SUPPLEMENTAL MATERIAL

Phosphodiesterase 3A and arterial hypertension

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Running title: **Phosphodiesterase 3A**

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German family with HTNB, substitution G449S

The 54 year-old female patient (Fig. 3; I/2) with a 17 year history of hypertension, cerebral seizures and mild hypercholesterolemia was admitted to hospital by an ambulance service due to severe hypertension (190/100 mmHg, HR 90/min). She initially presented with low sodium (128 mEq/l) and low potassium (3,4 mEq/l) presumably as a result of an antihypertensive treatment including hydrochlorothiazide. A 24 h blood pressure record with concomitant medication showed marked hypertension with missing nightly dipping. Laboratory results did not reveal any other reasons for secondary hypertension. She exhibited no signs of sleep apnea.

Antihypertensive treatment has proven difficult. Today she is treated by seven antihypertensive drugs including an aldosterone-antagonist and minoxidil to achieve a quite normal blood pressure of 130/80 mmHg.

Supplemental Table I. Treatment regime of an 54 year-old HTNB patient with a G449S substitution in PDE3A.

Drug	**Intake**
RAMIPRIL 10 mg	0-0-1-0
SPIRONOLACTONE 50 mg	1-0-0-0
AMLODIPINE 10 mg	1-0-0-0
CARVEDILOL 25 mg	1-0-1-0
DOXAZOSIN 4 mg	1-0-0-1
MOXONIDINE 0,2 mg	0-0-0-1
MINOXIDIL 2,5 mg	1-0-1-0

We did not find any hypertensive target organ complications. She did not show microalbuminuria, renal failure, left-ventricular hypertrophy or macrovascular damage. She reported not being diagnosed with fundus hypertonicus.

Due to her radiologically verified brachydactyly type E she was suspected of having HTNB, confirmed by a heterozygous G to A substitution in the *PDE3A* gene (c.1345G>A; cDNA. 1385G<A; Genebank transcript ID NM_000921) leading to an exchange of amino acid Glycine 449 to Serine (G449S).

Her 23-years-old daughter (Figure 3; II/2) exhibits also brachydactyly type E. Mutation testing revealed the identical mutation in exon 4 of the *PDE3A* gene. 24 h blood pressure recording showed mean blood pressure values SBP/DBP of 132.2 / 91.0 mmHg during daytime and 126.8 / 83.8 mmHg during night time, representing moderate age-related hypertension.

Expanded Methods

Generation of mouse model overexpressing PDE3A2-T445N in smooth muscle

State of Berlin authorities approved the mouse studies (license no. G 0046/17). The inducible mouse model (B6N-Gt(ROSA) 26Sor$^{tm1(CAG-PDEA3A*T445N)Geno}$) was custom-made by Genoway (Lyon, France). The insertion of the human transgene was targeted to the Rosa26 locus *via* homologous recombination in embryonic stem cells (Supplemental Figure IIA). The *PDE3A2* cDNA encoding the T445N substitution[4] and the polyA signal of the human growth hormone (hGH pA) was placed downstream of the inverted strong and ubiquitous CAG promotor, a fusion of the CMV immediate early enhancer and the chicken β-actin promotor, flanked by a combination of *lox*P and *lox*2272 sites. The inverted promoter is activated in the presence of Cre. To generate mice expressing smooth muscle cell specifically the human PDE3A2-T445N transgene (B6N-Gt(ROSA)26Sor$^{tm2(CAG-PDEA3A*T445N)Geno}$), male hemizygous mice for the smMHC/Cre/eGFP transgene (Jackson laboratory stock number 7742) were crossed with female hemizygous B6N-Gt(ROSA)26Sor$^{tm1(CAG-PDEA3A*T445N)Geno}$) mice.

Echocardiography

Rats were anesthetized by inhaling a 1.5-2 % isoflurane oxygen mixture in a heat-controlled chamber to keep the body temperature stable. After reaching full anesthesia, rats were fixed on a heated plate with electrodes on their paws to control heart and respiratory rate, as well as to record the electrocardiogram. Body temperature was monitored by a rectal sensor and corrected by a heating lamp if necessary. With the ultrasonic detector MS-250 collected data were visualized and analyzed *via* the VEVO 2100 high-resolution imaging system (Visualsonics Fujifilm,

VisualSonics, Toronto, Ca). Stroke volume and cardiac output was measured by tracing the endocardium in systole and diastole of a parasternal long axis view of the left ventricle.

Perfusion with Microfil for µCT

The rats were deeply anaesthetized with isoflurane (4-5 %) and metamizol (100 µg/kg) was administered for analgesia 30 min before the procedure. Heparin was administered *s.c.* (1000 IU in 200 µL). The animal was intracardially perfused with approximately 50 mL of warm heparanized normal saline solution, followed by 8 mL of Microfil (Flow Tech, Inc., Carver, Massachusetts, USA) solution. After the perfusion, 90 min was allowed for the contrast agent to harden. The brain was gently removed and kept in 4 % paraformaldehyde solution overnight.

µCT was performed on the next day using a Skyscan 1276 scanner (Bruker, Kontich, Belgium) and the following acquisition parameters: source voltage of 85 kV, source current of 200 µA, Al+Cu filter, exposure time of 590 ms, 5 μm^3 voxel size, rotation step of 0.15°, and frame averaging of 4. The images were reconstructed with Nrecon (Bruker), applying the ring artefact correction = 7 and the beam hardening correction = 8. CTVox (Bruker) was used for image display.

Treatment of rats with the sGC stimulator, BAY 41-8543

Rats were treated with the sGC stimulator, BAY 41-8543, purchased from Cayman Chemical and used in a formulation of Transcutol/Cremophor-EL/water (10 %:20 %:70 %). The solution was prepared at 37 °C as described.[9] The material was initially dissolved in Transcutol, Cremophor was added, and the solution stirred for 30

minutes. Prior to administration, saline was added to produce the desired dilution. The solution was administered per gavage to reach a dose of 3 mg/kg.

Measurements of isometric contractions of blood vessels

First-order mouse mesenteric arteries or rat aorta were removed immediately after sacrificing the animals under inhalation anesthesia with isoflurane by cervical dislocation, quickly transferred to cold (4 °C), oxygenated (95 % O_2/ 5 % CO_2) physiological salt solution (PSS) containing (in mmol/L) 119 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 25 $NaHCO_3$, 1.2 $MgSO_4$, 11.1 glucose, 1.6 $CaCl_2$. The vessels were dissected into 2 mm (mesenteric artery) or 5 mm (rat aorta) rings whereby perivascular fat and connective tissue were removed without damaging the adventitia. Mesenteric rings were positioned on two stainless steel wires (diameter 0.0394 mm) in a 10-ml organ bath of a Mulvany Small Vessel Myograph (DMT 610 M; Danish Myo Technology, Denmark). The organ bath was filled with 5 ml PSS. The bath solution was continuously oxygenated with a gas mixture of 95 % O_2 and 5 % CO_2 and kept at 37 °C (pH 7.4). The mesenteric rings were placed under a tension equivalent to that generated at 0.9 times the diameter of the vessel at 100 mm Hg by stepwise distending vessels using LabChart DMT Normalization module. This normalization procedure was performed to obtain the passive diameter of the vessel at 100 mm Hg. The basal tone of aortic rings was continuously monitored and adjusted to 1 g using the Organ Bath System (AD Instruments Ltd. Spechbach, Germany). The software Chart5 (AD Instruments Ltd. Spechbach, Germany) was used for data acquisition and display. After 60 min equilibration, vessels were pre-contracted with phenylephrine (PE) until a stable resting tension was acquired. Forskolin was directly added to the bath solution.

Tension is expressed as a percentage of the steady-state tension (100 %) obtained with PE. Salts and other chemicals were obtained from Sigma-Aldrich (Germany). Forskolin was dissolved according to the material sheet with 95 % ethanol.

Telemetric blood pressure measurements in mice

Male mice (16–26 g, 12–15 weeks) were used in all experiments. They were anesthetized with isoflurane (CuraMed Pharma GmbH, Karlsruhe, Germany). The pressure-sensing catheter of the TA11-PAC10 device (Data Science International) was placed into the left carotid artery in mice. As in rats, blood pressure and heart rate was recorded using the DATAQUEST software (A.R.T. 2.1, Data Sciences International). Also, the data were evaluated as of rats.

Histological staining and detection of cAMP in second-order mesenteric arteries

Formalin-fixed, paraffin-embedded sections (2 µm) of mouse and rat aortas were subjected to hematoxylin and eosin stain using standard protocols. Slides were scanned by Pannoramic MIDI automatic digital slide scanner (3DHISTECH) and analyzed by CaseViewer 2.3 (3DHISTECH). Media thickness was measured at 5-9 places in mice and 18-25 places in rats at positions with clear media-intima border with perfect transversal cut and at straight parts of the media.

Second-order rat mesenteric arteries were removed immediately after sacrificing the animals under inhalation anesthesia with isoflurane, quickly transferred to cold (4 °C), oxygenated (95 % O_2/5 % CO_2) physiological salt solution (PSS) containing (in mmol/L) 119 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 25 $NaHCO_3$, 1.2 $MgSO_4$, 11.1 glucose, 1.6 $CaCl_2$. The vessels were dissected into 4 mm rings whereby perivascular fat and

connective tissue were removed without damaging the adventitia. Arterial rings were incubated at 37 °C for 30 minutes. After removing the PSS, samples were frozen using liquid nitrogen and stored at -80 °C until they were subjected to radioimmunoassays (RIA).

Arterial rings were homogenized in 500 µl ice-cold 70 % ethanol by 20 strokes of a glass/glass Potter-Elvehjem homogenizer. After centrifugation (20,000 x g, 15 min, 4 °C), supernatants were dried at 100 °C under nitrogen, dissolved in 50 µl 100 mM sodium acetate, pH 6.0, and cAMP content was determined by RIA (Brooker 1979, pubmed.gov/222120). Pellets were dried at 60 °C, dissolved in 300 µl 0.1 M NaOH, 0.1 % sodium dodecyl sulfate and protein content was determined using Pierce BCA protein assay (Thermo Fisher Scientific).

The mesenteric arteries were dissected and fixed in 4 % buffered formalin and second-order mesenteric arteries were isolated keeping the surrounding fat tissue intact, processed with graded alcohol, paraffin-embedded, and stored at room temperature. Five-micrometer sections were cut using a rotary microtome (Microm HM355s, Thermo Fisher Scientific, Germany) and placed on Superfrost/Plus microscope slides (Thermo Fisher Scientific, Germany) and allowed to air dry. For morphological analysis, slides were deparaffinized in xylene, rehydrated in graded ethanols, and stained with Hematoxylin-Eosin (Sigma, Germany). The slides were then dehydrated in graded alcohols and xylene and coverslipped using Eukit (Sigma). Microscopic images were taken using an inverted microscope (Keyence BZ-9000, Germany) measurement and morphological analysis (media-to-lumen area) was performed using Keyence BZ-9000 Analysis Software System (Keyence, Germany) and quantified using Excel software followed by GraphPad Prism.

Cell proliferation assay

Thoracic aorta was obtained from male SD rats and VSMC were isolated as previously described.[13] The culture medium was Dulbecco's modified Eagle's media (DMEM; Gibco 21885-025) with 100 U/ml penicillin/100 µg/ml streptomycin (Biochrom (Merck) A2213), containing 10 % fetal bovine serum (Biochrom (Merck) S0615). Cells were maintained at 37 °C and 5 % CO_2. VSMCs of passages 5–9 were trypsinized and seeded in 12 well-plates at a density of 1 x 10^4 cells/ml. The cells were counted using a scepter device (Millipore Scepter Handheld automated cell counter, # PHCC00000) 3-7 days after the seeding.

Fluorescence resonance energy transfer (FRET) measurements

HEK293 cells (3 x 10^5) were seeded on glass coverslips, pre-coated with poly-L-lysine hydrobromide (Sigma, P5899-5MG) in 6-well plates. Transient transfections were performed after 24 hours using 2 µl Lipofectamine (Invitrogen, 11668-019) and 1 µg ICUE3 and PDE3A2-mCherry constructs, or the controls, cyan fluorescent protein (CFP) and Venus either alone or together, or a CFP-yellow fluorescent protein (YFP) tandem construct.

The cytosolic Epac-based cAMP sensor, ICUE3, consists of a cAMP binding domain of Epac flanked by CFP and Venus. Upon cAMP binding, the sensor undergoes a conformational change that increases the distance between CFP and Venus and thus decreases FRET. FRET measurements to determine PDE3A activity were carried out 24 hours after transfection. For this purpose, the sensor was co-expressed with WT or mutant (T445N, G449S, and Δ3aa deletion) PDE3A2-mCherry in HEK293 cells. Measurements of CFP or Venus alone as well as non-interacting co-

expressed CFP and Venus served as negative controls, while a CFP-YFP tandem construct served as a FRET positive control set to 100 %. Furthermore, independent measurements of the ICUE3 sensor under basal conditions as well as upon stimulation were performed (Supplemental Figure III).

The FRET measurements were performed in the presence of forskolin alone or in the presence of combinations of forskolin with milrinone, cilostamide or Bay 41-8543. Coverslips were placed on an inverted confocal laser scanning microscope (CLSM510-META-NLO; Carl Zeiss) equipped with a Plan-Neofluar 40x/1,3 (Oil). The excitation of CFP was at 810 nm (Chameleon^{TM} diode-pumped laser; Coherent), while the emission was detected from 430 to 505 nm. For the excitation of Venus or YFP, an argon laser at 514 nm was used and the fluorescence was measured between 520 and 560 nm. The excitation of mCherry was at 543 nm and an LP 560 nm long-pass emission filter was used for the fluorescence detection. In order to measure the FRET effect, the excitation was performed with the wavelength of 810 nm and the emission was detected in the spectral region from 430 to 655 nm. To calculate the FRET-based fluorescence, a λ-stack with a linear spectral unmixing mode was used in order to correct any CFP fluorescence cross-talking into the FRET channel (523-532 nm). The λ-stack is an integral part of the confocal laser system software. Furthermore, YFP correction was carried out in order to correct for direct excitation of the acceptor during donor excitation. The percentage change in the FRET signal (ΔFRET (%)) upon treatment was plotted. There was a direct correlation between the ΔFRET and the cAMP concentration.

Immunoprecipitation and phosphorylation detection

HEK293 cells (4 x 10^6) were seeded in a 10-cm petri dish and cultured for 24 h. Cells were transfected with 30 µl PEI (1 mg/ml; Linear Polyethylenimine 25,000, Polysciences, Inc.) and 5 µg PDE3A2-Flag constructs (WT, T445N, and Δ3aa) and cultured for another 24 h. Cells were stimulated for 30 min with 20 µM forskolin (Sigma-Aldrich), 100 ng/ml PMA (Sigma-Aldrich) or with 0.1 % EtOH (Sigma-Aldrich; for controls). Cells were scraped in mild lysis buffer (2 mM EDTA, 2 mM EGTA, 0.2 % Triton X in 1x PBS) supplemented with protease and phosphatase inhibitors (Complete and PhosSTOP, Roche Diagnostics) and lysed by use of 26G needles (Sterican, Braun). Lysates were cleared by centrifugation (21,250 x g, 10 min, 4 °C) and protein concentration was determined with Bradford reagent (Thermo Scientific). Anti-Flag M2 Magnetic Beads (Sigma-Aldrich) were washed with TBS, supplemented with protease and phosphatase inhibitors. The beads (40 µl) were added to 2 mg of protein and incubated overnight at 4 °C on a rotating device. The supernatant was denatured with 4x Lämmli sample buffer (50 mM Tris-HCl, pH 6.8, 4 % glycerol, 1.6 % SDS with 4 % β-mercaptoethanol) and the beads were washed with cold TBS, supplemented with protease and phosphatase inhibitors (Complete and PhosSTOP, Roche Diagnostics). 4x Lämmli sample buffer was added and the samples were boiled at 95 °C for 5 min. Western Blotting and detection with both 14-3-3θ antibody and phospho-site-directed antibodies was performed as described below.

Detection of PDE3A2 in murine and rat tissue

Bladder and skeletal muscle from mice and aorta from rat were sheared in Speed Mill tubes (Speed Mill P12, Analytik Jena) filled with RIPA buffer (50 mM Tris pH 7.8,

10 % glycerol, 150 mM NaCl, 1 % Triton X, 0.025 % sodium deoxycholate and 1 mM EDTA), supplemented with protease and phosphatase inhibitors (Complete and PhosSTOP, Roche Diagnostics) and lysed by freezing in liquid nitrogen and thawing. Lysates were cleared by centrifugation (21,250 x g, 10 min, 4 °C) and protein concentrations were determined with Bradford Reagent (Thermo Scientific). 4x Lämmli sample buffer (50 mM Tris-HCl, pH 6.8, 4 % glycerol, 1.6 % SDS with 4 % β-mercaptoethanol) was added to 20 µg of protein. The samples were boiled at 95 °C for 5 min and subjected to Western blotting as described below.

Western Blotting

Proteins were separated by 8 % or 10 % SDS-PAGE and transferred to PVDF membranes (0.45 mM, #T830.1; Carl Roth) by Turbo Blotting (30 min, 25 V; (Turbo Blot, Biorad) in SemiDry Buffer (480 mM Tris-HCl, 390 mM Glycine, 0.375 % SDS). The membranes were blocked in 5 % skimmed milk powder (Sigma-Aldrich) for 1 hour at room temperature. Primary antibodies were incubated overnight at 4 °C in 5 % skimmed milk and 0.1 % Tween. Membranes were washed 3x in TBS / 0.05 % Tween and incubated with secondary antibody for 2 hours at room temperature. Membranes were washed 3x in TBS / 0.05 % Tween and proteins were detected after short incubation in Immobilon™ Western ECL substrate (#WBKLS0500; Millipore) at an Odyssey FC device (Li-cor Biosciences). Signals were quantified and semi-quantitatively analyzed with Image J software and Graph Pad Prism.

qRT-PCR

Total RNA from snap-frozen tissues was isolated using the RNeasy RNA isolation kit (Qiagen) according to manufacturer's instruction after homogenization with a Precellys 24 homogenizator (Peqlab). In case of mesenterial arteries, the samples were pooled in order to ensure a sufficient total RNA concentration for a successful cDNA transcription. Quality and quantity of isolated RNA were determined by NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific). RNA (2 µg) was transcribed into cDNA (Applied Biosystems). Quantitative analysis of target mRNA expression was performed with qRT-PCR using the relative standard curve method. TaqMan and SYBR green analysis was conducted using QuantStudio 3 RT-PCR System (Applied Biosystems).

Primers for the detection of mouse and human PDE3A:

NM_018779.2 Mus musculus *Pde3a*, mRNA

product length = 86
Forward primer 1 CCAAGACCTAGCCAAGCCG 19
Template bp345 - 363

Reverse primer 1 GAGAGGACTCGCCACGATG 19
Template bp430 - 412

NM_000921.4 Homo sapiens *PDE3A*, transcript variant 1, mRNA

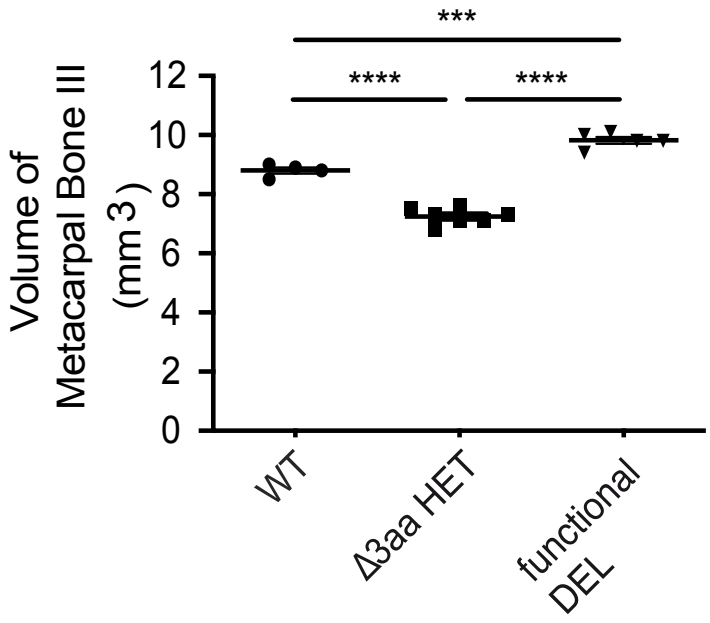
product length = 115
Forward primer 1 ACGGTTGGCAGGCATAGAAA 20
Template bp3322 - 3341

Reverse primer 1 CTCGCCTCTTGGTTTCCCTT 20
Template bp3436 – 3417

Supplemental Figures

Supplemental Figure I

A.



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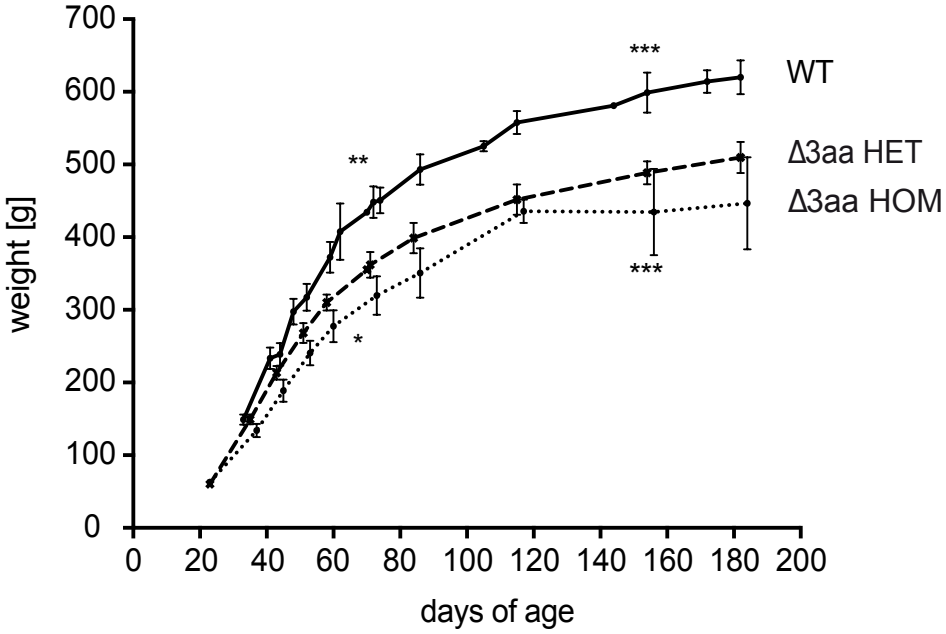
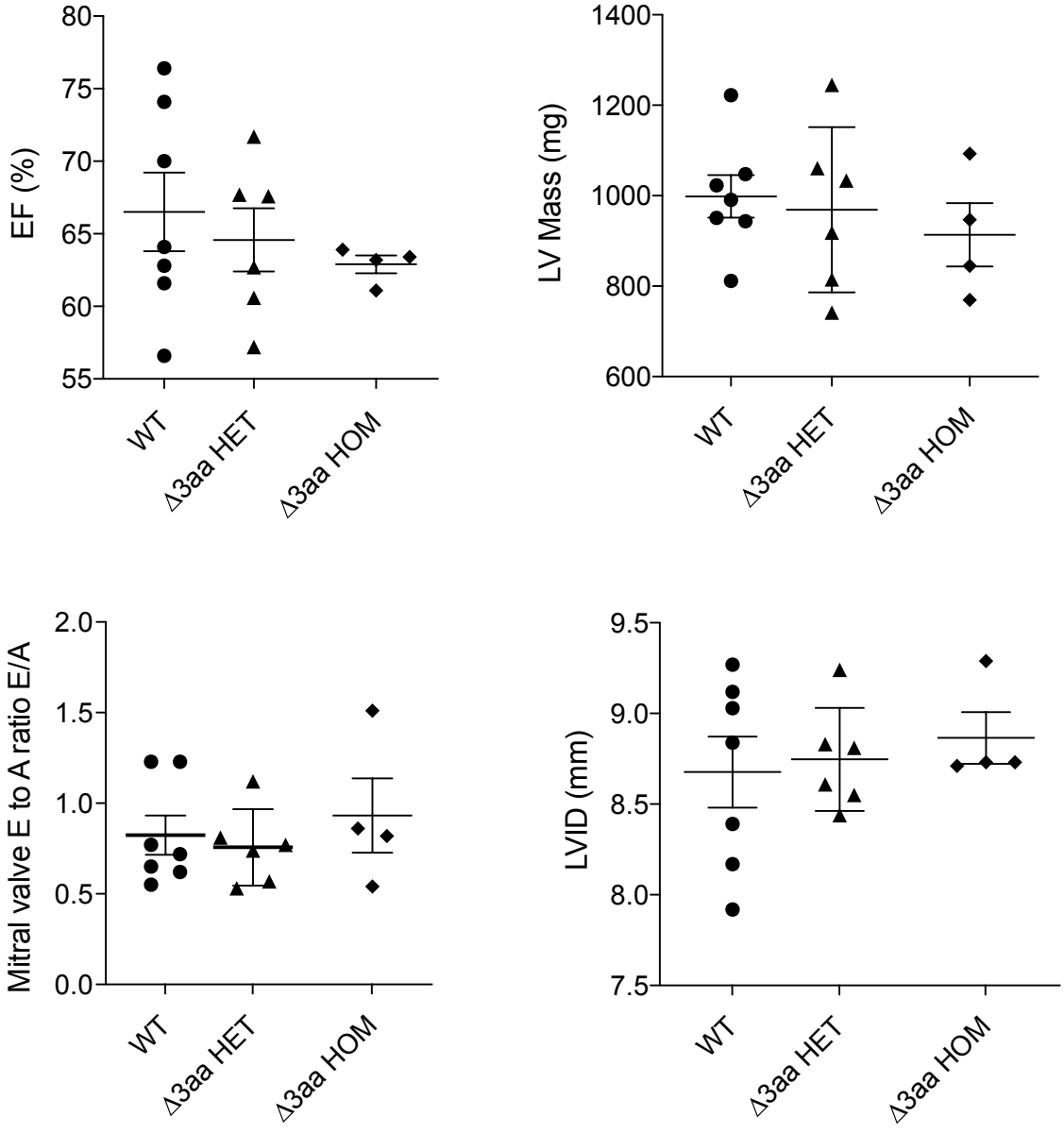
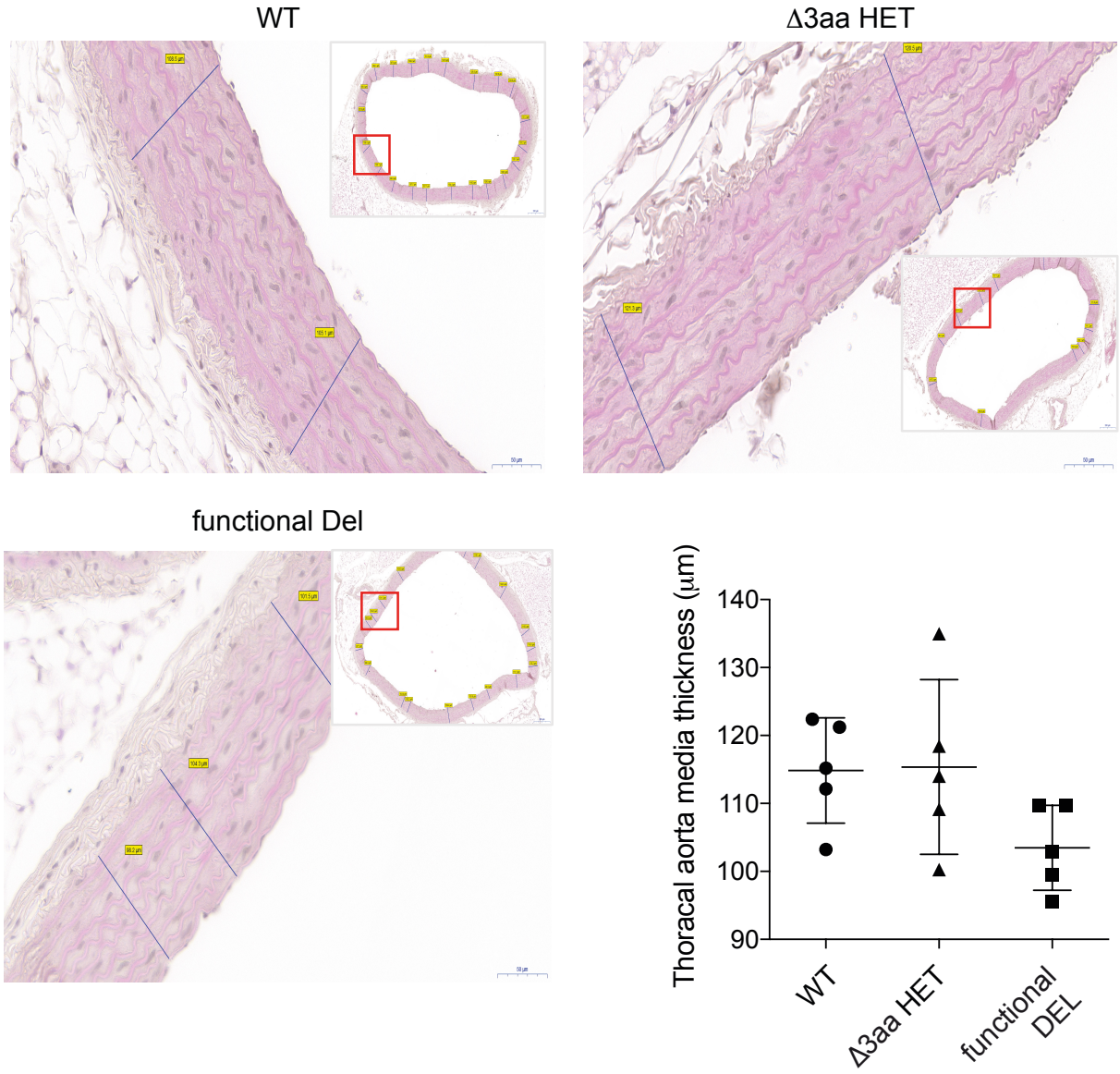


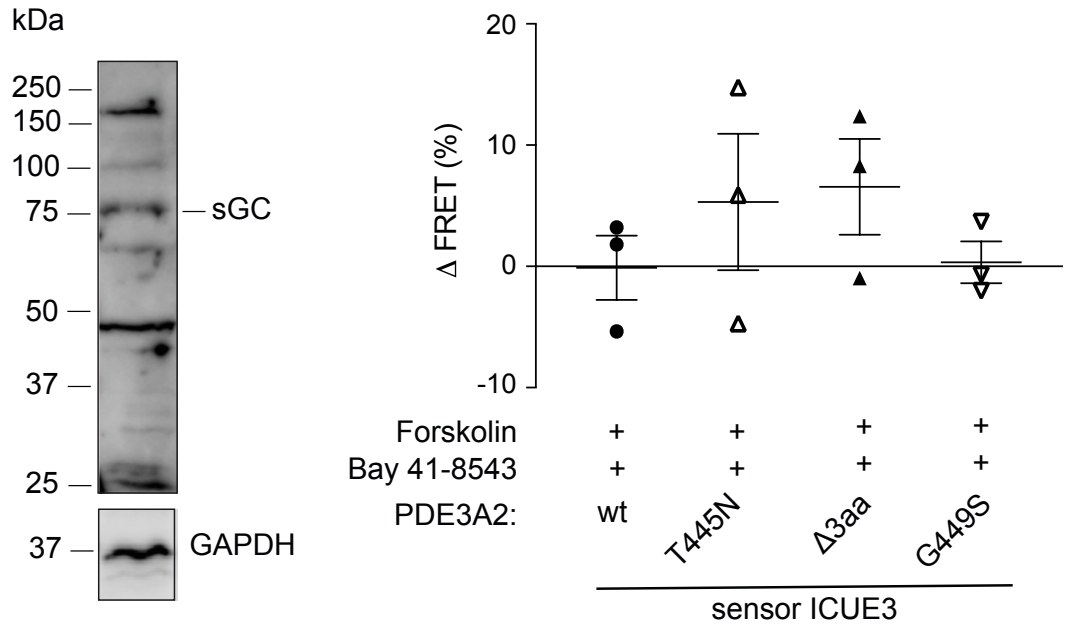
C.


EF (%)
80
75
70
65
60
55
WT
Δ3aa HET
Δ3aa HOM
LV Mass (mg)
1400
1200
1000
800
600
WT
Δ3aa HET
Δ3aa HOM
Mitral valve E to A ratio E/A
2.0
1.5
1.0
0.5
0.0
WT
Δ3aa HET
Δ3aa HOM
LVID (mm)
9.5
9.0
8.5
8.0
7.5
WT
Δ3aa HET
Δ3aa HOM

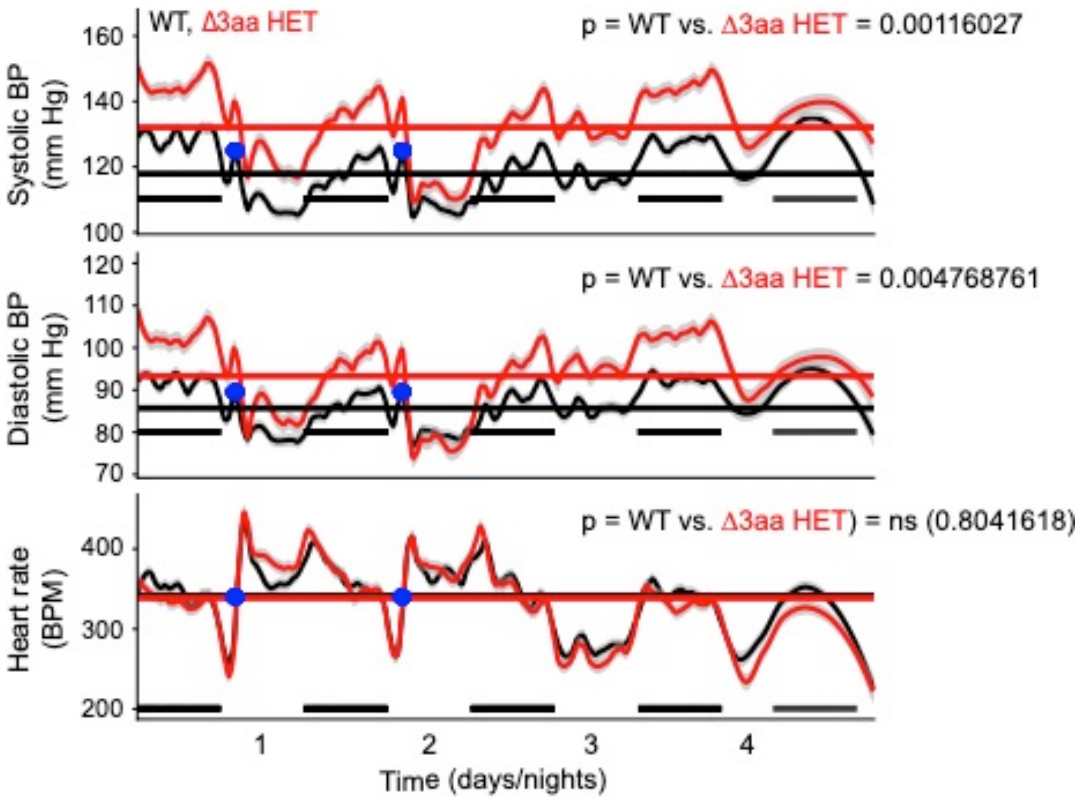
D.


WT
Δ3aa HET
functional Del
Thoracal aorta media thickness (µm)
140
130
120
110
100
90
WT
Δ3aa HET
functional DEL

E.


kDa
250
150
100
75
50
37
25
37
sGC
GAPDH
Δ FRET (%)
20
10
0
-10
Forskolin
Bay 41-8543
PDE3A2:
wt
T445N
Δ3aa
G449S
sensor ICUE3

F.


WT, Δ3aa HET
p = WT vs. Δ3aa HET = 0.00116027
Systolic BP (mm Hg)
160
140
120
100
p = WT vs. Δ3aa HET = 0.004768761
Diastolic BP (mm Hg)
120
110
100
90
80
70
p = WT vs. Δ3aa HET) = ns (0.8041618)
Heart rate (BPM)
400
300
200
1
2
3
4
Time (days/nights)

G.

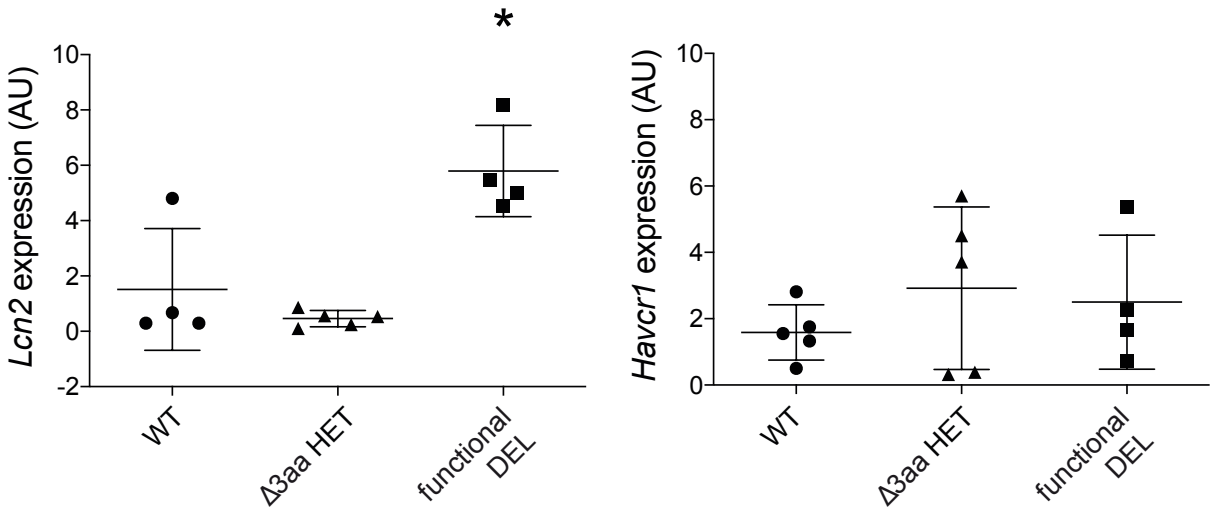


Figure I. Δ3aa rats recapitulate the HTNB phenotype and their blood pressure can be lowered by sGC stimulation. A. Shown is the volume of metacarpal bone III of the left forepaw. n = 4-6 animals per genotype. **B.** The Δ3aa HET rats are lighter than wild type animals. n = 2-6 animals per genotype were weighed at the indicated time points. Data are mean ± SEM, significant differences from WT are indicated, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. **C.** The indicated cardiac parameters of WT, heterozygous Δ3aa (HET) and homozygous Δ3aa (HOM) PDE3A rats were estimated by echocardiography. The differences were statistically not significant. **D.** HE staining of aorta from WT, Δ3aa HET and functional DEL rats with quantification of the media thickness are shown. Representative images of stainings from 5 animals per group are shown (mean ± SD). Significant differences were not observed. **E.** The left panel shows sGC expression in HEK293 cells detected by Western blotting. Right panel, FRET measurement to analyze the effect of the sGC activator Bay 41-8543 on PDE3A2 WT and the indicated mutant versions. The cells were incubated with Bay 41-8543 (15 µM, 15 min), forskolin (30 µM) was added, and the measurement immediately started. Bay 41-8543 inhibited the Δ3aa PDE3A2 mutant apparently stronger than the WT enzyme. However, the difference did not reach statistical significance. n = 3 independent experiments per PDE3A2 version. **F.** Telemetric blood pressure measurements of WT (black) and rats Δ3aa HET rats (red) treated with single-dose gavage (3 mg/kg); blue dots) on days 1 and 2. n = 5 WT and 4 Δ3aa HET; p values are indicated. A summary of the data is shown in the main manuscript as Figure 4J.

G. Detection of mRNA expression of the renal damage markers Lcn2 and Havcr1 in WT, Δ3aa HET and functional DEL rats. n = 4 or 5 animals per group. mean ± SD, p values are indicated.

Supplemental Table II. Statistical comparison between WT, Δ3aa HET and HOM and functional DEL rat models. Shown are the p values for the data depicted in Fig. 4C of the main manuscript.

P (genotype rats, systolic) = 3.371249e-08
P (genotype rats, diastolic) = 1.127179e-17
P (genotype rats, heart rate) = 0.00034653

Systolic WT *vs.* functional DEL = 0.000781638481121916
Systolic WT *vs.* Δ3aa HET = 1.87298941789806e-05
Systolic WT *vs.* Δ3aa HOM = 0.00390622244654646
Systolic functional DEL *vs.* Δ3aa HET =? 6.46910576721325e-08
Systolic functional DEL *vs.* Δ3aa HOM = 5.63714446462809e-06
Systolic Δ3aa HET *vs.* Δ3aa HOM = 0.664605889221758

Diastolic WT *vs.* functional DEL = 0.0471172223084532
Diastolic WT *vs.* Δ3aa HET = 1.82252752067942e-06
Diastolic WT *vs.* Δ3aa HOM = 8.257144939323e-07
Diastolic functional DEL *vs.* Δ3aa HET = 1.80855674803871e-06
Diastolic functional DEL *vs.* Δ3aa HOM = 3.21764541459098e-12
Diastolic Δ3aa HET *vs.* Δ3aa HOM = 0.00186857643850765

HR WT *vs.* functional DEL = 1.24113096775992e-05
HR WT *vs.* Δ3aa HET = 0.375719381357057
HR WT *vs.* Δ3aa HOM = 0.905779123838626
HR functional DEL *vs.* Δ3aa HET = 2.20296081439421e-06
HR functional DEL *vs.* Δ3aa HOM = 0.0183140364364043
HR Δ3aa HET *vs.* Δ3aa HOM = 0.793912351589182

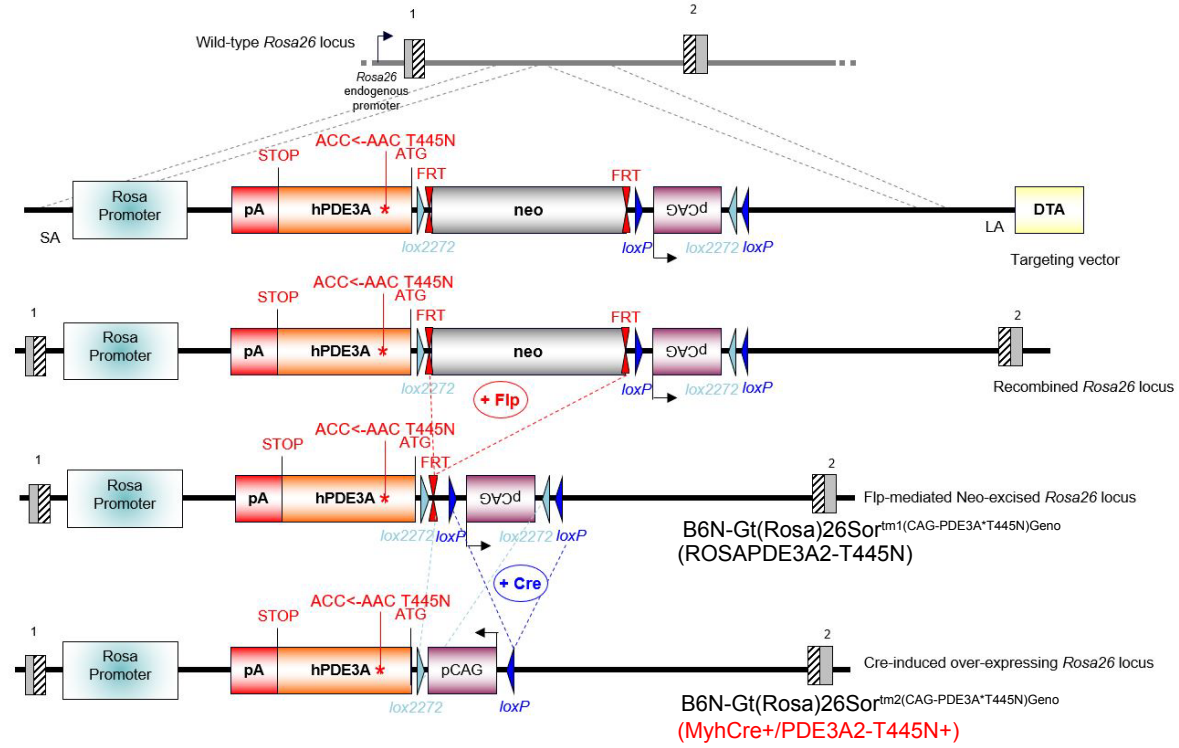
Overexpression of hyperactive PDE3A2 causes hypertension in mice

The T445N-encoding mutation as a human PDE3A2 transgene was expressed in VSMC (MyhCre+/PDEA3A2-T445N+) by crossing with a smooth-muscle myosin heavy-chain Cre strain (Supplemental Figures IIA). Hemizygous transgenic mice were used for the analysis. Of 50 litters, 180 mice (45.6 % male, 54.4 % female) were born resulting in a mean litter size of 3.6 offspring. 17.2 % of the offspring were negative for both the smMHC/Cre/eGFP and the PDE3A2-T445N, 38.9 % were positive for smMHC/Cre/eGFP transgene and 17.2 % for the PDE3A2-T445N transgene only and 26.7 % were positive for both transgenes. However, only 35 % of the double-positive mice showed Cre-mediated promoter cassette inversion (17 of the 180 animals). They were reduced in bodyweight (weight of WT males at 70 days around 25 g, of transgenes around 15 g) and lived shorter than the control animals. Compared to controls, about 50 % of females and 80 % of males were alive at day 25 (Supplemental Figure IIB). The reason for the smaller litter sizes and shorter survival rates of the mutants is unclear. Overexpression of the human PDE3A2 protein in the transgenic animals was initially confirmed using smooth muscle bladder tissue (Supplemental Figure IIC). Due to the low level of protein expression of PDE3A in vessels, mRNA expression was evaluated. The human transgene *PDE3A2* mRNA was detected in bladder, aorta and first- and second-order mesenteric arteries of the mutant animals, but not in the controls (Supplemental Figure IID). However, leaky expression of the transgene in skeletal muscle was observed. mRNA expression of the endogenous mouse *Pde3a* was detected in all tissues to similar degrees in transgenic and control animals (Supplemental Figure IID). The transgenic mice were hypertensive with SBP of about 126 mm Hg, DBP of about 85 mm Hg while controls were 115/84 mm Hg and

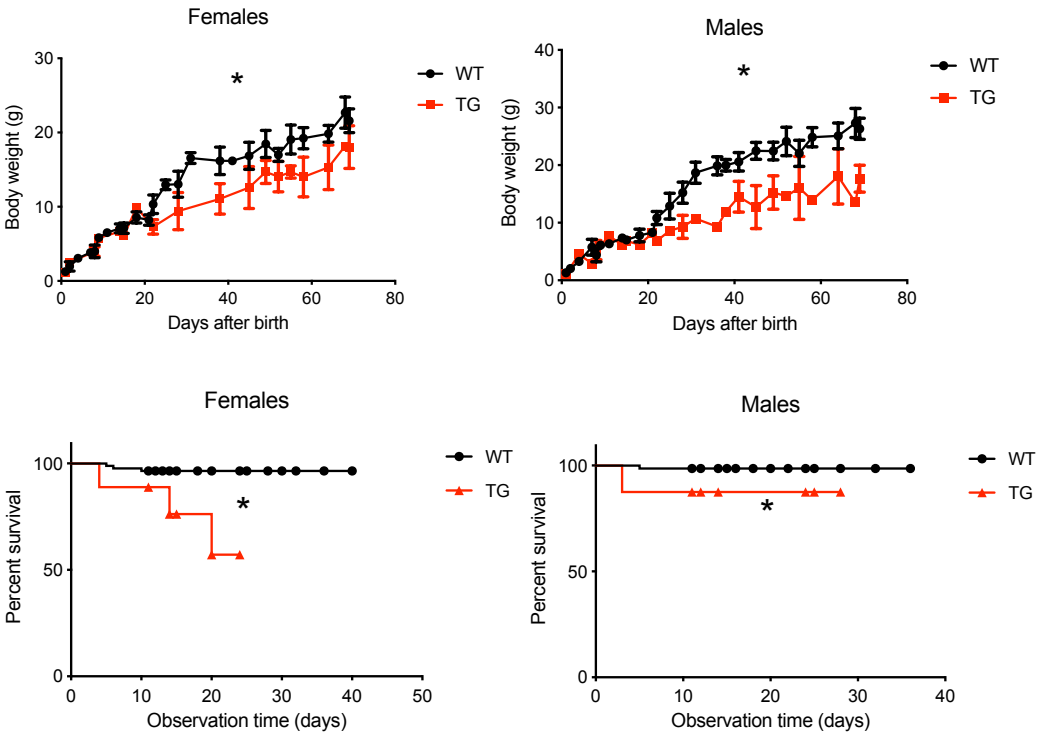
their heart rate was decreased (Supplemental Figure IIE). Blood pressure data of two mice, not included in the analysis because they did not survive the 10-day radio-telemetry observation, are documented in Supplemental Figure IIF. One mouse had a blood pressure of 155/125 mm Hg for 5 days before it died. Another mouse was fairly normotensive for 5 days and then developed a massive increase in blood pressure to 270/200 mm Hg before it died. Thus, the mouse model underscores that mutant PDE3A causes the hypertension. The media of aorta from the transgenes was not different from the control animals (Supplemental Figure IIG). We analyzed contractility of mesenteric arteries from the transgene-expressing mice. The vessels, pre-constricted with the adrenergic agent phenylephrine, reached around 25 % less dilation than the arteries from control animals in response to stimulation with forskolin (Supplemental Figure IIH), suggesting an enhanced level of cAMP degradation. Parameters of the RAAS system (Supplemental Figure III) and the renal damage markers Lcn2 and Havcr1 (Supplemental Figure IIJ) were similar in control and transgenic mice. The results indicate that overexpression of hyperactive PDE3A2 in VSMC results in hypertension.

Supplemental Figure II

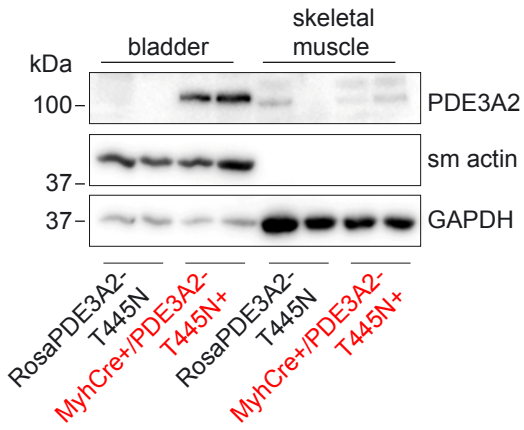
A.



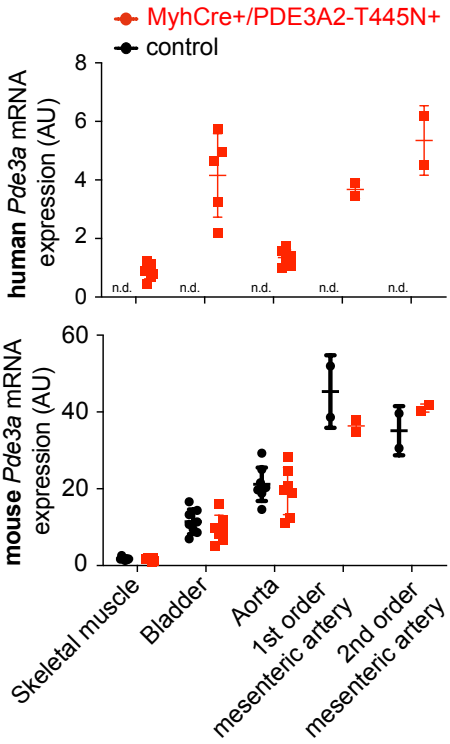
B.



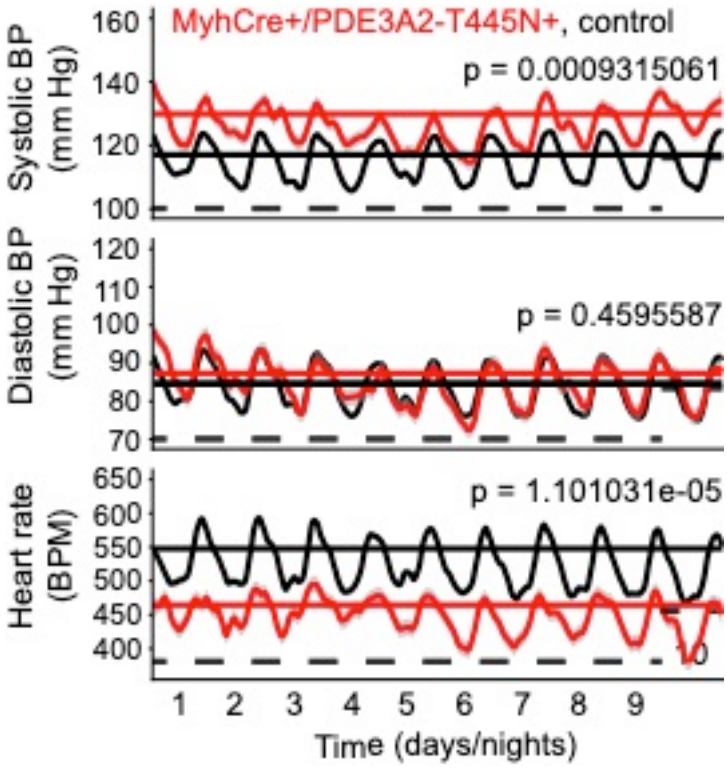
C.


bladder
skeletal muscle
kDa
100
37
37
PDE3A2
sm actin
GAPDH
RosaPDE3A2-T445N
MyhCre+/PDE3A2-T445N+
RosaPDE3A2-T445N
MyhCre+/PDE3A2-T445N+

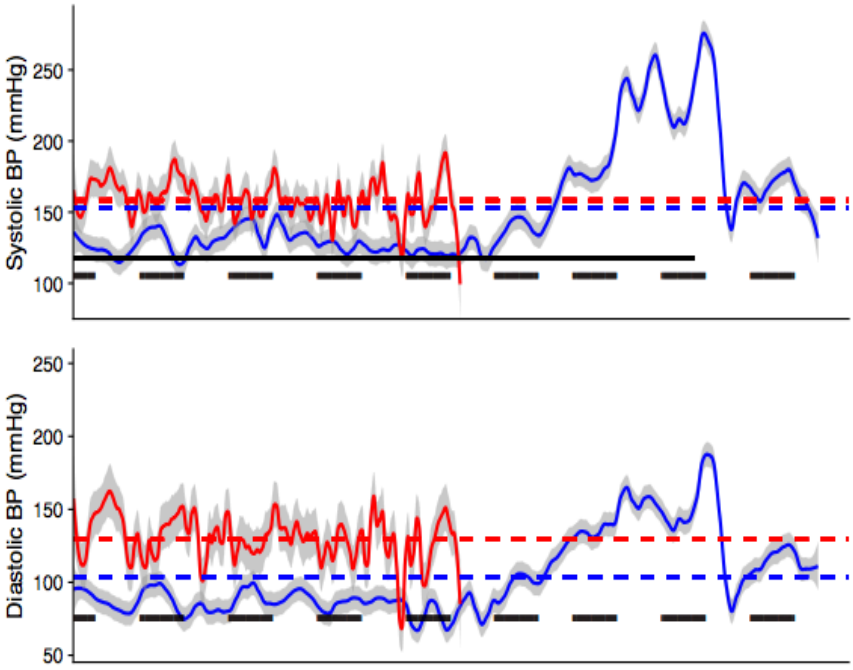
D.


MyhCre+/PDE3A2-T445N+
control
human Pde3a mRNA expression (AU)
mouse Pde3a mRNA expression (AU)
n.d.
Skeletal muscle
Bladder
Aorta
1st order mesenteric artery
2nd order mesenteric artery

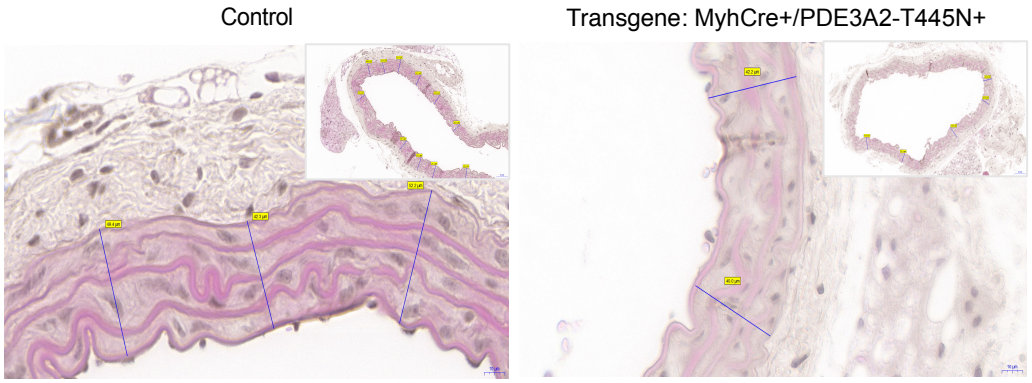
E.

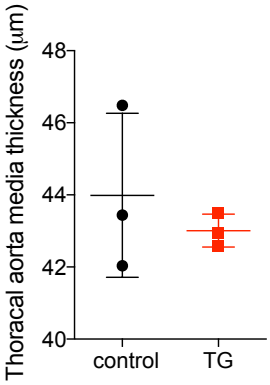

MyhCre+/PDE3A2-T445N+, control
p = 0.0009315061
p = 0.4595587
p = 1.101031e-05
Systolic BP (mm Hg)
Diastolic BP (mm Hg)
Heart rate (BPM)
Time (days/nights)

F.

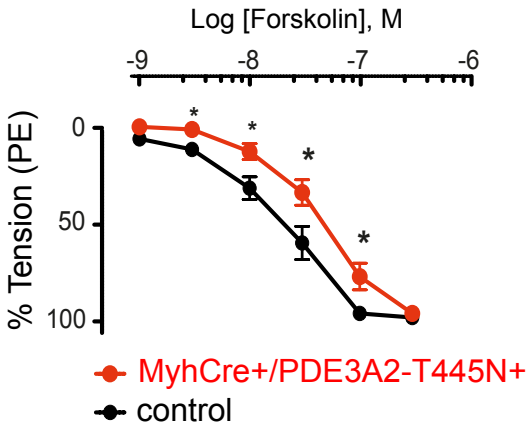

Systolic BP (mmHg)
250
200
150
100
Diastolic BP (mmHg)
250
200
150
100
50

G.

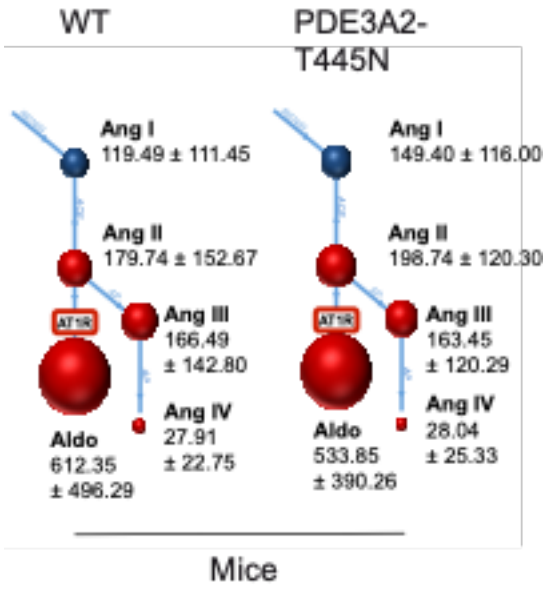

Control
Transgene: MyhCre+/PDE3A2-T445N+


Thoracal aorta media thickness (µm)
48
46
44
42
40
control
TG

H.


Log [Forskolin], M
-9
-8
-7
-6
% Tension (PE)
0
50
100
*
*
*
*
MyhCre+/PDE3A2-T445N+
control

I.


WT
PDE3A2-T445N
Ang I
119.49 ± 111.45
Ang II
179.74 ± 152.67
AT1R
Ang III
166.49
± 142.80
Ang IV
27.91
± 22.75
Aldo
612.35
± 496.29
Ang I
149.40 ± 116.00
Ang II
198.74 ± 120.30
AT1R
Ang III
163.45
± 120.29
Ang IV
28.04
± 25.33
Aldo
533.85
± 390.26
Mice

J.

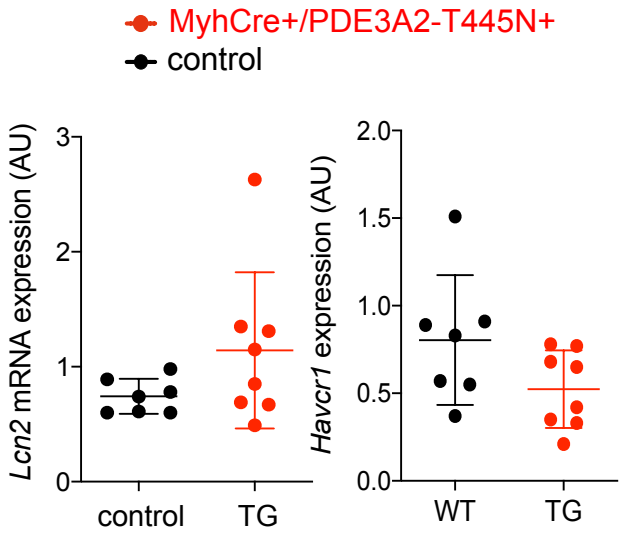


Figure II. Overexpression of human PDE3A2-T445N in vascular smooth muscle cells causes hypertension in mice. A. A transgenic mouse model, (B6N-Gt(ROSA)26Sort$^{m1(CAG-PDEA3A*T445N)Geno}$; RosaPDE3A2-T445N), for overexpression of human PDE3A2-T445N was generated according to the scheme. When crossed with the smooth muscle myosin heavy chain Cre (B6.Cg-Tg(Myh11-cre,-EGFP)2Mik/J) line, the transgene is activated in all smooth muscle cells by inversion of the CAG-promotor (B6N-Gt(ROSA)26Sor$^{tm2(CAG-PDEA3A*T445N)Geno}$ MyhCre+/PDE3A2-T445N+). Hemizygous animals were used for the analysis. **B.** Weight and Kaplan-Meier survival curves are shown in WT and transgenic PDE3A2-T445N overexpressing mice (MyhCre+/PDE3A2-T445N+). **C.** Bladder is rich in smooth muscle tissue and was therefore compared with skeletal muscle and analyzed for human PDE3A2 expression by Western blotting. A representative blot shows expression patterns of two transgenes (MyhCre+/PDE3A2-T445N+) compared to two control animals (RosaPDE3A2-T445N) without expression. Smooth muscle actin (sm actin) indicates smooth muscle tissue and GAPDH is the loading control. **D.** Detection of overexpressed human *PDE3A* (upper panel) and endogenous mouse *Pde3a* (lower panel) mRNA in the indicated organs by qRT-PCR. RNA was isolated from n = 8 control and transgenic mice. For the detection of expression in first- and second-order arteries, 4 samples were pooled. **E.** Mice overexpressing the human PDE3A under the promoter of the smooth muscle myosin heavy chain (MyhCre+/PDE3A2-T445N+) are hypertensive as measured by radio-telemetry and have a reduced beat-per-minute

(BPM) heart rate. The plots show time (horizontal axis, night phases marked in black) and blood pressure (systolic/diastolic mm Hg). Curves show *loess* fits made using R package ggplot2 with gray intervals indicating 95 % confidence intervals for *loess* parameters. Horizontal lines show model expectation value for WT (black, n = 5) and PDE3A2 mice (red, n = 3), respectively. Significance (nested model likelihood ratio test p value) and effect size (model fit parameter, same unit as vertical axis) is noted in the figure. **F.** We show two examples from the transgenic mouse model. In red is a mouse that we could observe for about 5 days. This animal had a systolic blood pressure of >150 mm Hg, and a diastolic blood pressure of 130 mm Hg. The animal died after 5 days. The mouse in blue was actually normotensive for 5 days and then exhibited an increase in blood pressure so that on days 7-8 values of 250-270/150-170 mm Hg were recorded until this animal died. **G.** HE staining of aorta from control and transgenic mice with quantification of the media thickness are shown. Representative images of stainings from 3 animals per group are shown (mean ± SD).

H. Phenylephrine-preconstricted mesenteric vessels were isolated and tested for their responsiveness to forskolin (adenylate cyclase stimulator). n = 8 rings, means ± SEM, *p<0.05. **I.** The indicated parameters of the renin-angiotensin-aldosterone system were investigated in wild-type (WT), mice and in mice with smooth muscle-specific overexpression of human PDE3A2-T445N. Similar sizes of spheres indicate similar relative concentrations (all in pmol/L). n = 9-10 per mouse group. Values are medians. n.d., not determinable; n.a., not available. **J.** Detection of mRNA expression of the renal damage markers Lcn2 and Havcr1 in control and transgenic (TG) mice. n = 7 or 8 animals per group.

Supplemental Table III. Plasma and urine parameters determined in PDE3A2-T445N transgenic mice. Plasma and urine parameters determined of control and transgenic mice (Transgene). Shown are plasma, urine and calculated values. Statistical comparison was carried out by unpaired t-test. The values determined in transgenic mice were not different from the control animals (n = 9-10). Values are means ± SD. FE, fractional excretion

Serum parameters	**Control**	**Transgene**
Sodium (mmol/L)	159.96 ± 3.33	157.39 ± 3.35
Potassium (mmol/L)	8.45 ± 0.56	7.95 ± 1.51
Chloride (mmol/L)	103.63 ± 1.73	106.02 ± 3.74
Urea nitrogen (mg/dL)	51.63 ± 7.86	49.94 ± 2.96
Creatinine (mg/dL)	0.10 ± 0.01	0.09 ± 0.01
Cystatin (mg/L)	0.07 ± 0.02	0.04 ± 0.02
Angiotensinogen (µg/ml)	28.31 ± 5.48	25.44 ± 7.26
Renin (ng/ AngI/ml/h)	33.51 ± 31.92	68.84 ± 61.98
Urine parameters		
Sodium (mmol/L)	125.54 ± 39.32	170.85 ± 92.36
Potassium (mmol/L)	173.20 ± 27.15	161.73 ± 27.86
Chloride (mmol/L)	165.60 ± 39.01	195.49 ± 105.40
Urea nitrogen (mg/dL)	7280.43 ± 1639.29	7758.23 ± 872.43
Creatinine (mg/dL)	24.10 ± 5.71	31.08 ± 8.47
Albumin (mg/dL)	0.32 ± 0.12	0.64 ± 0.55
Calculated parameters		
FENa (%)	0.25 ± 0.13	0.28 ± 0.17
FEK (%)	5.74 ± 2.28	5.71 ± 3.63
FECl (%)	0.45 ± 0.21	0.55 ± 0.29
Urine/ albumin creatinine (mg/mmol)	1.46 ± 0.43	2.06 ± 1.48

Supplemental Figure III

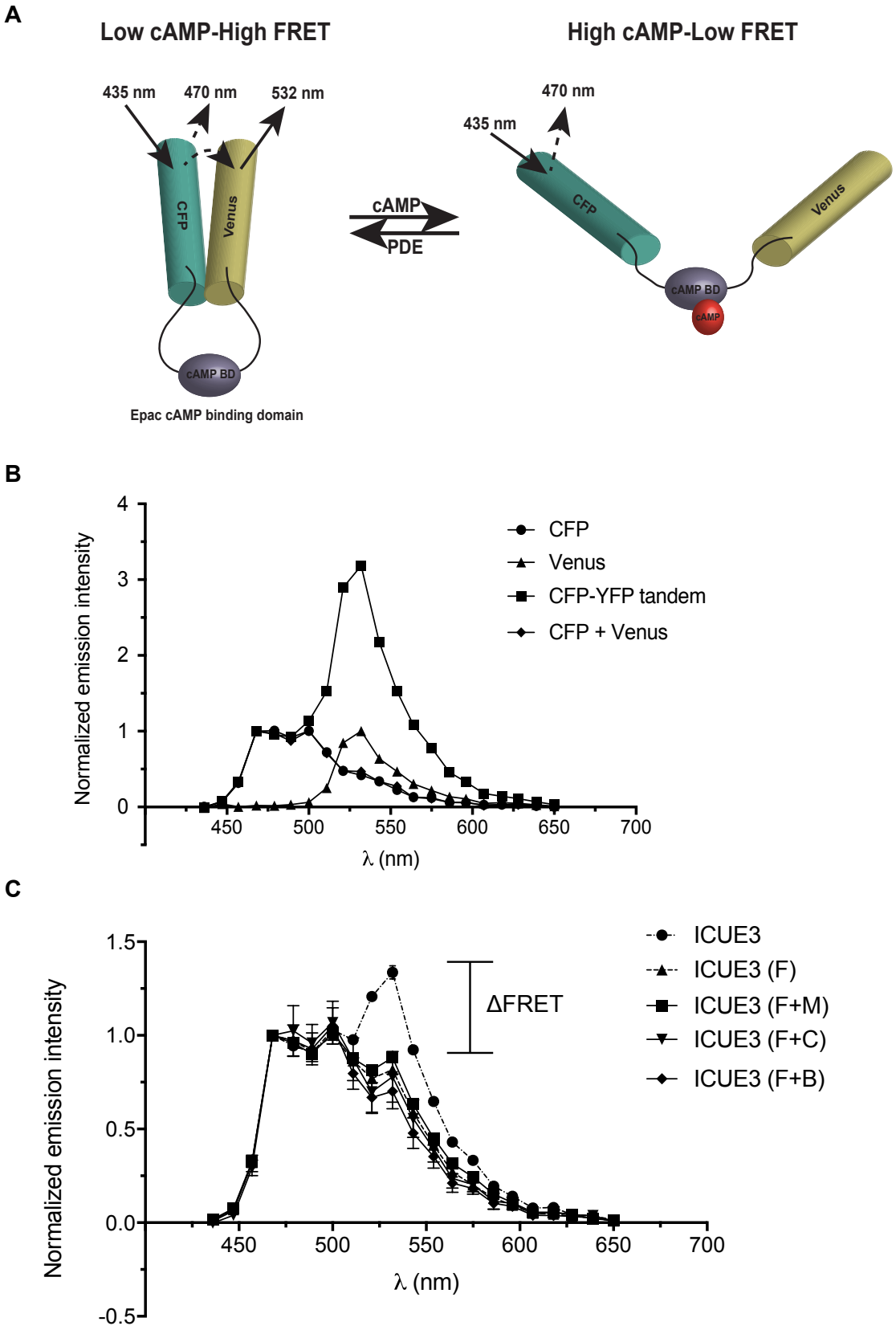


Figure III. Fluorescence resonance energy transfer (FRET) is illustrated. A. The cytosolic FRET sensor, ICUE3, consists of the cAMP binding domain of exchange factor activated by cAMP (Epac) flanked by the yellow fluorescent protein (YFP) variant, Venus, and cyan fluorescent protein (CFP). Under resting conditions, the conformation of the construct promotes a spatial arrangement of Venus and CFP that brings the two into close proximity (<10 nm). This conformation allows for a FRET signal to occur, i.e. excitation of CFP at 435 nm leads to an emission at a wavelength of 532 nm. Elevation of cAMP and subsequent binding of cAMP to the Epac moiety causes a conformational change of the sensor that separates CFP and YFP and thereby prevents FRET. **B.** Spectra show emission of CFP and Venus alone, upon co-expression and expression of a fusion of YFP and CFP that was used to calibrate the system. The indicated constructs were transiently expressed in HEK293 cells and spectra recorded. Shown are emission intensities. **C.** Spectra show emission of ICUE3 sensor under resting conditions and upon treatment with forskolin (F) and treatment with the indicated combinations of forskolin and milrinone (F+M), cilostamide (F+C) or Bay 41-8543 (F+B), measured in HEK293 cells upon transient expression. Shown are emission intensities of n = 40-60 cells per condition.

Supplemental Figure IV

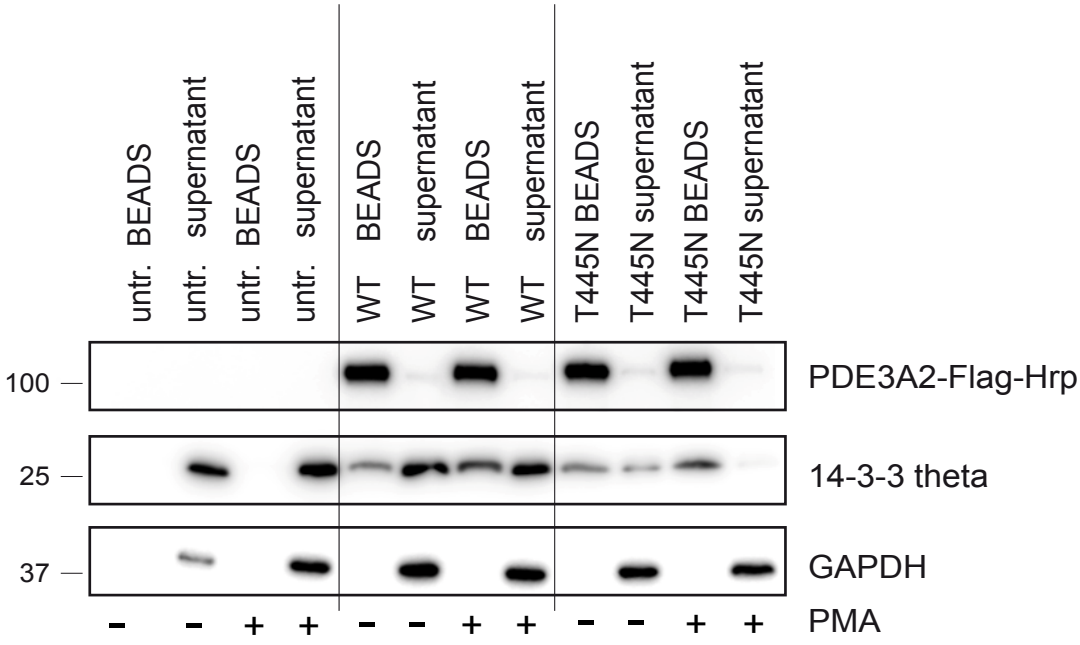


Figure SIV. Controls for co-Immunoprecipitation of PDE3A2 variants with 14-3-3θ. Untransfected (untr.) HEK293 cells and cells transiently expressing PDEA2-wild-type (WT) Flag or PDEA2-T445N-Flag were left untreated or were stimulated with PMA. The cells were lysed and the PDE3A2 versions were precipitated with Anti-FLAG M2 Magnetic Beads. Precipitated PDE3A2 and co-precipitated 14-3-3θ were detected with Western blotting. As expected, the beads did not precipitate proteins from untransfected cells. From the PDE3A2-expressing cells, the beads precipitated both the PDE3A2-Flag versions and the 14-3-3θ. The supernatant fractions indicate 14-3-3θ expression in all sample lysates.