Supplemental Material

Supplemental Methods

Constructs and Cell Culture – Human IP or TPα, N-terminally tagged with a hemagglutinin tag (3xHA), were from Missouri S&T cDNA Resource Center (Rolla, MO). IP and TPα fused at their C-termini to renilla luciferase (rLUC) or yellow fluorescent protein (YFP) were described previously[1]. COS-7, Raw 264.7 (American Type Tissue Culture Collection, Rockville, MD) and human aortic smooth muscle cells (Lonza, Basel, Switzerland) were maintained as described[2]. Transfections were with Fugene 6 (Roche, Indianapolis, IN) as described[2]. Where indicated, cells were cholesterol depleted (2-hydroxypropyl-β-cyclodextrin; 20mM, 1hr), or loaded (cholesterol-methyl-β-cyclodextrin complex; 80 μg/ml, 1hr). Peritoneal macrophages were isolated from female mice as previously described[3].

Subcellular fractionation – COS-7 cells were transfected with the IP or TPα tagged at the N-terminus with 3 copies of the hemagglutinin epitope tag (3xHA). Cells were harvested, 48hrs later, without the use of enzymes and homogenized in sodium carbonate (500 mM, pH 11) using a polytron tissue grinder (15s), followed by 3x20s sonication on ice. Homogenates were adjusted to 35% iodixanol using Optiprep™, a 60% iodixanol solution (Sigma, St Louis, MO), placed in the bottom of Ultra-Clear centrifuge tubes (Beckman, Indianapolis, IN) and overlayered with 30% Optiprep and 0% (buffer alone). Samples were centrifuged at 200,000xg for 2h using an SW40Ti rotor (Beckman, Indianapolis, IN). 11x1ml fractions will be taken from the top of the gradient and subjected to NUPAGE electrophoretic separation.

Bioluminescence Resonance Energy Transfer – Receptor dimerization was examined by measuring bioluminescence resonance energy transfer (BRET) from a donor (rLUCfused) receptor to an acceptor (YFP-fused) receptor following addition of an rLUC substrate (coelenterazine H; 5μM; Molecular Probes, Grand Island, NY). Cells were transfected with a fixed amount of donor together with increasing amounts of acceptor. BRET measurements were performed, as we described previously[1], 48hrs later. Cells were harvested without the use of enzymes and redistributed into 96-well plates (black, clear bottom, 100,000 cells/well). Donor and acceptor emissions were gathered sequentially from each well, at 485nm and 555nm, respectively, using a luminescence multi-plate reader (VICTOR3, Perkin Elmer, Waltham, MA) at 37°C. BRET is calculated as the ratio of Em555 over Em485 nm corrected for cells expressing donor alone. Each experimental condition included 4-6 replicates.

Membrane labeling for localization: membrane localization of IP and TPα was examined using a modified BRET assay. COS-7 cells were transfected with a fixed amount of rLUC-receptor. Cells were fluorescently labeled with increasing concentrations of a redorange fluorescent, lipophilic carbocyanine DiIC16 (Molecular Probes, Grand Island, NY) that labels the L_o membrane phase[4], at 37°C for 2 min. Cells were washed with PBS, treated with colenterazine H as above and energy transfer to DiIC16 measured at 530nm.

Membrane cholesterol measurement: COS-7 cells were harvested without the use of enzymes and homogenized in 10 mM Tris buffer using a polytron tissue grinder (15s), followed by 3x20s sonication on ice. After discarding the post-nuclear fraction, the lysate was centrifuged at 50.000 rpm at 4°C for 1h using an Optima TLX ultracentrifuge (Beckman). Membrane cholesterol was measured by Cholesterol E enzymatic colorimetric assay (Wako Diagnostics).

cAMP Assay: cAMP production was measured using the LANCE cAMP assay (PerkinElmer, Waltham, MA) according to manufacturer instructions. Briefly, cells dispensed in 384-well white optiplate (12,000 cells/well) were treated with vehicle (control), the PGI_2 analog cicaprost, or TxA_2 analog U46619, for 1h at room temperature in the presence of 3-isobutyl-1-methylxanthine (IBMX) to inhibit cAMP degradation by phodphodiesterases. The LANCE signal was quantified by measuring time resolved fluorescence resonance energy transfer (TR-FRET) between cAMP-biotin-streptavidin associated Europium and Alexa Fluor 647 conjugated to an anti-cAMP antibody measured at 665 nm using an Envision 2103 Multilabel Reader (PerkinElmer, Waltham, MA).

Inositol Phosphate Measurement – Cellular inositol phosphate (InsP) generation was quantified by IP-One Tb assay (CisBio, Bedford, MA) according to manufacturer instructions. Cells dispensed in 384-well white optiplate (12,000 cells/well) were treated with vehicle (control) or TxA_2 analog U46619, for 1h at 37°C. The signal was quantified by measuring TR-FRET (665nm, as above) between d2 labeled InsP1 and Lumi4-terbium cryptate conjugated anti-InsP1 antibody.

Surface expression of receptors – COS-7 cells transfected with 3xHA-IP or 3xHA-TPα were labeled with Alexa Fluor 488-anti-HA antibody (Molecular Probes, Grand Island, NY). Native IP was labeled with rabbit anti-IP antibody (Millipore) and with Alexa Fluor 488-goat-anti-rabbit (Molecular Probes). Surface fluorescence was measured by FACSCalibur flow cytometer (BD Biosciences, Billerica, MA).

Animal Experiments – Peritoneal macrophages were isolated from female mice between 6 and 12 months of age as previously described[3]. Briefly, 10ml ice-cold PBS were injected to the mouse peritoneal cavity, harvested few minutes later, and centrifuged (1000g, 4°C, 10min). Cells were resuspended and incubated in Dulbecco's Modified Eagle's Medium overnight at 37°C to allow cells to adhere.

Statistical Analysis: Data were analyzed using GraphPad Prism. Comparisons were made using a one-sample t-test or by ANOVA with suitable post-hoc multiple comparison testing.

Supplemental Figures

Fig.I

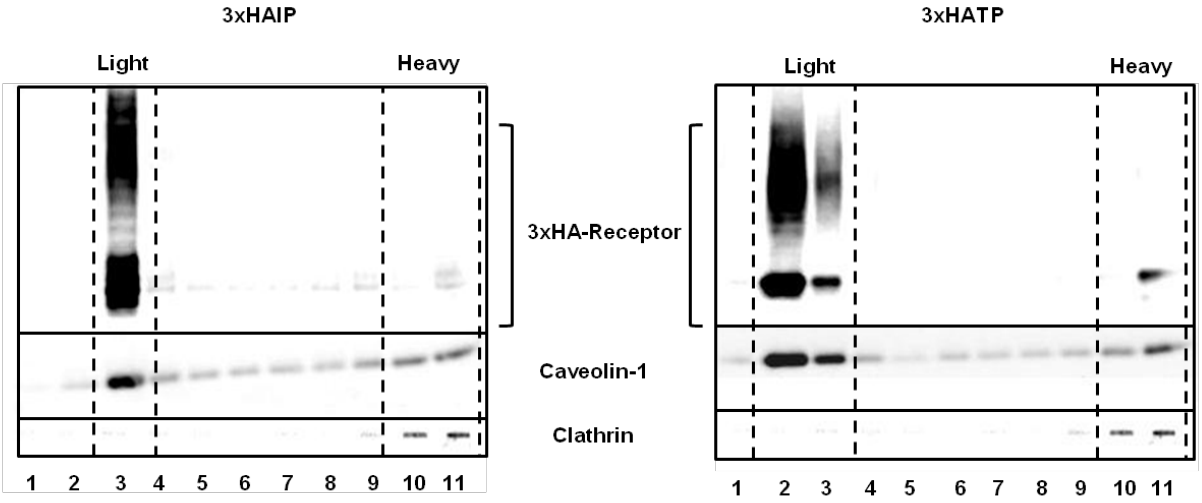

Fig.I. IP and TP membrane distribution by fractionation. COS-7 cells were transfected with 3xHA-tagged IP (left panel) or 3xHA-tagged TP (right panel). 48 hours later cells were homogenized in detergent-free conditions and subjected to iodixanol gradient fractionation. Fractions (1ml) collected from the top (1, light) to bottom (11, heavy) of the gradient were probed by immunoblotting for the HA-tagged receptor, caveolin-1 or clathrin, commonly used raft and non---raft markers. Blots are representative of at least n=3

Fig.**II**

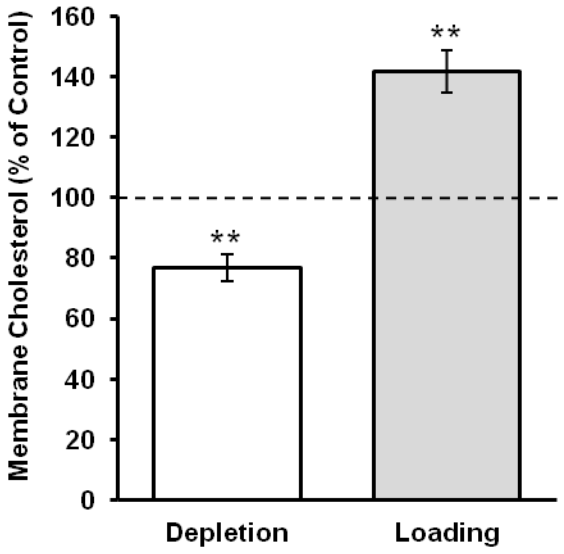

Fig.II. Membrane cholesterol. COS-7 cells were subjected to no treatment (control), cholesterol depletion (2-hydroxypropyl-β-cyclodextrin; 20mM, 1hr), or Cholesterol loading (cholesterol-methyl-β-cyclodextrin complex; 80 µg/ml, 1hr). Membrane cholesterol was measured and data expressed as % change of control $\pm$ sem from n=3. ** $p<0.005$

Fig.III

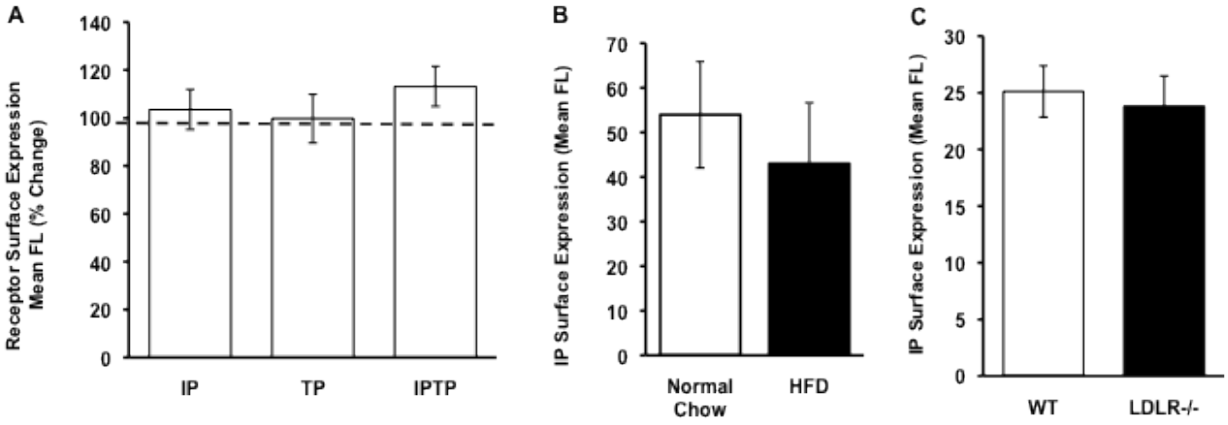

Fig.III. Receptor surface expression. (A) COS-7 cells were transfected with 3xHAIP alone, 3xHATP alone, or IP plus 3xHATP, then subjected to no treatment (control) or cholesterol loading (cholesterol-methyl-β-cyclodextrin complex; 80 μg/ml, 1hr). Cells were labelled with Alexa Fluor 488-anti-HA antibody. **(B, C)** Peritoneal macrophages were harvested from normo- or hyper-cholesterolemic mice. In (B) mice were fed normal chow or a high fat diet (42% fat) for 8 months; in (C) mice were wild type (WT) or low density lipoprotein receptor deficient (LDLR-/-; ~6 months of age). IP was labeled with anti-IP antibody and an Alexa Fluor 488-conjugated secondary antibody. Surface receptor expression measured in all cells by flow cytometry. Data are (A) % change of control mean fluorescence $\pm$ sem from n=4, or (B, C) mean fluorescence $\pm$ sem. In (B) n=3-5, in (C) n=6.

1. Ibrahim S, Tetruashvily M, Frey AJ, Wilson SJ, Stitham J, Hwa J, Smyth EM. Dominant negative actions of human prostacyclin receptor variant through dimerization: implications for cardiovascular disease. *Arterioscler Thromb Vasc Biol.* 2010;30:1802-1809.
2. Wilson SJ, Roche AM, Kostetskaia E, Smyth EM. Dimerization of the human receptors for prostacyclin and thromboxane facilitates thromboxane receptor-mediated cAMP generation. *J Biol Chem.* 2004;279:53036-53047.
3. Zhang X, Goncalves R, Mosser DM. The isolation and characterization of murine macrophages. *Curr Protoc Immunol.* 2008;Chapter 14:Unit 14 11.
4. Pontier SM, Percherancier Y, Galandrin S, Breit A, Gales C, Bouvier M. Cholesterol-dependent separation of the beta2-adrenergic receptor from its partners determines signaling efficacy: insight into nanoscale organization of signal transduction. *J Biol Chem.* 2008;283:24659-24672.