Supplemental Data

Adiponectin/T-cadherin system enhances exosome biogenesis and decreases cellular ceramides by exosomal release

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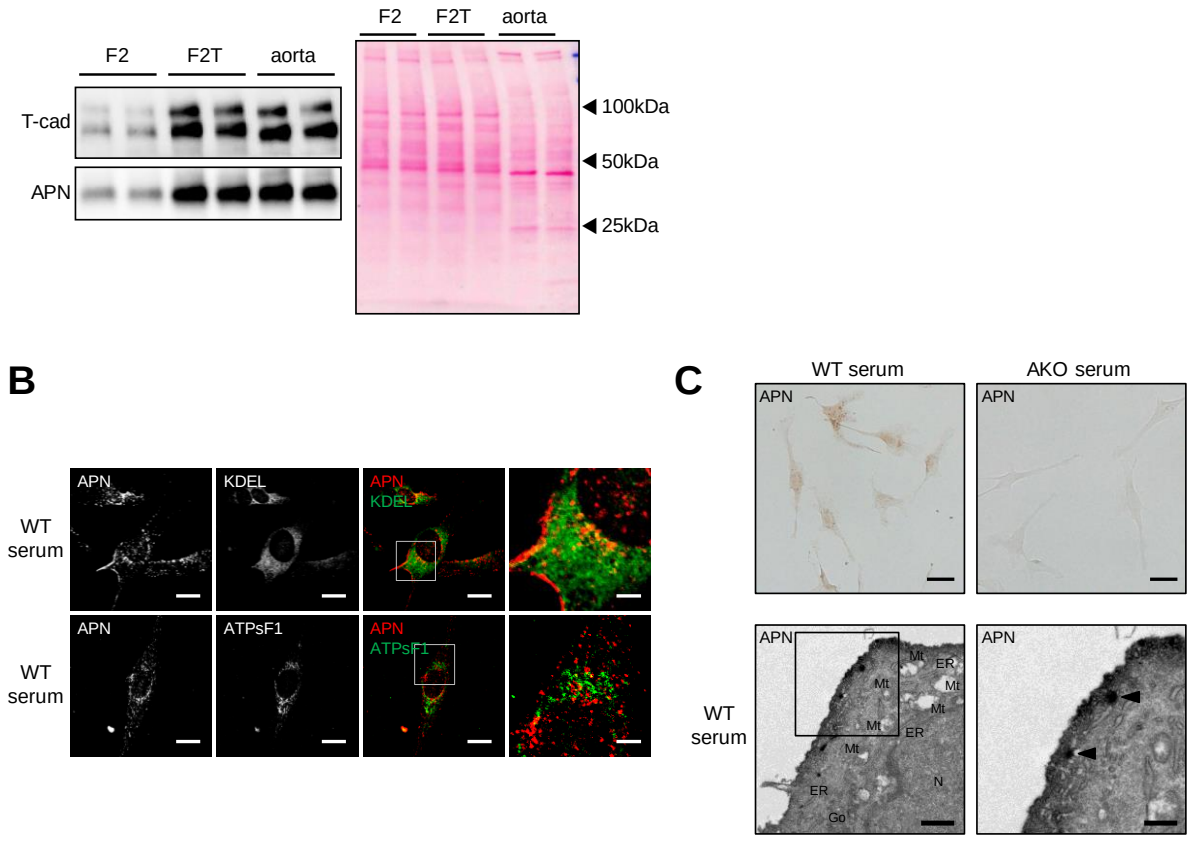
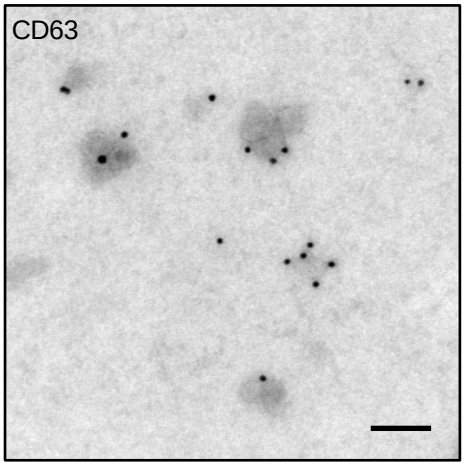
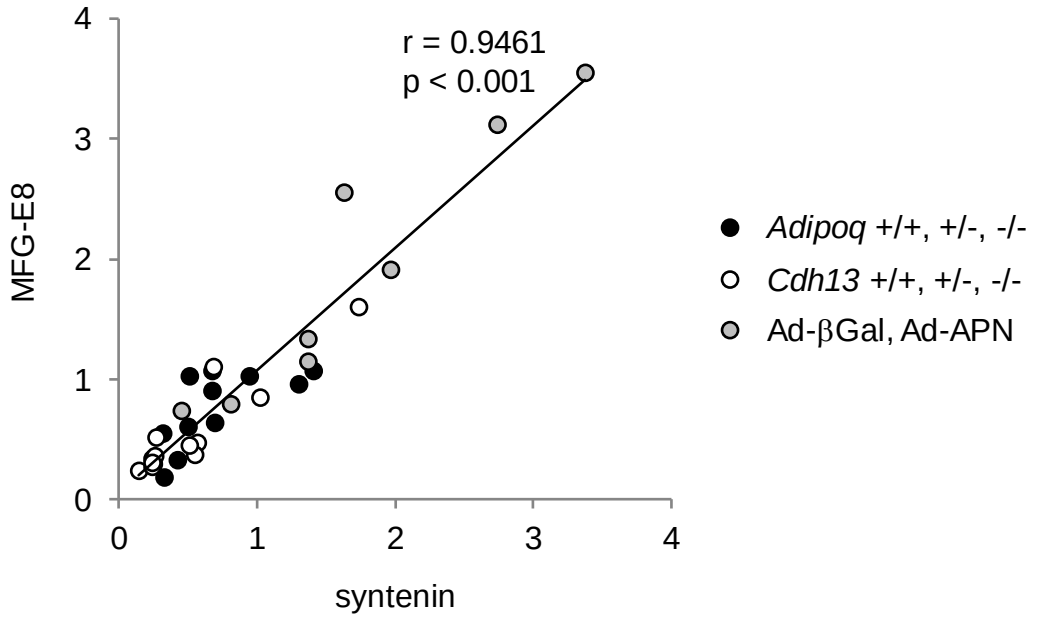
Supplemental Figures and Figure legends



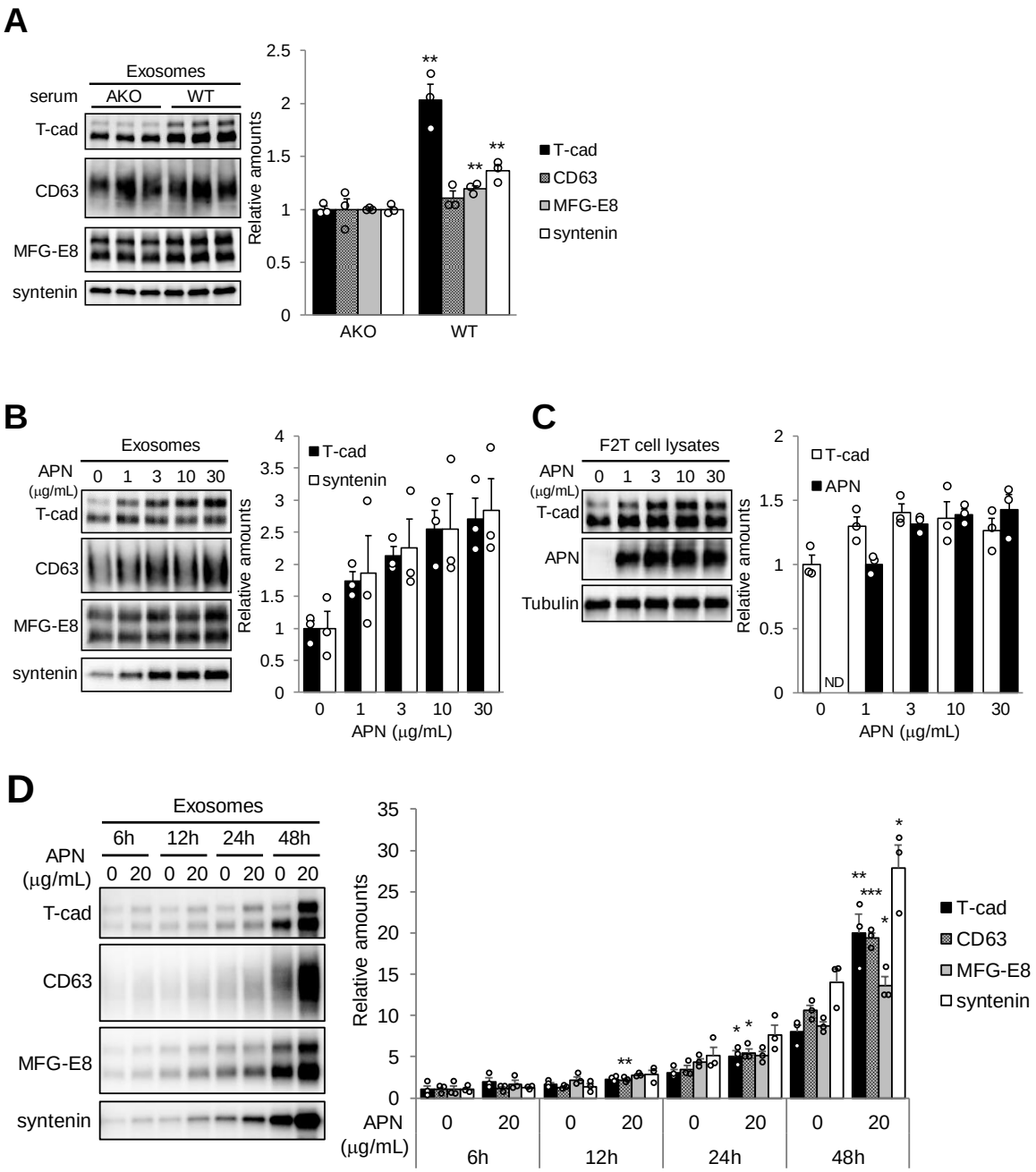
Supplemental Figure 1. Intraluminal accumulation of adiponectin in vesicles of multivesicular bodies (MVBs) in T-cadherin-expressing endothelial cells. (**A**) Comparison of T-cadherin protein and accumulated adiponectin in parental endothelial F2 cells, F2 cells stably expressing T-cadherin (F2T cells), and aorta tissue of WT mice. Cells were treated with medium containing 5% WT mouse serum. Equal protein amounts of whole cell lysates were loaded onto SDS-PAGE gels. Ponceau S staining as a loading control is shown in the right panel. *n* = 2. Representative results of 3 experiments with similar findings. (**B**) Confocal immunofluorescence micrographs of F2T cells. Cells cultured with WT mouse serum were stained with anti-adiponectin, anti-KDEL (endoplasmic reticulum), or anti-ATP synthase IF1 (ATPsF1, mitochondria) antibodies. Scale bar, 20 μm. Higher magnifications of the indicated areas are shown in the right panels. Scale bar, 5 μm. (**C**) Immunostaining using 3,3'-diaminobenzidine tetrahydrochloride (top panels) and immunoelectron micrographs using the pre-embedding immunoperoxidase technique (bottom panels) for adiponectin in F2T cells cultured with WT mouse serum or adiponectin knockout (AKO) mouse serum. Bottom right panel: higher magnification of the region outlined in the bottom left panel. Arrowhead, endocytic vesicles; N, nucleus; Mt, mitochondria; ER, endoplasmic reticulum; Go, Golgi body. Scale bar, 40 μm (top panels), 1.0 μm (bottom left panel), and 0.5 μm (bottom right panel).



Supplemental Figure 2. Presence of CD63 in exosomes from F2T cells. Immunoelectron micrograph of exosomes from F2T cells, labeled with anti-CD63 antibody and visualized by 10 nm-gold conjugated secondary antibody. Scale bar, 100 nm.

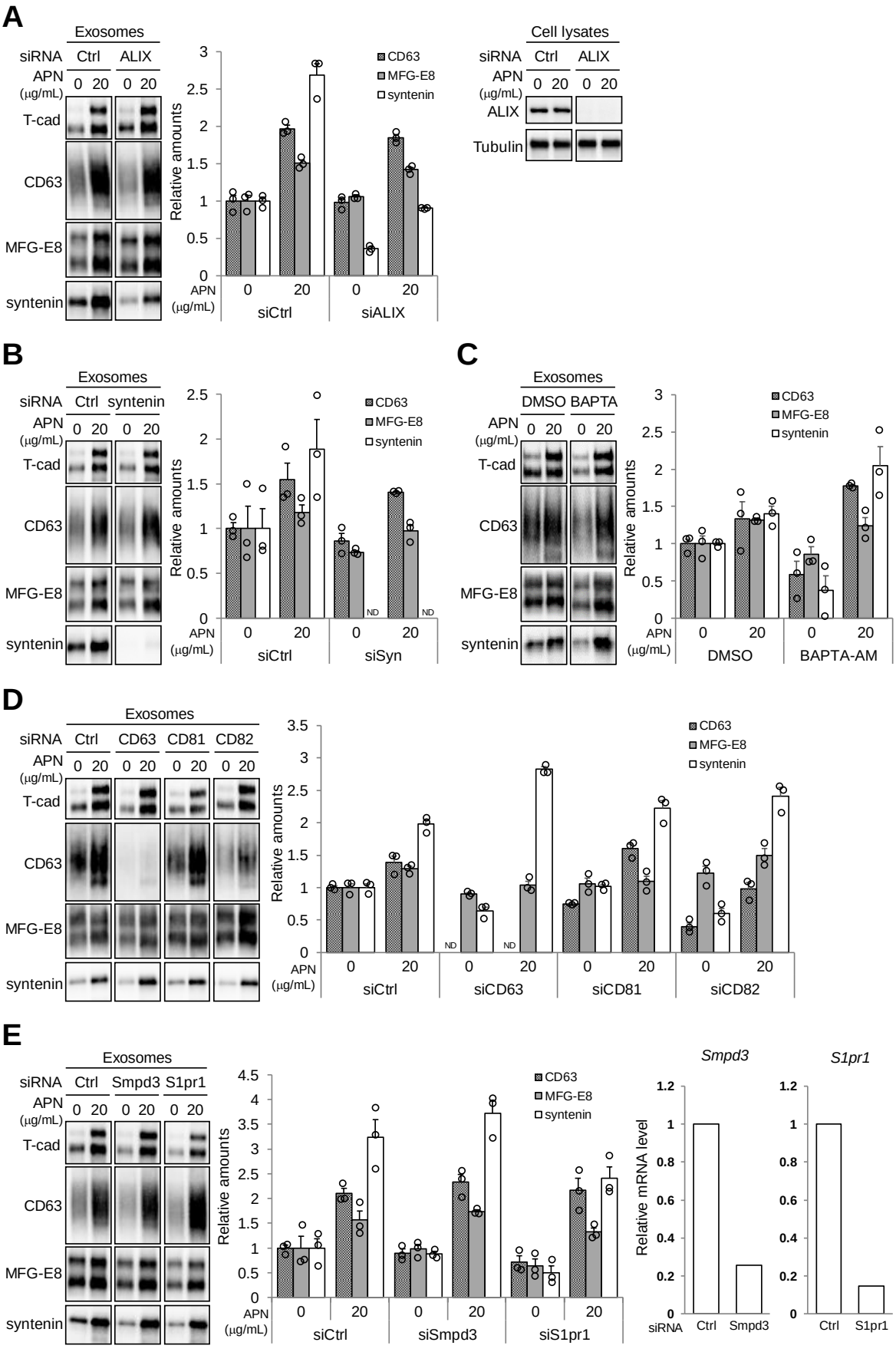


Supplemental Figure 3. Correlation between plasma exosome MFG-E8 and syntenin. Correlation between plasma exosome MFG-E8 and syntenin levels in *Adipoq* littermates (Figure 3A), *Cdh13* littermates (Figure 3B), and adiponectin overexpression experiment (Figure 3C). The mean values of WT controls in each experiment were set to 1.0. Statistical analysis by Pearson correlation test.



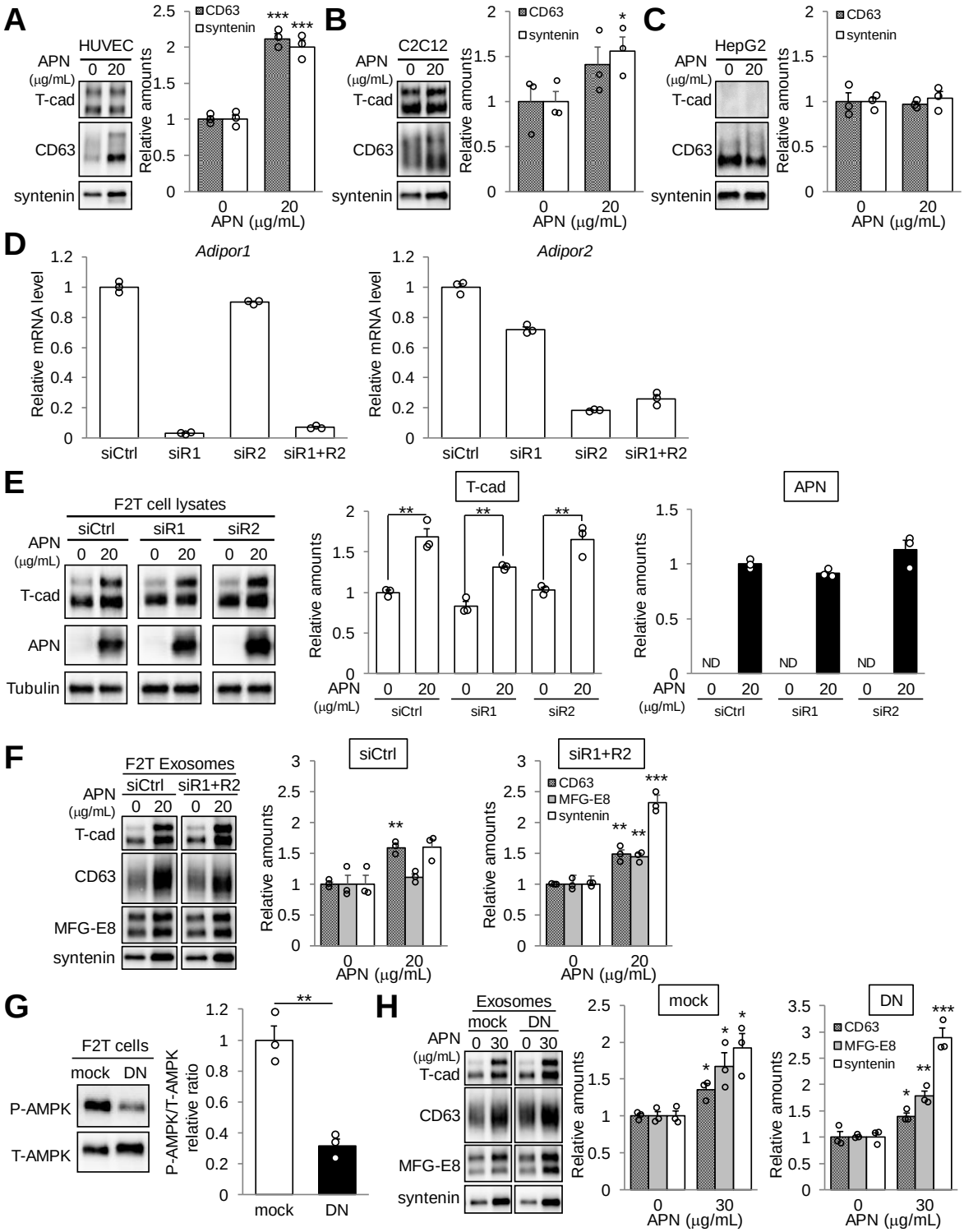
Supplemental Figure 4. Adiponectin increases exosome production from T-cadherin-expressing cells. (**A**) Western blots of exosome cargos. F2T cells were treated with serum from WT or AKO mice. Exosomes isolated by differential UC were analyzed by western blotting. $n = 3$. Representative results of 5 experiments with similar findings. $^{**}P < 0.01$ versus AKO serum (unpaired t-test). (**B**) Non-saturable increase in exosome yields by adiponectin. F2T cells were treated with the indicated adiponectin concentrations, and exosomes isolated by differential UC were analyzed by western blotting. The histogram (right) shows relative amounts of T-cad and syntenin as representatives, respectively. $n = 3$. Representative results of 2 experiments with similar findings. (**C**) Dose-dependent accumulation of adiponectin in F2T cells. The histogram (right) shows relative amounts of adiponectin and T-cadherin proteins. $n = 3$. Representative results of 3 experiments with similar findings. ND, not detected. (**D**) The time-course analysis of adiponectin effect on exosome production. F2T cells were treated with 0 or 20 μg/mL of adiponectin for the

indicated hours (h), and exosomes isolated by differential UC were analyzed by western
65 blotting. *n*=3. Representative results of 2 experiments with similar findings. **P* < 0.05, ***P* < 0.01 versus APN 0 μg/mL in each incubation time (unpaired t-test). Data are mean ± SEM.



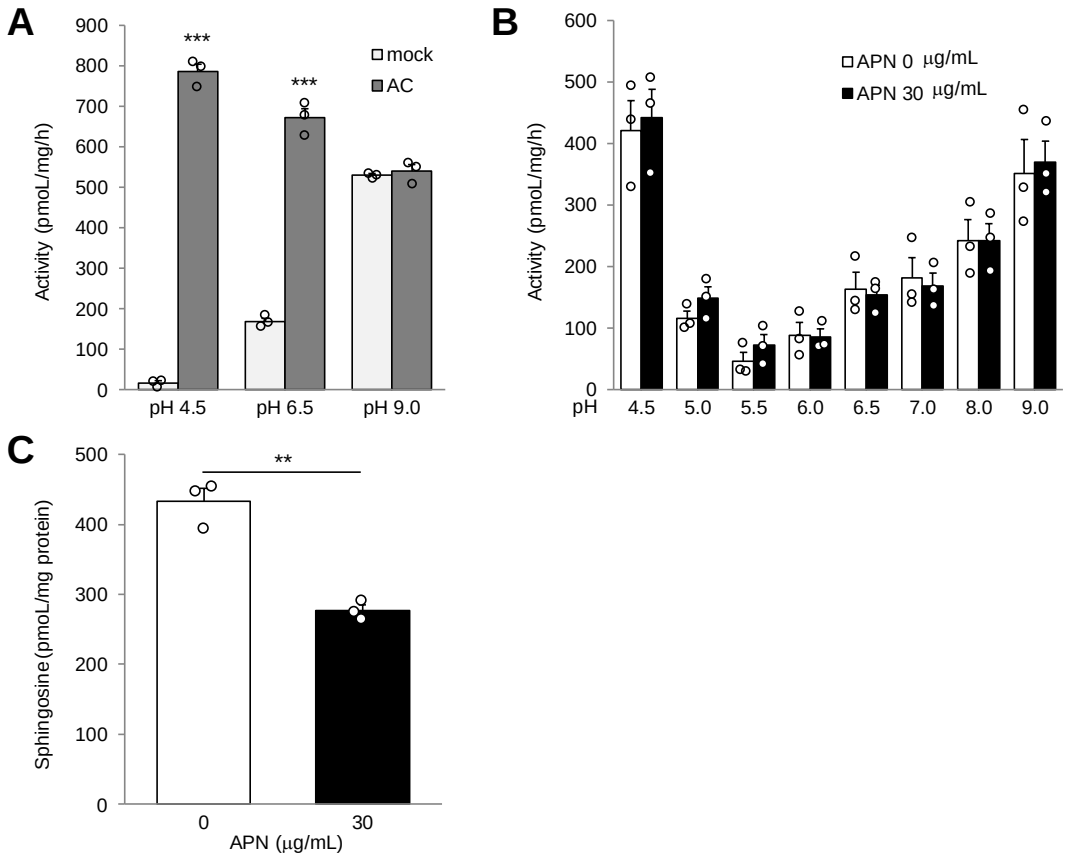
Supplemental Figure 5. Association between adiponectin-mediated exosome production

and known machineries for exosome biogenesis. (**A**-**E**) F2T cells with treated as below were incubated with 0 or 20 μg/mL of adiponectin, and exosome cargo levels were analyzed in exosome pellets by western blotting. *n* = 3 for each experiment. (**A**) siRNA knockdown of ALIX. (**B**) siRNA knockdown of syntenin. (**C**) treatment with BAPTA-AM (30μM). (**D**) siRNA knockdown of CD63, CD81, or CD82. (**E**) siRNA knockdown of neutral sphingomyelinase (Smpd3), or sphingosine 1-phosphate receptor 1 (S1pr1). Right panel: Relative mRNA levels of Smpd3 and S1pr1 in F2T cells transfected with respective siRNAs. Data are mean ± SEM.



Supplemental Figure 6. The effect of adiponectin on exosome production is dependent on T-cadherin, but not AdipoRs. (**A-C**) Effect of adiponectin on exosome production in different cells, expressing T-cadherin or not. HUVEC (**A**), C2C12 differentiated myotubes (**B**), and HepG2 hepatocytes (**C**). Each cell type was treated with 0 or 20 μg/mL of adiponectin. *n* = 3. Representative results of 2 experiments with similar findings, respectively. $*P < 0.05$, $***P < 0.001$ versus APN 0 μg/mL (unpaired t-test). (**D**) Relative mRNA levels of AdipoR1 and AdipoR2 in F2T cells transfected with AdipoR1 siRNA (siR1) or AdipoR2 siRNA (siR2). *n*=3. (**E**) AdipoRs-knockdown had little effect on adiponectin accumulation

and cellular T-cadherin protein. Western blots of F2T cell lysates. F2T cells transfected with control siRNA (siCtrl), AdipoR1 siRNA, or AdipoR2 siRNA were treated with 0 or 20 μg/mL of adiponectin. The histograms (right) show relative amounts of adiponectin (APN) and T-cadherin (T-cad) proteins. *n*=3. Representative results of 2 experiments with similar findings. ND, not detected. ***P* < 0.01 (unpaired t-test). (**F**) Effect of AdipoRs on exosome stimulation by adiponectin. F2T cells transfected with control siRNA (siCtrl), or AdipoR1 siRNA and AdipoR2 siRNA (siR1+siR2) were treated with 0 or 20 μg/mL of adiponectin, and exosome cargo levels were analyzed in exosome pellets by western blotting. *n*=3. Representative results of 2 experiments with similar findings. ***P* < 0.01, ****P* < 0.001 versus APN 0 μg/mL (unpaired t-test). (**G**) AMPK phosphorylation. AMPK phosphorylation was significantly suppressed in F2T cells stably overexpressing dominant-negative AMPK (DN). Representative results of 2 experiments with similar findings. ***P* < 0.01 (unpaired t-test). (**H**) AMPK activity and exosome stimulation by adiponectin. F2T cells stably overexpressing DN were treated with 0 or 30 μg/mL of adiponectin, and exosome cargo levels were analyzed in exosome pellets by western blotting. *n*=3. Representative results of 2 experiments with similar findings. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 versus APN 0 μg/mL (unpaired t-test). Data are mean ± SEM.



Supplemental Figure 7. Ceramidase activities and sphingosine level. (**A**) Lysates from HEK293 cells transfected with acid ceramidase (AC) expression plasmids showed increased ceramidase activity in acidic pH. Hydrolysis of C12-NBD-ceramide was evaluated by reverse-phase HPLC equipped with fluorescence detector. n=3. ***P < 0.001 versus mock (unpaired t-test). (**B**) Ceramidase activity, determined in lysates from F2T cells treated with 0 or 30 μg/mL of adiponectin, under ranges of pH condition. n=3. Representative results of 2 experiments with similar findings. There was no statistically significant difference between APN 0 μg/mL and 30 μg/mL in each pH condition (unpaired t-test). (**C**) Effect of adiponectin on cellular sphingosine level, determined by liquid chromatography and tandem mass spectrometry, in F2T cells. n=3. **P < 0.01 (unpaired t-test). Data are mean ± SEM.