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HIGHLIGHTS
- Glioma C6 extracellular vesicle protein mapping
- Quick steps protocols to map outer/inner membrane EV proteins
- C6 glioma cell line EVs contain ghost proteins
Reference and Ghost Proteins Identification in Rat C6 Glioma Extracellular Vesicles

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SUMMARY
Extracellular vesicles (EVs) mediate intercellular communication and regulate a broad range of biological processes. Novel therapeutic strategies have emerged based on the use of EVs as biological nanoparticles. To separate isolated EVs from protein aggregates and the external part of EVs membrane proteins, we performed a Trypsin/Lys C digestion treatment of EVs pellets, followed by Amicon filtration. After these steps, all the fractions have been subjected to proteomic analyses. Comparison between 6 h Trypsin/Lys C treatment or non-treated EVs revealed a quantitative variation of the surface proteins. Some surface proteins have been demasked after 6 h enzymatic digestion like CD81, CD82, Ust, Vcan, Lamp 1, Rab43, Annexin A2, Synthetin, and VSP37b. Moreover, six ghost proteins have also been identified and one corresponds to a long noncoding RNA. We thus demonstrate the presence of ghost proteins in EVs produced by glioma cells that can contribute to tumorigenesis.

INTRODUCTION
Extracellular vesicles (EVs) were identified in 1983 (Harding et al., 1983; Pan et al., 1985; Pan and Johnstone, 1983). The authors have shown that culture of immature red blood cells—reticulocytes—labeled transferrin receptors were internalized within the reticulocytes and then were repackaged into small (~50-nm) vesicles and secreted out of the maturing blood reticulocytes into the extracellular space. The name of these vesicles “exosomes” was given by Johnstone et al. (Johnstone et al., 1989). Exosomes belong to a large family of membrane vesicles referred to as EVs, which generally include microvesicles (100–350 nm), apoptotic blebs (500–1,000 nm), and exosomes (30–150 nm) (Johnstone, 2005, 2006). The EVs are involved in many biological processes as intercellular communication messengers (Johnstone, 2005, 2006). Their pathophysiological roles have been characterized by various diseases including cancer (Reclusa et al., 2017; Ruvo et al., 2017; Zhou et al., 2017). Recently, EVs and especially exosomes have been used as therapeutic targets (Harrell et al., 2018; Jing et al., 2018; Kojima et al., 2018; Yamashita et al., 2018; Zhou et al., 2017) or nanotherapeutic agents (Murgoci et al., 2018).

Ultracentrifugation is the most common approach for EVs isolation (Thery et al., 2001; Lane et al., 2017; Mommen-Heravi, 2017). Recently, size exclusion chromatography has been introduced after ultracentrifugation (Benedikter et al., 2017) and other separation techniques have been tested, including OptiPrep density-based separation or immune affinity capture using anti-EpCAM-coated magnetic beads (Tauro et al., 2012). Validation of EVs isolation is often based on fluorescent microscopy, scanning electron microscopy, and nanoparticle tracking analysis (NanoSight) (Murgoci et al., 2018). Under an electron microscope, exosomes show characteristic cup-shaped morphology, appearing as flattened spheres with diameters ranging from 30 to 150 nm (Murgoci et al., 2018). To identify the proteins, present at the surface of EVs and the ones within EVs, we performed a proteomic approach after Trypsin/Lys C treatment and Amicon filtration. Using this procedure, we were able to identify plasmatic membranes as well as luminal proteins.

At the same time, we were interested in looking for ghost proteins present in and on the surface of vesicles. Ghost proteins or alternative proteins (AltProts) represent the invisible part of the proteome, because they are not currently identified, annotated or added to the current database using bottom-up proteomics (Deldcourt et al., 2018). In fact, mass spectrometric (MS) proteomic strategies are based on protein identification against databases of annotated proteins. However, it has been shown that a large number of proteins have not yet been referenced to these databases, in particular because of the rules used to predict the proteins.
Thus, AltProts represent proteins of lower molecular weight than reference proteins (RefProts) and are derived from regions of the RNA described as non-coding. AltProts can be translated from 5’ UTR or 3’ UTR or even by shifting on the open reading frame by +2 or +3. Proteins from these regions do not share common sequences with the RefProts produced from the same mRNA and are therefore not isoforms. Some of these AltProts are also described as encoded by non-coding (ncRNA) and long non-coding RNA. Although the role of these proteins remains largely unexplored, some studies have shown their roles in crucial cell processes (Delcourt et al., 2018; Cardon et al., 2019a, 2019b), but as of today they have not been studied in vesicles.

RESULTS
Rat C6 Glioma EVs Purification

We previously published a protocol based on the ultracentrifugation approach for EVs isolation from microglia cells (Murgoci et al., 2018). Here, we present an updated version of this strategy by including an enzymatic digestion step using Trypsin/Lys C for 6 h validated by shotgun proteomics analysis. We thus performed such an approach on vesicles derived from rat C6 glioma cells. Therefore, for C6 glioma EVs isolation, multiple centrifugation steps were used to eliminate debris and other macrovesicles, with the final ultracentrifugation steps to collect EVs pellet. Once EVs were isolated, we have continued their purification by treatment with Trypsin/Lys C. For non-treated EVs (T-0h), the enzyme activity was stopped immediately after its introduction in the tube, so it was considered as a control group compared with EVs incubated for 6 h with enzymes (T-6h) (Figure 1). After enzymatic treatment, the EVs were controlled.

Figure 1. Scheme of Trypsin/LysC Digestion on EVs
The trypsin enzyme cleaves peptide chains at the amino acid residues lysine or arginine, except when either is followed by proline, and lysine-C can cleave lysine followed by proline. After 6 h of Trypsin/Lys C treatment, the exterior parts of exosomes membrane proteins are cleaved as well as protein aggregates in the pellet.
by nanoparticle tracking analysis (Figure S1) and then subjected to an Amicon filtration step to separate EVs from the digested peptides coming from protein aggregates and the external part of EVs membrane proteins. The next step was to extract EVs proteins using radioimmunoprecipitation assay buffer (RIPA) buffer, and by filter aided sample preparation (FASP), the proteins were digested and prepared for tandem MS (MS/MS) analyses (Figure 2).

Two groups of proteins were identified, one corresponding to EVs membrane proteins (external fragment) and protein aggregates and the other corresponding to EVs membrane proteins (internal fragment) and internal EVs proteins (Figure 1). Using this procedure, we can purify EVs released from cells in the cell culture medium and can enrich the list of EVs proteins identified by shotgun proteomics (Data S1, S2, S3, and S4).

**Identification of Glioma C6 EVs Proteins by Shotgun Proteomics**

A total of 115 proteins have been identified from all EVs released by glioma C6 cells (Table S1). String analysis revealed the presence of two major pathways; the first one is centered on Fn1 and includes, e.g., TGFα1, Notch1, Notch2, Adam10, Syndecan-1, and Syndecan-2, and the second is centered on CD63 and included, e.g., CD81, CD14, CD9, and annexins. Both pathways are connected by CD44, CD9, and RhoA proteins (Figure S2A). All these proteins are known to be implicated in tumorigenesis, therefore the EVs carry a cell-type-specific signature (Mathivanan et al., 2010). KEGGS analyses revealed 11 proteins involved in cancer (CD44, CD63, RhoA, Socd3, Sdc1, Itgb1, Fn1, Tgfb1, Vcam, Ras, Rac1) and 14 in glioblastoma (CD44, FnA, Itgb1, Tgfb1, ECm1, App, Anxa1, HSP90aa1, Flot1, HSP90ab1, Rab7a, Pdcd6ip, Dnja2, Mvp). From the 115 identified proteins, whatever the fractions (internal or external), 15 proteins are unique to EVs not treated with Trypsin/Lys C (T-0h) and 100 proteins are specific to EVs treated for 6 h (T-6h) (Figure S2B).

Among the 15 proteins identified from non-treated EVs, a pathway including GABA(A) receptor-associated protein-like 1 and 2 and their receptors were identified, linked to Tollip, Ran, PSMA1, and PSMA4. By contrast for T-6h-treated EVs, a unique pathway is found centered on the guanine nucleotide-binding protein family. After MaxQuant followed by Perseus analyses on the triplicate experiments conducted with a p value 1%, a heatmap has been constructed (Figure S3). From the Heatmap, good separation can be observed between the isolated...
EVs and the secretome (proteins that passed the Amicon filter, EV). Two branches separating the EVs treated (T-6h) or non-treated (T-0h) are observable (Figure S3). One of the two branches separates the secretome proteins from the EVs T-6h proteins. The secretome proteins is then separated by two sub-branches between T-0h and T-6h. However, some T-0h EVs proteins are masked by the T-0h secretome proteins but can be separated from them in subsidiary branches. This indicates that trypsin digestion is necessary to better separate the EVs from the rest of the cellular secreted factors (Data S1, S2, S3, and S4). We then decided to separate the EVs proteins that are at the membrane and the ones that are extracellular to those that are inside. ANOVA tests were performed with non-supervised clustering of samples (Figure 3A). Three clear clusters were highlighted. The first is vertical separating samples between membrane proteins and internal proteins; the second and the third clusters are horizontal describing proteins groups. Cluster 1 regroups 29 proteins, Cluster 2 groups 17 proteins, and Cluster 3 groups only eight proteins (Table 1). Based on the identified proteins in the different samples, schemes of EVs protein contents treated or non-treated have been drawn (Figure 4). Enrichment analyses of each of these three clusters showed that cluster 1 corresponds to proteins involved in cell proliferation, growth, differentiation, adhesion, migration, and apoptosis (Figure 4). Cluster 2 corresponds to proteins involved in cell transformation, tumor microenvironment, and cancer. Cluster 3 regroups proteins involved in cell migration, internalization, proliferation, and tumor microenvironment in the C6 glioma (Figure 3B and Data S5, S6, and S7). Comparison between clusters suggests a strong pro-tumoral potential of C6 glioma EVs (Figure S2 and Data S5, S6, and S7). Pre-treated and non-pre-treated EVs revealed that the pre-clearance of extravesicular proteins enhanced our ability to identify a broader range of EVs-specific protein species. As a matter of example, we identified a variation of expression of the EVs surface proteins CD9, CD63, Mac2 BP, lactadherin, poly-ubiquitin b, neurensin, Celsr1, and Denn5b in non-treated EVs, whereas in treated EVs, we detected CD81, CD82, Ust, Vcan, Lamp 1, rab43, Cspg4, Annexin A2, Synthenin 1, and VSP37b. Similarly, Actb12, P-deshydrogenase, Acan, albumin, osteopotin, Sec14-like2, and pdc6ip are found only at T-6h (Figure 4). These results establish evolution of surface protein nature in EVs produced by glioma cells over time.

Ghost Protein Identification

The internal fraction of proteomic analysis shows some AlProt in the internal part of the EVs. In total six AlPros are identified with minimum one unique peptide (Table 2), showing different expression after trypsin digestion (Table 3). IP_2634467 (AltOdf3l1) is identified in both conditions, treated and non-treated EVs; other AlPros like IP_2656216 (AltKlhl29) are recovered only after a 6 h digestion. Finally, the majority of AlPros are identified at 0 h, e.g., IP_2659453, an AltProt formed from an ncRNA (LOC103695286) specifically identified in MS/MS. This AltProt has some homology with the predicted, but not observed,
Table 1. Clusters of Specific Proteins Identified in the Internal Part of Glioma C6 EVs in the Time Course of Trypsin/Lys C Digestion after Permutation-Based FDR 0.01

| Cluster 1 | Cluster 2 | Cluster 3 |
|-----------|-----------|-----------|
| H2afj; H2afz | H2afj; H2afz | Fn1 |
| Pcolce | Lgals3bp | Uba52; Rps27a; Ubb; Ubc |
| Lgals3bp | Fn1 | Itgb1 |
| Fn1 | Uba52; Rps27a; Ubb; Ubc | Hspa8 |
| Spp1 | Lamp1 | Mfge8 |
| Rab7a | Cd63 | Anxa2 |
| Uba52; Rps27a; Ubb; Ubc | Cd9 | Vcan |
| Ncam1 | Itgb1 | Pdcd6ip |
| Lamp1 | Hist1h4b | |
| Cd63 | Hspa8 | |
| Cd9 | Mfge8 | |
| Itgb1 | Ltbp1 | |
| Hist1h4b | Anxa2 | |
| Rap1a, Rap1b | Vcan | |
| Hspa8 | Sdcbp | |
| Mfge8 | Pdcd6ip | |
| Ltbp1 | Htra1 | |
| Anxa2 | | |
| Tmem55a | | |
| Ecm1 | | |
| Pttg1ip | | |
| Pscrb3 | | |
| Vcan | | |
| Sdcbp | | |
| Notch2 | | |
| Pdcd6ip | | |
| Htra1 | | |
| Flot1 | | |
| Flot2 | | |

DISCUSSION

Taken together, our results confirm that C6 glioma EVs carry a majority of proteins that are involved in tumor transformation and proliferation. Specific proteins identified in EVs are involved in tumor progression...
and bad prognosis. For example, Mac-2 BP binds with galectin-1, galectin-3, and galectin-7 and is associated with shorter survival, occurrence of metastasis, and/or a reduced response to chemotherapy in patients with different types of malignancy (Grassadonia et al., 2002). Similarly, Notch1, Notch2, and osteopontin are also bad prognostic factors in glioma (Saito et al., 2015; Zhao et al., 2015).

We also identified Dennd5b. Dennd5b and Dennd5a are markers of glioma according to the Brain Atlas (https://www.proteinatlas.org/). This protein family is also known to be epileptic marker (Han et al., 2016). These results can explain why some patients present epileptic behavior linked to glioma development. Finally, we detected six AltProts in glioma EVs. One corresponds to a non-coding RNA. The other five have their RNA also coding for reference proteins known to be involved in glioma: Kelch like family member 29 (Klhl29), OTU domain-containing protein 7B (Otud7b) known to act as a negative regulator of the B-cell response, outer dense fiber protein 3-like protein 1 (Odf3l1) marker of glioma hypoxia, THO complex subunit 7 homolog (Thoc7), and MIR1 (allergin-1), which plays an inhibitory role in the degranulation of mast cells. Taken together, we demonstrate and describe for the first time the presence of AltProts, which can be specific biomarkers and may be useful to target glioma EVs in body fluids for diagnosis.

**LIMITATIONS OF THE STUDY**

In agreement with the fact that the AltProts are not referenced, it is complicated at present to monitor and quantify the AltProts by classical approach such as the western blot based on the use of antibodies. The MS strategy remains the best method for identifying this type of protein. The expression of RNA transcripts can also provide information on the expression capacity of these proteins, although the presence of an RNA does not guarantee the production of the protein. The MS strategy uses AltProt prediction; if the databases are getting richer and safer, certain models like the RAT still present a not well-annotated genome. Thus the AltProt database based on Rnor_6.0 genomic data needs to evolve. Finally, if we demonstrate the presence of AltProt in variable amounts under different conditions, their present function remains. Studies of the AltProt interactome have previously been undertaken (Cardon et al., 2019a, 2019b) and have given some clues and showed that they may be involved in phenotypic changes, and in the signaling pathways of gene expression (Chen et al., 2020).
| AltProt Accession | Gene    | Transcript Accession | Unique Peptide | PSM | Protein Sequence | DNA Sequence | Type    | Coverage% |
|-------------------|---------|----------------------|----------------|-----|------------------|--------------|---------|-----------|
| IP_2656216        | Klhl29  | XM_008764550.2       | 1              | 2   | MGQAVWRCV5WTC    | ATGGGACAAGCTG| 5’UTR   | 24        |
|                   |         | XM_017594070.1      |                |     | SYHSCVTMTCTRRLELSLENRV | TATGGAGATGTGAG |         |           |
|                   |         |                      |                |     |                  | CTGGACTTGTTCTTAC |         |           |
|                   |         |                      |                |     |                  | CATTCCTGTGCACAGA |         |           |
|                   |         |                      |                |     |                  | TGACATGCCGAAAGGC |         |           |
|                   |         |                      |                |     |                  | TGGAGCTGTCCCTAG |         |           |
|                   |         |                      |                |     |                  | AGAACAGGGTGTA  |         |           |
| IP_2659453        | LOC103695286 | XR_001838245.1 | 1              | 1   | MFCFCFVLFCFFLELRT | ATGGTTTGGTTTGGTT |         | 41        |
|                   |         |                      |                |     | EPRALCLLGKRSTTELPPIRDF | TTTTTCTGGAGCTGAGG |         |           |
|                   |         |                      |                |     |                  | ACCGAAACCAGGGCTTTT |         |           |
|                   |         |                      |                |     |                  | GTGCTTGCTAGGCAAGCG |         |           |
|                   |         |                      |                |     |                  | CTCTACCACTGAACTACC |         |           |
|                   |         |                      |                |     |                  | CCGGATTAGGGACTTTTA |         |           |
| IP_2613134        | Otud7b  | XM_006232957.3      | 1              | 2   | MIKPLTSVTLPSVPT  | ATGATCAAACCTTA | 3’UTR   | 9         |
|                   |         |                      |                |     | PQDTEKQFEALRRKV  | CCTCCGTTCTGACCATT |         |           |
|                   |         |                      |                |     | VERKRVLSSLHELFOKTLR | CCCCTCTATTCTCCGCT |         |           |
|                   |         |                      |                |     | CVGWWVSSAARFTS   | TCCACACCTCAAGAC |         |           |
|                   |         |                      |                |     | TIRLGALASY        | ACTAAAGAACAGAAAGG |         |           |
|                   |         |                      |                |     |                  | AGGCCTTAAAGAAAA |         |           |
|                   |         |                      |                |     |                  | AGAAGTGAGAGAGAGA |         |           |
|                   |         |                      |                |     |                  | AAAGTACTAATTCCTTA |         |           |
|                   |         |                      |                |     |                  | CATACATATTCCAGAA |         |           |
|                   |         |                      |                |     |                  | ACACTAGAGTGTTGAGG |         |           |
|                   |         |                      |                |     |                  | TGGGTTGTTTGTTGTC |         |           |
|                   |         |                      |                |     |                  | AGCAGCCTTGAGGACTTT |         |           |
|                   |         |                      |                |     |                  | CTACAATCAACCTCTA |         |           |
|                   |         |                      |                |     |                  | GCCCTGTTGCTAGCAAA |         |           |
|                   |         |                      |                |     |                  | GTACATACCTGTTTGTCC |         |           |
|                   |         |                      |                |     |                  | AGACCTGTCTCATGCTAG |         |           |

Table 2. Description of the AltProt Identified in the EVs

(Continued on next page)
| AltProt Accession | Gene | Transcript Accession | Unique Peptide | PSM | Protein Sequence | DNA Sequence | Type | Coverage% |
|------------------|------|----------------------|----------------|-----|------------------|--------------|------|----------|
| IP_2634467       | Odf3l1 | XM_008766272.2, XM_017595650.1 | 1              | 5   | MTTDRGWGLKLCRLLOQDGR | ATGACCCACAGACAGAGG ATGGGTTTTGGAAGTTGTG CAGGCTTCTGCTCCAGC AGGACTCAAGGAGGGA CCATTCCCAGGCCCCCT CCCCCACCCCCAGGCTC TAAGGAAGTGCT GTGGGATCTCTGA | 3'UTR | 20       |
| IP_2683599       | Thoc7 | XM_017599648.1       | 1              | 1   | MIKRSKLIFSRTLWVY LVPCCFLHLHCLTS5HM GCKLSLGAESRTW VLRKSRMH5S | ATGATCATAAAAGCGAAG GTATAAGATTGTTAGT CGCAGTGGGTTCGG TTTGATTTAGTCCTCCGC TGTGCCTACTGCATTAT AGTGTGACATCCCACAT GGCGTGAAACTCTCAGA CTTGAGTGAGAGGAGCC GACCTGGTCCTCCAGG AAAGACCAGAAGTGACTCTCTAA | 3'UTR | 16       |
| IP_2577320       | Milr1 | XM_017597497.1, XM_008768388.2 | 1              | 1   | MCMGQHMPRYYHVA GRTSWGSLSPS5YS MRPSWHTLIKIVKFGG QHGWTSPQHT | ATGTGCATGGGCAAC ATATGCAAGTATG TGCAAGCGGTTGGAAGG ACGAGTTGGGGGAGTCA GCTTTCTCTCTACTCCA TGAACCCCTGCGCATGA ACTAAGGCACTCAATGGTT GGGTGCACGACGGTTGGA ACCCGAGGCAACGACACCTGA | 3'UTR | 18       |

Table 2. Continued

AltProts identification: accession number according to OpenProt annotation correlated to the gene produced the AltProt; column “type” lists the kind of AltProt based on the location in the RNA: AltProts come from the 5' and 3' UTRs, by a shift in the CDS, or from ncRNA. The transcript accession is also mentioned permitting the identification of the RNA and gene; in the AltProt sequence in amino acid and nucleic acid, the sequence of the unique peptide identified has been underlined.
METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101045.

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AUTHOR CONTRIBUTIONS

A.-N.M. performed all EVs purification, S.A. performed the proteomic studies. M.S. and T.C. analyzed proteomic data. M.S., I.F., and D.C. obtained funding for the project. A.-N.M. and M.S. designed the study and wrote the manuscript with contributions from all authors. A.-N.M. drew Figures 1, 2, and 4.

DECLARATION OF INTERESTS

The authors declare no competing interest.

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| AltProt Accession | Internal EVs 0 h | External Membrane 0 h | Internal EVs 6 h | External Membrane 6 h |
|-------------------|-----------------|----------------------|-----------------|----------------------|
| IP_2656216        | 0               | 0                    | 2               | 0                    |
| IP_2659453        | 1               | 0                    | 0               | 0                    |
| IP_2613134        | 2               | 0                    | 0               | 0                    |
| IP_2634467        | 3               | 0                    | 2               | 0                    |
| IP_2683599        | 1               | 0                    | 0               | 0                    |
| IP_2577320        | 1               | 0                    | 0               | 0                    |

Table 3. Repartition of AltProts Identification on the Samples Condition

Number of identifications in the different conditions: internal/external at 0 h or 6 h of treatment by trypsin. The external parts are related to the first elution after treatment of the intact EVs by trypsin (n = 3), the internal part combined a triplicate passing the filter after the digestion and the top part of the Amicon (n = 6).
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Supplemental Information

Reference and Ghost Proteins Identification
in Rat C6 Glioma Extracellular Vesicles

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Figure S1. NanoSight analyzes, related to Figure 1. Isolated vesicles were analyzed using the NanoSight to characterize size and concentration. After the last ultracentrifugation step followed by Tryspin/Lys C digestion, the pellet obtained was diluted in particle-free PBS (1:100). To analyze the particles, 5 videos of 60 s for each sample were recorded. A monochromatic laser beam at 488nm was used for analyses. Particle movement was investigated with NTA software (version 3.2, NanoSight). NTA post-acquisition settings were kept constant between samples.
Figure S2. String analysis for glioma EVs proteins, related to Figure 2 and Figure 3. (a) String analysis for all proteins identified for glioma EVs by shotgun proteomics. (b) String analysis results for unique proteins identified from EVs not treated with trypsin/Lys C (T-0h) and for EVs that were 6h treated (T-6h).
Figure S3. Heatmap from Shot gun proteomics, related to Figure 2 and Figure 3. For identified proteins of EVs treated or not with Trypsin/Lys C was performed quantitative proteomics analyses using MaxQuant software, after ANOVA tests with a p value >0.05,
Table S1. List of the statistically validated identified proteins by shotgun proteomics from purified glioma C6 EVs proteins, related to Figure 2.

| Protein ID | Protein names | Gene names |
|------------|---------------|------------|
| B5DEN9     | Vacuolar protein sorting-associated protein 28 homolog | Vps28 |
| D328L7     | Ras-related protein R-Ras | Rras |
| M0RC99     | Ras-related protein Rab-5A | Rab5a |
| O35276     | Neuropilin-2 | Nrp2 |
| P62494;O35509 | Ras-related protein Rab-11A;Ras-related protein Rab-11B | Rab11a;Rab11b |
| O35806     | Latent-transforming growth factor beta-binding protein 2 | Ltbp2 |
| O35824     | Dnaj homolog subfamily A member 2 | Dnaja2 |
| O70352     | CD82 antigen | Cd82 |
| P02793;Q7TP54 | Ferritin light chain 1;Protein FAM65B | Ftl1;Fam65b |
| P04094     | Proenkephalin-A | Penk |
| P04642;P19629;P42123 | L-lactate dehydrogenase A chain | Ldha |
| P04797;Q9ESV6 | Glyceraldehyde-3-phosphate dehydrogenase | Gapdh;Gapdhs |
| P05371     | Clusterin;Clusterin beta chain;Clusterin alpha chain | Clu |
| P05712     | Ras-related protein Rab-2A | Rab2a |
| P06685;P06687;P06686;Q64541;P54708 | Sodium/potassium-transporting ATPase subunit alpha 1 | Atp1a1;Atp1a3;Atp1a2 |
| P07150     | Annexin A1 | Anxa1 |
| P07314     | Gamma-glutamyltranspeptidase 1 | Ggt1 |
| P07897;P07897-2 | Aggrecan core protein | Acan |
| P08592;P08592-2 | Amyloid beta A4 protein | App |
| P08753     | Guanine nucleotide-binding protein G(k) subunit alpha | Gna13 |
| P10960     | Sulfated glycoprotein 1 | Psap |
| P11167     | Solute carrier family 2, facilitated glucose transporter member 1 | Scl2a1 |
| P11762     | Galectin-1 | Lgals1 |
| P11980-2;P11980;P12928-2;P12928 | Pyruvate kinase PKM | Pkm |
| P13635     | Ceruloplasmin | Cp |
| P17046     | Lysosome-associated membrane glycoprotein 2 | Lamp2 |
| P17246     | Transforming growth factor beta-1;Latency-associated peptide | Tgfb1 |
| P19132     | Ferritin heavy chain;Ferritin heavy chain, N-terminally processed | Fth1 |
| P26051-2;P26051 | CD44 antigen | Cd44 |
| P26260     | Syndecan-1 | Sdc1 |
| P26453-2;P26453 | Basigin | Bsg |
| P27615     | Lysosome membrane protein 2 | Scarb2 |
| P28073     | Proteasome subunit beta type-6 | Psmb6 |
| P30121     | Metalloproteinase inhibitor 2 | Timp2 |
| P31000     | Vimentin | Vim |
| P34058;Q66HD0-2;Q66HD0 | Heat shock protein HSP 90-beta | Hsp90ab1 |
| P34064     | Proteasome subunit alpha type-5 | Psma5 |
| P34900     | Syndecan-2 | Sdc2 |
| Accession | Gene Name               | Description                                      |
|-----------|-------------------------|--------------------------------------------------|
| P47853    | Biglycan                | Bgn                                             |
| P52796    | Ephrin-B1               | Efnb1                                           |
| P54290    | Voltage-dependent calcium channel subunit alpha-2 | Cacna2d1                                      |
| P55260    | Annexin A4              | Anxa4                                           |
| P58195    | Phospholipid scramblase 1 | Plscr1                                          |
| P58751-3; P58751-2; P58751 | Reelin                  | Reln                                            |
| P60901    | Proteasome subunit alpha type-6 | Psma6                                        |
| P84082; P84079; P61206; P84083; P61751 | ADP-ribosylation factors | Arf2; Arf1; Arf3; Arf5; Arf4                        |
| P61589    | Transforming protein RhoA | Rhoa                                          |
| P62836; Q62636 | Ras-related protein Rap-1A; Ras-related protein Rap-1b | Rap1a; Rap1b                                  |
| P63036    | DnaJ homolog subfamily A member 1 | Dnaja1                                         |
| P82995    | Heat shock protein HSP 90-alpha | Hsp90aa1                                      |
| Q6LED0; P84245 | Histone H3.1; Histone H3.3 | H3f3b                                         |
| P85972    | Vinculin                | Vcl                                             |
| Q00657    | Chondroitin sulfate proteoglycan 4 | Csgp4                                         |
| Q07008    | Notch 1 intracellular domain | Notch1                                         |
| Q08420    | Extracellular superoxide dismutase [Cu-Zn] | Sod3                                          |
| Q10743    | Disintegrin and metalloproteinate domain-containing protein 10 | Adam10                                        |
| Q5BK83    | Transmembrane protein 106A | Tmem106a                                       |
| Q62632    | Follistatin-related protein 1 | Fstl1                                         |
| Q62667    | Major vault protein | Mvp                                             |
| Q62745    | CD81 antigen            | Cd81                                            |
| Q62786    | Prostaglandin F2 receptor negative regulator | Ptgfrn                                       |
| Q63083    | Nucleobindin-1          | Nucb1                                           |
| Q63691    | Monocyte differentiation antigen CD14 | Cd14                                         |
| Q66HA6    | ADP-ribosylation factor-like protein 8B | Arl8b                                         |
| Q6AYA5    | Transmembrane protein 106B | Tmem106b                                       |
| Q6IRE4    | Tumor susceptibility gene 101 protein | Tsg101                                       |
| Q6P6T1    | Complement C1s subcomponent | C1s                                          |
| Q6P777    | Multivesicular body subunit 12A | Mvb12a                                        |
| Q6Q0N0    | Calsyntenin-1; Soluble Aic-alpha; CTF1-alpha | Clstn1                                        |
| Q6RU5V; Q8CFN2-2; Q8CFN2; Q9JL4 | Ras-related C3 botulinum toxin substrate 1 | Rac1                                          |
| Q6VBQ5    | Myeloid-associated differentiation marker | Myadm                                         |
| Q6X936    | Kin of IRRE-like protein 1 | Kirrel                                         |
| Q794F9    | 4F2 cell-surface antigen heavy chain | Slc3a2                                        |
| Q7TNK0    | Serine incorporator 1   | Serinc1                                         |
| Q80WK7    | Equilibrative nucleoside transporter 3 | Slc29a3                                        |
| Q8VII6-2; Q8VII6 | Choline transporter-like protein 1 | Slc44a1                                        |
| Q9QW30    | Notch 2 intracellular domain | Notch2                                         |
| Q9QZA6    | CD151 antigen           | Cd151                                           |
TRANSPARENT METHODS

Experimental design and statistical rationale
Shotgun proteomics experiments on Amicon filtered glioma EVs, FASP and Trypsin/Lys C digestion were conducted in biological triplicates.

Reagents
The rat C6 glioma cell line was kindly provided by Prof. Dr. Bernd Kaina (Institute of Toxicology, University Medical Center, Mainz, Germany). Ham’s F12K, puromycin, phosphate buffer saline (PBS), fetal bovine serum (FBS), Trypsin/Lys C were all obtained from Promega (USA).

EVs purification
Glioma C6 cells were cultured in high-glucose Dulbecco’s Modified Eagle’s Medium (DMEM) and supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine (2 mM) and 1% gentamicin (50 units per ml), all from Sigma-Aldrich. Confluent population of cells were incubated without FBS for 24h to obtain cells conditioned media (CM) free of FBS. Afterwards, CM was cleared of cells and debris by centrifugation at 350×g for 10 min at 4°C, followed by filtration with nylon filter membranes, pore size 0.2 μm. Membranes and debris were discarded from the CM by centrifugation for 30 min at 2 000×g at 4°C. Then the withdrawn supernatant was centrifuged at 10 000×g for 30 min at 4°C to remove larger vesicles, followed by ultracentrifugation (Beckman Optima XPN80 Ultracentrifuge, USA) at 100 000×g for 70 min, 4°C. The pellet was washed in PBS in order to eliminate contaminating proteins and re-ultracentrifuged at 100 000×g for 120 min, 4°C.

Trypsin/Lys C digestion treatment
Isolated EVs were resuspended in Trypsin/Lys C 0.2 µg/mL solution diluted in NH4HCO3 50mM. For T0 condition (non-treated), the enzyme activity was immediately stopped with TFA 0.5%, for rest times points, the EVs were incubated at 37°C for 6h, then TFA 0.5% was added to stop enzyme activity.

Nanoparticle tracking analysis (NTA)
Isolated vesicles were analyzed using the NanoSight LM 10 instrument (Merkel technologies LTD., UK) to characterize size and concentration. After the last ultracentrifugation step followed by Trypsin/Lys digestion, the pellet obtained was diluted in particle-free PBS (1:100). To analyze the particles, 5 videos of 60 s for each sample were recorded. A monochromatic laser beam at 488nm was used for analyses. Particle movement was investigated with NTA software (version 3.2, NanoSight). NTA post-acquisition settings were kept constant between samples.

Samples preparation for mass spectrometry analysis
EVs digested with Trypsin/Lys C for 6h (T-6h) or non-digested samples (T-0h), were subjected to a filtration step using Amicon Ultra-0.5 mL Centrifugal Filters 10 KDa for 20min for 14 000xg at 4°C. In the Amicon “Filtrate” tube, peptides from protein aggregates and from external part of EV membrane proteins were recovered. The vesicles were recovered from Amicon filter by 4°C centrifugation for 5min at 1 000xg. EV proteins were extracted using RIPA buffer. Samples were prepared for mass spectrometry analysis by Filter-Aided Sample Preparation (FASP) as described in Duhamel et al., 2018. Briefly, proteins from all fractions were denatured with 2 M urea in 10 mM HEPES, pH 8.0 by sonication. Then they were reduced with 10 mM DTT for 40 min at 56°C followed by alkylation with 55 mM iodoacetamide for 40 min in the dark. The iodoacetamide was quenched with 100 mM thiourea. The proteins were digested in an Amicon 30KD (Millipore) overnight using 1
μg Trypsin/LysC mixture at 37°C. In a new tube, the Amicon is centrifuge at 14000 Xg to liberate the peptides then the digestion was stopped with 0.5% TFA and dry. The peptides were desalted with a Millipore ZipTip-C18 device. The solution was then dried using the SpeedVac. Dried samples were solubilized in 2% ACN / 98% of 0.1% formic acid in water, before nLC MS/MS analysis.

**LC MS/MS analysis**

Samples were separated by online reversed-phase chromatography using a Thermo Scientific Proxeon Easy-nLC system equipped with a Proxeon trap column (100 µm ID x 2 cm, Thermo Scientific) and a C18 packed-tip column (75 µm ID x 50 cm, Thermo Scientific). Peptides were separated using an increasing amount of acetonitrile (5–35% for 100 min) at a flow rate of 300 nL/min. The LC eluent was electrosprayed directly from the analytical column and a voltage of 1.7 kV was applied via the liquid junction of the nanospray source. The chromatography system was coupled with a Thermo Scientific Q Exactive mass spectrometer programmed to acquire using the data dependent Top 10 method. Survey scans were acquired at a resolution of 70 000 at m/z 400.

**Data analyses**

For the Alternative Proteins (AltProt), Proteome Discoverer V2.3 (Thermo Scientific) was used, the protein database was downloaded from Openprot (https://openprot.org/) and includes RefProts, novel isoforms and AltProts predicted from both Ensembl and RefSeq annotations (Rnor_6.0.84, Rnor_6.0) for a total of 293303 entries. The following processing and consensus parameters are used with: Trypsin as enzyme, 2 missed cleavages, methionine oxidation as variable modification and carbamidomethylation of cysteines as static modification, Precursor Mass Tolerance: 10 ppm and Fragment mass tolerance: 0.1 Da. The validation was performed using a Percolator with a protein strict FDR set to 0.001%, according to Brunet et al., for AltProt database, the FDR needs to be more restrictive to remove maximum of false positive (Brunet et al., 2019). A consensus workflow was then applied for the statistical arrangement, using the high confidence protein identification. Results are filtered to keep master protein and high confidence protein FDR. For the Reference Proteins (RefProts) the MS data was processed with MaxQuant (version 1.5.8.3) (Cox and Mann, 2008) using the Andromeda search engine. Proteins were identified by searching MS and MS/MS data against the Decoy version of the complete proteome for *Rattus norvegicus* of the UniProt database (UniProt Consortium, 2012) (Release June 2017, 8022 entries reviewed). Trypsin specificity was used for the digestion mode with N-terminal acetylation and methionine oxidation selected as the variable. Carbamydymethylation of cysteines was set as a fixed modification and we allowed up to two missed cleavages. For MS spectra, an initial mass accuracy of 6 ppm was selected, and the MS/MS tolerance was set to 20 ppm for HCD data. For identification, the FDR at the peptide spectrum matches (PSMs) and protein level was set to 0.01. Relative, label-free quantification of proteins was performed using the MaxLFQ algorithm (Cox et al., 2014) integrated into MaxQuant with the default parameters. Analysis of the proteins identified was performed using Perseus software (http://www.perseus-framework.org/) (version 1.6.0.7). The file containing this information from identification was used with hits to the reverse database, and proteins only identified with modified peptides and potential contaminants removed. Then, the LFQ intensity was transformed by the log2[x] function. Categorical annotation of rows was used to define different groups depending on the following criteria: 1) localization on the exosome and 2) time of enzyme treatment. Multiple-sample tests were performed using an ANOVA test with a p value > 0.05% and preserved grouping in randomization. To determine enrichment of categorical annotations (Gene Ontology terms and KEGG pathway), a Fisher’s exact test was used, taking in account the results of the ANOVA test for each group. Normalization was achieved using a Z-score with matrix access by rows. Only proteins significant by the ANOVA tests were used for statistical analysis. A hierarchical clustering was first performed using the Euclidean parameter for distance calculation and an average option for linkage in row and column trees using a maximum of 300 clusters. To quantify fold changes of proteins across samples, we used MaxLFQ. To visualize these fold changes in the context of individual protein abundance in the proteome, we projected them onto the summed
peptide intensities normalized by the number of theoretically observable peptides. Datasets including MaxQuant files and annotated MS/MS datasets, were uploaded to ProteomeXchange Consortium via the PRIDE database, and was assigned the dataset identifier PXD016944 (Username: reviewer95445@ebi.ac.uk, Password: wHS6rIOg). Functional annotation and characterization of identified proteins were obtained using PANTHER software (version 9.0, http://www.pantherdb.org) and STRING (version 9.1, http://string-db.org). The Elsevier's Pathway Studio version 9.0 (Ariadne Genomics/Elsevier) was used to deduce statistically significant altered biological and functional pathways pertaining to each identified set of protein hits. SNEA utilizes Fisher's statistical test used to determine if there are non-randomized associations between two categorical variables organized by specific relationships. SNEA starts by creating a central "seed" from all relevant entities in the database, and retrieving associated entities based on their relationship with the "seed" (i.e. binding partners, expression targets, protein modification targets, regulation). The algorithm compares the sub-network distribution to the background distribution using one-sided Mann-Whitney U-Test and calculates a p-value indicating the statistical significance of the difference between two distributions.

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