Supplementary Information

Heat Shock Protein A2 is a novel extracellular vesicle-associated protein

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Table S1. List of reference genes used in qPCR analysis.

Cell Line	Treatment	Analyzed gene	Reference genes
NCI-H1299	MA	HSPA2	*B2M, RPL13*
	MA	HSPA1	*B2M, RPL13,TMEM43*
	MG132	HSPA2	*RPL13,TMEM43*
	MG132	HSPA1	*RPL13,TMEM43*
	BTZ	HSPA2	*B2M*
	BTZ	HSPA1	*RPL13,TMEM43*
NCI-H23	MA	HSPA2	*RPL13*
	BTZ	HSPA2	*RPL13,TMEM43*
MCF7	MG132	HSPA2	*TMPR1, B2M*

Abbreviations: BTZ, bortezomib; MA, manumycin A; *RPL13*, Ribosomal Protein L13; *B2M*, Beta-2-Microglobulin; *TMEM43*, Transmembrane Protein 43.

Table S2. List of antibodies used in Western blot and EVs immunocapture analyses.

	Host/Clonality	Clone	Catalog Number/ RRID	Source	Dilution
Western blot					
HSPA1	Mo/M	C92F3A-5	ADI-SPA-810-F/AB_311860	Enzo, Life Sciences, Famingdale, NY, USA	1:5000 or 1:3000 for EVs samples
HSPA2	Rb/M	EPR4596	Ab108416/AB_10862351	Abcam, Cambridge, UK	1:5000 or 1:3000 for EVs samples
HSPA5	Mo/M	A-10	Sc-376768/nd	Santa Cruz Biotechnology, Inc., Dallas, USA	1:1000
HSPA8	Mo/M	B-6	Sc-7298/AB_627761	Santa Cruz Biotechnology, Inc., Dallas, USA	1:7500 or 1:3000 for EVs samples
HSPC	Mo/M	AC88	ADI-SPA-830-F/AB_11181197	Enzo, Life Sciences, Famingdale, NY, USA	1:2000
p62	Mo/M	D-3	Sc-28359/AB_628279	Santa Cruz Biotechnology, Inc., Dallas, USA	1:2000
β-actin (HRP)	Mo/M	AC15	A3854/AB_262011	Merck KGaA, Darmstadt, Germany	1:20000
CD63	Mo/M	MX-49.129.5	sc-5275/ AB_627877	Santa Cruz Biotechnology, Inc., Dallas, USA	1:1500
CD63[a]	Rb/M	SY21-02	MA5-32085/ AB_2809379	Thermo Fisher Scientific, Waltham, USA	1:1000
CD81	Mo/M	1.3.3.22	orb388959	Biorbyt Ltd.5, Cambridge, United Kingdom	1:500
CD81[a]	Rb/M	D3N2D	56039	Cell Signaling Technology, Danvers, USA	1:800
CD9	Mo/M	C-4	sc-13118/ AB_627213	Santa Cruz Biotechnology, Inc., Dallas, USA	1:800

CD9[a]	Rb/M	D8O1A	3174/ AB_2798139	Cell Signaling Technology, Danvers, USA	1:1000
TSG101	Mo/M	51	612697/ AB_399937	BD Biosciences, Franklin Lakes, USA	1:1000
TSG101	Rb/M	JJ0900	MA5-32463/ AB_2809740	Thermo Fisher Scientific, Waltham, USA	1:1000
PSMA	Rb/M	D4S1F	12702/ AB_2797998	Cell Signaling Technology, Danvers, USA	1:1000
GM-130	Rb/M	D6B1	12480/ AB_2797933	Cell Signaling Technology, Danvers, USA	1:1000
Secondary					
Anti-Mo IgG (HRP)	Go		AP124P/AB	Millipore, Billerica, MA, USA	1:5000
Anti-Ra IgG (HRP)	Go		AP132P/AB	Millipore, Billerica, MA, USA	1:2000
Anti-Mo IgG (HRP)	Go		31430/AB_228307	Thermo Fisher Scientific, Waltham, USA	1:10000
Anti-Ra IgG (HRP)	Go		111-035-003/ AB_2313567	Jackson ImmunoResearch Laboratories Inc., Ely, Cambridgeshire,, UK	1:10000

EVs immunocapture:

	Host/Clonality	**Clone**	**Catalog Number/ RRID**	**Source**	**Conjugate**
CD63	Mo/M	C047-1	MEX002-6/ nd	Medical & Biological Laboratories (MBL), Nagoya, Japan	Biotin
CD9	Mo/M	A100-4	MEX001-6/ nd	Medical & Biological Laboratories (MBL), Nagoya, Japan	Biotin
CD81	Mo/M	A103-10	MEX003-6/ nd	Medical & Biological Laboratories (MBL), Nagoya, Japan	Biotin
PSMA (FOLH1)	Mo/M	LNI-17	342510/ AB_2563243	BioLegend, San Diego, USA	Biotin

Abbreviations: RRID, Research Resource Identifier; EVs, Extracellular vesicles; M, monoclonal; Go, goat; Mo, mouse; nd, no data; Rb, Rabbit; HRP, horseradish peroxidase, [a] – antibodies used in Western Blot analysis of proteins in EVs isolated by immunocapture method

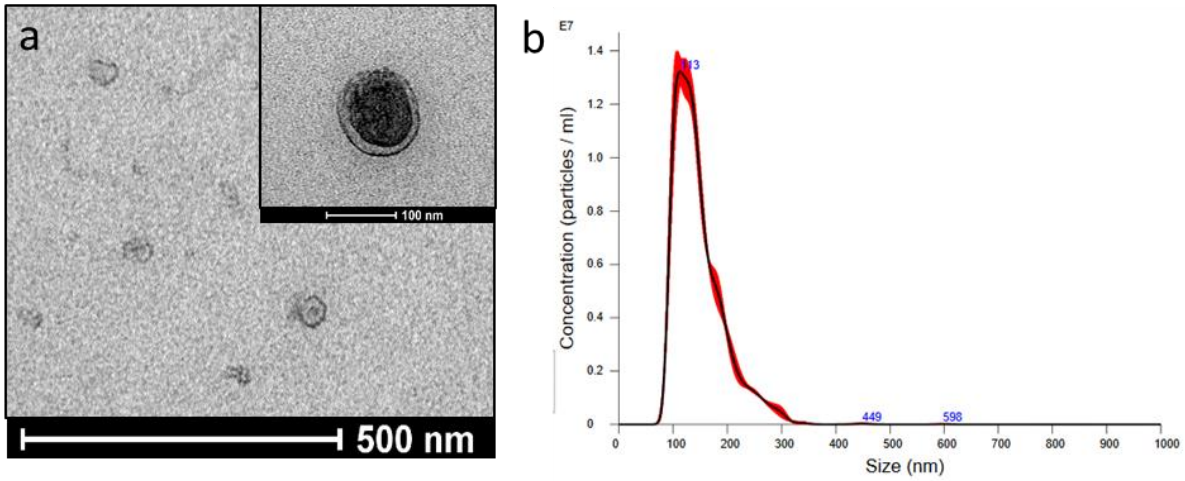


Figure S1. Characteristic of urinary EVs isolated by size exclusion chromatography. **(a)** Representative images from transmission electron microscopy. Two images with different magnifications are shown. Scale bar has been marked under the picture. **(b)** Representative histogram of particle size distribution of urinary EVs, mode: 112,3 nm.

Directly after size exclusion chromatography suspension containing urinal EVs fraction was mixed with equal volume of 4% PFA and stored at 4ºC until analysis in transmission electron microscope (TEM). For TEM analysis 5µl of the mixture was deposited onto a carbon-coated EM grid and incubated for 20 min in a dry environment. Subsequently, the grid has been washed for 2 min in PBS droplet placed on a sheet of Parafilm (Bemis Co. Inc., Neenah, WI). Afterwards, grid was successively transferred to the droplets of 1% glutaraldehyde (5 min,) and distilled water (2 min, 7 times), uranyl-oxalate solution (pH 7; 5 min) and methyl-cellulose-UA (10 min) prepared by mixing a 4% uranyl acetate solution and a 2% methyl-cellulose solution at the ratio of 1:9 (v/v). Finally the air-dried grids were visualized using a Tecnai G2 Spirit BioTWIN (FEI Companye, Hillsboro, OR) at 120-kV acceleration.

Fig. 1A

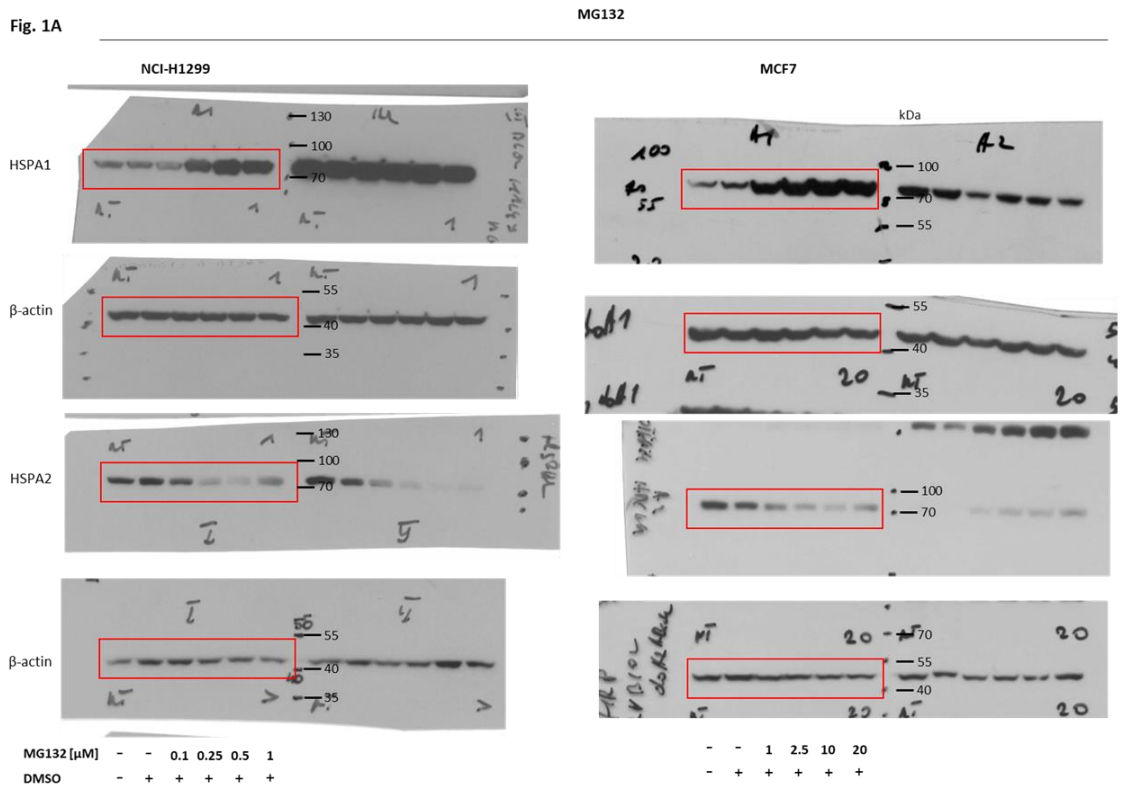
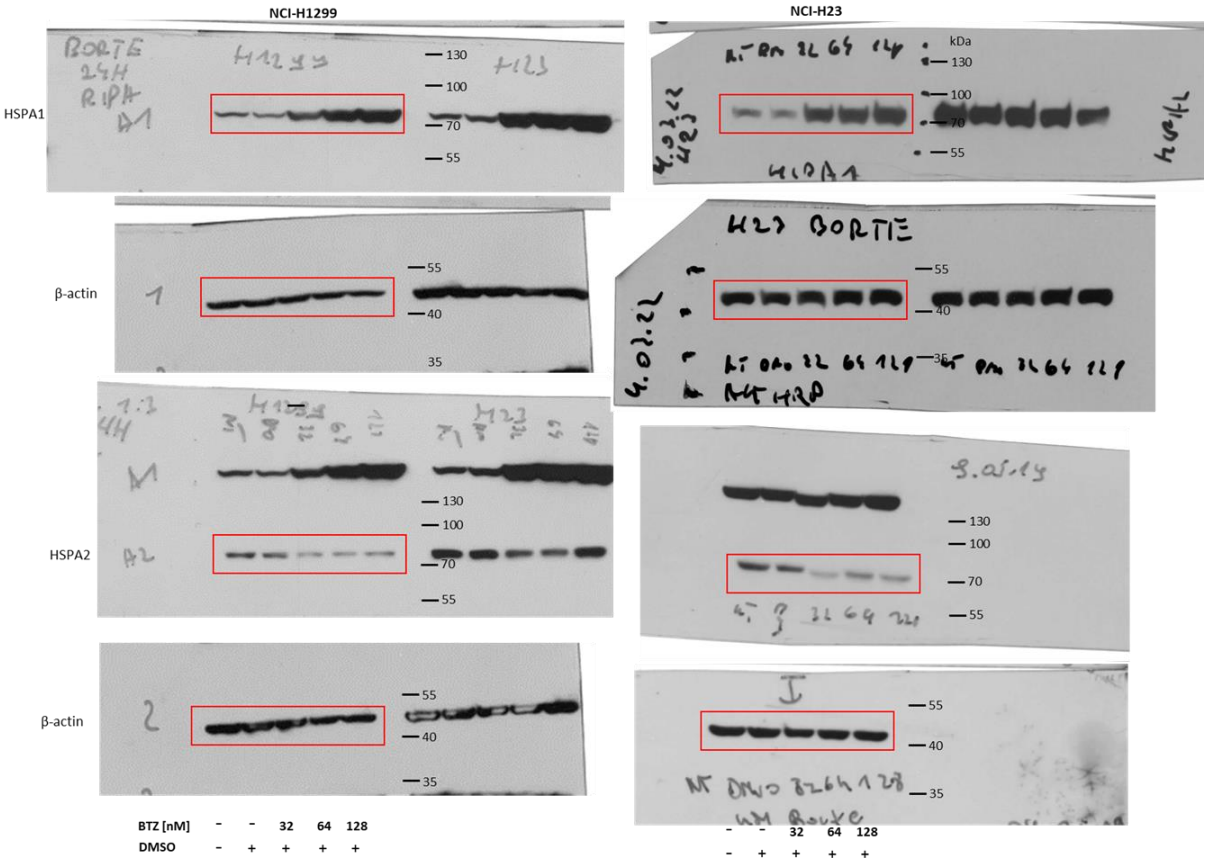

MG132
NCI-H1299
MCF7
HSPA1
β-actin
HSPA2
β-actin
kDa
MG132 [µM] - - 0.1 0.25 0.5 1
DMSO - + + + + +
- - 1 2.5 10 20
- + + + + +

Fig. 1B


Bortezomib
NCI-H1299
NCI-H23
HSPA1
β-actin
HSPA2
β-actin
kDa
BTZ [nM] - - 32 64 128
DMSO - + + + +
- - 32 64 128
- + + + +

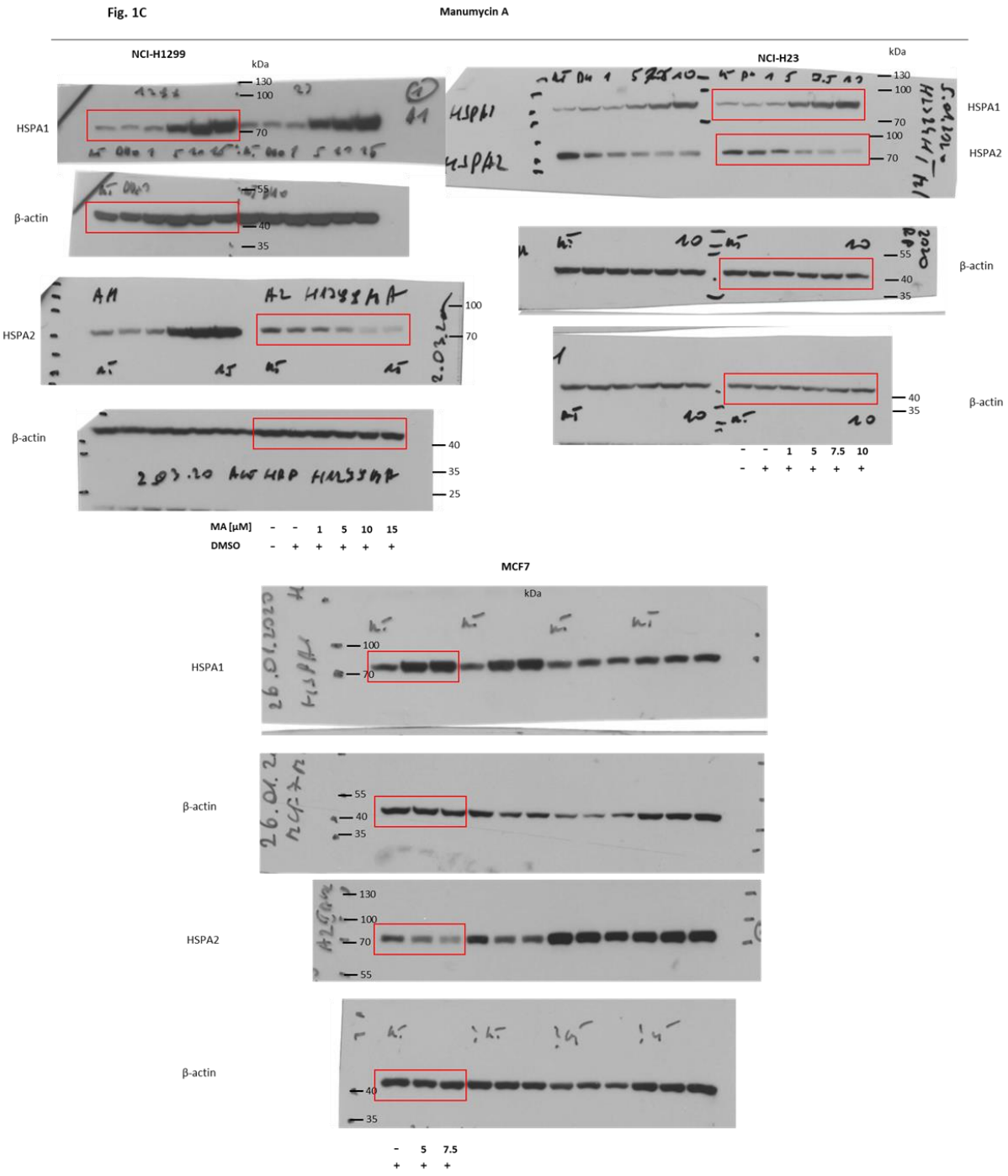


Figure S2. Unprocessed original scans of autoradiograms (immunoblots) included in Fig. 1 A-C. After protein transfer, membranes were cut according to 55 kDa molecular ladder band and fragments were incubated with appropriate primary antibodies. X-ray film was used for detection of chemiluminescent signal (the membrane edges are not visible on developed film due to detection of strong and specific signal)**.** Red lines identify cropped areas.

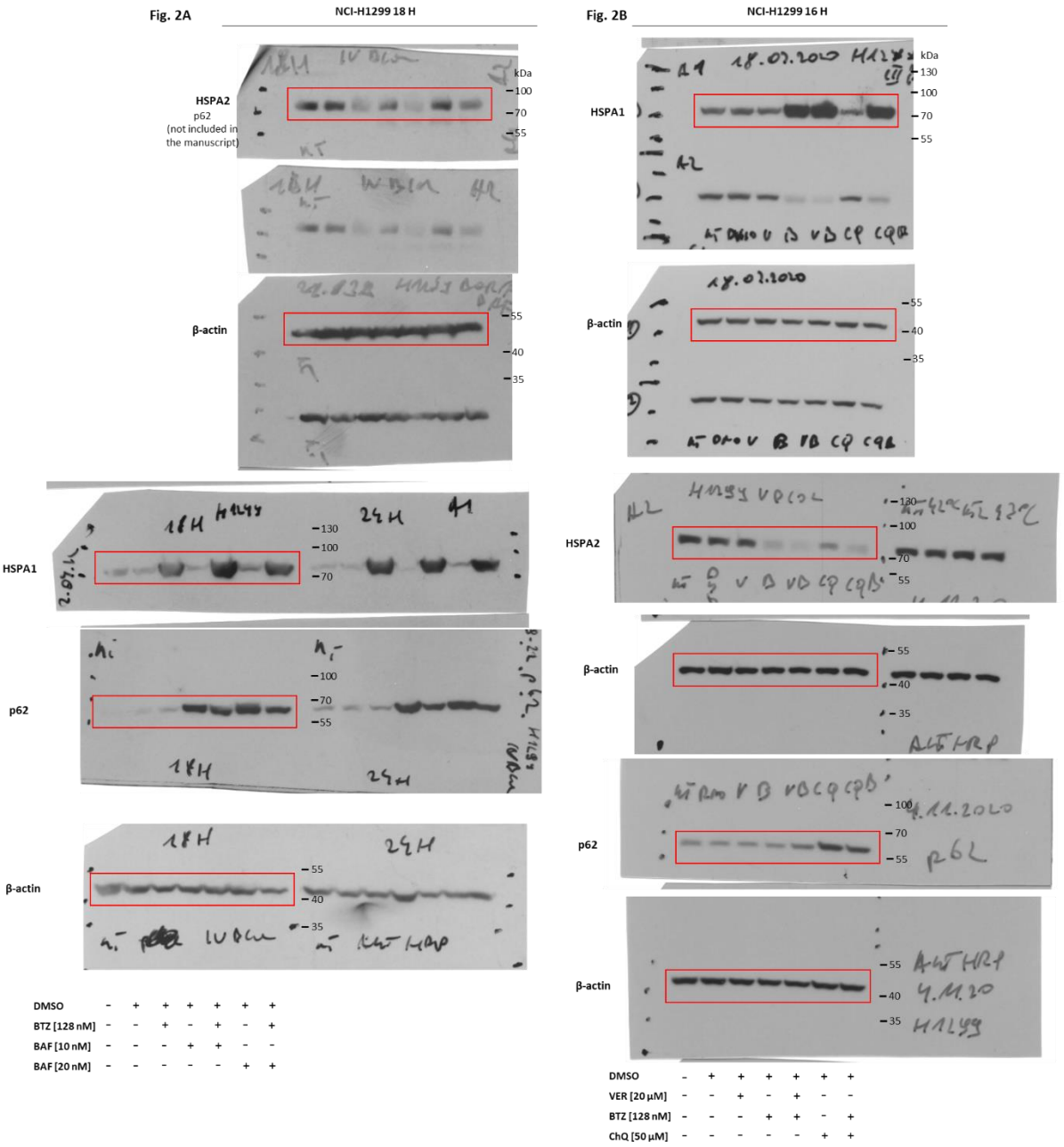


Figure S3. Unprocessed original scans of autoradiograms (immunoblots) included in Fig. 2 A and 2 B. After protein transfer, membranes were cut according to the 55 kDa molecular ladder band and each fragment was incubated with primary antibody. Chemiluminescent signal was detected using X-ray film (the membrane edges are not visible on developed film due to detection of strong and specific signal). Red lines identify cropped areas. In Fig. 2A the same membrane was used for HSPA1 and p62 detection after antibodies stripping in Restore Western Blot Stripping Buffer (Thermo Fisher Scientific, Waltham, MA, USA).

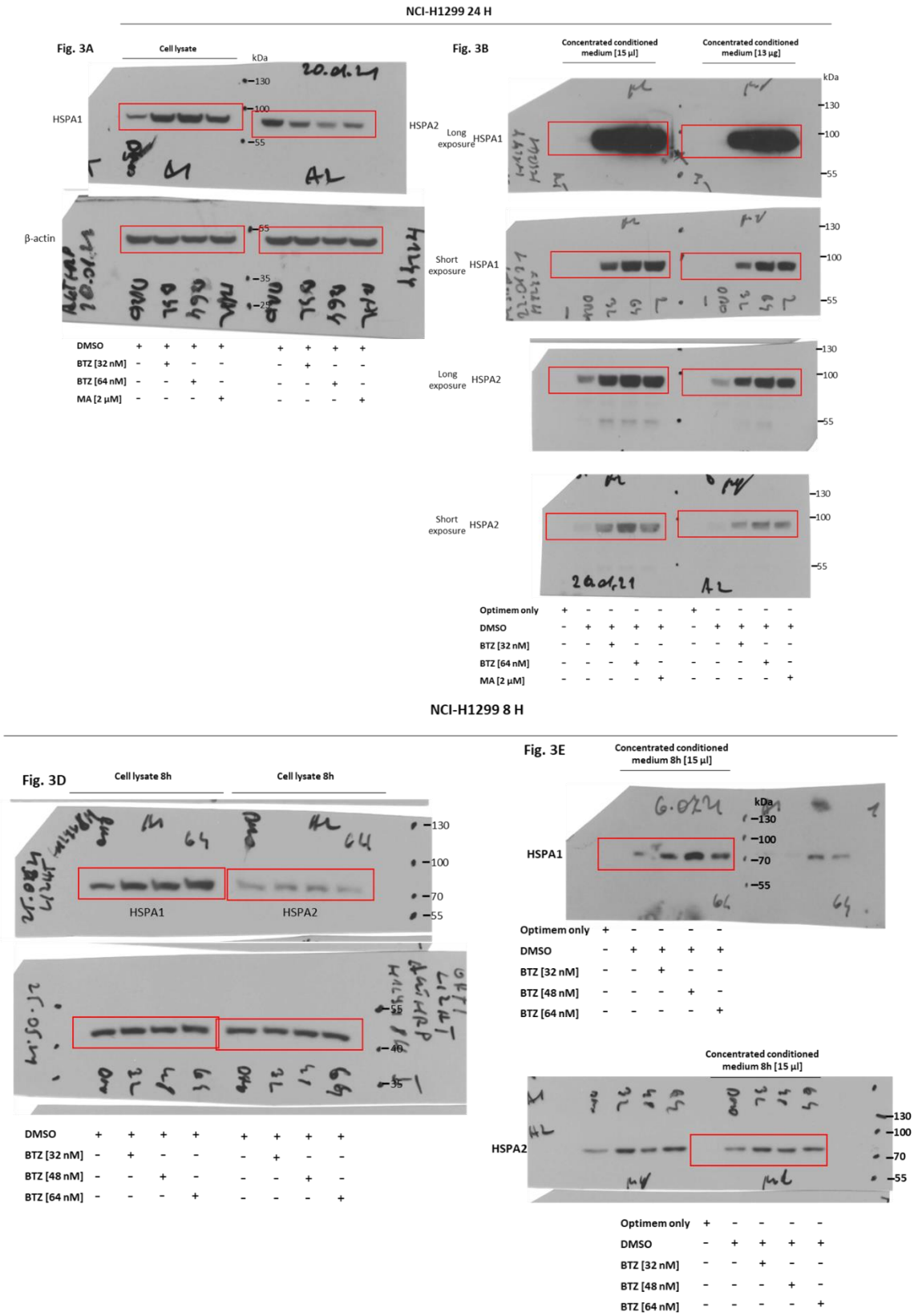


Figure S4. Unprocessed original scans of autoradiograms (immunoblots) included in Fig. 3 A-B and Fig. 3 D-E. After protein transfer, membranes were cut according to the 55 kDa molecular ladder band and were incubated with primary antibodies. Chemiluminescent signal was detected using X-ray film (blot edges are not visible on developed film due to detection of strong and specific signal). Red lines identify cropped areas.

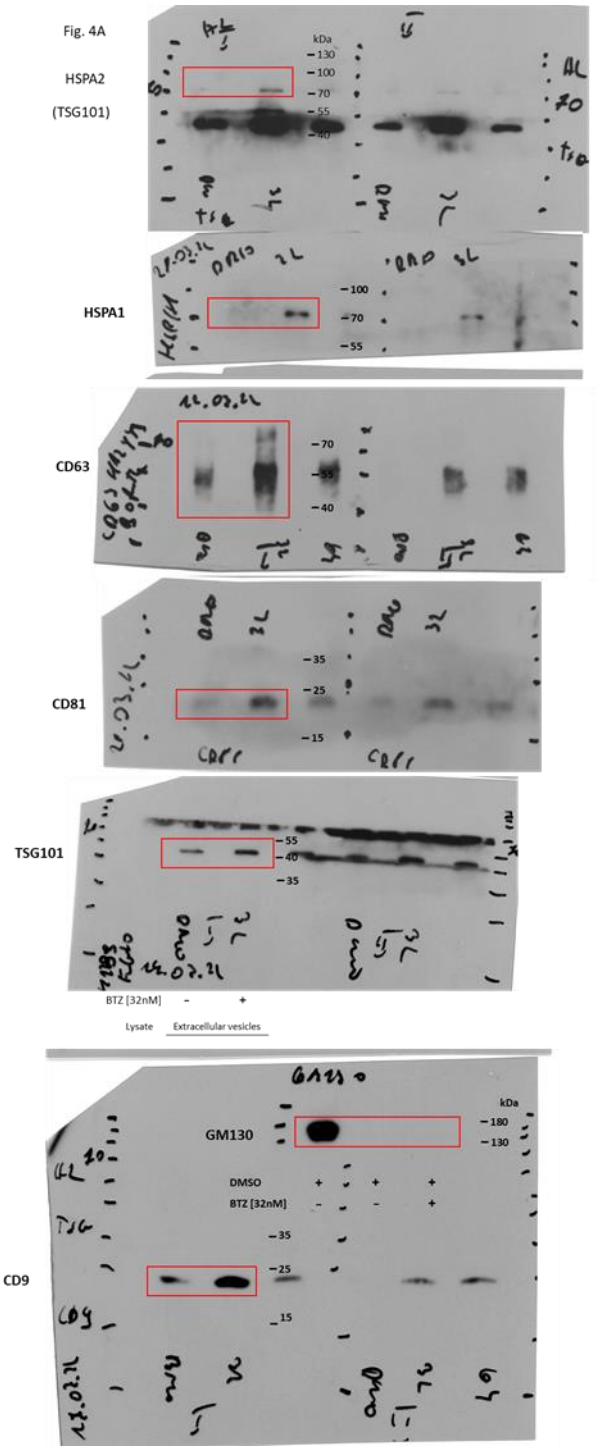


Figure S5. Unprocessed original scans of autoradiograms (immunoblots) included in Fig. 4 A. After protein transfer, membranes were cut according to the proteins' molecular weight and each fragment was incubated with appropriate primary antibody. X-ray film was used for chemiluminescent signal detection (blot edges are not visible on developed film). Red lines identify cropped areas.

HSPA2-knockout in NCI-H1299 cells

Fig. 5A

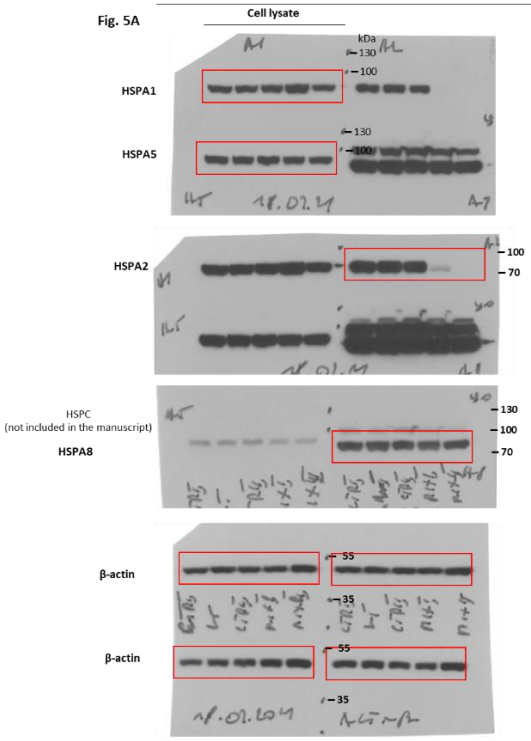
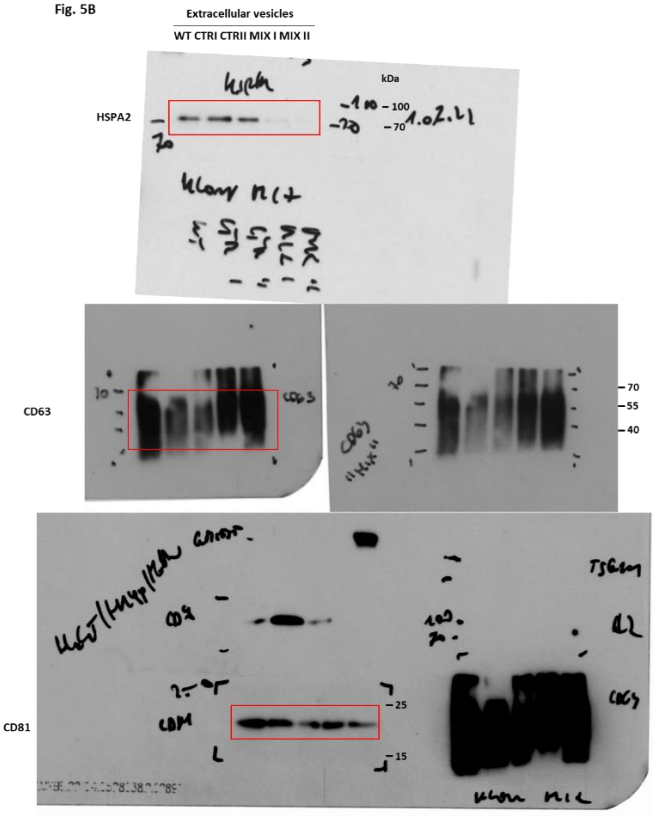
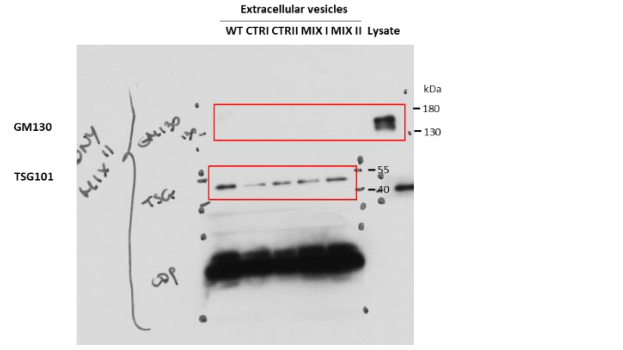


Fig. 5B





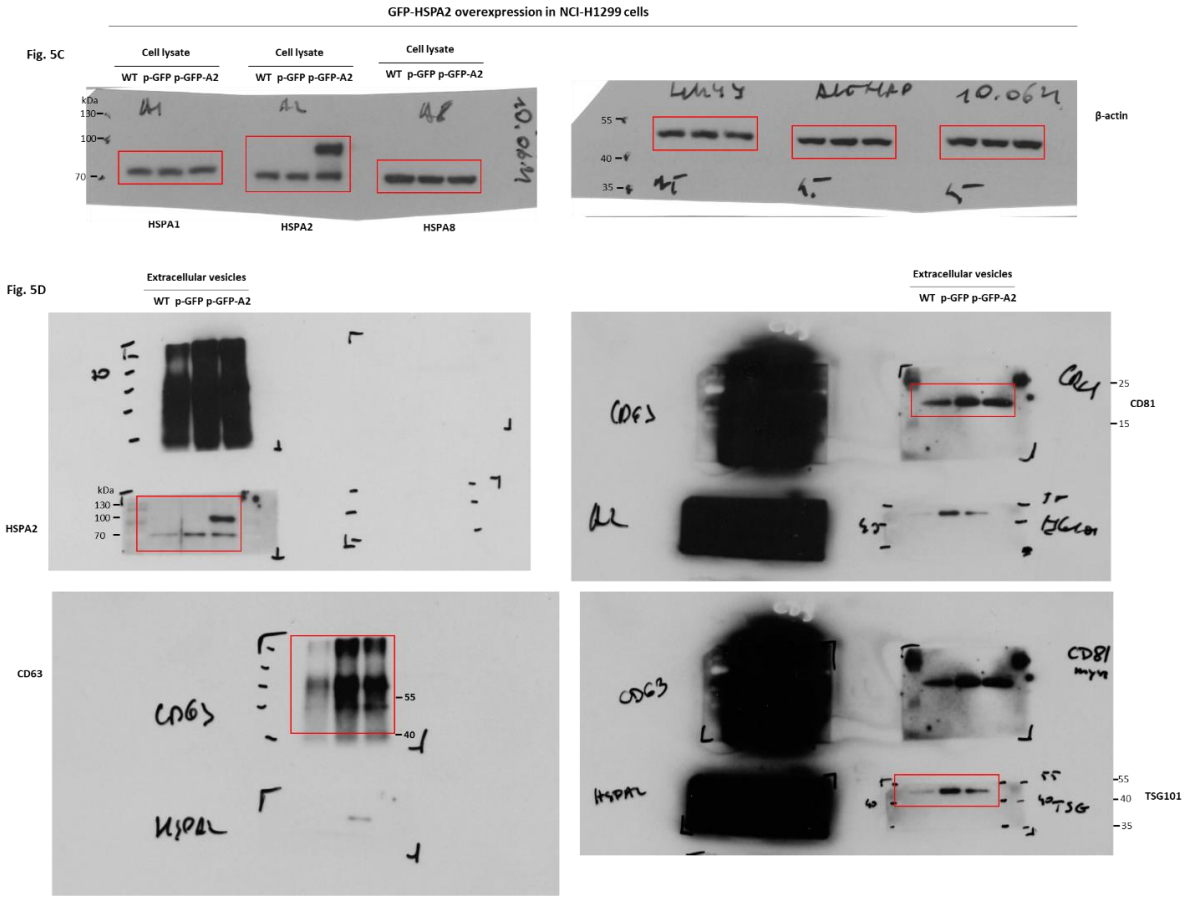


Figure S6. Unprocessed original scans of autoradiograms (immunoblots) included in Fig. 5 A-5D. After protein transfer, membranes were cut into two (or three) fragments according to the proteins' molecular weight and each fragment was incubated with primary antibody. For chemiluminescent signal detection X-ray film was used. Red lines identify cropped areas.

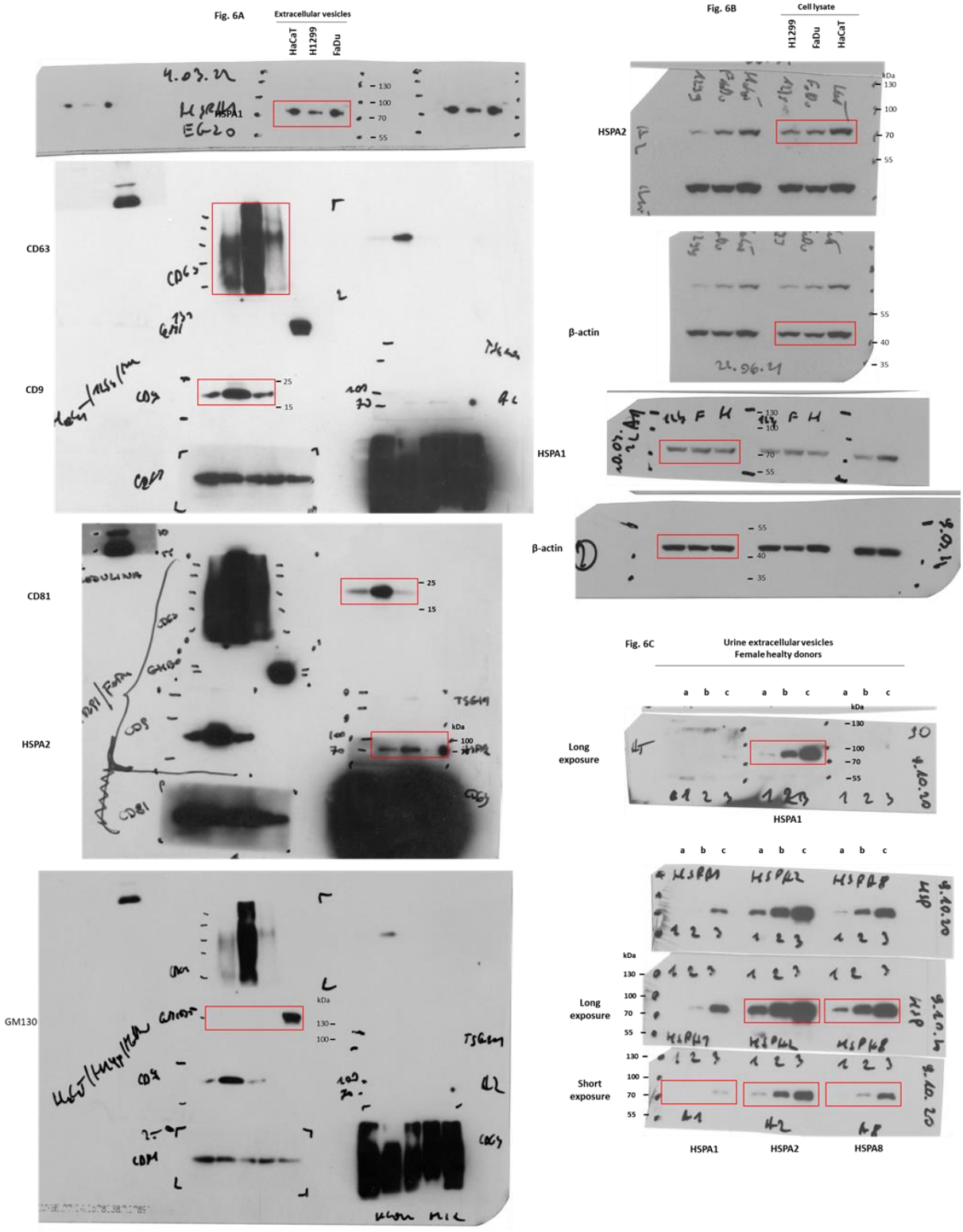


Fig. 6A
Extracellular vesicles
HaCaT
H1299
FaDu
HSPA1
CD63
CD9
CD81
HSPA2
GM130
Fig. 6B
Cell lysate
H1299
FaDu
HaCaT
HSPA2
β-actin
HSPA1
β-actin
Fig. 6C
Urine extracellular vesicles
Female healty donors
Long exposure
HSPA1
Long exposure
Short exposure
HSPA1
HSPA2
HSPA8

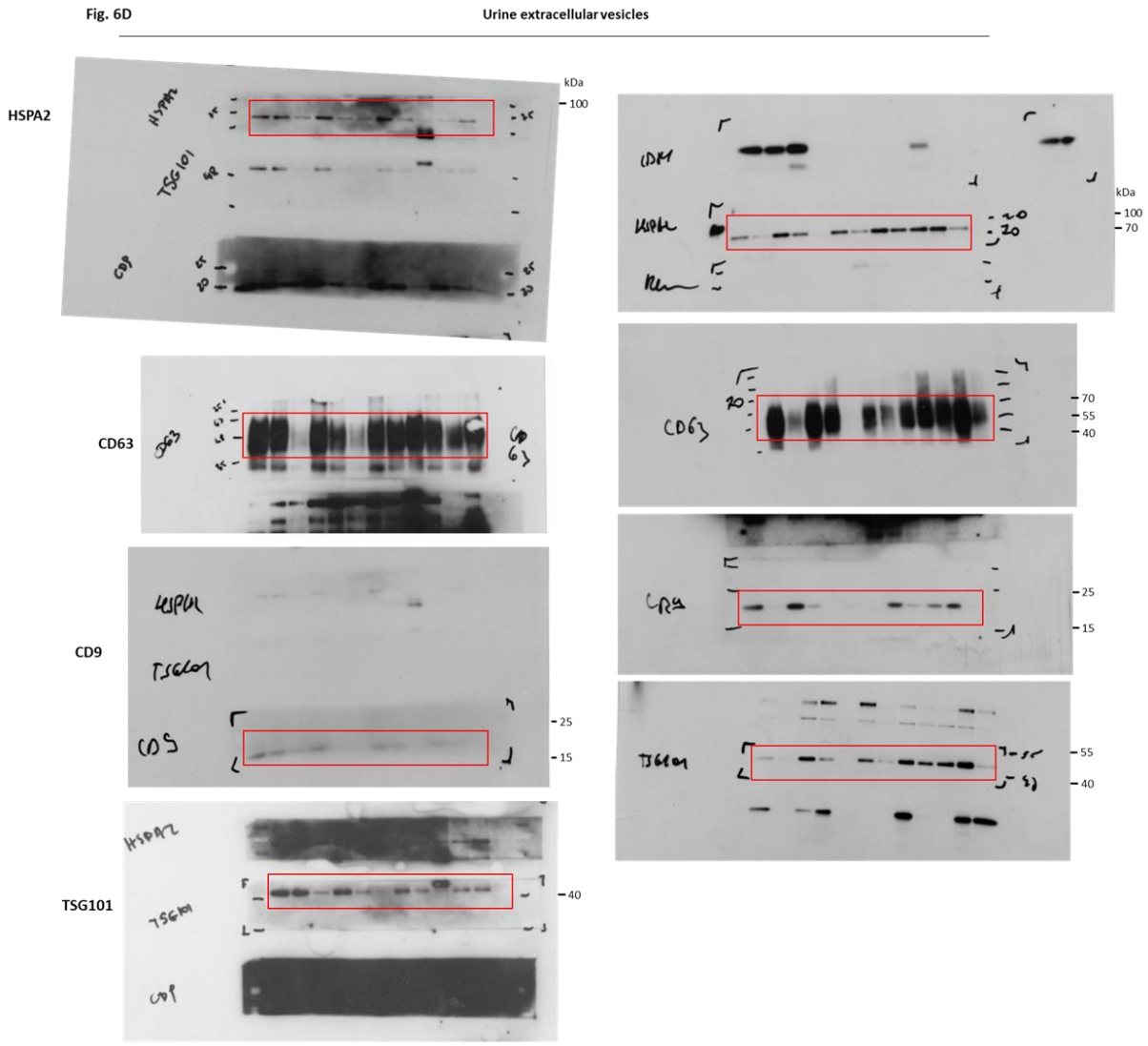


Figure S7. Unprocessed original scans of autoradiograms (immunoblots) included in Fig. 6 A-D. After protein transfer, membranes were cut into two (or more) fragments according to the proteins' molecular weight and each fragment was incubated with primary antibody. For chemiluminescent signal detection X-ray film was used. Red lines identify cropped areas.

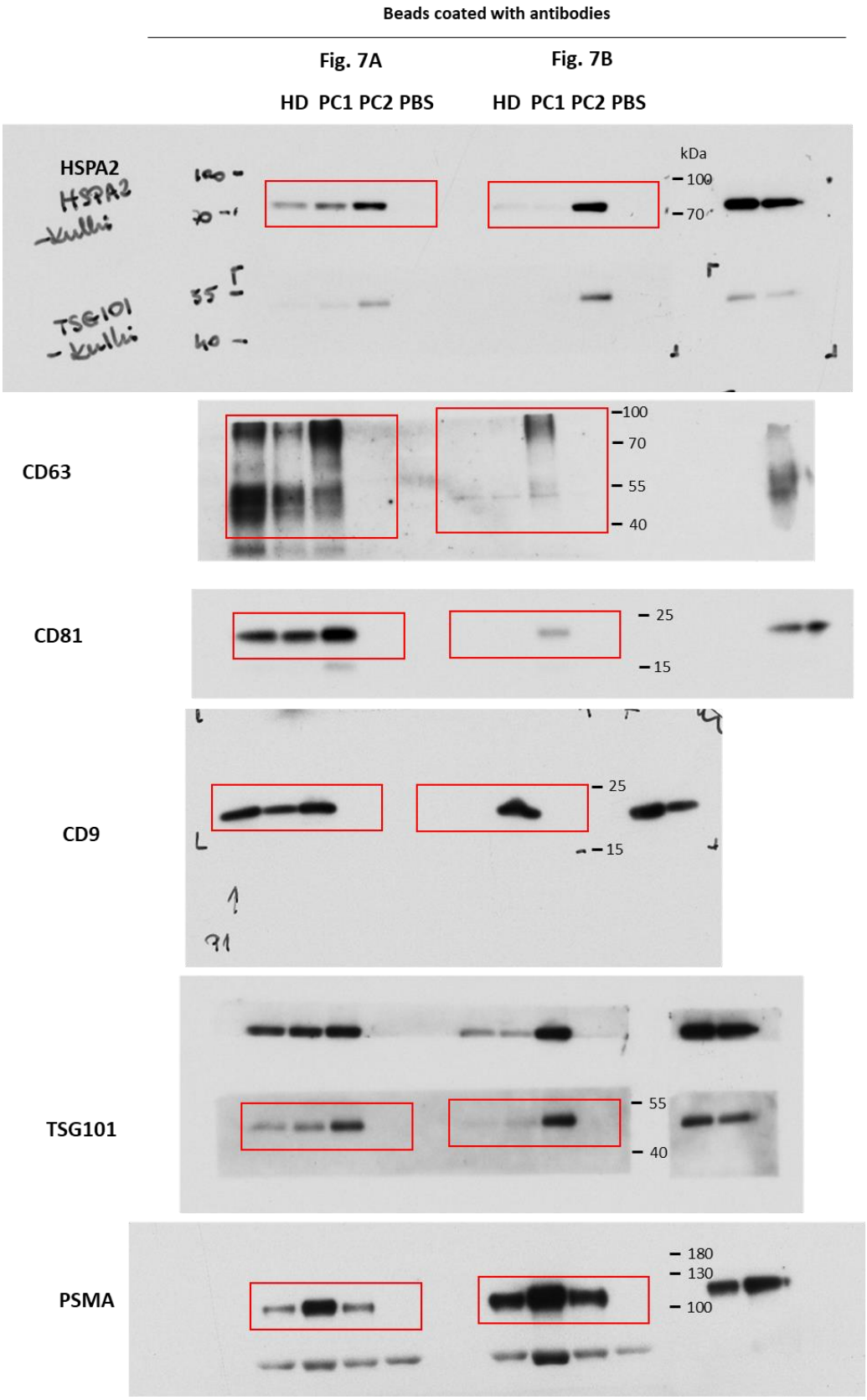


Figure S8. Unprocessed original scans of autoradiograms (immunoblots) included in Fig. 7 A. and 7 B. After protein transfer, were cut into two or more fragments according to the proteins' molecular weight. Each fragment was incubated with primary antibody. X-ray film was used for chemiluminescent signal detection. Red lines identify cropped areas.