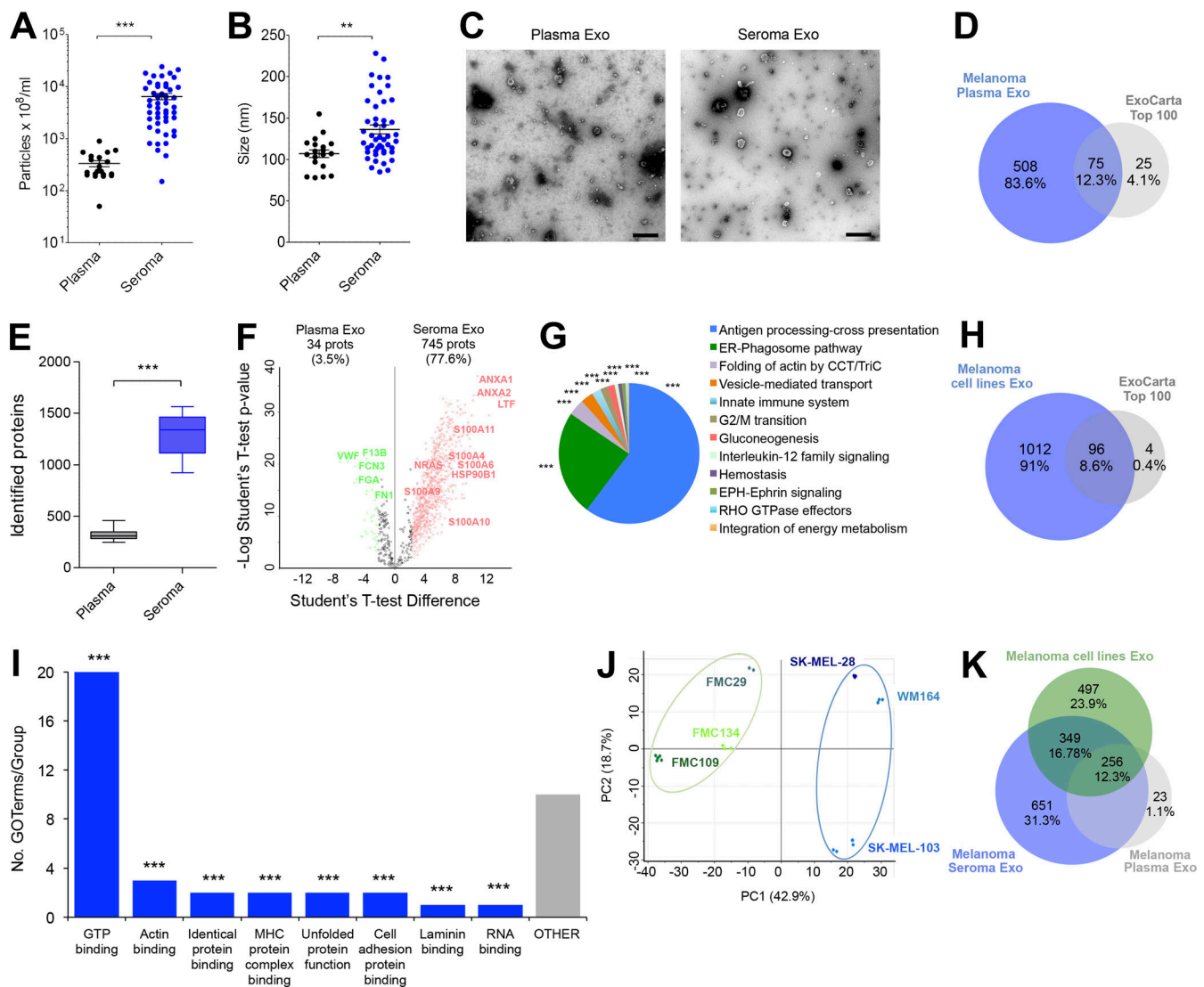# Supplemental material

Figure S1. **Characterization of ES-derived exosomes. (A and B)** Concentration of particles (A) and size (diameter, mean) of exosomes (B) purified from plasma (*n* = 20) and ES (*n* = 40) from melanoma patients. **(C)** Representative EM images of exosomes from plasma and ES. Bars, 500 nm. **(D)** Overlap between proteins in plasma-derived exosomes and the top 100 proteins in the ExoCarta database. **(E)** Number of proteins identified by LC-MS/MS in plasma- and ES-derived exosomes. **(F)** Volcano plot of differentially expressed proteins in plasma- and ES-derived exosomes. Red and green circles represent proteins differentially expressed with P < 0.05. **(G)** Reactome pathways associated with upregulated proteins in ES- compared with plasma-derived exosomes. **(H)** Venn diagram of proteins in melanoma cell line–derived exosomes overlapping with the top 100 proteins in the ExoCarta database. **(I)** Significant GOTerms (molecular function) enriched in melanoma cell line–derived exosomes. **(J)** PCA of melanocyte- and melanoma cell line–derived exosome samples. **(K)** Venn diagram of proteins in exosomes purified from ES, plasma, and melanoma cell lines. Dot plots represent mean ± SEM. ***, P < 0.001; **, P < 0.01, as determined by unpaired Student's *t* test (A and B). In pie charts, *** indicates group-corrected P < 0.001 as determined by Bonferroni stepdown test applied to pathway networks (G). Exo, exosomes; prots, proteins.
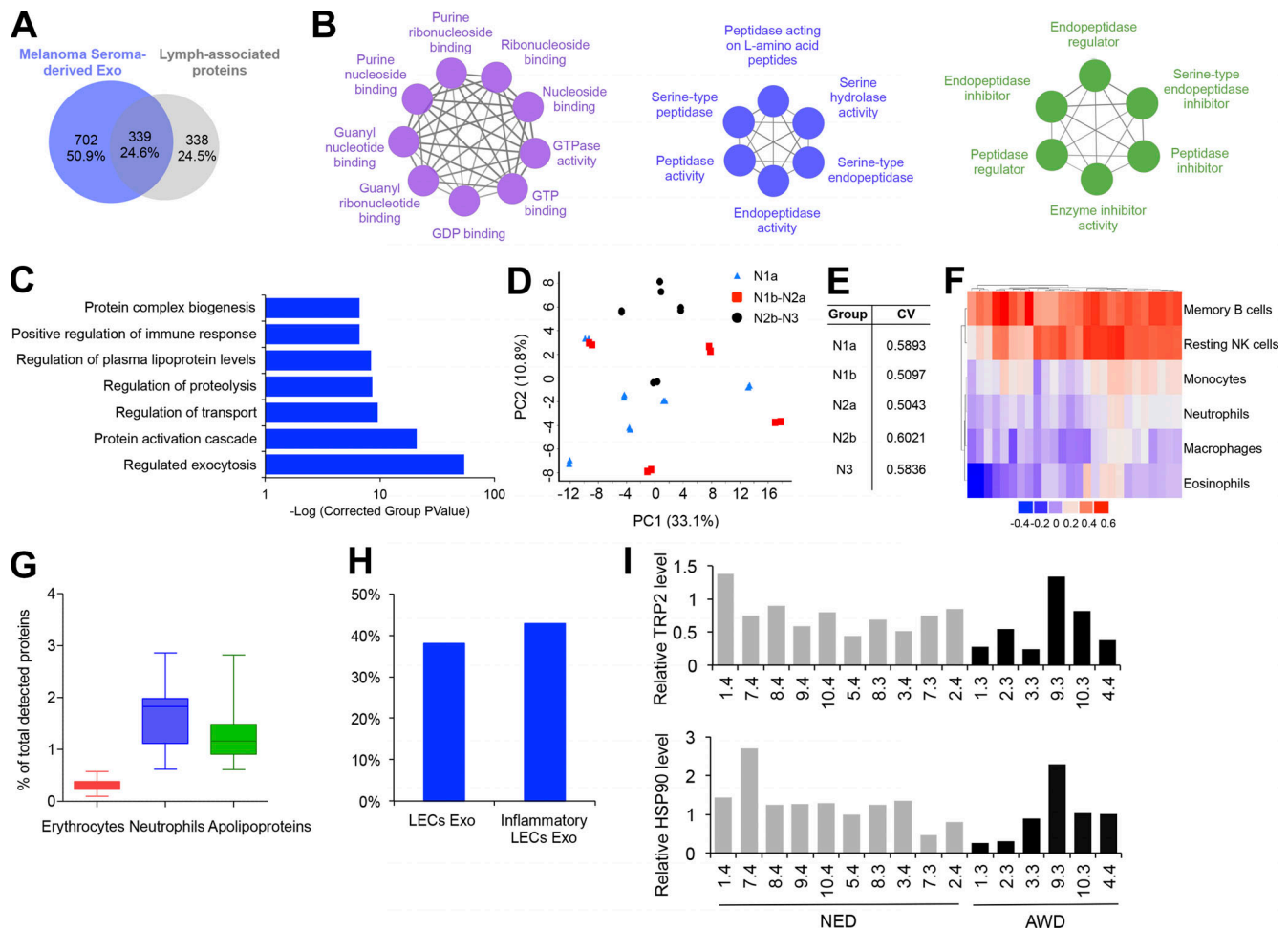
Figure S2.   **Characterization of ES-derived exosomes. (A)** Venn diagram of proteins in ES-derived exosomes and the proteins identified in human lymph. **(B)** GOTerms (molecular function) enriched in ES-derived exosomes by ClueGO and plotted with Cytoscape. **(C)** GOTerms (biological process) significantly enriched in ES-derived exosomes analyzed by ClueGO. **(D)** PCA of ES-derived exosome samples from stage III melanoma patients with different LN involvement. **(E)** Coefficients of variation (CV) for the proteomic samples grouped by substage. **(F)** Gene set enrichment analysis of ES-derived exosomes for the indicated hematopoietic cell types. **(G)** Analysis of contaminants in ES-derived EVs. **(H)** Percentage of proteins identified in human lymphatic endothelial cell exosomes in steady state (LECs Exo) and in inflammatory conditions (Inflammatory LECs Exo) that were present in ES-derived exosomes. **(I)** Quantification of Western blot shown in Fig. 3 G. AWD, alive with disease; Exo, exosomes; NED, no evidence of disease.
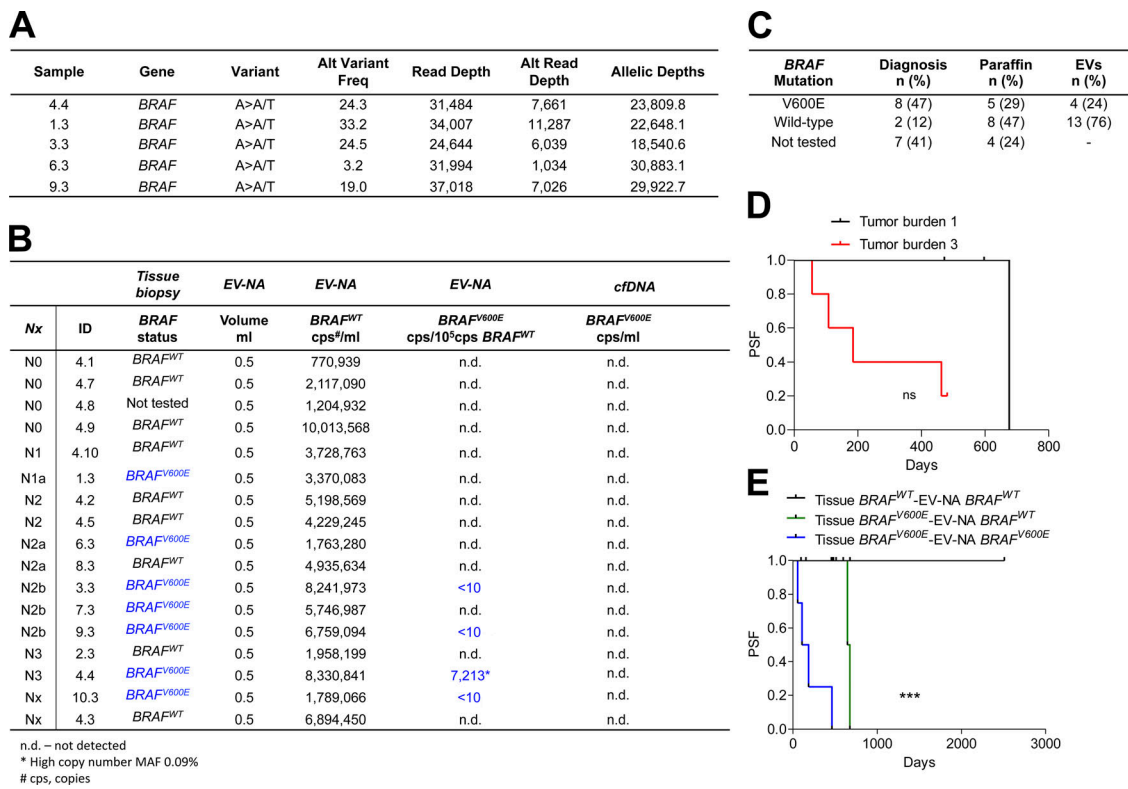
A

Sample	Gene	Variant	Alt Variant Freq	Read Depth	Alt Read Depth	Allelic Depths
4.4	BRAF	A>A/T	24.3	31,484	7,661	23,809.8
1.3	BRAF	A>A/T	33.2	34,007	11,287	22,648.1
3.3	BRAF	A>A/T	24.5	24,644	6,039	18,540.6
6.3	BRAF	A>A/T	3.2	31,994	1,034	30,883.1
9.3	BRAF	A>A/T	19.0	37,018	7,026	29,922.7

C

BRAF Mutation	Diagnosis n (%)	Paraffin n (%)	EVs n (%)
V600E	8 (47)	5 (29)	4 (24)
Wild-type	2 (12)	8 (47)	13 (76)
Not tested	7 (41)	4 (24)	-

B

		Tissue biopsy	EV-NA	EV-NA	EV-NA	cfDNA
Nx	ID	$BRAF$ status	Volume ml	$BRAF^{WT}$ cps#/ml	$BRAF^{V600E}$ cps/10⁵cps $BRAF^{WT}$	$BRAF^{V600E}$ cps/ml
N0	4.1	$BRAF^{WT}$	0.5	770,939	n.d.	n.d.
N0	4.7	$BRAF^{WT}$	0.5	2,117,090	n.d.	n.d.
N0	4.8	Not tested	0.5	1,204,932	n.d.	n.d.
N0	4.9	$BRAF^{WT}$	0.5	10,013,568	n.d.	n.d.
N1	4.10	$BRAF^{WT}$	0.5	3,728,763	n.d.	n.d.
N1a	1.3	$BRAF^{V600E}$	0.5	3,370,083	n.d.	n.d.
N2	4.2	$BRAF^{WT}$	0.5	5,198,569	n.d.	n.d.
N2	4.5	$BRAF^{WT}$	0.5	4,229,245	n.d.	n.d.
N2a	6.3	$BRAF^{V600E}$	0.5	1,763,280	n.d.	n.d.
N2a	8.3	$BRAF^{WT}$	0.5	4,935,634	n.d.	n.d.
N2b	3.3	$BRAF^{V600E}$	0.5	8,241,973	<10	n.d.
N2b	7.3	$BRAF^{V600E}$	0.5	5,746,987	n.d.	n.d.
N2b	9.3	$BRAF^{V600E}$	0.5	6,759,094	<10	n.d.
N3	2.3	$BRAF^{WT}$	0.5	1,958,199	n.d.	n.d.
N3	4.4	$BRAF^{V600E}$	0.5	8,330,841	7,213*	n.d.
Nx	10.3	$BRAF^{V600E}$	0.5	1,789,066	<10	n.d.
Nx	4.3	$BRAF^{WT}$	0.5	6,894,450	n.d.	n.d.

n.d. – not detected
* High copy number MAF 0.09%
# cps, copies

D

E

Figure S3. **Detection of the $BRAF^{V600E}$ mutation in melanoma patient samples. (A)** $BRAF$ status and the percentage of allelic variation in LN paraffin sections obtained by deep sequencing in patients when available. **(B)** $BRAF^{WT}$ and $BRAF^{V600E}$ copies found in ES-derived EV-NAs and cfDNA by quantitative PCR. **(C)** Summary of analyzed patients. **(D)** Kaplan–Meier plot showing PSF of $BRAF^{V600E}$ patients classified according to the tumor burden criteria specified in Fig. 4 B. **(E)** Kaplan–Meier curve showing PFS of patients stratified according to $BRAF$ status both in tissue and liquid biopsy. Mantel-Cox log-rank test was applied to the survival curves. ns, not significant, P = 0.0532 (D), and ***, P < 0.0001 (E). cps, copies; MAF, mutant allele frequency.

Table S1. **Clinicopathologic characteristics of melanoma patients for matched plasma and ES samples**

Nx	CNIO ID	Age (yr)	T	Primary tumor location	LN.D. location	Metastatic LNs (n)	Tumor burden[a]
N1a	5.1	73	T3a	Back	Axillary	1	No
N0	5.2	38	T3b	Breast	Axillary	0	1
N1a	5.3	75	T3b	Leg (shank)	Inguinal	1	1
N0	5.4	64	T4	Leg (thigh)	Inguinal	0	1
N0	5.5	58	T3b	Loins	Axillary	0	1
N1b	5.6	66	T3a	Arm	Axillary	1	No
N2b	5.7	72	T1b	Abdomen	Axillary	3	1
N3a	5.8	68	T3b	Back	Axillary	4	1
N2b	5.9	35	T4b	Leg (thigh)	Inguinal	2	3
N3b	5.10	85	T3b	Leg (shank)	Inguinal	4	3
N1b	5.11	70	T3b	Chest	Axillary	1	3

CNIO, Spanish National Cancer Research Center; LN.D. location, lymphadenectomy location; Nx, AJCC stage; T, tumor classification.
[a]Tumor burden: 1, low (micrometastases detected in sLN biopsy only); 3, high (clinically detected macrometastases).

Table S2. **Clinicopathologic characteristics of melanoma patients for independent plasma and ES samples**

Variable	Value
Age (median), yr	61
Primary tumor stage	No. of patients
T1	5
T2	9
T3	9
T4	10
Tx	7
LN involvement	No. of patients
N0	4
N1	15
N2	11
N3	8
Nx	2
Primary tumor location	No. of patients
Arm	5
Back	4
Head	4
Heel	2
Leg	3
Neck	1
Thorax/shoulder	4
Thigh	1
Trunk	9
Location of lymphadenectomy	No. of patients
Axillary	24
Inguinal	10
Neck	5
Unknown	1
Number of metastatic LNs	No. of patients
0	6
1	15
2	7
>3	10
Extracapsular involvement	No. of patients
No	28
Yes	10
Unknown	2
BRAF status at diagnosis	No. of patients
V600−	4
V600+	14
V600E+	3
Not tested	19

Table S6. **Clinicopathologic characteristics of the melanoma patients included in the proteomic profile of ES samples with different LN involvement**

Nx	CNIO ID	Age (yr)	T	Primary tumor location	LN.D. Location	Metastatic LNs (n)	Extracapsular involvement	Excised nodes (n)
N1a	2.2	33	T3a	Thorax/shoulder	Axillary	1	No	9
N1a	3.2	71	T4b	Back	Axillary	1	No	13
N1a	7.2	61	T1a	Back	Axillary	1	No	15
N1a	1.3	54	T3b	Head	Iliac-inguinal	1	No	14
N1b	4.2	85	T1b	Arm	Axillary	1	No	12
N1b	5.2	67	Tx	N.D.	Axillary	1	No	18
N2	5.3	71	T2a	Trunk	Axillary	N.D.	No	N.D.
N2a	4.3	49	T2a	L. arm	Axillary	2	No	12
N2a	6.3	40	T2a	Trunk	Axillary	2	No	13
N2a	8.3	70	T3a	Trunk	Axillary	3	Yes	12
N2b	11.2	46	T2a	Arm	Axillary	2	Yes	18
N2b	3.3	57	Tx	N.D.	Axillary	2	Yes	6
N3	8.2	59	T4a	Thorax	Axillary	18	Yes	32
N3	2.3	69	T3b	Back	Axillary	4	No	14

Gray shading indicates that patients were used also for *BRAF*[V600E] analysis. CNIO, Spanish National Cancer Research Center; L., left; LN.D. location, lymphadenectomy location; N.D., not determined/unknown; Nx, AJCC stage; T, tumor classification.

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Table S10. **Proteins differentially expressed in melanoma AJCC stage III LNs found in melanoma patient–derived seroma exosomes**

Entry	Entry name	Gene	Protein names
P23526	SAHH_HUMAN	AHCY	Adenosylhomocysteinase
Q09666	AHNK_HUMAN	AHNAK	Neuroblast differentiation-associated protein AHNAK
P04083	ANXA1_HUMAN	ANXA1	Annexin A1
P01024	CO3_HUMAN	C3	Complement C3
P27797	CALR_HUMAN	CALR	Calreticulin
P78371	TCPB_HUMAN	CCT2	T-complex protein 1 subunit beta
P10909	CLUS_HUMAN	CLU	Clusterin
P02452	CO1A1_HUMAN	COL1A1	Collagen alpha-1
Q14019	COTL1_HUMAN	COTL1	Coactosin-like protein
Q08211	DHX9_HUMAN	DHX9	ATP-dependent RNA helicase A
Q96C19	EFHD2_HUMAN	EFHD2	EF-hand domain-containing protein D2
P60842	IF4A1_HUMAN	EIF4A1	Eukaryotic initiation factor 4A-I
O75367	H2AY_HUMAN	H2AFY	Core histone macro-H2A.1
P61978	HNRPK_HUMAN	HNRNPK	Heterogeneous nuclear ribonucleoprotein K
Q00839	HNRPU_HUMAN	HNRNPU	Heterogeneous nuclear ribonucleoprotein U
P00738	HPT_HUMAN	HP	Haptoglobin
P02790	HEMO_HUMAN	HPX	Hemopexin
P08238	HS90B_HUMAN	HSP90AB1	Heat shock protein HSP 90-beta
P11142	HSP7C_HUMAN	HSPA8	Heat shock cognate 71-kD protein
P35527	K1C9_HUMAN	KRT9	Keratin
P13796	PLSL_HUMAN	LCP1	Plastin-2
P00338	LDHA_HUMAN	LDHA	L-lactate dehydrogenase A chain
P07195	LDHB_HUMAN	LDHB	L-lactate dehydrogenase B chain
P33241	LSP1_HUMAN	LSP1	Lymphocyte-specific protein 1
P51884	LUM_HUMAN	LUM	Lumican
P29966	MARCS_HUMAN	MARCKS	Myristoylated alanine-rich C-kinase substrate
P40926	MDHM_HUMAN	MDH2	Malate dehydrogenase
P26038	MOES_HUMAN	MSN	Moesin
P19105	ML12A_HUMAN	MYL12A	Myosin regulatory light-chain 12A
P07237	PDIA1_HUMAN	P4HB	Protein disulfide-isomerase
Q99497	PARK7_HUMAN	PARK7	Protein/nucleic acid deglycase DJ-1
P30048	PRDX3_HUMAN	PRDX3	Thioredoxin-dependent peroxide reductase
P10644	KAP0_HUMAN	PRKAR1A	cAMP-dependent protein kinase type I-alpha regulatory subunit
P14314	GLU2B_HUMAN	PRKCSH	Glucosidase 2 subunit beta
Q06323	PSME1_HUMAN	PSME1	Proteasome activator complex subunit 1
P62826	RAN_HUMAN	RAN	GTP-binding nuclear protein Ran
P23396	RS3_HUMAN	RPS3	40S ribosomal protein S3
P31949	S10AB_HUMAN	S100A11	Protein S100-A11
P01009	A1AT_HUMAN	SERPINA1	Alpha-1-antitrypsin
P04179	SODM_HUMAN	SOD2	Superoxide dismutase [Mn]
P53999	TCP4_HUMAN	SUB1	Activated RNA polymerase II transcriptional coactivator p15
P37802	TAGL2_HUMAN	TAGLN2	Transgelin-2
O15533	TPSN_HUMAN	TAPBP	Tapasin
P02787	TRFE_HUMAN	TF	Serotransferrin

Table S10. **Proteins differentially expressed in melanoma AJCC stage III LNs found in melanoma patient–derived seroma exosomes (*Continued*)**

Entry	Entry name	Gene	Protein names
P19971	TYPH_HUMAN	TYMP	Thymidine phosphorylase
P13010	XRCC5_HUMAN	XRCC5	X-ray repair cross-complementing protein 5
P12956	XRCC6_HUMAN	XRCC6	X-ray repair cross-complementing protein 6
P63104	1433Z_HUMAN	YWHAZ	14-3-3 protein zeta/delta
Q92820	GGH_HUMAN	GGH	Gamma-glutamyl hydrolase
O75431	MTX2_HUMAN	MTX2	Metaxin-2
P09874	PARP1_HUMAN	PARP1	Poly [ADP-ribose] polymerase 1
P30046	DOPD_HUMAN	DDT	D-dopachrome decarboxylase
Q99798	ACON_HUMAN	ACO2	Aconitate hydratase
Q15233	NONO_HUMAN	NONO	Non-POU domain–containing octamer-binding protein
P35580	MYH10_HUMAN	MYH10	Myosin-10
Q99832	TCPH_HUMAN	CCT7	T-complex protein 1 subunit eta
P22087	FBRL_HUMAN	FBL	rRNA 2′-O-methyltransferase fibrillarin
Q9Y3E5	PTH2_HUMAN	PTRH2	Peptidyl-tRNA hydrolase 2
Q15121	PEA15_HUMAN	PEA15	Astrocytic phosphoprotein PEA-15
P78417	GSTO1_HUMAN	GSTO1	Glutathione S-transferase omega-1
O75368	SH3L1_HUMAN	SH3BGRL	SH3 domain-binding glutamic acid-rich–like protein
P23381	SYWC_HUMAN	WARS	Tryptophan–tRNA ligase
P28838	AMPL_HUMAN	LAP3	Cytosol aminopeptidase
P42224	STAT1_HUMAN	STAT1	Signal transducer and activator of transcription 1-alpha/beta
Q07021	C1QBP_HUMAN	C1QBP	Complement component 1 Q subcomponent-binding protein
P24821	TENA_HUMAN	TNC	Tenascin
Q9ULZ3	ASC_HUMAN	PYCARD	Apoptosis-associated speck-like protein containing a CARD
P99999	CYC_HUMAN	CYCS	Cytochrome c
Q27J81	INF2_HUMAN	INF2	Inverted formin-2
Q53HJ8	Q53HJ8_HUMAN	Unknown	PKCI-1–related HIT protein variant
Q9UL46	PSME2_HUMAN	PSME2	Proteasome activator complex subunit 2
P07093	GDN_HUMAN	SERPINE2	Glia-derived nexin
P55008	AIF1_HUMAN	AIF1	Allograft inflammatory factor 1

Gray shaded rows indicate common proteins between ES exosomes and differentially expressed proteins in LNs from poor and good prognosis patients described in Mactier et al. (2014). Unshaded rows indicate proteins exclusively found in Mactier et al. (2014).

Table S11.   **Prognostic markers in melanoma AJCC stage III found in melanoma patient–derived seroma exosomes**

Entry	Entry name	Protein names	Gene names
Q15063	POSTN_HUMAN	Periostin (PN)	POSTN
P08238	HS90B_HUMAN	Heat shock protein HSP 90-beta (HSP 90)	HSP90AB1
P11142	HSP7C_HUMAN	Heat shock cognate 71-kD protein	HSPA8
P60842	IF4A1_HUMAN	Eukaryotic initiation factor 4A-I	EIF4A1
P23526	SAHH_HUMAN	Adenosylhomocysteinase (AdoHcyase)	AHCY
P12956	XRCC6_HUMAN	X-ray repair cross-complementing protein 6	XRCC6
P13010	XRCC5_HUMAN	X-ray repair cross-complementing protein 5	XRCC5
P00338	LDHA_HUMAN	L-lactate dehydrogenase A chain (LDH-A)	LDHA
Q08211	DHX9_HUMAN	ATP-dependent RNA helicase A	DHX9
P40227	TCPZ_HUMAN	T-complex protein 1 subunit zeta	CCT6A
P06576	ATPB_HUMAN	ATP synthase subunit beta, mitochondrial	ATP5F1B
P27797	CALR_HUMAN	Calreticulin (CRP55)	CALR
P04179	SODM_HUMAN	Superoxide dismutase	SOD2
P10909	CLUS_HUMAN	Clusterin	CLU
Q06323	PSME1_HUMAN	Proteasome activator complex subunit 1	PSME1
O75367	H2AY_HUMAN	Core histone macro-H2A.1	H2AFY
P13796	PLSL_HUMAN	Plastin-2	LCP1
P35527	K1C9_HUMAN	Keratin, type I cytoskeletal 9	KRT9
P09874	PARP1_HUMAN	Poly [ADP-ribose] polymerase 1 (PARP-1)	PARP1
P17844	DDX5_HUMAN	Probable ATP-dependent RNA helicase DDX5	DDX5
Q99798	ACON_HUMAN	Aconitate hydratase	ACO2
Q9UL46	PSME2_HUMAN	Proteasome activator complex subunit 2	PSME2
Q9ULZ3	ASC_HUMAN	Apoptosis-associated speck-like protein containing a CARD (hASC)	PYCARD

Gray shaded rows indicate common proteins between ES exosomes and prognostic candidates in LNs from poor and good prognosis patients described in Mactier et al. (2014). Unshaded rows indicate proteins exclusively found in Mactier et al. (2014).

Table S12. **Details of the cohort of patients included in the proteomic analysis for different LN involvement and the analysis of *BRAF^V600E* in EV-NAs**

Nx	CNIO ID	Age (yr)	T	Primary tumor location	LN.D. location	Metastatic LNs (n)	Extracapsular involvement	Excised nodes (n)
N0	4.1	60	T1a	Back	Axillary L.	0	No	2
N0	4.7	60	T1b	N.D.	Axillary L.	0	No	3
N0	4.8	86	T1b	L. leg	Inguinal	0	No	3
N0	4.9	57	T2a	Shoulder	Axillary	0	No	4
N1	4.10	73	T2a	L. arm	Axillary	1	No	5
N1a	10.1	31	T2a	Trunk	Axillary	1	No	12
N1a	1.2	72	T4b	Lower Leg	Inguinal	1	No	10
N1a	2.2	33	T3a	Thorax/shoulder	Axillary	1	No	9
N1a	3.2	71	T4b	Back	Axillary	1	No	13
N1a	6.2	44	T3b	Head	Neck	1	No	22
N1a	7.2	61	T1a	Back	Axillary	1	No	15
N1a	10.2	56	T3b	Arm	Axillary	1	No	8
N1a	1.3	54	T3b	Head	Iliac-inguinal	1	No	14
N1b	5.1	63	Tx	N.D.	Iliac-inguinal	1	Yes	16
N1b	4.1	72	T4b	Trunk	Iliac-inguinal	1	No	9
N1b	6.1	37	Tx	N.D.	Iliac-inguinal	1	N.D.	7
N1b	4.2	85	T1b	Arm	Axillary	1	No	12
N1b	5.2	67	Tx	N.D.	Axillary	1	No	18
N1b	9.2	67	T4b	Thigh	Iliac-inguinal	1	No	14
N2	5.3	71	T2a	Trunk	Axillary	N.D.	No	N.D.
N2	4.2	28	T2	R. shoulder	Axillary R.	2	No	2
N2	4.5	58	T2b	L. heel	N.D.	N.D.	No	2
N2a	4.3	49	T2a	L. arm	Axillary	2	No	12
N2a	6.3	40	T2a	Trunk	Axillary	2	No	13
N2a	8.3	70	T3a	Trunk	Axillary	3	Yes	12
N2b	8.1	61	T4a	Trunk	Axillary	3	Yes	10
N2b	11.2	46	T2a	Arm	Axillary	2	Yes	18
N2b	3.3	57	Tx	N.D.	Axillary	2	Yes	6
N2b	7.3	62	Tx	Neck	Neck	2	No	10
N2b	9.3	64	T4b	Trunk	Iliac-inguinal	2	Yes	10
N3	1.1	81	T4a	Head	Neck bilateral	7	Yes	28
N3	2.1	31	Tx	N.D.	Neck L.	4	No	14
N3	3.1	52	T3a	Trunk	Axillary bilateral	6	No	31
N3	7.1	88	T4b	Lower leg	Inguinal	7	No	10
N3	9.1	82	T3b	Trunk	Axillary	5	Yes	N.D.
N3	8.2	59	T4a	Thorax	Axillary	18	Yes	32
N3	2.3	69	T3b	Back	Axillary	4	No	14
N3	4.4	74	Tx	N.D.	Axillary R.	15	Yes	39
Nx	10.3	45	T4b	Head	Neck	N.D.	N.D.	N.D.
Nx	4.3	73	T3	R. heel	Inguinal	0	No	2

Patients in gray shading were used for *BRAF^V600E* analysis in EV-NAs. CNIO, Spanish National Cancer Research Center; L., left; LN.D. location, lymphadenectomy location; N.D., not determined/unknown; Nx, AJCC stage; R., right; T, tumor classification.

Tables S3, S4, S5, S7, S8, and S9 are provided online as separate PDF files. Table S3 lists proteins identified by LC/MS-MS in EVs from plasma and ES paired samples and in three human melanoma cell lines. Table S4 lists proteins identified in melanoma cell line–derived exosomes. Table S5 lists differentially expressed proteins between melanoma and melanocyte–derived exosomes. Table S7 lists proteins found in melanoma patient seroma–derived exosomes. Table S8 lists proteins found in melanoma patient ES–derived exosomes not found in human lymph data sets. Table S9 lists proteins upregulated in ES-derived exosomes from N3 versus N1a melanoma patients not found in human lymph datasets.

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Mactier, S., K.L. Kaufman, P. Wang, B. Crossett, G.M. Pupo, P.L. Kohnke, J.F. Thompson, R.A. Scolyer, J.Y. Yang, G.J. Mann, and R.I. Christopherson. 2014. Protein signatures correspond to survival outcomes of AJCC stage III melanoma patients. *Pigment Cell Melanoma Res.* 27:1106–1116. https://doi.org/10.1111/pcmr.12290