SUPPLEMENTAL MATERIAL

Detailed Methods

Generation of mutant mice. The VM substitution was introduced into the cardiac α- MHC gene using standard gene targeting techniques (Fig. S1).[1, 2] In brief, mutagenesis PCR was used to introduce the mutation into the targeting vector containing positive (neomycin resistance) and negative (thymidine kinase) selection markers. Neomycin-resistant embryonic stem cell clones (129/SvEv background) were screened for homologous recombination by Southern blot analyses and picked-out mutant cells injected into mouse blastocysts. Chimeric mice were mated with EIIa-Cre transgenic mice (129/SvEv background) for deletion of the neomycin resistance gene. RC and RW mutant mice have been described previously.[3, 4] Homozygous cardiac α-MHC mutants (VM/VM, RC/RC, RW/RW) were generated by crossbreeding of heterozygous mice. Double heterozygous mice (VM/RC) originated from crossbreeding of VM/VM and RC/+ animals. All animals were maintained and studied using protocols approved by the responsible ethics committees and government agencies (Regional Government of Lower Franconia, Germany). Inbred male mice in an 129/SvEv background were used in all studies. For cyclosporine treatment of mice 15 µg/g body weight cyclosporine A (CsA) in PBS were injected subcutaneously twice daily for up to 4 weeks.

Mouse heart weight, histopathology, cell size, fibrosis assessment and TUNEL staining. Mouse hearts were perfused with 4% paraformaldehyde at physiological pressures, blotted dry on tissue paper and weighed. Next, they were embedded in paraffin and sections were stained with hematoxylin and eosin (H & E), Masson's trichrome, Sirius Red, vonKossa or wheat germ agglutinin (WGA, Alexa Fluor 488, Molecular probes Inc., Eugene, OR) according to the manufacturer's protocol and as described previously.[5] For fibrosis assessment, the relative proportion of red staining was determined in at least four whole transverse sections. Sections were derived from different regions of left ventricles

>100μm apart from each other and stained with Sirius Red. Perivascular collagen was excluded from the analyses. Cell size was determined from >100 WGA stained myocytes per study group. Only round cells with a nucleus (visualized with Hoechst 33258 stain) present in the center of the myocyte were chosen. Measurements were performed using ImageJ software. TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining was performed on mouse heart sections using the *In Situ* Cell Death Detection Kit, Fluorescein (Roche Applied Science, Penzberg, Germany) according to the manufacturer's protocol.

qPCR. After extraction of total RNA from mouse ventricular tissue according to the manufacturer's protocol (Qiagen, Hilden, Germany), cDNA was synthesized from 1μg total RNA by *in vitro* transcription using Superscript II (Invitrogen) and oligo dt primers. Relative quantification of mRNA expression levels was performed by qPCR (Thermal Cycler CFX96, BioRad, Hercules, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous control.

Echocardiography. Mice were anesthetized with pentobarbital (30μg/g body weight), placed on a warm plate and attached to an ECG monitor. Using a Vevo 2100 High-Resolution In Vivo Micro-Imaging System and RMV 707B Scanhead (VisualSonics Inc., Toronto, Canada), M-mode traces of left parasternal long and short axes were obtained at a minimal heart rate 450 bpm, as described previously.[6] Left ventricular volumes, left ventricular mass, stroke volume fractional shortening and ejection fraction were calculated from measurements of left ventricular dimensions and wall thicknesses. E/A ratios were determined by Doppler echocardiography of left ventricular inflow velocities. A single individual performed all echocardiographic studies and cardiac measurements, without knowledge of the genotype.

Hemodynamic measurements. Hemodynamic measurements were performed as described previously.[6, 7] Briefly, the right carotid artery of anesthetized mice (300 μg/g body

weight tribromoethanol) was exposed and ligated. Proximal of the ligature a 1.4 F pressure catheter (Millar Instruments) was inserted and advanced to the left ventricle. Dobutamine was applied continuously via a polyethylene tubing placed in the left jugular vein and connected to a microinfusion pump (Braun, Melsungen, Germany). Pressure loops were recorded and analyzed for heart rate, maximal pressure, and the derivatives of pressure loops using a PowerLab system and Chart software (Chart 5.4, AD Instruments, Colorado Springs, CO).

Tissue bath experiments. For measurements of force generation in isolated left atria, 6 to 8 weeks-old mice were sacrificed, the hearts rapidly excised and placed in carbogenated modified Tyrode's solution (119 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 1 mM $MgCl_2$ 22.6 mM, $NaHCO_3$, 0.42 mM NaH_2PO_4, 0.025 mM EDTA, 10 mM glucose, 0.2 mM ascorbic acid, pH 7.4). Left atria were isolated, tied with two 6-0 silk sutures and the ends attached to a force transducer. Atria were kept in a tissue bath (IOA-5301, Föhr Medical Instruments, Seeheim, Germany) with carbogenated 37°C modified Tyrode's solution and electrically paced with 10 mV at 10 ms cycle length (Stimulator Type 215/I, HSE, March-Hugstetten, Germany). Signals from isometric force transducers were fed into a PowerLab system and analyzed using Chart software (Version 5.4, AD Instruments Colorado Springs, CO).

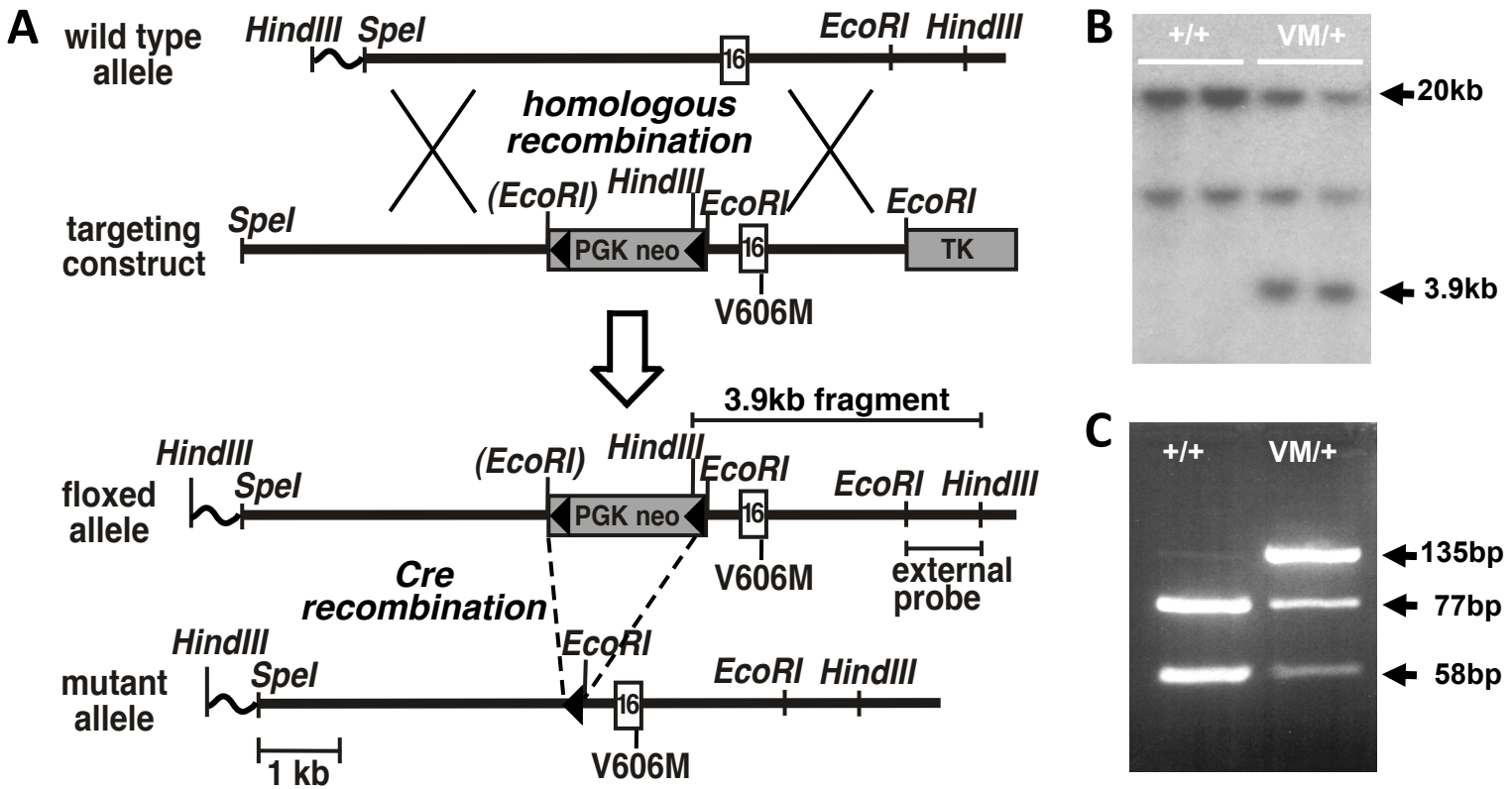
Transcriptional profiling. RNA was extracted from ventricles of 3 VM/RC and 4 wild-type male 129SvEv mice at the age of 7 weeks (RNeasy Midi kit, Qiagen, Hilden, Germany). RNA integrity was confirmed by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and quantified by photometric Nanodrop measurement. Synthesis of cDNA and subsequent biotin labeling of cRNA was performed according to the manufacturer's protocol (WT Plus Kit; Affymetrix, Inc., Santa Clara, CA). After fragmentation, labeled cRNA was hybridized to Affymetrix Mouse Gene 2.0 ST Gene Expression Microarrays, stained by

streptavidin/phycoerythrin conjugate and scanned as described in the manufacturer's protocol.

Statistical analysis. Normality testing using Prism software (Version 6.0) provided no indication of nonparametric data distribution. Thus, unpaired Student's t tests were used for comparisons of two groups and ANOVA for multiple comparisons. Survival curves were compared using log rank tests. $P<0.05$ was considered significant. Data are presented as mean ± SEM.

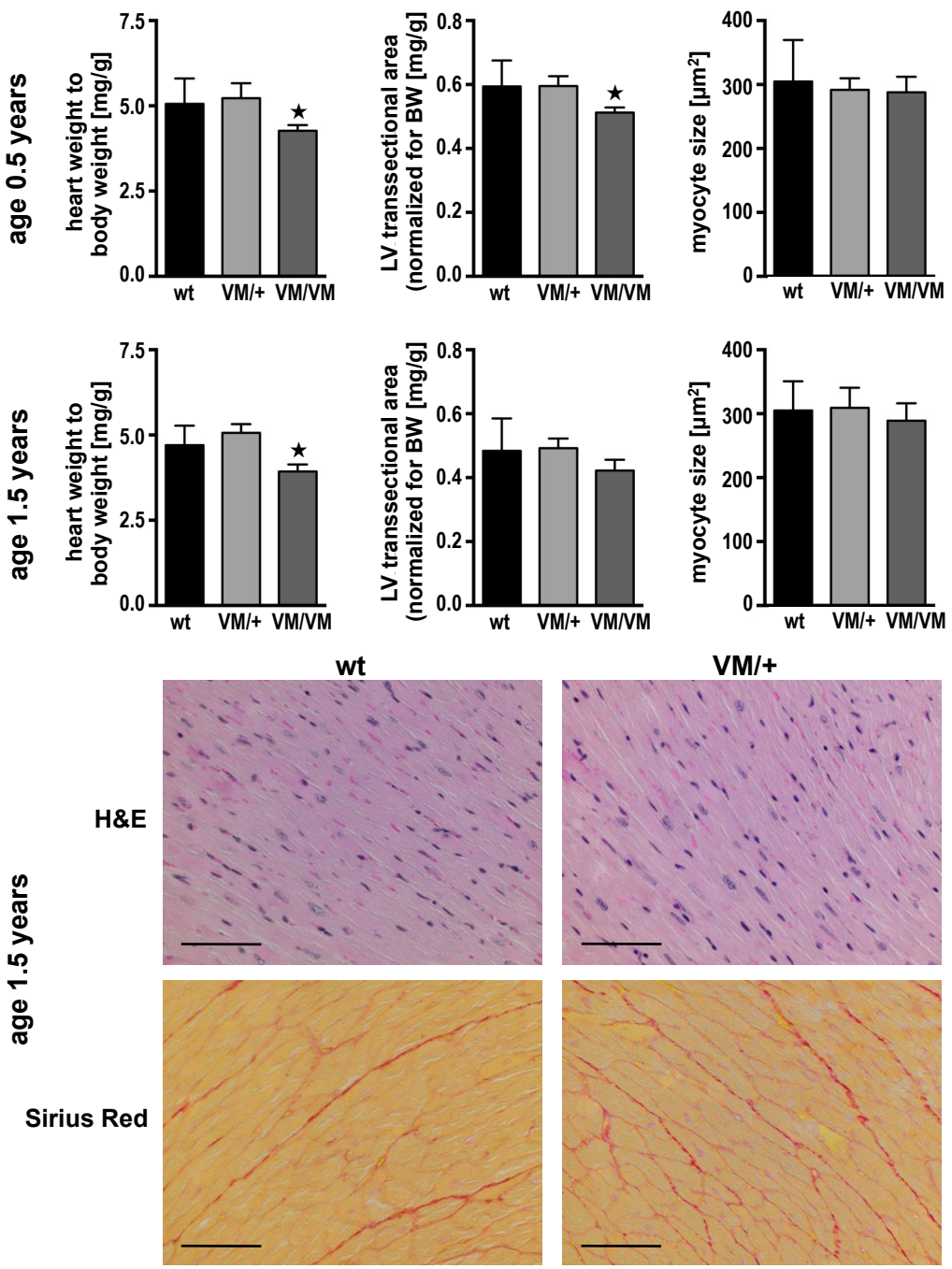
Affymetrix CEL files were analysed using Partek Genomics Suite software (v6.6; Partek Inc., St. Louis, MO). Raw expression values were background corrected by Robust Multi-array Average (RMA) and quantile normalized.[8] Partek standard One-way ANOVA on log2 transformed expression data was used to determine differential gene expression with significance levels set at $P<0.05$ and $P<0.01$, respectively. Differentially expressed genes passing a fold-change cut-off >1.5 were further classified using Partek´s Gene Ontology (GO) enrichment tool in order to cluster differentially expressed genes into functional GO classes. A modified Fisher's exact test was used to assess the probability that differentially expressed genes are statistically overrepresented within a GO class, expressed by a corresponding enrichment p-value. An enrichment P-value <0.05 was used to focus on highly enriched GO classes. Hierarchical clustering of differentially expressed genes enriched in distinct GO classes was performed using Euclidean similarity measures with Ward´s linkage.

Online Figure I



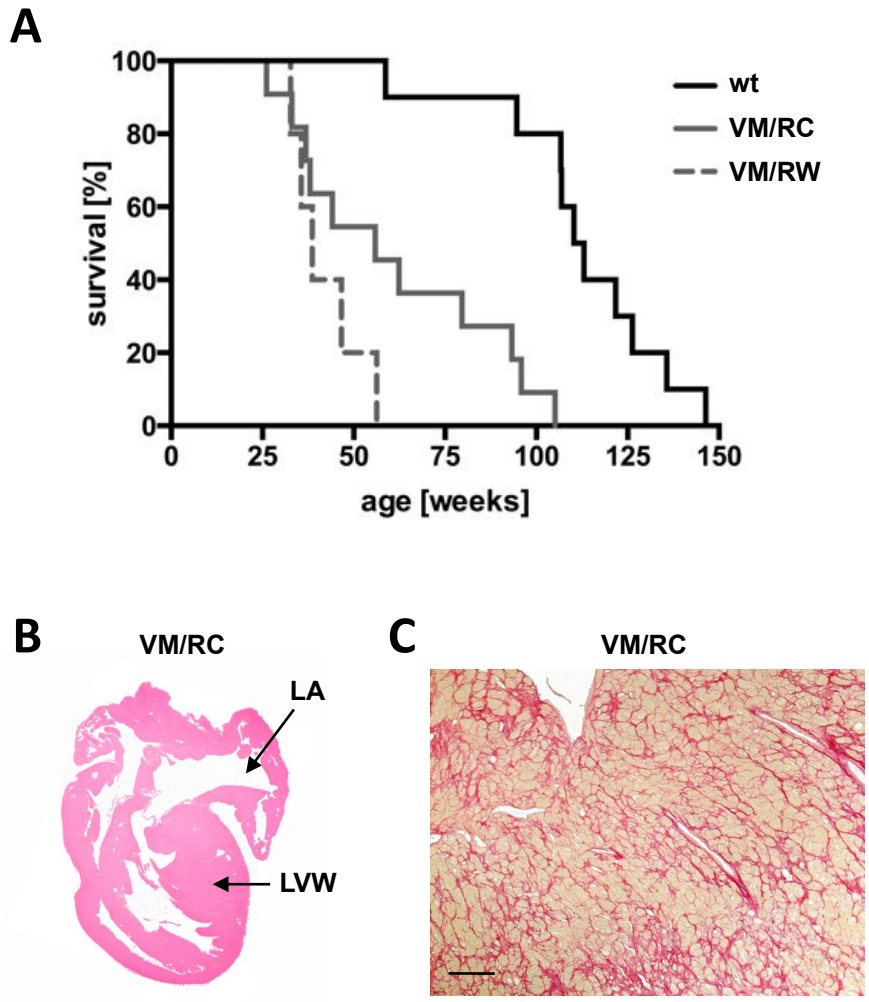
Online Figure I. Generation of knockin mice carrying the human Val 606 Met (V606M) substitution in the cardiac α-myosin heavy chain gene. A, targeting strategy; the targeting construct carries a neomycin resistance gene (PGK neo) flanked by loxP sites (black triangles), the V606M mutation engineered into exon 16, and a thymidine kinase cassette (TK) as negative selection marker; below, after homologous recombination (large crosses) properly targeted sequence (floxed allele) before and after loxP/Cre-mediated deletion of the neomycin resistance cassette (PGK neo). B, floxed alleles were identified by Southern blot analysis; Southern blotting of Hind III-digested DNA from targeted embryonic stem cells, probed with the external EcoR I / Hind III fragment; blot showing two examples of properly targeted clones containing both the 20kb wild-type and the engineered 3.9kb fragments (right two lanes). C, gel electrophoresis of RT-PCR fragments after mutation-specific restriction digest with Rsa I confirming the missense mutation (V606M) in the targeted allele. The mutation abolishes an Rsa I site.

Online Figure II



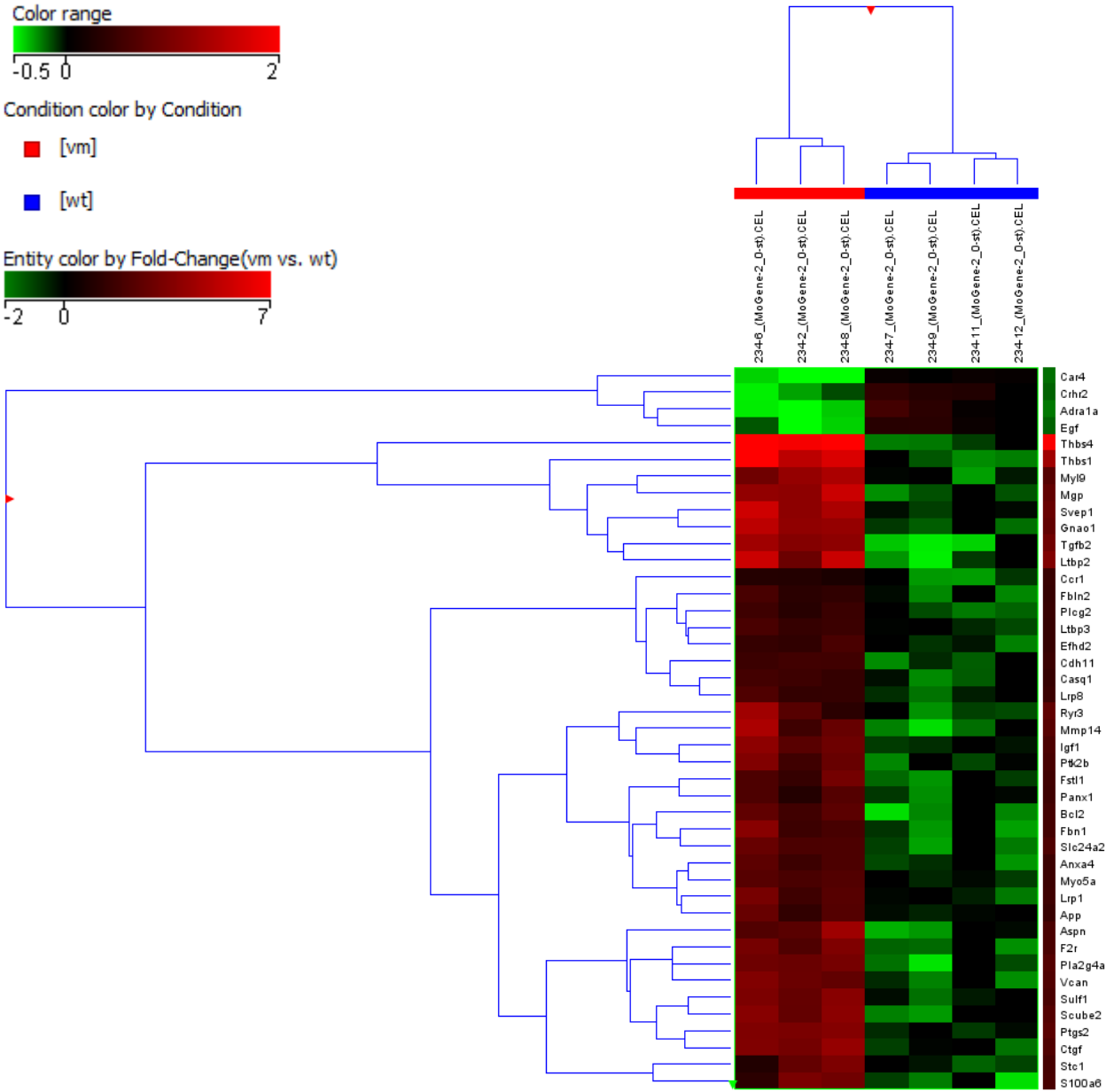
Online Figure II. Morphology of middle-aged (0.5 years) and old mouse hearts (1.5 years) carrying the human V606M substitution in the cardiac α-myosin heavy chain gene. A, heart-to-body weight ratios, left ventricular (LV) transsectional areas and myocyte size of 0.5 and 1.5 years-old wild-type, VM/+ and VM/VM mice. LV transsectional areas were determined as the area of the LV wall on a transverse heart sections just below the atrioventricular valves. BW, body weight. B, Hematoxylin and Eosin (H&E) and Sirius Red staining of wild-type and VM/+ mouse hearts aged 1.5 years.

Online Figure III



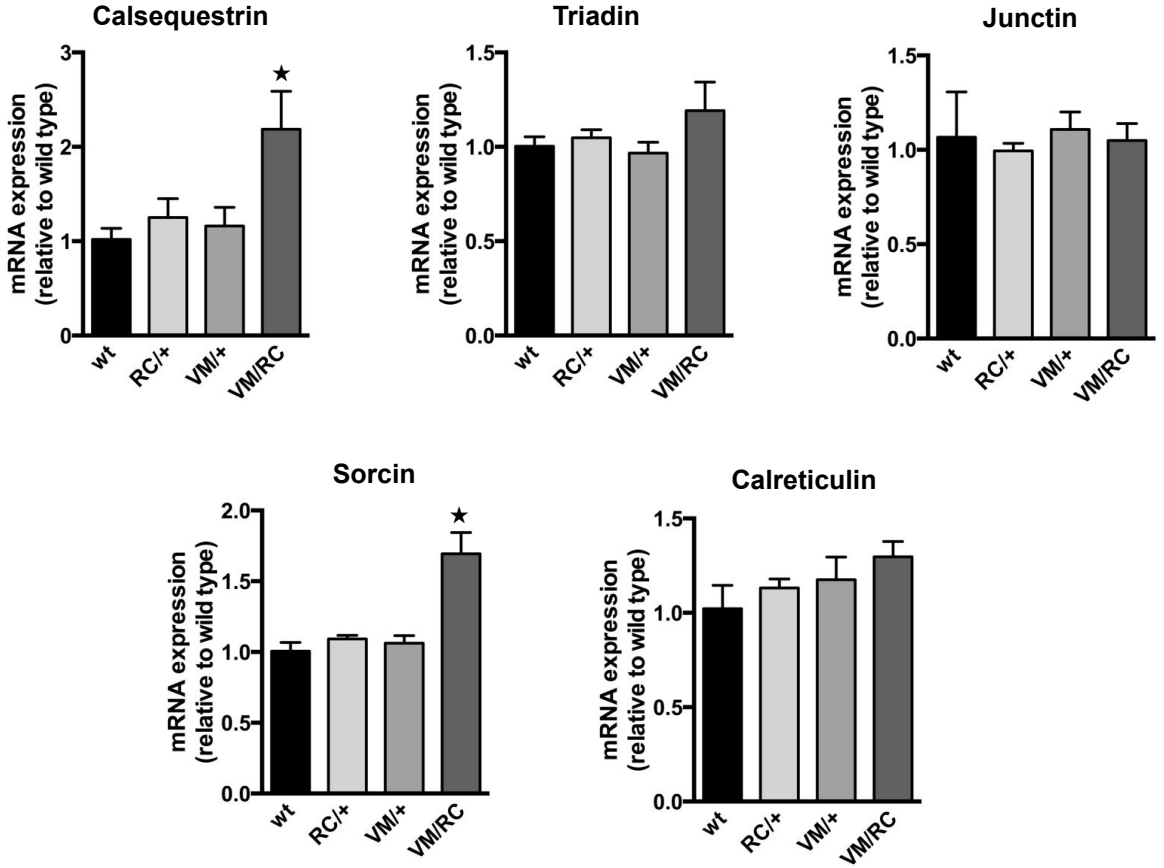
Online Figure III. Characterization of compound heterozygous VM/RW mouse hearts. A, survival curves of VM/RW mice compared to compound heterozygous VM/RC and wild type mice (wt); log rank-test of VM/RW vs. wt, $P < 0.0001$. B, Longitudinal section through a 25 weeks-old VM/RW mouse heart and H & E staining. Note the thick left ventricular free wall (LVW) and the big left atrium (LA). C, Sirius Red staining of the same heart as in B demonstrates marked collagen depositions in the myocardium. scale bar, 100 µm;

Online Figure IV



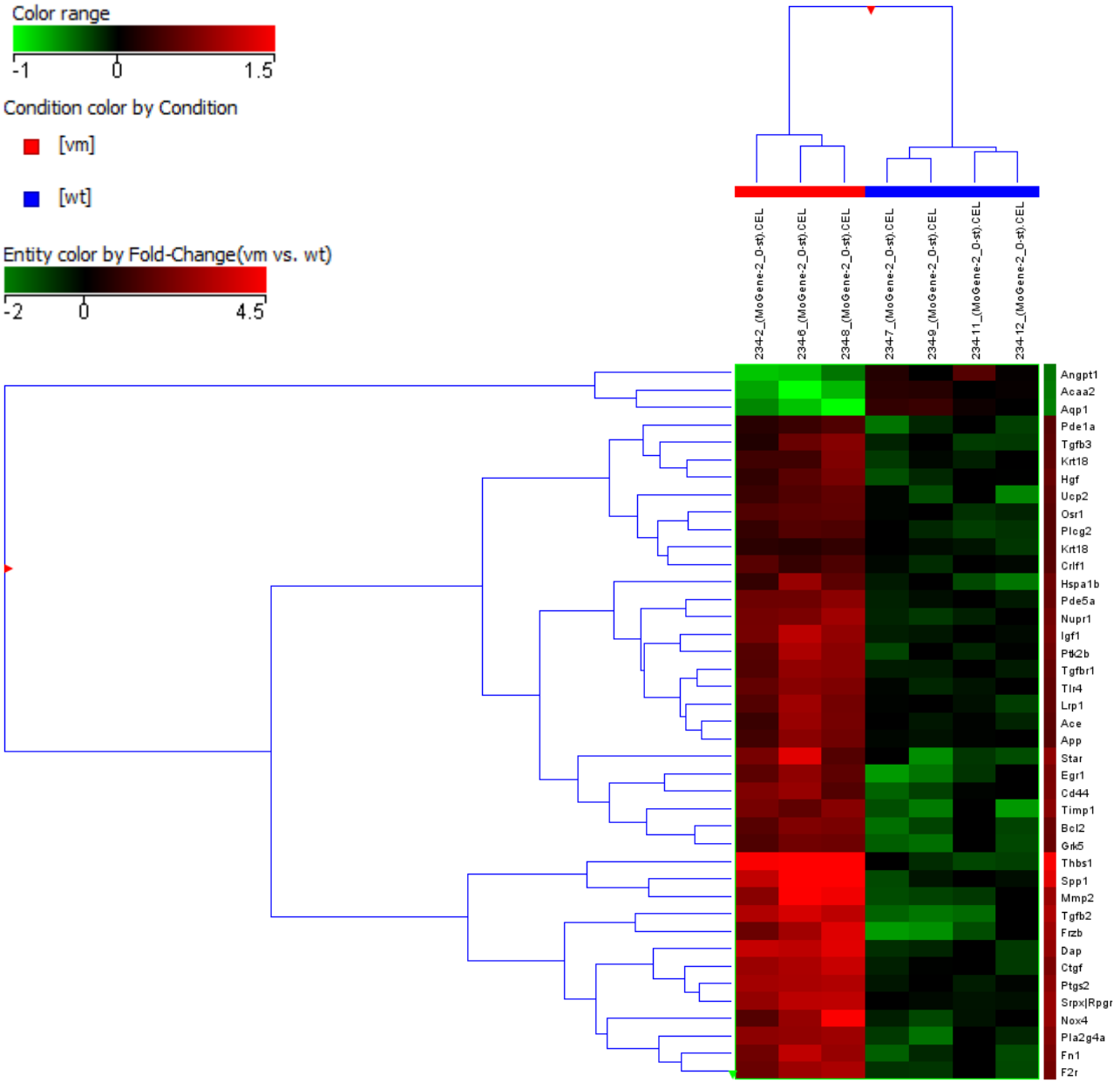
Online Figure IV. Gene expression profile of calcium-related genes in VM/RC compared to wild type mouse hearts. Heatmap and hierarchical clustering of genes involved in cellular calcium homeostasis. 39 genes were upregulated and 4 genes were downregulated in microarray analyses of 3 VM/RC compared to 4 age-, strain- and gender-matched wild-type mouse hearts ($p<0.05$). Color range displays the fold-change for each mRNA sample. Gene symbols and average fold change of each gene are displayed. Hierarchical clustering groups most similarly expressed genes and gene clusters.

Online Figure V



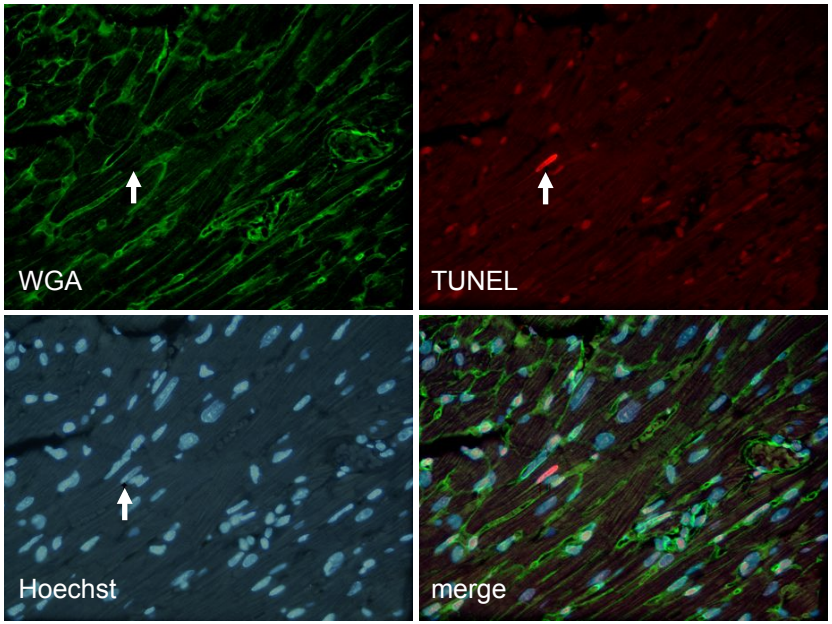
Online Figure V. mRNA expression of calcium-binding genes of the sarcoplasmic reticulum (SR) in 7 weeks-old wild type, (wt), RC/+, VM/+ and VM/RC mouse hearts as assessed by qPCR. SR calcium-binding genes have been proposed to play an important role in the pathogenesis of HCM.[9] Significant upregulation of calsequestrin and sorcin was found only for compound heterozygous VM/RC hearts. * $P<0.05$ vs. wt; n = 4 animals per genotype.

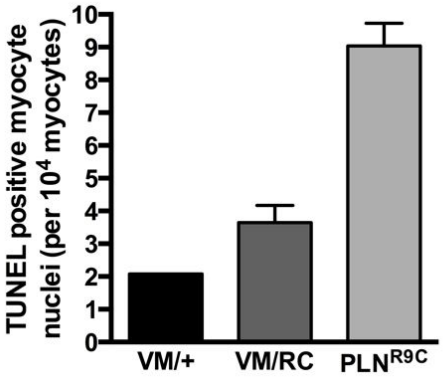
Online Figure VI



Online Figure VI. Gene expression profile of death-related genes. Heatmap and hierarchical clustering of death-related genes that were differentially regulated in microarray analyses of 3 VM/RC and 4 age-, strain- and gender-matched wild-type mouse hearts ($p<0.05$). 38 genes were up-regulated and 3 genes were down-regulated. Color range displays the fold-change for each mRNA sample. Gene symbols and average fold change of each gene are displayed. Hierarchical clustering groups most similarly expressed genes and gene clusters.

Online Figure VII





Online Figure VII. TUNEL staining of mouse hearts carrying the VM/+, VM/RC or a DCM causing human Arg9Cys mutation in the gene phospholamban (PLN^{R9C}).[10] Left, heart section showing an example of a TUNEL positive myocyte; WGA, fluorescently tagged wheat germ agglutinine for staining of cellular membranes; Hoechst, staining of DNA with Hoechst 33258 stain; the arrow points at the TUNEL-positive nucleus of a myocyte. right, counting results of TUNEL-positive myocytes.

Online Table I

Online Table I: Gene Ontology (GO) terms with significant enrichment of differentially expressed genes comparing 7 weeks-old compound heterozygous VM/RC and VM/+ mouse hearts.

Enriched GO terms	Enrichment p-value	number of genes	total genes in group
cellular component			
extracellular matrix	1.99E-36	51	298
collagen	7.17E-15	18	89
biological process			
positive regulation of fibroblast proliferation	8.13E-04	5	44
regulation of fibroblast proliferation	1.16E-04	7	66
regulation of cell-matrix adhesion	3.98E-05	7	56
cell proliferation	5.80E-04	17	409
regulation of cardiac muscle hypertrophy	7.23E-03	3	24
positive regulation of MAPK cascade	2.81E-05	13	196
positive regulation of ERK1 and ERK2 cascade	2.19E-05	9	82
regulation of cell death	4.59E-05	40	1229
regulation of apoptotic process	3.18E-05	39	1166
inflammatory response	1.43E-03	12	258
regulation of inflammatory response	2.48E-04	11	181
positive regulation of immune system process	3.99E-05	20	428
positive regulation of immune response	2.27E-03	11	237
response to cytokine stimulus	3.12E-03	13	321
regulation of cytokine production	1.50E-03	15	369
regulation of heart contraction	1.68E-04	9	118
calcium ion binding	4.55E-07	27	538
calcium ion transport	9.18E-03	8	174

Online Table II

Online Table II: Increased expression of TGF-beta in VM/RC

gene	fold increase	p-value	method
TGFB1	1.36	0.045	gene array
TGFB2	2.37	0.037	gene array
TGFBR1	1.67	0.033	gene array
TGFBR2	1.40	0.038	qPCR

TGFB, transforming growth factor beta (TGF); TGFBR, TGFB receptor; fold increase vs. VM/+ (gene array) or vs. wild-type (qPCR)

Supplemental References

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