solution. Coverslips were then mounted on slides with Fluoromount-G with DAPI (Invitrogen). Images were acquired on a Zeiss LSM 780 confocal microscope using a 63x objective with 1.46 size aperture, with the following acquisition parameters: frame rate 1 Hz, average per line 2, pixel size depending on the sample between 65 and 85 nm, pixel dwell 1.12 μsec, z-step 0.33 μm for stack imaging. At least 5 fields were captured to image each sample at least 10 cells per experiment (two independent experiments).

**Live imaging of RUSH constructs.** Cells were transfected 24 h before imaging and washed with PBS before adding fresh complete culture medium 6 h later. To analyze early-state distribution of the RUSH constructs, cells were transfected and cultured throughout in the presence of 40 μM biotin. For synchronized analysis of RUSH constructs, cells were transfected and cultured in complete medium until the time of biotin addition. Live imaging was performed with an Eclipse 80i microscope (Nikon) equipped with spinning disk confocal head (Yokogawa), using a 60x objective with 1.4 size aperture and a x40 UltraFluor camera (312 × 512 μm, pixel size 13 μm, Andor). 25 mm-diameter coverslips with the transfected cells were put in a L-shape tubing Chamable (Live Cell Instrument), filled with pre-warmed carbonate independent Leibovitz’s medium (Invitrogen) with 1% FCS. Medium was replaced by pre-warmed Leibovitz’ medium (Gibco) with 1% FCS and 40 μM biotin. For experiments with NH4Cl, medium was replaced by NH4Cl in the absence of biotin for medium with biotin and 50 mM NH4Cl. Movies were acquired using Metamorph software, imaging every 1 min for single color imaging, 2 min for two colors imaging, and an exposure time of 100 ms for each channel. 3 fields were imaged per replicate to image at least a total of 10 cells per replicate. For quantifications, manual image analysis was performed with Metamorph software. Each channel was used to calculate Manders’s coefficients in each individual cell. Intensity thresholds to measure Manders’s coefficients were set manually for each cell, according to the level of intensity of each channel. For colocalization between three channels in individual cells, a homemade macro was used to compute Manders’s coefficients. First, 3D masks of Rab7-, eGFP-, and mCherry-positive compartments were performed with a user-defined intensity threshold after background subtraction as previously. Then, Manders’s colocalization coefficients were calculated based on the percentage of overlapping volume for those masks.

**Western blot.** Cell lysates (CL) for Western blot were obtained by incubating cell pellets at a concentration of 1 × 10^6 cells in 25 mL of lysis buffer (50 mM Tris pH 7.5, 0.15 M NaCl, 1% Triton X-100) with 2% complete protease inhibitor (Roche) for 20 min on ice, followed by a 18,516× g centrifugation for 15 min at 4 °C to recover the supernatant. EVs from 20 × 10^6 cells (or specified number of particles) and CL from 0.2 × 10^6 cells were mixed with Laemmli sample buffer (BioRad), without reducing agent. After boiling for 5 min at 95 °C, samples were loaded on a 4–15% Mini-protean TGX stain-free gels (BioRad). Total proteins were imaged from the stain-free gels with the ChemiDoc Touch Imager (BioRad). Transfer was performed on Immuno-Blot PVDF membranes (BioRad), with the Trans-Blot Turbo Transfer System (BioRad) during 7 min. Blocking was performed during 30 min with a blocking solution of TBS 0.1% Tween for most antibodies or with 5% milk in PBS 0.1% Tween for the anti-GFP antibody. Primary antibodies were incubated overnight at 4 °C and secondary antibodies during 1 h at room temperature (RT). Development was performed using either the BM Chemiluminescence Western blotting Substrate (POD) (Roche), Clarity Western ECL Substrate (BioRad) or the Immobilon Forte Western HRP substrate (Millipore), and the ChemiDoc Touch Imagier (BioRad). Intensity of the bands was quantified using ImageJ.

**Immunostaining of EVs by anti-CD63 and -CD9 for Western blot.** Concentrated medium (CMC) was prepared using serum-free DMEM incubated with secreting cells during 24 h. This medium was centrifuged at 300 g for 20 min to remove floating cells and filter-concentrated using Sartortus Vivasan 100K (Sartorius). Culture medium (less than 100%) 150 cm dish centrifuged at 1000 g, gain of 26 ± 7% of harvest. Measurements were done at 11 different positions (2 cycles per position) and frame rate of 30 frames per second. Image evaluation was done on particles with minimum brightness: 20, minimum area: 10, maximum area: 500, maximum brightness: 255. Tracking radius was 100, and minimum trace length: 7.

**Antibodies:** Mouse anti-CD63 (TS63 Diaclone 857.770.000 1/200) in PBS-BSA 1% for 30 min, rabbit anti-CD9 (Abcam ab236630 1/80) for 30 min, 10 nm protein-A gold (CMC, Utrecht, The Netherlands) for 20 min, fixed for 5 min with 1% glutaraldehyde (Electron Microscopy Sciences), followed by mouse anti-CD63 (TS63 Diaclone 857.770.000 1/200) in PBS-BSA 1% for 30 min, rabbit anti-mouse (Sigma SAB3701080 1/1000) for 30 min, 15 nm protein-A-Gold (CMC, Utrecht, The Netherlands) for 20 min, and fixed for 5 min with 1% (w/v) glutaraldehyde/4% (v/v) paraformaldehyde (EMS) during 15 min at 4 °C. Secondary antibodies were successively incubated during 1 h each at RT in PBS containing 0.05% saponin and 0.2% BSA, except for anti-Rab7: cells were permeabilized in 0.3% Triton X-100 + 5% BSA (in PBS) for 15 min at RT, blocked in 5% BSA (in PBS) for 30 min at RT, primary antibody was incubated overnight at 4 °C and secondary antibody 1 h at RT, both in blocking solution. Coverslips were then mounted on slides with Fluoromount-G with DAPI (Invitrogen). Images were acquired on a Zeiss LSM 780 confocal microscope using a 63x objective with 1.46 size aperture, with the following acquisition parameters: frame rate 1 Hz, average per line 2, pixel size depending on the sample between 65 and 85 nm, pixel dwell 1.12 μsec, z-step 0.33 μm for stack imaging. At least 5 fields were captured to image each sample at least 10 cells per experiment (two independent experiments). Image analysis was performed with ImageJ. To focus on small compartments, a median filter of 1 pixel radius was first applied to remove noise, then a subtract background with a small rolling radius (between 10 and 50 pixels) was done to remove intensities from the large Golgi area. Then, to measure colocalization between three channels in individual cells, a homemade macro was used to compute Manders’s coefficients. First, 3D masks of Rab7-, eGFP-, and mCherry-positive compartments were performed with a user-defined intensity threshold after background subtraction as previously. Then, Manders’s colocalization coefficients were calculated based on the percentage of overlapping volume for those masks.
Electron microscopy on cells. Sample preparation, ultrathin cryosectioning, and immunolabelling were performed as already described. In brief, HeLa cells were grown on culture dishes and fixed by the addition of freshly prepared 4% PFA in 0.1 M phosphate buffer (pH 7.4) to an equal volume of culture medium for 10 min, followed by post-fixation with fresh 4% PFA overnight at 4 °C. After rinsing with PBS, the blocks were embedded in 12% gelatin, cryoprotected with 2.3 M sucrose, and frozen in liquid nitrogen. Ultrathin cryosections were cut on a Leica ultratome UC6 cryoultramicrotome and picked up in a pre-cooled 1:1 mixture of 2.3 M sucrose and 1.8% methylcellulose, thawed and collected on formvar-coated grids. After washing with PBS containing 0.02 M glycine, sections were incubated with primary antibodies and protein A-gold conjugates (PAG) (Utrecht University, The Netherlands) according to the manufacturer’s instructions. The pooled fractions were finally concentrated using 500UL. 100 kDa molecular weight cut-off concentrators (Sartorius). The percentage of transfected cells was determined by flow cytometry and the concentration of recovered EVs was measured by NTA. For 3 h samples, EVs from 20 × 10^9 GFP-positive cells were incubated with anti-GFP antibody and finally hereto with PBS 0.01% Tween overnight at 4 °C in rotation. One PD duplicate was used for Western blot and the other one for mass-spectrometry. For Western blot (Fig. 5b), the bands were then washed three times with PBS 0.01% Tween with 300 mM NaCl and three times with PBS. The flow through pooled with the first three washes was concentrated using 500UL. 100 kDa molecular weight cut-off concentrators. All the sample volume recovered from the PD and the FT was loaded on the gel. For mass-spectrometry, immuno-isolated vesicles on beads were eluted with 100 µL 80/20 MeCN/H2O + 0.1% TFA. Dry pellets were solubilized and reduced in 20 µL 8 M urea, 200 mM ammonium bicarbonate, 5 mM dithiothreitol, pH 8 with 1 h of heating at 100 °C. After cooling down room temperature, cysteines were alkylated by adding 10 M iodoacetamide for 30 min in the dark. After diluting to 1 M urea with 200 mM ammonium bicarbonate pH 8.0, samples are analyzed by LC-MS/MS. The results were normalized according to the CRAPome and enriched ions were selected as described. The FDR was calculated using FDR-corrected XICs, computed with MassChroQ version 2.2.143. XICs from proteotypic peptides were selected against Q ExactiveOrbitrap mass analyzer in ranges m/z 375–1500 with a resolution of 120,000 at m/z 200. The top 20 intense ions were subjected to Orbitrap for readout of MS/MS fragments from the high-energy collision dissociation cell (HCD), analyzed at 15,000 resolution, with the intensity threshold kept at 1.3 × 10^5. We selected ions with charge state from 2+ to 6+ for screening. Normalized collision energy (NCE) was set at 27 and the dynamic exclusion of 40 s. For identification, the data were searched against the Homo sapiens (UP000005640) or Orthologous (OrthoMcl) databases through ProteomeXchange (https://proteomeexchange.cocke. 2.4). Enzyme specificity was set to trypsin and a maximum of two-missed cleavage sites was allowed. Oxidized methionine, carbamidomethyl cysteines, and N-terminal acetylation were set as variable modifications. Maximum allowed mass deviation was set to 10 ppm for monoisotopic precursor ions and 0.02 Da for MS/MS peaks. The resulting files were further processed using myProMS41 v3.9. FDR calculation was performed using Percolator42 and was set to 1% at the peptide level for the whole study. The label-free quantification was performed by peptide Extracted Ion Chromatograms (XICs), computed with MassChroQ version 2.2.145. XICs from proteotypic peptides between compared conditions (TopN matching) with missed cleavages were used. Median and scale normalization was applied on the total signal to correct the XICs for each biological replicate (N = 4). To estimate the significance of the protein change abundance, a linear model (adjusted on peptides and biological replicates of an experimental condition and that showed a log2(fold change) > 0 in the samples containing GFP versus NT samples were selected. Proteins recovered in more than 200/411 IP analyses according to the contaminant repository for affinity purification (the CRAPathome46), hence the contaminant coefficient is isolated or identified using the background isolation method, was therefore excluded from further analysis. Proteins selected according these two criteria (log2(fold change) GFP/NT > 0 and not contaminant according to the CRAPathome) are highlighted in green in Supplementary data File 1, and those showing at least 2 peptides in at least 2 replicates of the CD9-eGFP and/or the CD63-eGFP samples were used for the quantitativeness of the quantitative data (Supplementary File 2 and File 6a). Proteins identified by at least 2 peptides identical in both CD9-eGFP and CD63-eGFP samples were considered enriched in one sample compared to the other if they showed a log2(fold change) ≥ 1 or ≤ −1 and an adjusted p-value of less than 0.05. Proteins with a log2(fold change) between −1 and 1 were considered as common to two samples. Proteins displaying peptides exclusively in CD63-eGFP or
in CD9-eGFP and with at least 2 peptides in 2 replicates were listed as unique to the corresponding sample.

GO term analysis was performed using the FunRich software\(^{27,28}\) using the HeLa Spatial Proteome database\(^{46}\). The hypergeometric uncorrected \(p\) value reflecting if there is an enrichment of proteins from one cellular component compared to the reference database, and the percentage of proteins compared to the total number of proteins in the list were calculated by the software for each cellular component.

**Statistical analyses.** Following the recommendation of D.L.Vaux\(^{45}\), for each experiment where number of biological replicates were 2 or 3, we displayed the results in a transparent manner, showing each individual biological replicate as a dot, so the readers could interpret the data for themselves. We (as suggested by D. L. Vaux) considered that same trends of results obtained independently 2–3 times were as informative as statistical tests to evaluate reproducibility of the experiments.

Nonetheless, we also performed statistical analyses with GraphPad Prism version 8.0.2 (GraphPad software, California USA), by one-way ANOVA followed by multiple comparison Tukey’s test (Figs. 2d, 4b, 5a, 7a, Supplementary Fig. 3c, Supplementary Fig. 4a,b,c,d,e), by two-tailed paired t-test (Fig. 5a,d), two-tailed unpaired t-test (Supplementary Fig. 3a) or one-sample two-tailed t-test (Figs. 5c and 7c, e).

**Data availability**

The mass spectrometry proteomics raw data have been deposited to the ProteomeXchange Consortium via the PRIDE\(^{47}\) partner repository with the dataset identifier PXD021515. Methods and/or raw data are available in the EV-TRACK platform\(^{45}\) (EV-TRACK ID: EV210105 [http://evtrack.epfl.ch]). All images (fluorescence and electron microscopy) have been deposited in BioImage via accession numberS-BIAD130. In-house generated ImageX macros have been deposited to Zenodo with the following references: 4768762 [https://doi.org/10.5281/zenodo.4768762]. This concerns the following figures: Figs. 1a–c, 2b–d, 3c, 4a–b, 4a–d, 7a–e,8, Supplementary Figs. 1a–b, 2a–b, 3a–c, 4a–e. The remaining data are available in the Article and Supplementary Information. Source data are provided with this paper.

Received: 22 September 2020; Accepted: 15 June 2021; Published online: 19 July 2021

**References**

1. Coccozza, F., Grisard, E., Martin-jaular, L., Mathieu, M. & Théry, C. SnapShot: extracellular vesicles. *Cell 182*, 262–262.e1 (2020).
2. Van Niel, G., D’Angelo, G. & Raposo, G. Shedding light on the cell biology of extracellular vesicles. *Nat. Rev. Mol. Cell Biol. 19*, 213–228 (2018).
3. Witwer, K. W. & Théry, C. Extracellular vesicles or exosomes? On primacy, precision, and popularity influencing a choice of nomenclature. *J. Extracell. Vesicles 8*, 1648167 (2019).
4. Mathieu, M., Martin-jaular, L., Lavieu, G. & Théry, C. Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. *Nat. Cell Biol. 21*, 9–17 (2019).
5. Escola, J. M. et al. Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B- lymphocytes. *J. Biol. Chem. 273*, 20121–20127 (1998).
6. Thery, C. et al. Molecular characterization of dendritic cell-derived exosomes. Selective accumulation of the heat shock protein hsc73. *J. Cell Biol. 147*, 599–610 (1999).
7. Kowal, J. et al. Proteome comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. *Proc. Natl Acad. Sci. USA 113*, E968–E977 (2016).
8. Boncompain, G. et al. Synchronization of secretory protein trafficking in CD63-containing extracellular vesicles. *PLoS One 16*, e0220807 (2019).
9. Itzhaki, D. N., Tyanova, S., Cox, J & Borner, G. H. H. Global, quantitative and dynamic mapping of protein subcellular localization. *Elife 5*, e16950 (2016).
10. Pathan, M. et al. FunRich: an open access standalone functional enrichment and interaction network analysis tool. *Proteomics 15*, 2597–2601 (2015).
11. Pathan, M. et al. A novel community driven software for functional enrichment analysis of extracellular vesicles data. *J. Extracell. Vesicles 6*, 1321455 (2017).
12. Trajkovic, K. et al. Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science 319*, 1244–1247 (2008).
13. Menck, K. et al. Neutral sphingomyelinases control extracellular vesicles budding from the plasma membrane. *J. Extracell. Vesicles 6*, 1378056 (2017).
14. Menck, K. et al. Microvesicles mediate breast cancer invasion through glycosylated EMMPRIN. *J. Mol. Cell Biol. 7*, 143–153 (2015).
15. Minciacchi, V. R. et al. Large oncosomes contain distinct protein cargo and represent a separate functional class of tumor-derived extracellular vesicles. *Oncotarget 6*, 11137–11141 (2015).
16. Keerthikumar, S. et al. Proteogenomic analysis reveals exosomes are more oncogenic than exosomes. *Oncotarget 6*, 13375–13396 (2015).
17. Zhang, H. et al. Identification of distinct nanoparticules and subsets of extracellular vesicles by asymmetric flow field-flow fractionation. *Nat. Cell Biol. 20*, 332–343 (2018).
18. Jeppesen, D. K. et al. Reassessment of exosome composition. *Cell 177*, 445–445.e18 (2019).
19. Mannioni, B. A. et al. The light chain of CD9 is identified as E16/TA1 protein. *J. Biol. Chem. 273*, 33127–33129 (1998).
20. Xu, D. & Hemler, M. E. Metabolic activation-related CD147-CD98 complex. *Mol. Cell. Proteom. 4*, 1061–1071 (2005).
21. Ip, H. & Sethi, T. CD98 signals controlling tumorigenesis. *Int. J. Biochem. Cell Biol. 81*, 148–150 (2016).
22. Muramatsu, T. Basigin (CD147), a multifunctional transmembrane glycoprotein with various binding partners. *J. Biochem. 159*, 481–490 (2016).
23. Cantor, J. M. & Ginsberg, M. H. CD98 at the crossroads of adaptive immunity and cancer. *J. Cell Sci. 125*, 1373–1382 (2012).
24. Arendt, B. K., Walters, D. K., Wu, X., Tschumper, R. C. & Jelinek, D. F. Multiple myeloma cell-derived microvesicles are enriched in CD147 expression and enhance tumor cell proliferation. *Oncotarget 5*, 5686–5699 (2014).
25. Abache, T. et al. The transferrin receptor and the tetraspanin web molecules CD9, CD81, and CD99-1 are differentially sorted into exosomes after TPA treatment of K562 cells. *J. Cell Biochem. 102*, 650–664 (2007).
26. Palokangas, H., Ying, M., Väänänen, K. & Saraste, J. Retrograde transport from the pre-Golg compartment and the golgi complex is affected by the vacuolar H+–ATPase inhibitor bafilomycin A1. *Mol. Biol. Cell 9*, 3561–3578 (1998).
27. Ran, F. A. et al. Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc. 8*, 2281–2308 (2013).
28. Charrin, S. et al. Rapid isolation of rare isotype-switched hybridoma variants: application to the generation of IgG2a and exosome marker. *Antibodies 9*, E29 (2020).
29. Le Naour, F. et al. Profiling of the tetraspanin web of human colon cancer cells. *Mol. Cell. Proteom. 5*, 845–857 (2006).
30. Thery, C., Amigorena, S., Raposo, G. & Clayton, D. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr. Protoc. Cell Biol. Chapter 3, Unit 3.22* (2006).
31. Slot, J. W. & Geuze, H. J. Cryosectioning and immunolabeling, *Nat. Protoc. 2*, 2490–2491 (2007).
32. Bolte, S. & Cordelières, F. P. A guided tour into subcellular colocalization analysis in light microscopy. *J. Microsc. 224*, 213–223 (2006).
33. Mayhew, T. M. The distributions and quantifying the labelling intensities of cell compartments by immunoelectron microscopy: progress towards a coherent set of methods. *J. Anat. 219*, 647–660 (2011).
34. Pouillet, P., Carpentier, S. & Barillot, E. myProMS, a web server for management and validation of mass spectrometry-based proteomic data. *Proteomics 7*, 2553–2556 (2007).
35. The, M., MacCoss, M. J., Noble, W. S. & Käll, L. Fast and accurate protein false discovery rates on large-scale proteomics data sets with Percolator 3.0. *J. Am. Soc. Mass Spectrom. 27*, 1719–1727 (2016).
36. Vaux, D. L. Research methods: know when your numbers are significant. *Nature 492*, 180–181 (2012).
37. Perez-Riverol, Y. et al. The PRIDE database and related tools and resources in 2019: improving support for quantification data. *Nucleic Acids Res. 47*, D442–D450 (2019).
47. Van Deun, J. et al. EV-TRACK: transparent reporting and centralizing knowledge in extracellular vesicle research. Nat. Methods 14, 228–232 (2017).

Acknowledgements
We thank for fruitful discussions several team members, especially Drs Mercedes Tkach, Eleonora Grisard, Lorena Martin-Jaular, Jason Ecard, and for helpful discussions and tools, Dr Aurélien Dauphin, Institut Curie, Paris, Dr P. Zimmermann, KU Leuven, Belgium and CRCM Marseille, France, Dr G. van Niel, IPN Paris, France, Dr P Benaroch, Institut Curie, Paris, France, Dr Suresh Mathivanan, LaTrobe University, Melbourne, Australia. The H4A3 monoclonal antibody (anti-human LAMP1), developed by August, J.T. and Hildreth, J.E.K. from Johns Hopkins University School of Medicine, was obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH, and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242. This work was funded by INSERM, CNRS, Institut Curie, French IdEx and LabEx (ANR-10-INSB-04, ANR-10-IDEX-0001-02 PSL, ANR-10-LABX-0038, ANR-11-LABX-0043, ANR-18-IDEX-0001 Université de Paris), grants from french ANR (ANR-18-CE13-0017-03; ANR-18-CE15-0008-01; ANR-18-CE16-0022-02), INCa (INCA-11548), Foundation ARC (PGrA18002009062), FRM (FRD201904007945, EQU201903007925 and DGGE20121125630), Cancéropôle Ille-de-France (2013-2-EML-02-ICR-1), USA NIDA (DA040385), and a Long-Term EMBO Fellowship (ALTF 607-2015) co-funded by the European Commission FP7 (Marie Curie Actions, LTFCOFUND2013, GA-2013-609409) to J.I.V. We also acknowledge the following platforms of Institut Curie: PICT-IBiSA, member of the France-BioImaging national research infrastructure (ANR-10-INBS-04) for fluorescence and electron microscopy, genomic for authentication of HeLa cells by STR analysis.

Author contributions
M. Mathieu and C.T. designed the study, interpreted the data, and wrote the article. M. Mathieu, N.N., M.J., J.J.V., F.D., and D. Lankar performed experiments. M. Mathieu, N.N., M. Maurin, and M.J. analyzed the data. M. Mathieu, N.N., F.V., R.P., E.R., and G.B. generated plasmids or KO cells. G.B. and F.P. designed and supervised the RUSH studies. D. Loew designed and supervised the proteomic study. E.R. interpreted data. All authors read and corrected the article.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-24384-2.

Correspondence and requests for materials should be addressed to C.T.

Peer review information Nature Communications thanks the anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.