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Supplemental Information

AdipoRon Attenuates Wnt Signaling by Reducing Cholesterol-Dependent Plasma Membrane Rigidity

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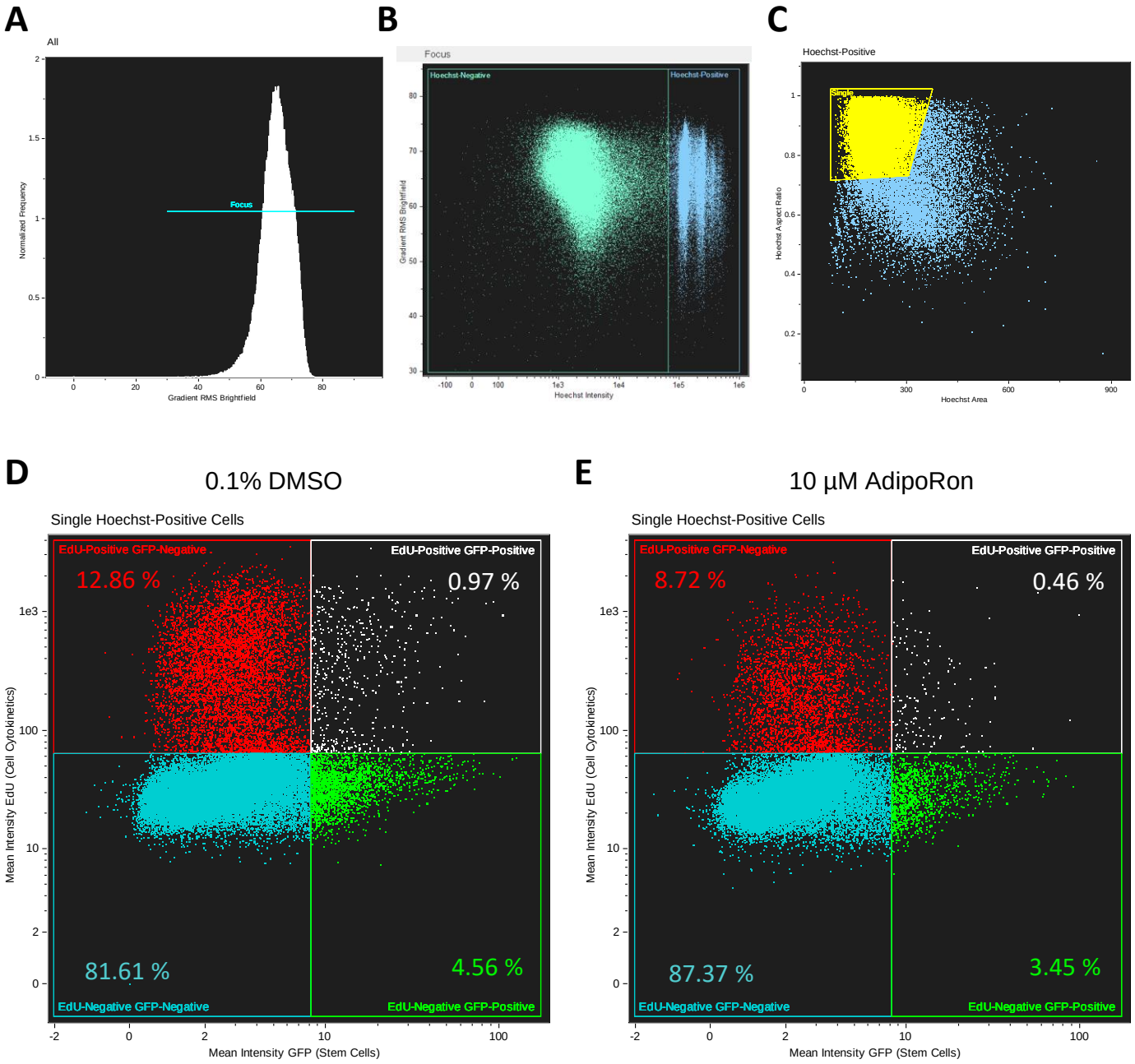

Figure S1. AdipoRon reduces stem cell proliferation. Colonic organoids derived from Lgr5-GFP mice were treated with 0.1% DMSO or 10 µM AdipoRon for 24 h prior to incubation with EdU for 1 h to label proliferating cells. Organoids were extracted from Matrigel, single cells were generated, fixed and labeled with Alexa Fluor 647 and Hoechst. Labeled cells were then analyzed on an Amnis FlowSight system. Gating strategies to identify (*A*) focused, (*B*) Hoechst positive, and (*C*) single Hoechst positive cells. Two dimensional dot plot of EdU-Alexa Fluor 647 (proliferation) and Lgr5-GFP (stem cells) from single Hoechst positive cells treated with (*D*) 0.1% DMSO or (*E*) 10 µM AdipoRon for 24 h. The percentage of cells that fall into each quadrant is displayed.

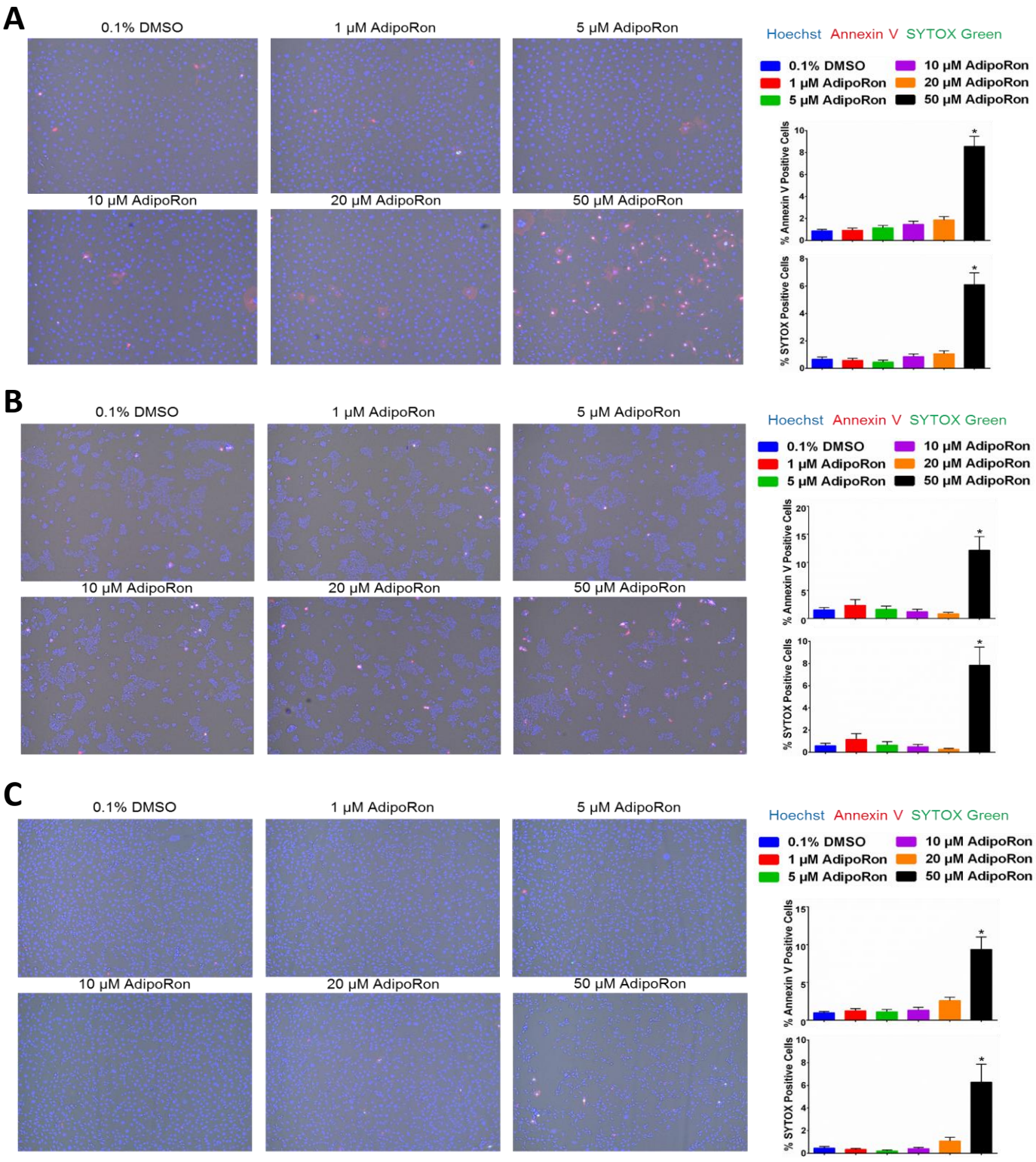

Figure S2. Assessment of apoptosis in AdipoRon treated YAMC, DKOB8 and Leading Light 3T3 cells. (*A*) YAMC, (*B*) DKOB8, or (*C*) Leading Light 3T3 cells were treated with the indicated dose of AdipoRon for 24 h, co-stained with Annexin V-Alexa Fluor 647 (PS/apoptosis), SYTOX Green (dead cells) and Hoechst (DNA/nucleus) before imaging on a Keyence wide-field microscope with 10X objective. n = 18 fields of view from two independent experiments. Statistical significance (*p < 0.001) between DMSO and indicated treatments was determined by one-way ANOVA with Dunnett's multiple comparisons test.

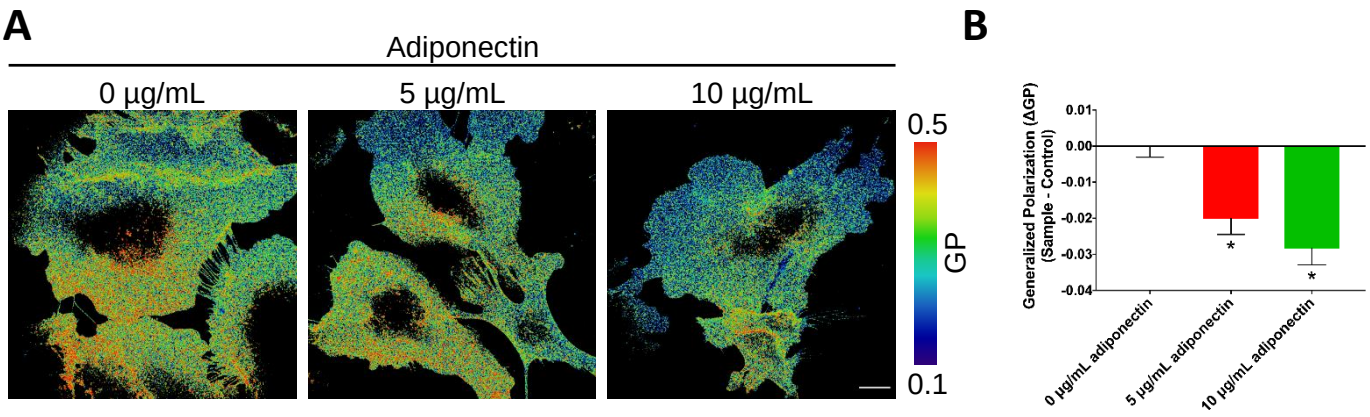

Figure S3. Adiponectin reduces membrane order in YAMC cells as determined by confocal imaging of Di-4-ANEPPDHQ. YAMC cells grown in 8-well dishes were treated with 0, 5 or 10 µg/mL adiponectin for 24 h. YAMC cells were imaged immediately after the addition of Di4. (*A*) Representative GP images of cells following treatment. (*B*) Quantification of cell membrane rigidity ΔGP (Sample - Control) values. Data are mean ± s. e. from 45 fields of view from two independent experiments. Statistical significance (*$p < 0.001$) between control and indicated treatments was determined by one-way ANOVA with Dunnett's multiple comparisons test. Scale bar = 20 µm.

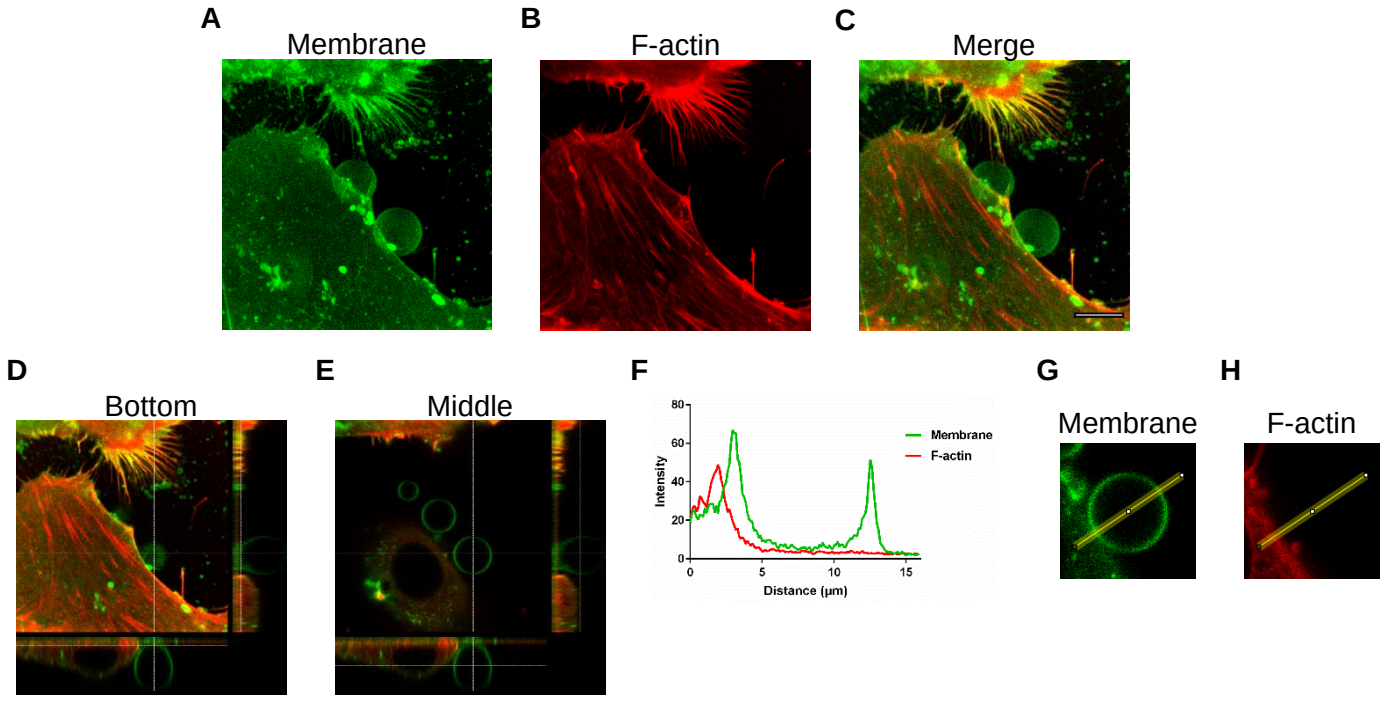

Figure S4. Isolated giant plasma membrane vesicles are devoid of filamentous actin. GPMVs were generated from YAMC cells as described in the Materials and Methods. Maximum intensity projection of (*A*) membrane visualized by green plasma membrane stain, (*B*) F-actin visualized by SiR-actin, and (*C*) merged. Orthogonal sections from the (*D*) bottom and (*E*) middle of representative GPMV. (*F*) Line scans of normalized intensity profile of GPMVs derived from (*G*) plasma membrane or (*H*) F-actin. Scale bar = 10 µm.

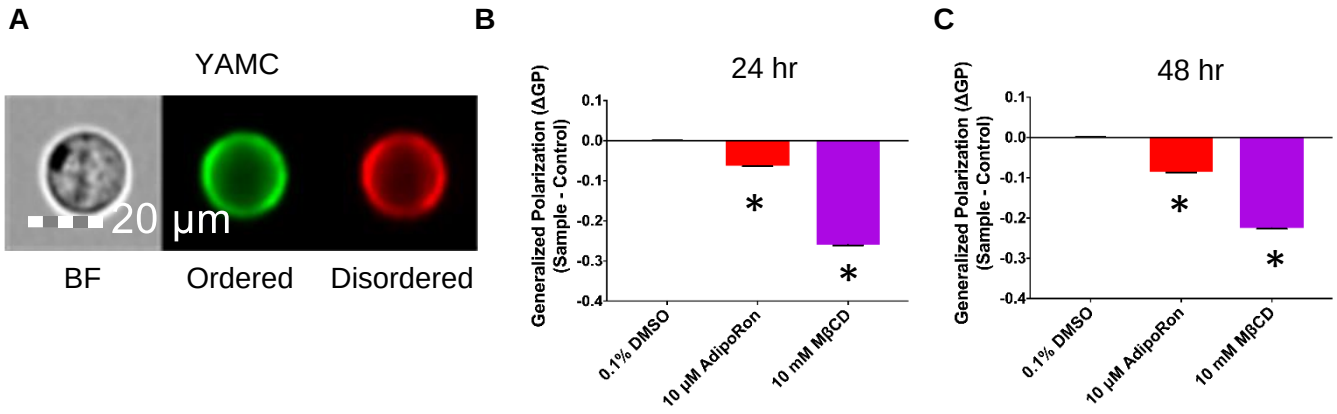

Figure S5. AdipoRon influences membrane order as determined by live cell imaging flow cytometry. YAMC cells were treated with 0.1% DMSO, 10 µM AdipoRon for 24 h or 48 h, or 10 mM MβCD for 30 min, then stained with 5 µM Di-4-ANEPPDHQ. (*A*) Representative FlowSight image of YAMC cells stained with Di-4-ANEPPDHQ: Brightfield (BF), Ordered, and Disordered. (*B*) Quantification of cell membrane rigidity ΔGP (Sample - Control) values for cells treated for 24 h (n = 3,149-3,325 cells per treatment). (*C*) ΔGP values of cells treated for 48 h (n = 2,845-2,952 cells per treatment). Unless otherwise indicated, data are mean ± s.e., statistical significance (*$p < 0.001$) between control and indicated treatments was determined by one-way ANOVA with Dunnett's multiple comparisons test.

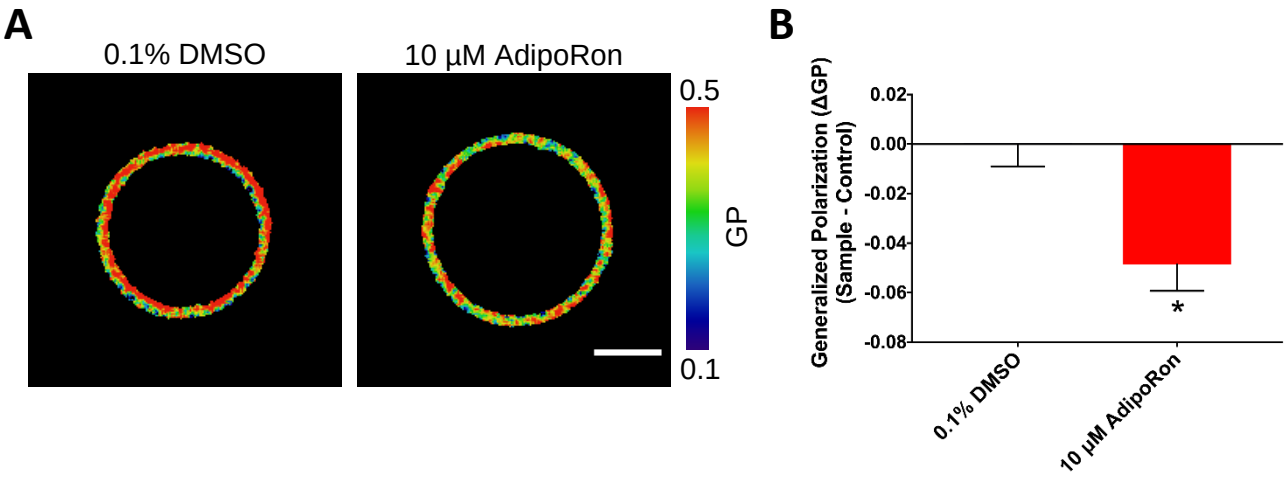

Figure S6. AdipoRon reduces plasma membrane rigidity as determined by C-laurdan labeled GPMVs. GPMVs were generated from YAMC cells grown in T-25 flask and treated with 0.1% DMSO or 10 µM AdipoRon for 24 h. Isolated GPMVs were labeled with 5 µM C-laurdan for 30 min and imaged on a Leica confocal microscope with a 63X objective. (A) Representative GP images of GPMVs from indicated treatments. (*B*) Quantification of cell membrane rigidity ΔGP (Sample - Control) values. Data are mean ± s. e. from at least 39 GPMVs from two independent experiments. Statistical significance between treatments (*$p < 0.001$) was determined using an unpaired *t*-test. Scale bar = 5 µm.

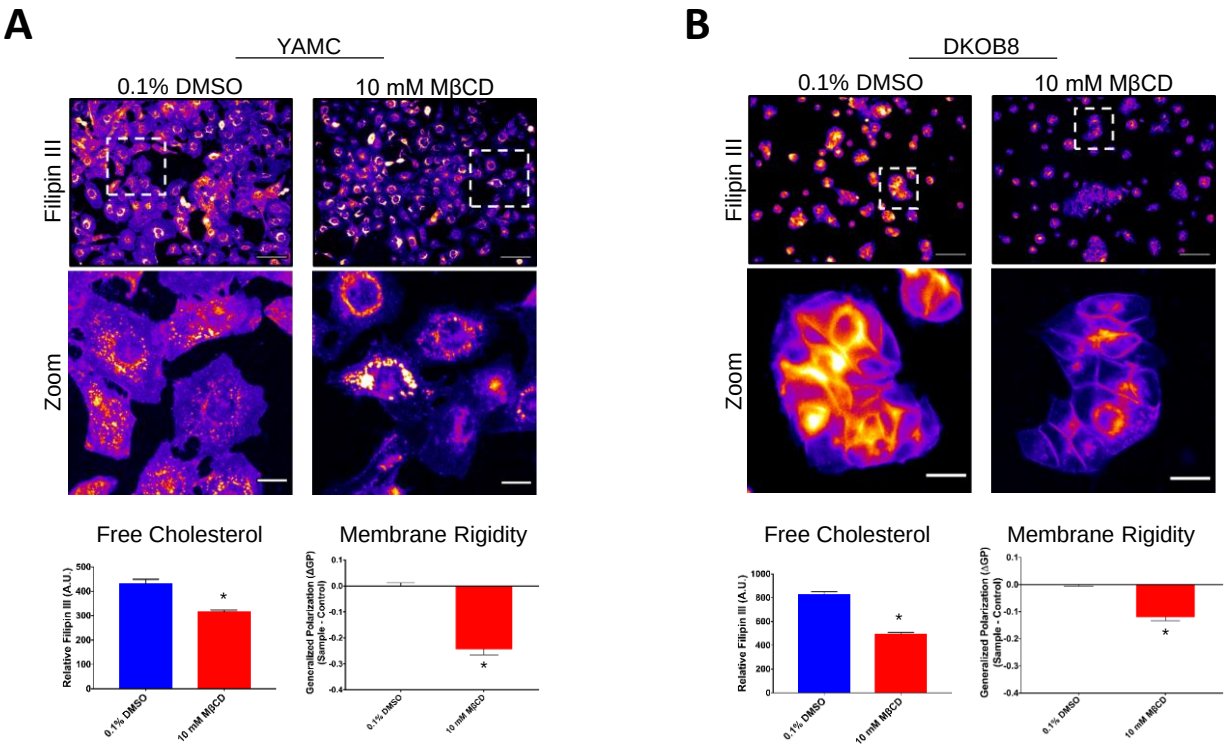

Figure S7. MβCD reduces membrane order and free cholesterol in YAMC and DKOB8 cells. (*A*) YAMC and (*B*) DKOB8 cells were incubated with complete media containing 10 mM MβCD for 30 min. To quantify free cholesterol, cells were fixed in 4% PFA for 15 min, followed by labeling of cholesterol with 50 µg/mL Filipin III for 45 min at room temperature in the dark. To quantify membrane order, cells were stained with 5 µM Di-4-ANEPPDHQ and imaged immediately. Unless otherwise indicated, data are mean ± s.e., statistical significance between treatments (**p* < 0.001) was determined using an unpaired *t*-test. Scale bar, (A and B) 100 µm and 20 µm (Zoom).

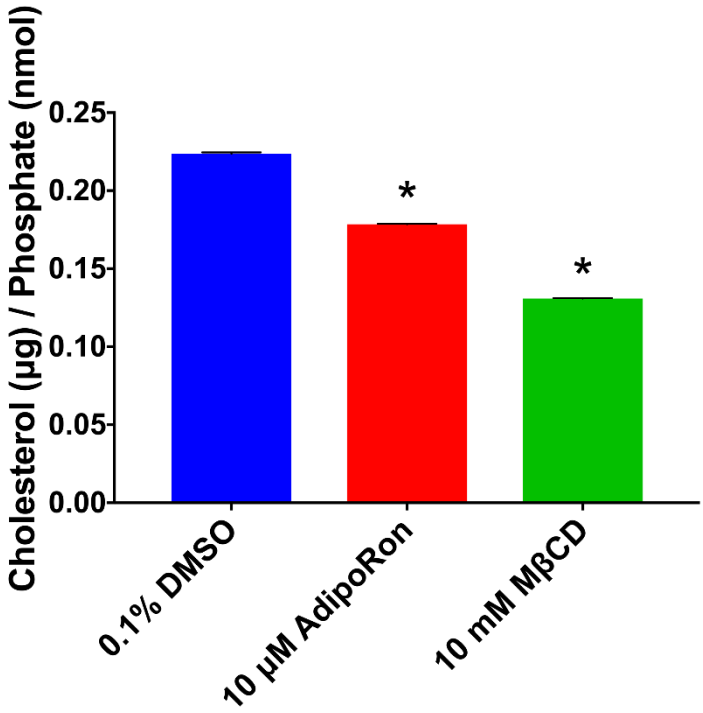

Figure S8. Amplex Red based quantification of free cholesterol content of GPMVs derived from DKOB8 cells. GPMVs were generated from DKOB8 cells treated with 0.1% DMSO, 10 µM AdipoRon for 24 h or 10 mM MβCD for 30 min. Lipids were extracted from GPMVs and used to quantify free cholesterol with an Amplex Red Cholesterol Assay Kit or inorganic phosphate with a Phosphate Colorimetric Assay Kit. Unless otherwise indicated, data are mean ± s.e., statistical significance (*$p < 0.001$) between DMSO and indicated treatments was determined by one-way ANOVA with Dunnett's multiple comparisons test.

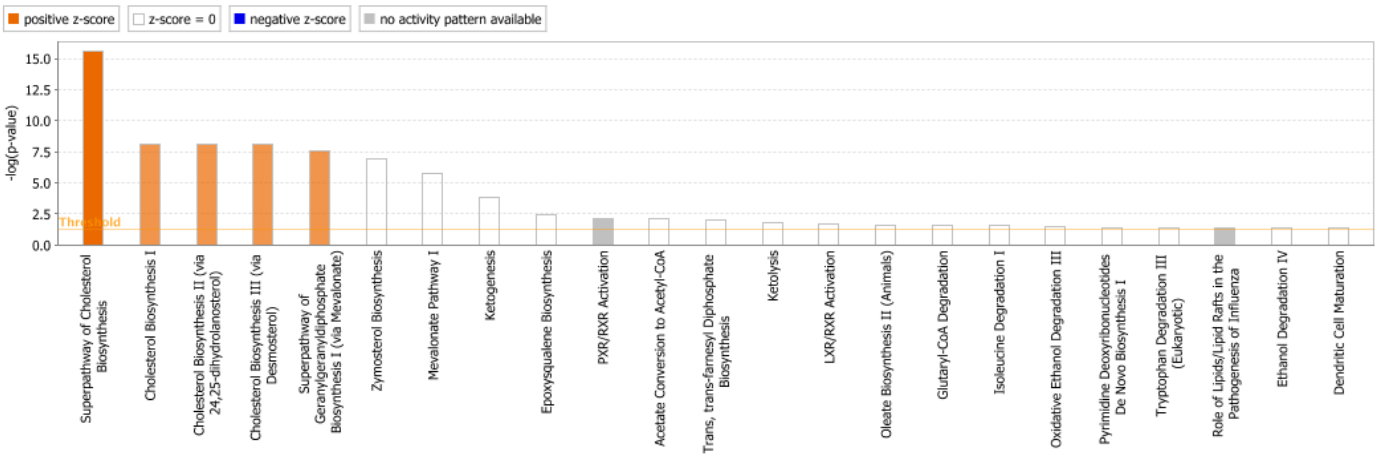

Figure S9. AdipoRon upregulates the cholesterol biosynthesis pathway at the RNA level. Ingenuity Pathway Analysis (IPA) of differentially expressed genes from DKOB8 cells treated with 0.1% DMSO or 20 µM AdipoRon for 24 h, analyzed using RNA-seq.