Red blood Cell-derived Extracellular Vesicles as biomaterials: the opportunity of freezing-induced accelerated aging

Lucia Paolini*[a,b], Miriam Romano*[b,c], Valentina Mangolini[d,e], Selene Tassoni[d, b], Shuhan Jiang[f,g], Elena Laura Mazzoldi[d,h], Angelo Musicò[d,b,i], Andrea Zendrini[b,d], Anna Kashkanova[f,g], Vahid Sandoghdar[f,g,l] Anna C. Berardi[m], Silvia Clara Giliani[d,h,n,o], Paolo Bergese[d,b,o] and Annalisa Radeghieri[§ d,b]

a Dept. of Medical and Surgical Specialties, Radiological Sciences and Public Health (DSMC), University of Brescia, Brescia, Italy.

b Center for Colloid and Surface Science, (CSGI), Sesto Fiorentino (FI), Italy.

c ASST – Spedali Civili di Brescia, Brescia, Italy;

d Dept. Molecular and Translational Medicine (DMMT), University of Brescia, Brescia, Italy

e IRCCS Fondazione Don Carlo Gnocchi ONLUS, Milano, Italy.

f Max-Planck-Institut für die Physik des Lichts, 91058 Erlangen, Germany

g Max-Planck-Zentrum für Physik und Medizin, 91058 Erlangen, Germany

h "Angelo Nocivelli" Institute for Molecular Medicine, ASST Spedali Civili, 25123, Brescia, Italy

i Instituto di Scienze e Tecnologie Chimiche "Giulio Natta"- National Research Council of Italy (SCITEC-CNR), Milan, Italy.

l Department Physik, Friedrich-Alexander Universität Erlangen, Nürnberg, 91058 Erlangen, Germany

m Laboratory of Stem Cells, U.O.C. of Immunohaematology and Transfusion Medicine, Santo Spirito Hospital, Pescara, Italy

n Section of Medical Genetics and Cytogenetics, ASST Spedali Civili of Brescia, 25123 Brescia, Italy

o National Center for Gene Therapy and Drugs based on RNA Technology, Padova, Italy

* These authors equally contributed to the work.

§ Corresponding author

Supplementary Info

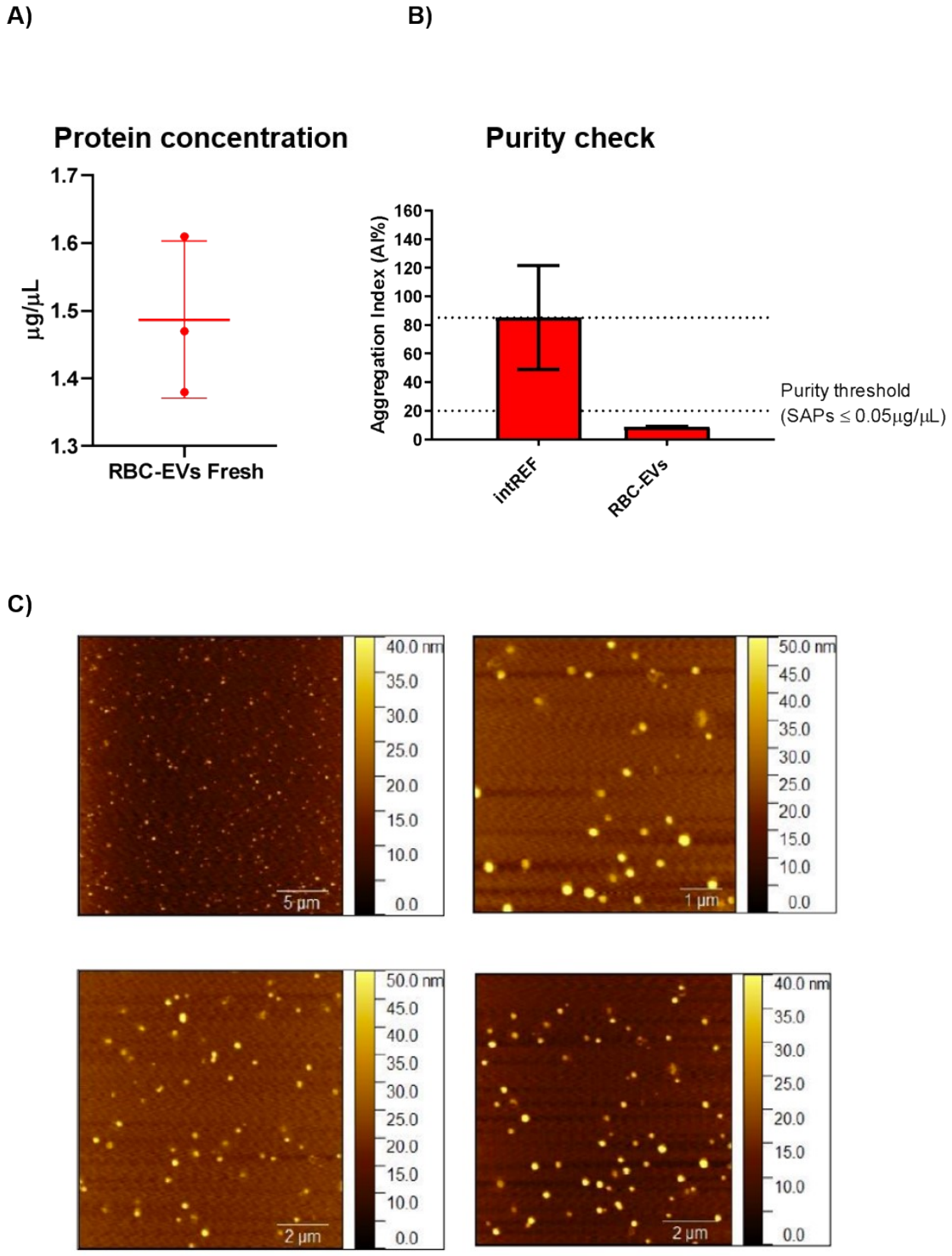
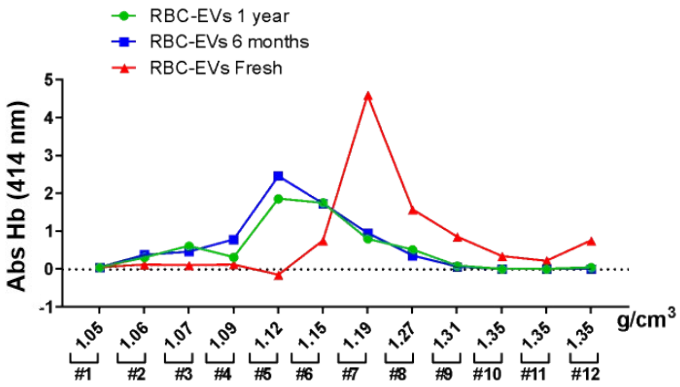


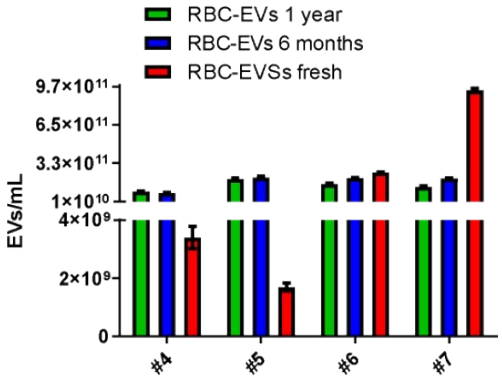
Figure S1. RBC-EV Fresh characterization. A) Protein concentration of Fresh RBC-EV calculated by Bradford assay. B) Purity check tested with the CONAN assay. IntREF: Internal reference control, aggregation index (AI) of AuNPs without Evs; RBC-EVs: AI of AuNPs after interaction with Fresh RBC-EV preparation. Purity determined with AI under 20% indicating a contamination of Single and

Aggregated Proteina (SAPs) below 0.05 ug/ul. (C) Representative images of Fresh RBC-EV sample by Atomic Force Microscopy (AFM) at different magnifications.

A)



B)



C)

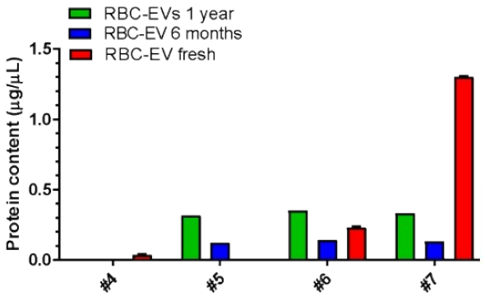


Figure S2. Analysis of the RBC-EV Fresh, 6-month, 1-year samples after DSG processing. A) Hemoglobin distribution. Hemoglobin distribution along DSG 12 fractions was checked determining hemoglobin absorbance at 414 nm for Fresh (red), 6-month (blue), 1-year (green) samples. C) Nanoparticle concentration from fraction 4 to fraction 7. RBC-EV concentration determined by Nanoparticle Tracking Analysis (NTA) for Fresh (red), 6 months (blue), 1-year (green) samples. D) Protein concentration from fraction 4 to fraction 7. RBC-EV protein concentration determined by Bicinchoninic acid (BCA) assay for Fresh (red), 6 month (blue), 1-year (green) samples.

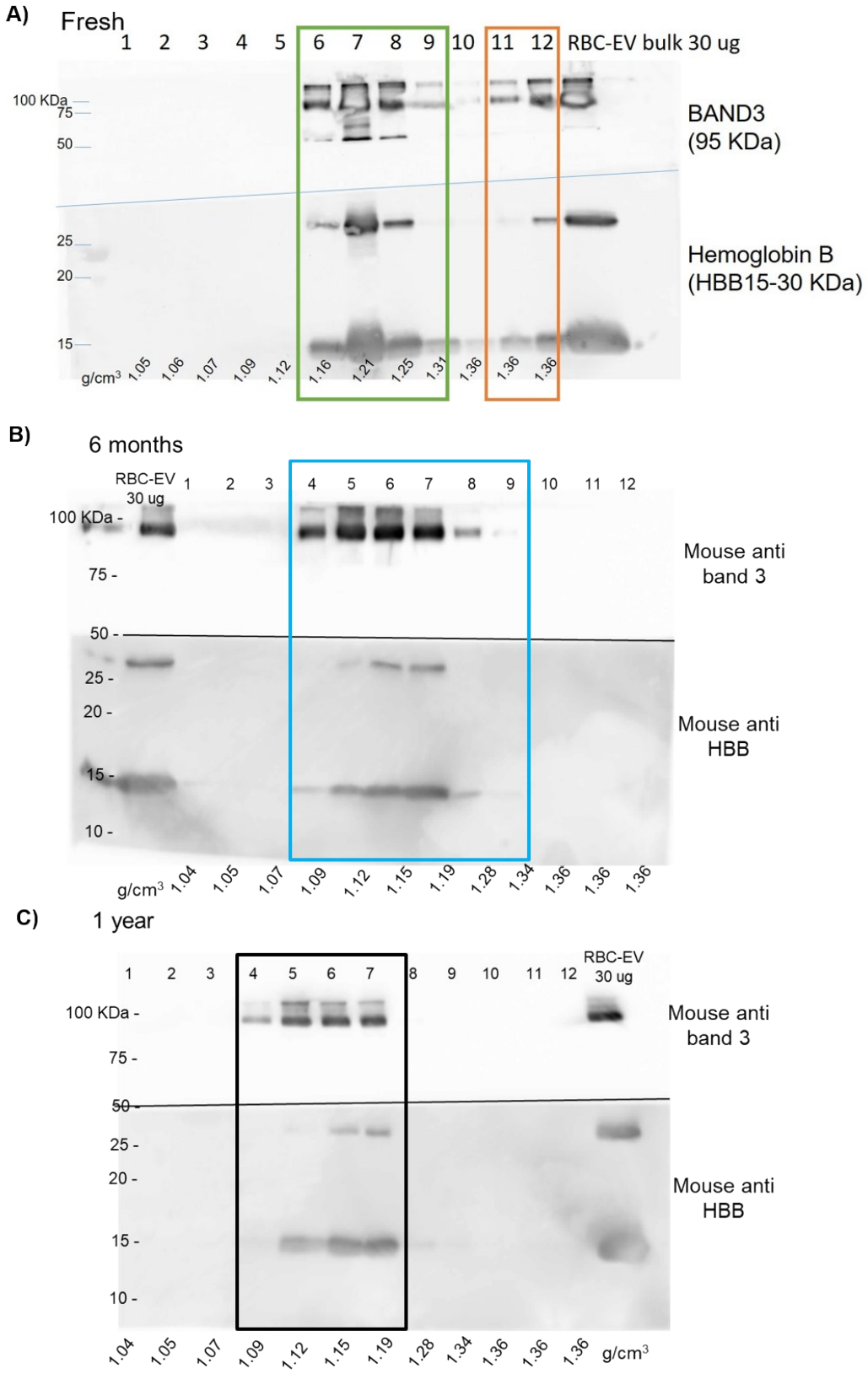


Figure S3. **Uncropped Western Blot images of gradient distribution of RBC-EVs after different storage conditions. A)** Fresh RBC-EVs**; B)** RBC-EVs stored for 6 months at -80; **C)** RBC-Evs stored for 1-year at -80°C**.**

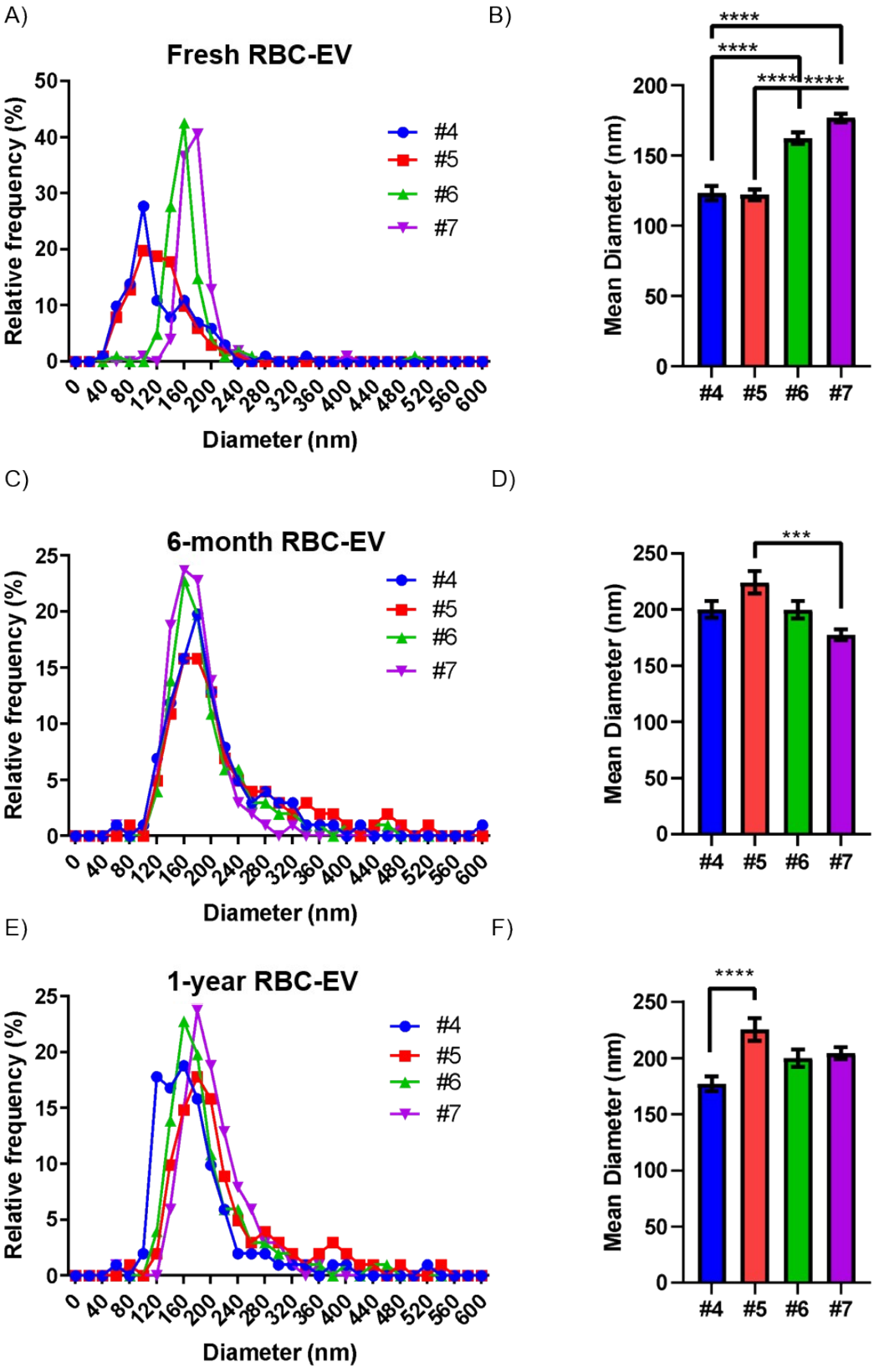


Figure S4. Size distribution of RBC-EVs storaged in different conditions determined by NTA. A) Size distribution nanoparticles in DSG fractions from 4 to 7 for Fresh RBC-Evs, stored at +4°C **B)** Size distribution of DSG fractions from 4 to 7 of 6-months RBC-Evs, stored at -80°C for 6 months **C)** Size distribution of DSG fractions from 4 to 7 of 1-year RBC-Evs, stored at -80°C for 1 year.

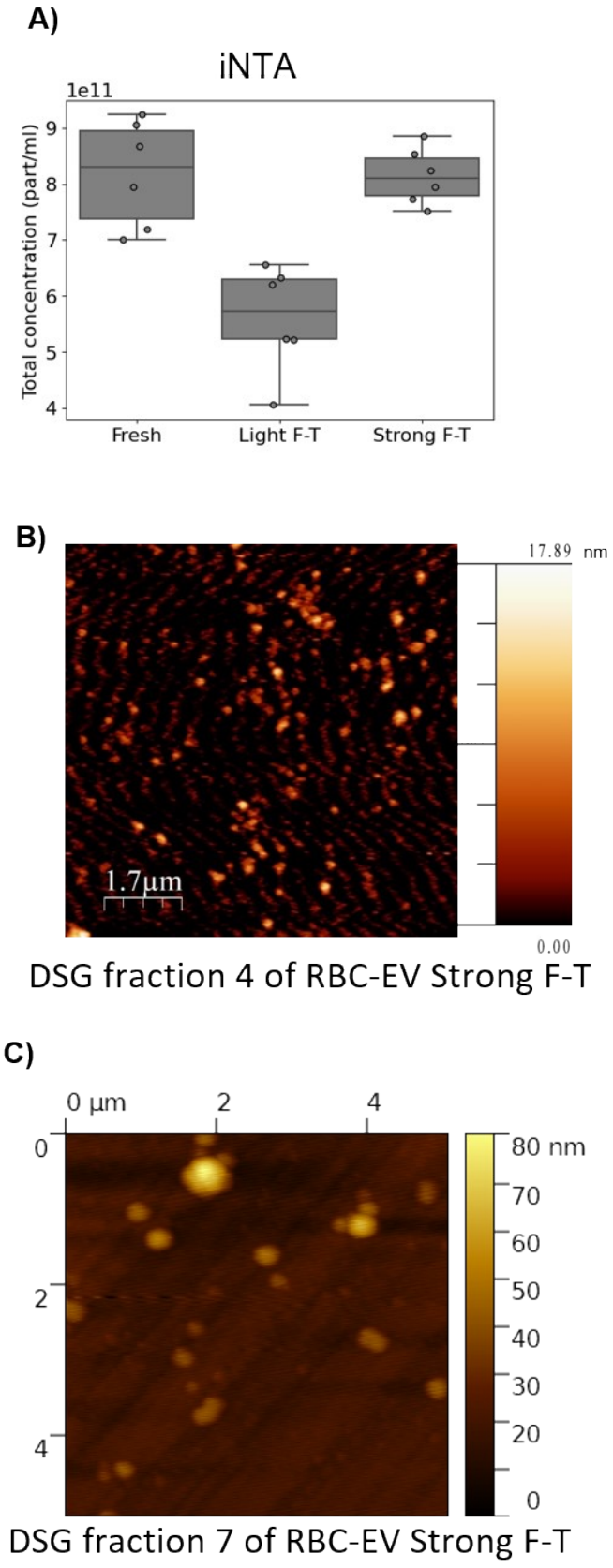
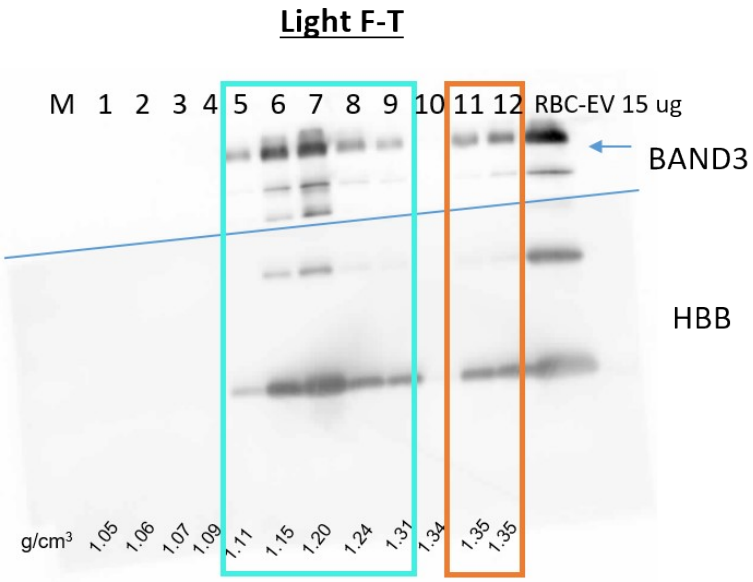


Figure S5: A) Particle concentration mesured by iNTA in Fresh sample and after Light and Strong Freeze and thaw (F-T) processing. B) AFM imaging of fraction 4 of DSG of Strong F-T sample. C) AFM imaging of fraction 7 of DSG of Strong F-T sample

A)



B)

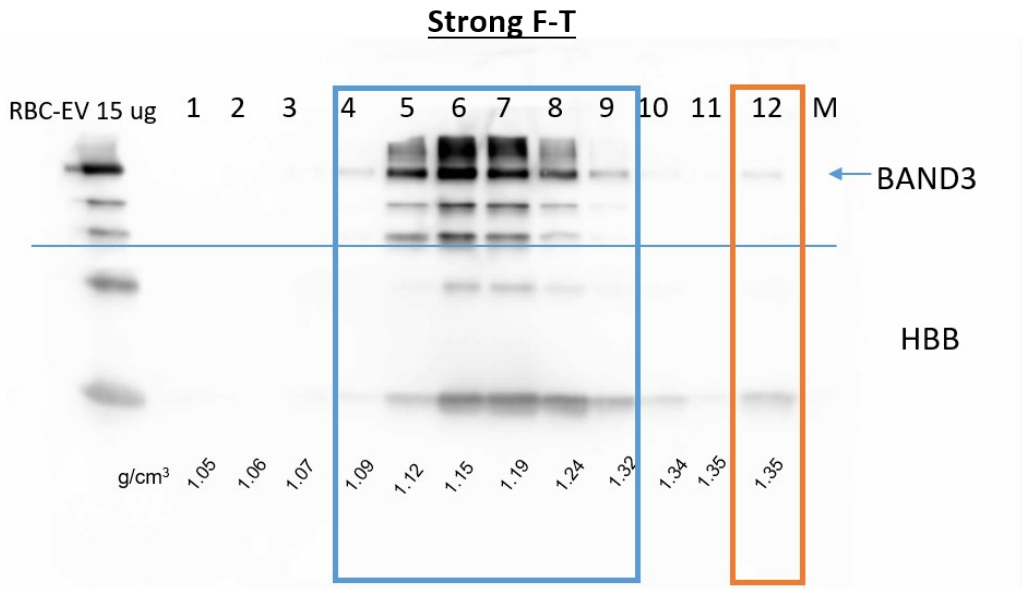


Figure S6. Uncropped Western Blot images of gradient distribution of RBC-EVs after Light Freeze and Thaw (Light F-T) (A) and Strong Freeze and Thaw (Strong F-T) (B) treatment.

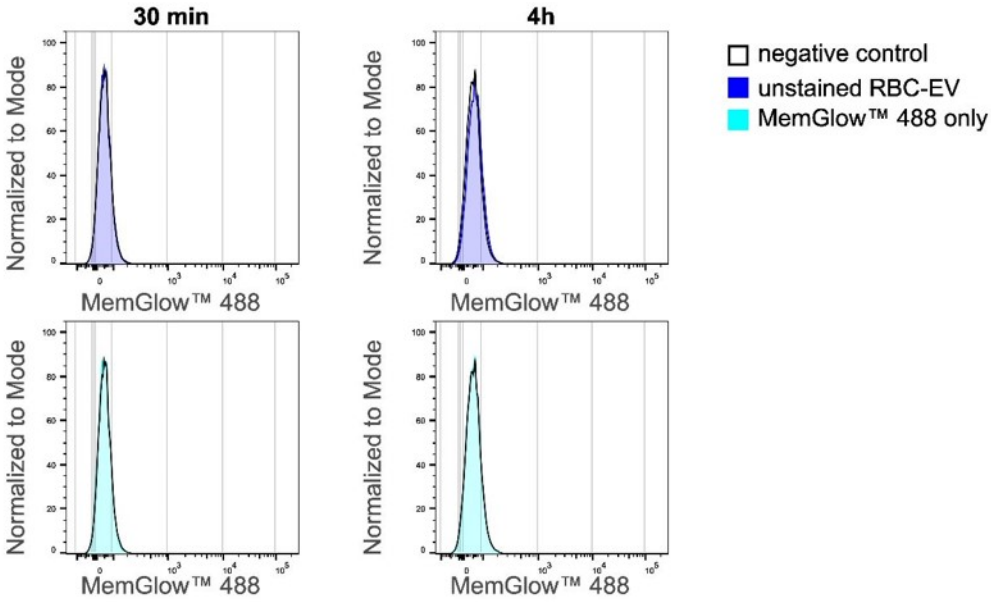


Figure S7: Flow cytometry analysis of RBC-EV uptake. Evaluation of unspecific fluorescent signal arising either from possibly increased cell autofluorescence (untreated cells, with histogram), due to EV internalization (unstained RBC-EV, blue hystogram) or to unbound MemGlow™ 488 fluorescent dye (MemGlow only, green histogram) incubated for 30 min or 4 hours with MDA-MB-231 cells.

X	Group A			Group B			Group C			Group D		
Minutes	PBS			RBC-EV Fresh			F-T strong			6 Months		
X	A:Y1	A:Y2	A:Y3	B:Y1	B:Y2	B:Y3	C:Y1	C:Y2	C:Y3	D:Y1	D:Y2	D:Y3
0	0.095	0.097	0.099	0.419	0.426	0.420	0.414	0.418	0.419	0.350	0.325	0.320
10	0.134	0.103	0.098	0.777	0.747	0.733	0.749	0.722	0.708	0.721	0.632	0.626
20	0.097	0.095	0.094	0.870	0.784	0.756	0.769	0.727	0.719	0.815	0.708	0.714
30	0.098	0.098	0.096	0.871	0.781	0.756	0.774	0.738	0.720	0.829	0.714	0.727
60	0.103	0.101	0.097	0.862	0.772	0.745	0.761	0.727	0.708	0.830	0.709	0.722

Figure S8: Achetylcolinesterase assay. Raw data of the Acetylcholinesterase enzymatic activity. Abs 415 nm for time and Samples indicated in the Figure.