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Research article

Comparative evaluation of five extracellular vesicle isolation methods using proteomic profiling

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Abstract: Extracellular vesicles (EVs) research has gained a significant amount of attention in recent years. EVs are a heterogeneous group of different vesicles that vary in their origin, size, and function. The very nature of EVs, from their biogenesis to the contents of their cargo, offers many options for exploration. Despite being studied for a few decades now, there has not been an established standardized approach to isolation. However, the future use of EVs in clinical practice is conditioned by the optimization and standardization of isolation methods. In this study, we systematically compared common EV isolation techniques such as ultracentrifugation, precipitation, tangential flow filtration, and affinity-based methods using conditioned cell culture media as the source. Isolated EVs were analyzed using mass spectrometry to characterize their proteomic profiles, and the shared protein content was evaluated across datasets. The gene ontology enrichment was further assessed using three bioinformatics platforms. Among the tested methods, ultracentrifugation emerged as the most effective isolation method in our analysis, thus underscoring its importance among commonly used techniques. Despite its analytical robustness, this method is unsuitable for routine clinical workflows due to its complexity and time demands. Our findings highlight the need for a standardized, less strenuous EV isolation method for clinical applications.

Keywords: extracellular vesicles, isolation methods, comparison, mass spectrometry, proteomic analysis

Appendix

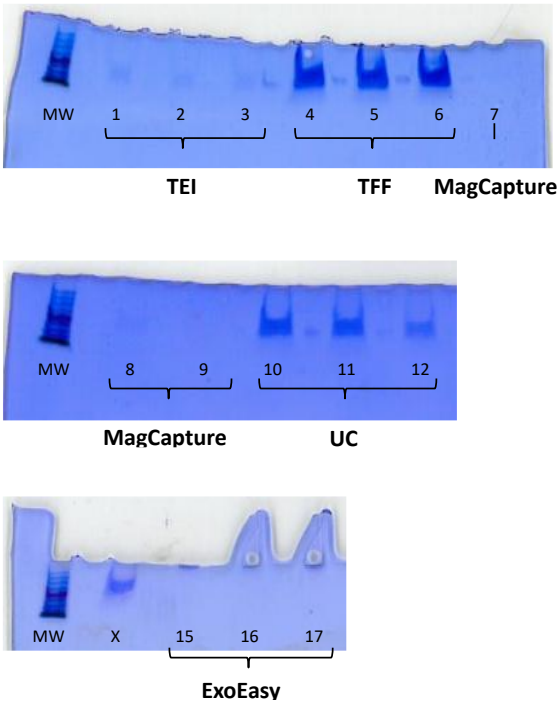


Figure S1. Visualization of EV proteins on gels. Since each isolation approach used different amounts of CCM, the amount of EVs and therefore proteins varied. (protein molecular weight standard (MW), ultracentrifugation (UC), tangential flow filtration (TFF), Total exosome isolation (TEI) (from cell culture media), exoEasy Maxi Kit (ExoEasy), MagCapture™ Exosome Isolation Kit PS Ver.2 (MagCapture), sample not relevant to this study (X))

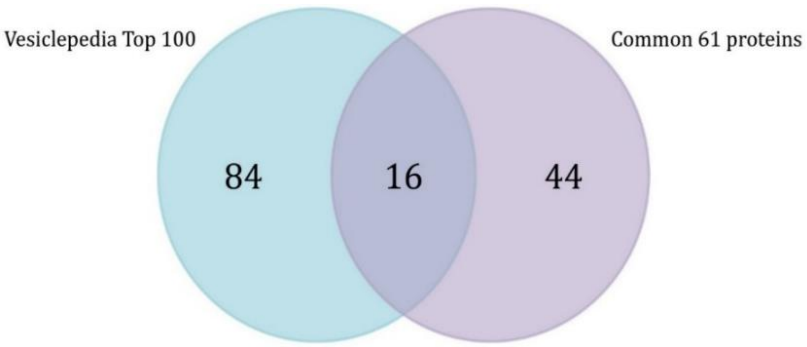
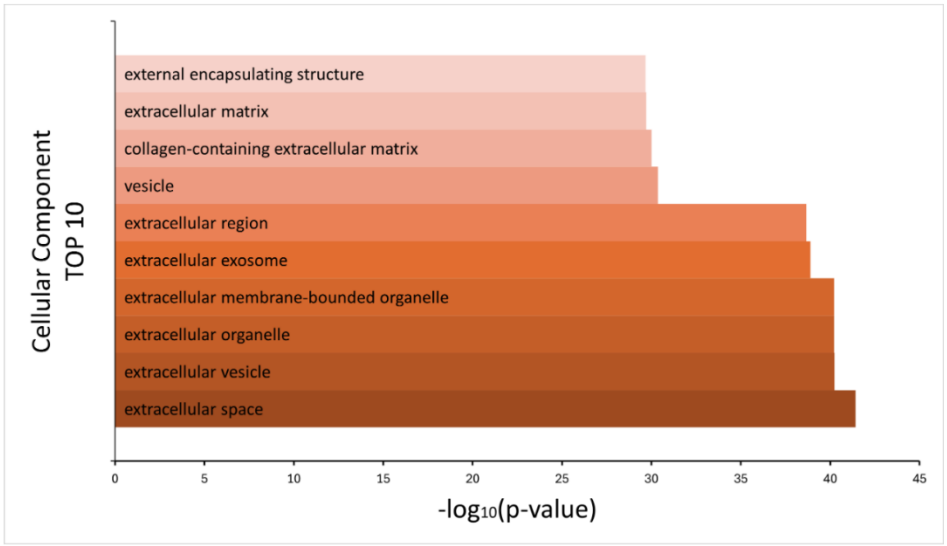


Figure S2. Comparison of 'Common 61 proteins' and 'Vesiclepedia Top 100' datasets. The 'Common 61 proteins' dataset includes Immunoglobulin kappa light chain, which lacks a gene symbol and was excluded from analysis, leaving 60 proteins (Venn diagram generated by FunRich tool (10.1016/j.jmb.2020.166747)).

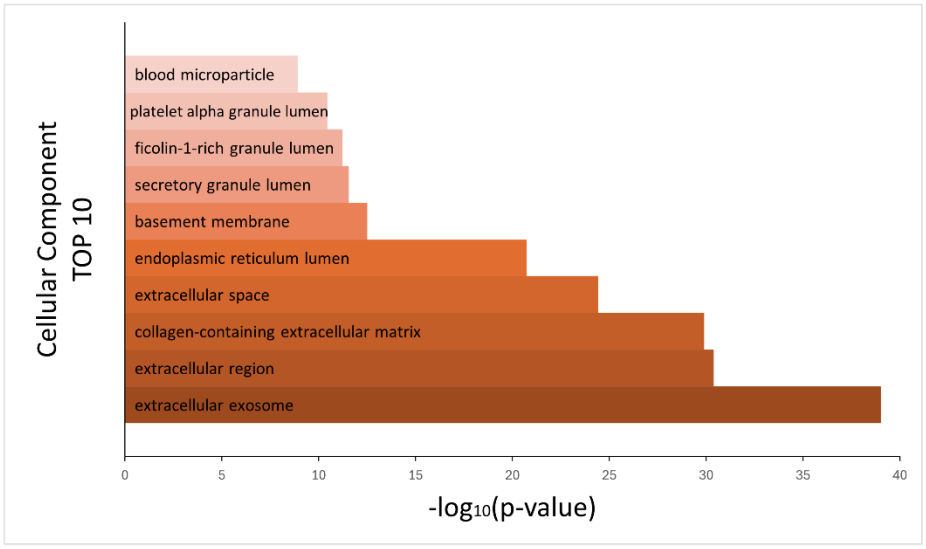
a

Cellular Component

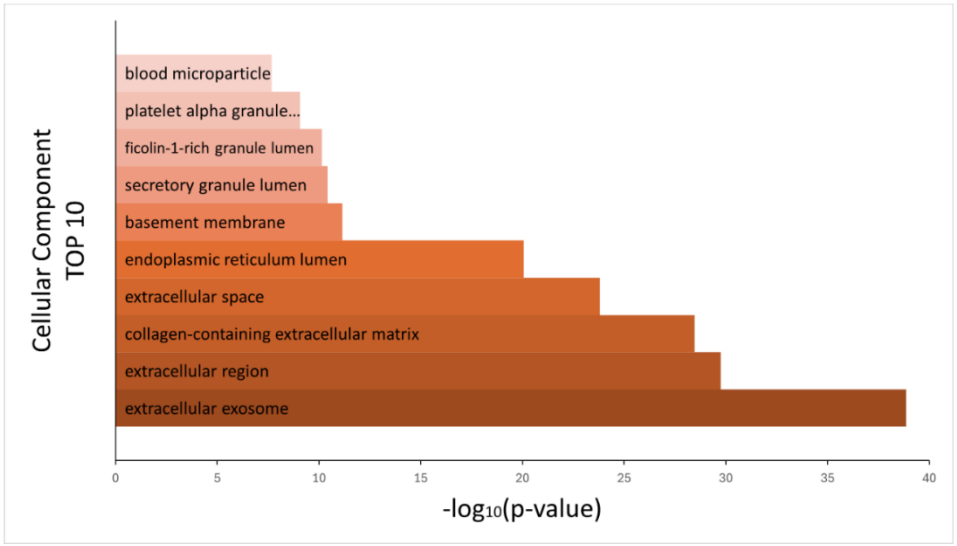
Gene Ontology

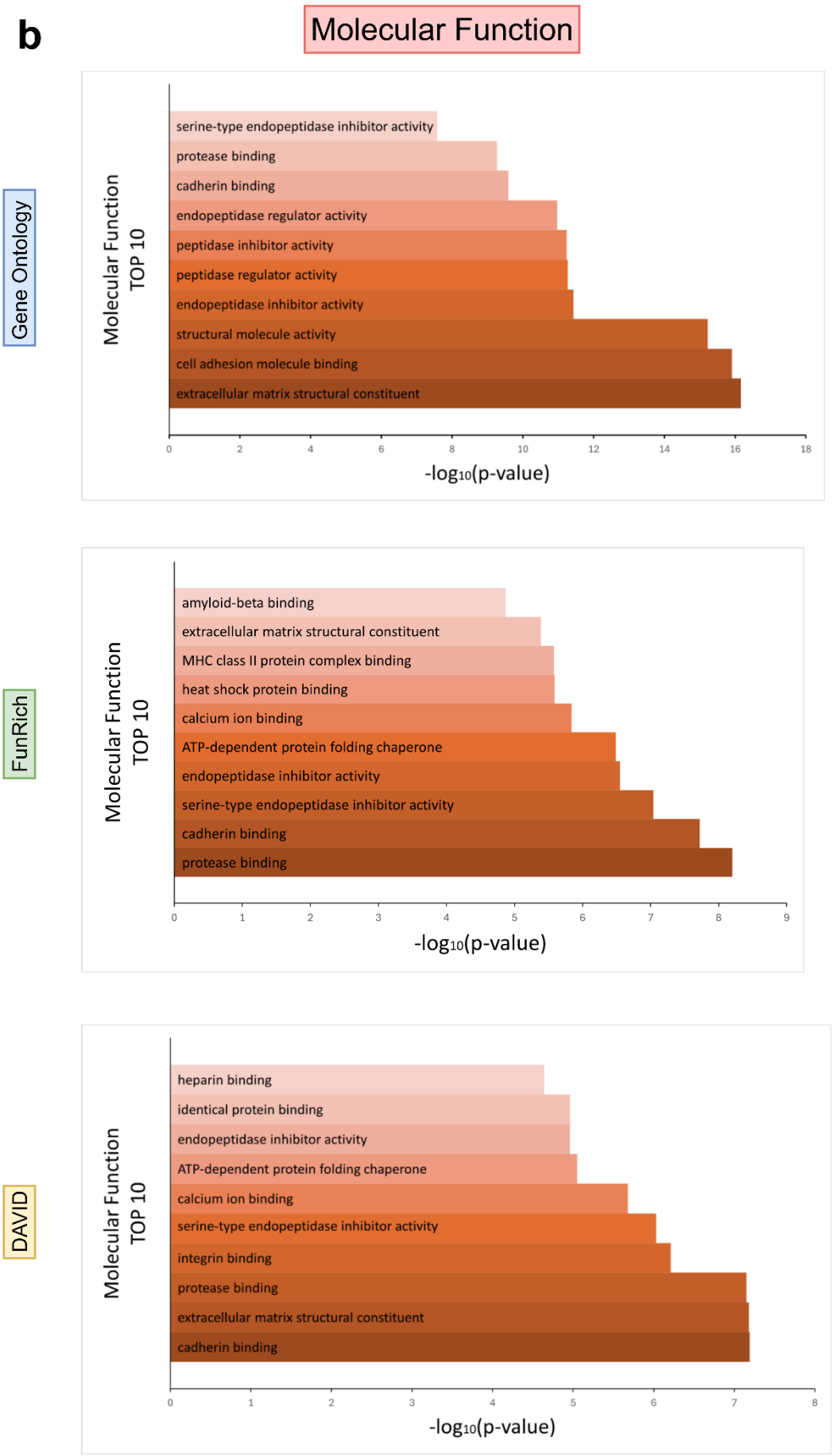


FunRich



DAVID




b
Molecular Function
Gene Ontology
Molecular Function TOP 10
serine-type endopeptidase inhibitor activity
protease binding
cadherin binding
endopeptidase regulator activity
peptidase inhibitor activity
peptidase regulator activity
endopeptidase inhibitor activity
structural molecule activity
cell adhesion molecule binding
extracellular matrix structural constituent
0 2 4 6 8 10 12 14 16 18
-log10(p-value)
FunRich
Molecular Function TOP 10
amyloid-beta binding
extracellular matrix structural constituent
MHC class II protein complex binding
heat shock protein binding
calcium ion binding
ATP-dependent protein folding chaperone
endopeptidase inhibitor activity
serine-type endopeptidase inhibitor activity
cadherin binding
protease binding
0 1 2 3 4 5 6 7 8 9
-log10(p-value)
DAVID
Molecular Function TOP 10
heparin binding
identical protein binding
endopeptidase inhibitor activity
ATP-dependent protein folding chaperone
calcium ion binding
serine-type endopeptidase inhibitor activity
integrin binding
protease binding
extracellular matrix structural constituent
cadherin binding
0 1 2 3 4 5 6 7 8
-log10(p-value)

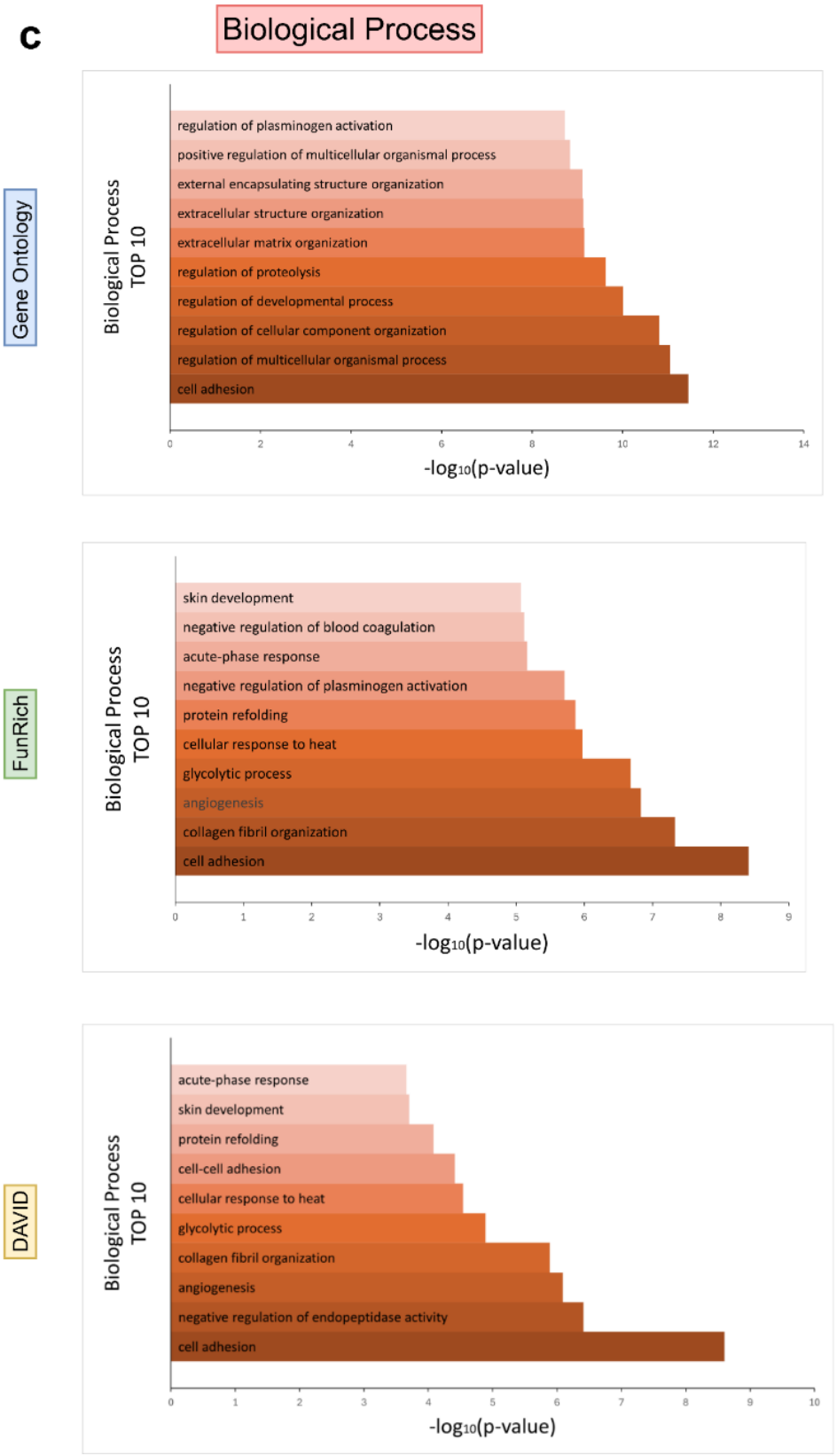


Figure S3. Top 10 entries given by each database. Results of the term analysis of gene ontology data. (a) cellular component, (b) molecular function, (c) biological process. Entries were aligned according to p-values from the most significant to the least.

a. Sample 1: SKOV3 EVs
Measurement: 1

System

Temperature (°C):	25.0	**Duration Used (s):**	60
Count Rate (kcps):	313.2	**Measurement Position (mm):**	4.65
Cell Description:	2µL quartz batch cell	**Attenuator:**	0

Results

			Size (d.nm):	**% Intensity:**	**St Dev (d.n...**
Z-Average (d.nm):	195.9	**Peak 1:**	236.5	98.9	111.4
PdI:	0.208	**Peak 2:**	4841	1.1	704.7
Intercept:	0.898	**Peak 3:**	0.000	0.0	0.000

Result quality : Good

Sample 1: SKOV3 EVs
Measurement: 2

System

Temperature (°C):	25.0	**Duration Used (s):**	60
Count Rate (kcps):	297.3	**Measurement Position (mm):**	4.65
Cell Description:	2µL quartz batch cell	**Attenuator:**	0

Results

			Size (d.nm):	**% Intensity:**	**St Dev (d.n...**
Z-Average (d.nm):	186.2	**Peak 1:**	214.3	98.5	87.08
PdI:	0.195	**Peak 2:**	4831	1.5	708.9
Intercept:	0.896	**Peak 3:**	0.000	0.0	0.000

Result quality : Good

Sample 1: SKOV3 EVs
Measurement: 3

System

Temperature (°C):	25.0	**Duration Used (s):**	60
Count Rate (kcps):	322.2	**Measurement Position (mm):**	4.65
Cell Description:	2µL quartz batch cell	**Attenuator:**	0

Results

			Size (d.nm):	**% Intensity:**	**St Dev (d.n...**
Z-Average (d.nm):	191.2	**Peak 1:**	209.5	97.3	78.98
PdI:	0.223	**Peak 2:**	4968	2.7	623.0
Intercept:	0.895	**Peak 3:**	0.000	0.0	0.000

Result quality : Good

Sample 2: SKOV3 EVs
Measurement: 1

System

Temperature (°C):	25.0	**Duration Used (s):**	60
Count Rate (kcps):	224.1	**Measurement Position (mm):**	4.65
Cell Description:	2µL quartz batch cell	**Attenuator:**	0

Results

			Size (d.nm):	**% Intensity:**	**St Dev (d.n...**
Z-Average (d.nm):	188.3	**Peak 1:**	199.5	93.8	83.83
PdI:	0.305	**Peak 2:**	4770	6.2	737.7
Intercept:	0.898	**Peak 3:**	0.000	0.0	0.000
Result quality :	Good				

Sample 2: SKOV3 EVs
Measurement: 2

System

Temperature (°C):	25.0	**Duration Used (s):**	60
Count Rate (kcps):	214.8	**Measurement Position (mm):**	4.65
Cell Description:	2µL quartz batch cell	**Attenuator:**	0

Results

			Size (d.nm):	**% Intensity:**	**St Dev (d.n...**
Z-Average (d.nm):	172.6	**Peak 1:**	177.9	97.3	53.78
PdI:	0.231	**Peak 2:**	5150	2.7	503.9
Intercept:	0.891	**Peak 3:**	0.000	0.0	0.000
Result quality :	Good				

Sample 2: SKOV3 EVs
Measurement: 3

System

Temperature (°C):	25.0	**Duration Used (s):**	60
Count Rate (kcps):	207.7	**Measurement Position (mm):**	4.65
Cell Description:	2µL quartz batch cell	**Attenuator:**	0

Results

			Size (d.nm):	**% Intensity:**	**St Dev (d.n...**
Z-Average (d.nm):	168.3	**Peak 1:**	197.9	100.0	72.91
PdI:	0.151	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.889	**Peak 3:**	0.000	0.0	0.000
Result quality :	Good				

Sample 3: SKOV3 EVs
Measurement: 1

System

Temperature (°C):	25.0	**Duration Used (s):**	60
Count Rate (kcps):	202.7	**Measurement Position (mm):**	4.65
Cell Description:	2µL quartz batch cell	**Attenuator:**	0

Results

			Size (d.nm):	**% Intensity:**	**St Dev (d.n...**
Z-Average (d.nm):	168.3	**Peak 1:**	183.5	98.8	59.02
PdI:	0.201	**Peak 2:**	5197	1.2	475.4
Intercept:	0.897	**Peak 3:**	0.000	0.0	0.000

Result quality : Good

Sample 3: SKOV3 EVs
Measurement: 2

System

Temperature (°C):	25.0	**Duration Used (s):**	60
Count Rate (kcps):	262.4	**Measurement Position (mm):**	4.65
Cell Description:	2µL quartz batch cell	**Attenuator:**	0

Results

			Size (d.nm):	**% Intensity:**	**St Dev (d.n...**
Z-Average (d.nm):	194.7	**Peak 1:**	218.0	98.1	90.83
PdI:	0.249	**Peak 2:**	5206	1.9	470.9
Intercept:	0.907	**Peak 3:**	0.000	0.0	0.000

Result quality : Good

Sample 3: SKOV3 EVs
Measurement: 3

System

Temperature (°C):	25.0	**Duration Used (s):**	60
Count Rate (kcps):	221.2	**Measurement Position (mm):**	4.65
Cell Description:	2µL quartz batch cell	**Attenuator:**	0

Results

			Size (d.nm):	**% Intensity:**	**St Dev (d.n...**
Z-Average (d.nm):	169.8	**Peak 1:**	200.4	100.0	76.11
PdI:	0.159	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.879	**Peak 3:**	0.000	0.0	0.000

Result quality : Good

b.

EV <30nm	
minimum	15.0
maximum	26.2
mean	19.2
median	19.8
SD	3.2
Number	13
% distribution	35.14
EV 30–160 nm	
minimum	46.3
maximum	138.0
mean	81.3
median	74.4
SD	28.0
Number	20
% distribution	54.05
EV > 160nm (250 nm max.)	
minimum	177.7
maximum	238.8
mean	204,6
median	200.9
SD	22.0
Number	4
% distribution	10.81

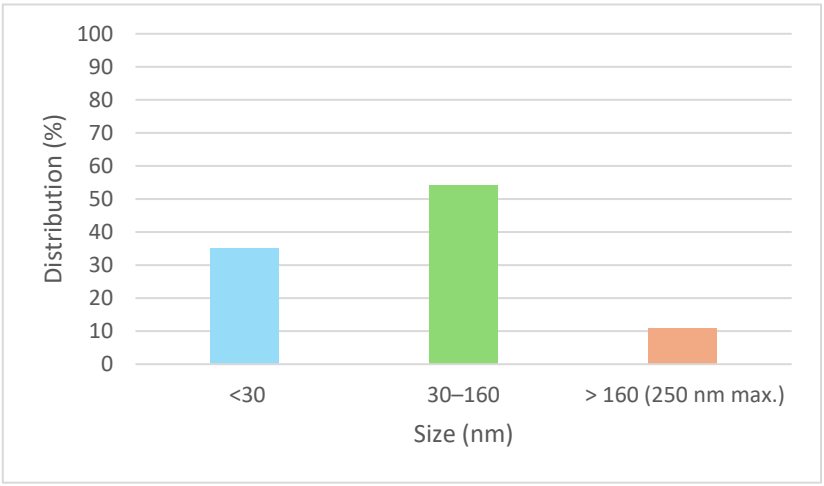


Figure S4. Characterization of EVs isolated by the ultracentrifugation – additional information. (a) the specific system settings for each DLS measurement and results for each sample. Every biological sample was measured in triplicate. (b) The size distribution in % of EVs measured by TEM (n = 37).

Table S1. List of 61 common proteins identified by all methods.

Gene symbol	Protein name
ANXA2	Annexin A2
DSP	Desmoplakin
VCAN	Versican core protein
ENO1	Alpha-enolase
HSPA8	Heat shock cognate 71 kDa protein
VIM	Vimentin
TF	Serotransferrin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
LDHA	L-lactate dehydrogenase A chain
DSG1	Desmoglein-1
EEF1A1	Elongation factor 1-alpha 1
A2M	Alpha-2-macroglobulin
APOA1	Apolipoprotein A-I
AHSG	Alpha-2-HS-glycoprotein
KPRP	Keratinocyte proline-rich protein
DCD	Dermcidin
KRT18	Keratin, type I cytoskeletal 18
	Immunoglobulin kappa light chain
AZGP1	Zinc-alpha-2-glycoprotein
CFL1	Cofilin-1
FABP5	Fatty acid-binding protein 5
APOD	Apolipoprotein D
PKM	Pyruvate kinase PKM
ALDOA	Fructose-bisphosphate aldolase A
JUP	Junction plakoglobin
TUBA1A	Tubulin alpha-1A chain
HSPA5	Endoplasmic reticulum chaperone BiP
DSC1	Desmocollin-1
APOE	Apolipoprotein E
SERPINA1	Alpha-1-antitrypsin
S100A8	Protein S100-A8
H4C1	Histone H4
AGRN	Agrin
SPTBN2	Spectrin beta chain, non-erythrocytic 2
FN1	Fibronectin
HSPG2	Basement membrane-specific heparan sulfate proteoglycan core protein
HSPA1B	Heat shock 70 kDa protein 1B
YWHAZ	14-3-3 protein zeta/delta
THBS1	Thrombospondin-1
COL1A1	Collagen alpha-1(I) chain

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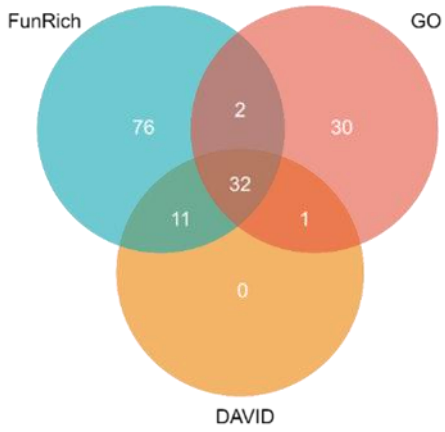
Gene symbol	Protein name
LAMC1	Laminin subunit gamma-1
FSTL1	Follistatin-related protein 1
COL18A1	Collagen alpha-1(XVIII) chain
COL5A1	Collagen alpha-1(V) chain
PLOD1	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1
ITIH2	Inter-alpha-trypsin inhibitor heavy chain H2
SPP1	Osteopontin
SERPINA4	Kallistatin
COL12A1	Collagen alpha-1(XII) chain
TGFBI	Transforming growth factor-beta-induced protein ig-h3
HSP90AB1	Heat shock protein HSP 90-beta
HSP90AA1	Heat shock protein HSP 90-alpha
MMP1	Interstitial collagenase
CLSTN1	Calsyntenin-1
MFGE8	Lactadherin
LOXL2	Lysyl oxidase homolog 2
QSOX1	Sulfhydryl oxidase 1
SERPINE1	Plasminogen activator inhibitor 1
SERPINE2	Glia-derived nexin
CST3	Cystatin-C
NID2	Nidogen-2

Table S2. Mutual terms identified by all databases. Separate analysis of gene ontology data: (a) cellular component, (b) molecular function, (c) biological process.

a	**Cellular Component**
	extracellular exosome
	extracellular region
	collagen-containing extracellular matrix
	extracellular space
	endoplasmic reticulum lumen
	basement membrane
	secretory granule lumen
	ficolin-1-rich granule lumen
	platelet alpha granule lumen
	blood microparticle
	vesicle
	melanosome
	extracellular vesicle
	cornified envelope
	desmosome
	extracellular matrix
	lysosomal lumen
	endocytic vesicle lumen
	focal adhesion
	cell surface
	fibrinogen complex
	dendritic growth cone
	tertiary granule lumen
	nuclear matrix
	chylomicron
	low-density lipoprotein particle
	ficolin-1-rich granule membrane
	platelet alpha granule
	plasma membrane
	endoplasmic reticulum
	membrane
	cytoplasm

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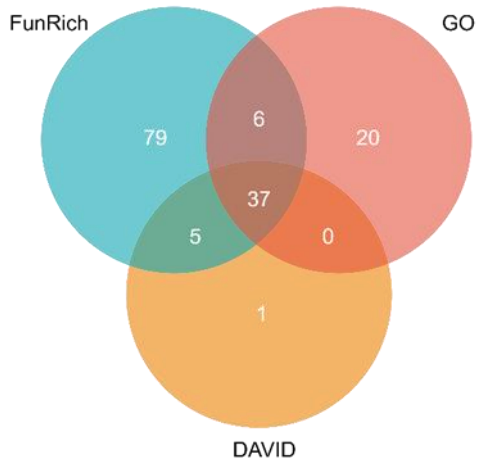
b	Molecular function
	protease binding cadherin binding serine-type endopeptidase inhibitor activity endopeptidase inhibitor activity ATP-dependent protein folding chaperone calcium ion binding heat shock protein binding MHC class II protein complex binding extracellular matrix structural constituent amyloid-beta binding proteoglycan binding identical protein binding extracellular matrix structural constituent conferring tensile strength integrin binding collagen V binding structural constituent of cytoskeleton heparin binding unfolded protein binding scaffold protein binding nitric-oxide synthase regulator activity cell adhesive protein binding involved in bundle of His cell-Purkinje myocyte communication phosphatidylcholine-sterol O-acyltransferase activator activity ATP-dependent protein disaggregase activity C3HC4-type RING finger domain binding disordered domain specific binding TPR domain binding signaling receptor binding RNA binding tau protein binding structural molecule activity platelet-derived growth factor binding protein folding chaperone ubiquitin protein ligase binding collagen binding enzyme binding lipid binding cell adhesion molecule binding

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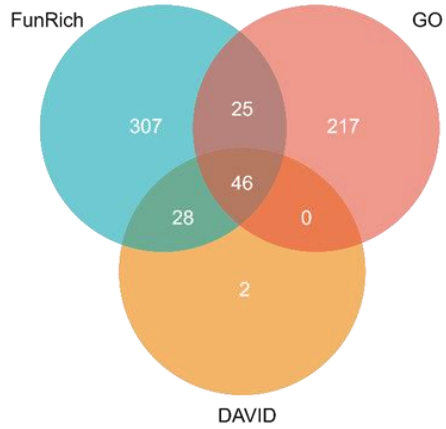
C	Biological Process
	cell adhesion collagen fibril organization angiogenesis glycolytic process cellular response to heat protein refolding negative regulation of plasminogen activation acute-phase response negative regulation of blood coagulation negative regulation of smooth muscle cell-matrix adhesion positive regulation of tau-protein kinase activity supramolecular fiber organization response to unfolded protein negative regulation of blood vessel endothelial cell migration regulation of protein-containing complex assembly peptide cross-linking positive regulation of phospholipid efflux telomerase holoenzyme complex assembly protein stabilization positive regulation of cholesterol metabolic process chaperone-mediated autophagy bundle of His cell-Purkinje myocyte adhesion involved in cell communication negative regulation of apoptotic process chaperone cofactor-dependent protein refolding defense response to fungus high-density lipoprotein particle clearance regulation of Cdc42 protein signal transduction positive regulation of CoA-transferase activity positive regulation of plasminogen activation negative regulation of protein metabolic process chronic inflammatory response high-density lipoprotein particle assembly collagen biosynthetic process regulation of ventricular cardiac muscle cell action potential positive regulation by host of viral process response to mechanical stimulus negative regulation of fibrinolysis phospholipid efflux glucose metabolic process

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C	Biological Process
	negative regulation of endopeptidase activity
	protein folding
	positive regulation of blood coagulation
	cell-cell adhesion
	regulation of protein stability
	cell migration
	extracellular matrix organization