SUPPLEMENTAL MATERIAL
Table S1. Basic information and supplementary findings from cardiac magnetic resonance imaging of α2+/G301R and wild type mice. Data are shown as mean ± standard mean error. *, P < 0.05 (unpaired t-test).

|                  | 3-month-old mice | 8-month-old mice |
|------------------|------------------|------------------|
|                  | wild type  | α2+/G301R | P value | wild type  | α2+/G301R | P value |
|                  | (n = 6)  | (n = 6)  |         | (n = 7)  | (n = 10) |         |
| Body weight (g)  | 20.02 ± 0.66 | 17.83 ± 1.19 | 0.14    | 27.96 ± 1.35 | 29.86 ± 1.23 | 0.32 |
| Heart rate (min⁻¹) | 434.9 ± 35.3 | 443.0 ± 20.6 | 0.85    | 485.0 ± 30.6 | 434.1 ± 12.5 | 0.10 |
| Respiration frequency (min⁻¹) | 147.3 ± 13.4 | 163.3 ± 5.6 | 0.30    | 132.6 ± 6.7 | 147.4 ± 11.4 | 0.13 |
| Left ventricular end-diastolic volume (ml) | 0.051 ± 0.005 | 0.047 ± 0.004 | 0.57    | 0.064 ± 0.006 | 0.080 ± 0.004 | 0.04* |
| Left ventricular end-systolic volume (ml) | 0.014 ± 0.002 | 0.016 ± 0.002 | 0.60    | 0.017 ± 0.003 | 0.026 ± 0.003 | 0.03* |
| Left ventricular ejection fraction (%) | 71.99 ± 2.09 | 66.52 ± 2.47 | 0.12    | 75.18 ± 2.57 | 67.09 ± 1.81 | 0.02* |
| Left ventricular stroke volume (ml•beat⁻¹) | 0.036 ± 0.004 | 0.031 ± 0.002 | 0.25    | 0.048 ± 0.004 | 0.053 ± 0.003 | 0.20    |
| Left ventricular cardiac output (ml•min⁻¹) | 15.90 ± 2.31 | 13.53 ± 0.62 | 0.35    | 23.24 ± 1.66 | 22.90 ± 1.91 | 0.87    |
| Left ventricular cardiac index (ml•min⁻¹•g⁻¹) | 0.793 ± 0.107 | 0.772 ± 0.051 | 0.87    | 0.837 ± 0.058 | 0.783 ± 0.062 | 0.55    |
| Left ventricular mass-to-body weight ratio (mm³•g⁻¹) | 3.844 ± 0.183 | 3.384 ± 0.174 | 0.10    | 3.325 ± 0.170 | 3.432 ± 0.130 | 0.62    |
| Right ventricular end-diastolic volume (ml) | 0.041 ± 0.003 | 0.038 ± 0.003 | 0.46    | 0.040 ± 0.005 | 0.053 ± 0.002 | 0.02* |
| Right ventricular systolic volume (ml) | 0.011 ± 0.002 | 0.009 ± 0.001 | 0.74    | 0.013 ± 0.003 | 0.017 ± 0.002 | 0.20    |
| Right ventricular ejection fraction (%) | 74.66 ± 3.63 | 74.17 ± 1.95 | 0.91    | 73.49 ± 3.52 | 69.59 ± 1.95 | 0.31    |
Table S2. Characteristics of spontaneous electrical activity of atrial and ventricular myocardium from 8-month-old α2+/G301R and wild type mice. APD50 and APD90 indicate action potential duration at the level of 50% and 90% repolarization. Data are shown as mean ± standard mean error. Groups were compared using unpaired t-test. See also Figure S3 for representative traces of membrane potentials.

|                          | Wild type (n = 9) | α2+/G301R (n = 8) | P value |
|--------------------------|------------------|-------------------|--------|
| **Right ventricle wall** |                  |                   |        |
| Heart rate (min⁻¹)       | 404.0 ± 11.9     | 422.2 ± 20.4      | 0.44   |
| Resting membrane potential (mV) | -80.9 ± 1.0  | -79.9 ± 1.5       | 0.54   |
| Resting potential interval (ms) | 147.3 ± 5.3    | 144.6 ± 4.8       | 0.71   |
| APD50 (ms)               | 8.79 ± 0.70      | 8.43 ± 0.44       | 0.68   |
| APD90 (ms)               | 33.02 ± 2.48     | 32.62 ± 2.13      | 0.91   |
| Action potential amplitude (mV) | 109.5 ± 2.3    | 109.9 ± 2.8       | 0.92   |
| Maximal depolarization (mV) | 30.4 ± 1.9     | 28.6 ± 1.5        | 0.46   |
| Maximal slope of depolarization (mV/s) | 375320 ± 27989  | 365007 ± 23912    | 0.78   |
| **Right atrial trabeculae** |                  |                   |        |
| Heart rate (/min)        | 409.3 ± 12.7     | 423.9 ± 13.4      | 0.44   |
| Resting membrane potential (mV) | -83.1 ± 0.8    | -83.4 ± 1.8       | 0.85   |
| Resting potential interval (ms) | 144.2 ± 4.0    | 143.8 ± 4.1       | 0.94   |
| APD50 (ms)               | 11.52 ± 1.84     | 10.44 ± 0.45      | 0.66   |
| APD90 (ms)               | 23.91 ± 2.19     | 28.64 ± 2.61      | 0.18   |
| Action potential amplitude (mV) | 115.6 ± 1.8    | 110.8 ± 3.9       | 0.25   |
| Maximal depolarization (mV) | 32.5 ± 1.4     | 29.8 ± 2.3        | 0.31   |
| Maximal slope of depolarization (mV/s) | 313687 ± 9511  | 317438 ± 27078    | 0.88   |
| **Right atrial wall**    |                  |                   |        |
| Heart rate (/min)        | 416.9 ± 13.9     | 424.2 ± 15.4      | 0.73   |
| Resting membrane potential (mV) | -82.0 ± 0.9    | -83.3 ± 1.4       | 0.43   |
| Resting potential interval (ms) | 143.1 ± 3.7    | 142.8 ± 4.4       | 0.95   |
| APD50 (ms)               | 5.74 ± 0.83      | 5.36 ± 0.54       | 0.71   |
| APD90 (ms)               | 15.44 ± 2.25     | 17.93 ± 0.95      | 0.35   |
| Action potential amplitude (mV) | 104.5 ± 2.1    | 101.1 ± 2.8       | 0.34   |
| Maximal depolarization (mV) | 22.3 ± 1.5     | 17.3 ± 2.3        | 0.08   |
| Maximal slope of depolarization (mV/s) | 247597 ± 18311  | 230319 ± 17148    | 0.51   |
| **Right atrial posterior part of intercaval region** |                  |                   |        |
| Heart rate (/min)        | 411.2 ± 15.3     | 449.1 ± 32.3      | 0.29   |
| Resting membrane potential (mV) | -83.3 ± 0.9    | -84.7 ± 1.2       | 0.37   |
| Resting potential interval (ms) | 145.3 ± 3.9    | 140.3 ± 4.5       | 0.40   |
| APD50 (ms)               | 12.88 ± 1.87     | 11.10 ± 0.88      | 0.42   |
| APD90 (ms)               | 34.19 ± 3.26     | 31.26 ± 2.22      | 0.48   |
| Action potential amplitude (mV) | 106.1 ± 1.9    | 108.9 ± 2.1       | 0.34   |
| Maximal depolarization (mV) | 25.2 ± 1.8     | 23.2 ± 1.9        | 0.47   |
| Maximal slope of depolarization (mV/s) | 234844 ± 16919  | 278884 ± 23057    | 0.14   |
A homology model of the human Na,K-ATPase α2 isoform (light blue) and the β isoform 1 (grey) is shown as cartoon representation from two different angles, rotated 90 degrees (A). The Familial Hemiplegic Migraine type 2-associated Gly301Arg25 mutation is plotted on the homology model and highlighted in red. A close up of the structural region around the mutation (B) show that Gly301Arg mutation is located in transmembrane helix (H3) and might interfere with the membrane helix arrangement around transmembrane helices 1, 4 and 5 (H1, H4, H5).
Circadian variations in blood pressure (A, C) and heart rate (B, D) of 3-month-old (A, B; \( n = 6 - 9 \)) and 8-month-old (C, D; \( n = 7 \)) \( \alpha_2^{+/G301R} \) and wild type mice. For statistical analyses, see Figure 3.
Representative traces of membrane potentials show that the waveform of spontaneous left ventricle membrane action potentials recorded in ventricular myocardium was similar in the hearts from α2+/G301R and wild type mice. See Table S2 for statistical analyses.
Citrate synthase activity as a marker enzyme for mitochondrial content suggested similar mitochondrial content in cardiac samples from 3-month-old ($n = 7$) and 8-month-old ($n = 12$) $\alpha_2^{+/-G301R}$ and wild type mice. Data compared with two-way ANOVA.
Figure S5.

Representative respirometry traces of hearts from 8-month-old wild type (A) and α2+/G301R (B) mice. Blue traces show O₂ concentration (hyperoxygenated environment), red traces show O₂ consumption.
Figure S6. Phospho-specific Western blot analysis did not suggest any changes in the expression of key molecules for the Na,K-ATPase-dependent signalling pathways in the hearts from 3-month-old α2+/G301R and wild type mice.

Semi-quantitative assessment of total Src kinase expression and the level of Src phosphorylation at Tyr418 (A), total Erk1/2 kinase expression and its Thr202/Tyr204 phosphorylation level (B), and total PLCγ expression and the level of its Tyr783 phosphorylation (C). Representative images for Src
(D), Erk1/2 (E), and PLCγ (F) semi-quantification Western blot experiments that are averaged in (A), (B) and (C), respectively. Left images correspond to total protein-of-interest (Src, Erk1/2 and PLCγ, respectively). Images in the center correspond to phosphorylated protein of interest (p-Src, p-Erk1/2 and p-PLCγ, respectively). Molecular weight markers and total protein load in the membrane detected with stain-free gels are shown to the right. All representative images were cropped to the size identified by molecular marker in (D), (E) and (F), respectively. All images are cropped to include at three molecular weight markers positioned both above and below the band. Prior to incubation with the antibodies, the membrane was divided into two parts; the upper part above 75 kDa was used to detect PLCγ and p-PLCγ, the lower part below 75 kDa was used to detect either Src and p-Src, or Erk1/2 and p-Erk1/2, respectively. The expression was normalized to total protein load for the corresponding probe. An average level for the wild type (WT) group was taken as 100%. \( n = 5 \). Data compared with unpaired \( t \)-test.
Figure S7. Morphology of left ventricle tissue was similar in 8-month-old mice of both genotypes.

Masson’s trichrome staining of left ventricle cross sections. Cytoplasm and muscle fibres appear red, nuclei are blue. The tissue was examined at x20 magnification. Representative images of myocardium with cardiomyocytes in cross sectional orientation from a wild type (WT) and α2+/G301R mouse (A). The area of cardiomyocytes in the cross-sectional orientation was similar between genotypes (B). Representative images of cardiomyocytes in long axis (C). Cardiomyocyte diameter measured in long-axis was similar between genotypes (D). No fibrosis was detected in the myocardium in any of the mice of both genotypes. $n = 4$. Bars in (A and C) correspond to 30 μm. Data compared with unpaired $t$-test.
Figure S8. Systolic dysfunction in 8-month-old $\alpha_2^{+/-}G301R$ mice may be a result of changes in the contractile machinery but not of Ca$^{2+}$ handling.

(A) The expression of several proteins important for the Ca$^{2+}$-dependent interaction of actin and myosin during cardiomyocyte contraction was modified in $\alpha_2^{+/-}G301R$ hearts, including suppressed components of the troponin-tropomyosin complex i.e. cardiac troponin I3 (Tnni3), troponin T2 (Tnnt2), $\alpha$-tropomyosin (Tpm1) and tropomodulin 1 (Tmod1). Reduced Tnnt2 was previously suggested to contribute to dilated cardiomyopathy-like changes in the mouse heart. An ablation of Tpm1 was shown to lead to degeneration of partially assembled sarcomeres due to unregulated actin-myosin interactions and its abnormal regulation is associated with dilated cardiomyopathy. Tmod1 is also essential for regulation of the thin filament elongation and depolymerization. Moreover, cardiac myosin-binding protein C (Mybpc3) is the thick filament associated protein that mediates regulation of acto-myosin cross-bridge cycling, i.e., regulation of cardiac contraction and relaxation. Mutations in Mybpc3 are associated with a large range of inherited cardiomyopathies. It has been shown that Mybpc3 haploinsufficiency leads to increased myofilament Ca$^{2+}$ sensitivity. It is therefore possible that increased Mybpc3 expression, as it is seen in $\alpha_2^{+/-}G301R$ mice, is associated with a reduction in Ca$^{2+}$ sensitivity of the myofilaments and contractility. Accordingly, the upregulation of myosin light chain 7 (Myl7), reduction of Tnni3 and increase in $\alpha$-actinin 2 (Actn2) are characteristic for the hearts with low Ca$^{2+}$ sensitivity. Reduction in cardiac $\alpha$-actin (Actc1), a major constituent of the cytoskeleton of cardiomyocytes, is associated with dilated cardiomyopathy. Profilin 2 (Pfn2), an actin-binding protein involved in the dynamic turnover of the actin cytoskeleton, was increased in $\alpha_2^{+/-}G301R$ cardiomyocytes. Pfn2 is known to modulate the sarcomere structure, partially via Erk1/2 signalling that, accordingly, was increased in 8-month-
old α2+/G301R mice (Fig. 6). An increased expression of collagen type VI α2 chain (Col6a2) was previously associated with worsened cardiac function and remodelling.75

(B) No difference in the Ca^{2+} transporting proteins was seen between the hearts from 8-month-old α2+/G301R and wild type (WT) mice. The similar expression of the Na,Ca-exchanger 1 (Slc8a1), the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase 2 (Atp2a2) and 3 (Atp2a3), the plasma membrane Ca^{2+}-transporting ATPase 1 (Atp2b1) and 4 (Atp2b4) was detected. Similar expression of the voltage-dependent Ca^{2+} channel subunit alpha-2/delta-1 (Cacna2d1), a subunit that regulates Ca^{2+} current density and activation/inactivation kinetics of the Ca^{2+} channel, and the voltage-dependent L-type Ca^{2+} channel subunit beta-2 (Cacnb2) suggests similar Ca^{2+} influx in cardiomyocytes from α2+/G301R and WT mice. n = 6, except Atp2a3 and Atp2b4, where the signal was only detected in 3 WT and 3 α2+/G301R hearts. *, P < 0.05 (unpaired t-test). See also Data S1-S3.
Figure S9. Proteomics data analysis suggested that changed expression of Na,K-ATPase isoforms was associated with amplified Src/Ras/Erk1/2 signalling in hearts from 8-month-old $\alpha_2^{+/G301R}$ mice.

An increased expression of the Na,K-ATPase $\alpha_1$ isoform and decreased expression of the $\alpha_2$ isoform was detected by the proteomics data analysis in hearts from $\alpha_2^{+/G301R}$ mice (A). The $\alpha_2^{+/G301R}$ hearts had upregulated protein phosphatase 1 regulatory subunit 3A (Ppp1r3a), protein phosphatase 2 regulatory subunit Bα (Ppp2r2a) and mitogen-activated protein kinase kinase 3 (Map2k3) suggesting increased signalling of the Src/Ras/Erk1/2 pathway (B). The Ras oncogene family members Rab4a, Rab11b and Rab14 were found upregulated, which further supported the finding of amplified Src/Ras/Erk1/2 signalling in the hearts from $\alpha_2^{+/G301R}$ mice (C). Interestingly, an increased expression of Rab4a was previously suggested to be responsible for hypersensitivity to β-adrenergic stimulation, metabolic remodelling and cardiac mitochondrial dysfunction.\textsuperscript{76}

$n = 6$, *, ** and ***, $P < 0.05$, $< 0.01$ and $< 0.001$ (unpaired $t$-test).

See also Data S1-S3.
Figure S10. Proteomics data analysis suggested upregulation of enzymes generating oxidative stress and enzymes involved in regulation of cellular redox state in the hearts from 8-month-old $\alpha_2^{+/G301R}$ mice.

Proteomics data analysis identified upregulation of several enzymes known to contribute to generation of reactive oxygen species including xanthine oxidoreductase/dehydrogenase (Xdh), nitric oxide synthase 3 (Nos3) and proline dehydrogenase (Prodh). An increased oxidative stress in the hearts of $\alpha_2^{+/G301R}$ mice was further supported by an increased expression of enzymes involved in regulation of cellular redox state, including thioredoxin related transmembrane protein 2 (Tmx2), microsomal glutathione S-transferase 1 (Mgst1), carbonyl reductase 3 (Cbr3) and ATP-hydrolysing 5-oxoprolinase (Oplah). Myoglobin (Mb), which acts as a short-term storage and reactive oxygen scavenger in cardiomyocytes, was reduced in the hearts of $\alpha_2^{+/G301R}$ mice in comparison with wild type (WT).

$n = 6$, except Cbr3 where the signal was only detected in 3 WT and 3 $\alpha_2^{+/G301R}$ hearts.

*, ** and ***, $P < 0.05$, $< 0.01$ and $< 0.001$ (unpaired $t$-test). For reference, see also Data S1-S3.
Data S1.

Proteins mapped by proteomics of left ventricles from 8-month-old α2+/G301R and wild type mice. Data compared using unpaired $t$-test.

Data S2.

Proteins relevant for the heart structure and function, which were significantly different between genotypes, suggested by Ingenuity Pathway Analysis of proteomics data from the hearts of 8-month-old α2+/G301R and wild type mice. Data compared using unpaired $t$-test.

Data S3.

The cardiac disease and altered function suggested by Ingenuity Pathway Analysis of proteomics data from the hearts of 8-month-old α2+/G301R and wild type mice. $P$ values were calculated based on one-sided Fisher’s exact test.

Data S4.

Whole uncropped Western blot gels
Data S4. Whole uncropped Western blot gels
Changed expression of the Na,K-ATPase α isoforms in the heart from α_2^{+/G301R} mice but similar expression of the Na,Ca-exchanger-1. Results are shown in Fig. 1.

Western blot α_2^{+/G301R} (Hz) vs. wild type (WT)
Changed expression of the Na,K-ATPase α isoforms in the heart from $\alpha_2^{+/G301R}$ mice but similar expression of the Na,Ca-exchanger-1. Results are shown in Fig. 1.

Western blot $\alpha_2^{+/G301R}$ (Hz) vs. wild type (WT)

Protein load, Stain-free gel
Changed expression of the Na,K-ATPase α isoforms in the heart from α$_2^{+/G301R}$ mice but similar expression of the Na,Ca-exchanger-1. Results are shown in Fig. 1.

**Na,Ca-exchanger**

3-month-old (3-m) and 8-month-old (8-m) mice

**Marker (kDa)**

Western blot $\alpha_2^{+/G301R}$ (Hz) vs. wild type (WT)

Protein load, Stain-free gel, from 250 kDa to 10 kDa
The hearts from 8-month-old $\alpha_2^{+/G301R}$ mice showed modified signalling of pathways downstream from the Na,K-ATPase. Results are shown in Fig. 6.

Western blot $\alpha_2^{+/G301R} (H_2)$ vs. wild type (WT)

Protein load, Stain-free gel, from 250 kDa to 10 kDa
The hearts from 8-month-old $\alpha_2^{+/G301R}$ mice showed modified signalling of pathways downstream from the Na,K-ATPase. Results are shown in Fig. 6.

**Western blot $\alpha_2^{+/G301R}$ (Hz) vs. wild type (WT)**

**Protein load, Stain-free gel, from 250 kDa to 10 kDa**
The hearts from 8-month-old $\alpha_2^{+/G301R}$ mice showed modified signalling of pathways downstream from the Na,K-ATPase. Results are shown in Fig. 6.

**Western blot $\alpha_2^{+/G301R} (Hz)$ vs. wild type (WT)**

**Protein load, Stain-free gel, from 250 kDa to 10 kDa**
Phospho-specific Western blot analysis did not suggest any changes in the expression of key molecules for the Na,K-ATPase-dependent signalling pathways in the hearts from 3-month-old $\alpha_2^{+/G301R}$ and WT mice. Results are shown in Fig. S6.

**G**

*Western blot $\alpha_2^{+/G301R}$ (Hz) vs. wild type (WT)*

*Protein load, Stain-free gel, from 250 kDa to 10 kDa*
Phospho-specific Western blot analysis did not suggest any changes in the expression of key molecules for the Na,K-ATPase-dependent signalling pathways in the hearts from 3-month-old α₂⁺/G301R and WT mice. Results are shown in Fig. S6.

**Western blot α₂⁺/G301R (Hz) vs. wild type (WT)**

Protein load, Stain-free gel, from 250 kDa to 10 kDa
Phospho-specific Western blot analysis did not suggest any changes in the expression of key molecules for the Na,K-ATPase-dependent signalling pathways in the hearts from 3-month-old $\alpha_2^{+/G301R}$ and WT mice. Results are shown in Fig. S6.

**Western blot $\alpha_2^{+/G301R}$ (Hz) vs. wild type (WT)**

**Protein load, Stain-free gel, from 250 kDa to 10 kDa**