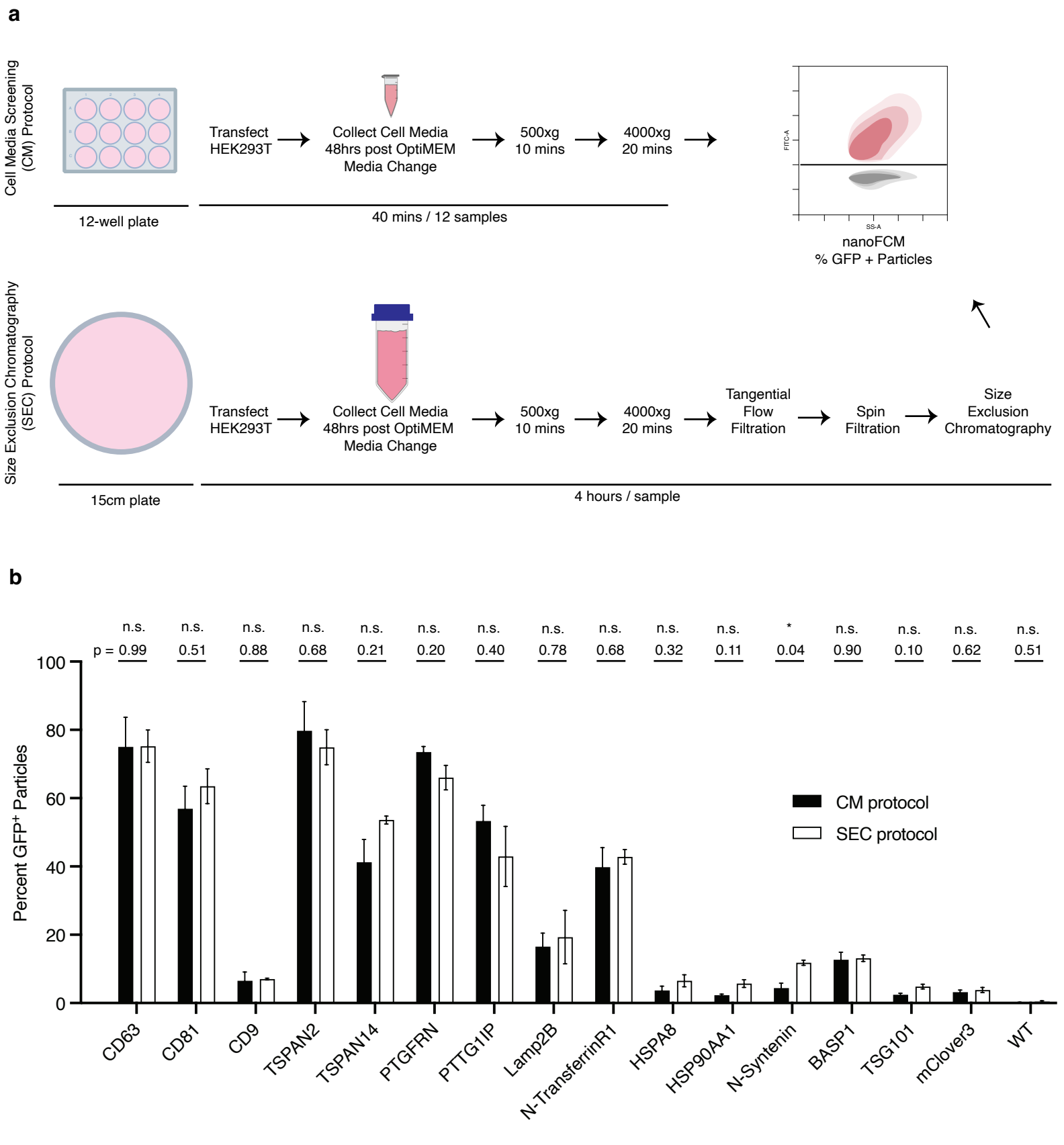

a
Cell Media Screening (CM) Protocol
Transfect HEK293T
Collect Cell Media 48hrs post OptiMEM Media Change
500xg 10 mins
4000xg 20 mins
12-well plate
40 mins / 12 samples
FITC-A
SS-A
nanoFCM
% GFP + Particles
Size Exclusion Chromatography (SEC) Protocol
Transfect HEK293T
Collect Cell Media 48hrs post OptiMEM Media Change
500xg 10 mins
4000xg 20 mins
Tangential Flow Filtration
Spin Filtration
Size Exclusion Chromatography
15cm plate
4 hours / sample
b
n.s. n.s. n.s. n.s. n.s. n.s. n.s. n.s. n.s. n.s. n.s. * n.s. n.s. n.s. n.s.
p = 0.99 0.51 0.88 0.68 0.21 0.20 0.40 0.78 0.68 0.32 0.11 0.04 0.90 0.10 0.62 0.51
Percent GFP+ Particles
100
80
60
40
20
0
CM protocol
SEC protocol
CD63
CD81
CD9
TSPAN2
TSPAN14
PTGFRN
PTTG1IP
Lamp2B
N-TransferrinR1
HSPA8
HSP90AA1
N-Syntenin
BASP1
TSG101
mClover3
WT

Figure S1. Validating an EV marker screening assay for single particle analysis.

(A) Schematic representing the EV marker screening assay protocol. (B) Percentage of GFP+ (mClover3) EVs from transiently transfected HEK-293T cells isolated by CM or SEC protocols. Data are presented as the mean ± SEM, n = 3 biological replicates, n.s. $P > 0.05$, * $P < 0.05$.

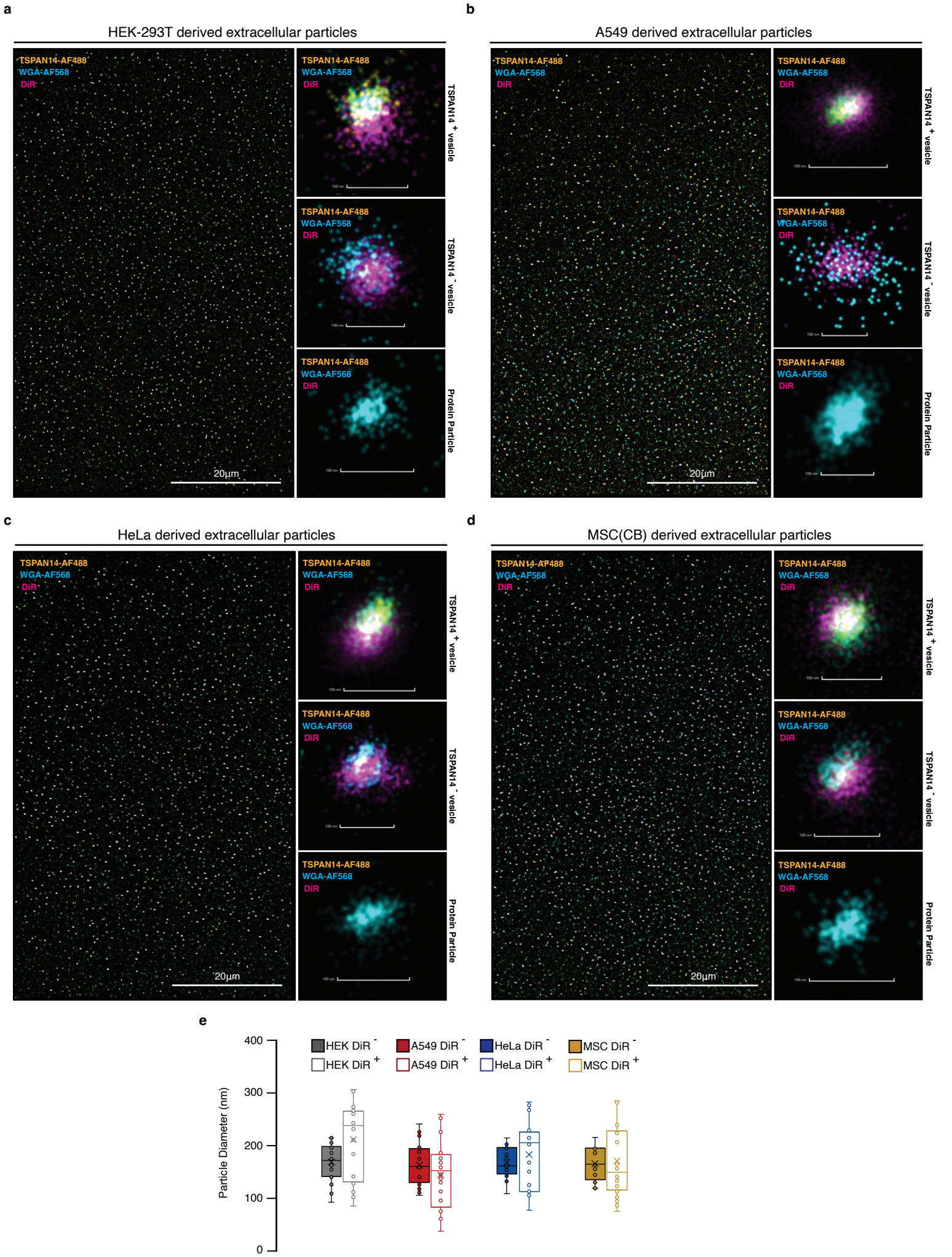

a
HEK-293T derived extracellular particles
TSPAN14-AF488
WGA-AF568
DiR
20μm
TSPAN14 + vesicle
TSPAN14 - vesicle
Protein Particle
b
A549 derived extracellular particles
c
HeLa derived extracellular particles
d
MSC(CB) derived extracellular particles
e
Particle Diameter (nm)
400
300
200
100
0
HEK DiR -
HEK DiR +
A549 DiR -
A549 DiR +
HeLa DiR -
HeLa DiR +
MSC DiR -
MSC DiR +

Figure S2. EP subpopulation dSTORM imaging confirms presence of glycosylated NVEPs. Single vesicle 2D SMLM dSTORM image analysis of nanoparticles from TSPAN14-mClover3 expressing stable cell lines in (A) HEK-293T, (B) A549, (C) HeLa, and (D) MSC(CB). Nanoparticles isolated by SEC are stained with DiR (far red membrane intercalating dye), WGA-AF568 (wheat germ agglutinin; binds N-linked glycosylated protein residues) to detect glycosylation PTMs, and anti-GFP-AF488. Scale bar 20μm in full field of view and 100nm in single particle zoom panels. Representative images of TSPAN14+ EVs, TSPAN14– EVs, and WGA+ spherical protein particles are shown. (E) Box plot of particle diameter (nm) calculated using the ONI CODI software are shown for EVs (DiR+ particles) and protein particles (DiR– particles) in each cell line (pair of bars from left to right correspond to HEK, A549, HeLa and MSC). Data represents n=20 nanoparticles per group.

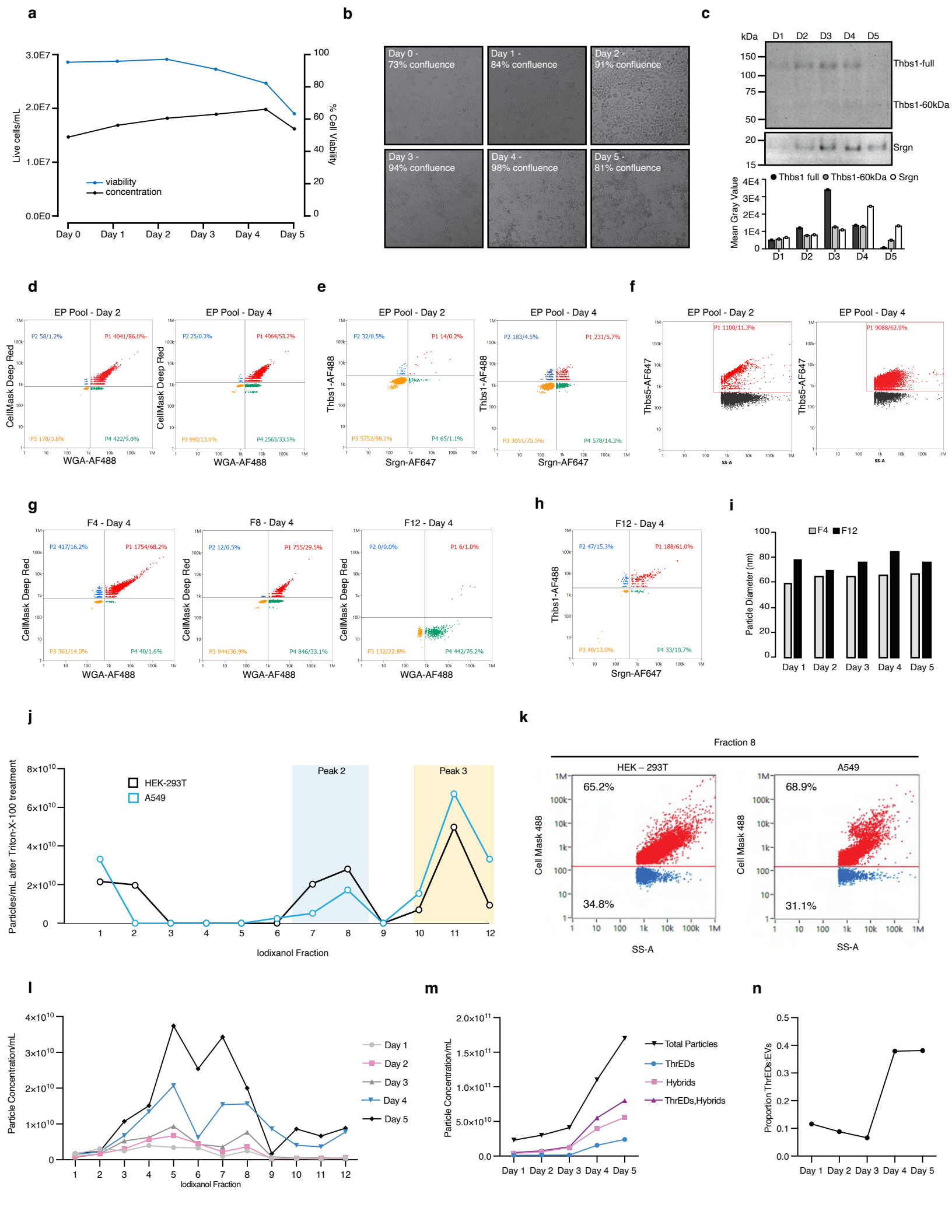
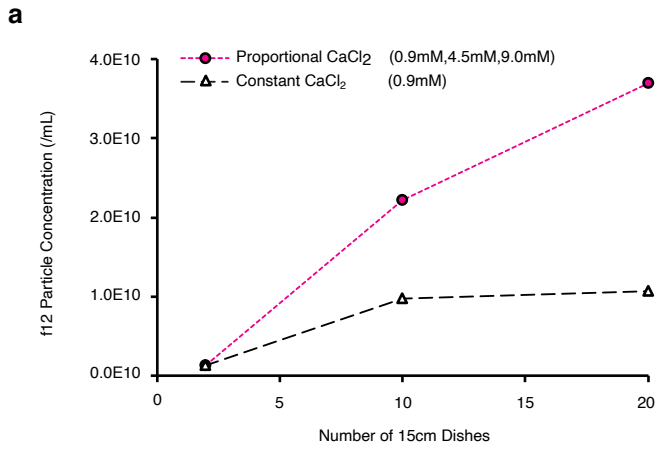
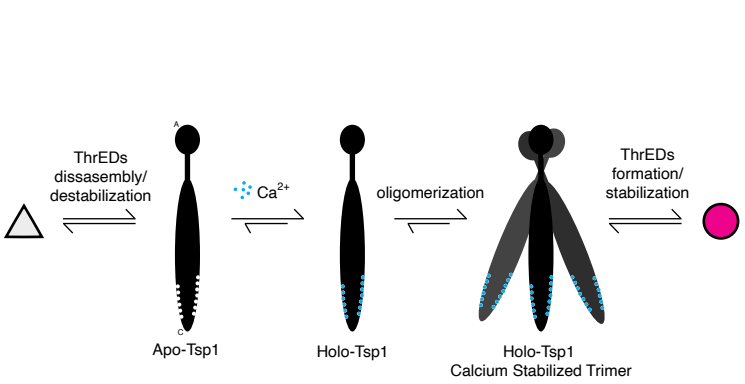
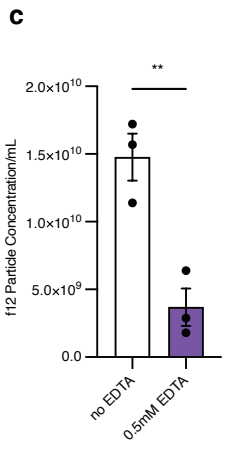

a
Live cells/mL
% Cell Viability
viability
concentration
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
b
Day 0 - 73% confluence
Day 1 - 84% confluence
Day 2 - 91% confluence
Day 3 - 94% confluence
Day 4 - 98% confluence
Day 5 - 81% confluence
c
kDa
Thbs1-full
Thbs1-60kDa
Srgn
Thbs1 full
Thbs1-60kDa
Srgn
Mean Gray Value
d
EP Pool - Day 2
EP Pool - Day 4
CellMask Deep Red
WGA-AF488
e
EP Pool - Day 2
EP Pool - Day 4
Thbs1-AF488
Srgn-AF647
f
EP Pool - Day 2
EP Pool - Day 4
Thbs5-AF647
g
F4 - Day 4
F8 - Day 4
F12 - Day 4
CellMask Deep Red
WGA-AF488
h
F12 - Day 4
Thbs1-AF488
Srgn-AF647
i
F4
F12
Particle Diameter (nm)
Day 1
Day 2
Day 3
Day 4
Day 5
j
HEK-293T
A549
Peak 2
Peak 3
Particles/mL after Triton-X-100 treatment
Iodixanol Fraction
k
Fraction 8
HEK – 293T
A549
65.2%
34.8%
68.9%
31.1%
Cell Mask 488
SS-A
l
Particle Concentration/mL
Iodixanol Fraction
Day 1
Day 2
Day 3
Day 4
Day 5
m
Particle Concentration/mL
Total Particles
ThrEDs
Hybrids
ThrEDs,Hybrids
n
Proportion ThrEDs:EVs

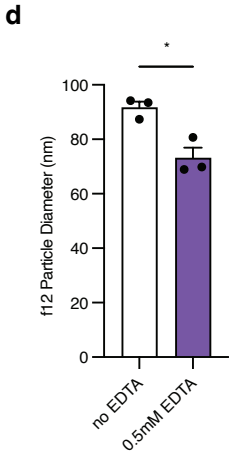
Figure S3. Release kinetics of Thrombospondin Encapsulated Dense particles.

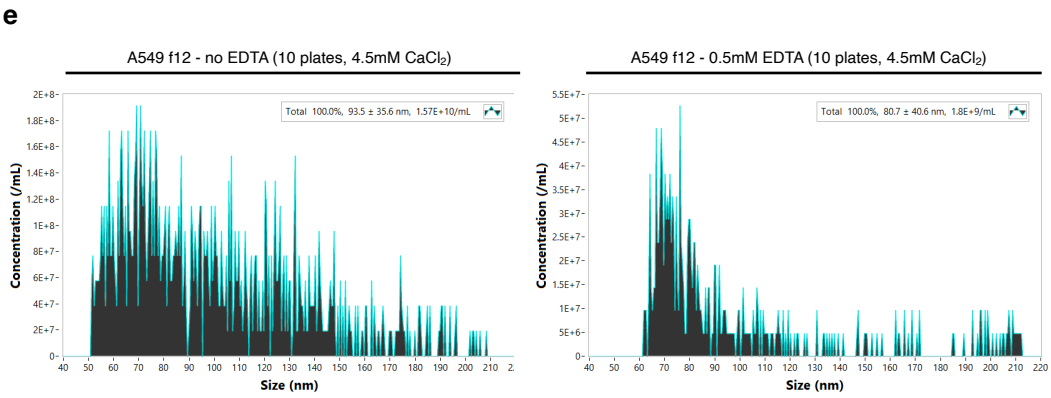
(A) Plot of A549 live cell concentration in cells/mL (bottom curve) and viability (top curve) at timepoints across 5 days of collection with (B) representative micrographs of cell confluence and morphology at each day. (C) Western blot whole cell lysates from day 1 through 5 probing Thrombospondin 1 and Serglycin with associated quantification of the mean grey value of each band. (D) Nanoflow cytometry analysis of extracellular particles pools from conditioned media at days 2 and 4 probing protein (WGA) and lipid (Cell Mask) biomolecules (E) TSP1 and SRGN and (F) COMP. (G) Nanoflow cytometry analysis of f4, F8 and f12 probing protein (WGA) and lipid (Cell Mask) biomolecules and (H) f12 probing Thbs1 and Srgn, all at the day 4 time point. (I) Size profiles of EVs in f4 and ThrEDs in f12 determined by side scatter using nanoflow cytometry across time points day 1 to 5. (J) EPs were isolated in 4.5mM calcium and then treated with 1% Triton-X-100. The EP Migration profile was achieved following iodixanol density-based ultracentrifuge fractionation for Triton-X-100 treated samples. (K) Following a typical protocol without Triton-X-100, f8 fractions from HEK- 293T and A549 were stained for Cell Mask which labelled lipids (EVs), showing two populations in f8. (L) Migration profile of Extracellular Particles (EP), isolated in isosmotic buffer with 4.5mM $CaCl_2$ at time points day 1 through 5, following iodixanol density-based ultracentrifuge fractionation. (M) Concentrations of f1-12 total particles, f12+11 ThrEDs, f7+8 Hybrids, across time points and (N) the proportion of f11+12 ThrEDs relative to the proportion of f4+5 EVs across time points.

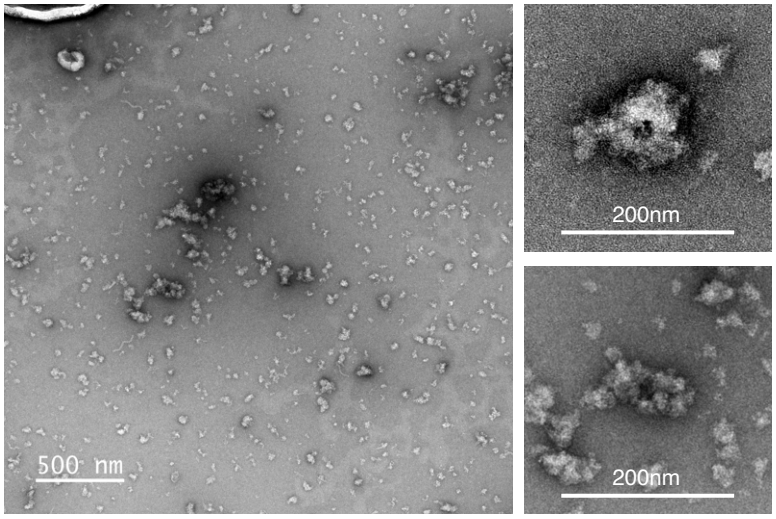

a
Proportional CaCl2 (0.9mM,4.5mM,9.0mM)
Constant CaCl2 (0.9mM)
f12 Particle Concentration (/mL)
4.0E10
3.0E10
2.0E10
1.0E10
0.0E10
0
5
10
15
20
Number of 15cm Dishes


b
ThrEDs dissassembly/ destabilization
Ca2+
oligomerization
ThrEDs formation/ stabilization
Apo-Tsp1
Holo-Tsp1
Holo-Tsp1 Calcium Stabilized Trimer


c
**
f12 Particle Concentration/mL
2.0x10^10
1.5x10^10
1.0x10^10
5.0x10^9
0.0
no EDTA
0.5mM EDTA


d
*
f12 Particle Diameter (nm)
100
80
60
40
20
0
no EDTA
0.5mM EDTA


e
A549 f12 - no EDTA (10 plates, 4.5mM CaCl2)
Total 100.0%, 93.5 ± 35.6 nm, 1.57E+10/mL
Concentration (/mL)
Size (nm)
A549 f12 - 0.5mM EDTA (10 plates, 4.5mM CaCl2)
Total 100.0%, 80.7 ± 40.6 nm, 1.8E+9/mL
Concentration (/mL)
Size (nm)


f
A549 F8 - no EDTA (10 plates, 4.5mM CaCl2)
500 nm
200nm
200nm

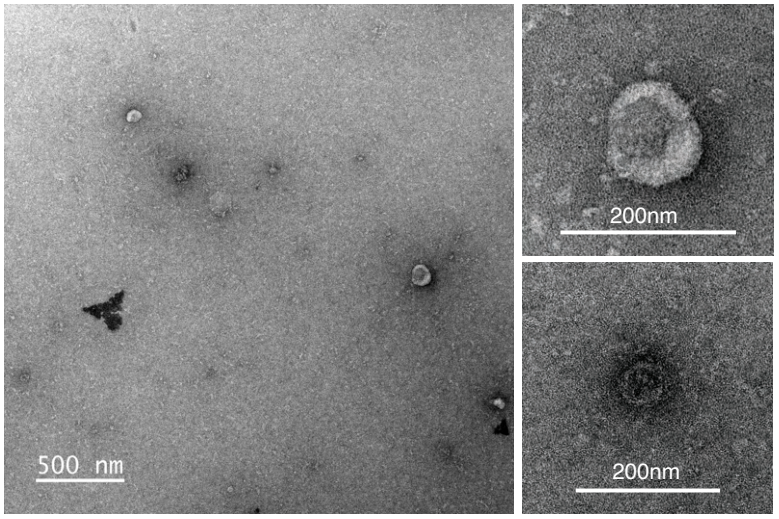

g
A549 F8 - 0.5mM EDTA (10 plates, 4.5mM CaCl2)
500 nm
200nm
200nm

Figure S4. Calcium is critical in the formation and stabilization of ThrEDs.

(A) The effect of increasing amount of $CaCl_2$ in an isosmotic buffer on ThrEDs f12 particle concentration. Samples were isolated from 2 plates, 10 plates and 20 plates, purified with constant calcium (0.9mM $CaCl_2$) or proportional amounts of calcium at 0.9mM, 4.5mM and 9mM for 2, 10 and 20 plates, respectively. (B) Diagram representing the homo-trimerization of Thrombospondin 1 induced by calcium and the effect on ThrEDs stability equilibrating between low concentration and destabilized and high concertation and stable. (C) f12 particle concentration following treatment with 0.5mM EDTA and the (D) associated change in particle size with raw size distribution plots shown in (E). (C-E) were generated from 10 plates isolated in 4.5mM CaCl2. (F-G) Demonstrate the effect of calcium stabilization on ThrEDs morphology in f8 before and after EDTA treatment. Calcium stabilization of Thrombospondin subunits is seen in (F) with dense particles abundant among EVs (hybrid population) and the loss of large ThrEDs following EDTA treatment while EVs morphology and concentration remain unchanged (G). n.s. $P > 0.05$, * $P < 0.05$, ** $P < 0.01$.(A) n=1, (C-G) n=3 biological replicates.

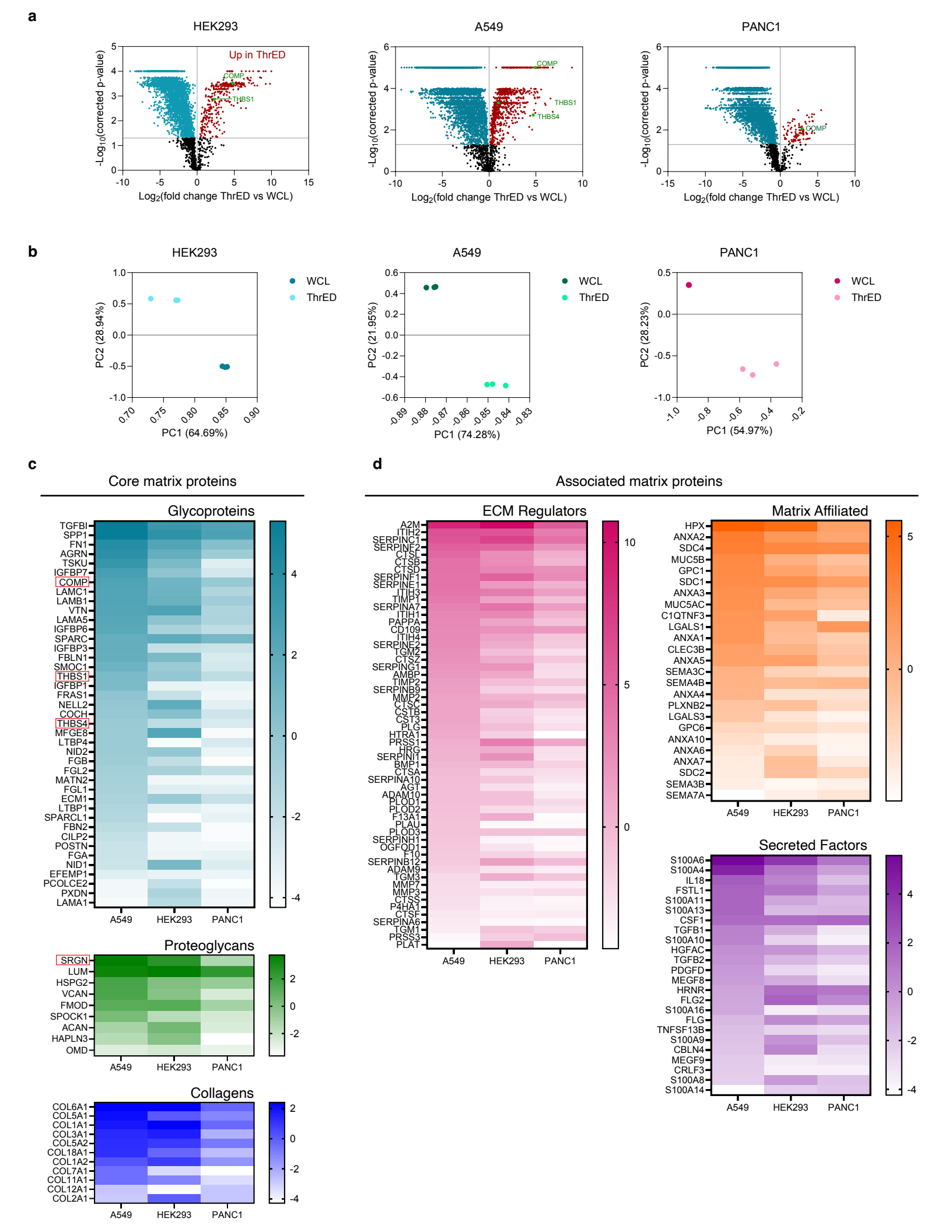

a
HEK293
A549
PANC1
Up in ThrED
COMP
THBS1
THBS4
-Log10(corrected p-value)
Log2(fold change ThrED vs WCL)
b
HEK293
A549
PANC1
WCL
ThrED
PC2 (28.94%)
PC1 (64.69%)
PC2 (21.95%)
PC1 (74.28%)
PC2 (28.23%)
PC1 (54.97%)
c
Core matrix proteins
Glycoproteins
TGFBI
SPP1
FN1
AGRN
TSKU
IGFBP7
COMP
LAMC1
LAMB1
VTN
LAMA5
IGFBP6
SPARC
IGFBP3
FBLN1
SMOC1
THBS1
IGFBP1
FRAS1
NELL2
COCH
THBS4
MFGE8
LTBP4
NID2
FGB
FGL2
MATN2
FGL1
ECM1
LTBP1
SPARCL1
FBN2
CILP2
POSTN
FGA
NID1
EFEMP1
PCOLCE2
PXDN
LAMA1
A549
HEK293
PANC1
Proteoglycans
SRGN
LUM
HSPG2
VCAN
FMOD
SPOCK1
ACAN
HAPLN3
OMD
Collagens
COL6A1
COL5A1
COL1A1
COL3A1
COL5A2
COL18A1
COL1A2
COL7A1
COL11A1
COL12A1
COL2A1
d
Associated matrix proteins
ECM Regulators
A2M
ITIH2
SERPINC1
SERPINF2
CTSF
CTSB
CTSD
SERPINF1
SERPINE1
ITIH3
TIMP1
SERPINA7
ITIH1
PAPPA
CD109
ITIH4
SERPINE2
TGM2
CTSZ
SERPING1
AMBP
TIMP2
SERPINB9
MMP2
CTSC
CSTB
CST3
PLG
HTRA1
PRSS1
HRG
SERPINI1
BMP1
CTSA
SERPINA10
AGT
ADAM10
PLOD1
PLOD2
F13A1
PLAU
PLOD3
SERPINH1
OGFOD1
F10
SERPINB12
ADAM9
TGM3
MMP7
MMP3
CTSS
P4HA1
CTSF
SERPINA6
TGM1
PRSS3
PLAT
A549
HEK293
PANC1
Matrix Affiliated
HPX
ANXA2
SDC4
MUC5B
GPC1
SDC1
ANXA3
MUC5AC
C1QTNF3
LGALS1
ANXA1
CLEC3B
ANXA5
SEMA3C
SEMA4B
ANXA4
PLXNB2
LGALS3
GPC6
ANXA10
ANXA6
ANXA7
SDC2
SEMA3B
SEMA7A
A549
HEK293
PANC1
Secreted Factors
S100A6
S100A4
IL18
FSTL1
S100A11
S100A13
CSF1
TGFB1
S100A10
HGFAC
TGFB2
PDGFD
MEGF8
HRNR
FLG2
S100A16
FLG
TNFSF13B
S100A9
CBLN4
MEGF9
CRLF3
S100A8
S100A14
A549
HEK293
PANC1

Figure S5. ThrED proteomics matrix highlight core and associated matrix proteins.
(A) Volcano plots of proteins identified in ThrEDs. Red and blue labelled proteins indicate those significantly upregulated in ThrEDs or whole cell lysate, respectively. (B) PCA plots for the proteins identified in f11+12 ThrEDs and whole cell lysates of HEK293, A549, and PANC1 cells. (C-D) Heat maps of proteins quantified (Log_2 abundance) in ThrEDs in A549, HEK293, PANC1 sorted into protein classes and functional groupings as determined by MatrisomeDB. n=3 biological replicates.

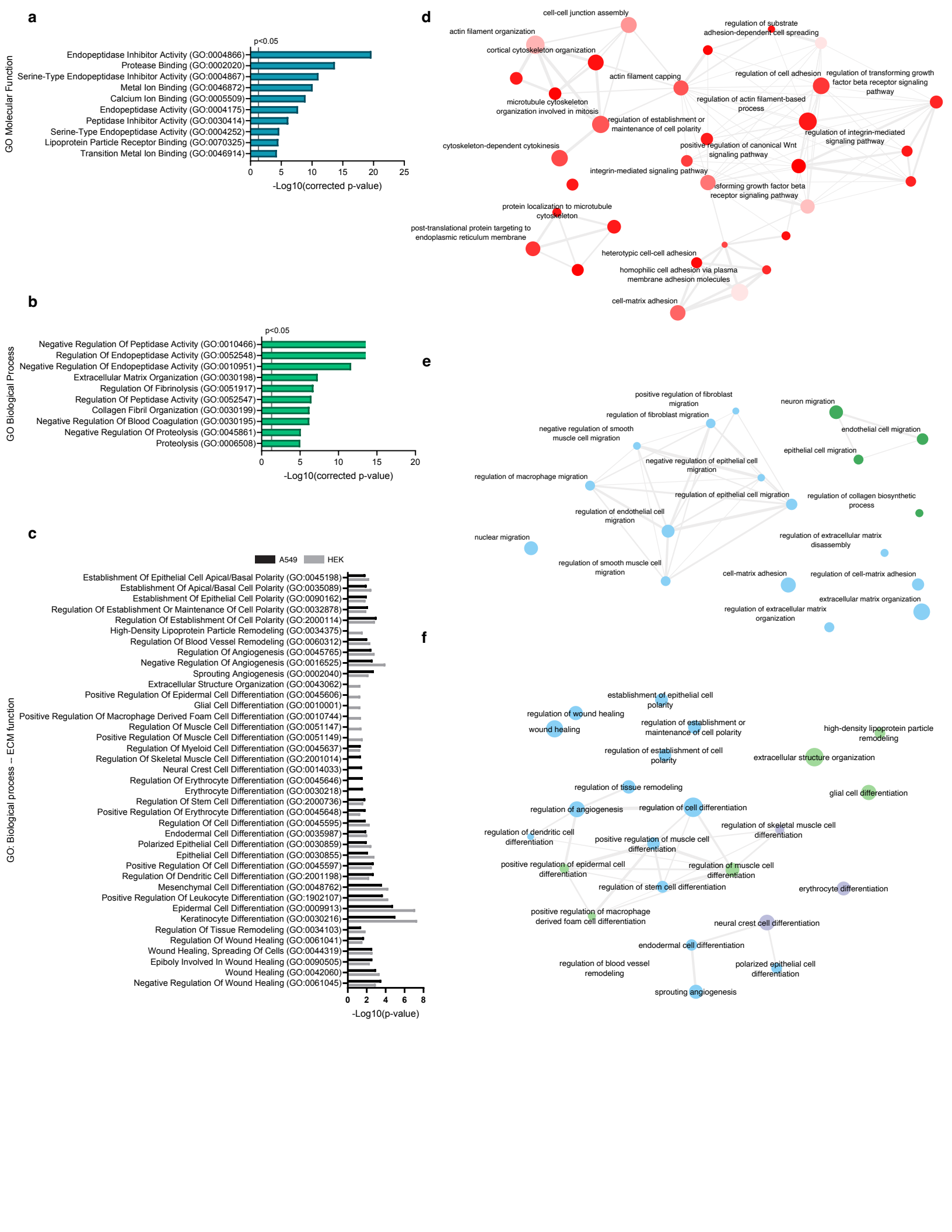

a
GO Molecular Function
p<0.05
Endopeptidase Inhibitor Activity (GO:0004866)
Protease Binding (GO:0002020)
Serine-Type Endopeptidase Inhibitor Activity (GO:0004867)
Metal Ion Binding (GO:0046872)
Calcium Ion Binding (GO:0005509)
Endopeptidase Activity (GO:0004175)
Peptidase Inhibitor Activity (GO:0030414)
Serine-Type Endopeptidase Activity (GO:0004252)
Lipoprotein Particle Receptor Binding (GO:0070325)
Transition Metal Ion Binding (GO:0046914)
0 5 10 15 20 25
-Log10(corrected p-value)
b
GO Biological Process
p<0.05
Negative Regulation Of Peptidase Activity (GO:0010466)
Regulation Of Endopeptidase Activity (GO:0052548)
Negative Regulation Of Endopeptidase Activity (GO:0010951)
Extracellular Matrix Organization (GO:0030198)
Regulation Of Fibrinolysis (GO:0051917)
Regulation Of Peptidase Activity (GO:0052547)
Collagen Fibril Organization (GO:0030199)
Negative Regulation Of Blood Coagulation (GO:0030195)
Negative Regulation Of Proteolysis (GO:0045861)
Proteolysis (GO:0006508)
0 5 10 15 20
-Log10(corrected p-value)
c
GO: Biological process -- ECM function
A549
HEK
Establishment Of Epithelial Cell Apical/Basal Polarity (GO:0045198)
Establishment Of Apical/Basal Cell Polarity (GO:0035089)
Establishment Of Epithelial Cell Polarity (GO:0090162)
Regulation Of Establishment Or Maintenance Of Cell Polarity (GO:0032878)
Regulation Of Establishment Of Cell Polarity (GO:2000114)
High-Density Lipoprotein Particle Remodeling (GO:0034375)
Regulation Of Blood Vessel Remodeling (GO:0060312)
Regulation Of Angiogenesis (GO:0045765)
Negative Regulation Of Angiogenesis (GO:0016525)
Sprouting Angiogenesis (GO:0002040)
Extracellular Structure Organization (GO:0043062)
Positive Regulation Of Epidermal Cell Differentiation (GO:0045606)
Glial Cell Differentiation (GO:0010001)
Positive Regulation Of Macrophage Derived Foam Cell Differentiation (GO:0010744)
Regulation Of Muscle Cell Differentiation (GO:0051147)
Positive Regulation Of Muscle Cell Differentiation (GO:0051149)
Regulation Of Myeloid Cell Differentiation (GO:0045637)
Regulation Of Skeletal Muscle Cell Differentiation (GO:2001014)
Neural Crest Cell Differentiation (GO:0014033)
Regulation Of Erythrocyte Differentiation (GO:0045646)
Erythrocyte Differentiation (GO:0030218)
Regulation Of Stem Cell Differentiation (GO:2000736)
Positive Regulation Of Erythrocyte Differentiation (GO:0045648)
Regulation Of Cell Differentiation (GO:0045595)
Endodermal Cell Differentiation (GO:0035987)
Polarized Epithelial Cell Differentiation (GO:0030859)
Epithelial Cell Differentiation (GO:0030855)
Positive Regulation Of Cell Differentiation (GO:0045597)
Regulation Of Dendritic Cell Differentiation (GO:2001198)
Mesenchymal Cell Differentiation (GO:0048762)
Positive Regulation Of Leukocyte Differentiation (GO:1902107)
Epidermal Cell Differentiation (GO:0009913)
Keratinocyte Differentiation (GO:0030216)
Regulation Of Tissue Remodeling (GO:0034103)
Regulation Of Wound Healing (GO:0061041)
Wound Healing, Spreading Of Cells (GO:0044319)
Epiboly Involved In Wound Healing (GO:0090505)
Wound Healing (GO:0042060)
Negative Regulation Of Wound Healing (GO:0061045)
0 2 4 6 8
-Log10(p-value)
d
cell-cell junction assembly
actin filament organization
regulation of substrate adhesion-dependent cell spreading
cortical cytoskeleton organization
actin filament capping
regulation of cell adhesion
regulation of transforming growth factor beta receptor signaling pathway
microtubule cytoskeleton organization involved in mitosis
regulation of actin filament-based process
regulation of establishment or maintenance of cell polarity
regulation of integrin-mediated signaling pathway
cytoskeleton-dependent cytokinesis
positive regulation of canonical Wnt signaling pathway
integrin-mediated signaling pathway
transforming growth factor beta receptor signaling pathway
protein localization to microtubule cytoskeleton
post-translational protein targeting to endoplasmic reticulum membrane
heterotypic cell-cell adhesion
homophilic cell adhesion via plasma membrane adhesion molecules
cell-matrix adhesion
e
positive regulation of fibroblast migration
neuron migration
regulation of fibroblast migration
negative regulation of smooth muscle cell migration
endothelial cell migration
epithelial cell migration
negative regulation of epithelial cell migration
regulation of macrophage migration
regulation of epithelial cell migration
regulation of collagen biosynthetic process
regulation of endothelial cell migration
nuclear migration
regulation of extracellular matrix disassembly
regulation of smooth muscle cell migration
cell-matrix adhesion
regulation of cell-matrix adhesion
extracellular matrix organization
regulation of extracellular matrix organization
f
establishment of epithelial cell polarity
regulation of wound healing
wound healing
regulation of establishment or maintenance of cell polarity
high-density lipoprotein particle remodeling
regulation of establishment of cell polarity
extracellular structure organization
regulation of tissue remodeling
glial cell differentiation
regulation of angiogenesis
regulation of cell differentiation
regulation of skeletal muscle cell differentiation
regulation of dendritic cell differentiation
positive regulation of muscle cell differentiation
positive regulation of epidermal cell differentiation
regulation of muscle cell differentiation
regulation of stem cell differentiation
erythrocyte differentiation
positive regulation of macrophage derived foam cell differentiation
neural crest cell differentiation
endodermal cell differentiation
regulation of blood vessel remodeling
polarized epithelial cell differentiation
sprouting angiogenesis

Figure S6. GO term analysis of ThrED proteins have complementary and opposing biological and molecular ECM function. Top ten GO terms from proteins identified in at least 2 ThrEDs sources of HEK, A549, PANC1 for (A) Molecular Function, (B) Biological Process, (C) Significant GO terms related to the formation of the ECM from proteins identified in ThrED particles in HEK and A549. (D) GO term network analysis of significant, non-redundant polarity terms identified in A549 whole cell lysates. The nodes are coloured by p-value where darker red indicated increased confidence, the size of each dot represents the number of annotations of the GO term. (E) GO term network analysis on ThrED proteins from HEK and A549 involved in general ECM biology and ECM formation and (F) ECM function. Blue points ae present in both HEK and A549. Green points are only in HEK and purple points are only in A549. The size of each dot describes the number of annotations for that GO term. n=3 biological replicates.

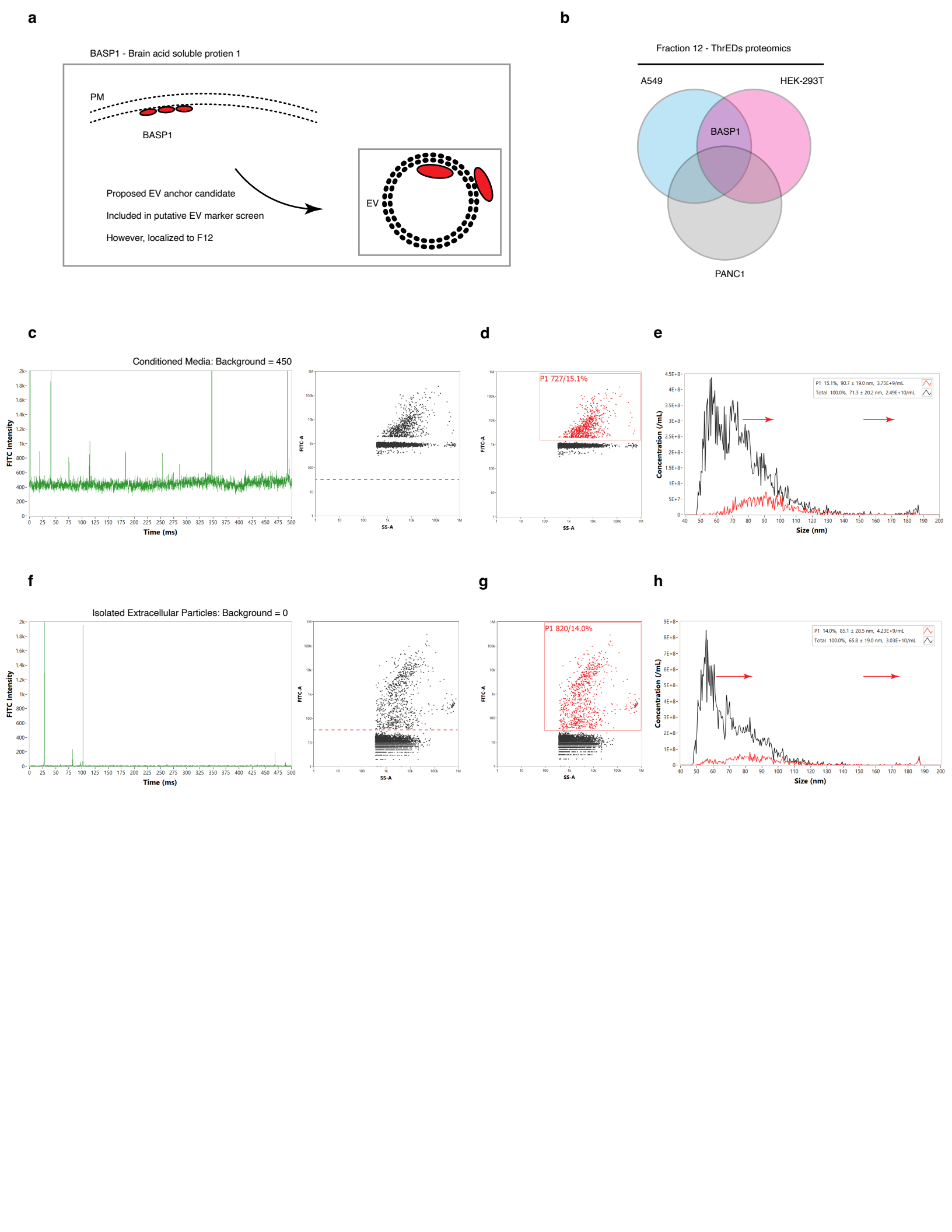
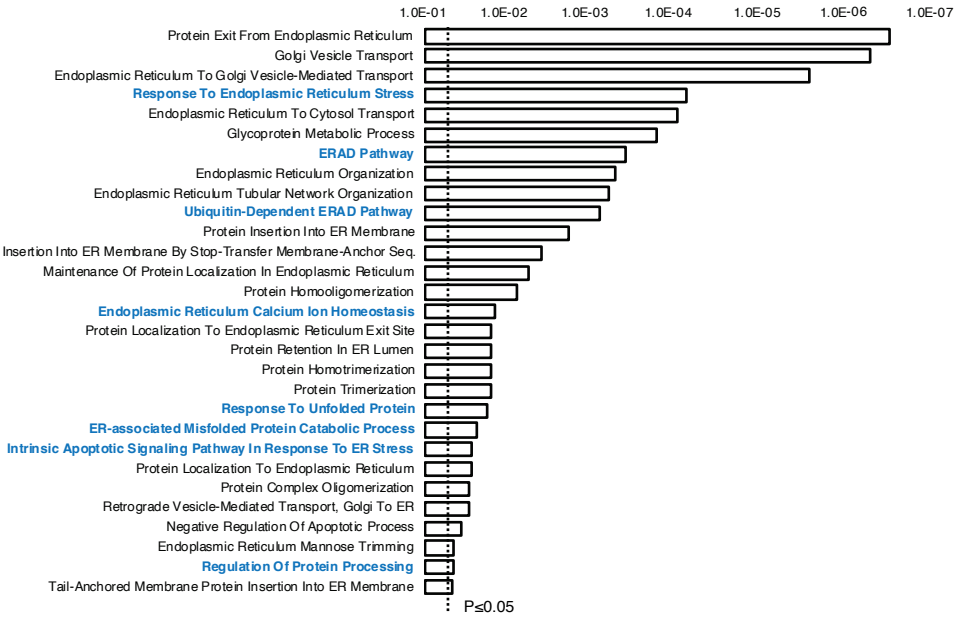

a
BASP1 - Brain acid soluble protien 1
PM
BASP1
Proposed EV anchor candidate
Included in putative EV marker screen
However, localized to F12
EV
b
Fraction 12 - ThrEDs proteomics
A549
HEK-293T
BASP1
PANC1
c
Conditioned Media: Background = 450
FITC Intensity
Time (ms)
FITC-A
SS-A
d
P1 727/15.1%
FITC-A
SS-A
e
P1 15.1%, 90.7 ± 19.0 nm, 3.75E+9/mL
Total 100.0%, 71.3 ± 20.2 nm, 2.49E+10/mL
Concentration (/mL)
Size (nm)
f
Isolated Extracellular Particles: Background = 0
FITC Intensity
Time (ms)
FITC-A
SS-A
g
P1 820/14.0%
FITC-A
SS-A
h
P1 14.0%, 85.1 ± 28.5 nm, 4.23E+9/mL
Total 100.0%, 65.8 ± 19.0 nm, 3.03E+10/mL
Concentration (/mL)
Size (nm)

Figure S7. BASP1 is a Thrombospondin Encapsulated Dense Particle protein.

(A) Diagram of Brain Acid Soluble Protein 1 (BASP1) a membrane associated cytosolic protein which has typically been described as an extracellular vesicle protein marker (was therefore included in the screen in Figure 1). (B) BASP1 is one of the abundant, common proteins present in ThrED f11-12 samples. (C) Shows the raw FITC readings from the nanoflow cytometer when measuring BASP1-mClover and associated dot plot. The horizontal dotted line represents where a gate would typically be placed resulting in the incorrect gating on BASP1-mClover3 presence in EP samples and a nearly 100% positive FITC population, as has been previously published. (D) Demonstrates where the gate should be placed based on the high background in the sample. (E) Shows the size profile of BASP1 positive and negative extracellular particles with a greater average diameter in positive particles. (F-H) Show the same data from samples isolated using Size Exclusion Chromatography instead of crude conditioned media. (F) Shows lower background when soluble BASP1 proteins are separated from EPs during purification and (G) shows the correct gating. (H) Identifies a similar size profile as in (E). n=3 biological replicates.

a


A549 Whole Cell Lysate - Proteostatic Stress Pathway Enrichment
1.0E-01
1.0E-02
1.0E-03
1.0E-04
1.0E-05
1.0E-06
1.0E-07
Protein Exit From Endoplasmic Reticulum
Golgi Vesicle Transport
Endoplasmic Reticulum To Golgi Vesicle-Mediated Transport
Response To Endoplasmic Reticulum Stress
Endoplasmic Reticulum To Cytosol Transport
Glycoprotein Metabolic Process
ERAD Pathway
Endoplasmic Reticulum Organization
Endoplasmic Reticulum Tubular Network Organization
Ubiquitin-Dependent ERAD Pathway
Protein Insertion Into ER Membrane
Insertion Into ER Membrane By Stop-Transfer Membrane-Anchor Seq.
Maintenance Of Protein Localization In Endoplasmic Reticulum
Protein Homooligomerization
Endoplasmic Reticulum Calcium Ion Homeostasis
Protein Localization To Endoplasmic Reticulum Exit Site
Protein Retention In ER Lumen
Protein Homotrimerization
Protein Trimerization
Response To Unfolded Protein
ER-associated Misfolded Protein Catabolic Process
Intrinsic Apoptotic Signaling Pathway In Response To ER Stress
Protein Localization To Endoplasmic Reticulum
Protein Complex Oligomerization
Retrograde Vesicle-Mediated Transport, Golgi To ER
Negative Regulation Of Apoptotic Process
Endoplasmic Reticulum Mannose Trimming
Regulation Of Protein Processing
Tail-Anchored Membrane Protein Insertion Into ER Membrane
P≤0.05

b

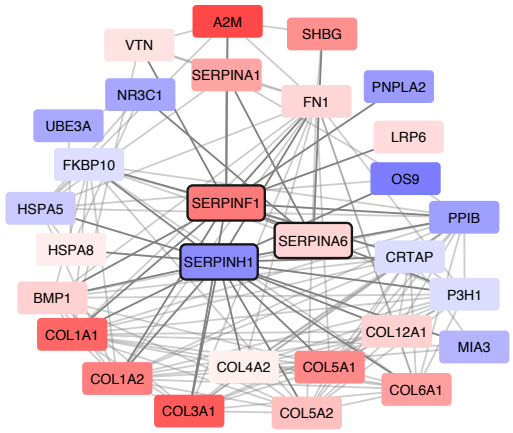
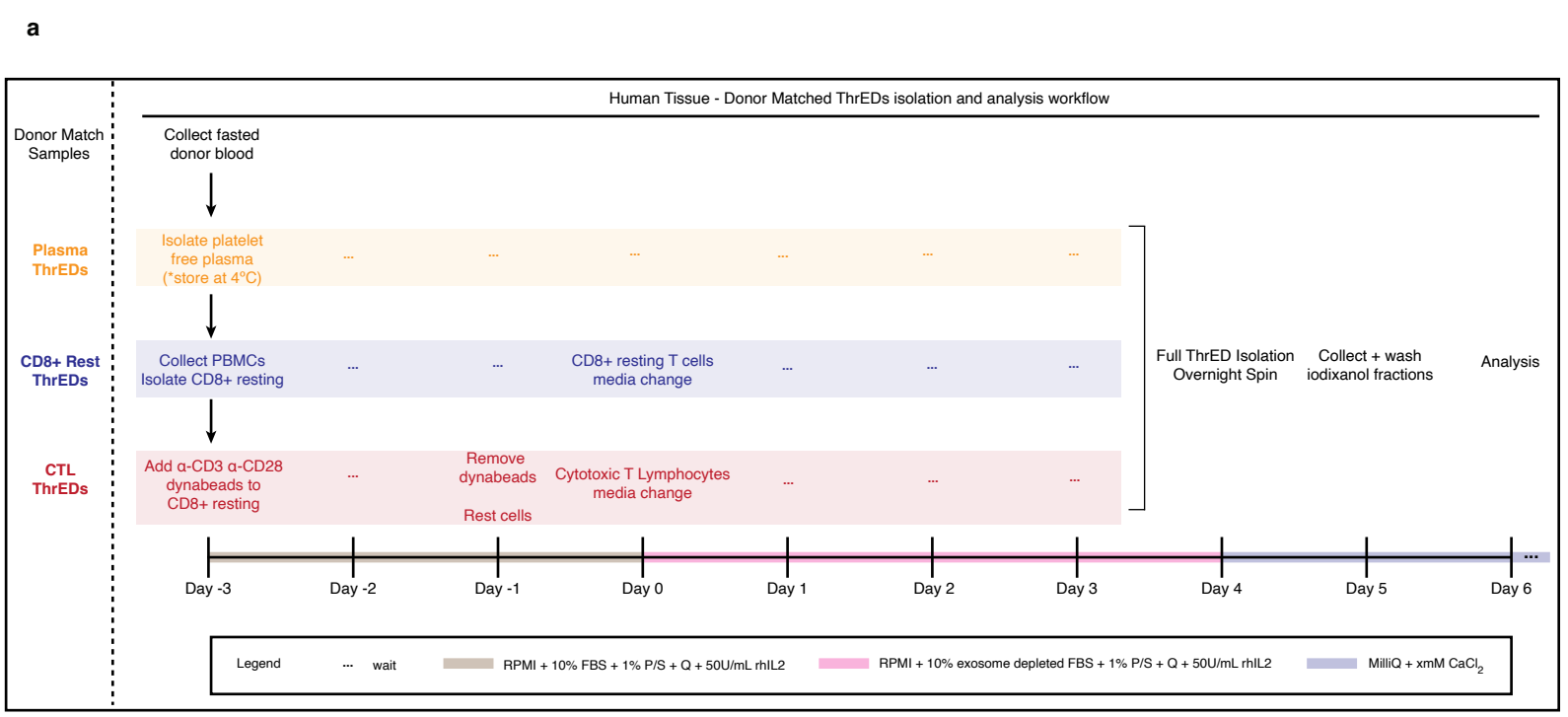
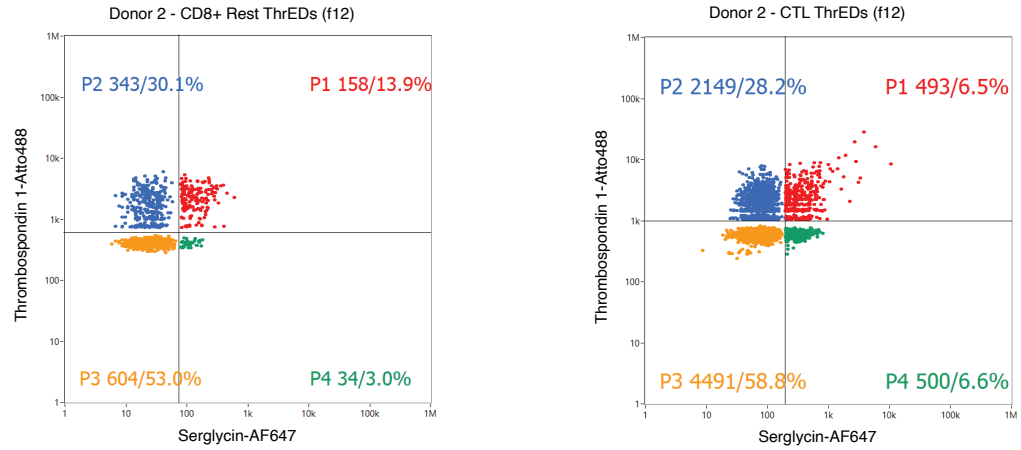
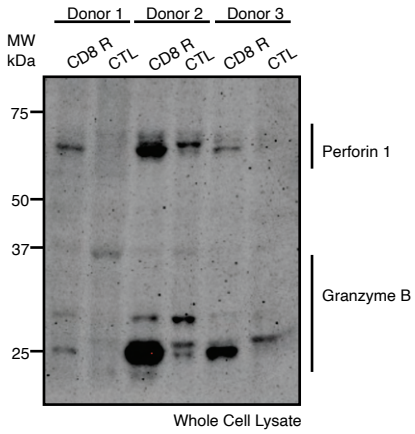

A549 f11-12 ThrEDs - Proteostatic Stress Protein Enrichment
A2M
SHBG
VTN
SERPINA1
NR3C1
FN1
PNPLA2
UBE3A
LRP6
FKBP10
OS9
HSPA5
SERPINF1
PPIB
SERPINA6
HSPA8
CRTAP
SERPINH1
BMP1
P3H1
COL1A1
COL12A1
MIA3
COL4A2
COL5A1
COL1A2
COL6A1
COL3A1
COL5A2

Figure S8. GO term enrichment analysis of proteostatic stress pathways in A549 lysates and ThrEDs

(A) Significant GO terms from proteomics analysis carried out on A549 whole cell lysates at day 4 involved in protein stress response and unfolded protein response and (B) Proteins significantly upregulated in ThrEDs involved in the UPR, cellular stress and ER stress pathways potentially involved in ThrEDs biogenesis and cargo loading. n=3 biological replicates.


a
Human Tissue - Donor Matched ThrEDs isolation and analysis workflow
Donor Match Samples
Collect fasted donor blood
Plasma ThrEDs
Isolate platelet free plasma (*store at 4°C)
CD8+ Rest ThrEDs
Collect PBMCs Isolate CD8+ resting
CD8+ resting T cells media change
CTL ThrEDs
Add α-CD3 α-CD28 dynabeads to CD8+ resting
Remove dynabeads
Rest cells
Cytotoxic T Lymphocytes media change
Full ThrED Isolation Overnight Spin
Collect + wash iodixanol fractions
Analysis
Day -3
Day -2
Day -1
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Legend
wait
RPMI + 10% FBS + 1% P/S + Q + 50U/mL rhIL2
RPMI + 10% exosome depleted FBS + 1% P/S + Q + 50U/mL rhIL2
MilliQ + xmM CaCl2


b
Donor 2 - CD8+ Rest ThrEDs (f12)
P2 343/30.1%
P1 158/13.9%
P3 604/53.0%
P4 34/3.0%
Thrombospondin 1-Atto488
Serglycin-AF647
Donor 2 - CTL ThrEDs (f12)
P2 2149/28.2%
P1 493/6.5%
P3 4491/58.8%
P4 500/6.6%
Thrombospondin 1-Atto488
Serglycin-AF647


c
Donor 1
Donor 2
Donor 3
MW kDa
CD8 R
CTL
75
50
37
25
Perforin 1
Granzyme B
Whole Cell Lysate

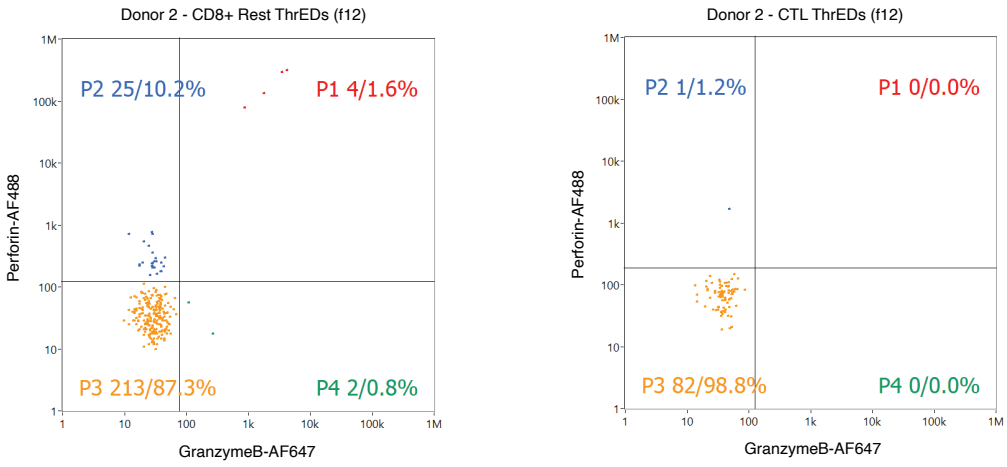

d
Donor 2 - CD8+ Rest ThrEDs (f12)
P2 25/10.2%
P1 4/1.6%
P3 213/87.3%
P4 2/0.8%
Perforin-AF488
GranzymeB-AF647
Donor 2 - CTL ThrEDs (f12)
P2 1/1.2%
P1 0/0.0%
P3 82/98.8%
P4 0/0.0%
Perforin-AF488
GranzymeB-AF647

Figure S9. ThrED isolation and characterization from donor matched human tissue.
(A) Schematic diagram of protocol timing for ThrEDs in Donor Matched tissues. Fasted blood is collected from donors to generate Platelet Free Plasma, resting CD8+ cells and Cytotoxic T lymphocyte (CTLs). Media used at each step and timing for ThrEDs collection is highlighted. (B) Nanoflow cytometry analysis of f12 ThrEDs from $CD8^+$ Resting population and CTLs probing Thrombospondin-1 and Serglycin. (C) Western blot of whole cell lysate from donor resting CD8+ T cells and cytotoxic T lymphocytes probed with Perforin 1 and Granzyme B antibodies to confirm positive cellular expression. (D) Nanoflow cytometry analysis of Donor 2 f12 ThrEDs samples from resting CD8+ cells and CTLs probing Granzyme B and Perforin 1. Data represents n=6 donors. Representative images are shown.

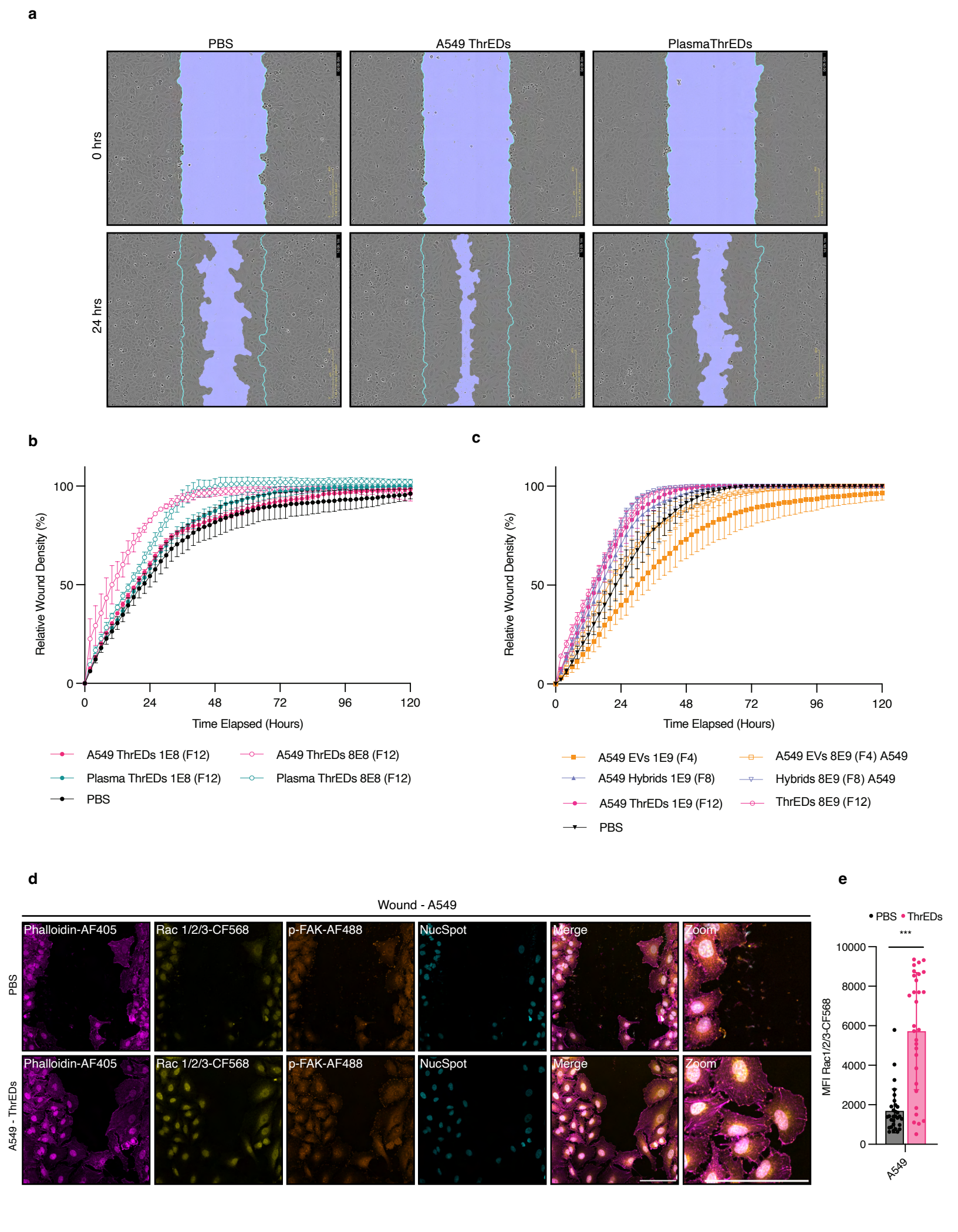

a
PBS
A549 ThrEDs
PlasmaThrEDs
0 hrs
24 hrs
b
Relative Wound Density (%)
Time Elapsed (Hours)
A549 ThrEDs 1E8 (F12)
A549 ThrEDs 8E8 (F12)
Plasma ThrEDs 1E8 (F12)
Plasma ThrEDs 8E8 (F12)
PBS
c
Relative Wound Density (%)
Time Elapsed (Hours)
A549 EVs 1E9 (F4)
A549 EVs 8E9 (F4) A549
A549 Hybrids 1E9 (F8)
Hybrids 8E9 (F8) A549
A549 ThrEDs 1E9 (F12)
ThrEDs 8E9 (F12)
PBS
d
Wound - A549
PBS
A549 - ThrEDs
Phalloidin-AF405
Rac 1/2/3-CF568
p-FAK-AF488
NucSpot
Merge
Zoom
e
PBS
ThrEDs
***
MFI Rac1/2/3-CF568
A549

Figure S10. **ThrED positive effectors of wound healing in lung adenocarcinoma cells.** (A) Representative images of scratch wounds in A549 cells treated with PBS, A549 ThrEDs and Plasma ThrEDs. Initial scratch is shown with a border line (cyan), and the wound area is shown in light purple (the central region). At the 24-hour time point the scratch wound mask fails to recognize certain migrating cells in ThrED treated samples because the cell morphology changes drastically following particle administration to promote migration. (B) Relative wound density of A549 wounds treated with A549 and Plasma ThrEDs from (A) at 2 different doses showing a dose response. (C) Relative wound density of A549 wounds treated with PBS, EVs, Hybrids and ThrEDs at different doses. (D) Confocal imaging of Rac1/2/3 and p-FAK in A549 wounds treated with PBS and ThrEDs at 24 hrs with associated MFI quantification of images (E). Scale bar=100μm. Data represents n=3 biological replicates.