

## Supplemental Information

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### Integrating Cardiac PIP<sub>3</sub> and cAMP Signaling through a PKA Anchoring Function of p110 $\gamma$

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#### SUPPLEMENTAL EXPERIMENTAL PROCEDURES

**Antibodies and reagents** - Monoclonal antibodies against p110 $\gamma$  were used as previously described (Hirsch et al., 2000). A new mouse monoclonal antibody against p110 $\gamma$  was produced, purified and used for immunoprecipitation. Briefly, a 630 bp fragment encoding amino acids 1-210 of p110 $\gamma$  was cloned into the EcoRI/XhoI-digested bacterial expression vector pGEX 5X-1 (Amersham Bioscience, Buckinghamshire, UK) and expressed in *Escherichia coli* Top F10 cells. The fusion protein was purified from bacterial lysates by affinity chromatography using a glutathione-Sepharose matrix and used as immunogen to generate N-term-p110 $\gamma$ -GST mouse monoclonal antibody with standard procedures (Harlow and Lane, 1988). Rabbit polyclonal antibodies against PDE3B were a kind gift from J.A. Beavo (University of Washington, Seattle, WA). Rabbit anti p84/87 absorbed serum, goat anti p84/87 antisera and rabbit anti p101 serum were kindly provided by M.P. Wymann (University of Basel, Basel, Switzerland). The following commercial antibodies were used: FLAG-M2, HRP-conjugated secondary antibodies (Sigma-Aldrich, Saint Louis, MO); PKA  $\alpha$ -catalytic subunit, PKA RII $\alpha$  subunit, Myc, goat p110 $\gamma$ , His-probe, p110 $\alpha$  and p110 $\beta$  (Santa Cruz Biotechnology, Santa Cruz, CA); PKA RII $\alpha$  subunit (BD Bioscience, San Jose, CA); phospho-Ser/Thr-PKA substrate, phospho-Akt (S473), Akt (Cell Signaling Technology, Danvers, MA); IgG (Jackson ImmunoResearch, West Grove, PA); GST (Molecular probes, Invitrogen Corporation, Carlsbad, CA); GFP (Abcam, Cambridge, UK); 488, 563 and 647 Alexa Fluor-conjugated secondary antibodies (Invitrogen Corporation, Carlsbad, CA).

Forskolin (FSK), H89, PKI and Alprenolol were purchased from Sigma-Aldrich (Saint Louis, MO); <sup>125</sup>I-labeled cyanopindolol was purchased from Amersham Bioscience (Buckinghamshire, UK); PKI-Myristoylated (PKI-Myr) was purchased from Calbiochem (Darmstadt, Germany).

**Constructs** – p110 $\gamma$ -pcDNA3, p110 $\gamma$ -Myc-pcDNA3, p110 $\gamma$ -KD-pcDNA3 and p110 $\gamma$  deletion fragments p110 $\gamma$ -Myc  $\Delta$ 114-280-pcDNA3 (lacking amino acids 114-280), p110 $\gamma$ -Myc  $\Delta$ RBD-pcDNA3 (lacking amino acids 220-331), p110 $\gamma$ -Myc  $\Delta$ PIK-pcDNA3 (lacking amino acids 541-736) were kindly provided by R. Wetzker (Friedrich Schiller University, Jena, Germany). p101-HA-pcDNA3, p84/87-HA-pcDNA3, 110 $\beta$ -HA-pcDNA3, p110 $\alpha$ -HA-pcDNA3 and p85 $\alpha$ -pcDNA3 were kindly provided by M.P. Wymann (University of Basel, Basel, Switzerland). PKA RII $\alpha$ -ECFP-pcDNA3, PKA RII $\alpha$   $\Delta$ 1-45-ECFP-pcDNA3, PKA RI $\alpha$ -GFP-pcDNA3 and PKA  $\alpha$ -catalytic-YFP-pcDNA3 were kindly provided by M. Zaccolo (University of Glasgow, Scotland, UK). AKAP18 $\alpha$ -pcDNA33.1D/V5-His-TOPO, AKAP79-pcDNA33.1D/V5-His-TOPO and AKAP-Lbc-Flag-PEGFP-N1 were kindly provided by J.D. Scott (University of Washington, WA, USA). PDE3B cDNA was obtained by reverse transcriptase-PCR strategy from mouse liver RNA and subcloned in Flag-pcDNA3 vector. PKA RII $\alpha$  was subcloned from pcDNA3 vector to a pQE31 vector.

p110 $\gamma$  K126A,R130A, p110 $\gamma$  S400A, p110 $\gamma$  T1024A and p110 $\gamma$  T1024D mutants were generated using the Quickchange Lightning Kit (Stratagene, La Jolla, CA), according to the manufacturer's protocol. The mutations were confirmed by DNA sequencing (BMR-Genomics, Padova, Italy).

p110 $\gamma$  126-150-PEGFP-N3 was generated by annealing of oligonucleotides encoding for aa 126-150 of p110 $\gamma$

(CGCGGATCCATGAAGGCCACGCACCGGAGCCCGGGCCAGATCCACCTGGTGCAGCGGCACC  
CGCCCTCCGAGGAGTCCCAAGCCTTCTGAGAAATTCCGG and  
CCGGAAATTCTTCAGAAGGCTTGGGACTCCT

CGGAGGGCGGGTGCCGCTGCACCAGGTGGATCTGGCCCCGGGCTCCGGTGCGTGGCCTTCATG  
GATCCGCG, restriction sites for BamHI and EcoRI underlined), and cloning into the pEGFP-N3 vector  
(Clontech, Mountain View, CA).

**Cell transfection** - Human embryonic kidney 293 T (HEK293T) cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA) supplemented with 10% Fetal Bovine Serum (FBS) and 5 mM penicillin/streptomycin (Gibco, Carlsbad, CA).

For transient expression, HEK293T cells plated at low density were transfected with the indicated plasmids with calcium phosphate or jetPEI (PolyPlus-transfection, Illkirch, France). 24 or 48 hours after transfection cells were washed with phosphate-buffered saline (PBS) and lysed as described below.

**Cell stimulation** – Cell medium was replaced with a serum-depleted medium 16 hours prior to the stimulation. Cells were stimulated with 20-50  $\mu$ M forskolin (FSK) for the indicated time points and lysed as described below. 1-5  $\mu$ M PKI-Myr or 5-10  $\mu$ M H89 were used on cells, before and together with forskolin when indicated.

**Hearts and cell lysis, protein immunoprecipitation and western blotting** - Hearts were rapidly removed, frozen, pestled in liquid nitrogen and homogenized in 120 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1% Triton X-100, protease inhibitor Complete (Roche Applied Science, Indianapolis, IN), 1 mM PMSF (Sigma-Aldrich, Saint Louis, MO) and phosphatase inhibitors (50 mM sodium fluoride, 1 mM sodium orthovanadate, and 10 mM sodium pyrophosphate). HEK293T transfected cells and adult rat cardiomyocytes were lysed using the same lysis buffer. Lysates were incubated on ice for 15 min and then detergent-insoluble material was precipitated by centrifugation at 13000 rpm for 15 min at 4°C. Protein concentration was determined by Bradford method. Supernatants were then analysed for immunoblotting or for immunoprecipitation with the indicated antibodies. Precleared extracts were incubated with 1:1

slurry of protein A- or G-Sepharose (Amersham Biosciences, Buckinghamshire, UK) and 1µg of antibody/mg of protein for 2 h or overnight at 4°C with rocking.

Immunocomplexes bound to protein A- or G-Sepharose beads were washed, subjected to SDS/PAGE and transferred onto polyvinylidene difluoride membranes (PVDF, Millipore, Billerica, MA) or assayed for activity measurement. Membranes were probed with the indicated antibodies and developed with enhanced chemiluminescence (ECL, Millipore, Billerica, MA).

***Real Time Polymerase Chain Reaction*** - 2-Step quantitative reverse transcription real time polymerase chain reaction (qRT-PCR) was performed with standard procedures. Briefly, total RNA was extracted from cardiac samples (20 mg of tissue per sample) using Trizol Reagent (Invitrogen). First strand cDNA was synthesized using 1 µg of total RNA per sample with M-MLV reverse transcriptase (Promega). Subsequently, qRT-PCR was performed using TaqMan chemistry on an Applied Biosystems ABI 7300 instrument. Primers and probes were designed according to the Universal Probe Library system (Roche). Gene expression levels were analyzed using the  $2^{-\Delta\Delta C_t}$  relative quantification method, using 18S as the endogenous control.

***PDE assay*** - PDE activity in immunoprecipitates was measured according to the two-step method as previously described (Patrucco et al., 2004; Thompson and Appleman, 1971) with small variations. Briefly, washed immunoprecipitates were assayed in a total volume of 200 µl of reaction mixture containing 40 mM Tris-HCl pH 8.0, 5 mM MgCl<sub>2</sub>, 10 mM 2-mercapto-ethanol, [<sup>3</sup>H]cAMP (0.1 µCi/tube, approximately 1x10<sup>5</sup> cpm/tube, Amersham Bioscience, Buckinghamshire, UK) with or without 10 µM cAMP (Sigma-Aldrich, Saint Louis, MO). Samples were incubated under agitation at 30°C for 5-15 minutes, boiled for 3 min to stop reaction and incubated with snake venom nucleotidase from Crotalus Atrox (Sigma-Aldrich, Saint Louis, MO). The products of the reaction were separated by the anion exchange chromatography through addition of a 30% (w/v) suspension of Dowex AG1-X8 resin (Bio-

Rad, Segrate, Milano, Italy). The amount of radiolabelled adenosine in the supernatant was quantified by scintillation counting (Ultima Gold scintillation liquid from Perkin Elmer, Waltham, MA by using a  $\beta$ -counter machine). PKI was added to the washed immunoprecipitate prior to the reaction, when indicated.

**PKA activity assay** - Immunoprecipitates were washed and incubated in PKA kinase buffer (50 mM Tris-HCl pH 7.5, 5 mM  $MgCl_2$ ) containing 5 mM kemptide, 0.3 mM cAMP, 100  $\mu$ M ATP and 5  $\mu$ M  $\gamma$ - $^{32}P$ -ATP with or without the PKA catalytic subunit for 20 min at 30°C. The reaction mixture was spotted onto a p81 phosphocellulose paper (Whatman, Scientific Ltd., UK), washed four times in 75 mM phosphoric acid and once in 95% ethanol. Filters were air-dried and radioactivity was quantified by liquid-scintillation counting.

**PKA kinase assay** - Immunoprecipitates were washed and incubated in PKA kinase buffer (50 mM MOPS pH 6.8, 50 mM NaCl, 2 mM  $MgCl_2$  and 1 mM DTT) containing 100  $\mu$ M ATP and 5  $\mu$ M  $\gamma$ - $^{32}P$ -ATP with or without the PKA catalytic subunit for 30 min at 30°C. Reactions were stopped by elution in Laemmli sample buffer and analyzed by SDS-PAGE and autoradiography to visualize  $^{32}P$ -labeled proteins.

**In vitro interaction assay** – p110 $\gamma$ -GST and GST purified from baculovirus-infected Sf9 cells were kindly provided by R. Wetzker (Friedrich Schiller University, Jena, Germany). PKA RII $\alpha$ -6-His protein was produced in *Escherichia coli*. Briefly, a PKA RII $\alpha$  (1301 bp fragment) was cloned KpnI/SacI into the bacterial expression vector pQE-31 (Amersham Bioscience, Buckinghamshire, UK), expressed in *Escherichia coli* Top F10 cells and purified using Ni-NTA resin (Amersham Bioscience, Buckinghamshire, UK) under native conditions.

Binding assays of immobilized 50 nM GST fusion proteins with 100 nM free PKA RII $\alpha$ -6-His were performed in binding buffer (50 mM Tris-HCl pH 7.4, 20 mM NaCl, 0.05% Triton X-100). The reactions

were incubated for 30 min at room temperature with shaking and the beads were washed four times with binding buffer. Proteins associated to the beads were eluted by treating the beads for 5 min at 95°C in 2x Laemmli sample buffer and subjected to SDS-PAGE.

***RII overlay assay*** - Immunoprecipitated proteins were separated by SDS-PAGE and electrotransferred to nitrocellulose. The membrane was incubated over-night with recombinant murine RII $\alpha$  phosphorylated by the catalytic subunit of PKA using  $\gamma^{32}\text{P}$ -ATP (Bregman et al., 1989; Coghlan et al., 1994; Lohmann et al., 1984; Scott et al., 1990) and with 50  $\mu\text{M}$  of either AKAP-*IS* or AKAP-*IS* scrambled peptide (Alto et al., 2003).

***Surface Plasmon Resonance*** - Surface plasmon resonance (SPR) was performed with a Biacore 2000 instrument (Biacore AB, Uppsala, Sweden). Recombinant p110 $\gamma$ -GST was immobilized onto CM5 Sensor Chips (Biacore AB, Uppsala, Sweden) by amine group coupling as indicated by manufacturer instructions (Biacore amine coupling kit). PKA RII $\alpha$ -6-His was used as soluble analyte and injected at different concentrations. All analytes were dissolved in running buffer and binding experiments were performed at 25°C in running buffer with a flow rate of 5  $\mu\text{l}/\text{min}$ . All the binding curves were corrected for the signal of GST alone coupled to the matrix and analyzed using the Langmuir model.

***Peptide array*** - Peptides arrays of p110 $\gamma$  on cellulose were generated and probed as previously described (Baillie et al., 2007; Bolger et al., 2006). Briefly, scanning libraries of overlapping 25-mer peptides covering the entire sequence of p110 $\gamma$  were produced by automatic SPOT synthesis and synthesized on continuous cellulose membrane supports on Whatman 50 cellulose using Fmoc (9-fluorenylmethyloxycarbonyl) chemistry with the AutoSpot-Robot ASS 222 (Intavis Bioanalytical Instruments). The interaction of PKA RII $\alpha$ -6-His with the p110 $\gamma$  array was investigated by overlaying membranes with 10  $\mu\text{g}/\text{ml}$  PKA RII $\alpha$ -6-His. Bound protein was detected with specific anti-His antibody

and a secondary anti-rabbit antibody coupled with horseradish peroxidase. Once the binding site of PKA-RII $\alpha$  on the full-length p110 $\gamma$  array was determined, specific double alanine scanning substitution arrays were generated for selected peptides using the same synthesis procedure. The same protocol was used to identify the minimal region essential for the binding. For that purpose, the peptide of interest was truncated both to the N-terminus and to the C-terminus.

Phosphorylation of peptide array was performed on a membrane activated in 100% ethanol. Briefly, the membrane was incubated for 1 hour at room temperature in phosphorylation buffer (20 mM Hepes, pH7.4, 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 1mM DTT, 0.2 mg/ml BSA) and then at 4°C overnight in blocking buffer (Phosphorylation buffer + 0.2 mg/ml BSA 100  $\mu$ M ATP). The next day, the array was incubated for 1 hour at 37°C with or without PKA (1g/ml) in phosphorylation buffer containing 100  $\mu$ M ATP (or 50 $\mu$ M and 10 $\mu$ Ci  $\gamma$ -<sup>32</sup>P-ATP). PKA phosphorylation was then assayed with the phospho Ser/Thr PKA substrate antibody or by autoradiography.

**Peptide competition assay** - 126-150 p110 $\gamma$  peptide (KATHRSPGQIHLVQRHPPSEESQAF Bachem, Weil am Rhein, Germany) was resuspended in PBS to a working concentration of 50  $\mu$ M or 250  $\mu$ M. An unrelated peptide was used as a control (KHPILAADWHPELGPDPGHPVPATA, home made). HEK293T cells transfected with p110 $\gamma$  were lysed and the extract was incubated with the 126-150 p110 $\gamma$  peptide or with the control peptide for 30 min at room temperature. PKA RII $\alpha$  was then immunoprecipitated with the appropriate antibody for 2 h at 4° in agitation and the presence of p110 $\gamma$  associated to PKA RII $\alpha$  was evaluated through SDS-PAGE and immunoblotting analysis. The same procedure was used for the AKAP-IS competition experiment.

**Lipid kinase assay** - Immunoprecipitated or recombinant p110 $\gamma$  was incubated in lipid kinase buffer (20 mM Hepes pH 7.4, 5 mM MgCl<sub>2</sub>) containing 10  $\mu$ g of phosphatidylinositol or PtdIns(4,5)P<sub>2</sub> (Avanti Polar

Lipids, Alabaster, AL), 40 µg of phosphatidylserine (Avanti Polar Lipids, Alabaster, AL), 10 µM of cold ATP and 5 µCi of  $^{32}\text{P}$ -ATP for 10 min at 30°C at 1200 rpm. 40 µM LY292004 was added to the reaction when indicated. The reaction was stopped by the addition of HCl 1N and lipids were extracted using chloroform/methanol (1:1). The organic phase, containing  $\text{PI}(3)^{32}\text{P}$  or  $\text{PI}(3,4,5)^{32}\text{P}_3$ , was spotted on thin-layer chromatography plates and resolved chromatographically with chloroform/methanol/ammonium hydroxide/water (45:35:1.5:8.5). Dried plates were exposed for autoradiography.

**PIP3** – PIP3 was extracted from  $5 \times 10^6$  transfected HEK-293T cells upon stimulation with indicated drugs or total hearts (20 mg of powder) and PIP3 measurement was carried out using an ELISA competitive kit (K-2500s, Echelon, Salt Lake City, UT ), following the manufacturer's instructions.

**Acute isoproterenol treatment** - Freshly prepared aqueous solution (PBS) of isoproterenol (1.25 mg/kg; Sigma-Aldrich, Saint Louis, MO) was administered intraperitoneally in isoflurane anesthetized mice. Hearts were rapidly excised after the indicated time points, washed in PBS and frozen in liquid nitrogen. Control unstimulated hearts were derived from mice injected with saline solution only.

**Langendorff** - Mouse hearts were rapidly excised under isoflurane anaesthesia (1.5% isoflurane, 66% nitrous oxide and 33% oxygen), the aorta cannulated and retrogradely perfused with oxygenated (5%  $\text{CO}_2$ , 95%  $\text{O}_2$ ) Tyrode solution (5 mM Hepes, 154 mM NaCl, 4 mM KCl, 2 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 5.5 mM D-glucose, pH 7.35) at a constant flow rate ( $4 \pm 0.5 \text{ ml} \cdot \text{min}^{-1}$ ). The temperature of the heart was maintained at 37 °C through water-jacketed heating. Hearts were exposed to 10 min stabilization prior to 5 min treatment with 13.4 µM isoproterenol (Sigma-Aldrich, Saint Louis, MO). 10 µM H89 was perfused for 5 min before and simultaneously with 13.4 µM isoproterenol when indicated.



***Adult rat cardiomyocyte culture*** - Adult Rat Ventricular Myocytes (ARVMs) were obtained from young adult (4–6 months) rats by enzymatic dissociation, as previously described (Cerra et al., 2008). Upon isolation, ARVMs were immediately stimulated for 3 min with 1  $\mu$ M isoproterenol with or without pre-incubation with 1  $\mu$ M PKI-Myr for 5 min. Untreated myocytes were used as a control. Cells were washed once with cold PBS and lysed with the same lysis buffer described above.

***Mouse adult cardiomyocytes*** - Cardiomyocytes were collected from adult mice euthanized by injection of sodium pentobarbital. The heart was removed rapidly and placed in ice cold digestion buffer, pH 7.4 (130 mM NaCl, 5m M KCl, 3 nM pyruvic acid, 25 mM Hepes, 0.5 mM MgCl<sub>2</sub>, 0.33 mM NaH<sub>2</sub>PO<sub>4</sub>, 22 mM dextrose, 5 mM taurine, 10 mM 2,3-Butanedione monoxime) with 100  $\mu$ M EGTA. The heart was then attached to a perfusion pipette via the aorta and perfused with digestion buffer with 1mg protease, 25 mg Collagenase and 50  $\mu$ M CaCl<sub>2</sub> for 10 minutes. The ventricle was then isolated, cut into small pieces and placed in digestion buffer with 0.5 mg protease, 15 mg collagenase, 125 mg BSA and 100  $\mu$ M CaCl<sub>2</sub>. A monocytic mixture was made by mechanical agitation of the tissue. Undigested tissue was then allowed to float to the top of the solution and removed, remaining cells were pelleted by gravity over a 15 minute period. Pellet was then washed and re-suspended in media and allowed to adhere to poly-d-lysine/laminin coated coverslips for 1 hour.

***Immunofluorescence*** – Adherent mouse adult cardiomyocytes were fixed by submersion in ice cold methanol with 2% paraformaldehyde for 5 minutes. Following fixation, coverslips were rinsed three times with PBS, permeabilized with PBS-T (0.1% Triton X-100) and blocked for 30 minutes in 3% BSA. Cells were incubated overnight at 4°C in primary antibody and in secondary for two hours at room temperature. Coverslips were mounted in ProLong Gold anti-fade reagent (Invitrogen Corporation, Carlsbad, CA). Primary antibodies used in this study include the mouse monoclonal anti-RII $\alpha$  antibody (1:200), affinity purified rabbit anti-PDE3B (1:200) and goat anti-p110 $\gamma$  (1:150). Primary antibodies were visualized using

488, 563 and 647 Alexa Fluor-conjugated secondary antibodies (1:500). Zeiss 510 Meta confocal microscope equipped with 488, 543 and 633 laser lines was used to acquire images.

***Human cardiac biopsies and immunohistochemistry*** - Cardiac biopsies of patients with severe pressure overload due to isolated aortic stenosis, and five controls were included. The patients with aortic stenosis underwent surgical aortic valve replacement. Five patients undergoing off-pump coronary artery bypass graft surgery (CABG) with normal ejection fraction, diastolic function, and without unstable angina, previous history of myocardial infarction, or LV hypertrophy at echocardiography or ECG measurements served as controls. Transmural true-cut needle biopsies, each weighing 1 to 2 mg, were taken from the anterior LV at close proximity between the left descending coronary artery and the circumflex coronary artery (Heymans et al., 2005). For immunohistochemical examination, hearts were cut transversely, fixed in 4% buffered paraformaldehyde and embedded in paraffin. To study ventricular morphology, hearts were histochemically stained with hematoxylin and eosin. The cardiomyocyte cell surface area was measured as the mean of >50 cross sectioned cardiomyocytes across the left ventricular wall. A primary antibody against p110 $\gamma$  (Hirsch et al., 2000) was used for immunostaining on 4  $\mu$ m thick paraffin sections. Relative surface area of p110 $\gamma$  immunoreactivity was expressed as percentage immunoreactive area divided by total area (Heymans et al., 2005).

***Transverse aortic constriction and AS605240 treatment*** - *In vivo* pressure overload was imposed on the left ventricle by surgical banding of the transverse aorta, as previously described (Patrucco et al., 2004). Sham operated animals underwent the same surgical procedure without transverse aortic constriction (TAC). 2D guided M-mode echocardiography was performed in anesthetized mice (i.p. injection of 2,2,2-tribromoethanol) with a Vevo 770 echocardiograph equipped with a 30-MHz probe (RMV-707B) (VisualSonics, Toronto, Canada). Peak blood flow velocities ( $V_{\max}$ ) at the site of TAC were measured by Doppler ultrasound and aortic pressure gradients (PG in mmHg) were calculated by the formula  $PG = 4 X$

$(V_{\max}/1000)^2$ . Two-dimensional M-mode images in the long axis view at the proximal level of the papillary muscles were obtained to determine the end-diastolic and end-systolic internal diameters (LVID<sub>ED</sub> and LVID<sub>ES</sub>), the end-diastolic septal and posterior wall thicknesses. Fractional shortening (FS) was calculated as  $FS (\%) = [(LVID_{ED} - LVID_{ES}) / LVID_{ED}] \times 100$ . FS lower than 30% was used as a threshold to discriminate between compensated and decompensated hearts.

Mice displaying a 25-30% FS were injected i.p. daily for one week with either 10 mg/kg AS605240 (Sigma-Aldrich, Saint Louis, MO; dissolved in 0.5% carbosimethylcellulose and 0.25% Tween 20) or vehicle alone. The cardiac function (FS measurement) was evaluated after the treatment.

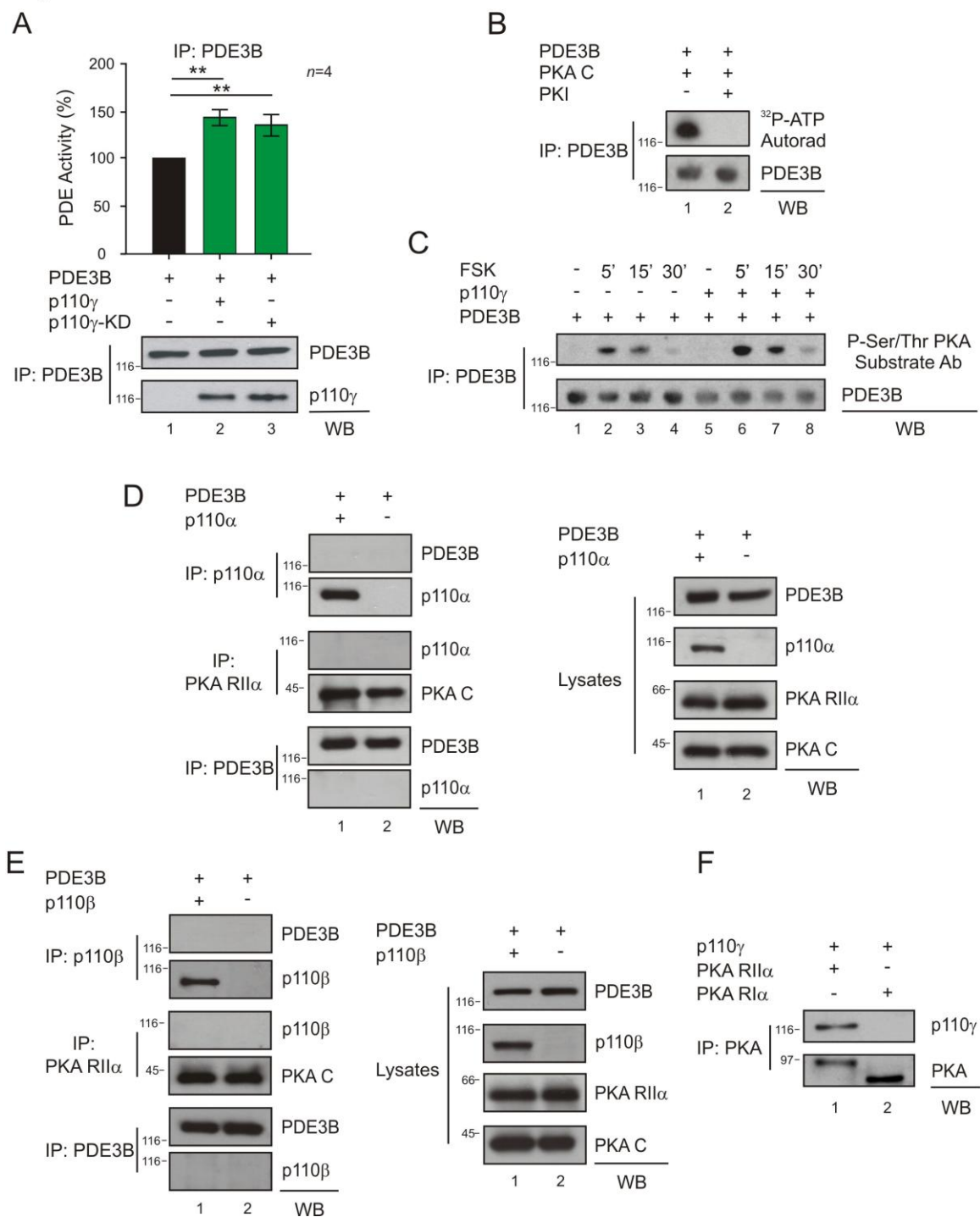
***β-adrenergic receptor density measurement*** - Mouse hearts were homogenized at 4°C in 15 volumes of 250 mM sucrose, 5 mM EDTA, 5 mM Tris-HCl pH 7.5, 2 μM leupeptin, 100 μM benzamidine and 100 μM PMSF. Samples were centrifuged at 800 g for 15 min at 4°C to clear the homogenate of cellular debris and nuclei. Supernatants were filtered through cheesecloth and centrifuged at 25,000 g for 30 min at 4°C. The pelleted myocardial sarcolemmal membranes were washed in acidified ice-cold β-AR binding buffer (75 mM Tris-HCl pH 2.5, 12.5 mM MgCl<sub>2</sub>, 2 mM EDTA) before being resuspended in neutral pH (7.5) binding buffer at [1 mg/ml]. Cardiac membrane total β-AR density was determined by incubation of 25 μg of membrane proteins with a saturating concentration of <sup>125</sup>I-labeled cyanopindolol. Nonspecific binding was determined in the presence of 10 μM alprenolol (nonselective β-AR antagonist), as previously described (Ciccarelli et al., 2008). Reactions were conducted in 100 μl of binding buffer at 37°C for 1 h and then terminated by vacuum filtration through glass-fiber filters. After extensive ice-cold washing (50 mM Tris buffer), bound radioactivity remaining on the filters was assessed on a gamma counter (Perkin Emler, Waltham, MA). All assays were performed in triplicate, and receptor density (in femtomoles) was normalized to milligrams of membrane protein.

When indicated, wild-type were injected i.p. daily for three days with either 50 mg/kg AS605240 (Sigma-Aldrich, Saint Louis, MO; dissolved in 0.5% carbosimethylcellulose and 0.25% Tween 20) or vehicle alone.

**Mice** - p110 $\gamma$  knock-out (Hirsch et al., 2000), p110 $\gamma$  kinase-dead (Patrucco et al., 2004) and p110 $\beta$  kinase-dead (Ciraolo et al., 2008) are all in a C57Bl/6J background. C57Bl/6J wild-type mice were used as controls. Mice were group-housed, provided free-access to standard chow and water in a controlled facility providing a 12 hour light/dark cycle and were used according to institutional animal welfare guidelines and legislation, approved by the local Animal Ethics Committee.

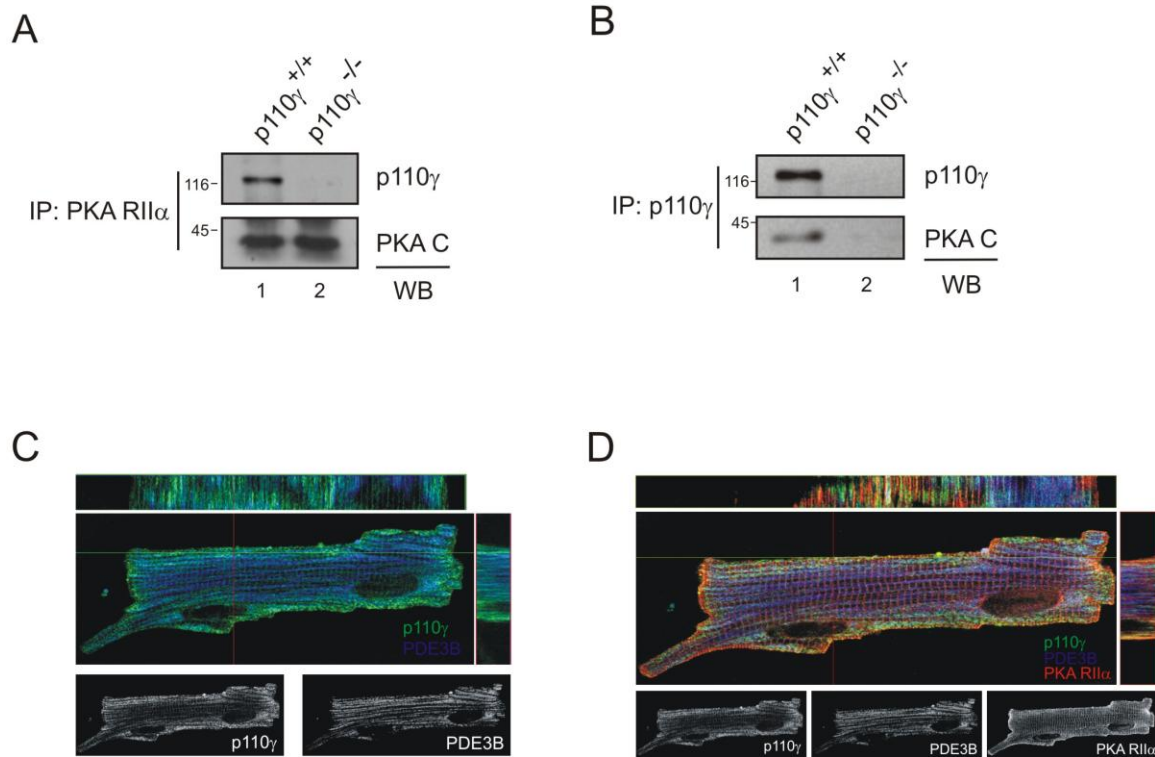
**Data and Statistical Analyses** - Prism software (GraphPad) was used for statistical analysis. All data were expressed as mean  $\pm$  standard error of the mean (SEM). *P* values were calculated by using Student *t* test, and one-way ANOVA test followed by Bonferroni's post hoc analysis when appropriate. *P* < 0.05 was considered significant (\*), *P* < 0.01 was considered very significant (\*\*) and *P* < 0.001 was considered extremely significant (\*\*\*)

Figure S1



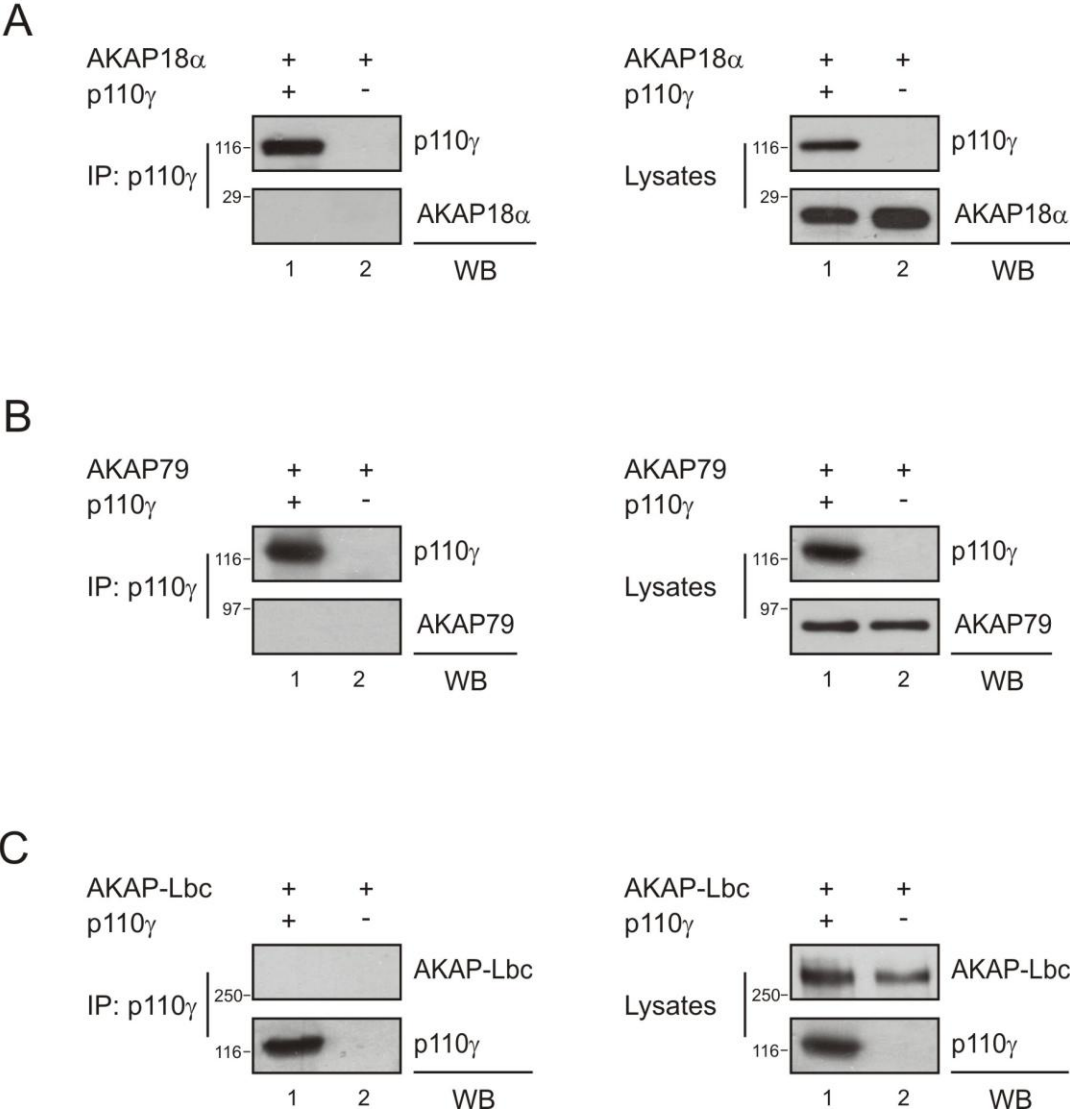
**Figure S1.** p110 $\gamma$  activates PDE3B in a PKA-dependent manner. Related to Figure 1. **(A)** Phosphodiesterase activity in PDE3B-Flag immunoprecipitates upon transfection of HEK293T cells with PDE3B-Flag alone or PDE3B-Flag and wild-type or kinase-dead p110 $\gamma$  (p110 $\gamma$ -KD). PDE activity (%) was calculated relative to the activity of single PDE3B transfectants. Values represent mean  $\pm$  SEM of four independent experiments.  $^{**}P < 0.01$ . A representative immunoprecipitation is presented. **(B)** Phosphorylation of PDE3B-Flag immunoprecipitated from HEK293T cells by recombinant PKA C in the presence of  $^{32}\text{P}$ -ATP. Control is provided by incubation with PKA inhibitor PKI (1  $\mu\text{M}$ ). A representative assay is presented. **(C)** PKA-mediated phosphorylation of PDE3B-Flag immunoprecipitated from HEK293T cells transfected with PDE3B-Flag alone or with PDE3B-Flag and p110 $\gamma$ . Cells were stimulated with the adenylyl cyclase activator forskolin (FSK 20  $\mu\text{M}$ ) for the indicated time points (5, 15 or 30 min.). A representative blot is presented. **(D)** p110 $\alpha$  does not co-immunoprecipitate with both PDE3B-Flag (PDE3B) and the PKA holoenzyme in transfected HEK293T cells. Representative reciprocal co-immunoprecipitations (left panel) and total extracts (right panel) are presented. **(E)** p110 $\beta$  does not co-immunoprecipitate with both PDE3B-Flag (PDE3B) and the PKA holoenzyme. Representative reciprocal co-immunoprecipitations (left panel) and total extracts (right panel) are presented. **(F)** Co-immunoprecipitation of p110 $\gamma$  with PKA RII $\alpha$  but not with PKA RI $\alpha$  in transfected HEK293T cells. A representative immunoprecipitation is presented.

## Figure S2



**Figure S2.** The p110γ-PKA-PDE3B complex in the heart. Related to Figure 1. **(A)** Co-immunoprecipitation of p110γ with PKA RIIα from myocardial tissue extracts of wild-type (p110γ<sup>+/+</sup>) but not of knock-out (p110γ<sup>-/-</sup>) mice. A representative immunoprecipitation is presented. **(B)** Co-immunoprecipitation of PKA C with p110γ from myocardial tissue extracts of wild-type (p110γ<sup>+/+</sup>) but not of knock-out (p110γ<sup>-/-</sup>) mice. A representative immunoprecipitation is presented. **(C)** Co-localization (light blue spots) of p110γ (green) and PDE3B (blue) by immunofluorescence in mouse adult cardiomyocytes. Longitudinal and transverse sections are shown in the upper and right panels, respectively. Single p110γ and PDE3B localizations are presented in the lower panels. **(D)** Co-localization (white spots) of p110γ (green), PDE3B (blue) and PKA RIIα (red) by immunofluorescence in mouse adult cardiomyocytes. Longitudinal and transverse sections are shown in the upper and right panels, respectively. Single p110γ, PDE3B and PKA RIIα localizations are presented in the lower panels.

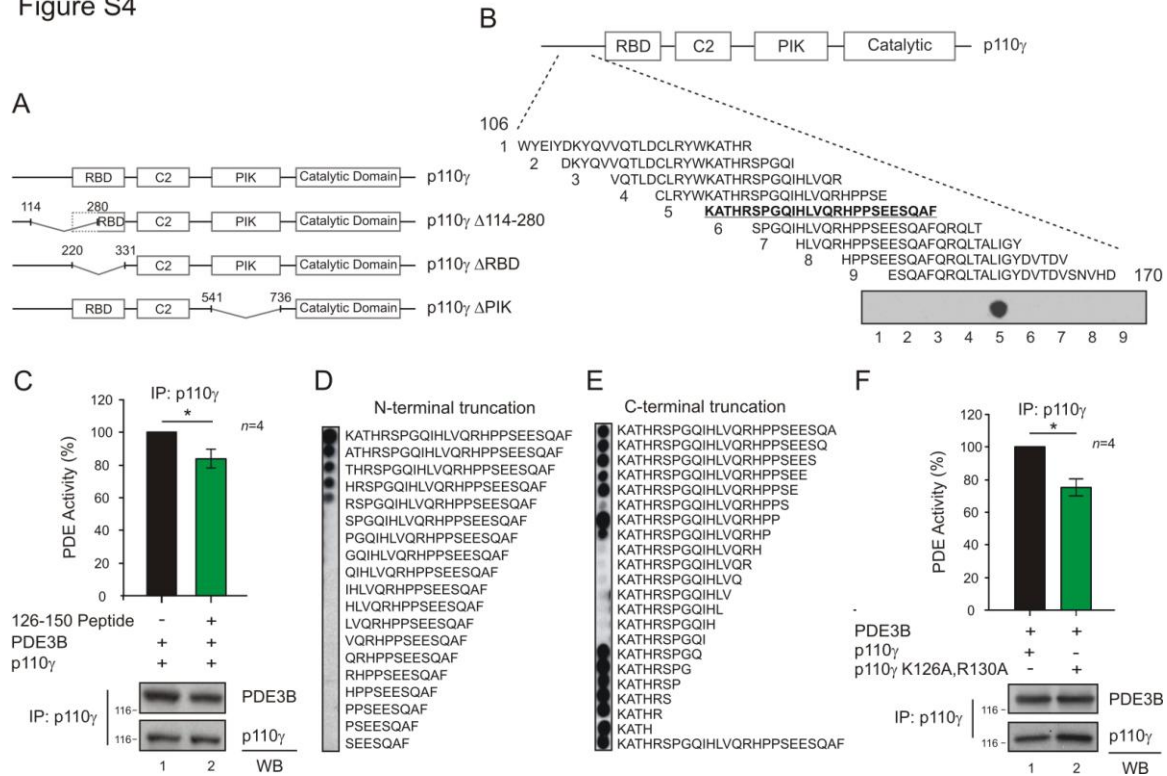
Figure S3





**Figure S3.** Three myocardial AKAPs do not interact with p110 $\gamma$ . Related to Figure 2. **(A)** AKAP18 $\alpha$ -His (AKAP18 $\alpha$ ) does not co-immunoprecipitate with p110 $\gamma$  in transfected HEK293T cells (left panel). Total extracts are shown (right panel). A representative immunoprecipitation is presented. **(B)** AKAP79-His (AKAP79) does not co-immunoprecipitate with p110 $\gamma$  in transfected HEK293T cells (left panel). Total extracts are shown (right panel). A representative immunoprecipitation is presented. **(C)** AKAP-Lbc-Flag (AKAP-Lbc) does not co-immunoprecipitate with p110 $\gamma$  in transfected HEK293T cells (left panel). Total extracts are shown (right panel). A representative immunoprecipitation is presented.

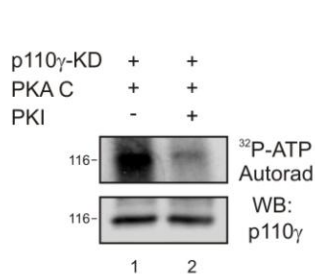
Figure S4



**Figure S4.** Mapping of the p110 $\gamma$ -PKA RII $\alpha$  interaction. Related to Figure 3. **(A)** Representative scheme of p110 $\gamma$ -Myc tagged deletion fragments transfected in HEK293T cells. Deletion fragments were used to map the binding of p110 $\gamma$  to PKA RII $\alpha$  in Fig. 3A. **(B)** Binding of PKA RII $\alpha$  to the 126-150 p110 $\gamma$  peptide in a solid-phase peptide array. Sequences of the peptides spotted on the array are represented on a scheme of p110 $\gamma$ . **(C)** Phosphodiesterase activity in p110 $\gamma$  immunoprecipitates upon transfection of HEK293T cells with PDE3B-Flag (PDE3B), p110 $\gamma$  and a 126-150 p110 $\gamma$  construct (126-150 Peptide) or empty pcDNA3 vector. PDE activity (%) was calculated relative to the activity of PDE3B-p110 $\gamma$  transfectants. A representative immunoprecipitation is presented. **(D)** Binding of PKA RII $\alpha$  to a series of N-terminal truncated 126-150 p110 $\gamma$  peptides in a solid-phase peptide array. **(E)** Binding of PKA RII $\alpha$  to a series of C-terminal truncated 126-150 p110 $\gamma$  peptides in a solid-phase peptide array. **(F)** Phosphodiesterase activity in p110 $\gamma$  immunoprecipitates upon transfection of HEK293T cells with PDE3B-Flag (PDE3B) and p110 $\gamma$  or p110 $\gamma$  K126A,R130A mutant. PDE activity (%) was calculated relative to the activity of PDE3B-p110 $\gamma$  transfectants. A representative immunoprecipitation is presented. For all bar graphs, values represent mean  $\pm$  SEM of four independent experiments. \* $P < 0.05$ .

Figure S5

A



B

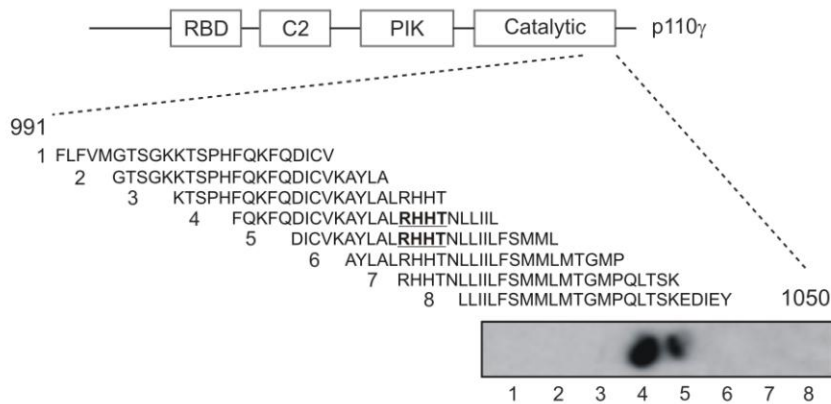
p110 $\gamma$  S400

Homo sapiens IPVLP~~PRNTDLTVFVEANIQHGGQVLCQ~~**RRTS**PKPFTEEVLWNVWLEFSIKI  
 Mus musculus IPVLP~~PRNTDLTVFVEANIQHGGQVLCQ~~**RRTS**PKPFEEVLWNVWLEFGIKI  
 Sus scrofa IPVLP~~PRTADLTVFVEANIYGGQVLCQ~~**RRTS**PKPFTEEVLWNVWLEFSIKI  
 Gallus gallus IPVLP~~PRNTDLTVFVEANIQHGGQLLSQ~~**RRTS**SKPFTEEVLWNIWLEFDIKI  
 Xenopus IPVLP~~KNTELIAFVEANIQHGGVLSQ~~**RRTT**CKPFTEEVLWNSWLEFDIKI  
 Danio Rerio IPVLP~~RNSELIVFVEASIFHGQQLLAQ~~**ERTS**SKPFTEEVIWNTWLEFNIKI  
 \*\*\*\*\*:..\* :\*\*\*\*\* \* :\*\*\*:\*.\*.\*\* : \*\*:\*:\*\*.\* \*\* \* \*\*

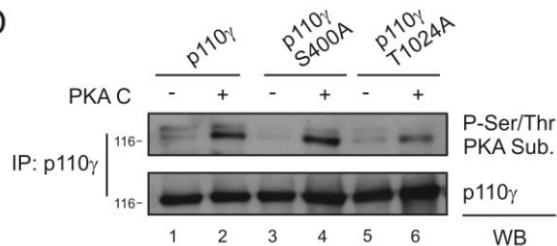
p110 $\gamma$  T1024

Homo sapiens LFV~~MGTS~~SGK~~KTSPHFQKFQD~~ICVKAYLAL**RHHT**NLLIILFSMMLMTGMPQ  
 Mus musculus LFV~~MGSS~~SGK~~KTSPHFQKFQD~~VCVRAYLAL**RHHT**NLLIILFSMMLMTGMPQ  
 Sus scrofa LFV~~MGTS~~SGK~~KTSLHFQKFQD~~VCVKAYLAL**RHHT**NLLIILFSMMLMTGMPQ  
 Gallus gallus LFV~~MGTS~~SGK~~KTSLHFHKFQD~~VCVKAYLAL**RHHT**NLLIILFSMMLMTGMPQ  
 Xenopus LYV~~MGTS~~SGK~~KTQYFNKFQD~~VCVKAYLAL**RHHT**NLMIILFSMMLMTGMPQ  
 Danio Rerio LYLM~~GTTG~~K~~SSPNFLQFQNV~~CLKAYLAL**RHHT**NLLIILFSMMLMTGMPQ  
 \*::\*\*::\*\*\*:\* \* :\*\*:::\*\*\*\*\*:\*\*\*\*\*

C



D



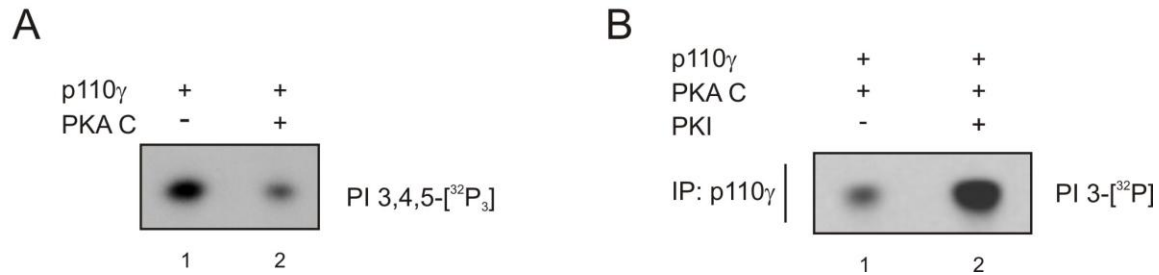
E

T1024

P110 $\gamma$  KKTS~~PH~~--FQ~~KFQD~~ICV~~KAYLALRHHT~~NLLIILFSMMLMTGMP~~QLTSK~~ED  
 p110 $\alpha$  QECT~~KTR~~EFERFQEMCY~~KAYLAI~~**RQHA**NLFINLFSMMLGSGMPELQSFDD  
 p110 $\beta$  TG~~NTEK~~--FGRFRQCCEDAYLIL**RRHG**NLFITL~~FALMLTAGL~~PELTSVKD  
 p110 $\delta$  TN~~NSEK~~--FERFRGYCERAYTIL**RRHG**LLFLHLFALMRAAGLPELSCSKD  
 : \* :\*: \* \*\* :\*: \* :\*: \* :\*: \* :\*: \* . . \*

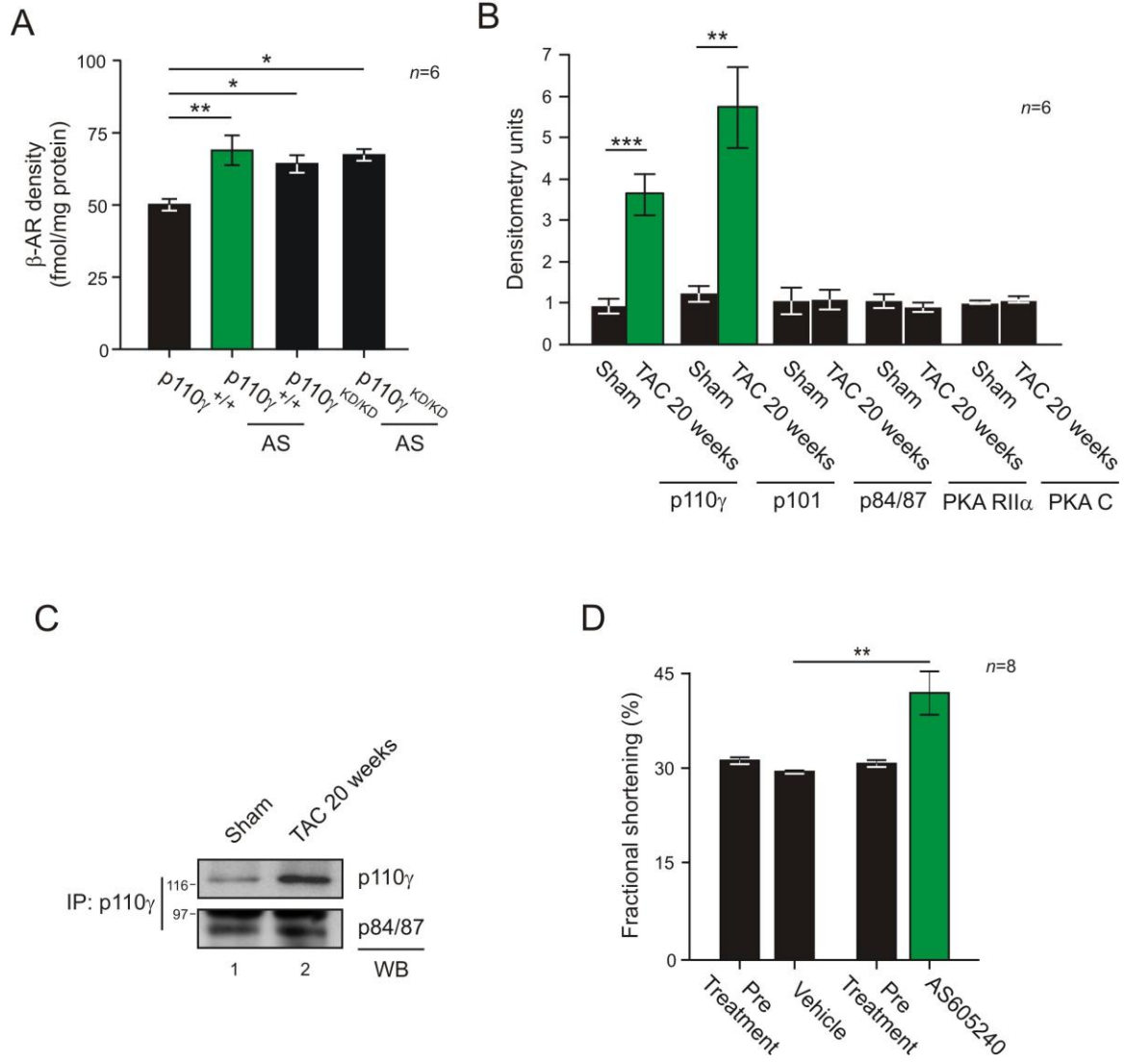
**Figure S5.** Recombinant p110 $\gamma$  is phosphorylated by PKA. Related to Figure 4. **(A)** *In vitro* phosphorylation of recombinant p110 $\gamma$ -GST kinase-dead (p110 $\gamma$ -KD) by recombinant PKA C in the presence of  $^{32}$ P-ATP. p110 $\gamma$ -KD was used instead of wild-type p110 $\gamma$  to avoid any interference by p110 $\gamma$  autophosphorylation. Control is provided by PKA inhibitor PKI (1  $\mu$ M). A representative assay is presented. **(B)** Evolutionary conservation of residues S400 and T1024 (underlined) of p110 $\gamma$ . Alignment of p110 $\gamma$  from the indicated species using ClustalW. **(C)** Binding of the Phospho-Ser/Thr PKA Substrate antibody to a solid-phase peptide array of p110 $\gamma$  incubated with recombinant PKA. Sequences of the peptides spotted on the array are represented on a scheme of p110 $\gamma$ . **(D)** PKA-mediated phosphorylation of p110 $\gamma$ , p110 $\gamma$  S400A or p110 $\gamma$  T1024A immunoprecipitated from HEK293T cells and incubated or not with active PKA (30 min.). **(E)** Alignment (ClustalW) of the four members of Class I PI3K. T1024 is not conserved in Class IA PI3K (p110 $\alpha$ , p110 $\beta$  and p110 $\delta$ ) but is present only in p110 $\gamma$  (underlined).

## Figure S6



**Figure S6.** PKA inhibits p110 $\gamma$  lipid kinase activity. Related to Figure 4. **(A)** Lipid kinase activity of recombinant p110 $\gamma$ -GST (p110 $\gamma$ ) incubated with recombinant PKA (PKA C) or vehicle. The ability of p110 $\gamma$  to phosphorylate PtdIns(4,5)P<sub>2</sub> (PI4,5P<sub>2</sub>) was detected by autoradiography following incubation with <sup>32</sup>P-ATP substrate. A representative assay is presented. **(B)** Lipid kinase activity of p110 $\gamma$  immunoprecipitated from transfected HEK293T cells after incubation with recombinant PKA C. PKA inhibitor PKI (1  $\mu$ M) or vehicle was added to the reaction as indicated. The ability of p110 $\gamma$  to phosphorylate PtdIns (PI) was detected by autoradiography following incubation with <sup>32</sup>P-ATP substrate. A representative assay is presented.

Figure S7



**Figure S7.** p110 $\gamma$  levels and function in the heart. Related to Figures 6 and 7. **(A)** Myocardial  $\beta$ -AR density (fmol/mg protein) of wild-type (p110 $\gamma^{+/+}$ ) or of p110 $\gamma$  kinase-dead (p110 $\gamma^{KD/KD}$ ) mice treated with selective p110 $\gamma$  inhibitor AS605240 (AS 10 mg/kg i.p. q.d. for 3 days) or vehicle. **(B)** Quantitative densitometry of the western blot presented in Fig. 6F. **(C)** Coimmunoprecipitation of p84/87 with p110 $\gamma$  from myocardial lysates of mice subjected to pressure overload for 20 weeks or to sham operation. A representative immunoprecipitation is presented. For all bar graphs, values represent mean  $\pm$  SEM of six independent experiments or six mice per group. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . **(D)** p110 $\gamma$  inhibition ameliorates heart contractility. Related to Figure 7. Left ventricular fractional shortening of wild-type mice subjected to aortic constriction for 20 weeks was measured before (pre treatment) and after one week treatment with vehicle or AS605240 (10 mg/kg i.p. q.d.). Values represent mean  $\pm$  SEM of eight mice per group. \*\* $P < 0.01$ .

**Table S1.** Putative PKA phosphorylation sites on p110 $\gamma$ . Related to Figure 4.

<u>MotifScan</u>	<u>NetPhosK</u>	<u>GPS 2.1</u>
		S27
S53		
		T155
	T227	
T228		
S257	S257	
	T377	
		T399
S400	S400	S400
	S582	
S915		
S1003		
	T1024	T1024

Putative PKA phosphorylation sites on p110 $\gamma$  as predicted by different algorithms.



**Table S2.** Effects of aortic stenosis on cardiomyocytes size.

	Control	Aortic Stenosis
Cardiomyocyte Size	$200.84 \pm 13.50 \mu\text{m}^2$	$381.77 \pm 16.37 \mu\text{m}^2$

Quantifications of cardiomyocytes size in controls and in patients with aortic stenosis (n=6) relative to the immunohistochemistry presented in Figure 7A.

**Table S3.** AS605240 treatment restores cardiac function. Related to Figure 7.

	Vehicle		AS605240	
	Pre Treatment	Post Treatment	Pre Treatment	Post Treatment
FS (%)	29.87 ± 0.74	27.48 ± 1.92	28.47 ± 0.93	38.30 ± 2.60 <sup>a,b</sup>
EF (%)	55.59 ± 1.07	53.74 ± 3.12	55.70 ± 1.36	69.16 ± 3.38 <sup>a,b</sup>
IVSTD (mm)	1.13 ± 0.05	1.17 ± 0.01	1.03 ± 0.03	1.10 ± 0.05
LVEDD (mm)	3.95 ± 0.09	3.94 ± 0.08	3.66 ± 0.14	3.44 ± 0.14
PWTD (mm)	1.14 ± 0.02	1.15 ± 0.02	1.00 ± 0.04	1.03 ± 0.04
RWT	0.58 ± 0.02	0.59 ± 0.01	0.56 ± 0.03	0.62 ± 0.03

Echocardiographic parameters before and after 7 days of treatment of wild-type mice subjected to 20 weeks TAC (FS < 30%) with vehicle (n=8) or p110 $\gamma$  selective inhibitor AS605240 (n=8). FS: percent fractional shortening; EF: percent ejection fraction; IVSTD: interventricular septum thickness in end diastole; LVEDD: left ventricle end diastolic diameter; PWTD: posterior wall thickness in end diastole; RWT: relative wall thickness. <sup>a</sup>*P* < 0.01 post treatment status vs. pre treatment status. <sup>b</sup>*P* < 0.01 AS605240 post treatment status vs. vehicle post treatment status.

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