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Supplementary Materials for

Annexin A1–dependent tethering promotes extracellular vesicle aggregation revealed with single–extracellular vesicle analysis

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Other Supplementary Material for this manuscript includes the following:

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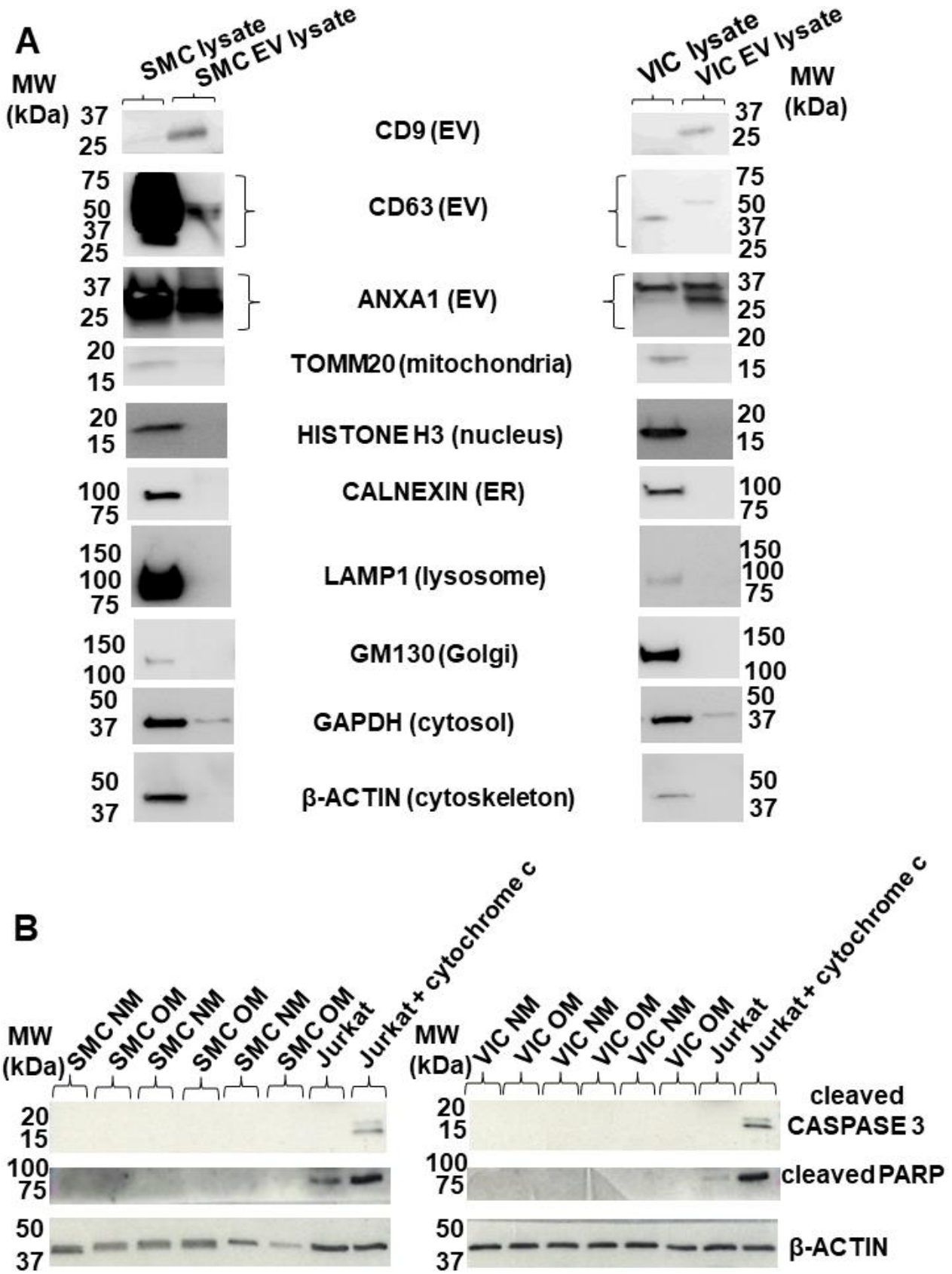

A
SMC lysate
SMC EV lysate
VIC lysate
VIC EV lysate
MW (kDa)
CD9 (EV)
CD63 (EV)
ANXA1 (EV)
TOMM20 (mitochondria)
HISTONE H3 (nucleus)
CALNEXIN (ER)
LAMP1 (lysosome)
GM130 (Golgi)
GAPDH (cytosol)
β-ACTIN (cytoskeleton)
B
SMC NM
SMC OM
Jurkat
Jurkat + cytochrome c
VIC NM
VIC OM
cleaved CASPASE 3
cleaved PARP
β-ACTIN

Fig. S1. EV isolation purity indicated by Western blot comparison of EV to whole cell lysate. (**A**) Western blot comparison of EV (CD9, CD63, ANXA1), mitochondria (TOMM20), nucleus (histone H3), endoplasmic reticulum (ER; calnexin), lysosome (LAMP1), Golgi (GM130), cytosol (GAPDH), and cytoskeleton (β-actin) markers in equal loaded (protein content) EV and whole cell SMC and VIC lysates (N = 3 donors with one representative blot of three shown). (**B**) Cleaved caspase 3 (not detected) and cleaved PARP (not detected) Western blots of whole cell lysates for SMC and VIC cultured in NM and OM for two weeks (N = 3 donors). Jurkat cell lysates with and without cytochrome c treatment included as antibody positive control.

SMC EV

Shared pathways enriched in OM

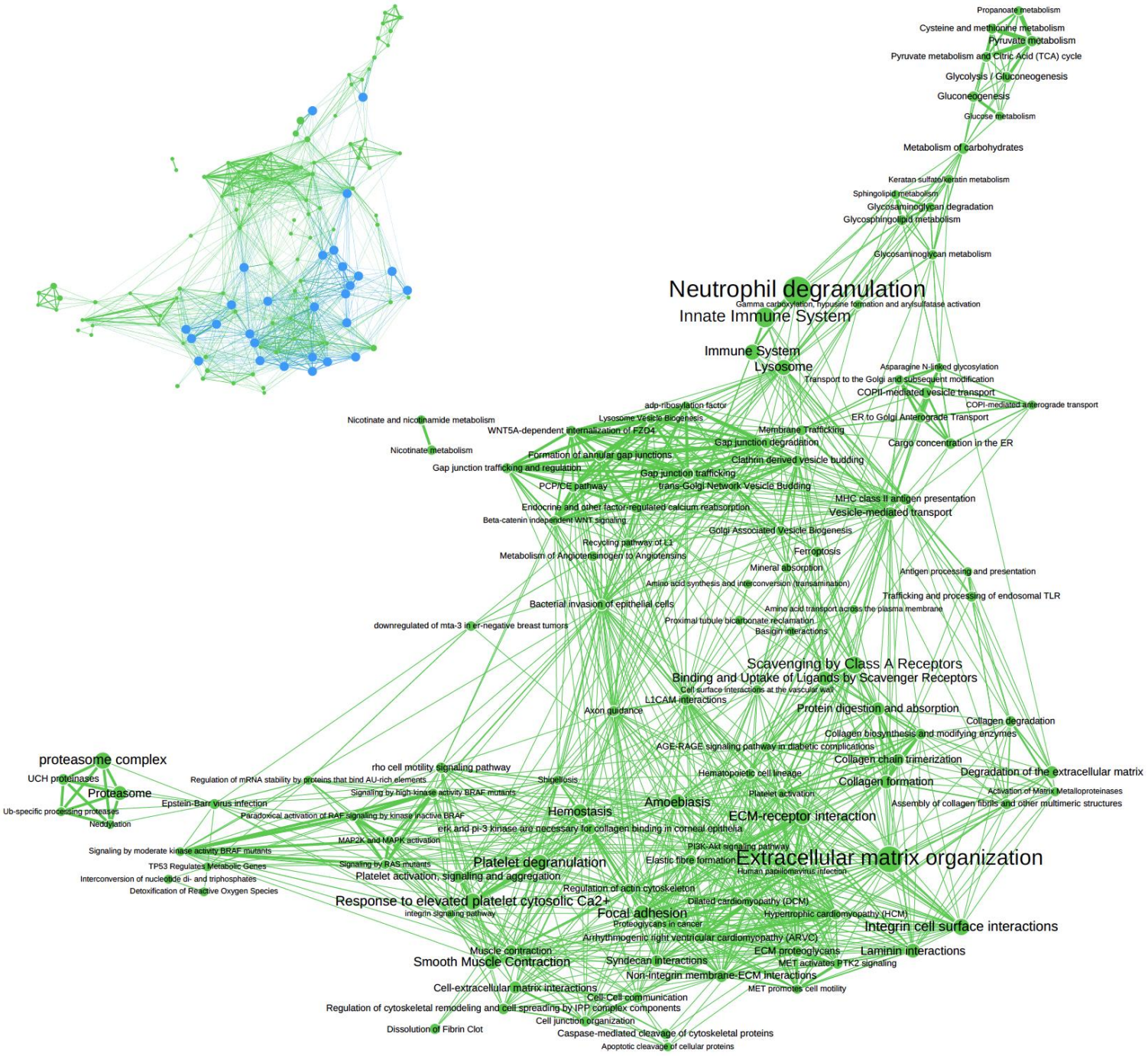


Fig. S2. Enriched pathways based on significantly changed proteins in OM from SMC EV. Connections in networks denote common proteins between pathways (more proteins equate to a thicker connection). Node sizes correspond to significance of enrichment (proportional to -log(q-value)); $N = 9$ donors. Shared pathways enriched in both SMC EV and VIC EV in OM indicated by blue nodes on the adjacent network.

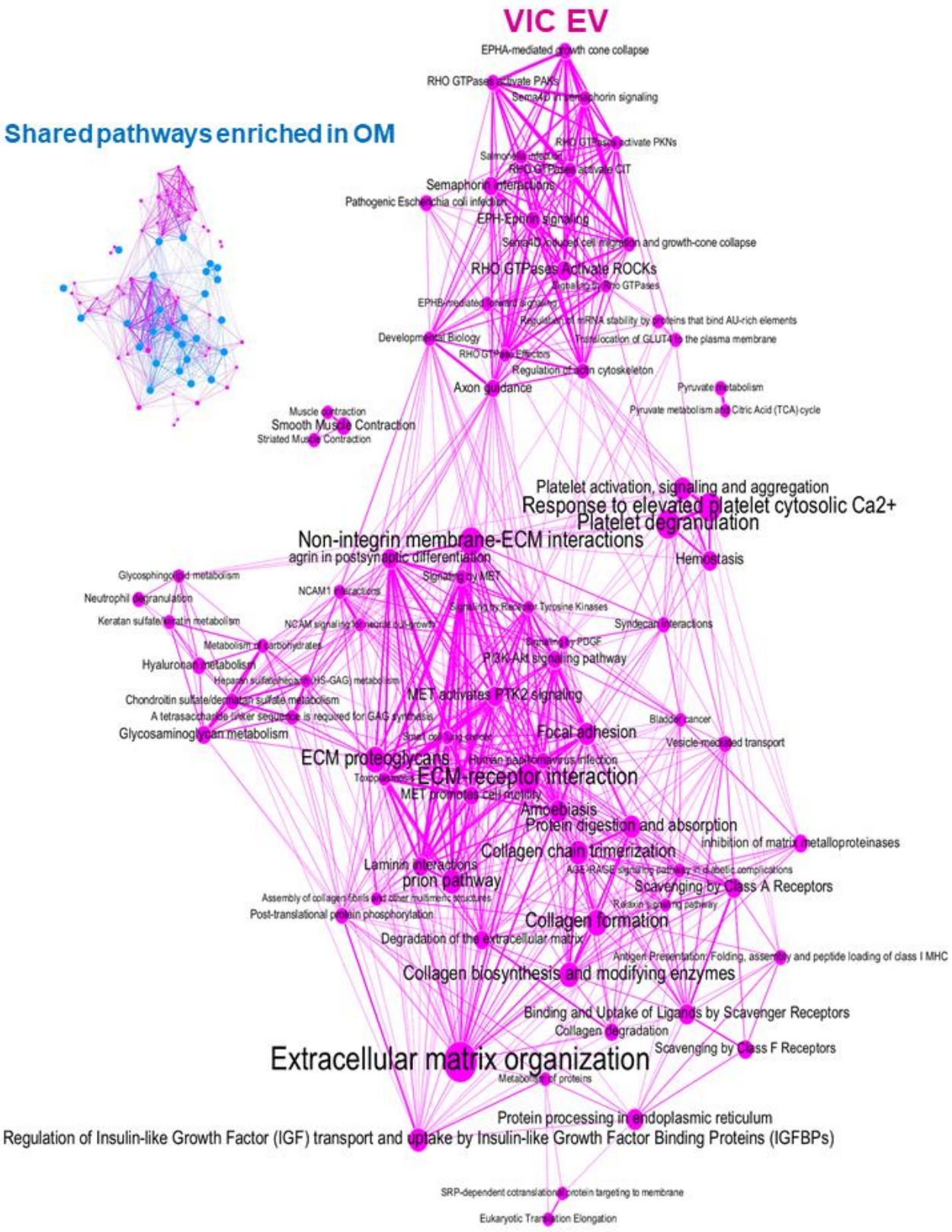

VIC EV
Shared pathways enriched in OM
EPHA-mediated growth cone collapse
RHO GTPases activate PAKs
Sema4D in semaphorin signaling
RHO GTPases activate PKNs
Salmonella infection
RHO GTPases activate CIT
Semaphorin interactions
Pathogenic Escherichia coli infection
EPH-Ephrin signaling
Sema4D induced cell migration and growth-cone collapse
RHO GTPases Activate ROCKs
Signaling by Rho GTPases
EPHB-mediated forward signaling
Regulation of mRNA stability by proteins that bind AU-rich elements
Developmental Biology
Translocation of GLUT4 to the plasma membrane
RHO GTPase Effectors
Regulation of actin cytoskeleton
Axon guidance
Pyruvate metabolism
Pyruvate metabolism and Citric Acid (TCA) cycle
Muscle contraction
Smooth Muscle Contraction
Striated Muscle Contraction
Platelet activation, signaling and aggregation
Response to elevated platelet cytosolic Ca2+
Platelet degranulation
Non-integrin membrane-ECM interactions
agrin in postsynaptic differentiation
Hemostasis
Glycosphingolipid metabolism
Signaling by MET
NCAM1 interactions
Neutrophil degranulation
Signaling by Receptor Tyrosine Kinases
Keratan sulfate/keratin metabolism
NCAM signaling for neurite out-growth
Syndecan interactions
Signaling by PDGF
Metabolism of carbohydrates
PI3K-Akt signaling pathway
Hyaluronan metabolism
Heparan sulfate/heparin (HS-GAG) metabolism
MET activates PTK2 signaling
Chondroitin sulfate/dermatan sulfate metabolism
A tetrasaccharide linker sequence is required for GAG synthesis
Bladder cancer
Glycosaminoglycan metabolism
Focal adhesion
Small cell lung cancer
Vesicle-mediated transport
ECM proteoglycans
Human papillomavirus infection
Toxoplasmosis
ECM-receptor interaction
MET promotes cell motility
Amoebiasis
Protein digestion and absorption
inhibition of matrix metalloproteinases
Collagen chain trimerization
Laminin interactions
AGE-RAGE signaling pathway in diabetic complications
prion pathway
Scavenging by Class A Receptors
Assembly of collagen fibrils and other multimeric structures
Relaxin signaling pathway
Post-translational protein phosphorylation
Collagen formation
Degradation of the extracellular matrix
Antigen Presentation: Folding, assembly and peptide loading of class I MHC
Collagen biosynthesis and modifying enzymes
Binding and Uptake of Ligands by Scavenger Receptors
Collagen degradation
Scavenging by Class F Receptors
Extracellular matrix organization
Metabolism of proteins
Protein processing in endoplasmic reticulum
Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)
SRP-dependent cotranslational protein targeting to membrane
Eukaryotic Translation Elongation

Fig. S3. Enriched pathways based on significantly changed proteins in OM from VIC EV. Connections in networks denote common proteins between pathways (more proteins equate to a thicker connection). Node sizes correspond to significance of enrichment (proportional to -log(q-value)); $N = 7$ donors. Shared pathways enriched in both SMC EV and VIC EV in OM indicated by blue nodes on the adjacent network.

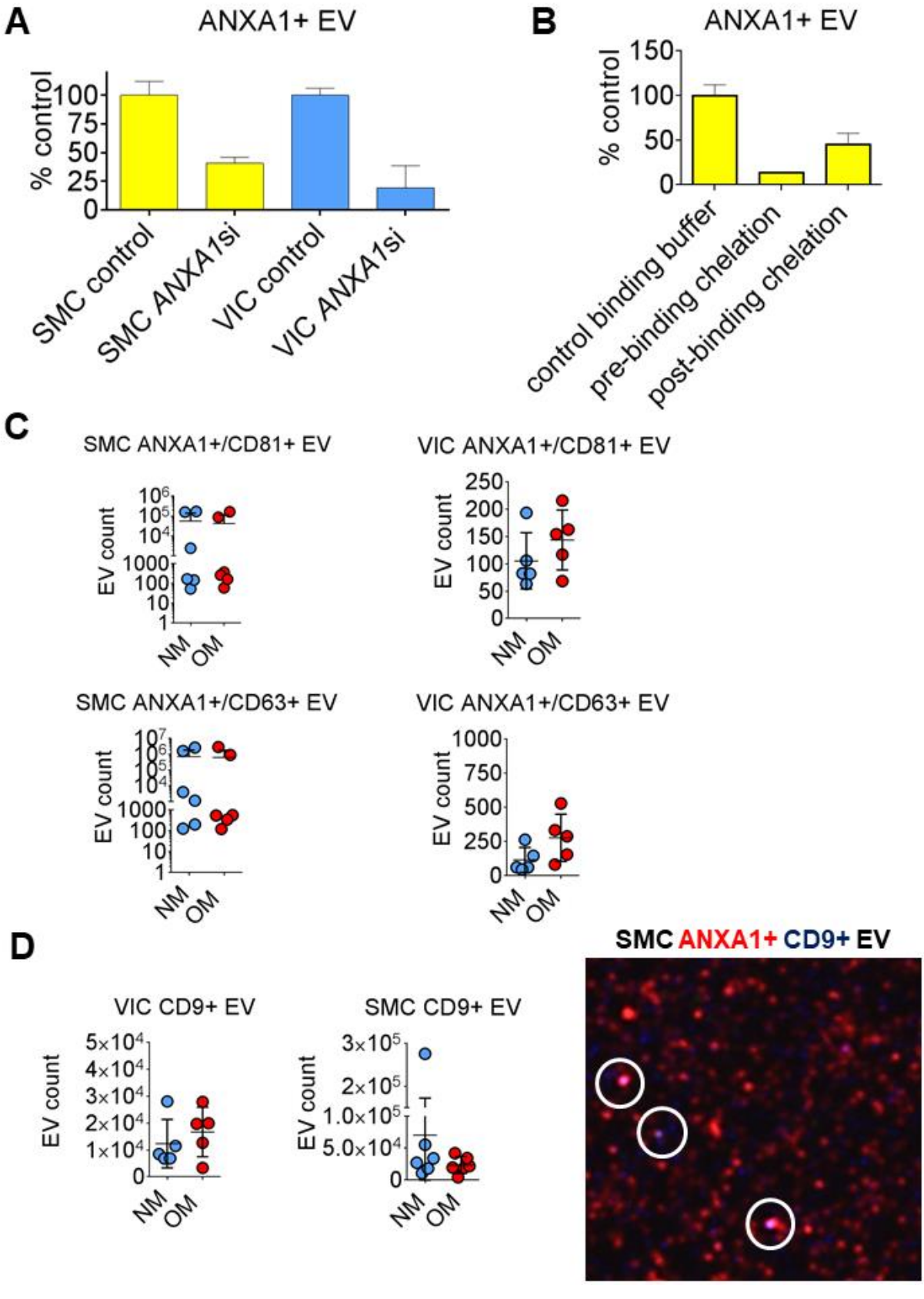

A
ANXA1+ EV
% control
100
75
50
25
0
SMC control
SMC ANXA1si
VIC control
VIC ANXA1si
B
ANXA1+ EV
% control
150
100
50
0
control binding buffer
pre-binding chelation
post-binding chelation
C
SMC ANXA1+/CD81+ EV
VIC ANXA1+/CD81+ EV
SMC ANXA1+/CD63+ EV
VIC ANXA1+/CD63+ EV
EV count
NM
OM
D
VIC CD9+ EV
SMC CD9+ EV
SMC ANXA1+ CD9+ EV

Fig. S4. Single-EV microarray assessment of ANXA1 detection on SMC EV and VIC EV.

(**A**) SMC and VIC ANXA1+ EV counts presented as percent control for SMC and VIC incubated with *ANXA1* siRNA (*ANXA1*si) or control siRNA (*N* = 3 technical replicates, error bars are mean ± SD). (**B**) ANXA1 detection on captured SMC EV with or without calcium chelation incubated pre- and post-EV binding to microarray chips; *N* = 3 technical replicates, error bars are mean ± SD. (**C**) SMC (*N* = 6 donors) and VIC (*N* = 5 donors) ANXA1+/CD63+ EV, ANXA1+/CD81+ EV and (**D**) CD9+ EV in NM and OM; error bars are mean ± SD. Representative single-EV microarray image for ANXA1+/CD9+ EV (white circles), with quantification of ANXA1+/CD9+ EV included in Fig. 3E.

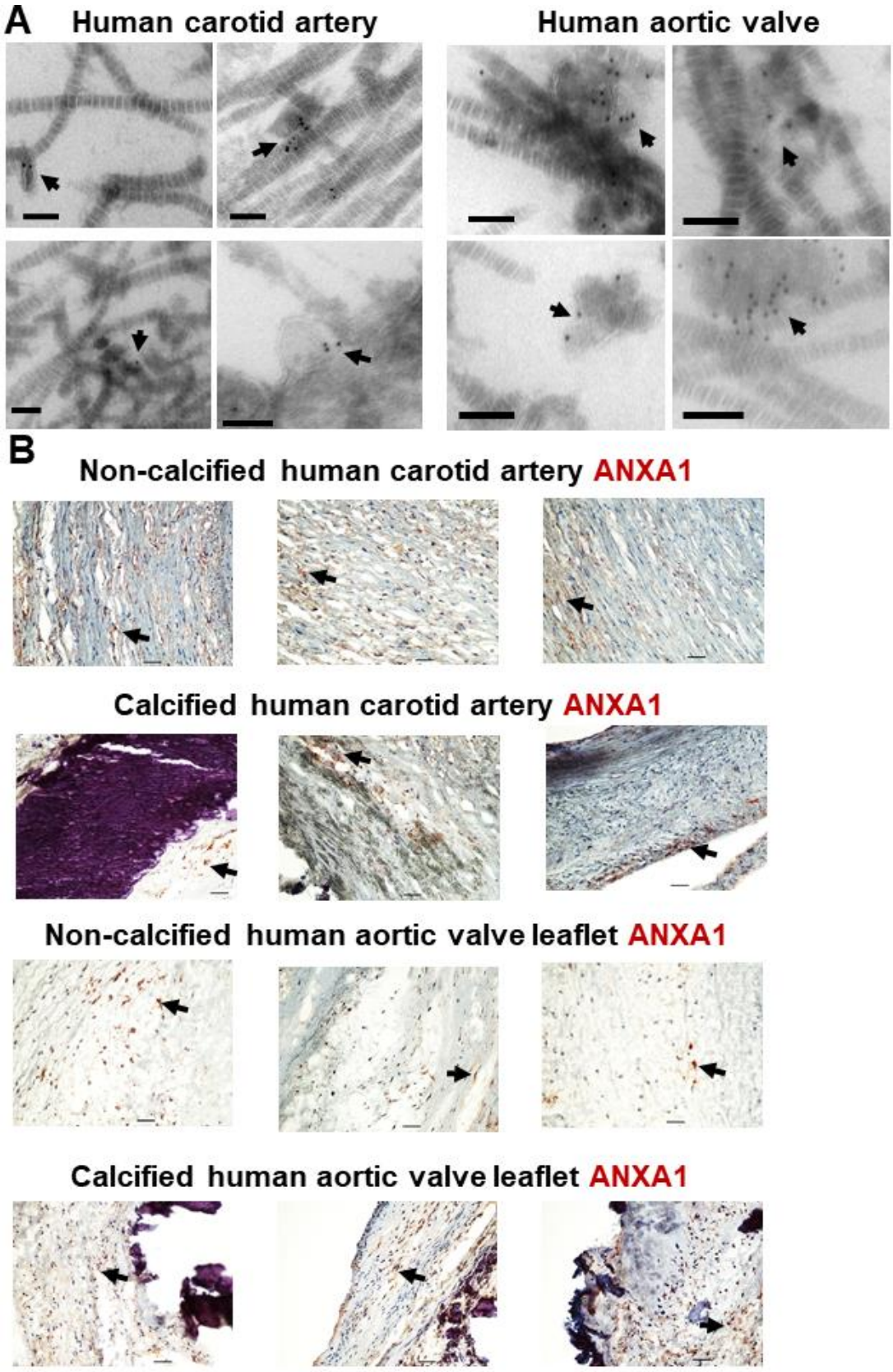

A
Human carotid artery
Human aortic valve
B
Non-calcified human carotid artery ANXA1
Calcified human carotid artery ANXA1
Non-calcified human aortic valve leaflet ANXA1
Calcified human aortic valve leaflet ANXA1

Fig. S5. ANXA1 is on tethered EV in human cardiovascular tissues. (**A**) Calcified human carotid artery and aortic valve tissue transmission electron microscopy with ANXA1 immunogold labeling (black dots) on tethered EV (arrows) in collagen ECM. Scale bars, 100 nm; *N* = 5 donors, with four representative images shown and an additional image included in Fig. 4A. (**B**) ANXA1 immunohistochemistry (red color) in non-calcified and calcified (hematoxylin stain, purple color) human carotid artery and aortic valve tissues. Scale bars, 50 μm; *N* = 5 donors, with 3 donors shown and an additional donor image included in Fig. 4A.

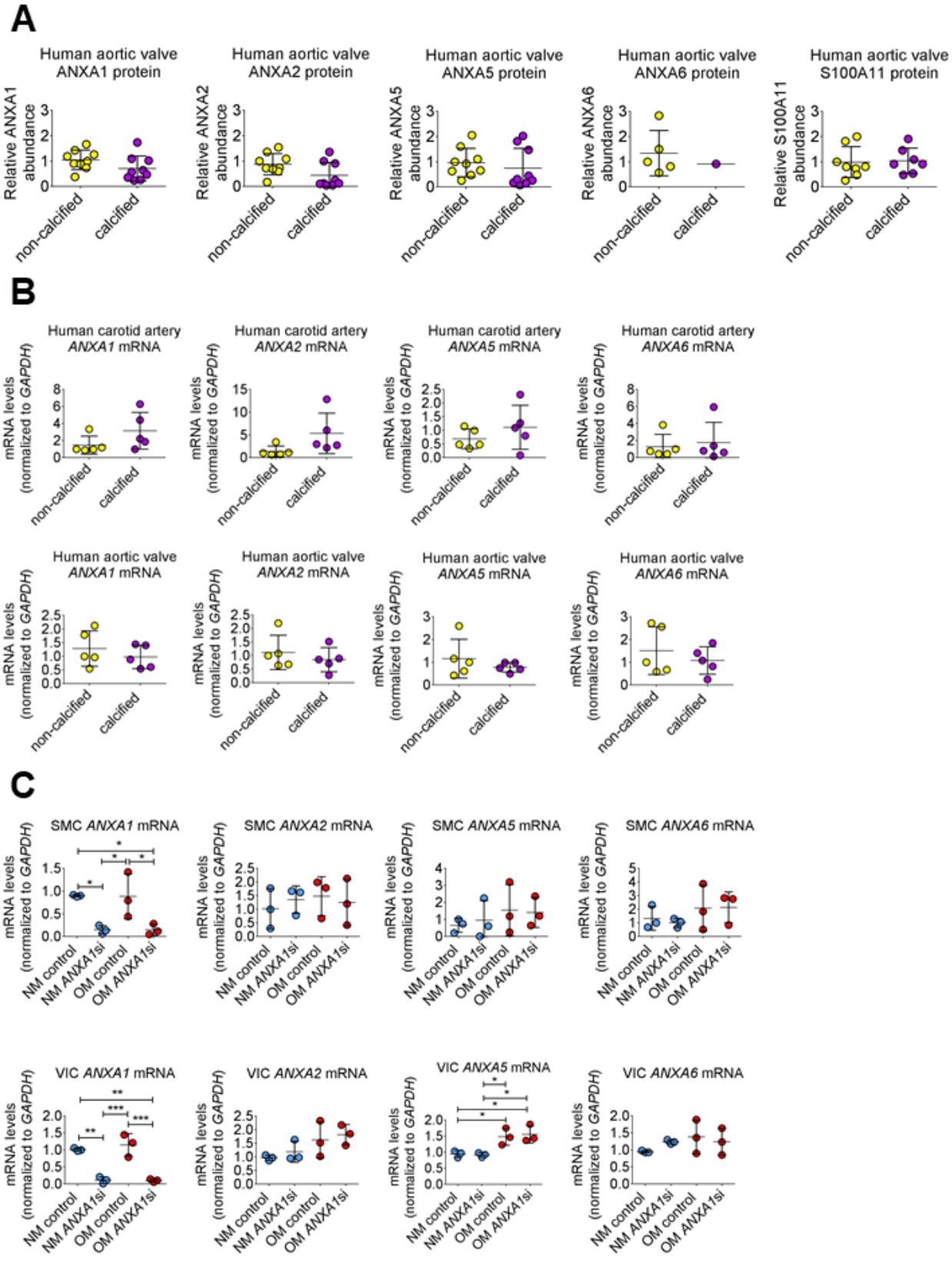

A
Human aortic valve ANXA1 protein
Relative ANXA1 abundance
Human aortic valve ANXA2 protein
Relative ANXA2 abundance
Human aortic valve ANXA5 protein
Relative ANXA5 abundance
Human aortic valve ANXA6 protein
Relative ANXA6 abundance
Human aortic valve S100A11 protein
Relative S100A11 abundance
non-calcified
calcified
B
Human carotid artery ANXA1 mRNA
Human carotid artery ANXA2 mRNA
Human carotid artery ANXA5 mRNA
Human carotid artery ANXA6 mRNA
Human aortic valve ANXA1 mRNA
Human aortic valve ANXA2 mRNA
Human aortic valve ANXA5 mRNA
Human aortic valve ANXA6 mRNA
mRNA levels (normalized to GAPDH)
C
SMC ANXA1 mRNA
SMC ANXA2 mRNA
SMC ANXA5 mRNA
SMC ANXA6 mRNA
VIC ANXA1 mRNA
VIC ANXA2 mRNA
VIC ANXA5 mRNA
VIC ANXA6 mRNA
NM control
NM ANXA1si
OM control
OM ANXA1si

Fig. S6. Annexin mRNA expression and protein total abundance is largely not altered in calcified human tissues and cells. (**A**) Unlabeled proteomics analysis of annexins A1, A2, A5, A6, and S100A11 in non-calcified and calcified human aortic valve tissue; *N* = 9 donors, with S100A11 detected in 8/9 non-calcified and 7/9 calcified donor tissues, ANXA1 detected in 9/9 donors, ANXA2 detected in 9/9 donors, ANXA5 detected in 9/9 donors, and ANXA6 detected in 5/9 non-calcified and 1/9 calcified donor tissues; error bars are mean ± SD, analyzed by Welch's t-test. (**B**) mRNA levels of annexins A1, A2, A5, A6 in non-calcified and calcified human carotid artery and aortic valve tissues; analyzed by Welch's t-test, *N* = 5 donors, mean ± SD. (**C**) Human SMC and VIC mRNA levels of annexins A1, A2, A5, A6 in NM or OM incubated with control siRNA (control) or *ANXA1* siRNA (*ANXA1*si) for two weeks; error bars are mean ± SD from 3 donors, analyzed by ANOVA, ***P <0.001, **P <0.01, *P <0.05.

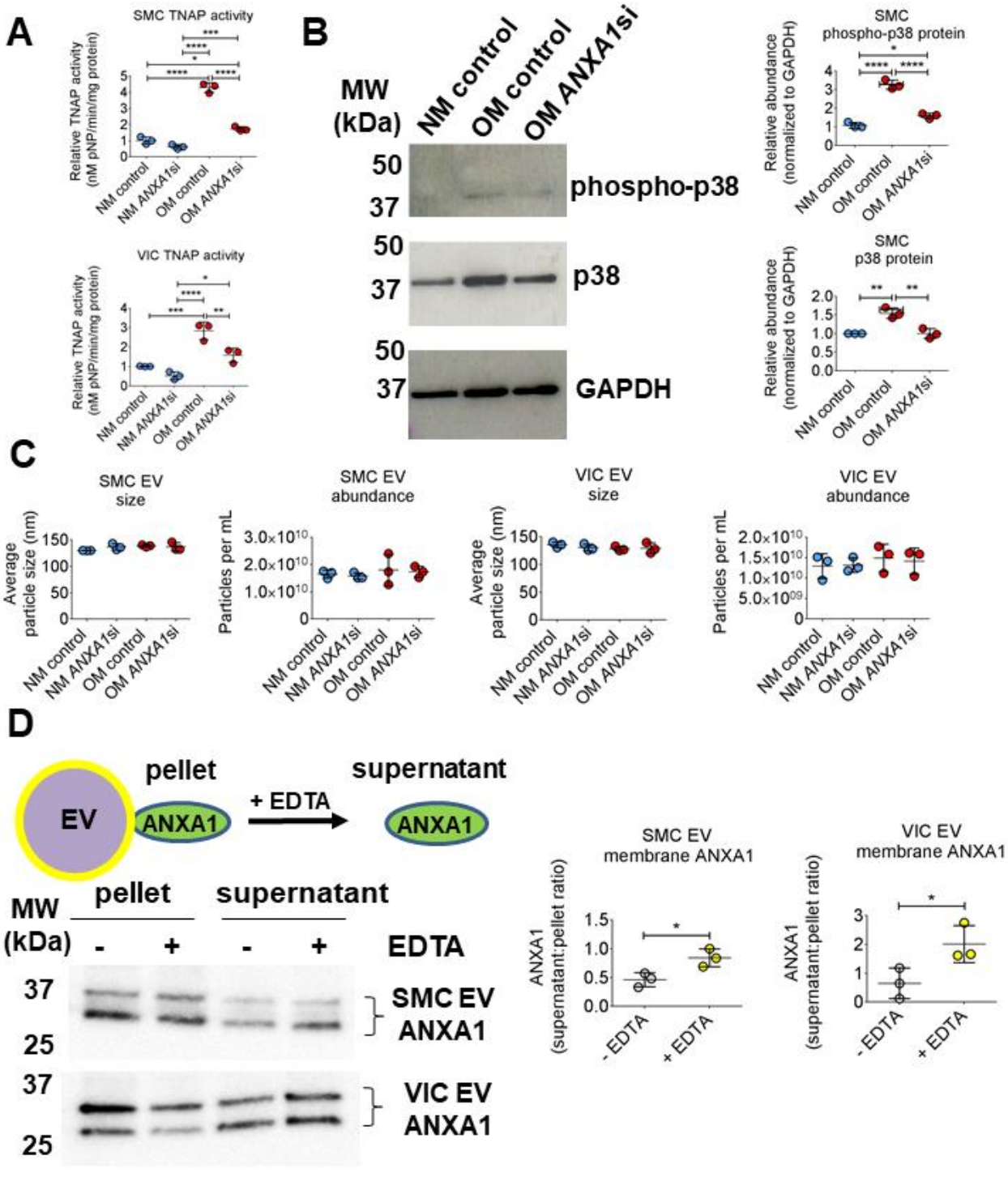
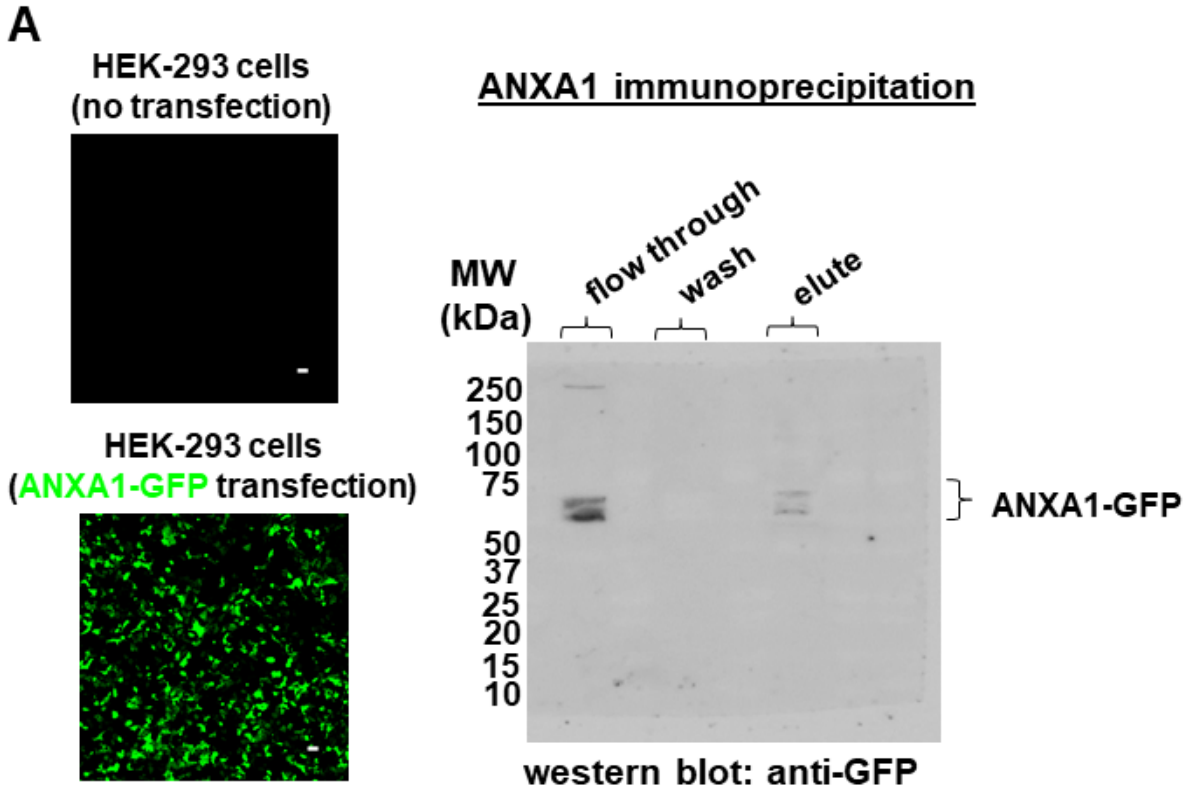
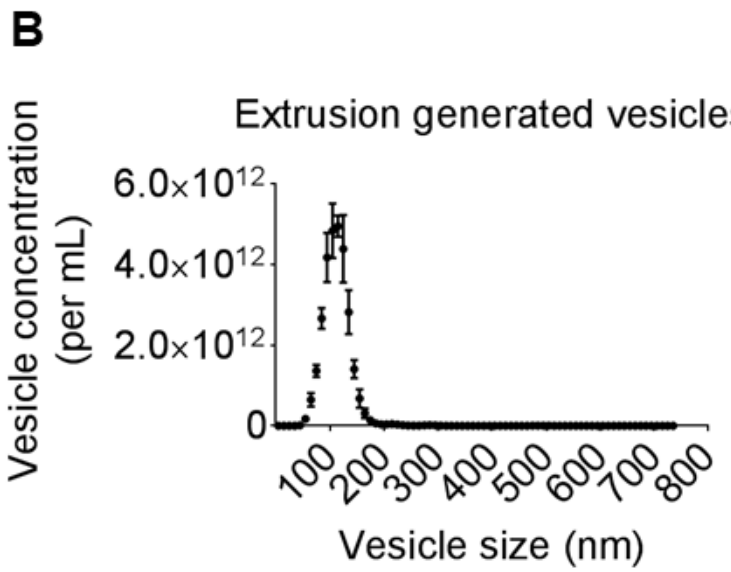

A
SMC TNAP activity
Relative TNAP activity (nM pNP/min/mg protein)
NM control
NM ANXA1si
OM control
OM ANXA1si
VIC TNAP activity
B
MW (kDa)
NM control
OM control
OM ANXA1si
50
37
phospho-p38
p38
GAPDH
SMC phospho-p38 protein
Relative abundance (normalized to GAPDH)
SMC p38 protein
C
SMC EV size
Average particle size (nm)
SMC EV abundance
Particles per mL
VIC EV size
VIC EV abundance
D
pellet
supernatant
EV
ANXA1
+ EDTA
MW (kDa)
- EDTA
SMC EV ANXA1
VIC EV ANXA1
SMC EV membrane ANXA1
VIC EV membrane ANXA1
ANXA1 (supernatant:pellet ratio)

Fig. S7. ANXA1 is on the surface of SMC and VIC EV. (**A**) TNAP activity in human SMC and VIC in NM or OM incubated with control siRNA (control) or *ANXA1* siRNA (*ANXA1*si) for two weeks. Error bars are mean ± SD from 3 donors; analyzed by ANOVA. (**B**) Total p38 and phospho-p38 protein in SMC cultured in NM or OM with control or *ANXA1*si for two weeks. Error bars are mean ± SD from 3 donors; analyzed by ANOVA. (**C**) Human SMC EV and VIC EV size and quantity in conditioned media from cells cultured in NM or OM with control or *ANXA1*si; $N = 3$ donors, error bars are mean ± SD. (**E**) Illustration showing experiment design to confirm EV surface localization of ANXA1. ANXA1 Western blots from human SMC EV and VIC EV incubated with EDTA to release ANXA1 from the EV surface into the supernatant; quantification is the sum of both bands, $N = 3$ donors, error bars are mean ± SD, analyzed by Welch's t-test. ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.


A
HEK-293 cells
(no transfection)
HEK-293 cells
(ANXA1-GFP transfection)
ANXA1 immunoprecipitation
MW
(kDa)
flow through
wash
elute
250
150
100
75
50
37
25
20
15
10
ANXA1-GFP
western blot: anti-GFP


B
Extrusion generated vesicles
Vesicle concentration
(per mL)
6.0×10^12
4.0×10^12
2.0×10^12
0
100
200
300
400
500
600
700
800
Vesicle size (nm)

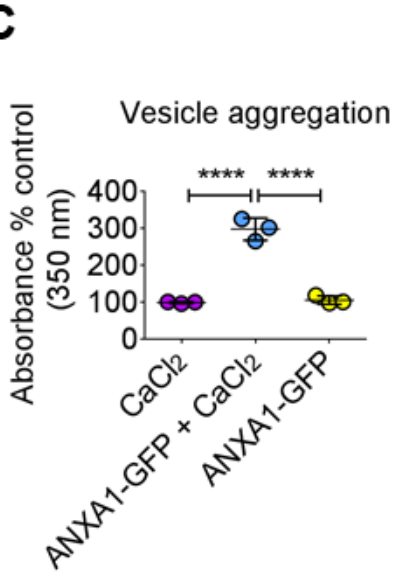

C
Vesicle aggregation
Absorbance % control
(350 nm)
****
****
400
300
200
100
0
CaCl2
ANXA1-GFP + CaCl2
ANXA1-GFP

Fig. S8. Immunopurification of ANXA1-GFP protein and extrusion generated vesicles nanoparticle tracking analysis. (**A**) Live cell confocal microscopy of HEK-293 cells transfected with human ANXA1-GFP tagged plasmid; scale bars, 50 μm. Immunoprecipitation Western blot of eluted ANXA1-GFP protein used for confocal imaging of ANXA1 binding to polyvinyl alcohol swelling generated phosphatidylserine vesicles. (**B**) Nanoparticle tracking analysis of filter extrusion generated phosphatidylserine vesicles size; error bars are mean ± SD from 5 replicate measurements. (**C**) Extrusion generated phosphatidylserine vesicle aggregation in buffer containing 1 mmol/L $CaCl_2$ with 0 μg ANXA1-GFP ($CaCl_2$) or 1 μg ANXA1-GFP (ANXA1-GFP + $CaCl_2$), or 1 μg ANXA1-GFP in buffer without $CaCl_2$ (ANXA1-GFP). Error bars are mean ± SD from 3 experimental replicates; analyzed by ANOVA, ****P <0.0001.

Movie S1. ANXA1 tethered vesicles. Confocal Z stack video of phosphatidylserine swelling generated vesicles (purple color) incubated with human green fluorescent protein tagged ANXA1 (green color). Width: 32.46 μm, height: 32.46 μm, depth: 18.90 μm. Corresponding image also included in Fig. 5A.

Dataset S1. Proteomics and network analysis. Human SMC EV (*N* = 9 donors) and VIC EV (*N* = 7 donors) proteomics datasets, statistical analysis, comparative analysis, and pathway network analysis.