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Supplementary Materials for

Endocytosis blocks the vesicular secretion of exosome marker proteins

Yiwei Ai *et al.*

Corresponding author: Stephen J. Gould, sgould@jhmi.edu

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Figure S1. Characterization of an AP2M1$^{-/-}$ derivative of 293F cells. (**A**) Amino acid alignment showing (top line) the 1 amino acid deletion (I63Δ) in the mu2 protein encoded by allele #2 of the AP2M1 gene and (lower lines) the same region of the AP-2 mu2 protein encoded by other metazoans. Please note the highly degree of sequence conservation in this region, as well as the presence of Ile or Val at position 63 in all metazoan homologs. (**B**) Immunoblot of cell lysates from 293F cells and the 293F AP2M1$^{-/-}$ cell line, probed with antibodies specific for AP2M1 and Hsp90. Protein molecular weight markers are listed by size in kDa. Note that the amount of AP2M1 protein present in the AP2M1$^{-/-}$ cell lines is reduced but not absent, as expected for a cell line that carries a null mutation on allele #1 of the AP2M1 gene (a 198 bp deletion that eliminates the 5' half of exon 3, the 3' end of intron 2, and precludes proper splicing of the AP2M1 transcript) and a 1 codon deletion on allele #2 that encodes a mutant AP2M1 protein of nearly normal length. This immunoblot was performed twice.

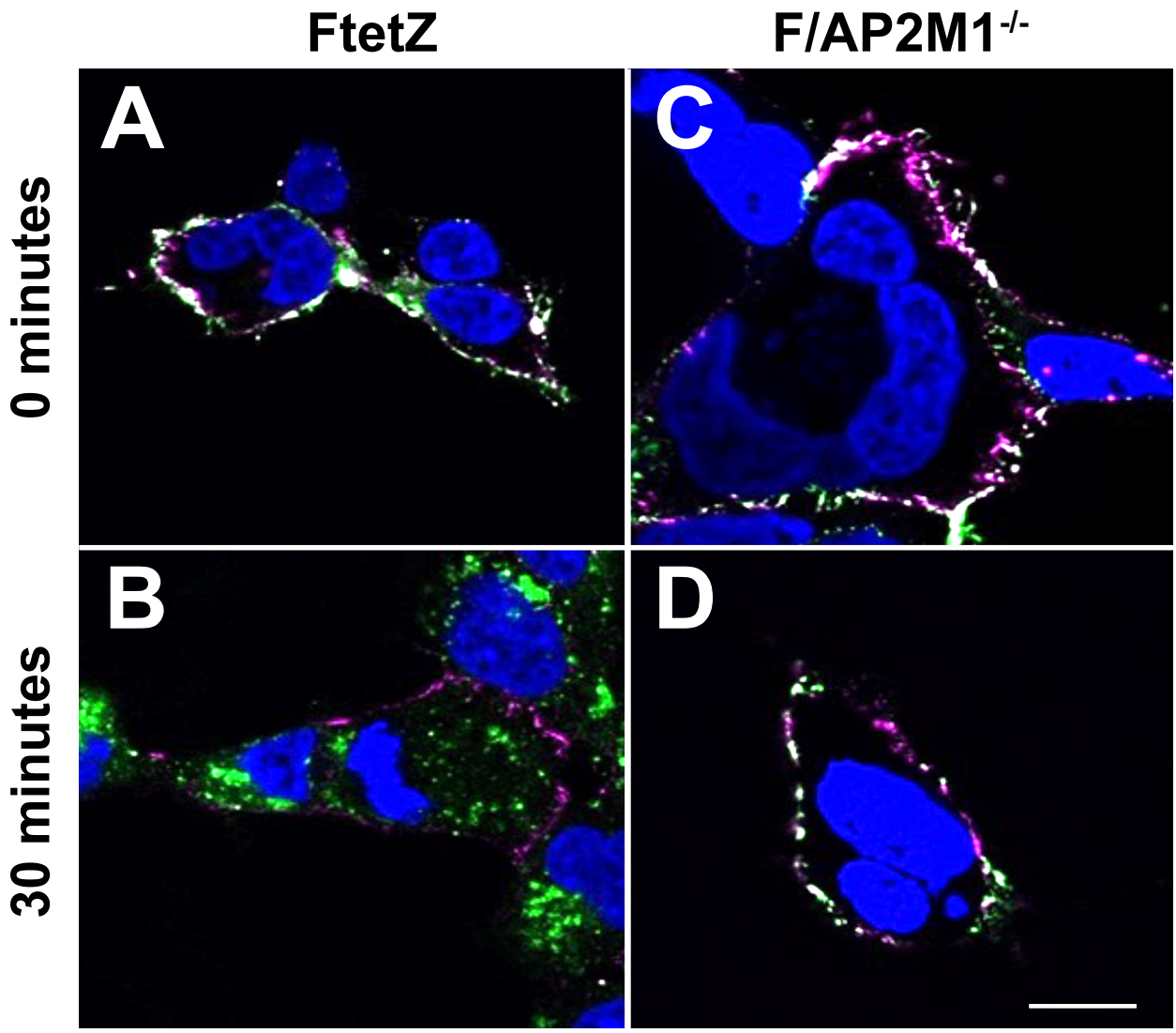

Figure S2. AP2M1$^{-/-}$ cells are defective in CD63 endocytosis. Confocal fluorescence micrographs of (A, B) 293F cells and (C, D) 293F/AP2M1$^{-/-}$ cells that had been chilled to 4°C, stained with fluorescently labeled antibodies to (green) CD63 or (pink) CD9, also at 4°C, then fixed immediately (0 minutes) or incubated for 30 minutes at 37°C and then fixed. Fixed cells were stained with DAPI and imaged by confocal fluorescence microscopy. These data are representative of the results from three independent trials. Bar, 10 μm

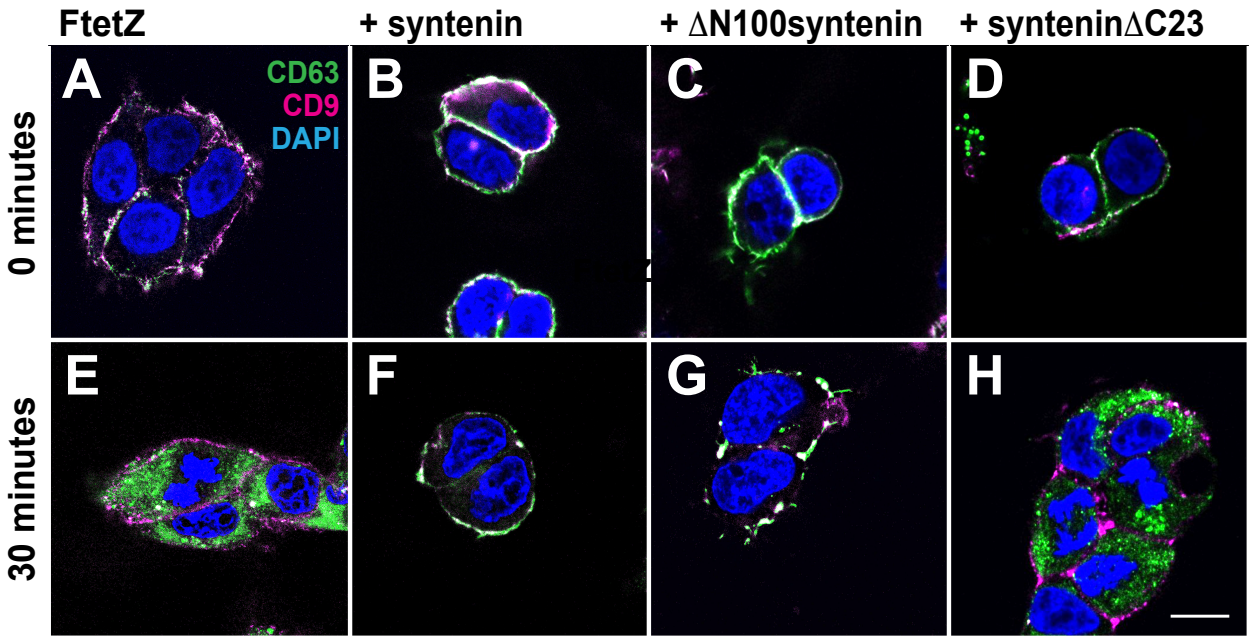

Figure S3. High level expression of syntenin or ΔN100 syntenin inhibits the internalization of CD63. Confocal fluorescence microscopy images of doxycycline-grown FtetZ cells and FtetZ cells carrying TRE3G-regulated transgenes encoding syntenin, ΔN100syntenin, or syntenin ΔC23 that had been chilled, stained with fluorescently labeled antibodies to (green) CD63 or (pink) CD9 also at 4°C, then fixed immediately (0 minutes) or incubated for 30 minutes at 37°C and then fixed. Fixed cells were stained with DAPI and imaged by confocal fluorescence microscopy. These data are representative of the results from three independent trials. Bar, 10 μm

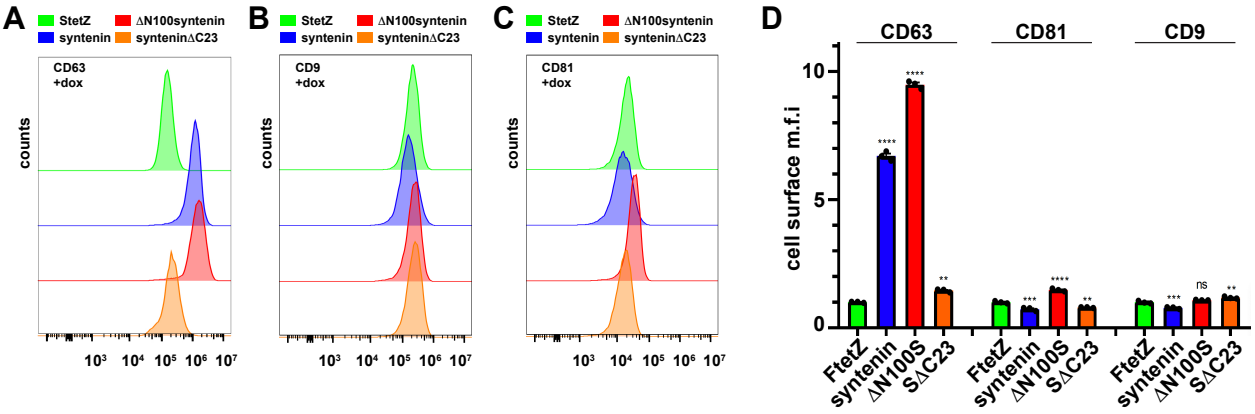

Figure S4. High-level expression of syntenin or ΔN100 syntenin induces the plasma membrane accumulation of CD63 in Hela cells. (**A-C**) Flow cytometry histograms showing the fluorescence intensity of chilled, antibody-stained (green) Tet-On Hela-S (StetZ) cells, and also for StetZ cells carrying TRE3G-regulated transgenes encoding (blue) WT human syntenin, (red) ΔN100syntenin, or (orange) syntenin ΔC23. Cells were grown overnight in the presence of doxycycline, then chilled to 4ºC and stained using (A) FITC-labeled antibody specific for CD63, (B) PE-labeled antibody specific for CD9, and (C) APC-labeled antibody specific for CD81. (**D**) Bar graph showing mean fluorescence intensity (m.f.i.) for CD63, CD81, and CD9 in each cell lines across three independent trials. ANOVA *p* values are denoted by ** for <0.01, *** for <0.005, and **** for <0.0005. These experiments werer repeated three times.

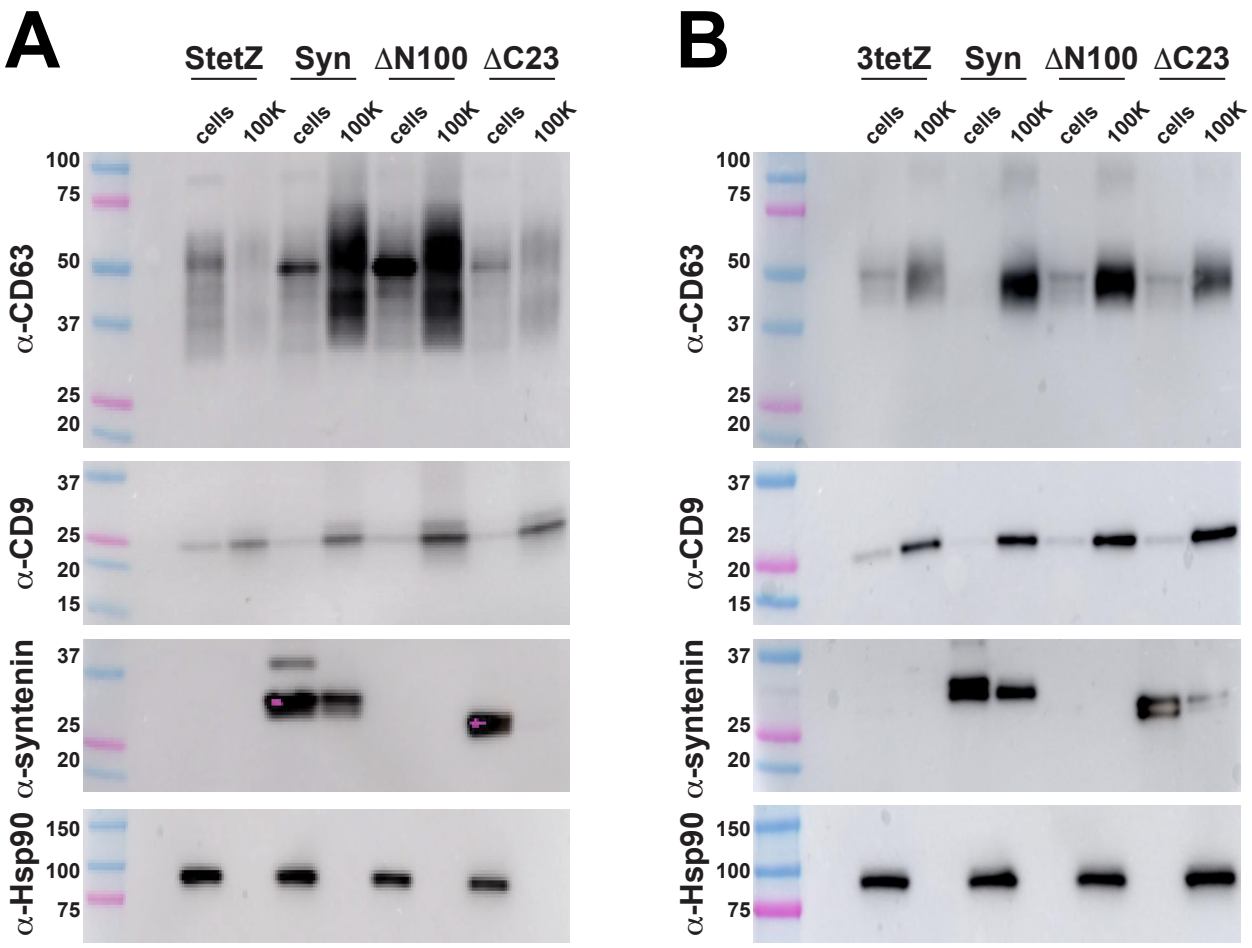

Figure. S5. Syntenin and ΔN100syntenin drive the exosomal secretion of CD63 from Hela cancer cells and mouse NIH3T3 fibroblasts. (**A**) Immunoblot analysis of cell lysates and exosome-containing fractions collected from the Tet-on Hela cell line StetZ carrying TRE3G-regulated transgenes encoding (Syn) WT human syntenin, (ΔN100) ΔN100syntenin, or (ΔC23) synteninΔC23. Cells were grown for three days in doxycycline-containing, chemically defined medium, exosome-containing fractions were collected by differential centrifugation, and immunoblots were probed using antibodies specific for CD63, CD9, syntenin, and the cytoplasmic chaperone Hsp90. MW markers are in kDa. (**B**) Immunoblot analysis of cell lysates and exosome-containing fractions collected from the Tet-on NIH3T3 cell line 3tetZ carrying TRE3G-regulated transgenes encoding (Syn) WT human syntenin, (ΔN100) ΔN100syntenin, or (ΔC23) synteninΔC23. Cells were grown for three days in doxycycline-containing complete medium (DMEM + 10% FCS), exosome-containing fractions were collected by differential centrifugation, and immunoblots were probed using antibodies specific for CD63, CD9, syntenin, and the cytoplasmic chaperone Hsp90. MW markers are in kDa. Data are from three independent trials.

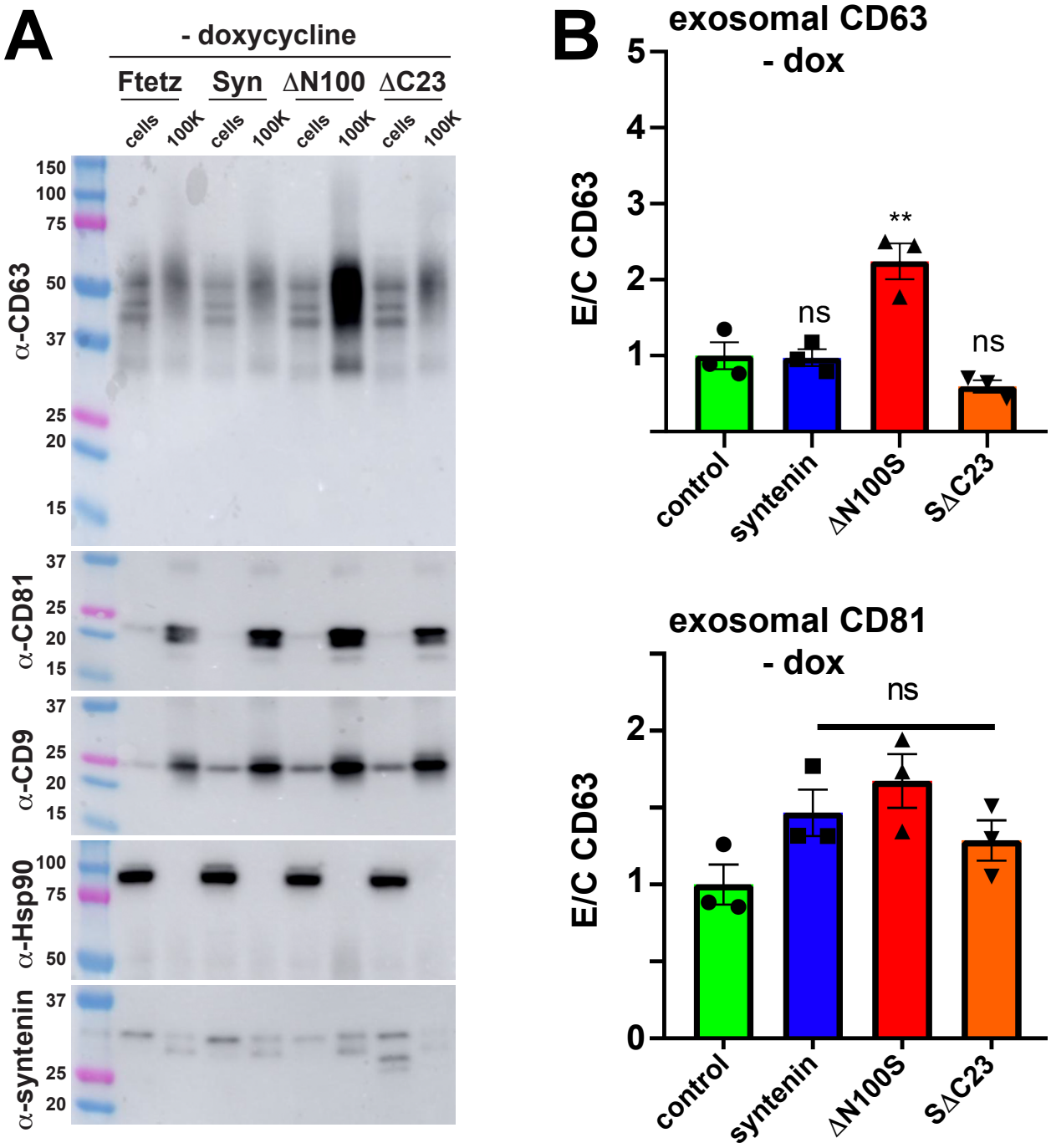

Figure S6. Low baseline expression of ΔN100syntenin can induce the exosomal secretion of CD63. (**A**) Immunoblot analysis of cell lysates and exosome-containing fractions collected from the Tet-on 293F cell line FtetZ and its syntenin transgenic cell line derivatives, grown in the absence of doxycycline for three days. MW markers are in kDa. (**B**) Bar graphs showing the relative exosomal secretion of (upper graph) CD63 and (lower graph) CD81 calculated from triplicate biological replicates of this experiment.

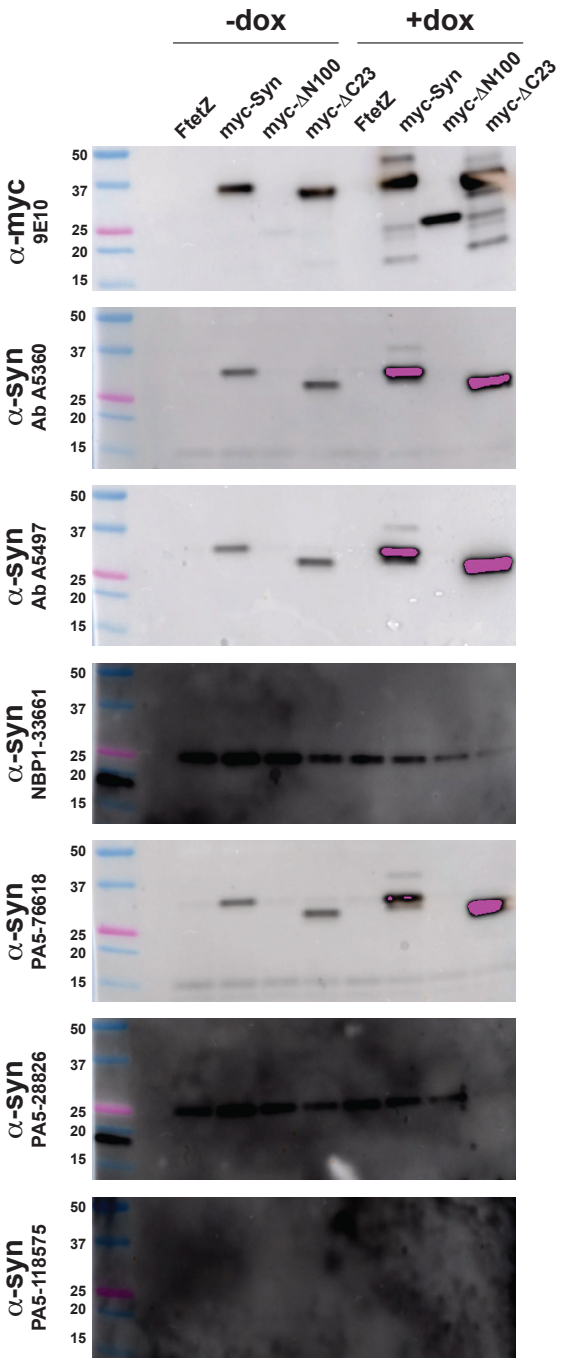

Figure S7. Antibody tests of myc-tagged syntenin cell lines. Immunoblot analysis of cell lysates collected from the Tet-on 293F cell lines (FtetZ) carrying no syntenin transgene or dox-inducible, TRE3G-driven transgenes designed to express N-terminally myc-tagged forms of syntenin, ΔN100syntenin, or syntenin ΔC23. Cells were grown overnight +/- dox, lysed, and processed for immunoblot using the 9E10 anti-myc monoclonal antibody or each of six different anti-syntenin antibodies. Note that none of the anti-syntenin antibodies were able to detect mycΔN100syntenin, even when its expression was induced by doxycycline. MW markers are in kDa.

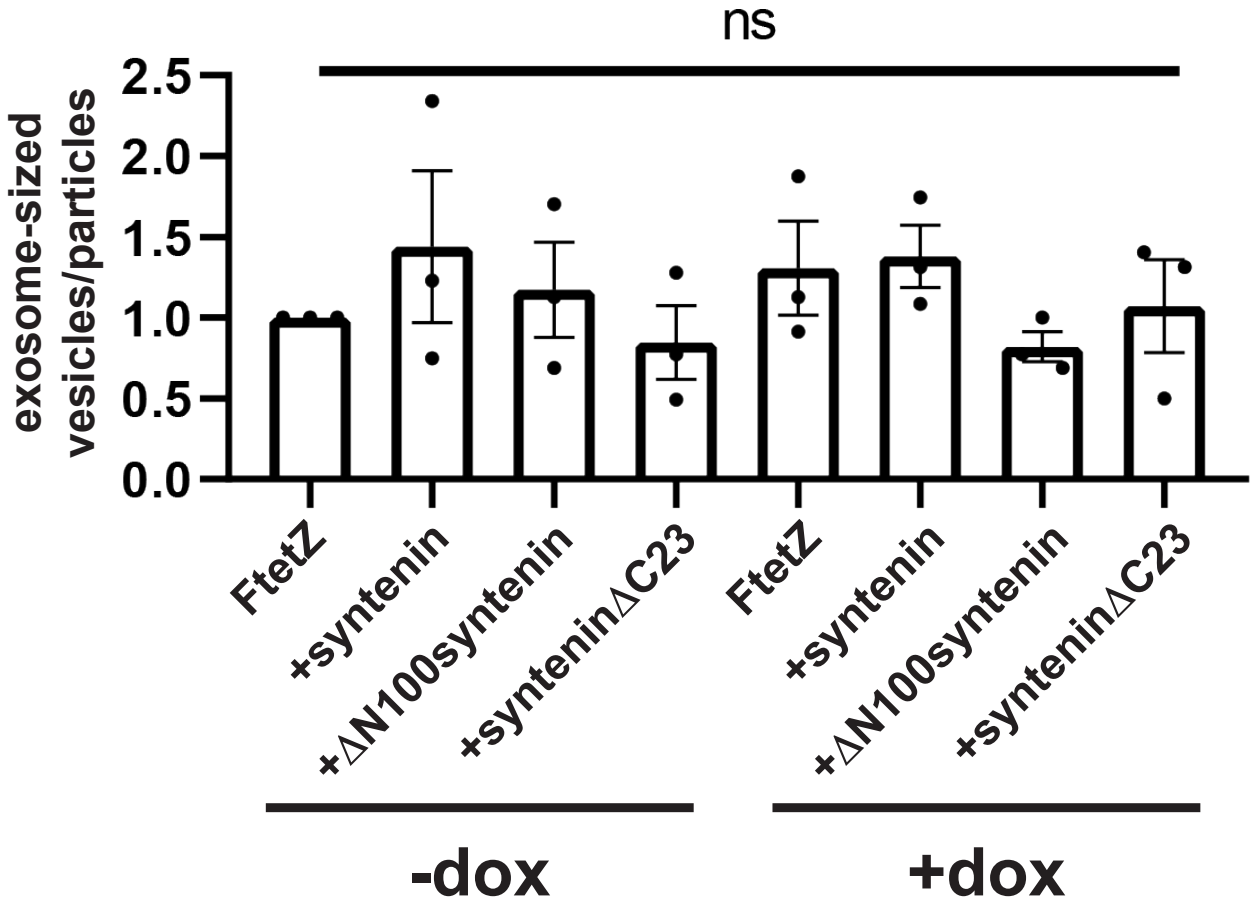

Figure S8. Yield of exosome-sized vesicles and particles is unchanged by syntenin expression. Exosome-sized vesicles were collected from Tet-on 293F (FtetZ) cell lines carrying transgenes that inducibly express syntenin (Syn), ΔN100syntenin (ΔN100), or syntenin ΔC23 (ΔC23) that had been grown in the absence or presence of doxycycline for three days. The concentration of small extracellular vesicles and particles in each preparation was determined by nanoparticle tracking analysis (NTA). Bar heights represent the mean, error bars represent the standard error of the mean, ANOVA was used to determine whether any of the observed differences were of high statistical significance, and ns refers to a *p* value >0.05. These results are from three independent trials.

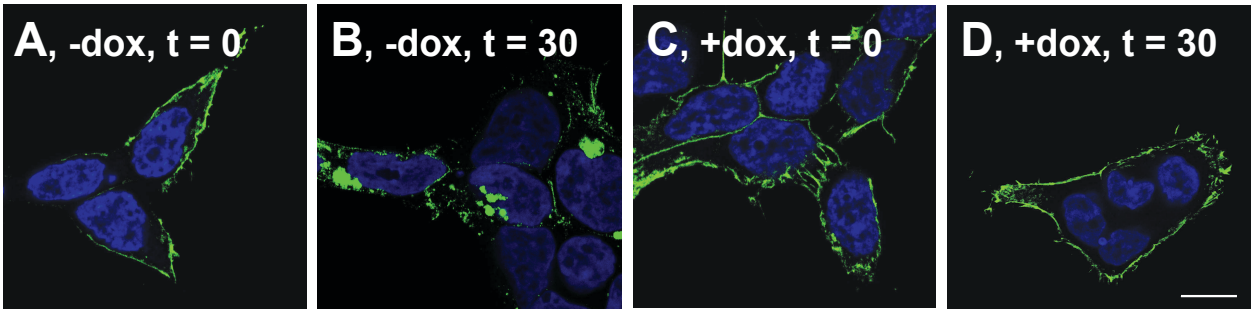

Fig. S9. **High-level expression of CD63 inhibits endocytosis**. Confocal fluorescence microscopy of images from the following experiment: 293F/CD63$^{-/-}$::TRE3G-CD63 cells were seeded onto glass coverslips, grown overnight in medium lacking or containing doxycycline, chilled to 4ºC, incubated with FITC-labeled anti-CD63 antibody for 30 minutes at 4ºC, washed, then incubated at 37ºC for 0 or 30 minutes. Cells from the -dox culture efficiently internalized CD63 during the 37ºC incubation (**A**, **B**), whereas cells from the +dox culture did not (**C**, **D**). Readers should note that image exposure for these different samples had to be adjusted for brightness, as otherwise the images of the -dox cells would have been underexposed and the images of the +dox cells would have been over-exposed. Bar, 10 μm

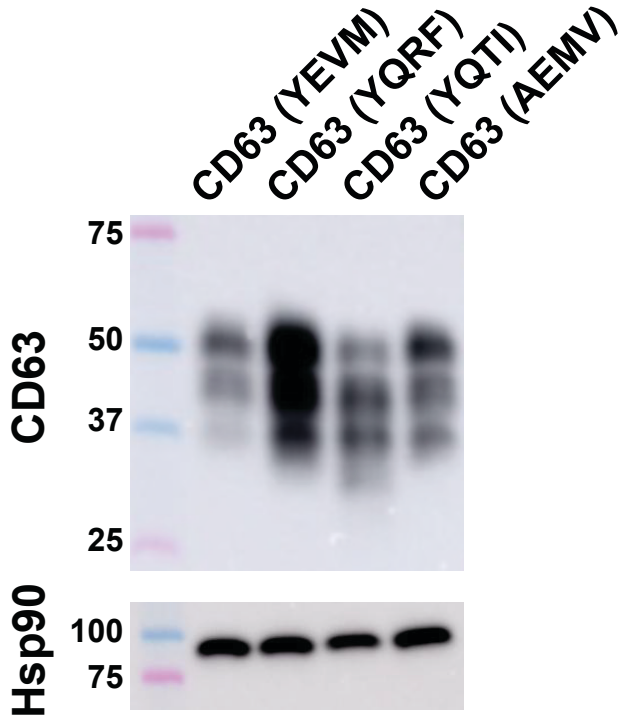

Fig. S10. Low-baseline expression of transgene-encoded CD63 proteins reveals a selective accumulation of CD63-YQRF. Tet-on CD63-/- knockout cells carrying TRE3G-regulated transgenes encoding WT CD63, CD63-YQRF, CD63-YQTI, or CD63-AEMV were grown in the absence of doxycycline, lysed, and processed for immunoblot using antibodies specific for CD63 and Hsp90. MW markers are in kDa.

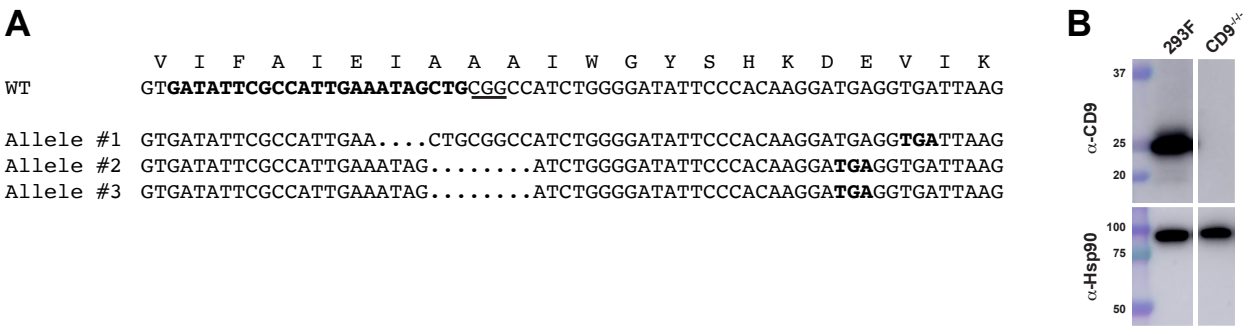

Figure S11. Genetic and biochemical validation of a 293F CD9$^{-/-}$ cell line. (**A**) Amino acid and DNA sequences of (top line) the WT human CD9 gene in the vicinity of the CD9-targeting gRNA target site (bold) and PAM site (underlined) that was used to mutate the CD9 gene, positioned above the DNA sequences of the three CD9 alleles in the 293F/CD9$^{-/-}$ cell line (293F cells carry three alleles of the CD9 gene). The sequences of the three mutated CD9 alleles were determined by amplifying a genomic DNA amplicon flanking the gRNA target site, followed by cloning the amplification product into a bacterial cloning vector, a then sequencing 25 separate clones of the amplicon, of which 6 clones carried the 4 bp deletion and 19 clones carried the 8 bp deletion. Bold TGA codons show the position of stop codons placed in frame by the frameshift mutations. (**B**) Immunoblot of cell lysates extracted from the parental 293F cell lines and the 293F/CD9$^{-/-}$ cell lines, probed with antibodies specific for CD9 and Hsp90. Protein molecular weight markers are listed by size in kDa. Data are from two independent trials.

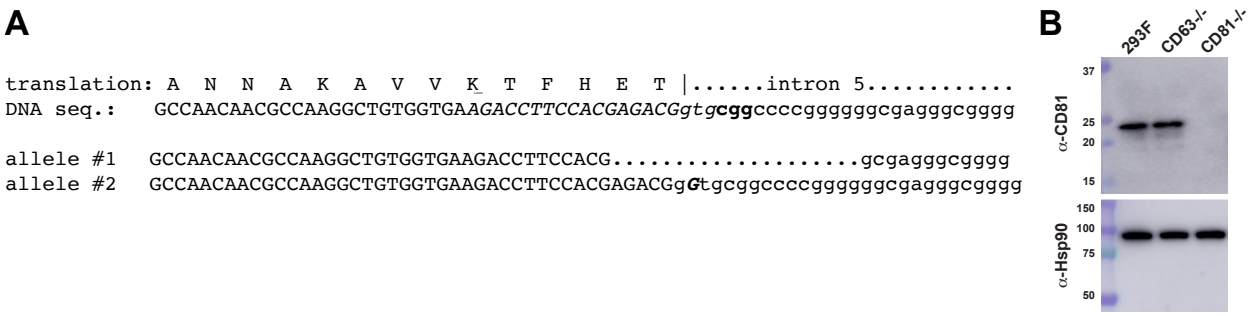

Figure S12. Genetic and biochemical validation of a 293F CD81$^{-/-}$ cell line. (**A**) Amino acid and DNA sequences of (top lines) the WT human CD81 gene in the vicinity of the gRNA target site (italics) and PAM site (bold) that was used to mutate the CD81 gene, positioned above the DNA sequences of the two CD81 alleles in the 293F/CD81$^{-/-}$ cell. The sequences of the mutated CD81 alleles were determined by amplifying a genomic DNA amplicon flanking the gRNA target site, followed by cloning the amplification product into a bacterial cloning vector. Note that both alleles eliminate the splice donor site at the 5' end of intron 5; allele #1 deletes the splice donor site entirely while allele #2 inserts a G that changes the GT donor site to a non-functional GG dinucleotide. (**B**) Immunoblot of cell lysates extracted from the parental 293F cell line, a CD63$^{-/-}$ derivative of 293F cells, and the 293F/CD81$^{-/-}$ cell line used in this study, probed with antibodies specific for CD81 and Hsp90. Protein molecular weight markers are listed by size in kDa. Data are from three independent trials.

Table S1. List of cell lines, Tet-on status, and their Sleeping Beauty transposon.

Cell line	Tet-on	Sleeping Beauty transposon
293F	no	none
Hela-S3	no	none
NIH3T3	no	none
293F/SDCBP-/-	no	none
293F/AP2M1-/-	no	none
293F/CD63-/-	no	none
293F/CD9-/-/-	no	none
293F/CD81-/-	no	none
FtetZ	Tet-on 293F (BleoR)	none
HtetZ	Tet-on Hela-S3 (BleoR)	none
3tetZ	Tet-on NIH3T3 (BleoR)	none
FtetZ::syntenin	Tet-on 293F (BleoR)	TRE3G-syntenin; EFS-HygR
FtetZ::ΔN100syntenin	Tet-on 293F (BleoR)	TRE3G-ΔN100syntenin; EFS-HygR
FtetZ::synteninΔC23	Tet-on 293F (BleoR)	TRE3G- synteninΔC23; EFS-HygR
FtetZ::mycsyntenin	Tet-on 293F (BleoR)	TRE3G-mycsyntenin; EFS-HygR
FtetZ::mycΔN100syntenin	Tet-on 293F (BleoR)	TRE3G-mycΔN100syntenin; EFS-HygR
FtetZ::mycsynteninΔC23	Tet-on 293F (BleoR)	TRE3G-mycsynteninΔC23; EFS-HygR
HtetZ::syntenin	Tet-on Hela-S3 (BleoR)	TRE3G-syntenin; EFS-HygR
HtetZ::ΔN100syntenin	Tet-on Hela-S3 (BleoR)	TRE3G-ΔN100synteni; EFS-HygR
HtetZ::synteninΔC23	Tet-on Hela-S3 (BleoR)	TRE3G- synteninΔC23; EFS-HygR
3tetZ/::yntenin	Tet-on NIH3T3 (BleoR)	TRE3G-syntenin; EFS-HygR
3tetZ::ΔN100syntenin	Tet-on NIH3T3 (BleoR)	TRE3G-ΔN100synteni; EFS-HygR
3tetZ::synteninΔC23	Tet-on NIH3T3 (BleoR)	TRE3G- synteninΔC23; EFS-HygR
FtetZ/CD63-/-	293F/CD63-/- + Tet-on (BleoR)	none
FtetZ/CD63-/-::CD63	293F/CD63-/- + Tet-on (BleoR)	TRE3G-CD63; EFS-HygR
FtetZ/CD63-/-::CD63-YQRF	293F/CD63-/- + Tet-on (BleoR)	TRE3G-CD63-YQRF; EFS-HygR
FtetZ/CD63-/-::CD63-YQTI	293F/CD63-/- + Tet-on (BleoR)	TRE3G-CD63-YQTI; EFS-HygR
FtetZ/CD63-/-::CD63-AEMV	293F/CD63-/- + Tet-on (BleoR)	TRE3G-CD63-AEMV; EFS-HygR
FtetZ/CD9-/-/-	293F/CD9-/-/- + Tet-on (BleoR)	none
FtetZ/CD9-/-/-::CD9	293F/CD9-/-/- + Tet-on (BleoR)	TRE3G-CD9; EFS-HygR
FtetZ/CD9-/-/-::CD9-YQRF	293F/CD9-/-/- + Tet-on (BleoR)	TRE3G-CD9-YQRF; EFS-HygR
FtetZ/CD9-/-/-::CD9-YQTI	293F/CD9-/-/- + Tet-on (BleoR)	TRE3G-CD9-YQTI; EFS-HygR
FtetZ/CD9-/-/-::CD9-AEMV	293F/CD9-/-/- + Tet-on (BleoR)	TRE3G-CD9-AEMV; EFS-HygR
FtetZ/CD9-/-/-::CD9-YEVM	293F/CD9-/-/- + Tet-on (BleoR)	TRE3G-CD9-YEVM; EFS-HygR
FtetZ/CD81-/-	293F/CD81-/- + Tet-on (BleoR)	none
FtetZ/CD81-/-::CD81	293F/CD81-/- + Tet-on (BleoR)	TRE3G-CD81; EFS-HygR
FtetZ/CD81-/-::CD81-YQRF	293F/CD81-/- + Tet-on (BleoR)	TRE3G-CD81-YQRF; EFS-HygR
FtetZ/CD81-/-::CD81-YQTI	293F/CD81-/- + Tet-on (BleoR)	TRE3G-CD81-YQTI; EFS-HygR
FtetZ/CD81-/-::CD81-AEMV	293F/CD81-/- + Tet-on (BleoR)	TRE3G-CD81-AEMV; EFS-HygR
FtetZ/CD81-/-::CD81-YEVM	293F/CD81-/- + Tet-on (BleoR)	TRE3G-CD81-YEVM; EFS-HygR

Table S2. Evidence that high-level expression of CD63 or other YxxΦ motif-containing proteins inhibits endocytosis, increases the plasma membrane staining of YxxΦ motif-containing proteins, or drives their vesicular secretion

Expressed Protein	Effect
CD63	Inhibited CD63 endocytosis
CD63, CD63-YQRF, CD63-YQTI	Increased the cell surface staining of CD63 proteins
CD63, CD63-YQRF, CD63-YQTI	Increased the cell surface staining of Lamp1
CD63, CD63-YQRF, CD63-YQTI	Increased the cell surface staining of Lamp2
CD63, CD63-YQRF, CD63-YQTI	Increased the vesicular secretion of CD63 proteins
CD63, CD63-YQRF, CD63-YQTI	Increased the vesicular secretion of Lamp1
CD63, CD63-YQRF, CD63-YQTI	Increased the vesicular secretion of Lamp2
CD63, CD63-YQRF, CD63-YQTI	Increased the vesicular secretion of AP-2 subunit mu2
CD9-YEVM, CD9-YQRF, CD9-YQTI	Inhibited the destruction of CD9-YxxΦ proteins
CD9-YEVM, CD9-YQRF, CD9-YQTI	Increased the vesicular secretion of CD9-YxxΦ proteins
CD9-YEVM, CD9-YQRF, CD9-YQTI	Increased the cell surface staining of CD9-YxxΦ proteins
CD81-YEVM, CD81-YQRF, CD81-YQTI	Inhibited the destruction of CD81-YxxΦ proteins
CD81-YEVM, CD81-YQRF, CD81-YQTI	Increased the vesicular secretion of CD81-YxxΦ proteins
CD81-YEVM, CD81-YQRF, CD81-YQTI	Increased the cell surface staining of C81-YxxΦ proteins
CD81-YEVM, CD81-YQRF, CD81-YQTI	Increased the vesicular secretion of Lamp2