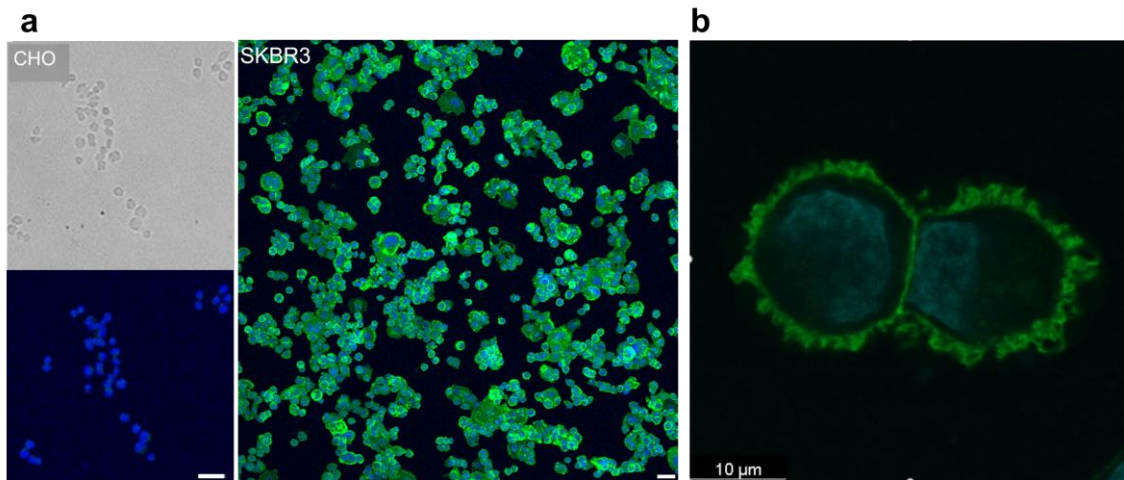


1 **Supplementary figures and tables**

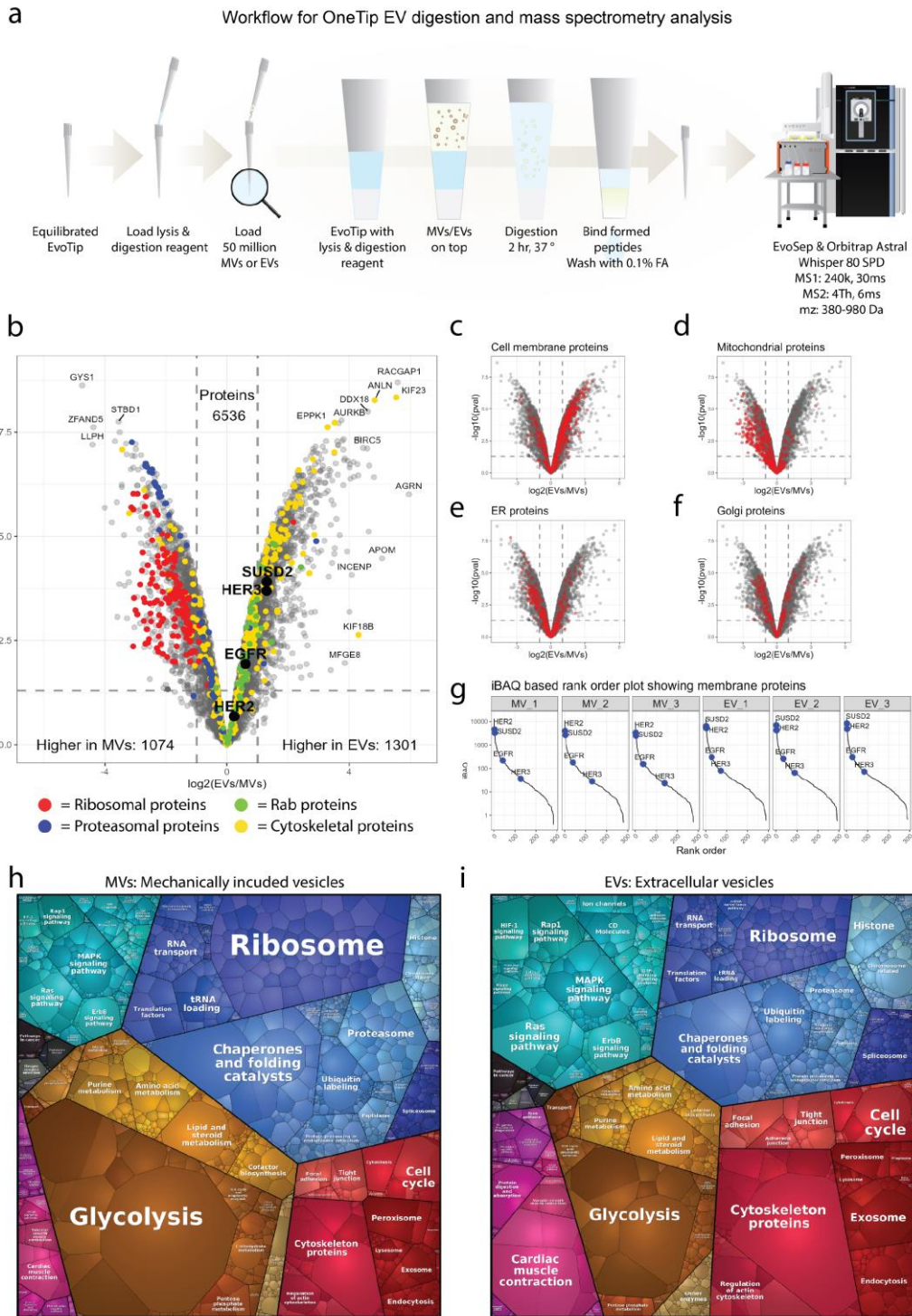
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4 **Figure S1. Immunofluorescence microscopy images showing presence of HER2 in the**  
5 **human breast cancer cell line SKBR3. a.** A strong HER2 signal (Green) is detected in SKBR3  
6 cells, using anti-HER2 antibody (Merck, MABE330), but no signal was detected in the control  
7 CHO cells (bright field and fluorescent channel are shown, up and bottom, respectively). Scale  
8 bar 40μm. **b.** The cellular ruffled plasma membrane of SKBR3 cells is revealed by confocal  
9 microscopy imaging. Chromatin (blue) stained using bisbenzimidide.

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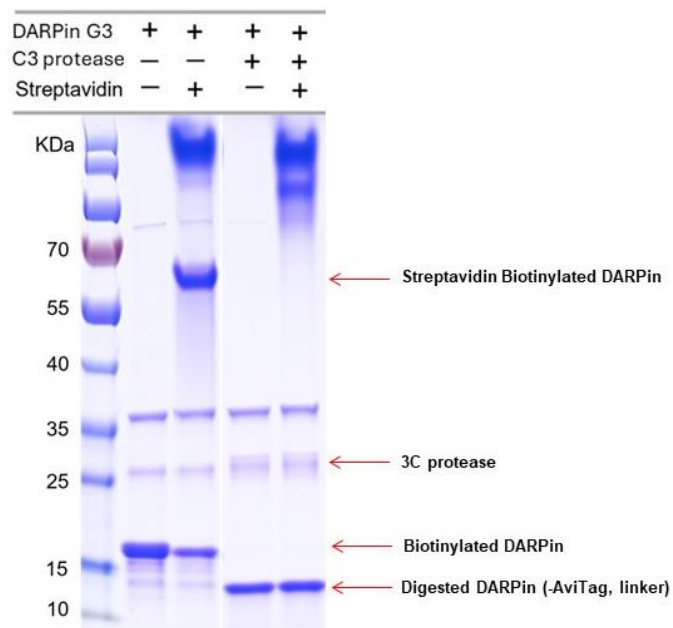
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13 **Supplementary Figure 2. Mass spectrometry-based proteome analysis of mechanically**  
 14 **induced vesicles (MVs) and extracellular vesicles (EVs) from SKBR3 cells. a.** Workflow for  
 15 mass spectrometry-based analysis of MVs and EVs. **b.** Volcano plot showing proteins with  
 16 different abundances in MVs and EVs; ribosomal proteins (red), proteasomal proteins (blue),  
 17 Rab proteins (green), cytoskeletal proteins (yellow). **c-f.** Same volcano plot as in b with cell  
 18 membrane proteins (c), mitochondrial proteins (d), endoplasmic reticulum proteins (e) and  
 19 Golgi apparatus proteins (f) in red. **g:** Cell membrane proteins visualized in an iBAQ based rank  
 20 order blot showing the abundance of HER2, SUSD2, EGFR and HER3 relative to all other cell

- 21 membrane proteins. **h-i.** Proteome composition of MVs (h) and EVs (i) visualized in Voronoi plots.
- 22 Each polygon represents the abundance of a quantified protein. The colors highlight different
- 23 functional groups of proteins

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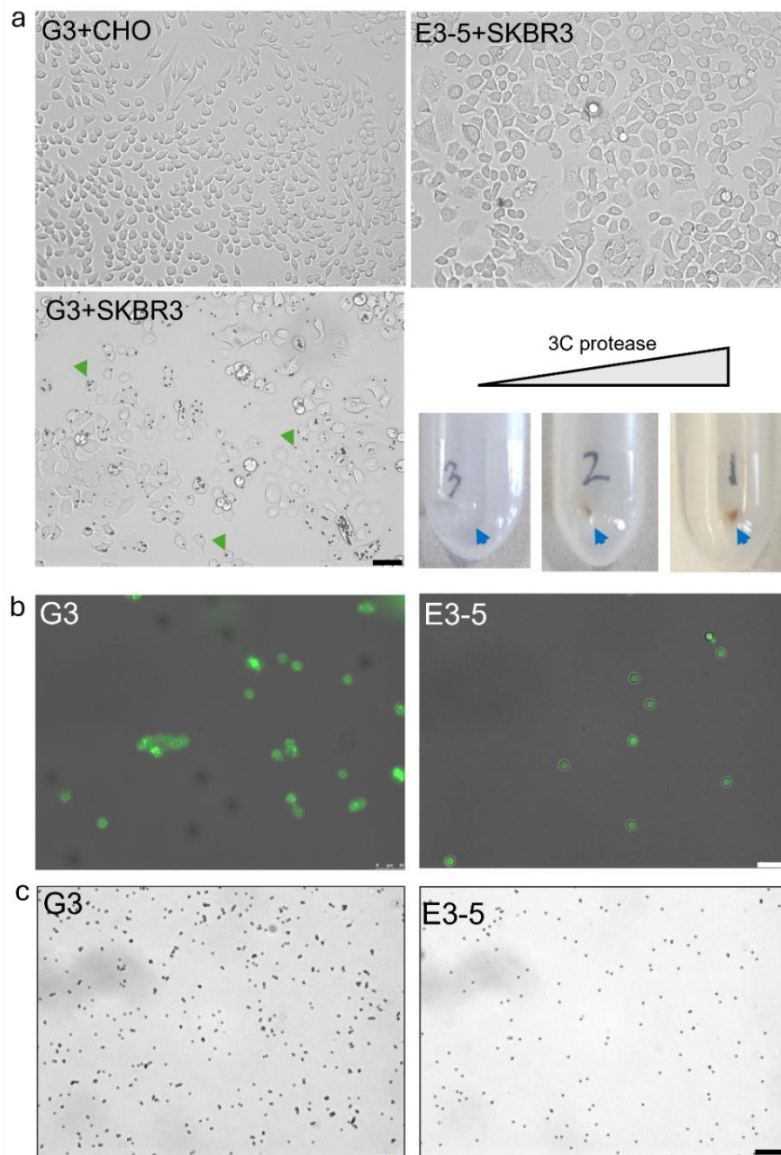
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27 **Figure S3. Expression and purification of the biotinylated DARPins.** SDS-PAGE confirms the  
28 ability to attach DARPin to streptavidin and release it by 3C protease cleavage.

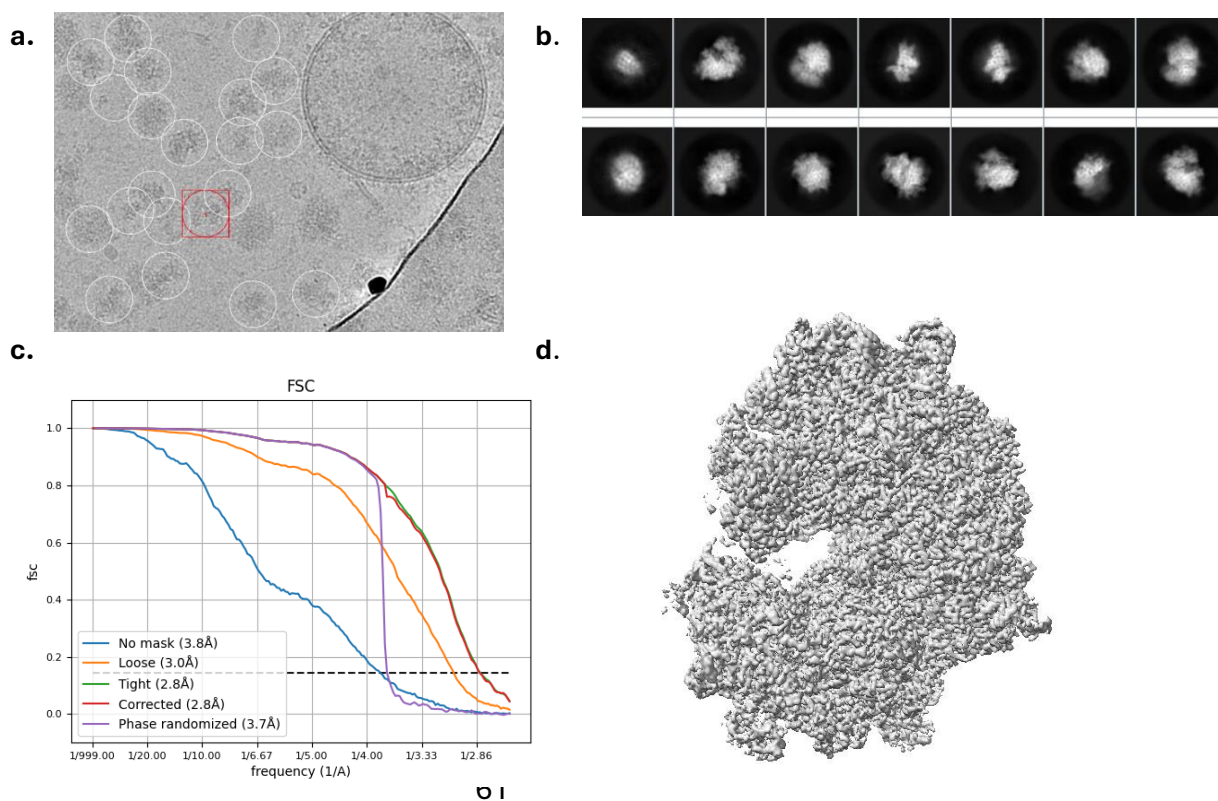
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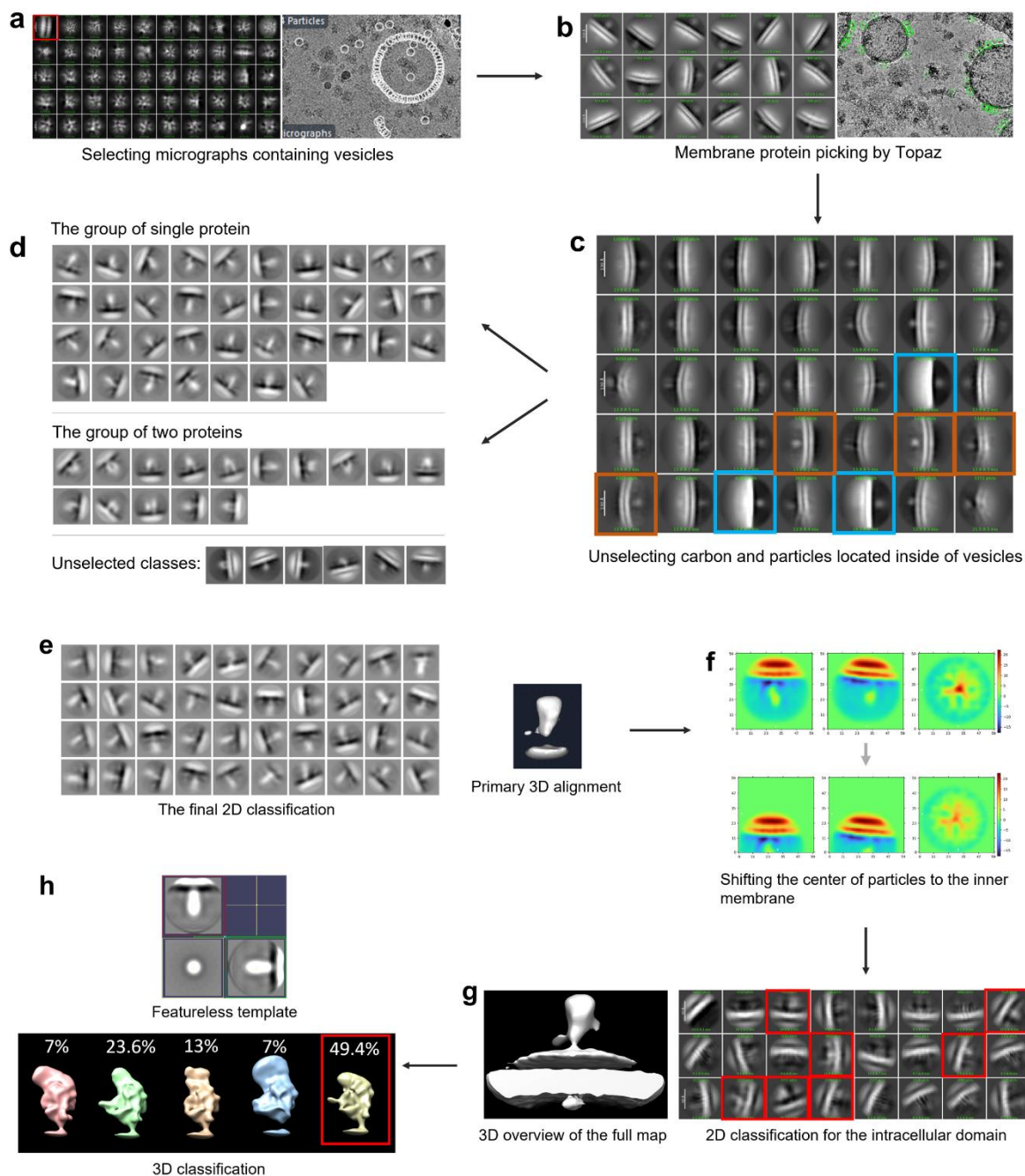
31 **Figure S4. The strategy used for purifying HER2 containing vesicles using different intact**  
 32 **cells - calibration and controls.** a. Bright field light microscopy images showing CHO cells  
 33 (HER2 negative, upper left), SKBR3 cells (HER2 positive, bottom left) incubated with magnetic  
 34 beads (green arrows) conjugated with DARPin G3, and SKBR3 cells incubated with magnetic  
 35 beads conjugated with DARPin E3-5 (not binding to HER2, control), upper right). The cells were  
 36 washed with PBS prior to imaging to remove unbound magnetic beads. Magnetic beads are  
 37 detected only after conjugation with DARPin G3 (Scale bar is 50  $\mu$ m). The magnetic beads can be  
 38 recovered by 3C protease followed by centrifugation, where increasing the amounts of 3C  
 39 protease increase the recovery of magnetic beads (bottom right, blue arrows, 0 uM, 50 uM and  
 40 80 uM 3C protease were applied, respectively). b. Mechanically induced vesicles (MVs),  
 41 produced from HER2-GFP expressing Sf9 cells, purified using magnetic force applied to  
 42 magnetic beads conjugated with DARPins. The fluorescence images indicate the specificity of  
 43 the process by the higher fluorescence from the HER2-GFP expressing Sf9 cells (Scale bar 10  
 44  $\mu$ m). c. DARPin G3-coated magnetic beads show a tendency to aggregate after incubation with  
 45 MVs from HER2-GFP expressing Sf9 cells, this was not observed with DARPin E3-5-coated beads  
 46 (Scale bar 50  $\mu$ m).

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62 **Figure S5. The quality of the dataset was evaluated by structural characterization of the**  
63 **ribosome at high resolution. a.** Micrograph showing a vesicle and many ribosomes in the  
64 background (indicated using white circles). **b.** Reference-free 2D classes of ribosomes in HER2-  
65 vesicles. **c.** FSC gold-standard curves in the non-uniform refinement using cryoSparc. **d.** Final  
66 map after sharpening.

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71 **Figure S6. Experimental workflow used for single particle cryo-EM analysis of receptors. a.**

72 Selecting the micrographs containing vesicles and initial 2D averaging (left). **b.** Densities

73 emanating from the membrane were picked by Topaz. **c.** Class averages which do not contain

74 membranes were removed (Blue), in addition to concave membranes with respect to protein

75 densities (red squares). **d.** Particle classification indicated a variety of protein architectures. The

76 classes which show two proteins or proteins with non-relevant dimensions were removed. **e.**

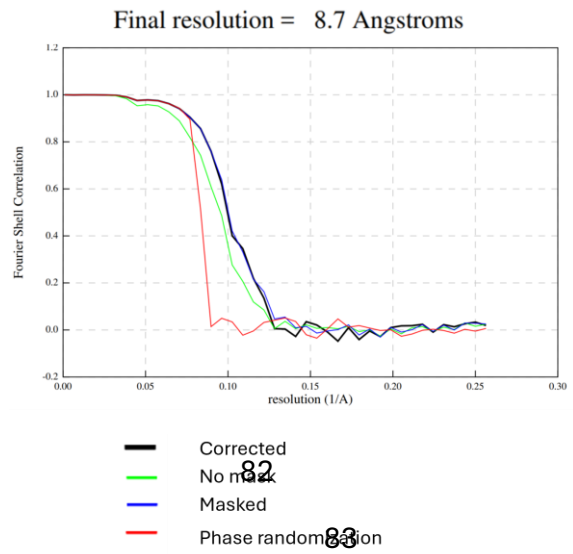
77 Resulting 2D classification of the dataset. **f.** The center of the images was shifted to the inner

78 membrane to focus the analysis on the intracellular densities. **g.** Only classes that exhibit

79 intracellular densities (the classes marked by the red squares) were selected for further

80 structural determination. **h.** A 3D classification resulted in structures that resemble HER2, even

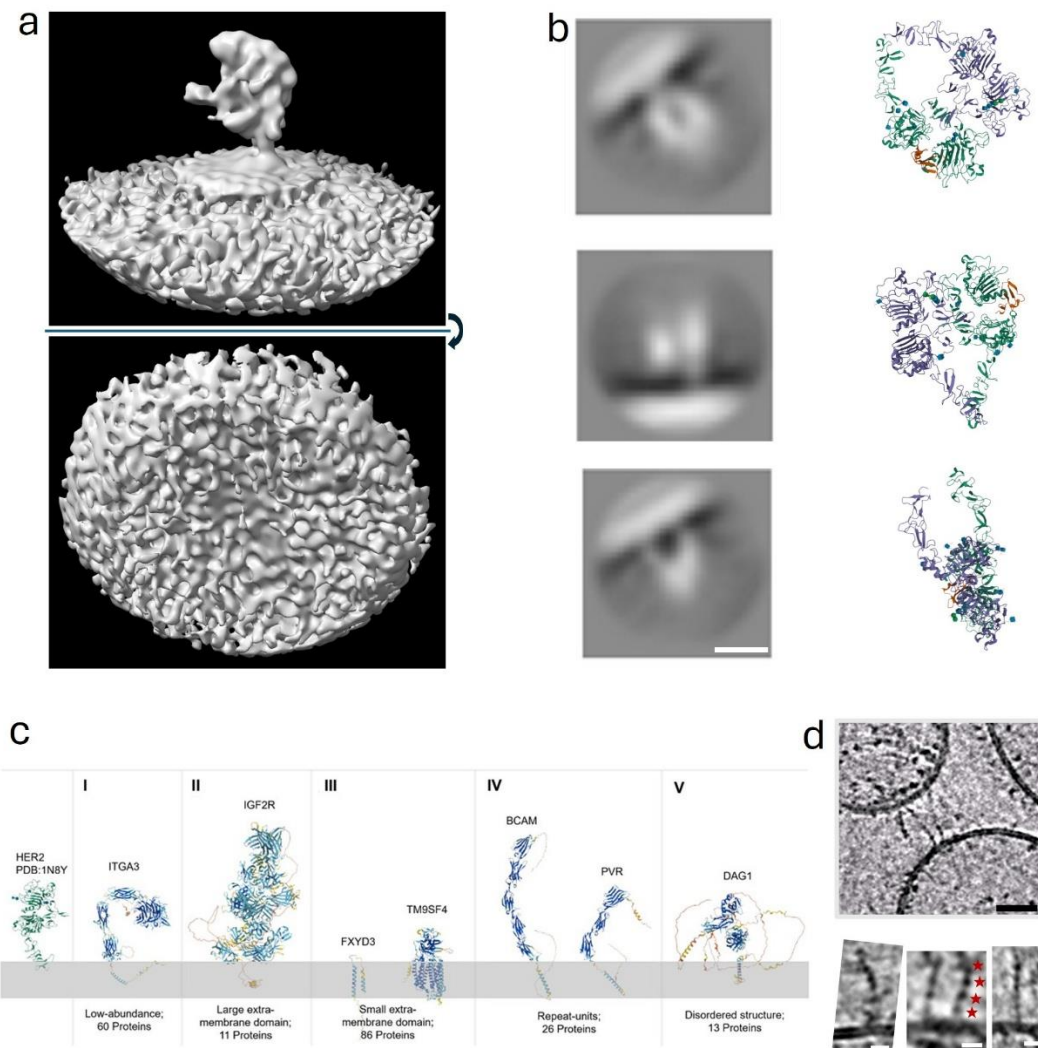
81 though the template was a featureless structure (upper part, perpendicular views are shown).



85 **Figure S7. The obtained structure of the HER2 receptor was resolved to 8.7 Å.** The rendered  
 86 view of the resulting structure (left), resembling the HER2 structure, was refined to a resolution  
 87 of 8.7 Å (right, gold-standard FCS resolution curves). The scale bar is 5 nm.

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**Figure S8. Additional structural observations.** **a.** The refined structure does not exhibit an intracellular density, supporting the notion that this region is highly flexible in relation to the ECD. **b.** Representative 2D classes show a dimeric-like appearance in agreement with 2D views of the HER2-HER3 heterodimer (PDB 7MN5). Scale bar is 5 nm. **c.** AlphaFold2 models of the different classes of membrane proteins which were identified in MVs by mass spectrometry (Table 1) and compared with the structure of HER2 (Fig. 4c). **d.** Elongated membrane proteins composed of globular units span between vesicles (upper panel). Three examples from our data set is showed with higher magnification (lower panel). Repeating globular densities (red stars). Black scale bar 20 nm, white scale bar 10 nm.

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101 **Table 1. Membrane proteins in diverse categories, correspond to Figure 4.** The gene  
 102 names and UniProt IDs are shown. Cross correlation analysis between HER2 and the 34  
 103 protein with some structural resemblance are shown in Fig. 4c

Membrane proteins with a large non-membrane domain (11 proteins)					
Gene	Uniprot ID	Gene	Uniprot ID	Gene	Uniprot ID
IGF2R	P11717	PTPRF	P10586	PLXNB1	O43157
PLXNB2	O15031	SORL1	Q92673	NOTCH3	Q9UM47
GLG1	Q92896	L1CAM	P32004	NUP210	Q8TEM1
PLXNA1	Q9UIW2	NOTCH2	Q04721		

Membrane proteins with a small non-membrane domain (87 proteins)					
Gene	Uniprot ID	Gene	Uniprot ID	Gene	Uniprot ID
FXYD3	Q14802	SLC35F6	Q8N357	LMAN2L	Q9H0V9
TM9SF2	Q99805	TMED1	Q13445	TMX2	G1K152
TMED10	P49755	VTCN1	Q7Z7D3	SMIM7	E9PP42
LMAN2	Q12907	MPZL1	O95297	SDC4	P31431
LMAN1	P49257	TMX1	Q9H3N1	TLCD1	Q96CP7
TMED2	Q15363	JTB	O76095	EMC10	Q5UCC4
CANX	P27824	SPINT2	O43291	TMEM9	B1ALM4
TM9SF4	Q92544	GPR107	Q5VW38	GPR180	Q86V85
TMED4	Q8R1V4	CD99	P14209	ACP2	P11117
M6PR	P20645	CCDC47	J3KRX4	TMX3	Q96JJ7
TACSTD2	P09758	GPR108	Q9NPR9	DDR1	Q08345
TMED7	Q9Y3B3	TMEM87A	Q8NBN3	MFAP3L	D6RCF0
TMEM165	D6RBL0	TM9SF1	O15321	F3	Q86SE7
TMED9	Q9BVK6	CD47	Q08722	SPPL2A	Q8TCT8
RPN1	P04843	LAMP2	P13473	MPZL2	O60487
EPCAM	C9JKY3	MLEC	F5H1S8	KITLG	P21583
LAMP1	P11279	TMEM194A	O14524	TMCO3	Q6UWJ1
DHCR24	Q15392	PTTG1IP	P53801	TMEM41A	Q96HV5
TM9SF3	Q9HD45	SSR1	P43307	HLA-E	P13747
SSR4	P51571	CD58	P19256	PGAP3	Q96FM1
SDC1	P18827	TMEM87B	Q96K49	TPBG	Q13641
TMED3	Q9Y3Q3	ATP6AP1	Q15904	TMEM9B	Q9NQ34
TMEM123	Q8N131	MAGT1	Q9H0U3	ICOSLG	O75144
DDOST	U3KQ84	PROCR	Q9UNN8	GLMP	Q8WVB7
F11R	Q9Y624	TGFBR1	P36897	UBAC2	Q8R944
PRSS8	Q16651	TNFRSF12A	Q9NP84	HLA-C	P10321
CA12	O43570	ATP6AP2	O75787	SIDT2	Q8N119
TMEM109	Q9BVC6	TUSC3	Q13454	SLC39A14	Q75T53
TMED5	Q9Y3A6	PIGK	Q92643	DCD	P81605

Membrane proteins with non-membrane repeating domains (26 proteins)					
Gene	Uniprot ID	Gene	Uniprot ID	Gene	Uniprot ID
BCAM	P50895	NOMO3	J3KN36	DSC2	Q02487
PTGFRN	Q9P2B2	DSG2	Q14126	PTK7	Q13308
PROM2	Q8N271	ALCAM	Q13740	PCDH1	Q08174
CD97	P48960	ITGA2	P17301	BSG	P35613
IGSF3	O75054	PTPRK	Q15262	PVR	P15151
CD46	P15529	PVRL4	Q96NY8	CADM1	Q9BY67
EPHB3	P54753	PVRL2	Q92692	EPHA1	P21709
NPTN	Q9Y639	PVRL1	Q15223	CD276	Q5ZPR3
IGSF8	Q969P0	EPHA2	P29317		

Membrane proteins with intrinsically disordered non-membrane domains (13 proteins)					
Gene	Uniprot ID	Gene	Uniprot ID	Gene	Uniprot ID
TGOLN2	O43493	SLC39A10	Q9ULF5	SYVN1	Q86TM6
DAG1	Q14118	SLC39A6	Q13433	MMP15	P51511
VASN	Q6EMK4	MIA3	Q5JRA6	CD44	P16070
CLCC1	Q96S66	STIM1	Q13586		
APP	P05067	APLP2	Q06481		

Membrane proteins with some resemblance to HER2 (34 proteins)					
Gene	Uniprot ID	Gene	Uniprot ID	Gene	Uniprot ID
HER2	P04626	ITGB1	P05556	PTPRA	P18433
SUSD2	Q9UGT4	NCSTN	Q92542	ENG	P17813
RPN2	Q3SZI6	GPR56	A0A024R6U7	NCLN	B2RA56
SORT1	Q99523	KIAA1324	A0A6P5P8N1	ADAM9	Q13443
EGFR	P00533	ITGB6	P18564	QSOX1	O00391
ADAM10	O14672	PIGT	Q969N2	LDLR	P01130
ALPP	P05187	QSOX2	Q6ZRP7	ADAM17	P78536
TAPBP	O15533	SEL1L	Q9UBV2	EMC1	Q8N766
ITGAV	P06756	KIAA2013	Q8IYS2	ATRN	O75882
ITGB5	P18084	TMEM132A	Q24JP5	NPC1	A0A193DRS0
CPD	O75976	ADAM15	Q13444		
ITFG1	Q8TB96	ERBB3	P21860		

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