SUPPORTING INFORMATION

A systematic evaluation of factors affecting extracellular vesicle uptake by breast cancer cells

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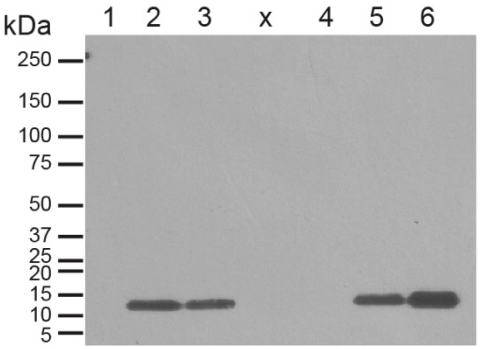
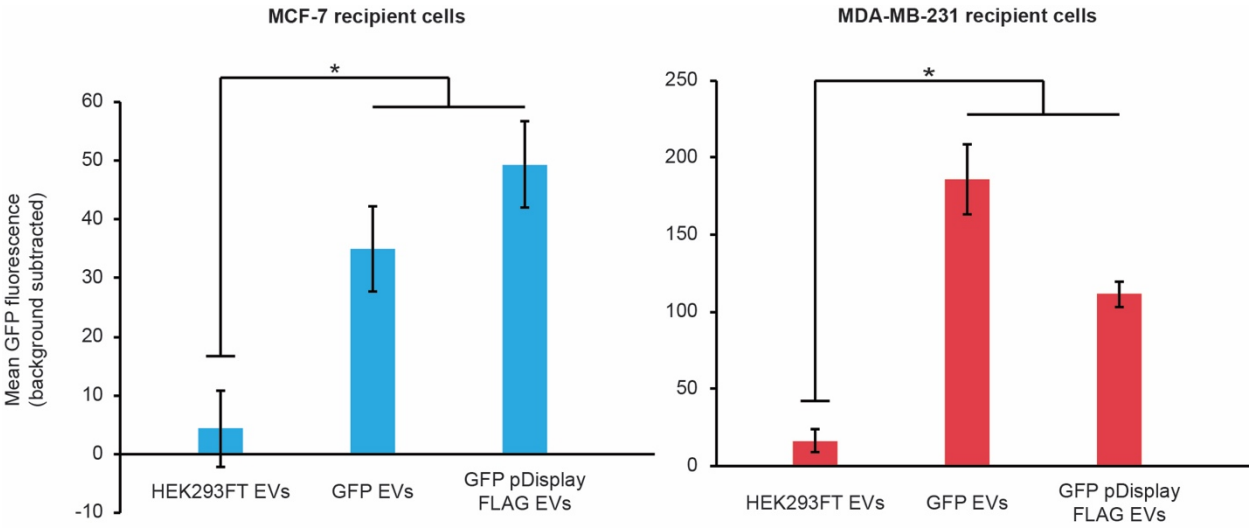
Contents

I. Supplementary Figures

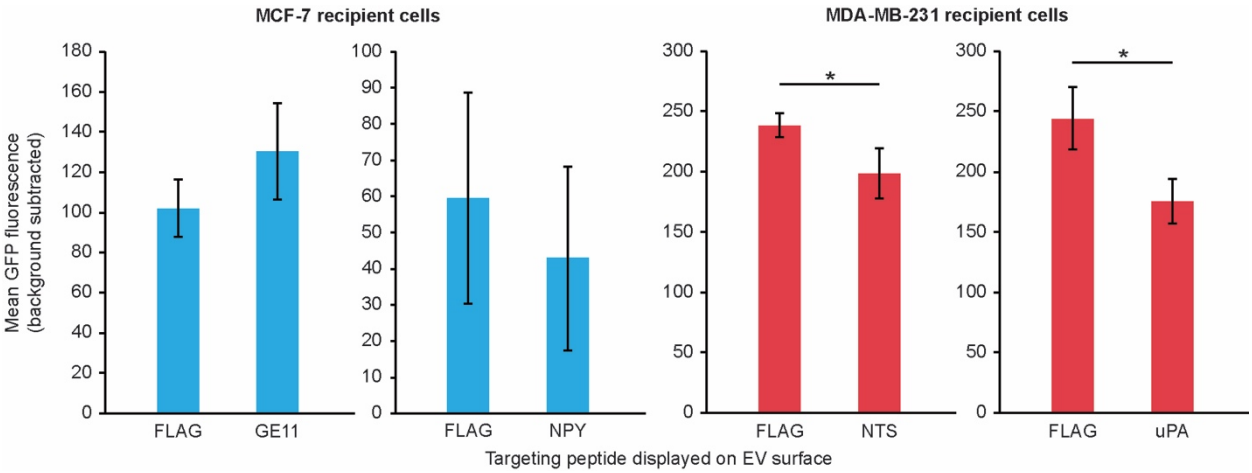


1. HEK293FT cell lysate
2. GNSTM-FLAG-PDGFR transmembrane domain cell lysate
3. GASTM-FLAG-PDGFR transmembrane domain cell lysate
x. Empty lane
4. HEK293FT EVs
5. GNSTM-FLAG-PDGFR transmembrane domain EVs
6. GASTM-FLAG-PDGFR transmembrane domain EVs

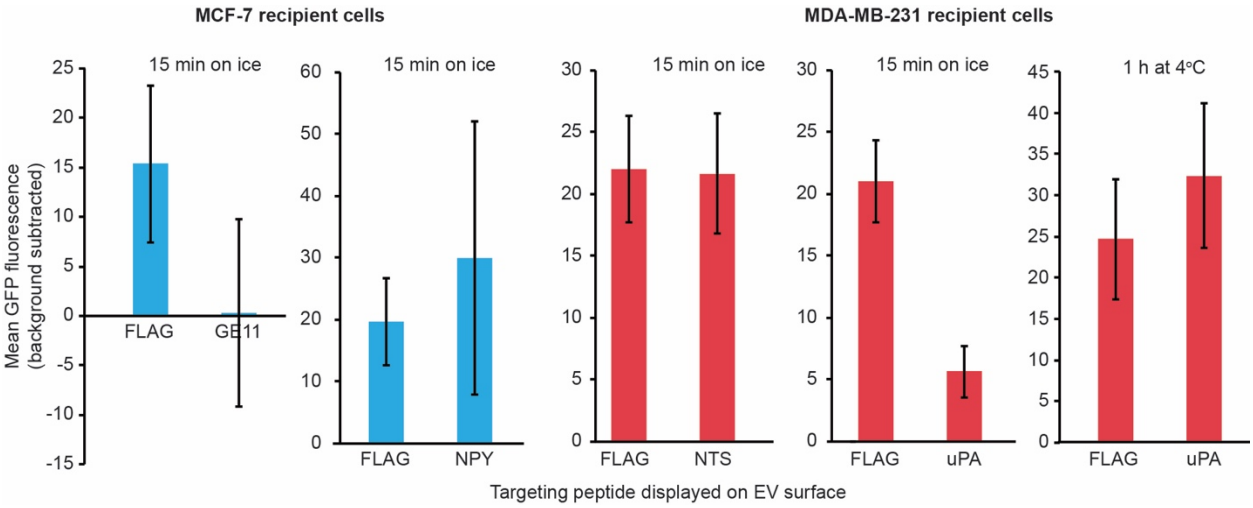
SUPPLEMENTARY FIGURE S1. Expression of glycosylated (GNSTM) or unglycosylated (GASTM) FLAG-PDGFR transmembrane domain peptides in cell lysates and EVs. 0.5 µg protein was loaded for each cell lysate sample, and 1.5 x 10^8 EVs were added for each EV sample. Samples were labeled with an anti-FLAG antibody.



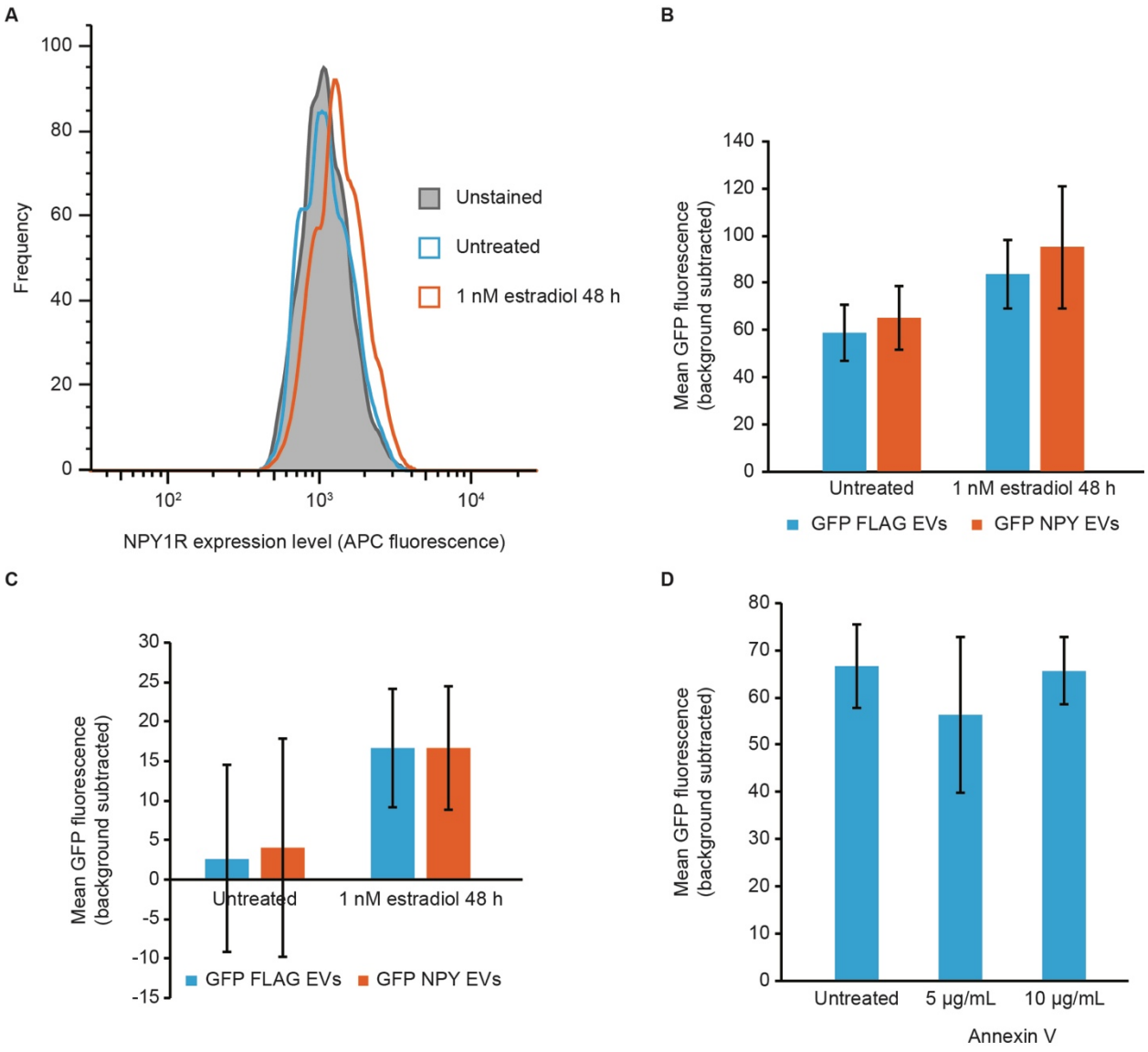
SUPPLEMENTARY FIGURE S2. Delivery of GFP-labeled EVs to MCF-7 and MDA-MB-231 cells. Cells were incubated with EVs for 2 h prior to analysis by flow cytometry. Experiments were performed in biological triplicate, and error bars indicate one standard deviation. Statistical tests comprise two-tailed Student's t-tests (* $p < 0.05$) comparing each GFP-labeled EV population to unlabeled HEK293FT EVs.



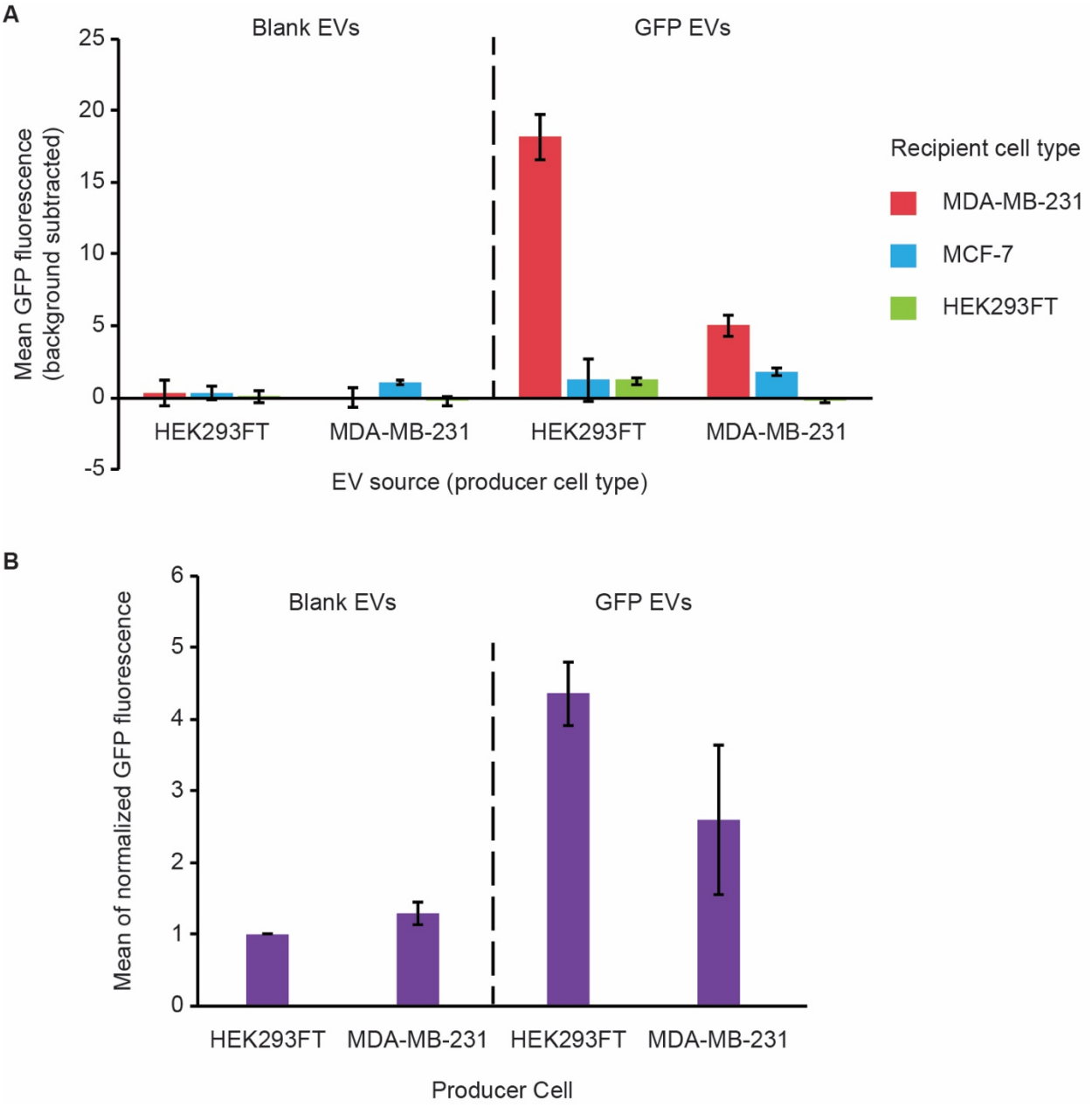
SUPPLEMENTARY FIGURE S3. Repeat of experiments shown in Figure 1B. Uptake of targeted, CD63-GFP-labeled EVs by MCF-7 cells or MDA-MB-231 cells following 2 h incubation. "FLAG" refers to EVs displaying a non-targeting FLAG tag as a negative control. Data for both MDA-MB-231 targeting peptides are compared to the same negative control. Experiments were performed in biological triplicate, and error bars indicate one standard deviation. Statistical tests comprise two-tailed Student's t-tests (* $p < 0.05$).



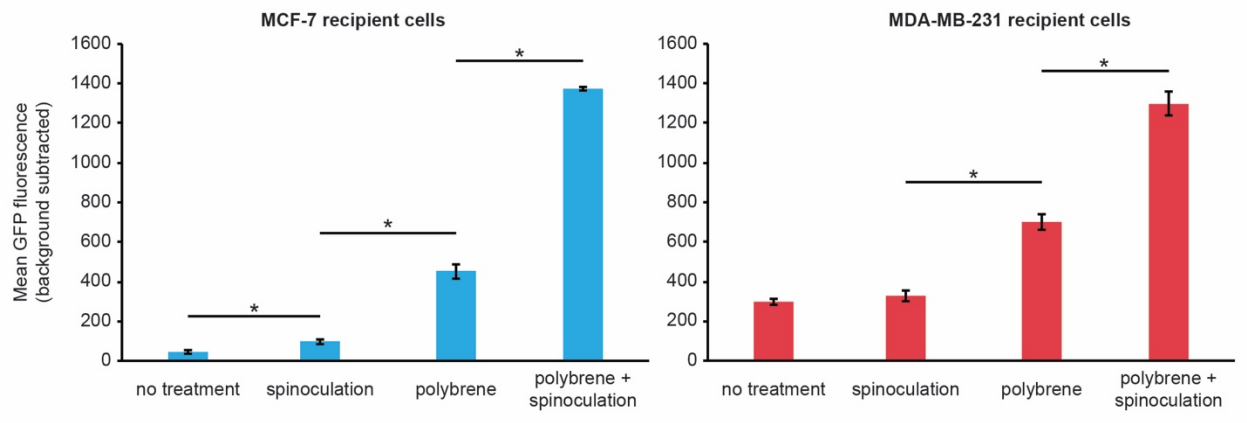
SUPPLEMENTARY FIGURE S4. Evaluation of binding of targeting peptide EVs to cells. Targeted, CD63-GFP-labeled EVs incubated with MCF-7 or MDA-MB-231 cells for 15 min on ice or for 1 h at 4° C to facilitate binding but not internalization. Cells were washed three times prior to analysis by flow cytometry. Experiments were performed in biological triplicate, and error bars indicate one standard deviation. “FLAG” refers to EVs displaying a non-targeting FLAG tag as a negative control.



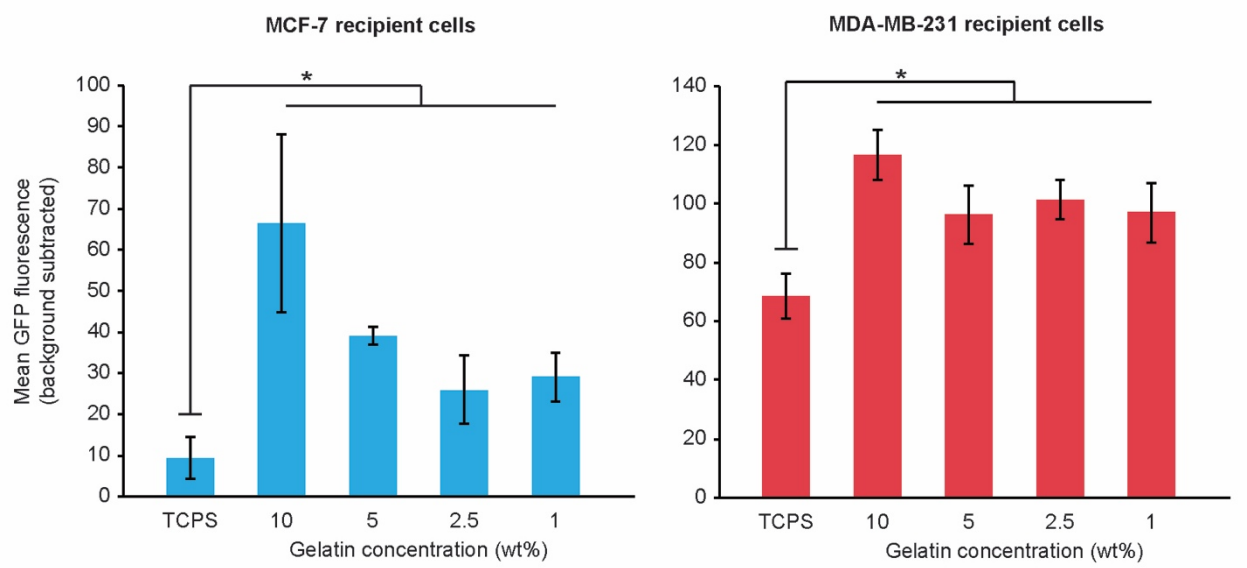
SUPPLEMENTARY FIGURE S5. Effect of receptor upregulation on targeting peptide-mediated EV delivery. (A) Surface staining of NPY1R on untreated MCF-7 cells or cells treated with 1 nM estradiol for 48 h. (B) NPY CD63-GFP-labeled EV uptake by MCF-7 cells treated with 1 nM estradiol for 48 h prior to EV delivery. (C) Repeat of (B) but with low dose of EVs (2.5 x 10^9 EVs per well, vs. ~ 1 x 10^{10} EVs per well in part (B)), a dose that is below the threshold where uptake of untargeted EVs is typically observed (as seen here). (D) Effect of annexin V treatment on CD63-GFP-labeled EV uptake. ~100 µL concentrated EVs were incubated with annexin V for 15 min at 37° C before being delivered to MCF-7 cells, which had been treated with 1 nM estradiol for 48 h. Annexin V concentrations indicated are the *final* concentrations after the EV-annexin V mixture was added to cell culture medium. Experiments were performed in biological triplicate, and error bars indicate one standard deviation. “FLAG” refers to EVs displaying a non-targeting FLAG tag as a negative control.



SUPPLEMENTARY FIGURE S6. (A) Repeat of experiments shown in Figure 2B: uptake of blank or CD63-GFP-labeled EVs harvested from HEK293FT or MDA-MB-231 cells and delivered to MDA-MB-231, MCF-7, or HEK293FT cells following 2 h incubation. Experiments were performed in biological triplicate, and error bars indicate one standard deviation. (B) Four types of CD63-GFP-labeled EVs used in cell delivery experiments were adsorbed onto latex beads and analyzed by flow cytometry. Blank bead autofluorescence was subtracted from EV samples, and all samples were normalized to beads bound to blank HEK293FT-derived EVs. At least 10,000 beads were quantified by flow cytometry in each experiment, and the means of two independent experiments were averaged together to generate this plot. Error bars represent one standard deviation (across experiments).



SUPPLEMENTARY FIGURE S7. Repeat of experiments shown in Figure 3B: uptake of CD63-GFP-labeled EVs by MCF-7 cells or MDA-MB-231 cells was quantified following incubation for 2 h under the delivery conditions indicated (for conditions including spinoculation, spinoculation was performed for 1 h, and then cells were returned to the incubator for 1 additional h prior to analysis). Experiments were performed in biological triplicate, and error bars indicate one standard deviation. Statistical tests comprise two-tailed Student's t-tests (* $p < 0.05$).



SUPPLEMENTARY FIGURE S8. Repeat of experiments shown in Figure 4C: CD63-GFP-labeled EV uptake by MCF-7 cells or MDA-MB-231 cells during culture on various substrates following 2 h incubation. Experiments were performed in biological triplicate, and error bars indicate one standard deviation. Statistical tests comprise paired two-tailed Student's t-tests, individually comparing each gelatin concentration condition to TCPS (* $p < 0.05$).

II. Supplementary Methods

Plasmid construction- To create a plasmid backbone lacking BsaI restriction sites, the BsaI restriction site in the Amp resistance gene of pcDNA3.1 + Hygro backbone (Clontech) was mutated. The Hygro resistance gene was deleted and the pieces reassembled via blunt end ligation. The SV40 promotor was then deleted. Single nucleotide polymorphisms were introduced to mutate the BpiI site and the BsaI site in the 5' UTR following the CMV promoter, and the plasmid was reassembled via Gibson assembly to create pD005. pDisplay-pHuji was obtained from Addgene (plasmid #61556), deposited by Robert Campbell, and was inserted into pD005 via restriction cloning with HindIII/XhoI to create pDMS1. pHuji was replaced with a FLAG tag (DYKDDDDK) flanked by a 3 amino acid spacer (upstream) and a 10 amino acid spacer (downstream) containing BsaI restriction sites. The sequence was preceded by either a glycosylation sequon (GNSTM),[1] yielding pDMS2 or a mutated glycosylation sequon (GASTM), yielding pDMS3. GE11 (YHWYGYTPQNVI) and NTS (RRPYIL) peptide sequences flanked by BsaI restriction sites were made by oligonucleotide annealing. The uPA peptide sequence (LHQVPSNCDCLNGGTCVSNKYFSNIHWCNCPKKFGGQHCE) flanked by BsaI restriction sites was generated by amplification of overlapping PCR of primers. NPY (YPSKPRNPGEDAPAEDLARYYSALRHYINLITRPRY) was amplified out of a plasmid encoding NPY tagged human Lamp2b cDNA clone 3543019 (Open Biosystems, now sold by Dharmacon) inserted into pcDNA3.1 + Hygro backbone. To create a plasmid encoding CD63-CD-UPRT-EGFP Human CD63 cDNA was amplified from HEK293FT cell lysate. CD-UPRT-EGFP was amplified out of pCD-UPRT-EGFP, gifted by Okay Saydam (Medical University of Vienna) and was inserted into the lentiviral pGIPZ backbone (obtained from Northwestern High Throughput Analysis Core Facility) in place of GFP between NheI and AscI. CD63 was inserted upstream of CD-UPRT-EGFP by restriction cloning with SnaBI and NheI, creating an alanine-serine linker between CD63 and CD-UPRT.

III. References Cited in Supplement

1. Bano-Polo, M., Baldin, F., Tamborero, S., Marti-Renom, M.A., andMingarro, I. N-glycosylation efficiency is determined by the distance to the C-terminus and the amino acid preceding an Asn-Ser-Thr sequon. Protein science : a publication of the Protein Society **20**, 179, 2011.