SUPPLEMENTAL MATERIAL

Supplemental methods:

Animals: Cardio-myocyte specific-GRK2 deletion was obtained ($GRK2^{f/f;Nkx2.5\text{-}cre}$ or GRK2 del) by breeding Nkx2.5-cre and GRK2 f/f mice[1] (from Dr. Gerald W. Dorn II) and myocytes were isolated for cardiac contractility studies.[2] Vascular aortic endothelial cells and cardiac lysates from TNFR1 (TNFα receptor 1) and TNFR2 (TNFα receptor 2) knockout mice[3] (from Dr. Paul E. DiCorleto) were used for the study. Animals were handled according to the approved protocols and animal welfare regulation of Institutional Review Board at Cleveland Clinic.

Cell culture and treatments: HEK 293 cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37 °C as described previously.[4] Cells were seeded at a density of ~1 x 10^6 cells/ 100 mm. Cells were serum-starved before agonist stimulation. HEK-FLAG-β_2AR cells were generously provided by Dr. Robert J. Lefkowitz, Duke University, Durham, NC. HEK 293 cells overexpressing HA-β_2AR and GFP-β-arrestin 2 were kindly provided by Dr. Marc G. Caron, Duke University, Durham, NC. HEK-FLAG-β_2AR cells or HEK 293 cells in serum free media were pre-treated with various cytokines TGFβ (10 ng/mL), TNFα (10 ng/mL), IL-6 (50 ng/mL) or IL-13 (50 ng/mL). ISO (10 μM) pre-treatment was carried out for 15 minutes. In all the studies we have used TNFα at 10 ng/ml and ISO at 10 μM unless mentioned otherwise. Cells were re-challenged with ISO (10 μM) for 5 minutes prior to cAMP measurement. Propranolol (100 μM) pre-treatment was done for 30 minutes prior to TNFα. "n" represents the number of independent experiments. Each experiment has been carried out in triplicate.

Endothelial cell culture: Isolated aortic endothelial cells were plated onto 0.1% gelatin-coated 60-mm culture dishes and grown in DMEM supplemented with 15% FBS, 1% penicillin-streptomycin, 90 mg/mL heparin, 60 mg/mL EC growth supplements, and 100 U/ml fungizone at 37°C in a 95% air/5% CO2 incubator as described before[3]. The subsequent passages were performed with 0.25% trypsin-EDTA, and cells were split in a 1:4 ratio. HL-1 myocytes culture: HL-1 cells were cultured[2] in Claycomb medium supplemented with 10% fetal bovine serum, 0.2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (all from Sigma). HL-1 cells were serum starved overnight prior to stimulation.

Immunoprecipitation and Immunoblotting: FLAG-β_2ARs, PI3Kγ or TNFα receptors R1 or R2 were immunoprecipitated by incubating 300 μg of plasma membranes with anti-FLAG antibody (Roche), anti-PI3Kγ antibody or anti-TNFα receptor antibodies respectively along with protein A/G agarose beads over night at 4 °C. The immunoprecipitates were washed with appropriate buffers before subjecting the beads to immunoblotting or lipid kinase assays. Hearts were homogenized or cells were harvested in lysis buffer containing 20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 1% NP-40, 1 mM PMSF, 20% glycerol, 10 mM NaF, 1 mM sodium orthovanadate, 2 μg/mL leupeptin and aprotinin. The lysates were cleared by centrifugation at 15,000X*g* for 15 min at 4 °C. The supernatants were used for immunoprecipitation. Washed immunoprecipitates were resolved by SDS-PAGE and blotted onto PVDF membranes (BIO-RAD). Blots were blocked with 5% milk and incubated with antibodies recognizing phospho-355/356-β_2AR (sc-22191-R, Santa Cruz Biotechnology)[2] at 1:1000 dilution, GRK 2, 3, 5 and 6 (Santa Cruz Biotechnology) at 1:1000 dilution, adenylyl cyclase V/VI at 1:1000 (Santa Cruz

Biotechnology), PI3Kγ at 1:100 (Santa Cruz Biotechnology) and FLAG (Roche)[2] at 1:1000 dilution. Appropriate secondary antibody (1:3000) was used and detection carried out using enhanced chemiluminescence. We tested the specificity of the phospho-355/356-β_2AR antibody using the blocking peptide (sc-22191 P, Santa Cruz Biotechnology) by pre-incubating the blots with excess of blocking peptide followed by anti-phospho-β2AR antibody at 4^{o}C in 5% BSA (Supplementary Fig. 3 D & E). We also tested the efficacy of the phospho-355/356-β_2AR (Santa Cruz Biotechnology) antibody by immunoblotting the cardiac lysates from wild type (Wt), β_1AR knockout (β_1AR KO) and β_2AR knockout (β_2AR KO) mice along with early endosomal lysate from β_2AR transgenic (β_2AR-Tg) mice (as positive control) with the respective antibodies (Supplementary Figure 3B). We also confirmed the βAR density on the plasma membranes isolated from the hearts of Wt, β_1AR KO or β_2AR KO and on endosomal fraction from β_2AR-Tg mice, (n=6) (Supplementary Figure 3C).

Glycosidase treatment: Cardiac lysates (30 μl) were incubated with glycoprotein denaturing buffer (NEB) for 10 minutes at 60^{0}C. Samples were then incubated with G7 buffer, NP-40 and 500 U PNGase F[4, 5] (NEB) for 2 hours at 37^{0}C, then blotted for phospho-355/356 β_2AR as described above.

Site Directed Mutagenesis: The phosphorylation deficient (S-A) mutants of serine 355/356 β_2AR were generated according to manufacturer's instructions (Quick change lightning site-directed mutagenesis kit, Agilent Technologies)[2]. The following primers were used to create respective mutations in pcDNA3.1 FLAG-β_2AR on serine 355/356. Forward primer (S355/356A): 5'-ctatgggaatggctacgccgccaacggcaacacaggg-3' and Reverse primer (S355/356A): 5'-ccctgtgttgccgttggcggcgtagccattcccatag-3'.

Purification of plasma membrane, early endosomes and late endosomes: Purification of plasma membrane, early endosomes and late endosomes was done as described previously.[2] Briefly, hearts or cells were homogenized in ice-cold lysis buffer containing 5 mM Tris-HCl pH 7.4, 5 mM EDTA, 1 mM PMSF, and 2 μg/mL leupeptin and aprotinin. Intact cell debris and nuclei were removed by centrifugation at 2,500Xg for 5 minutes and the supernatant was subjected to centrifugation at 37,000Xg for 20 minutes. The pelleted plasma membrane was resuspended in binding buffer (75 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 12.5 mM $MgCl_2$) for measuring βAR density and adenylyl cyclase activity, and in triton re-suspension buffer (20 mM Tris-HCl, pH 7.4, 300 mM NaCl, 1.0 % triton X-100, 1 mM PMSF, 20% glycerol, 10 mM NaF, 1 mM sodium orthovanadate, 2 μg/mL leupeptin and aprotinin) for lipid kinase assays. Early and late endosomal fractions were recovered following centrifugation of the supernatant cytosolic fraction for 1 hour at 300,000Xg and 200,000Xg respectively.

Adenylyl cyclase assay: Adenylyl cyclase assays were carried out by incubating 20 μg of membranes at 37 $^{\circ}$C for 15 min with isoproterenol or NaF in 50 μL of assay mixture containing 20 mM Tris-HCl, 0.8 mM $MgCl_2$, 2 mM EDTA, 0.12 mM ATP, 0.05 mM GTP, 0.1 mM cAMP, 2.7 mM phosphoenolpyruvate, 0.05 IU/mL myokinase, 0.01 IU/mL pyruvate kinase and ^{32}P-α-ATP and generated cAMP quantified.[2]

Lipid kinase assay: Lipid kinase assays were performed on immunoprecipitated proteins as previously described.[6] Briefly, pelleted beads were resuspended in 50 μl of reaction buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA and 100 μM sodium-orthovandate) and 10 μl of 100 mM $MgCl_2$, and 10 μl of 2 mg/ml PtdIns (20 μg) sonicated in TE buffer (10 mM Tris-HCl, pH 7.4 and 1 mM EDTA) was added to each

reaction. Reactions were started by adding 10 µl of 440 µM ATP and 10 µCi ^{32}P-γ-ATP, and were incubated at 25 °C for 10 min with continuous agitation, then stopped with 6N HCl. Lipids were extracted by adding 160 µl of chloroform:methanol (1:1). After centrifugation, 30 µl of the organic phase was spotted onto 200 µm silica-coated TLC plates (Selecto-flexible; Fischer Scientific, Pittsburgh, PA) that were pre-coated with 1% potassium oxalate, and was resolved using chromatography with 2N glacial acetic acid:1-propanol (1:1.87). The plates were dried, exposed, and lipid phosphorylation was assessed by autoradiography.

Confocal Microscopy: Confocal microscopy was performed as previously described.[2] Briefly, HEK-FLAG-β_2AR cells were plated onto poly L-lysine treated cover slips. Cells were serum starved for 3 hours, treated with endocytosis inhibitors 0.45M sucrose and 2% β-cyclodextrin for 1 hour and stimulated with 10 µM isoproterenol for 10 min or TNFα (10 ng/ml) for 1 hour. Cells were fixed in 4% paraformaldehyde for 30 min, permeabilized with 0.1% triton-X 100 and incubated in 1% BSA in PBS for 1 hour. After washing in PBS they were incubated with anti-phospho-355/356-β_2AR polyclonal antibody (1:500; Santa Cruz Biotechnology) in 1% BSA in PBS for 1hour. Washed cells were incubated with goat anti-rabbit IgG conjugated with AlexaFlour 488 (1:1000; Molecular probes, Eugene, OR) for 1hour. Samples were visualized using sequential line excitation at 488 for green, with appropriate emission filters.

Cardiac contractility: Peak contraction was measured as the percentage of peak cell shortening. Myocytes were pre-treated with ISO for 30 min or TNFα for 45 minutes and stimulated with ISO to assess myocyte contractility. Each data point represents myocytes isolated from 5 different animals from each of the genotypes with at least ~30 cells

averaged/treatment. Isolated myocytes were maintained in high calcium containing buffer and contractility of the myocytes was assessed using IonOptix System.

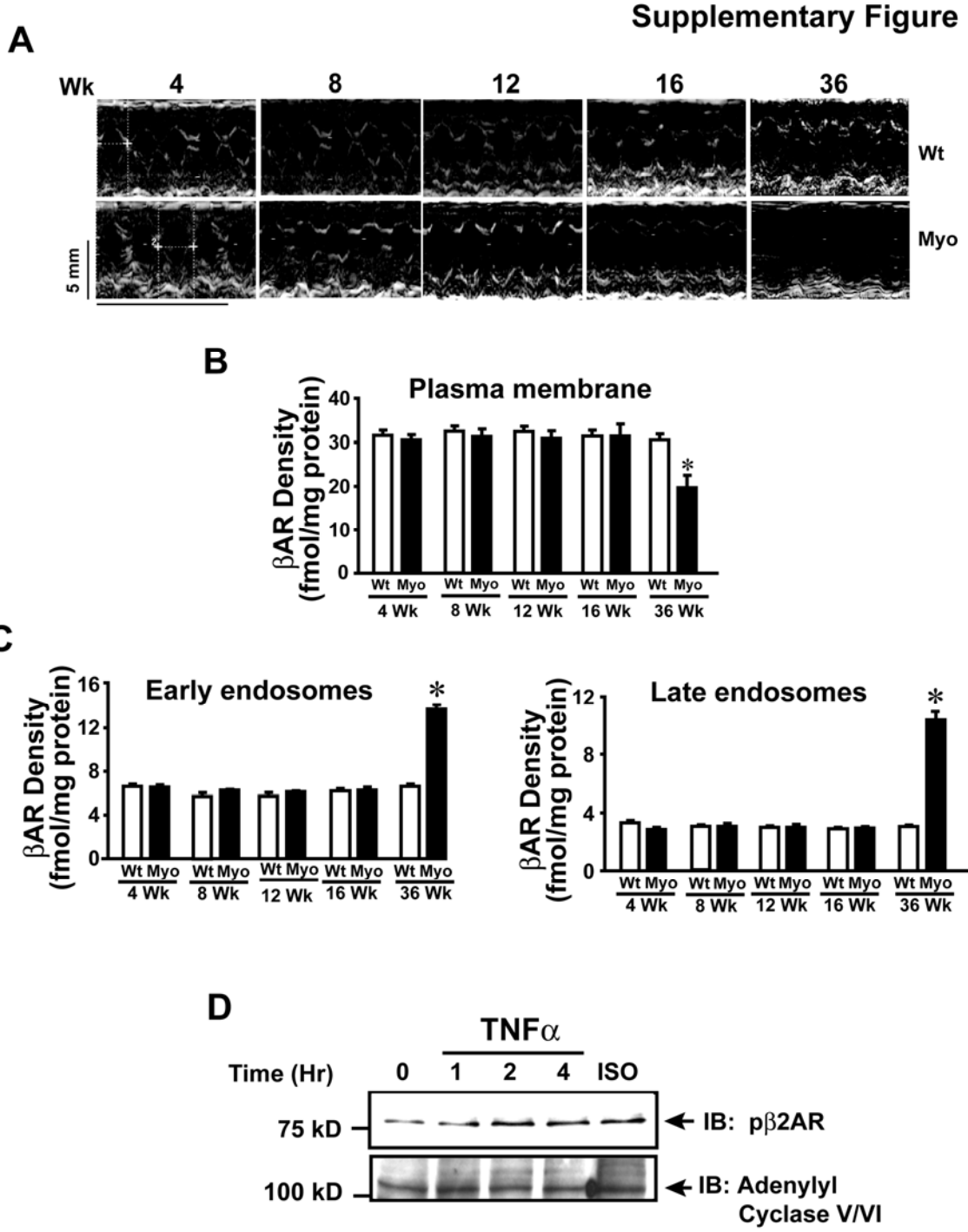
Supplemental Table:

Supplementary Table 1: Morphometric and echocardiographic analysis of Myo-Tg and wild type littermate controls

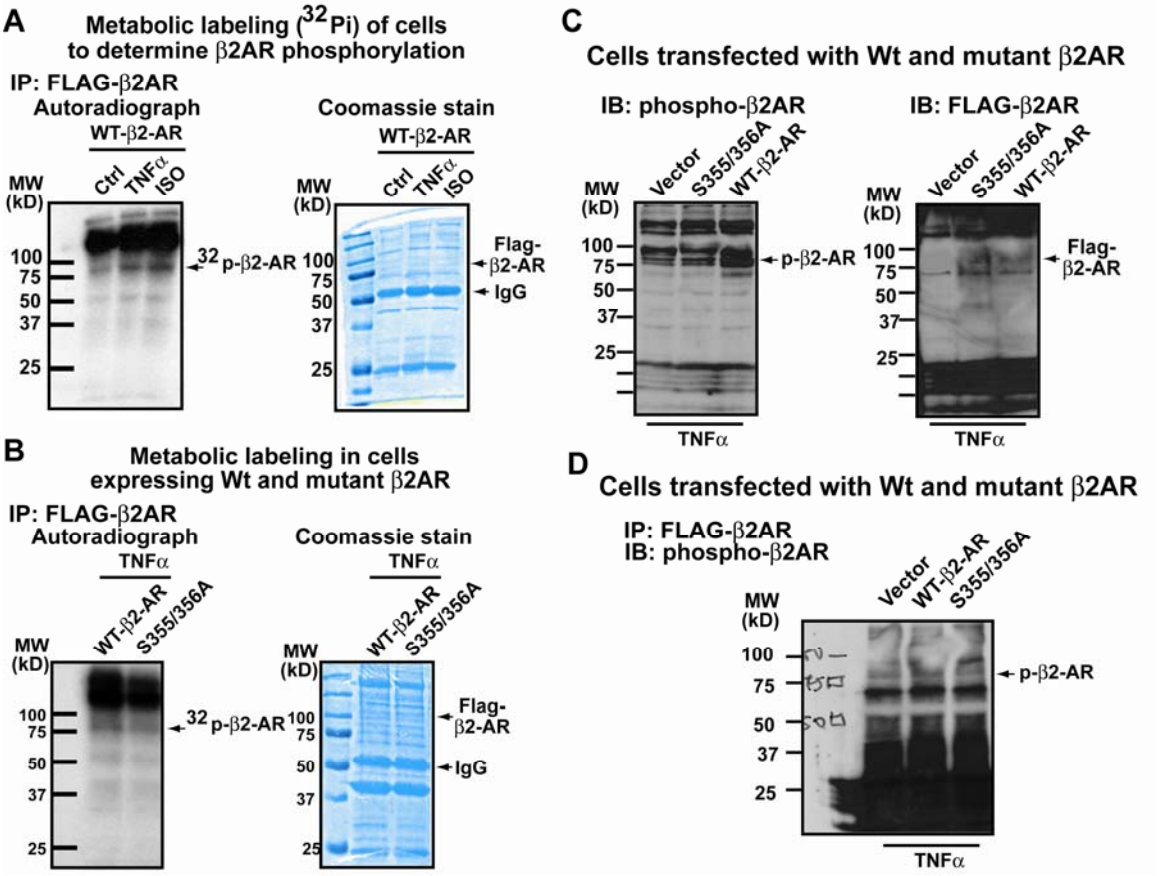
	4 weeks		8 weeks		12 weeks		16 weeks		36 weeks	
	Wt	Myo-Tg	Wt	Myo-Tg	Wt	Myo-Tg	Wt	Myo-Tg	Wt	Myo-Tg
HW/BW (mg/g)	4.71 ± 0.3	5.51 ± 0.3[a]	4.77 ± 0.4	5.91 ± 0.4[a]	4.80 ± 0.8	6.50 ± 0.7[a,b]	4.91 ± 0.6	7.32 ± 0.81[a,b]	5.10 ± 0.8	9.91 ± 0.65[a,b]
LVEDD (mm)	2.38 ± 0.3	2.80 ± 0.3 [a]	2.39 ± 0.4	2.90 ± 0.3[a]	2.41 ± 0.2	3.09 ± 0.7[a,b]	2.43 ± 0.2	3.69 ± 0.1[a,b]	2.45 ± 0.2	5.47 ± 0.4[a,b]
LVESD (mm)	0.93 ± 0.1	1.26 ± 0.3 [a]	0.94 ± 0.2	1.36 ± 0.5[a]	0.99 ± 0.2	1.66 ± 0.3[a,b]	0.97 ± 0.1	2.39 ± 0.2[a,b]	1.07 ± 0.1	4.57 ± 0.2[a,b]
IVS (mm)	0.74 ± 0.1	0.89 ± 0.2 [a]	0.76 ± 0.3	0.91 ± 0.4[a]	0.79 ± 0.2	1.01 ± 0.1[a,b]	0.86 ± 0.3	1.19 ± 0.1[a,b]	1.01 ± 0.1	0.71 ± 0.2[b]
% FS	60.1 ± 1.4	55.9 ± 1.5 [a]	58.1 ± 1.9	53.9 ± 1.3[a]	57.6 ± 1.7	46.3 ± 1.1[a,b]	59.3 ± 2.0	35.6 ± 1.7[a,b]	56.3 ± 1.7	16.0 ± 1.8[a,b]

HW, heart weight, BW, body weight, LVEDD, left ventricular end diastolic diameter, LVESD, left ventricular end systolic diameter
% FS, percent fractional shortening, [a] $p<0.05$ versus respective Wt, [b] $p<0.05$ versus 4 & 8 weeks Myo-Tg. Comparisons were made using student's *t* test with Bonferoni correction for multiple comparisons.

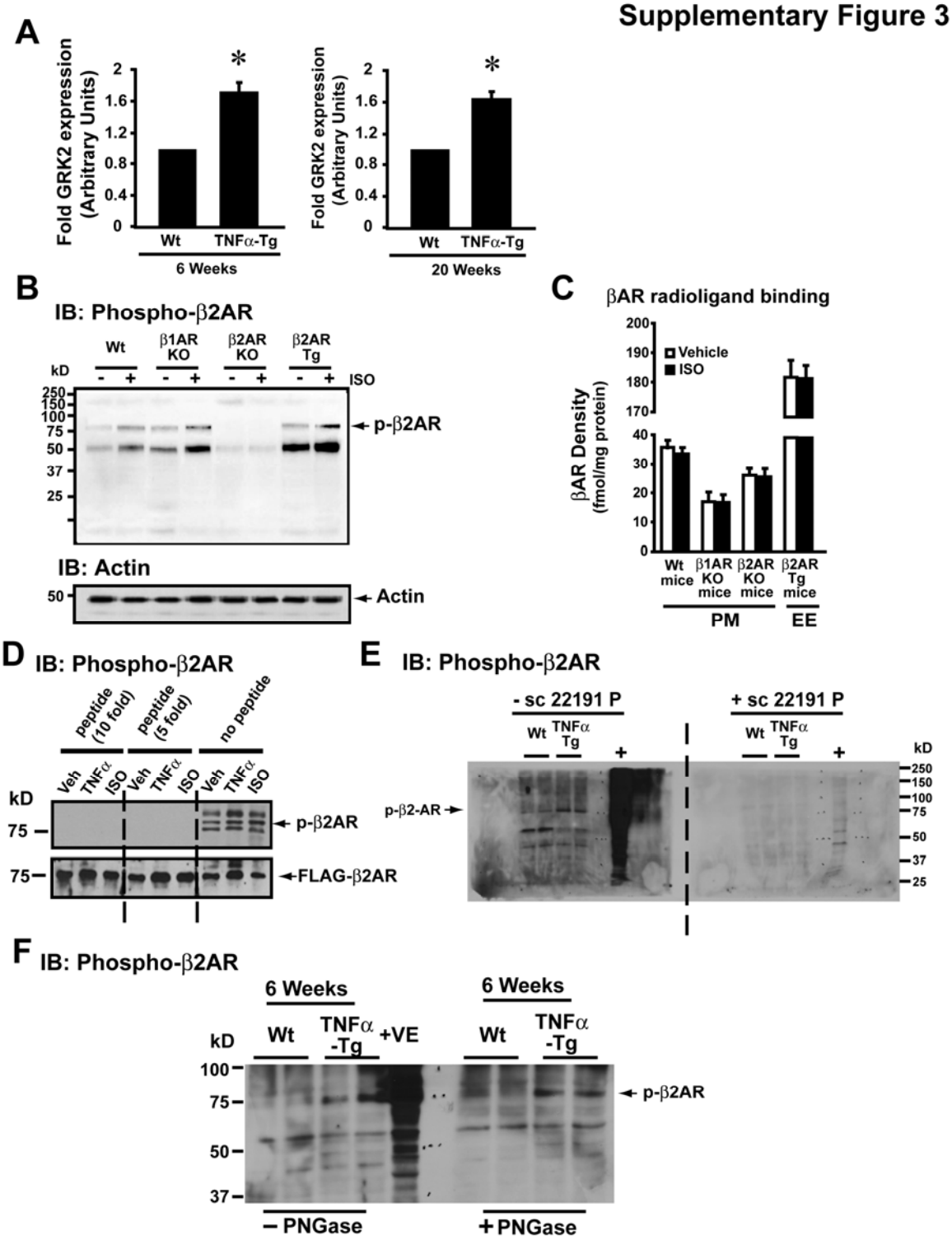
Supplementary Figure 1



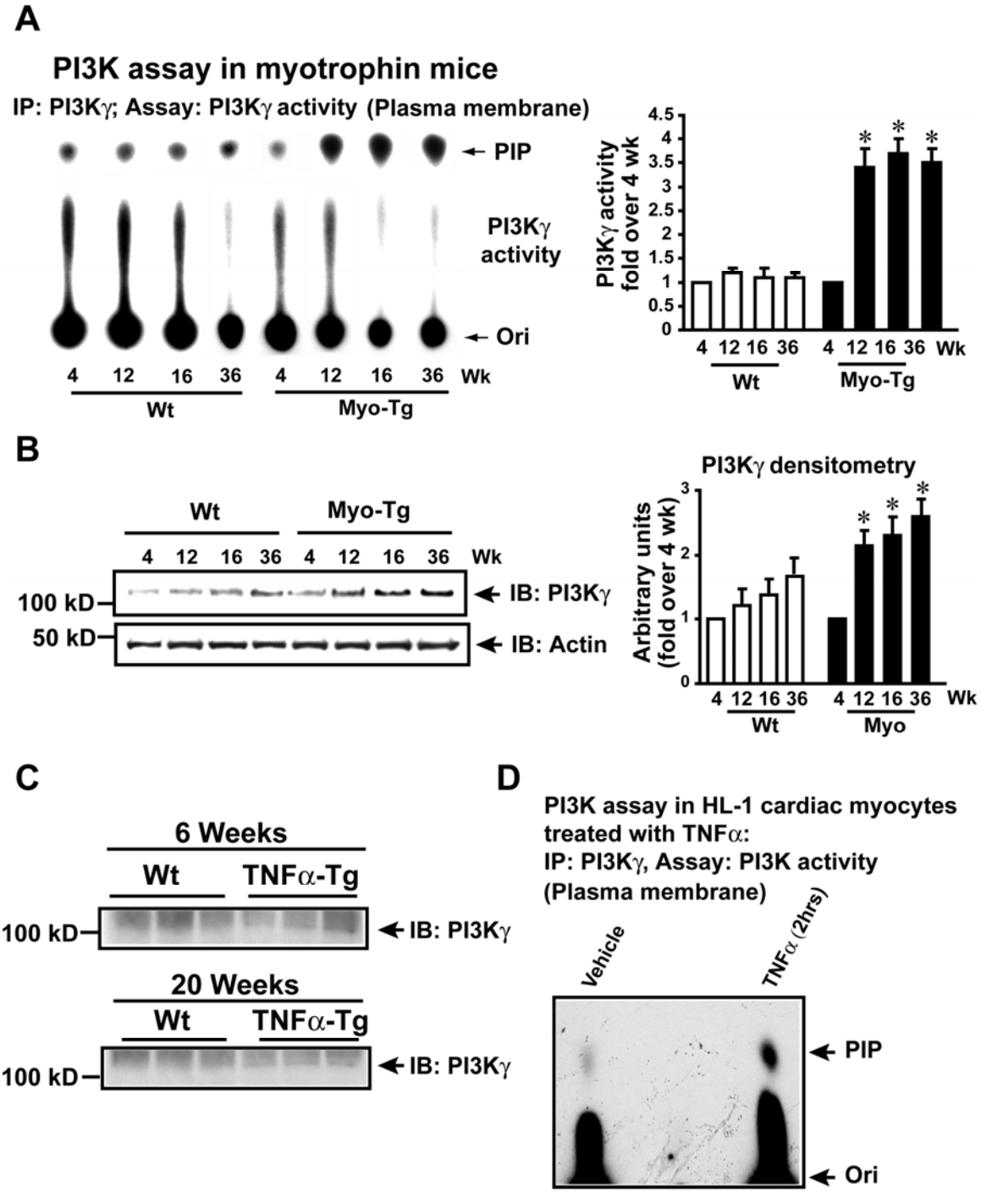
Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure Legends:

Supplementary Figure 1. (A) Echocardiographic images of Wt and Myo-Tg mice at 4, 8, 12, 16 and 36 weeks of age. (B) βAR density on the plasma membranes isolated from the hearts of Wt and Myo-Tg mice at 4, 8, 12, 16 and 36 weeks of age, (n=6), *p< 0.001 versus 36 weeks Wt. (C) βAR density on the early endosomes (left panel) and late endosomes (right panel) from the hearts of Wt and Myo-Tg mice at 4, 8, 12, 16, and 36 weeks of age. (n=6), *p< 0.001 versus 36 weeks Wt. (D) Upper panel: Lysates from HL-1 cardiac myocytes following vehicle or TNFα treatment were immunoblotted for phospho-β_2AR. Lower Panel: Lysates from HL-1 cardiac myocytes treated with Vehicle or TNFα for 1, 2 and 4 hours were immuno blotted for adenylyl cyclase V/VI. Lysates from ISO treated HEK-FLAG-β_2AR cells were used as control.

Supplementary Figure 2. (A) HEK-FLAG-β_2AR cells were metabolically labeled with $^{[32]}$Pi, treated with TNFα or ISO (n=4). β_2ARs were immunoprecipitated using anti-FLAG antibody, resolved on SDS-PAGE and autoradiography performed (n=4). Coomassie stained gel showing equal IP is shown on the right side panel. (B) HEK 293 cells were transfected with FLAG-β_2AR Wt or serine 355/356 mutant β_2AR cDNA constructs, metabolically labeled with $^{[32]}$Pi, followed by TNFα or ISO (n=4). Wt or mutant β_2ARs were immunoprecipitated using anti-FLAG antibody, resolved on SDS-PAGE and autoradiography performed (n=4). Coomassie stained gel showing equal IP is shown on the right side panel. (C) HEK 293 cells were transfected with FLAG-β_2AR Wt or serine 355/356 mutant β_2AR cDNA constructs and treated with TNFα. Following

treatment, the lysates were immunoblotted with anti-phospho-β2AR antibody (left panel) stripped and re-probed with anti-FLAG antibody (right). (D) HEK 293 cells were transfected with FLAG-β_2AR Wt or serine 355/356 mutant β_2AR cDNA constructs, treated with TNFα and β2ARs were immunoprecipitated using anti-FLAG antibody. The immunoprecipitates were resolved and immunoblotted with anti-phospho-β2AR antibody.

Supplementary Figure 3. (A) Densitometric analysis of the GRK2 immunoblots shown in Fig. 6A from Wt and TNFα-Tg mice at 6 or 20 weeks of age, *$p< 0.001$ versus Wt. (B) Cardiac lysates from Wt, β_1AR KO or β_2AR KO along with early endosomal fraction from β_2AR-Tg mice were immunoblotted for phospho-β_2AR. The blot was stripped and re-probed for β-actin. (C) βAR density on the plasma membranes isolated from the hearts of Wt, β_1AR KO or β_2AR KO and early endosomes from β_2AR-Tg mice, (n=6). (D) Specificity of the phospho-355/356 β_2AR antibody was confirmed by immunoblotting the lysates from TNFα or ISO treated β_2AR cells using the blocking peptide (sc-22191 P, Santa Cruz Biotechnology). (E) Cardiac lysates from 6 weeks old Wt and TNFα-Tg mice were tested for the specificity of phospho-355/356 β_2AR antibody by immunoblotting in presence and absence of the blocking peptide (sc-22191 P, Santa Cruz Biotechnology). (F) Cardiac lysates from 6 weeks old Wt and TNFα-Tg mice were treated with the glycosidase enzyme PNGase F and immunoblotted for phospho-β_2AR.

Supplementary Figure 4. (A) Left Panel: Representative PI3Kγ activity in cardiac plasma membranes from the hearts of Wt and Myo-Tg mice at 4, 12, 16 and 36 weeks of age. Summary data (n=6) are on the right panel. Ori, origin; PIP, phosphatidylinositol mono-phosphate. **p < 0.01 versus Myo-Tg 4 weeks. (B) Left Panel: Immunoblotting for PI3Kγ and actin from cardiac lysates of Wt and Myo-Tg mice at 4, 12, 16 and 36 weeks of age. Right Panel: Summary data of densitometric analysis of PI3Kγ. (n=6) *p< 0.001 versus Myo-Tg 4 weeks. (C) Cardiac lysates from Wt and TNFα-Tg mice at 6 or 20 weeks of age immunoblotted for PI3Kγ. (D) Representative PI3Kγ activity at the plasma membranes from HL-1 cardiac myocytes following TNFα for 2 hours.

Supplemental References:

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