# Basic Research in Cardiology - Online Supplement

## Conserved expression and functions of PDE4 in rodent and human heart

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### Methods

Heart tissue

Human myocardium was obtained from the left ventricular free walls of the hearts of organ donors for whom no suitable recipients were identified on the United Network for Organ Sharing (UNOS) waiting list at the time of organ procurement ('normal' hearts) and of the explanted hearts of patients with idiopathic dilated cardiomyopathy (IDC) undergoing cardiac transplantation (failing hearts). The use of human tissues was approved by the University of Utah internal review board and conformed to the principles outlined in the 1964 Declaration of Helsinki. All animal studies were performed according to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee of the University of California San Francisco. Human and rodent hearts were quickly dissected, the tissue sections flash-frozen in liquid nitrogen, and stored at -80°C until further use.

Preparation of protein extracts and immunoprecipitation from heart tissue

For generation of protein extracts, frozen heart tissues were homogenized in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.2 mM EGTA, 150 mM NaCl, 1.34 mM β-mercaptoethanol, 10% glycerol, 0.2% NP-40 (nonylphenoxypolyethoxylethanol), 1 µM microcystin-LR, Complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), and 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF; Roche Diagnostics, Indianapolis, IN). After a rotation for 60 min at 4°C, cell debris was pelleted by centrifugation at 20,000 *g* for 30 min and soluble extracts were precleared with a 1 h incubation with 50 µl ProteinG sepharose. After a second centrifugation at 20,000 *g* for 10 min, detergent extracts were subjected to immunoprecipitation (IP) using 30 µl ProteinG sepharose and 2 µg of the respective antibody or normal IgG as a control. After incubation for 4 h at 4°C, the resin was pelleted, washed three times with lysis buffer and PDE recovered in the IP pellet was detected by PDE activity assay or Western blotting.

Cell culture and harvest

Dividing human cardiomyocytes (DHCMs) were obtained from PromoCell GmbH (Heidelberg, Germany). The cells were cultured using PromoCell's Myocyte Growth Medium containing 5% fetal calf serum, 30 µg/ml penicillin, 100 µg/ml streptomycin, 0.5 ng/ml epidermal growth factor (EGF), 2 ng/ml basic fibroblast growth factor (BFGF), and 5 µg/ml insulin. Ventricular cardiac myocytes were isolated from the excised hearts of 1–2 day old neonatal mice as described previously [8]. They were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Nu Serum IV (BD Falcon, Franklin Lakes, NJ), 5% fetal bovine serum (FBS), 1 mM glutamine, 20 µg/ml gentamycin, and 1x ITS media supplement (Sigma-Aldrich,

St. Louis, MO) on plates precoated with 10 μg/ml laminin. Experiments were carried out on day 4 of culture. All cells were cultured at 37°C under a 5% $CO_2$ atmosphere. The day before the experiment, cells were rinsed twice with Hank's balanced salt solution and then cultured in serum-free medium overnight. Cells were pretreated for 5 min with rolipram (10 μM), and subsequently stimulated with isoproterenol (ISO, 1 μM) for another 5 min. Cells were then lysed in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.2 mM EGTA, 150 mM NaCl, 1.34 mM β-mercaptoethanol, 10% glycerol, 0.2% NP-40 (nonylphenoxypolyethoxylethanol), 1 μM microcystin-LR, Complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), and 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF; Roche Diagnostics, Indianapolis, IN).

PDE activity assay

PDE activity was measured according to the method of Thompson and Appleman [11] as described in detail previously [7]. In brief, samples were assayed in a reaction mixture of 200 μl containing 40 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 1 μM cAMP, 0.75 mg/ml bovine serum albumin, and 0.1 μCi of [3H]cAMP for 10 min at 33°C. The reaction was terminated by adding 200 μl of 10 mM EDTA in 40 mM Tris-HCl (pH 8.0) followed by heat inactivation in a boiling water bath for 1 min. The PDE reaction product 5'-AMP was then hydrolyzed by incubation of the assay mixture with 50 μg of *Crotalus atrox* snake venom (Sigma-Aldrich, St. Louis, MO) for 20 min at 33°C, and the resulting adenosine was separated by anion exchange chromatography using 1 ml of AG1-X8 resin (BioRad, Hercules, CA) and quantitated by scintillation counting. PDE subtype activities were defined as the fraction of cAMP-PDE activity inhibited by 20 μM vinpocetine (PDE1), 100 nM Bay60-7550 (PDE2), 1 μM cilostamide (PDE3) or 10 μM rolipram (PDE4), respectively.

Adenylyl cyclase activity assay

Heart tissues were homogenized in ice-cold buffer containing 25 mM Hepes (pH 7.4), 20% w/v sucrose, 150 mM NaCl, 2 mM EDTA, 0.2 mM EGTA, 1 μM microcystin-LR, Complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), and 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF; Roche Diagnostics, Indianapolis, IN) using a glass homogenizer. The cell extracts were then spun for 20 min at 15,000 *g* and 4°C. The resulting pellets were resuspended in lysis buffer and subjected to adenylyl cyclase activity assays according to the method of Alvarez and Daniels [1] with minor variations. In brief, samples were assayed in a reaction mixture of 100 μl containing 40 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 0.2 mM cAMP, 10 mM phosphoenol pyruvate, 3 units of pyruvate kinase, 10 μM GTP, 1 mM ATP, and 2 μCi of [$\alpha^{32}$P]-ATP for 20 min at 37°C. The reaction was terminated with the addition of 20 μl of 2.2 N HCl containing 0.01 μCi [$^3$H]-cAMP (added to determine the recovery of cAMP from subsequent column chromatography) followed by boiling for 2 min. Cyclic AMP was then separated from the substrate ATP by column chromatography using 2.5 $cm^3$ Alumina WN-6. The column was eluted into scintillation vials with 5 ml of 0.1 M ammonium acetate (pH 6.5), the eluate was mixed with 12 ml of Aquasol-2 scintillation fluid (PerkinElmer, Waltham, MA) and the eluted cAMP quantified by scintillation counting.

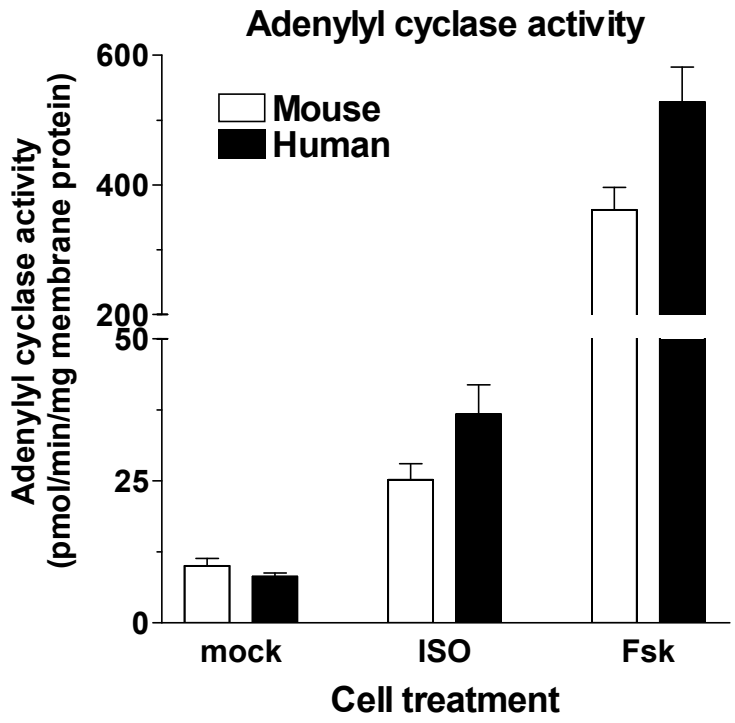
Antibodies

The following antibodies were used in this study: antibody against sarcomeric actinin generated in rabbits (Abcam Inc., Cambridge, MA), calsequestrin and phospholamban antibodies (Affinity

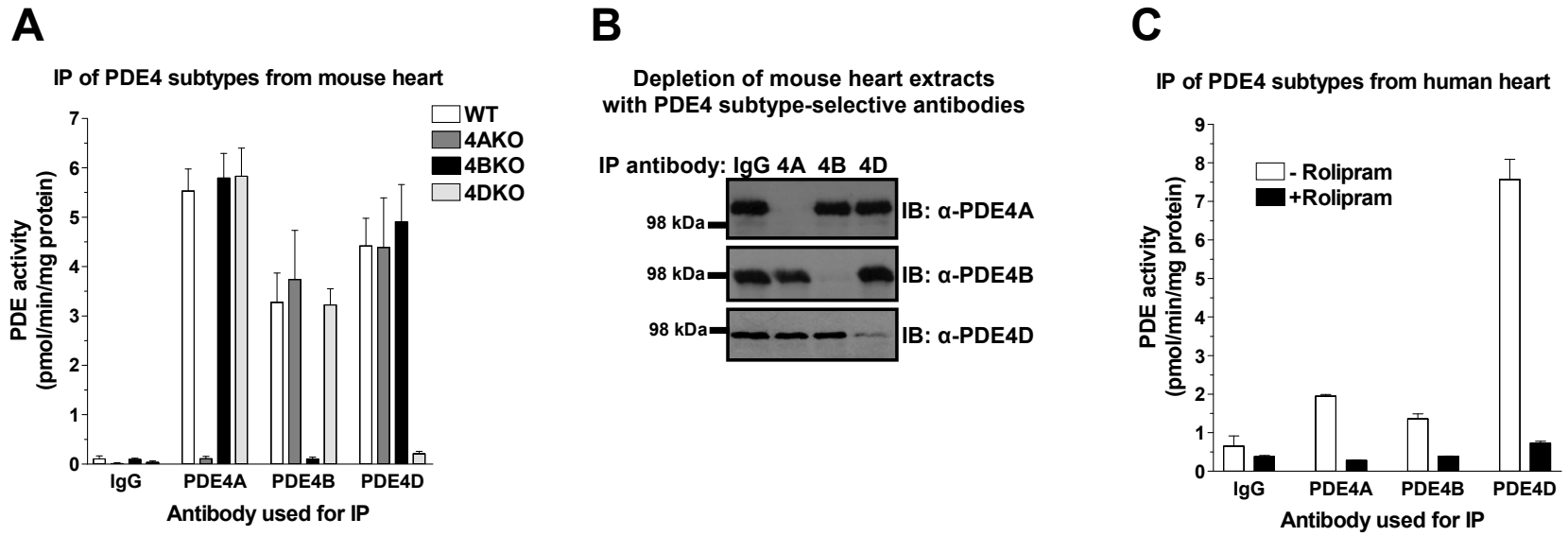
BioReagents, Golden, CO), PKA substrate antibody (Cell Signaling Technology, Danvers, MA), Ser16-phospho-phospholamban antibody (Upstate, Lake Placid, NY), $\beta_1$-adrenergic receptor antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA; sc-568), antibody against sarcomeric actinin generated in mice, α-Flag(M1) resin, α-Flag(M2) antibody and the antibody against α-tubulin (Sigma-Aldrich, St. Louis, MO), and antibodies against individual PDE4A and PDE4B variants (Fabgennix Inc., Frisco, TX). PAN-PDE4 (K116) as well as PDE4 subtype-selective antibodies against PDE4A (AC55), PDE4B (K118), and PDE4D (M3S1) and splice variant selective antibodies against PDE4D3, PDE4D4, PDE4D5, PDE4D8, and PDE4D9 have been described previously [9]. The antibody against PDE4D was a gift from ICOS Corp. (Bothell, WA).

Immunocytochemistry

Portions of human myocardium were fixed in 4% paraformaldehyde at 4°C overnight followed by a 24 h incubation in 50% sucrose in PBS at 4°C. The tissue was then frozen in Tissue-Tek®O.C.T. (Optimal Cutting Temperature) Compound (Sakura Finetek, Torrance, CA) and subsequently sectioned at 7 μm onto Superfrost plus slides (ThermoFisher, Waltham, MA). The slides were stored at -80°C until further use. For immunocytochemistry, sections were re-fixed with 4% paraformaldehyde for 20 min at RT followed by a 5-min incubation with 0.5% Triton X-100 in PBS. Slides were subsequently blocked for 60 min at room temperature with PBS containing 10% normal goat serum, 1% bovine serum albumin, and 0.1% Triton X-100. Slides were then incubated for 2 h at room temperature with anti-sarcomeric actinin antibody and either PAN-PDE4 antiserum (K116) or normal rabbit serum as a control, all diluted 1:500 in blocking buffer. After three washes with blocking buffer, the sections were incubated for 1 h with Fluorescein isothiocyanate (FITC)-labeled anti-rabbit-IgG and cyanine dye 3 (Cy3)-labeled anti-mouse-IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Sections were then washed three times with blocking buffer and one time with PBS, dehydrated and mounted in VectaShield mounting medium (Vector Laboratories, Burlingame, CA).



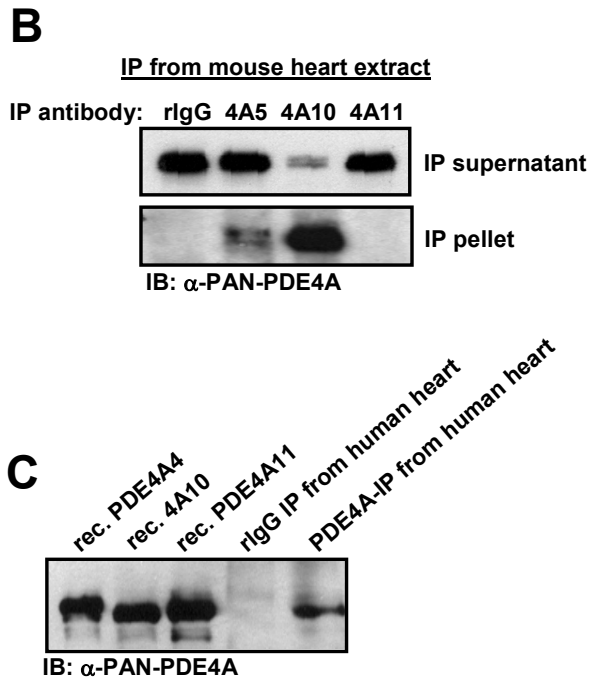

**Supplementary Fig. 1** Comparison of adenylyl cyclase activity in mouse and human heart.
Membrane preparations of mouse and failing human myocardium were subjected to adenylyl cyclase assays in the presence or absence of isoproterenol (ISO; 10 μM) or forskolin (Fsk; 100 μM). Data represent the mean ± S.E.M. of four experiments




**Supplementary Fig. 2** Characterization of PDE4 subtype-selective antibodies.
**a** Detergent extracts prepared from wild type as well as PDE4A-, PDE4B-, and PDE4D-knock out mouse hearts were subjected to immunoprecipitations with PDE4 subtype-selective antibodies or normal IgG as a control. PDEs recovered in the IP pellets were detected by PDE activity assay using 1 μM cAMP as substrate. Data represent the mean ± S.E.M. of three experiments. The data suggest that IP with PDE4 subtype-selective antibodies is highly specific for the targeted PDE4 subtype as IP of PDE activity is completely ablated when the respective PDE4 knock-out mouse tissues are used. **b** Detergent extract prepared from WT mouse heart was subjected to IP with PDE4 subtype-selective antibodies or normal IgG as a control. The amount of PDE4 remaining in the post-IP supernatants after IP depletion was detected by Western blotting. The data suggest that IP with PDE4 subtype-selective antibodies is highly efficient as the targeted PDE4 subtypes are efficiently and selectively depleted from tissue extracts. **c** Detergent extracts prepared from human myocardium were subjected to IP with PDE4 subtype-selective antibodies or normal IgG as a control. PDE activity recovered in the IP pellets was measured in the absence or presence of the PDE4-specific inhibitor rolipram. Shown is one representative IP experiment with PDE activity measured in triplicate. Although PDE4 represents only a minor portion of total cAMP-PDE activity expressed in human heart, the PDE activity recovered in IP pellets with PDE4 subtype-selective antibodies is solely due to the rolipram-sensitive PDE4. This suggests that IP with subtype-selective PDE4 antibodies is also highly specific using human heart samples

**A**

| PDE4A variant | Amino acid residues | Migration in SDS/PAGE (kDa) | mRNA tissue distribution |
|---|---|---|---|
| PDE4A1 | 647 | 83 | brain |
| PDE4A4/5 | 886 | 125 | brain |
| PDE4A7 | 324 | 37 | widely distributed |
| PDE4A8 | 763 | 125 | brain, skeletal muscle, testis |
| PDE4A10 | 825 | 121 | widely distributed |
| PDE4A11 | 860 | 126 | widely distributed |




**Supplementary Fig. 3** PDE4A10 is the predominant PDE4A variant expressed in heart tissue.
Six variants of PDE4A, PDE4A1, PDE4A4/5, PDE4A7, PDE4A8, PDE4A10 and PDE4A11, are known to date [6,12]. PDE4A4 and PDE4A5 designate the human and mouse orthologs of the same variant, respectively. **a** Characterization of PDE4A variants. Amino acid residues, migration in SDS/PAGE and mRNA tissue distribution refer to the human variants and were taken from [2,4-6,12]. Due to shorter C-terminal sequences, the apparent migration of rodent PDE4A variants is 10-15 kDa lower compared to human variants. **b** Detergent extracts prepared from mouse heart were subjected to pull-down assays using variant-specific antibodies for PDE4A4/5, PDE4A10, PDE4A11 or normal rabbit IgG as a control. PDE4A variants recovered in the IP pellet (bottom panel) and those remaining in the post-IP supernatant (top panel) are detected by immunoblotting using a PAN-PDE4A antibody. PDE4A10 is the predominant PDE4A variant expressed in mouse heart. PDE4A4/5 contributes a minor portion to total PDE4A protein in the heart. **c** Comparison of SDS/PAGE migration of recombinant human PDE4A4, PDE4A10 and PDE4A11, with endogenous PDE4A immunoprecipitated from human heart. Migration of the endogenous PDE4A band is consistent with that of PDE4A10

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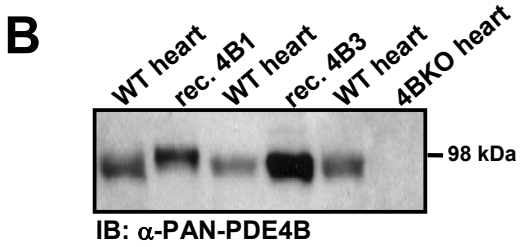
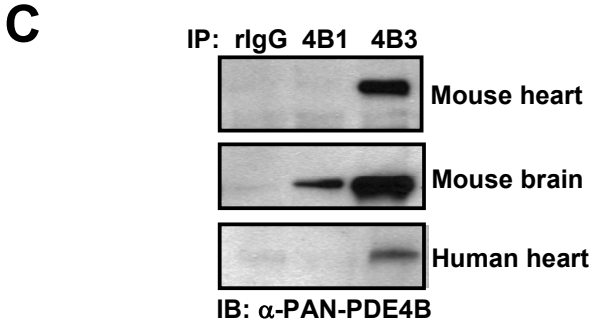
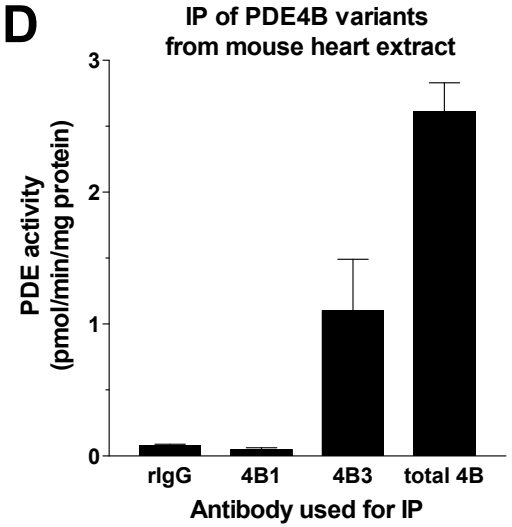
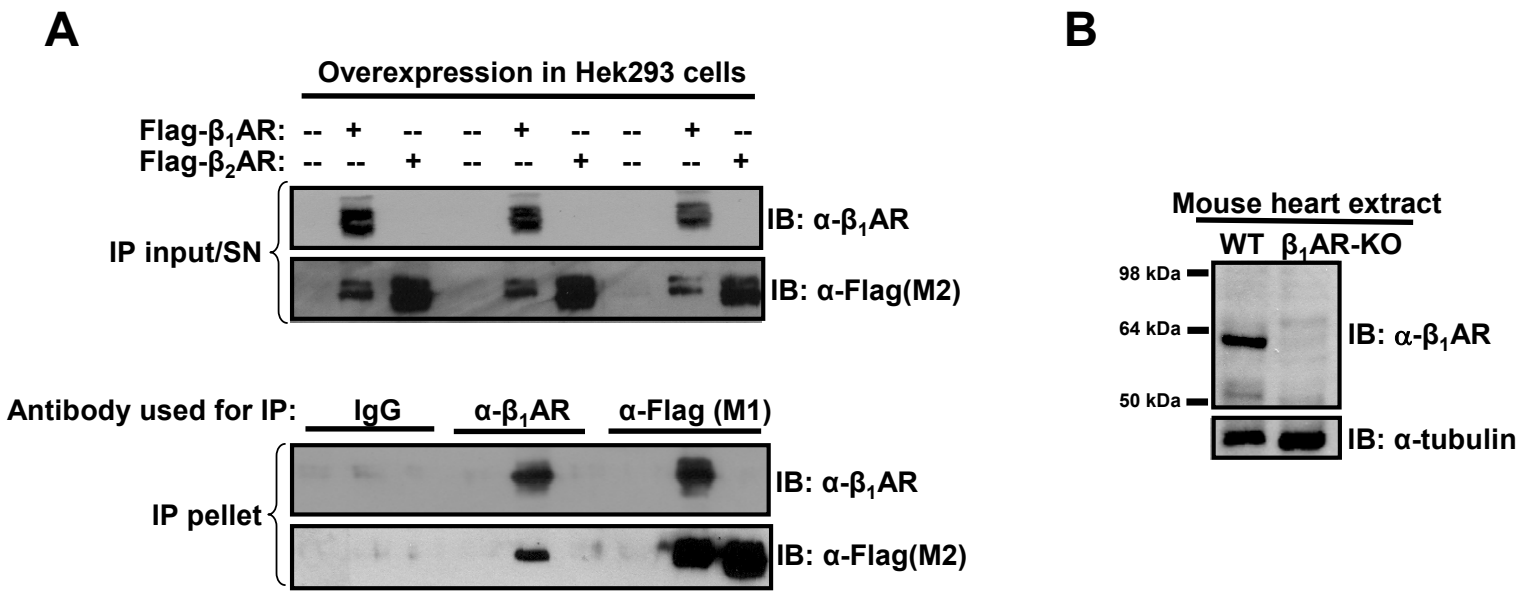
| PDE4B variant | Migration in SDS/PAGE (kDa) According to [3] | According to * |
|---|---|---|
| PDE4B1 | 104 | 107 |
| PDE4B2 | 68 | 78 |
| PDE4B3 | 103 | 100 |
| PDE4B4 | 84 | 66 |
| PDE4B5 | 58 | |




**Supplementary Fig. 4** PDE4B3 is the predominant PDE4B variant expressed in heart tissue.
Thus far, five PDE4B variants, PDE4B1-PDE4B5, have been reported [3]. **a** Published data on the migration of distinct PDE4B variants in SDS/PAGE taken from [3] or the Fabgennix Inc. (Frisco, TX) website *. Migration of the 95 kDa PDE4B-immunoreactive band observed in the hearts of rodents and humans in the present study (Fig. 3A) is most compatible with the reported migration for PDE4B3. **b** Comparison of the SDS/PAGE migration of recombinant PDE4B1, recombinant PDE4B3 and endogenous PDE4B detected in heart extracts from wild type and PDE4BKO mice. The endogenous PDE4B migrates similar to recombinant PDE4B3, and both migrate faster than recombinant PDE4B1. **c** PDE4B3, but not PDE4B1, can be immunoprecipitated from mouse and human heart. **d** Comparison of the amount of PDE4B activity immunoprecipitated with a PDE4B3-selective antibody and a PAN-PDE4B antibody. The PDE4B3 antibody immunoprecipitates about half of the total PDE4B activity from mouse heart. This observation is consistent with the level of PDE4B protein detected in IP pellets by immunoblotting. This suggests that either part of the PDE4B3 protein is not accessible to IP with the PDE4B3 antibody or that there is an additional PDE4B variant present in mouse heart




**Supplementary Fig. 5** Characterization of $\beta_1$AR antibodies.
**a** Detergent extracts prepared from Hek293 cells overexpressing Flag-tagged $\beta_1$AR or Flag-tagged $\beta_2$AR were subjected to IP with normal IgG as a control, the α-$\beta_1$AR antibody from Santa Cruz Biotechnology Inc. (sc-568; Santa Cruz, CA) or α-Flag(M1) antibodies as described previously [8]. Both the extracts used as IP input and the βAR protein recovered in IP pellets were probed by Western blotting with either α-$\beta_1$AR or α-Flag(M2) antibodies. The $\beta_1$AR antibody from Santa Cruz Biotechnology Inc. interacts efficiently with $\beta_1$AR in both Western blots and pull down assays, and does not cross-react with the $\beta_2$AR. **b** Detergent extracts prepared from whole hearts of wild type or $\beta_1$AR-knock-out mice [10] were probed with the $\beta_1$AR antibody from Santa Cruz Biotechnology Inc. in Western blots

Supplementary Table 1 Donor information for the left ventricular tissues analyzed in Fig. 5.

| Donor | Age | Gender | Total PDE Activity | PDE4A Activity | PDE4D Activity |
|---|---|---|---|---|---|
| | (Y) | (M/F) | (% of average IDC) | | |
| IDC1 | 54 | M | 59.6 | 136.7 | 98.5 |
| IDC2 | 62 | M | 136.2 | 96.1 | 103.3 |
| IDC3 | 35 | F | 141.8 | 67.9 | 119.2 |
| IDC4 | 46 | M | 117.6 | 122.5 | 107.3 |
| IDC5 | 39 | M | 78.6 | 114.8 | 108.4 |
| IDC6 | 49 | M | 84.4 | 95.1 | 69.5 |
| IDC7 | 24 | M | 81.7 | 62.6 | 77.9 |
| Normal1 | 61 | F | 85.1 | 176.9 | 151.4 |
| Normal2 | 55 | M | 86.7 | 193.4 | 97.2 |
| Normal3 | 40 | F | 83.5 | 136.9 | 131.1 |
| Normal4 | 15 | M | 124.9 | 90.4 | 134.8 |

**Supplementary References**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