Expanded View Figures

Figure EV1. Structure of MVBs and sEVs in polarized MDCK cells.

A Scheme of sEV and exosome isolation methods used in this study.
B Polarized MDCK cells were immunostained with anti-CD63 and anti-CD9 antibodies. Arrowheads, CD63- and CD9-double-positive dots; arrows, CD63-positive dots; and double-headed arrows, CD9-positive dots. Scale bar, 20 μm.
C Polarized MDCK cells were analyzed by conventional electron microscopy. Small ILVs and a large ILV were indicated by arrowheads and an arrow, respectively. It is noteworthy that almost all of the ILVs measured < 100 nm in diameter. Scale bar, 100 nm.
D MDCK cells were cultured on cell culture inserts for 4 days. On the last day, the culture medium was replaced with EV-depleted medium. sEVs released from the apical and basolateral side of the MDCK cells were purified by PEG precipitation and analyzed by NTA. Representative NTA traces were shown. Note that both PEG pellets showed a broad particle size distribution, suggesting that PEG pellets contain various types of sEVs.
E Quantification of the NTA data obtained in five independent experiments of (D). Mean ± s.e.m. was shown.
F PEG pellets prepared as in (D) were immunonegative stained with anti-CD9 antibody and analyzed by electron microscopy. Scale bar, 100 nm.
G The percentages of CD9-positive sEVs in 10 images were calculated. Mean ± s.e.m. was shown.

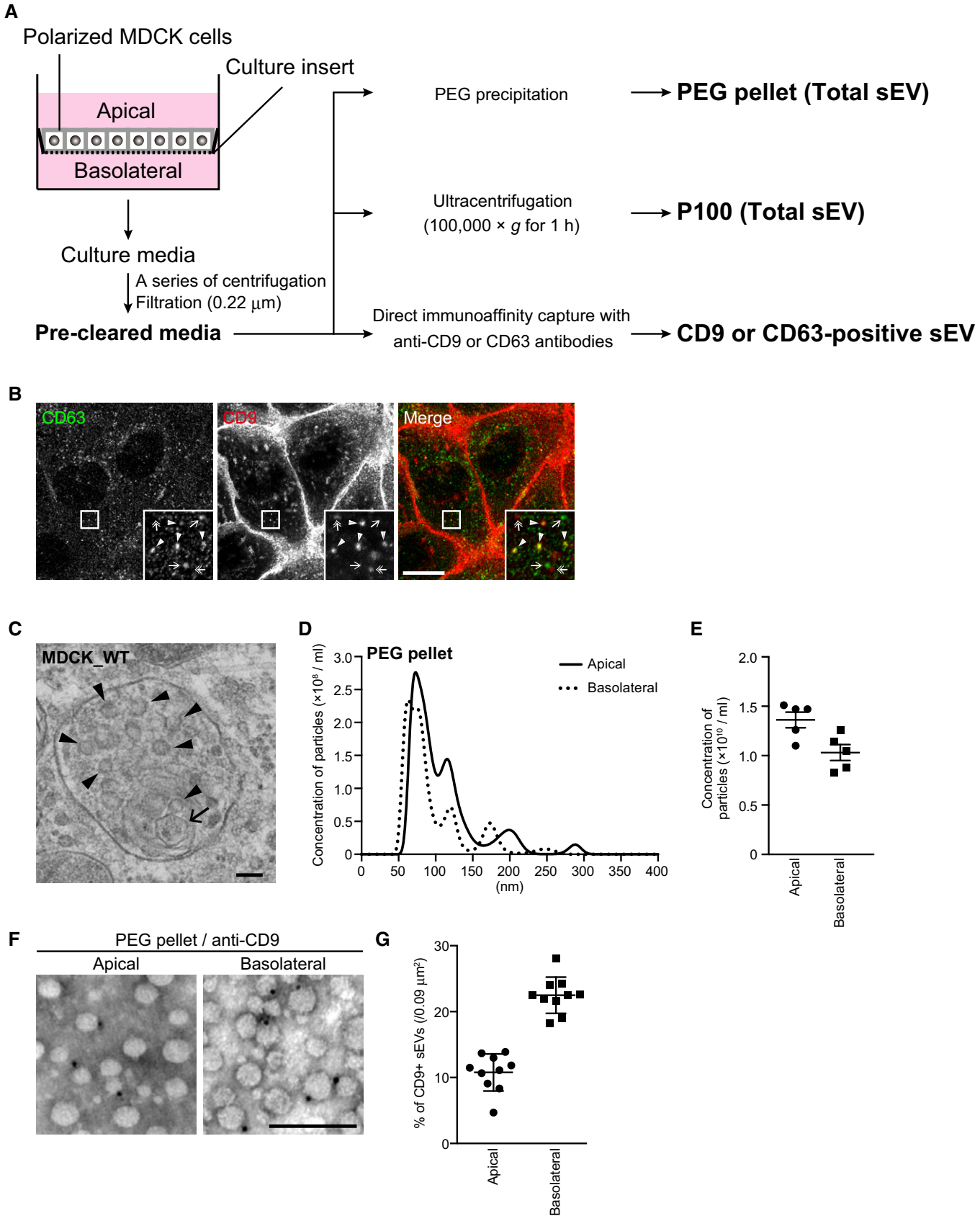
A
Polarized MDCK cells
Culture insert
Apical
Basolateral
Culture media
A series of centrifugation
Filtration (0.22 μm)
Pre-cleared media
PEG precipitation
PEG pellet (Total sEV)
Ultracentrifugation
(100,000 × g for 1 h)
P100 (Total sEV)
Direct immunoaffinity capture with
anti-CD9 or CD63 antibodies
CD9 or CD63-positive sEV
B
CD63
CD9
Merge
C
MDCK_WT
D
PEG pellet
Apical
Basolateral
Concentration of particles (×10⁸ / ml)
(nm)
E
Concentration of particles (×10¹⁰ / ml)
Apical
Basolateral
F
PEG pellet / anti-CD9
Apical
Basolateral
G
% of CD9+ sEVs (/0.09 μm²)
Apical
Basolateral

Figure EV1.

Figure EV2. Exosome release is upregulated by inhibition of lysosomal function.

A MDCK cells were cultured on cell culture inserts for 4 days. On the last day, the cells were cultured in EV-depleted medium with or without 100 nM bafilomycin A_1 (Baf A_1) for the times indicated until the medium was harvested. sEVs were isolated from the pre-cleared medium by direct immunoaffinity capture using anti-CD9 antibody. Cell lysates and sEV samples were analyzed by immunoblotting with the antibodies indicated.

B The intensity of the bands shown in (A) was measured in three independent experiments.

C sEVs prepared as in (A) were eluted from the beads with a glycine buffer and analyzed by NTA. Representative NTA traces were shown.

D Quantification of the NTA data obtained in five independent experiments of (C).

E Polarized MDCK cells were cultured in EV-depleted medium with or without 100 nM Baf A_1 for 6 h and cells were immunostained with anti-CD63 antibody. Scale bars, 20 μm (1 μm, insets).

F MDCK cells were transfected with siControl or the siRNAs indicated. After 3 days, the culture medium was replaced with EV-depleted medium. One day later, the cells were immunostained with anti-CD63 antibody. Scale bars, 20 μm (1 μm, insets).

G MDCK cells were transfected with the siRNAs indicated, cultured as in (E), and analyzed by conventional electron microscopy. Scale bars, 400 nm. Note that in addition to normal MVBs, enlarged and ILV-less MVB-like structures were often observed in HRS-KD cells.

H MDCK cells were transfected with siControl or two independent siALIX. The cells were then transferred to cell culture inserts and cultured for 4 days. On the last day, the culture medium was replaced with EV-depleted medium. sEVs were isolated from the pre-cleared medium by direct immunoaffinity capture using anti-CD9 antibody. Cell lysates and sEV samples were analyzed by immunoblotting with the antibodies indicated.

I The intensity of the bands shown in (H) was measured in three independent experiments.

Data information: (A and H) CD63 blots were separately obtained on different days using the same samples. (B, D and I) $*P < 0.05$, $**P < 0.01$ (one-way ANOVA and Tukey's test). Mean ± s.e.m. was shown.

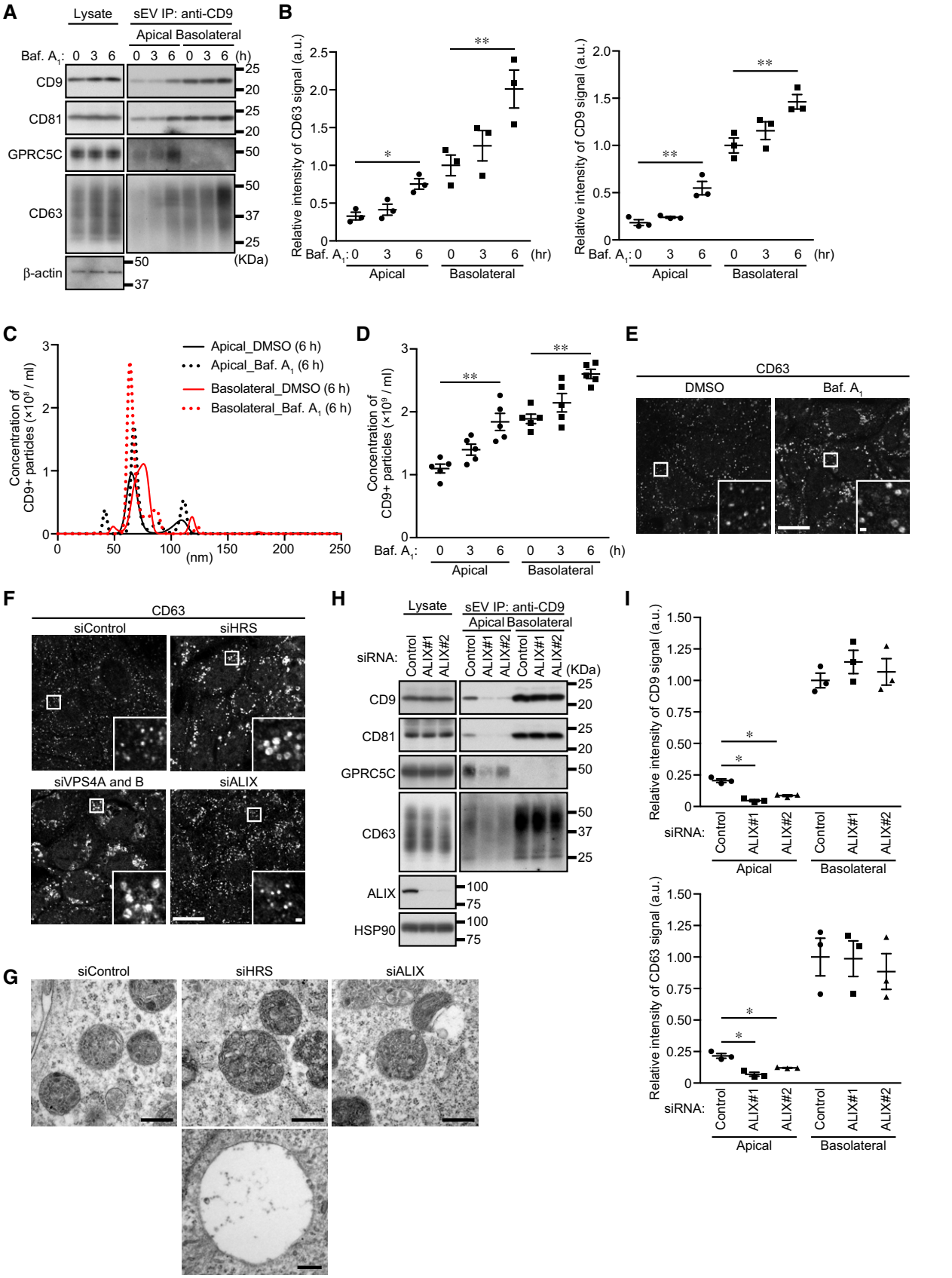
A
Lysate
sEV IP: anti-CD9
Apical Basolateral
Baf. A1: 0 3 6 0 3 6 0 3 6 (h)
CD9
CD81
GPRC5C
CD63
β-actin
25
20
25
20
50
50
37
25
50
37
(KDa)
B
Relative intensity of CD63 signal (a.u.)
Relative intensity of CD9 signal (a.u.)
Baf. A1: 0 3 6 0 3 6 (hr)
Apical
Basolateral
C
Apical_DMSO (6 h)
Apical_Baf. A1 (6 h)
Basolateral_DMSO (6 h)
Basolateral_Baf. A1 (6 h)
Concentration of CD9+ particles (×10^8 / ml)
(nm)
D
Concentration of CD9+ particles (×10^9 / ml)
E
CD63
DMSO
Baf. A1
F
CD63
siControl
siHRS
siVPS4A and B
siALIX
G
siControl
siHRS
siALIX
H
Lysate
sEV IP: anti-CD9
Apical Basolateral
siRNA: Control ALIX#1 ALIX#2 Control ALIX#1 ALIX#2
(KDa)
CD9
CD81
GPRC5C
CD63
ALIX
HSP90
I
Relative intensity of CD9 signal (a.u.)
Relative intensity of CD63 signal (a.u.)
siRNA: Control ALIX#1 ALIX#2 Control ALIX#1 ALIX#2
Apical
Basolateral

Figure EV2.

Figure EV3. The effect of ALIX-KD and GW4869 treatment on release of CD63-positive sEVs and all sEVs.

A MDCK cells stably expressing human CD63 were transfected with siControl or siALIX, and the cells were transferred to cell culture inserts and cultured for 4 days. On the last day, the culture medium was replaced with EV-depleted medium with or without 10 nM GW4869. sEVs were isolated from the pre-cleared medium by direct immunoaffinity capture using anti-CD63 antibody or ultracentrifugation. Cell lysates and sEV samples were analyzed by immunoblotting with the antibodies indicated.

B The intensity of the bands shown in (A) was measured in three independent experiments.

C sEVs prepared as in (A) were eluted from the beads with a glycine buffer and analyzed by NTA. Representative NTA traces were shown.

D Quantification of the NTA data obtained in five independent experiments of (C).

Data information: (B and D) *$P < 0.05$, **$P < 0.01$ (one-way ANOVA and Tukey's test). Mean ± s.e.m. was shown.

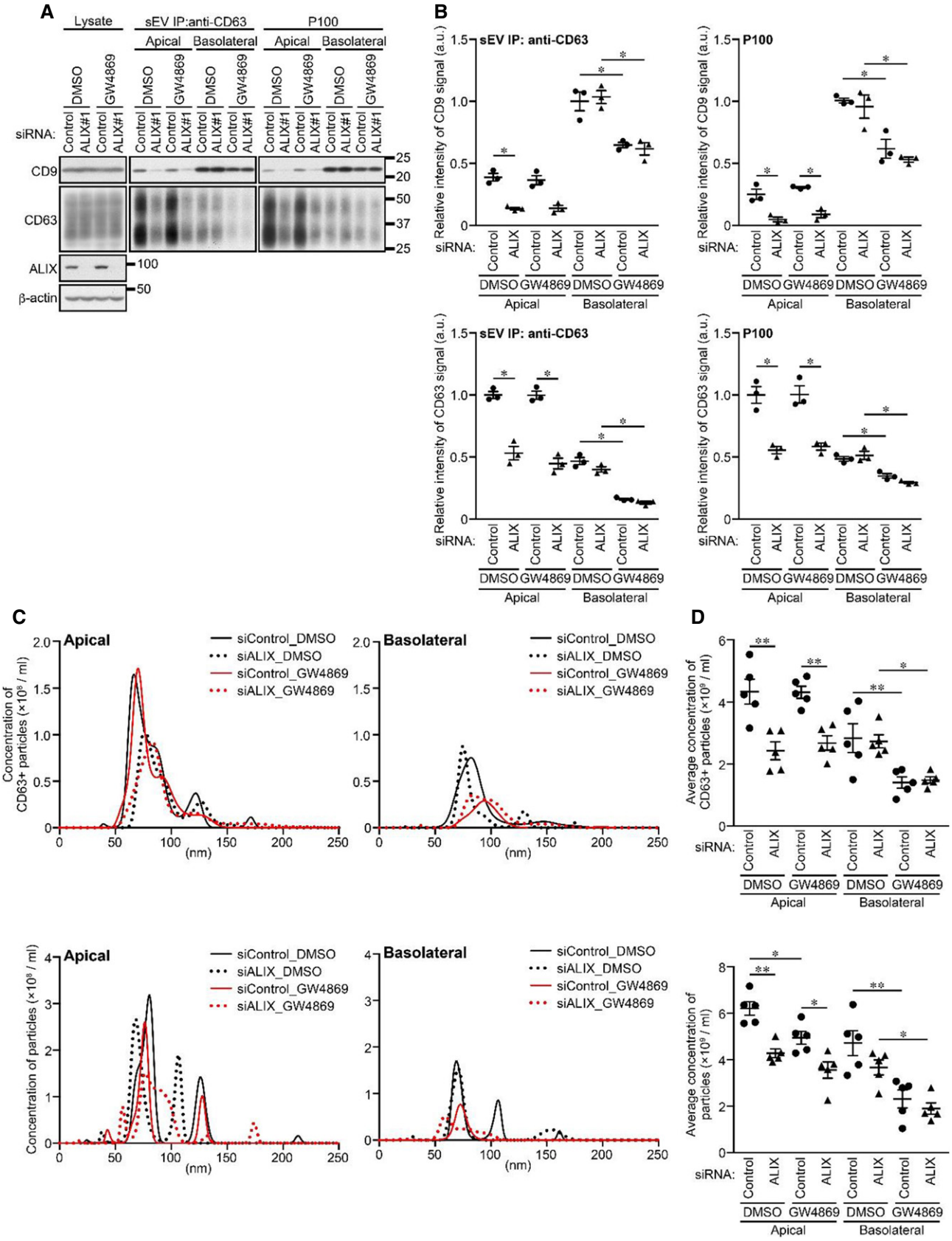
A
Lysate
sEV IP:anti-CD63
P100
Apical
Basolateral
DMSO
GW4869
siRNA:
Control
ALIX#1
CD9
CD63
ALIX
β-actin
B
sEV IP: anti-CD63
P100
Relative intensity of CD9 signal (a.u.)
Relative intensity of CD63 signal (a.u.)
ALIX
C
Apical
Basolateral
siControl_DMSO
siALIX_DMSO
siControl_GW4869
siALIX_GW4869
Concentration of CD63+ particles (×10⁸ / ml)
Concentration of particles (×10⁸ / ml)
(nm)
D
Average concentration of CD63+ particles (×10⁹ / ml)
Average concentration of particles (×10⁹ / ml)

Figure EV3.

Figure EV4. Syntenin1 and Syndecan1 are specifically involved in the apical exosome release.

A MDCK cells were transfected with siControl or the siRNAs indicated. The cells were then transferred to cell culture inserts and cultured for 4 days. On the last day, the culture medium was replaced with EV-depleted medium. sEVs were isolated from the pre-cleared medium by direct immunoaffinity capture using anti-CD9 antibody. Cell lysates and sEV samples were analyzed by immunoblotting with the antibodies indicated.

B The intensity of the bands shown in (A) was measured in three independent experiments.

C EVs prepared as in (A) were eluted from the beads with a glycine buffer and analyzed by NTA. Representative NTA traces were shown.

D Quantification of the NTA of data obtained in five independent experiments of (C).

Data information: (A) CD63 blots were separately obtained on different days using the same samples. (B and D) *$P < 0.05$, **$P < 0.01$ (one-way ANOVA and Tukey's test). Mean ± s.e.m. was shown.

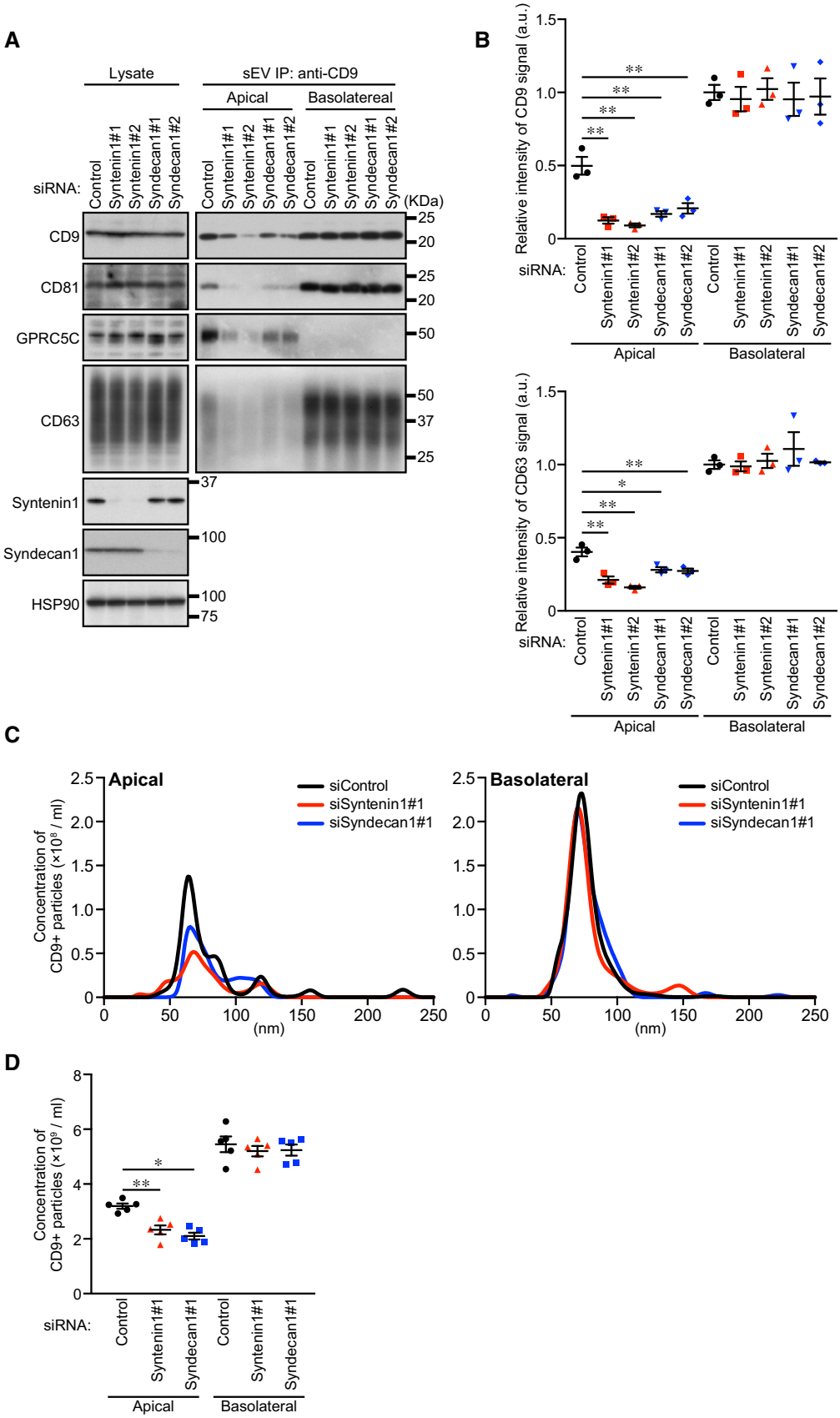
A
Lysate
sEV IP: anti-CD9
Apical
Basolatereal
siRNA:
Control
Syntenin1#1
Syntenin1#2
Syndecan1#1
Syndecan1#2
(KDa)
CD9
CD81
GPRC5C
CD63
Syntenin1
Syndecan1
HSP90
B
Relative intensity of CD9 signal (a.u.)
Relative intensity of CD63 signal (a.u.)
Apical
Basolateral
C
Apical
Basolateral
siControl
siSyntenin1#1
siSyndecan1#1
Concentration of CD9+ particles (×10⁸ / ml)
(nm)
D
Concentration of CD9+ particles (×10⁹ / ml)

Figure EV4.

Figure EV5. Ceramide is specifically involved in the basolateral exosome release.

A MDCK cells were cultured on cell culture inserts for 4 days. On the last day, the cells were cultured in EV-depleted medium with or without 10 nM GW4869. sEVs were isolated from the pre-cleared medium by direct immunoaffinity capture using anti-CD9 antibody. Cell lysates and sEV samples were analyzed by immunoblotting with the antibodies indicated.

B The intensity of the bands shown in (A) was measured in three independent experiments.

C sEVs prepared as in (A) were eluted from the beads with a glycine buffer and analyzed by NTA. Representative NTA traces were shown.

D Quantification of the NTA data obtained in five independent experiments of (C).

E MDCK cells were transfected with siControl or the sinSMase2. The cells were then transferred to cell culture inserts and cultured for 4 days. On the last day, the culture medium was replaced with EV-depleted medium. sEVs were prepared and analyzed as in (A).

F The intensity of the bands shown in (E) was measured in three independent experiments.

G sEVs prepared as in (E) were eluted from the beads and then analyzed as in (C).

H Quantification of the NTA of data obtained in five independent experiments of (G).

Data information: (A and E) CD63 blots were separately obtained on different days using the same samples. (B, D, F and H) $*P < 0.05$, $**P < 0.01$ (one-way ANOVA and Tukey's test). Mean ± s.e.m. was shown.

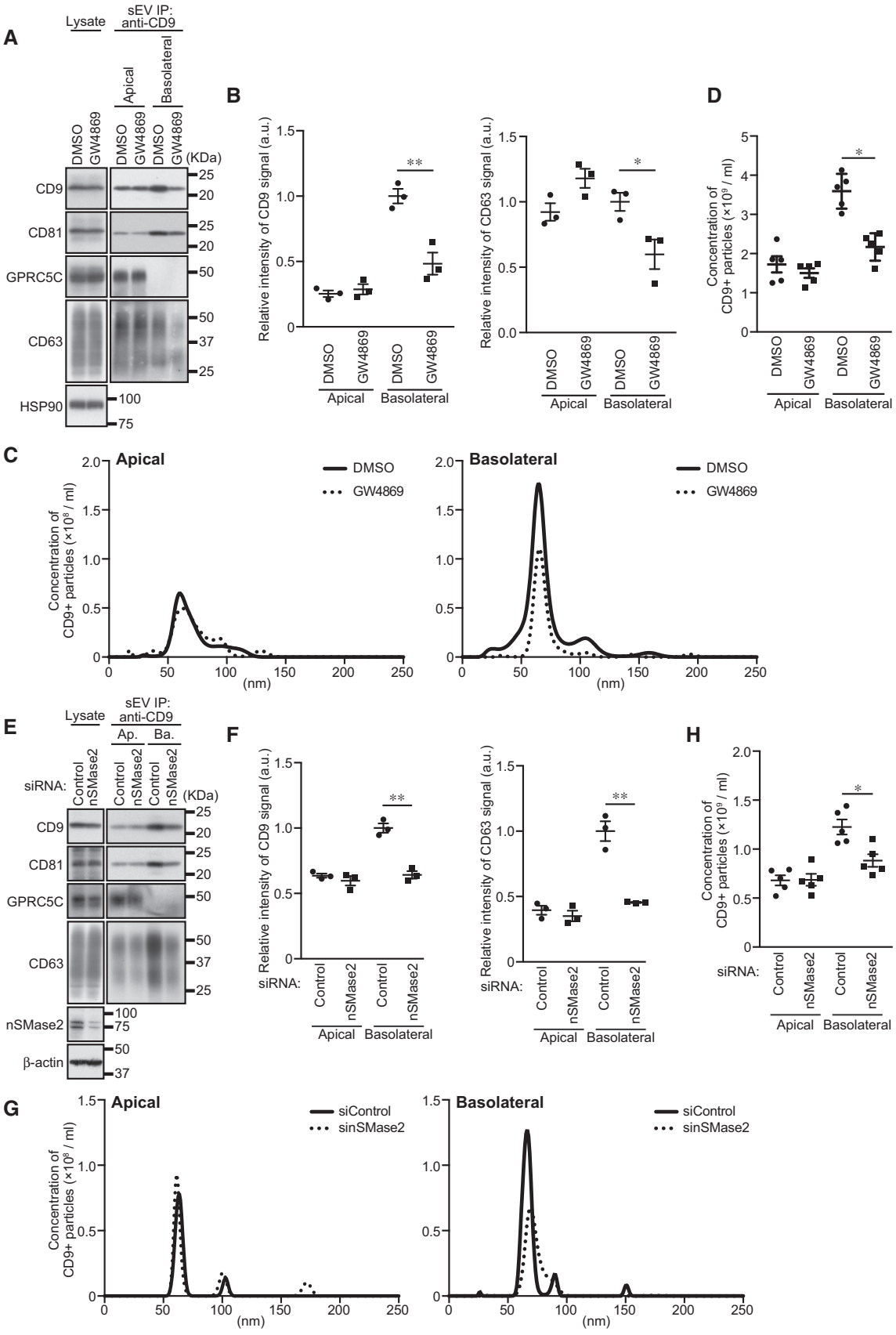
A
Lysate
sEV IP: anti-CD9
Apical
Basolateral
DMSO
GW4869
(KDa)
CD9
CD81
GPRC5C
CD63
HSP90
B
Relative intensity of CD9 signal (a.u.)
Relative intensity of CD63 signal (a.u.)
Apical
Basolateral
D
Concentration of CD9+ particles (×10⁹ / ml)
C
Apical
Basolateral
Concentration of CD9+ particles (×10⁸ / ml)
(nm)
E
Ap.
Ba.
siRNA:
Control
nSMase2
nSMase2
β-actin
F
H
G
siControl
sinSMase2

Figure EV5.