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## Supplemental information

# Astrocyte-derived small extracellular vesicles promote synapse formation via fibulin-2-mediated TGF-β signaling

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**Figure S1:**

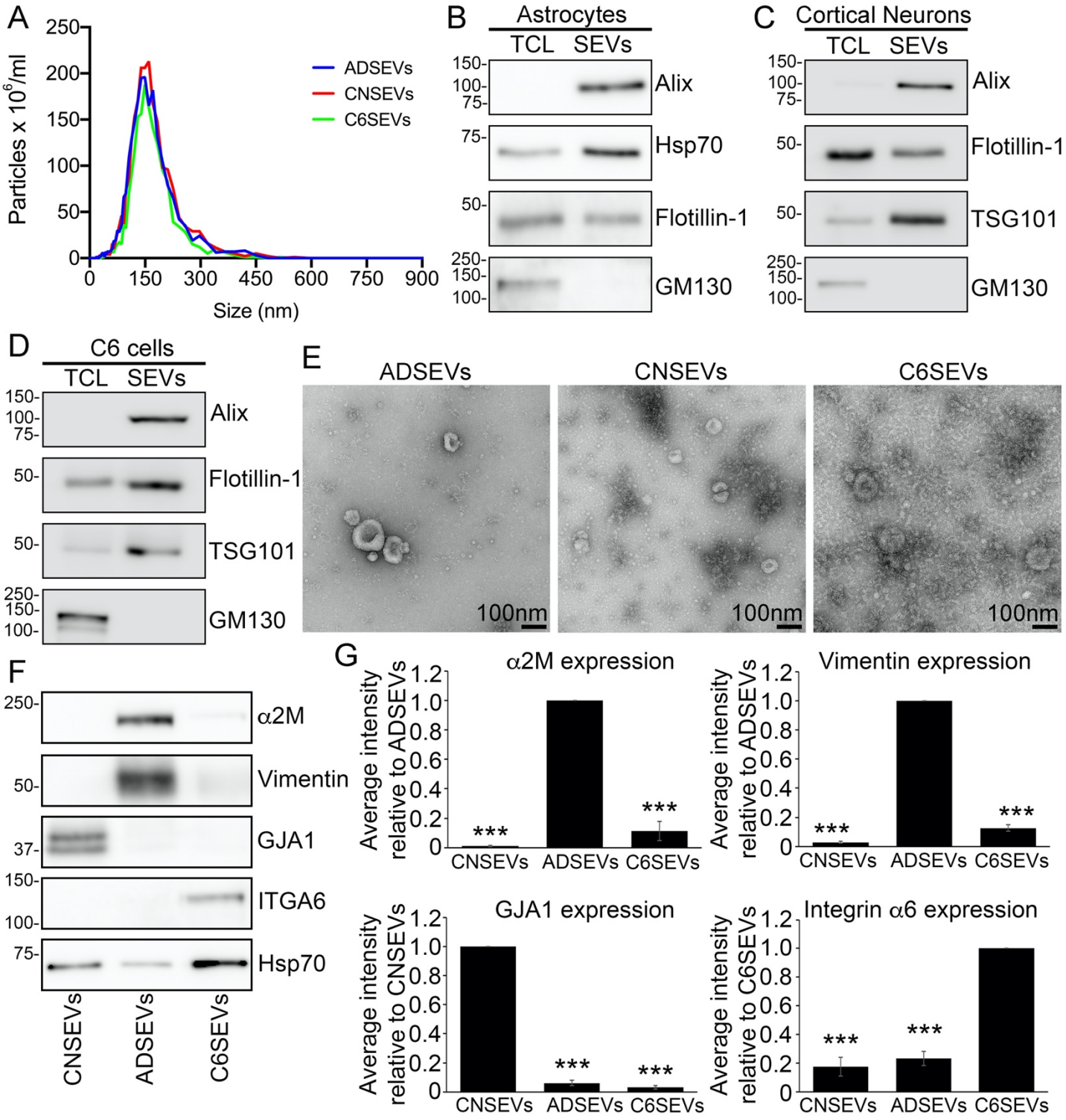
A
Particles x 10^6/ml
Size (nm)
ADSEVs
CNSEVs
C6SEVs
B
Astrocytes
TCL SEVs
Alix
Hsp70
Flotillin-1
GM130
C
Cortical Neurons
TCL SEVs
Alix
Flotillin-1
TSG101
GM130
D
C6 cells
TCL SEVs
Alix
Flotillin-1
TSG101
GM130
E
ADSEVs
CNSEVs
C6SEVs
100nm
F
α2M
Vimentin
GJA1
ITGA6
Hsp70
CNSEVs
ADSEVs
C6SEVs
G
α2M expression
Vimentin expression
GJA1 expression
Integrin α6 expression
Average intensity relative to ADSEVs
Average intensity relative to CNSEVs
Average intensity relative to C6SEVs
***

**Figure S1: SEV characterization and iTRAQ validation. Related to STAR Methods and Figures 1 and 2.**

(A) Representative particle size traces from nanoparticle tracking analysis of SEVs isolated from primary astrocytes (ADSEVs), cortical neurons (CNSEVs) and C6 glioma cells (C6SEVs).

(B, C and D) Representative Western blots showing the presence of SEV markers Alix, Flotillin-1, TSG101 or HSP70 and absence of the Golgi marker GM130 in ADSEVs, CNSEVs and C6SEVs. TCL and SEVs loaded at equal protein concentration. TCL, total cell lysate.

(E) TEM images of negative stained ADSEVs, CNSEVs and C6SEVs. Scale bar, 100nm.

(F) Western blot of CNSEVs, ADSEVs and C6SEVs stained for alpha-2-macroglobulin ($\alpha$2M), vimentin, gap junction alpha-1 (GJA1), integrin alpha-6 (ITGA6) and Hsp70.

(G) Quantification of $\alpha$2M, vimentin, GJA1 and ITGA6 expression from 3 independent experiments. Data represented as mean$\pm$SEM. ***$p<0.001$.

**Figure S2:**

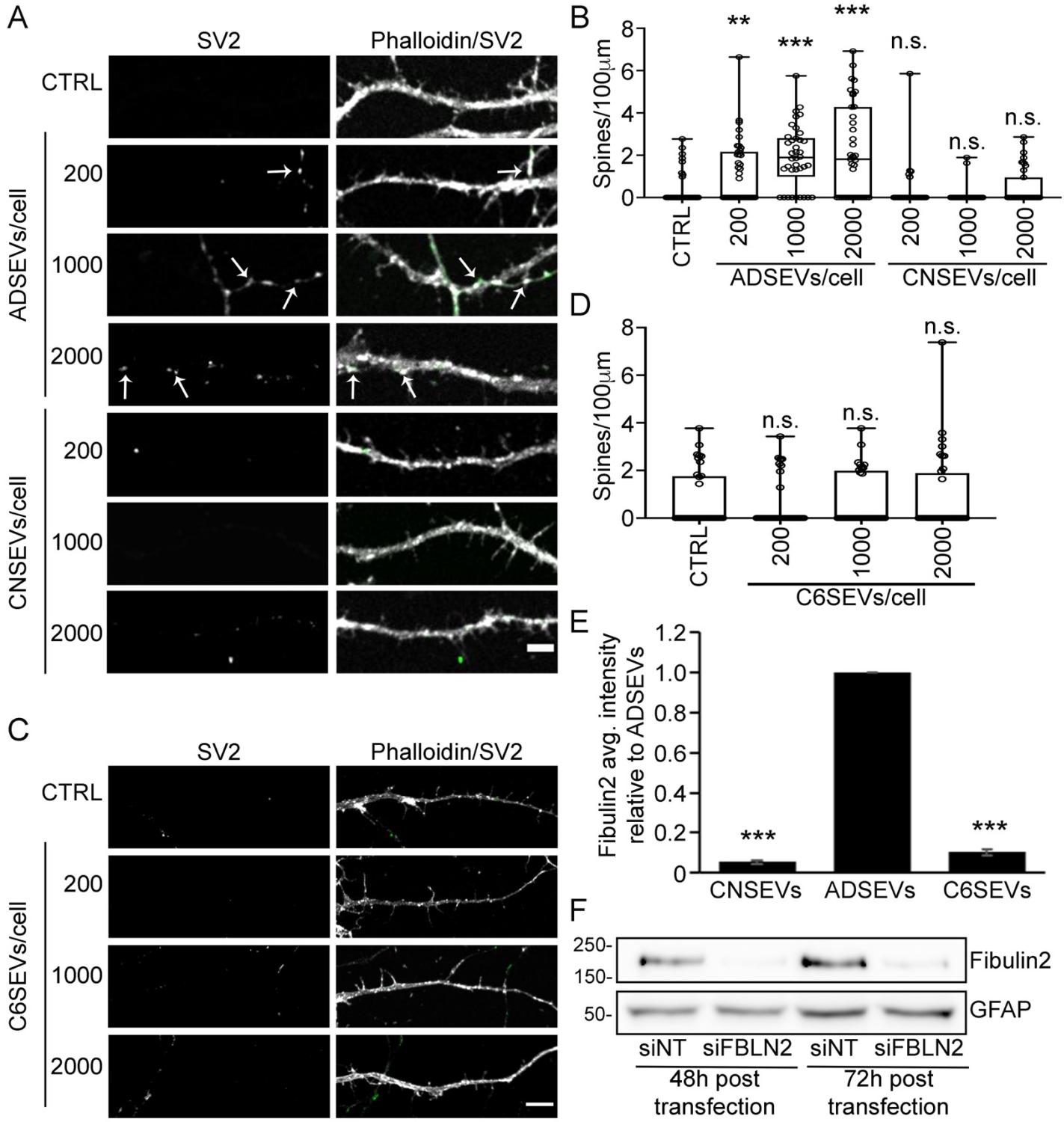
A
SV2
Phalloidin/SV2
CTRL
200
1000
2000
ADSEVs/cell
CNSEVs/cell
B
Spines/100µm
**
***
***
n.s.
n.s.
n.s.
CTRL
200
1000
2000
200
1000
2000
ADSEVs/cell
CNSEVs/cell
C
SV2
Phalloidin/SV2
CTRL
200
1000
2000
C6SEVs/cell
D
Spines/100µm
n.s.
n.s.
n.s.
CTRL
200
1000
2000
C6SEVs/cell
E
Fibulin2 avg. intensity relative to ADSEVs
***
***
CNSEVs
ADSEVs
C6SEVs
F
250-
150-
50-
Fibulin2
GFAP
siNT
siFBLN2
siNT
siFBLN2
48h post transfection
72h post transfection

**Figure S2: Characterization of astrocyte-derived SEVs for dendritic spine formation at an early developmental stage and fibulin-2 levels. Related to Figures 1 and 3.**

(A) Representative SV2 and Phalloidin/SV2 overlay immunofluorescence images of neuronal dendrites at day 6 in control neurons or neurons treated with 200, 1000 or 2000 ADSEVs or CNSEVs/neuron.

(B) Quantification of dendritic spine density from images. n $\geq$ 36 primary or secondary dendrites from 3 independent experiments.

(C) Representative SV2 and Phalloidin/SV2 overlay immunofluorescence images of neuronal dendrites at day 6 in control neurons or neurons treated with 200, 1000 or 2000 C6SEVs/neuron.

(D) Quantification of dendritic spine density from images. n $\geq$ 36 primary or secondary dendrites from 3 independent experiments.

(E) Quantification of fibulin-2 in CNSEVs, ADSEVs and C6SEVs from 3 independent experiments. Data represented as mean$\pm$SEM. ***p<0.001.

(F) Western blot of siNT and siFBLN2 astrocyte total cell lysates at 48 h and 72 h post transfection stained for fibulin-2 and GFAP loading control.

For better visualization of SV2 colocalization on dendritic spines, images in A and C were adjusted for brightness and contrast and cropped to same size. Left panel: SV2 only. Right panel: Overlay of phalloidin (gray-scale) and SV2 (green). Some example dendritic spines are indicated with arrows. Data represented as box and whiskers plots with all data points shown, bar indicating the median, and the box showing interquartile range. Scale bar, 5 $\mu$m. **p<0.01, ***p<0.001, n.s. – not significant.

**Figure S3:**

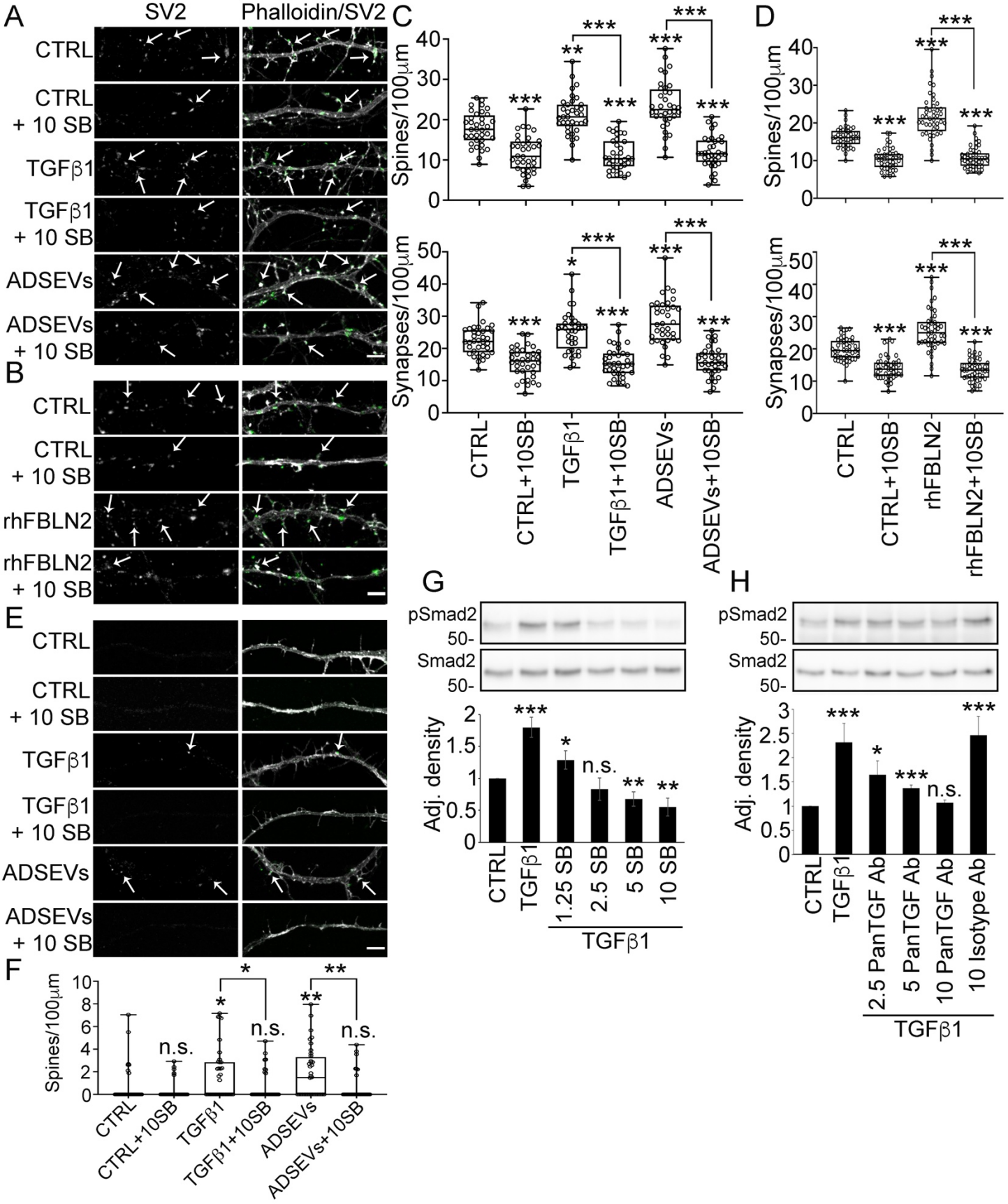
A
SV2
Phalloidin/SV2
CTRL
CTRL + 10 SB
TGFβ1
TGFβ1 + 10 SB
ADSEVs
ADSEVs + 10 SB
B
CTRL
CTRL + 10 SB
rhFBLN2
rhFBLN2 + 10 SB
C
Spines/100μm
Synapses/100μm
CTRL
CTRL+10SB
TGFβ1
TGFβ1+10SB
ADSEVs
ADSEVs+10SB
D
Spines/100μm
Synapses/100μm
CTRL
CTRL+10SB
rhFBLN2
rhFBLN2+10SB
E
CTRL
CTRL + 10 SB
TGFβ1
TGFβ1 + 10 SB
ADSEVs
ADSEVs + 10 SB
F
Spines/100μm
n.s.
CTRL
CTRL+10SB
TGFβ1
TGFβ1+10SB
ADSEVs
ADSEVs+10SB
G
pSmad2
50-
Smad2
50-
Adj. density
n.s.
CTRL
TGFβ1
1.25 SB
2.5 SB
5 SB
10 SB
TGFβ1
H
pSmad2
50-
Smad2
50-
Adj. density
n.s.
CTRL
TGFβ1
2.5 PanTGF Ab
5 PanTGF Ab
10 PanTGF Ab
10 Isotype Ab
TGFβ1

**Figure S3: ADSEVs and fibulin-2 promote dendritic spine and synapse formation dependent on TGF-β signaling. Related to Figure 4.**

(A and B) Representative SV2 and Phalloidin/SV2 overlay immunofluorescence images of day 12 cortical neurons treated with 10 ng/ml TGF-β1 or 2000 ADSEVs/cell (A) or 2 μg/ml rhFBLN2 (B) in the absence or presence of 10 μM SB431542 (SB).

(C and D) Quantification of spine and synapse density from images of day 12 cortical neurons treated with 10 ng/ml TGF-β1, or 2000 ADSEVs/cell (C) or with soluble recombinant human fibulin-2 (D) in the absence or presence of 10 μM SB. n=45 primary or secondary dendrites from three independent experiments.

(E) Representative SV2 and Phalloidin/SV2 overlay immunofluorescence images of day 6 cortical neurons treated with 10 ng/ml TGF-β1 and 2000 ADSEVs/cell in the absence or presence of 10 μM SB.

(F) Quantification of dendritic spine density from images. n=45 primary or secondary dendrites from 3 independent experiments.

All images are adjusted for brightness and contrast and cropped to same size for better visualization of SV2 colocalization on dendritic spines. Left panel: SV2 only. Right panel: Overlay of phalloidin (gray-scale) and SV2 (green). Some example dendritic spines are indicated with arrows. Scale bar, 5 μm. Data represented as box and whiskers plots with all data points shown, bar indicating the median, and the box showing interquartile range.

(G) Western blot analysis of pSmad2 and Smad2 in day 10 cortical neurons after treatment of 10 ng/ml TGF-β1 for 1 h in the absence or presence of 1.25, 2.5, 5 or 10 μM SB. Representative western blot (top) and quantification of absolute density of pSmad2 compared to total Smad2 (bottom). n=3. Data represented as mean ± SEM.

(H) Western blot analysis of pSmad2 and Smad2 in day 10 cortical neurons after treatment of 10 ng/ml TGF-β1 for 1 h in the absence or presence of 2.5, 5 or 10 μg/ml pan-TGFb-neutralizing antibody or 10 μg/ml isotype control antibody. Representative western blot (top) and quantification of absolute density of pSmad2 compared to total Smad2 (bottom). n=3. Data represented as mean ± SEM.

*p<0.05, **p<0.01, ***p<0.001, n.s. – not significant.

**Figure S4:**

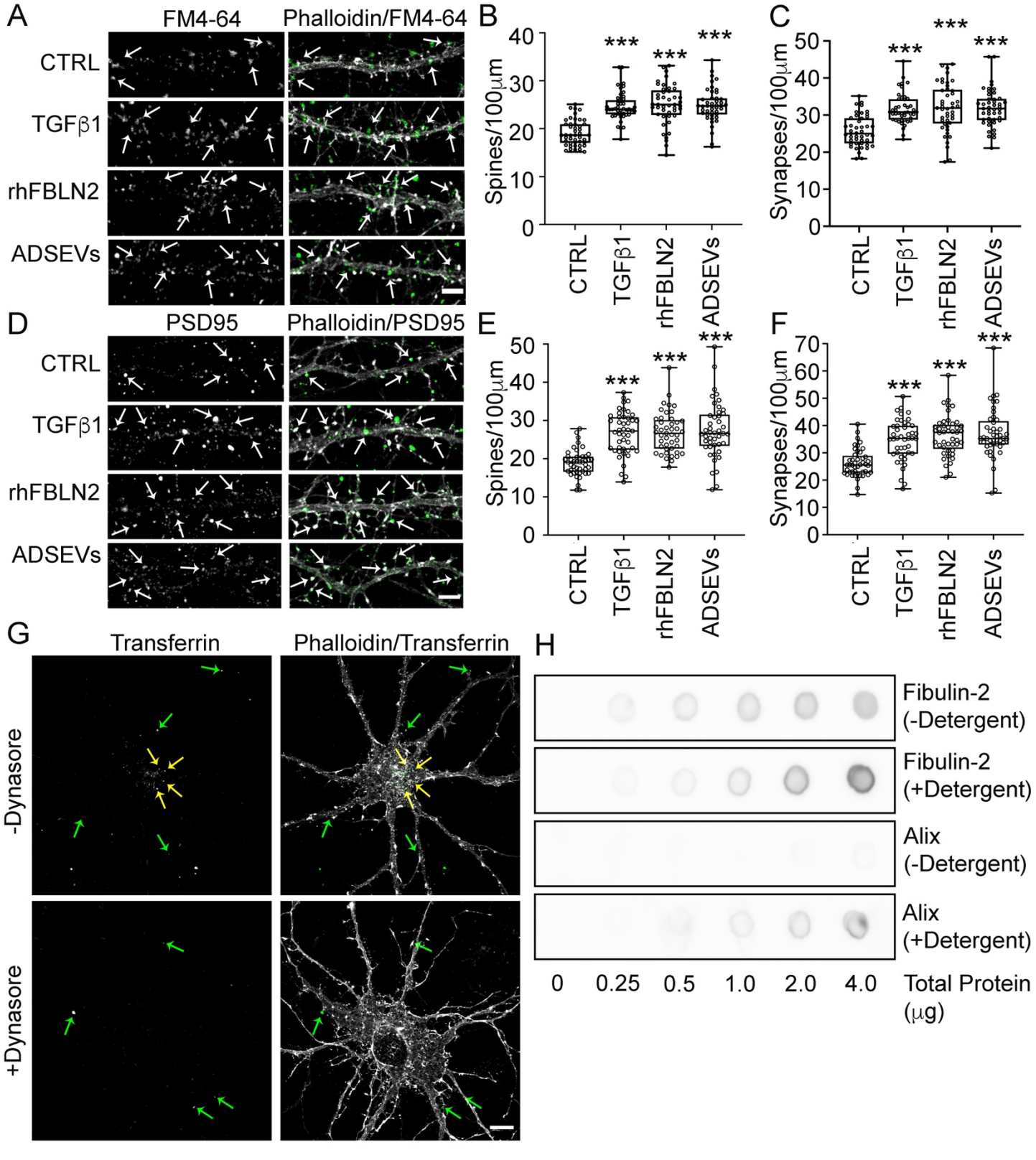
A
FM4-64
Phalloidin/FM4-64
CTRL
TGFβ1
rhFBLN2
ADSEVs
B
Spines/100μm
***
CTRL
TGFβ1
rhFBLN2
ADSEVs
C
Synapses/100μm
D
PSD95
Phalloidin/PSD95
E
F
G
Transferrin
Phalloidin/Transferrin
-Dynasore
+Dynasore
H
Fibulin-2
(-Detergent)
Fibulin-2
(+Detergent)
Alix
(-Detergent)
Alix
(+Detergent)
0
0.25
0.5
1.0
2.0
4.0
Total Protein
(μg)

**Figure S4: Fibulin-2 promotes formation of active synapses and is present on the outside of ADSEVs. Related to Figure 4.**

(A) Representative FM4-64 (grayscale) and Phalloidin (gray)/FM4-64 (green) overlay images of day 12 neurons treated with 10 ng/ml TGF-β1, 2 μg/ml rhFBLN2 or 2000 ADSEVs/cell.

(B and C) Quantification of spine and synapse density from images in A. n=45 primary or secondary dendrites from three independent experiments.

(D) Representative PSD95 (grayscale) and Phalloidin (gray)/PSD95 (green) overlay images of day 12 neurons treated with 10 ng/ml TGF-β1, 2 μg/ml rhFBLN2 or 2000 ADSEVs/cell.

(E and F) Quantification of spine and synapse density from images in D. n=45 primary or secondary dendrites from three independent experiments.

(G) Representative Transferrin (grayscale) and Phalloidin (gray)/Transferrin (green) overlay images of Transferrin internalization in the absence or presence of 100 μM Dynasore. n≥12 images from 3 independent experiments. Yellow arrows show internalized transferrin. Green arrows show transferrin at the surface of or outside of the neuron. Scale bar, 10 μm.

(H) Dot blot assay of astrocyte-derived SEVs at 0, 0.25, 0.5, 1, 2 and 4 μg concentrations stained for fibulin-2 or Alix in the absence or presence of 0.1% (v/v) Tween-20 detergent in 1x TBS. Representative of three independent experiments.

All images are adjusted for brightness and contrast and cropped to same size. For images in A and D, Left panel: FM4-64/PSD95 only. Right panel: Overlay of phalloidin (gray-scale) and FM4-64/PSD95 (green). Some example dendritic spines are indicated with arrows. Data are represented as box and whiskers plots with all data points shown, bar indicating the median, and the box showing interquartile range. Scale bar 5 μm. ***p<0.001.

**Table S1: Proteins with more than 2-fold change in ADSEVs compared to both C6SEVs and CNSEVs. Related to Figure 2.**

| Protein Name | Fold change compared to | |
|---|---|---|
| | C6-SEVs | CN-SEVs |
| Collagen alpha-1(I) chain* | 8.65 | 8.50 |
| Alpha-2-macroglobulin* | 8.57 | 9.03 |
| Microfibril-associated glycoprotein 4-like* | 7.76 | 12.04 |
| Fibulin 2* | 6.64 | 5.29 |
| Protein S100-A6* | 6.03 | 13.53 |
| Epoxide hydrolase 1* | 5.86 | 4.29 |
| Atrial natriuretic peptide receptor 3* | 5.80 | 5.00 |
| Low-density lipoprotein receptor-related protein 2* | 5.77 | 4.04 |
| Tubulin tyrosine ligase-like 12* | 5.51 | 6.25 |
| Annexin A3* | 5.37 | 3.97 |
| Complement C3* | 5.09 | 7.49 |
| Coiled-coil domain-containing 150* | 5.08 | 5.96 |
| Pyruvate dehydrogenase phosphatase regulatory subunit | 4.55 | 3.91 |
| Vimentin | 4.54 | 2.96 |
| LSM4 homolog, U6 small nuclear RNA and mRNA degradation-associated | 4.53 | 7.59 |
| Metalloendopeptidase | 4.41 | 5.51 |
| Collagen alpha-1(III) chain | 4.36 | 4.13 |
| Adipocyte enhancer-binding protein 1 | 4.33 | 4.35 |
| Receptor expression-enhancing protein 5 | 4.23 | 2.12 |
| Glycogen [starch] synthase, muscle | 4.05 | 4.12 |
| Cell migration-inducing hyaluronan-binding protein | 3.77 | 3.50 |
| Family with sequence similarity 171, member A2 | 3.66 | 2.54 |
| Aa1018 | 3.59 | 4.70 |
| Cystathionine beta-synthase | 3.55 | 4.55 |
| Chromodomain helicase DNA-binding protein 1-like | 2.91 | 26.10 |
| Alpha-1-macroglobulin | 2.82 | 9.70 |
| Citrate synthase, mitochondrial | 2.63 | 2.69 |
| Creatine kinase S-type, mitochondrial | 2.60 | 2.35 |
| WD repeat-containing protein 1 | 2.50 | 2.15 |
| Intercellular adhesion molecule 1 | 2.49 | 2.66 |
| Maltase-glucoamylase | 2.31 | 5.30 |
| Cleavage and polyadenylation-specific factor 1 | 2.19 | 5.74 |
| Cerebellar degeneration-related protein 2-like | 2.11 | 5.01 |

*Significantly higher in ADSEVs compared to C6SEVs.
Proteins with a Benjamini-Hochberg (BH) FDR $p<0.05$ were defined as significantly changed.