SUPPLEMENTAL MATERIAL

DWORF extends life span in a PLN-R14del cardiomyopathy mouse model by reducing abnormal sarcoplasmic reticulum clusters

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Supplementary methods

Animals

Mice were housed in a cage with wood chips bedding, on a 12 h light/12 h dark cycle with ad libitum access to standard mouse chow (ssniff®) and water. The PLN-R14$^{\Delta/\Delta}$ mouse model (C57BL/6J background) has been extensively described previously [12]. The DWORF transgenic (Tg) mouse line 1 (C57BL/6J background) with cardiac-specific DWORF overexpression, has been described previously [14]. Genotyping of the mice was performed according to the methods described in the original articles of both mouse lines. To compare the reduction of DWORF gene expression in general heart failure (HF) to PLN-R14del cardiomyopathy, left ventricular (LV) tissues from mice that underwent ischemia/reperfusion (I/R; temporal ligation of the left anterior descending coronary artery (LAD) for 60 minutes; n=8) injury or myocardial infarction (MI; permanent LAD ligation; n=9) were included as ischemic HF samples together with mice that underwent sham treatment (n=7) as corresponding controls (samples from Du et al. [19]). Minimal group sizes for all animal experiments were determined using power calculations with the significance level at 5% and power at 80%. Exclusion criteria set before the start of the study were the presence of the following genetic deviations: malocclusion/teeth overgrowth, hydrocephalus or growth retardation. However, mice did not have these genetic deviations, hence all mice with the correct genotypes were included in the study.

Euthanasia

Mice were euthanized either when the predetermined endpoint (an age of interest) was reached or, in case of survival studies, when mice reached the humane endpoint due to severe HF, which was evaluated in a blinded fashion (EF<10%, or strongly reduced activity with clearly elevated respiration). Mice were anesthetized using a mixture of oxygen and isoflurane (2.5%). The abdomen and thoracic cavity were opened, the abdominal aorta was cut and the circulation was flushed with saline via the heart. Subsequently, the heart was excised and rinsed in ice-cold 1 M potassium chloride (KCl) (Merck Millipore, Germany) solution. A

transverse mid-slice was taken for histological processing, several small pieces (1 mm^3) from the left ventricle (LV) myocardium were collected for electron microscopy processing and the remaining LV tissue was snap-frozen in liquid nitrogen for molecular analysis.

Patient Material

Post-mortem end-stage HF formalin-fixed paraffin-embedded human cardiac tissue samples from PLN-R14del (n=5) and non-PLN-R14del DCM patients (n=5) were provided by the Pathology biobank from the University Medical Center of Groningen (UMCG). Material was acquired in accordance with international regulations and professional guidelines (the Declaration of Helsinki and the International Conference on Harmonization Guidelines for Good Clinical Practice). Material used in this project (RR#201800551) was obtained from anonymous excess material and the Institutional Review Board (IRB) of the UMCG approved the use of the tissues for explorative tissue-based studies. The medical ethics review board (Central Ethics Review Board non-WMO studies, UMCG) waives the need for approval if excess material is used, under the law in the Netherlands and waives the need for informed consent when patient anonymity is assured.

Histological analysis

To determine the degree of cardiac fibrosis, Masson's trichrome stain for collagen deposition detection was performed following standard procedures. The entire stained sections were imaged using a NanoZoomer 2.0-HT digital slide scanner (Hamamatsu Photonics, Japan). Subsequently the degree of fibrosis was quantified as a percentage of the entire section surface using the Positive Pixel Count v9 algorithm of Aperio's ImageScope software (version 12.4.0; Leica Microsystems).

To determine the cell size of cardiomyocytes, fluorescein isothiocyanate (FITC)-conjugated wheat germ agglutinin (WGA; Sigma-Aldrich, MO, USA) was used to stain the extracellular matrix to visualize cell boundaries. The entire stained sections were imaged using an Olympus

VS200 ASW digital fluorescent slide scanner (Olympus - Life Science, Japan) at 40x magnification. QuPath Open Software for Bioimage Analysis (version 0.4.3) was used to measure the surface area of 30-50 transversally cut cardiomyocytes per mouse (n=4-5 mice per group per timepoint). Relative cardiomyocyte size is displayed as a fold change difference to age-matched WT controls.

To determine the abundance of PLN-containing protein clusters, immunofluorescent (IF) staining for PLN was performed using the 2D12 anti-PLN antibody (Invitrogen, CA, USA). This antibody has successfully been used to visualize normally distributed PLN and aggregated PLN clusters in patients and mice carrying the R14del deletion [11,12,29]. To prevent unspecific background staining from the secondary anti-mouse antibody, the primary anti-PLN antibody was labeled with Alexa Fluor 555 (red) using an APEX antibody labeling kit (Invitrogen). In addition, the extracellular matrix was stained green in these tissue sections using WGA and nuclei were stained blue with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, CA, USA). For the counting of PLN cluster positive cells, 2 representative images of longitudinal sectioned cardiomyocytes were taken at 10x magnification (Leica AF6000 fluorescence imaging system, Leica Microsystems) via a blinded procedure performed by an unbiased person. Counts were corrected for selected surface area measured by ImageJ software (National Institutes of Health, MD, USA) and averaged per mouse. Data is presented as cluster positive cells per 0.1 mm^2. For determination of percentage cells with and without PLN stained speckles or clusters, stained sections were imaged using an Olympus VS200 ASW digital fluorescent slide scanner at 40x magnification. Longitudinal sectioned cardiomyocytes were counted and clusters were arbitrarily defined as any densely stained PLN structures with a radius above 1,8 µm and speckles with diameters below this size, although in reality these form a continuum. The abundance of p62 positive cardiomyocytes was determined. Cardiac tissue sections were stained with Alexa Fluor 488 (green) labeled (Invitrogen APEX antibody labeling kit) anti-p62 antibody (ab56416, Abcam) and anti-cardiac troponin I (troponin, ab47003, Abcam) with secondary antibody donkey anti-rabbit Alexa Fluor™ 555 (A31572,

Invitrogen) and DAPI. The entire stained sections were imaged using an Olympus VS200 ASW digital fluorescent slide scanner at 40x magnification. OlyVIA image viewer software (version 3.3, Olympus Soft Imaging Solutions GmbH, Germany) was used to view the images and count the number of p62+troponin-positive cardiomyocytes within a selected and subsequently measured surface area. Data is presented as cluster positive cells per 0.1 mm^2. In addition the number of longitudinal cardiomyocytes positive for p62, but negative for troponin, were counted within the cardiac section as an indication for the number of death cells.

To show the localization of PLN and other S/ER proteins in mouse cardiac tissue samples, IF staining was performed. The anti-PLN antibody (clone 2D12, Invitrogen) was used together with anti-SERCA2 (MA3-919, ThermoFisher), anti-histidine-rich calcium binding protein (HRC, HPA004833, Merck, Germany), anti-calnexin (ab22595, Abcam, UK) or anti-atlastin-3 (ATL3, 16921-1-AP-s, Proteintech, UK) anti-cardiac troponin I (troponin, ab47003, Abcam), anti-Tom20 (sc-11415, Santa Cruz). Since the host of the anti-PLN antibody is mouse, the antibody was labeled using the APEX™ Alexa Fluor™ 488 Antibody Labeling Kit (A10468, Invitrogen). For HRC, calnexin, ATL-3, troponin and Tom20 a secondary donkey anti-rabbit Alexa Fluor™ 555 (A31572, Invitrogen) was used. To amplify the signal of anti-SERCA antibody, which was generated in mouse, the antibody was labeled using the APEX™ Biotin-XX Antibody Labeling Kit (A10495, Invitrogen) and a secondary Streptavidin Alexa Fluor™ 555 conjugate was used (S21381, Invitrogen). For the biotin-streptavidin-based staining, endogenous biotin and streptavidin were blocked before primary antibody incubation, using a blocking kit (E21390, Invitrogen). Mounting medium with DAPI was used to stain nuclei blue (ab104139, Abcam).
The same antibodies were used to show the localization of PLN and other S/ER proteins in post-mortem end-stage HF formalin-fixed paraffin-embedded human cardiac tissue samples from PLN-R14del and non-PLN-R14del DCM patients. Only if both antibodies were derived from the same host, antibodies were labeled using the APEX Alexa Fluor Labeling Kit. Otherwise, the secondary donkey anti-mouse Alexa Fluor™ 488 (A21202, Invitrogen) was used together with donkey anti-rabbit Alexa Fluor™ 555 (A31572, Invitrogen). The primary and

secondary antibodies that were used are presented in Table S1. Fluorescent imaging was performed using Leica DMI 8CS DLS confocal microscope (63x magnification, numerical aperture 1.4, pinhole 0.85) and deconvolution was performed with Imaris (version 9.7.2, non-iterative deconvolution algorithm).

Antibodies directed against ATL3, calnexin, HRC, p62 and SERCA were knockdown/knockout validated by the providing company. For the anti-troponin antibody, the company demonstrated cardiac positive and liver negative signal. For the anti-PLN antibody, cell treatment validation was performed by the providing company to ensure that the antibody binds to the antigen stated. Moreover, for all antibodies we confirmed that the staining pattern we observed when staining healthy cardiac tissue, corresponded to the staining patterns described in literature. In addition, secondary antibody only controls were used to distinguish genuine target staining from background staining.

Quantitative polymerase chain reaction

TRI Reagent (Sigma-Aldrich, MO, USA) was used to isolate RNA from powdered snap-frozen left ventricle (LV) tissues according to the manufacturer's protocol. Subsequently, cDNA synthesis was performed using the QuantiTect RT kit (Qiagen, Germany). Quantitative polymerase chain reaction (qPCR) was performed with iQ SYBR green supermix (Bio-Rad, CA, USA) and CFX384 Touch real-time PCR detection system (Bio-Rad) with recommended settings and protocol. CFX Manager software (version 3.0; Bio-Rad) was used for data processing. The exported values of the genes of interest were normalized to the expression level of housekeeping gene *Rplp0* (36B4) and these ΔCt values were presented as a fold change compared to the age-matched control group. Primer sequences are listed in Table S2.

Western blot analysis

The protein concentration was determined using a Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, MA, USA). Protein samples were adjusted to a fixed concentration and prepared for Western blot. The samples were heated at 40°C for 10 minutes and 5µg of protein was loaded onto a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) system. Subsequently, the separated proteins were transferred onto an Immun-Blot polyvinylidene fluoride (PVDF) membrane using a semi-dry transfer system (Amersham Biosciences, UK). The membranes were incubated with a primary antibody overnight at 4°C, followed by one hour of incubation at room temperature (RT) with a horseradish peroxide (HRP)-linked secondary antibody. Subsequently, detection was performed using Immobilon Classico Western HRP substrate (Merck Millipore) and an ImageQuant LAS 4000 digital imaging system (GE Healthcare, IL, USA).

Evans blue staining

To evaluate the occurrence of necrosis of cardiomyocytes in WT, $R14^{\Delta/\Delta}$ and $R14^{\Delta/\Delta}DWORF^{Tg}$ mice, 100 µl of 16.7 mg/ml Evans Blue dissolved in 0.9% NaCl was administered to mice via intraperitoneal injection. After 24 hours, mice we euthanized under anesthesia (2.5% isoflurane mixed with oxygen) by flushing the circulation with saline via the heart and subsequent removal of the heart. The heart was rinsed in ice-cold 1 M KCl solution and a transverse mid-slice was taken and embedded in Tissue-Tek O.C.T. compound, after which the samples were frozen. Frozen tissue specimens were sectioned using a cryostat (4 µm), cardiac sections were fixed for 10 minutes in 4% buffered formaldehyde solution (4% formalin; Klinipath, the Netherlands), incubated with (FITC)-conjugated WGA for 30 minutes and mounted using mounting medium with DAPI. The entire stained sections were imaged using an Olympus VS200 ASW digital fluorescent slide scanner at 40x magnification (Evans Blue staining was detected using excitation at 632 nm and emission at 647 nm). OlyVIA image viewer software was used to view the images, to export the acquired virtual digital slides and to count the number of Evans Blue

positive cardiomyocytes within a selected and subsequently measured surface area. Data is presented as Evans Blue positive cells per 0.1 mm^2.

Calcium transient and contraction measurements in isolated adult mouse cardiomyocytes

Cardiomyocyte isolation was performed protocol of Ackers-Johnson et al. [22]. In brief, mice were anesthetized and the descending aorta was cut. Subsequently, the heart was flushed by injection of 7 mL EDTA buffer (130 mmol/l NaCl, 5 mmol/l KCl, 0.5 mmol/l NaH_2PO_4, 10 mmol/l HEPES, 10 mmol/l glucose, 10 mmol/l BDM, 10 mmol/l taurine, 5 mmol/l EDTA, pH 7.8) into the right ventricle. The ascending aorta was clamped, the heart was excised and transferred to a dish with fresh EDTA buffer. By sequential injection of 10 mL EDTA buffer, 3 mL perfusion buffer (130 mmol/l NaCl, 5 mmol/l KCl, 0.5 mmol/l NaH_2PO_4, 10 mmol/l HEPES, 10 mmol/l glucose, 10 mmol/l BDM, 10 mmol/l taurine, 1 mmol/l $MgCl_2$, pH 7.8) and 30 to 50 mL collagenase buffer (0.5 mg/ml collagenase 2, 0.5 mg/ml collagenase 4, 0.05 mg/ml protease XIV) into the LV, digestion was accomplished. Tissue was gently pulled into 1-mm pieces using forceps and cellular dissociation was completed by gentle trituration using a wide bore pipette tip. Enzyme activity was inhibited by adding 5 mL stop buffer (perfusion buffer containing 5% fetal calf serum), after which cell suspension was passed through a 100-µm filter. Calcium concentration was gradually restored to physiological levels by 4 sequential rounds of gravity settling, using 3 intermediate calcium reintroduction buffers and a highly pure cardiomyocyte fraction was obtained. Medium was replaced by Tyrode solution (134 mM NaCl, 5 mM KCl, 5 mM sodium pyruvate, 12 mM HEPES, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 2 mM $CaCl_2$ and 11 mM glucose, pH 7.4) for calcium transient and excitation-contraction coupling measurements. Cardiomyocytes (~50.000 cells) were loaded with Fura-2 (1 µg/mL; F1221 Invitrogen™) using 1X PowerLoad™ Concentrate (P10020 Invitrogen™) for 30 minutes at 25°C. After washing, plating and acclimatization of cells to 37°C, contraction was evoked via electrical field stimulation (IonOptix MyoPacer; 2 Hz, 4 ms pulse duration, 20 V). An IonOptix Calcium and

Contractility System (IonOptix corporation, Milton, MA, United States) was used to measure calcium transients and video-based sarcomere length (SL) at 250 Hz sampling frequency. Per mouse the process of loading and measuring cardiomyocytes was performed 2 sequential times. Each time ~10 cardiomyocytes (~20 cells per mouse) were imaged for ~15 contraction-relaxation cycles. Subsequently, isoproterenol was added to induce β-adrenergic stimulation (final concentration 100 nM, I6504 Sigma-Aldrich) and measurements were performed on ~5 stimulated cardiomyocytes (~10 cells per mouse). We like to note that cardiomyocyte yield was much lower for $R14^{\Delta/\Delta}$ mice at 6 weeks of age, indicative for a high vulnerability of these cardiomyocytes as compared to WT and $R14^{\Delta/\Delta}DWORF^{Tg}$ cardiomyocytes at this age.

Electron microscopy

LV myocardial tissue pieces were fixed in EM fixative (4% paraformaldehyde and 0.1% glutaraldehyde in 0.1M sodium cacodylate (pH 7.4)) overnight at 4˚C. Subsequently EM fixative was diluted four times with 0.1M cacodylate buffer and samples were stored at 4˚C for further processing. Post fixation was performed with 1% osmium tetroxide and 1.5% potassium ferrocyanide. Next, samples were dehydrated, embedded in EPON epoxy resin, and ultrathin sections (80 nm) were cut, collected on single slot nickel grid and contrasted using 4% neodymium acetate. For immunolabeling, samples were etched with 1% periodic acid for 10 minutes, followed by a 30-minute blocking step using 1% bovine serum albumin (BSA; Sanquin, the Netherlands) in tris-buffered saline (TBS), pH 7.4. Next, PLN primary antibody (clone 2D12, Invitrogen, dilution 1:200) was incubated for 2 hours followed by washing and subsequent incubation for 1 hour with biotinylated horse anti-mouse secondary antibody (VEC.BA-2001, Vector Laboratories, dilution 1:400), followed by washing steps. Finally, streptavidin conjugate Qdot™ 655 (Q10123MP, Invitrogen, dilution 1:1000) was incubated for 1 hour. Sections were imaged using scanning transmission electron microscopy (STEM) (Zeiss Supra55, Germany).

Measurement of troponin in plasma

To determine plasma cardiac troponin I levels, blood was collected via the descending aorta. Plasma was diluted 1:4 and using the Muscle Injury Panel 3 Mouse Kit (K15186C, Meso Scale Diagnostics, MD, USA) cardiac troponin I level (and skeletal troponin I as a control) was measured on the MESO QuickPlex SQ 120 instrument (Meso Scale Diagnostics). Analysis was performed using MSD Discovery Workbench 4.0 Analysis Software (Meso Scale Diagnostics). Values below the limit of detection are replaced by zero.

Supplementary figures

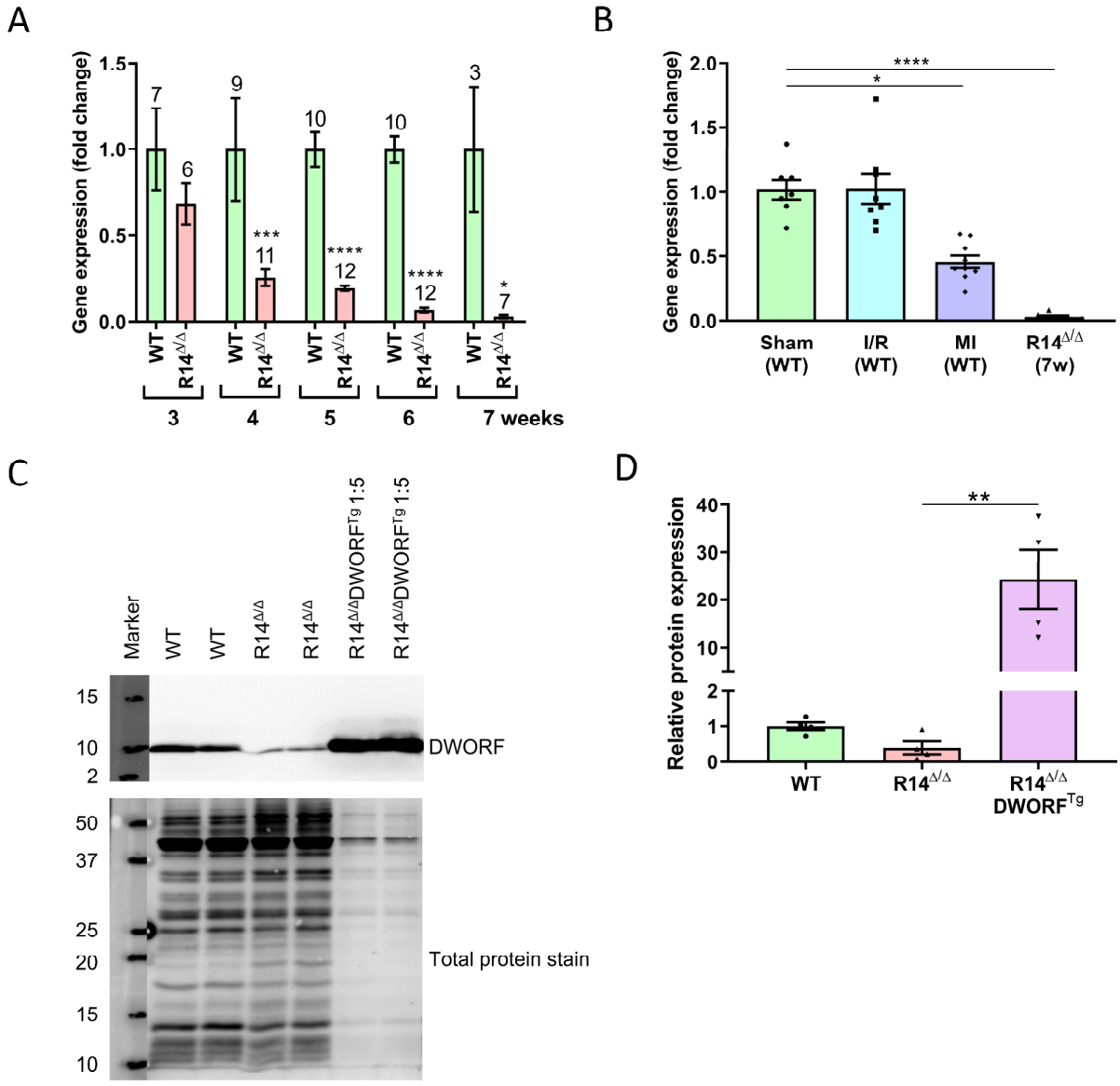

Figure S1 Cardiac DWORF expression and overexpression in R14$^{\Delta/\Delta}$ mice. A, Relative cardiac DWORF gene expression over time in 3- to 7-week-old R14$^{\Delta/\Delta}$ mice, displayed as fold change difference to age-matched WT controls. The numbers of mice used per group and timepoint are listed above each bar. **B,** Relative cardiac DWORF gene expression in mice with myocardial ischemia reperfusion injury (I/R), mice that underwent myocardial infarction (MI) and 7-week-old R14$^{\Delta/\Delta}$ mice, displayed as a fold change to WT sham mice (n=7-9). **C,** Representative (average-based) Western blot image of DWORF protein levels in 7-week-old WT, R14$^{\Delta/\Delta}$ and R14$^{\Delta/\Delta}$DWORF^{Tg} mice (R14$^{\Delta/\Delta}$DWORF^{Tg} is diluted 1:5 to maintain linear range of chemiluminescent signal). **D,** Quantification of DWORF protein levels, confirming DWORF overexpression in R14$^{\Delta/\Delta}$DWORF^{Tg} mice (n=4 per group). * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. Significance was examined by Kruskal-Wallis with Dunn's multiple comparisons test, or the Mann-Whitney U test when two groups were compared.

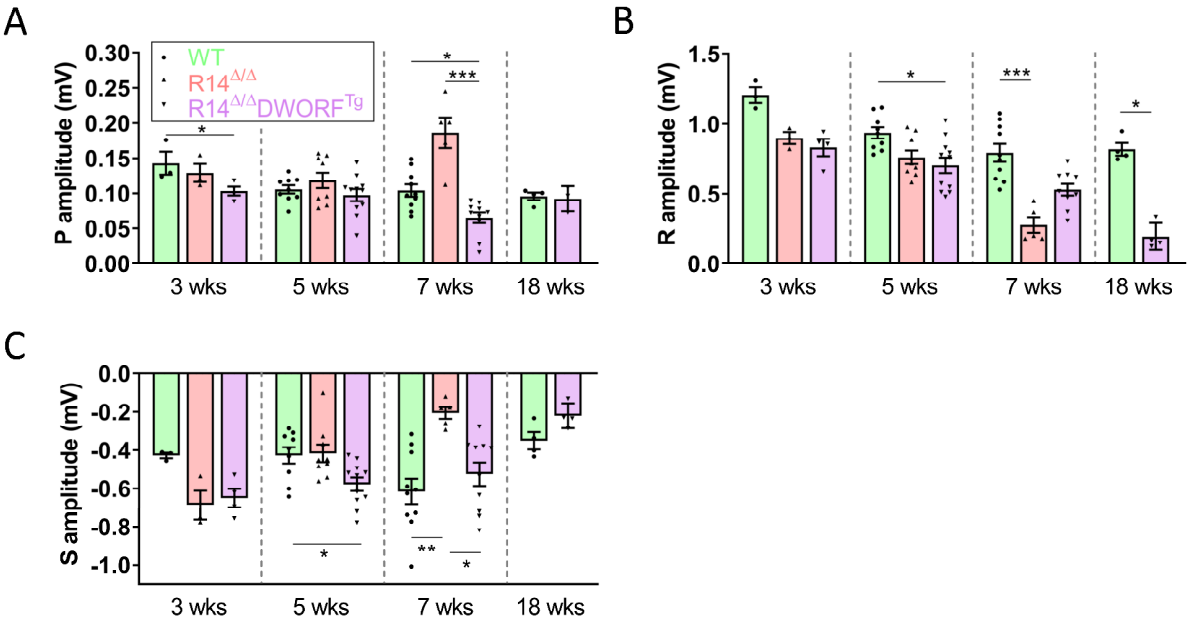

Figure S2 Surface electrocardiography of WT, R14$^{\Delta/\Delta}$ and R14$^{\Delta/\Delta}$DWORF^{Tg} mice. (**A-C**) P, R and S amplitudes of ECGs that are displayed in Figure 1G of 3- to 18-week-old R14$^{\Delta/\Delta}$, R14$^{\Delta/\Delta}$DWORF^{Tg} and WT control mice. (n=3-4 at 3 weeks, n=9-11 at 5 weeks, n=5-10 at 7 weeks, n=4 at 18 weeks). * P<0.05, ** P<0.01, *** P<0.001. Significant differences were examined by Kruskal-Wallis with Dunn's multiple comparisons test, or the Mann-Whitney U test when only two groups were compared.

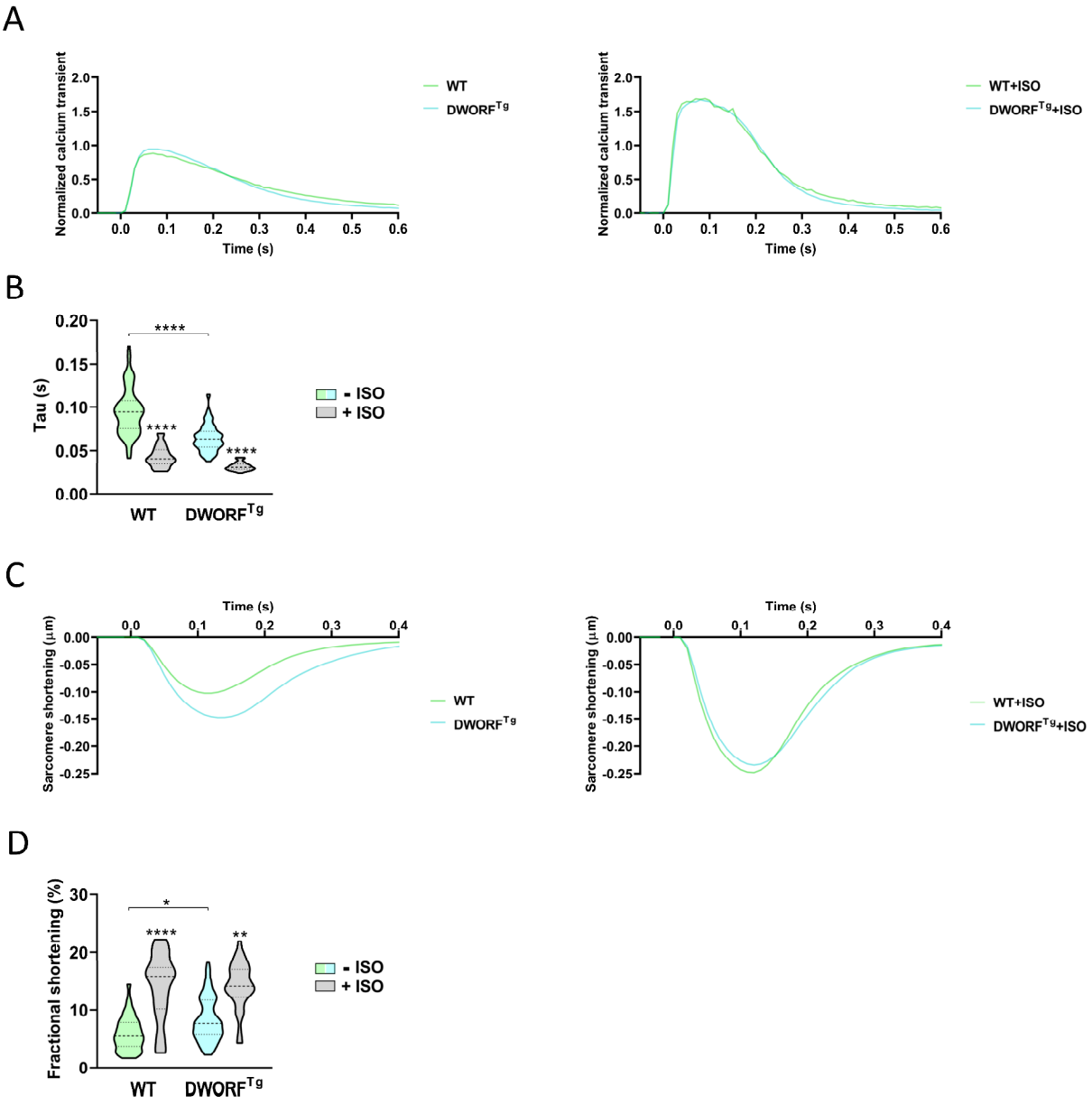

Figure S3 Confirmation of calcium handling and sarcomere function data in isolated DWORF^{Tg} cardiomyocytes. A, Normalized calcium transients under pacing-conditions with and without ISO stimulation in isolated mouse cardiomyocytes from WT and DWORF^{Tg} mice and (**B**) quantification of Tau (-ISO: n = 60–100 cells from 4 mice per group, +ISO: n = 25–40 cells from 4 mice per group). **C,** Normalized pacing-induced sarcomere contraction-relaxation curves with and without ISO stimulation and (**D**) quantification of fractional shortening. ISO= isoproterenol, ISO condition was compared with the untreated matched genotype. * P<0.05, ** P<0.01, **** P<0.0001. Significance was examined by Hierarchical cluster analysis using ANOVA with Bonferroni correction.

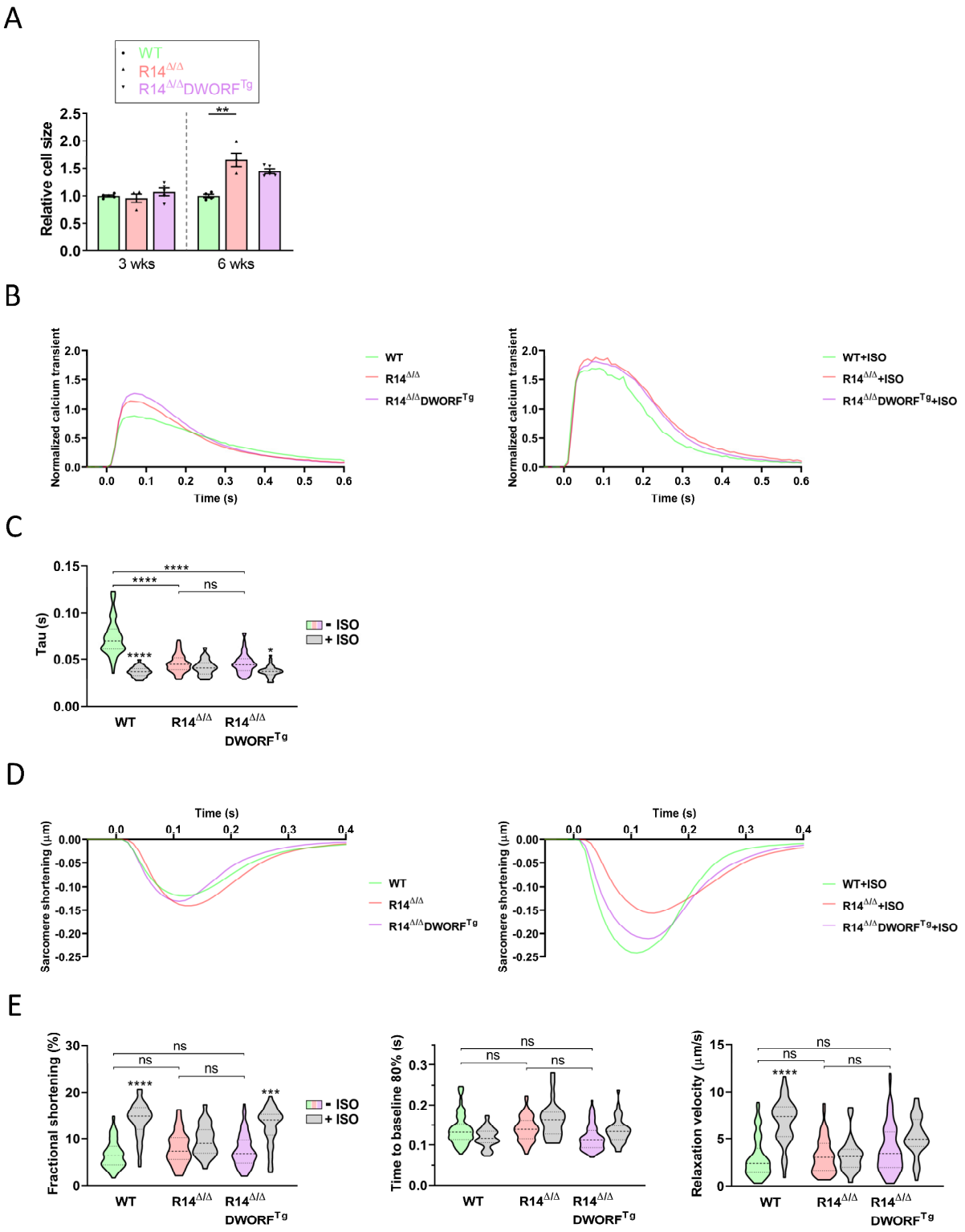

Figure S4 Calcium handling and sarcomere function parameters in cardiomyocytes isolated from 6-weeks-old mice. A, Relative cardiomyocyte size of WT, R14$^{\Delta/\Delta}$ and R14$^{\Delta/\Delta}$DWORF^{Tg} mice at about 3 or 6 weeks of age, displayed as a fold change difference to age-matched WT controls. **B**, Normalized calcium transients under pacing-conditions with and without ISO stimulation from isolated mouse cardiomyocytes of 6-week-old R14$^{\Delta/\Delta}$, R14$^{\Delta/\Delta}$DWORF^{Tg} and WT control mice, and (**C**) the corresponding quantification of decay time constant (Tau). **D,** Normalized pacing-induced sarcomere contraction-relaxation curves with and without ISO stimulation, with quantification of (**E**) fractional shortening, time to reach 80% of the diastolic sarcomere length after systole and the relaxation velocity. Calcium and contraction measurements were simultaneously performed in isolated adult mouse cardiomyocytes from

6-week-old WT, $R14^{\Delta/\Delta}$ and $R14^{\Delta/\Delta}DWORF^{Tg}$ mice (-ISO: n = 80–100 cells from 4 mice per group, +ISO: n = 30–40 cells from 3-4 mice per group). ISO= isoproterenol, ISO condition was compared with the untreated matched genotype. * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001. Significance for cell size was examined by Kruskal-Wallis with Dunn's multiple comparisons test. Significance for calcium and sarcomere function parameters was examined by Hierarchical cluster analysis using ANOVA with Bonferroni correction.

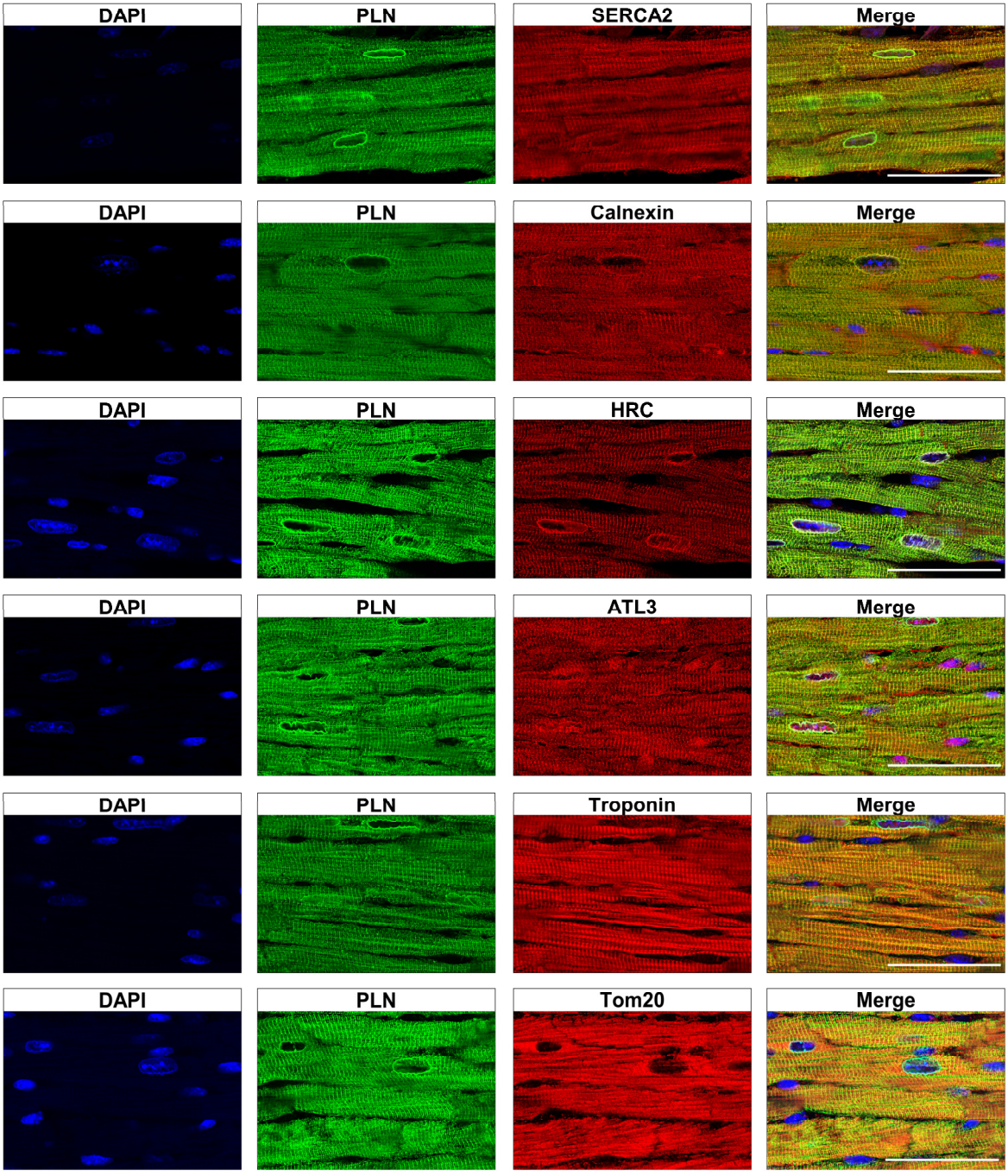

Figure S5 Representative (average-based) IF double staining for PLN together with SERCA2, calnexin, HRC, ATL3, troponin or Tom20 in LV tissue sections from 7-week-old WT mice (n=6; scale bar: 50 µm).

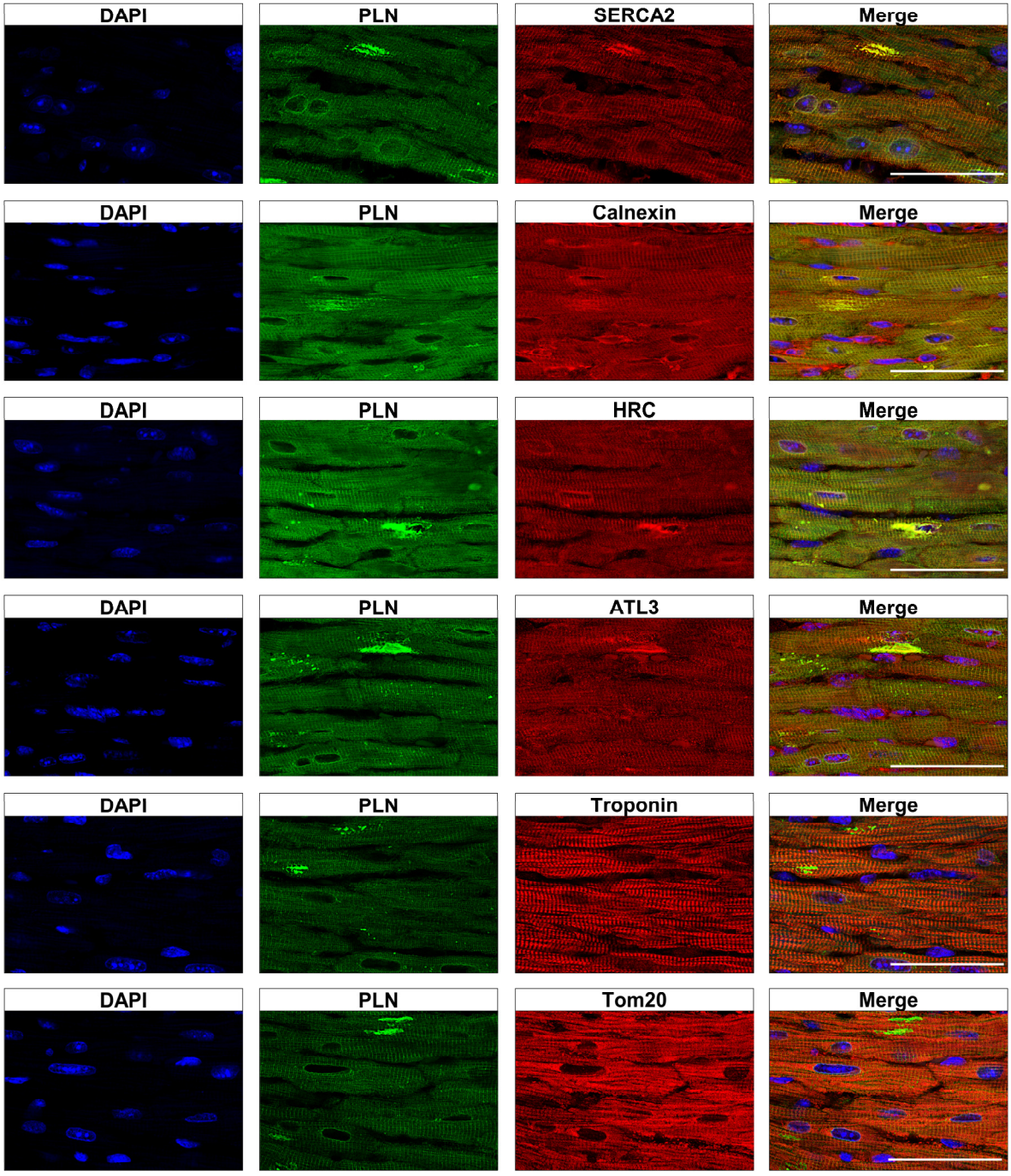

Figure S6 Representative (average-based) IF double staining for PLN together with SERCA2, calnexin, HRC, ATL3, troponin or Tom20 in LV tissue sections from 3-week-old R14$^{\Delta/\Delta}$ mice (n=4; scale bar: 50 µm).

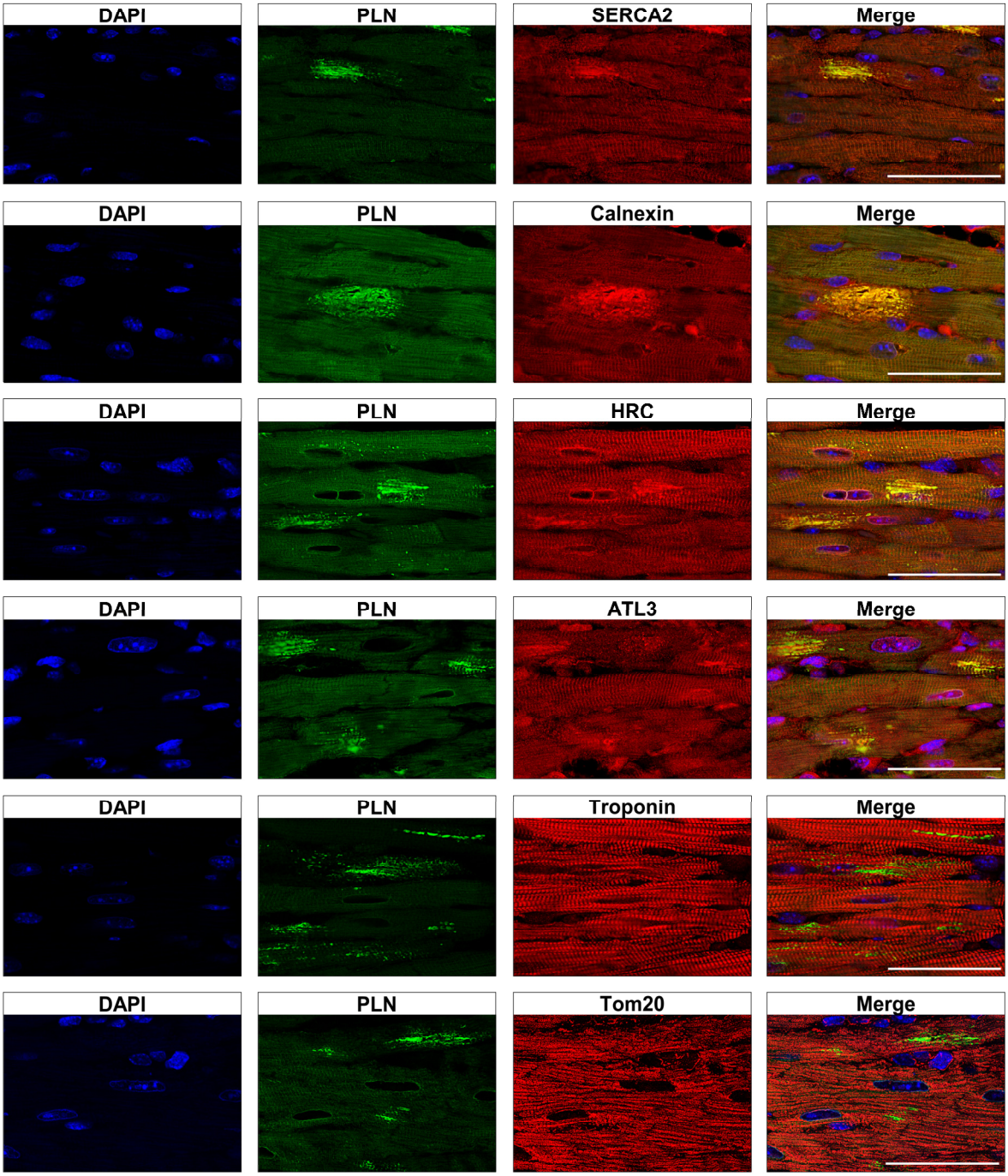

Figure S7 Representative (average-based) IF double staining for PLN together with SERCA2, calnexin, HRC, ATL3, troponin or Tom20 in LV tissue sections from 5-week-old R14$^{\Delta/\Delta}$ mice (n=4; scale bar: 50 µm).

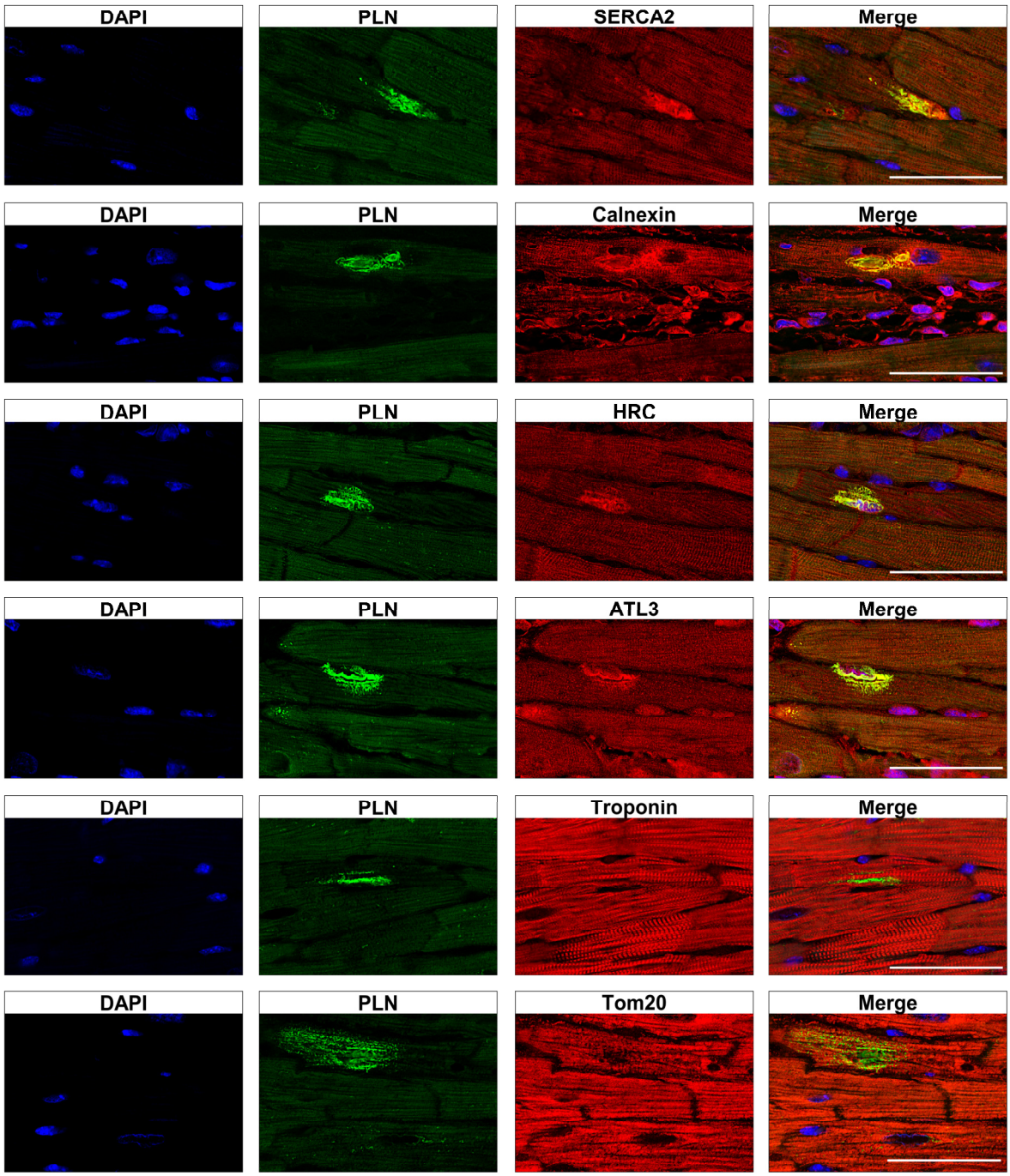

Figure S8 Representative (average-based) IF double staining for PLN together with SERCA2, calnexin, HRC, ATL3, troponin or Tom20 in LV tissue sections from 18-week-old R14$^{\Delta/\Delta}$DWORF^{Tg} mice (n=5; scale bar: 50 µm).

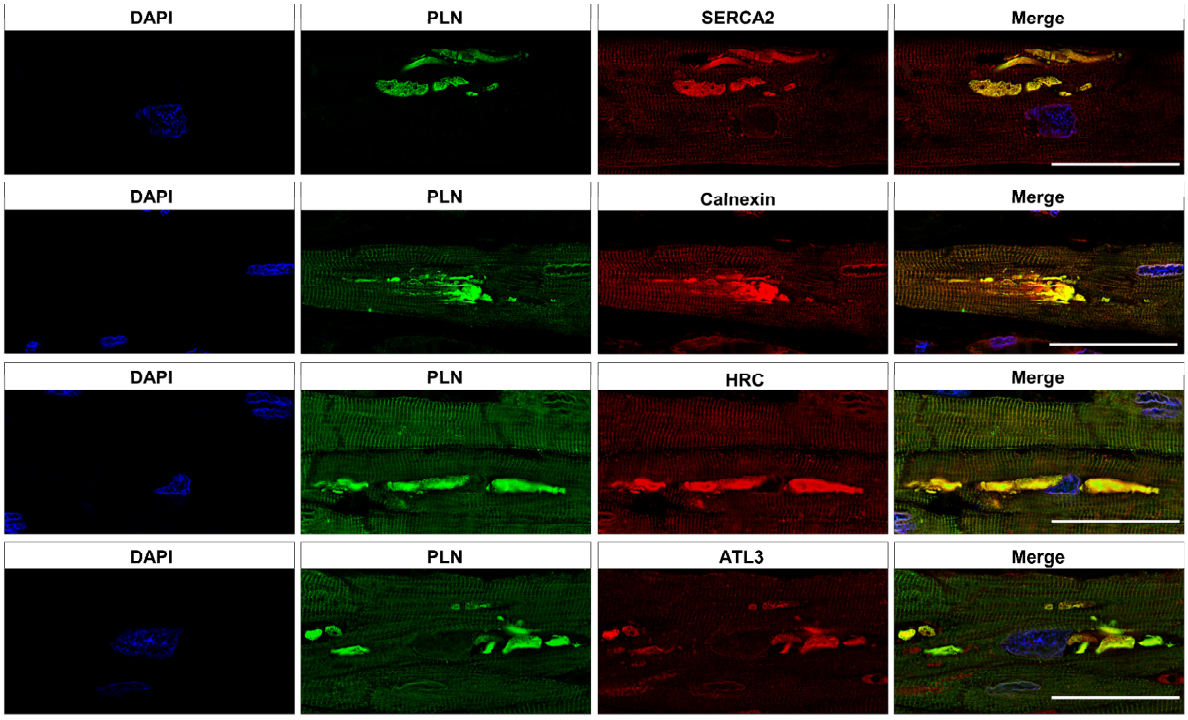

Figure S9 Clustering of the S/ER in patients with PLN-R14del cardiomyopathy. Representative (average-based) IF double staining for PLN together with SERCA2, calnexin, HRC or ATL3 in LV tissue sections from PLN patients (n=5; scale bar: 50 µm).

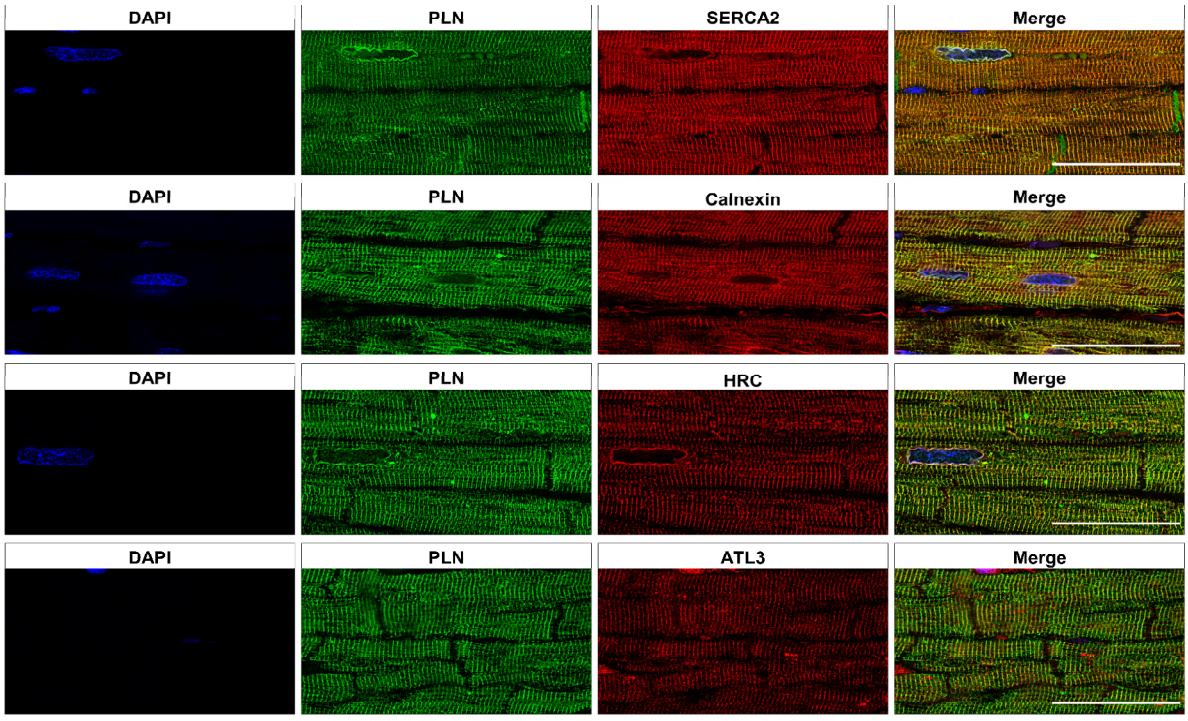

Figure S10 Representative (average-based) IF double staining for PLN together with SERCA2, calnexin, HRC or ATL3 in LV tissue sections from non-PLN-R14del DCM patients (n=5; scale bar: 50 µm).

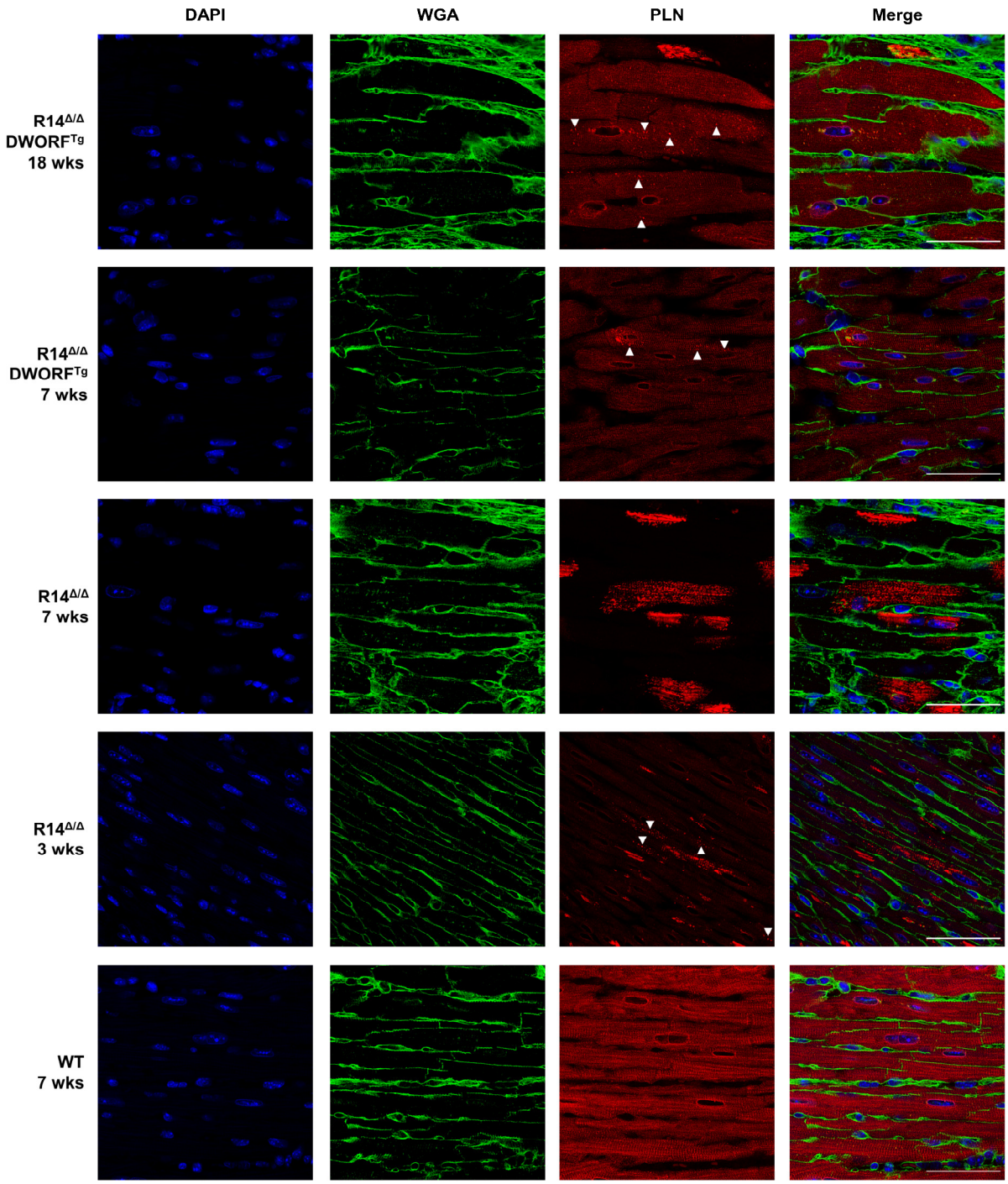

Figure S11 DWORF overexpression inhibits PLN cluster size increase. Representative (average-based) IF double staining for PLN (red) together with together with WGA (green) and DAPI (blue) in LV tissue sections of 7- and 18-week-old R14$^{\Delta/\Delta}$DWORF^{Tg} mice, 3- and 7-week-old R14$^{\Delta/\Delta}$ mice and 7-week-old WT mice. White arrowheads emphasize small sized PLN clusters (n=5; scale bar: 50 µm).

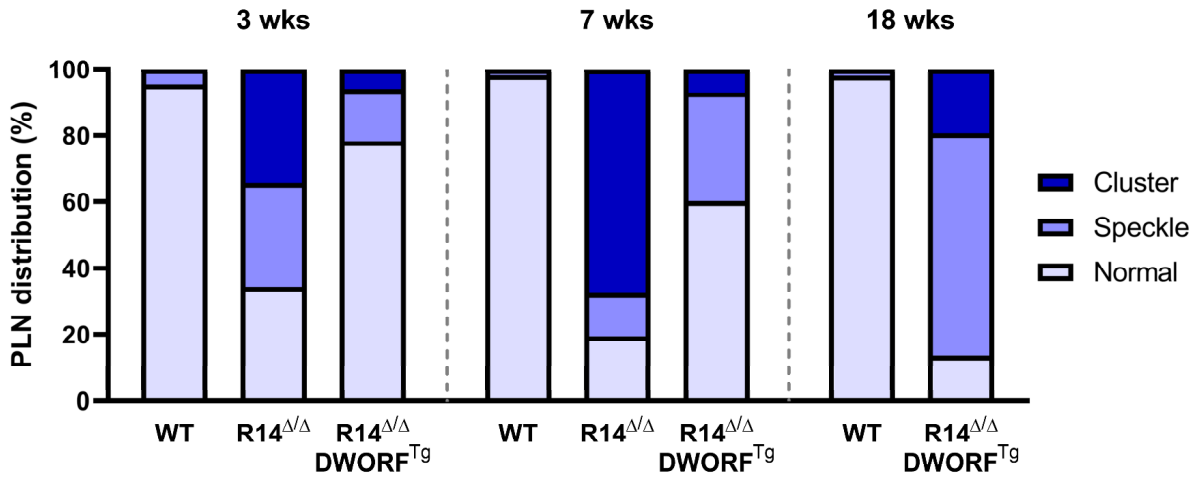

Figure S12 PLN distribution quantified in WT, $R14^{\Delta/\Delta}$ and $R14^{\Delta/\Delta}DWORF^{Tg}$ mice at 3, 7 and 18 weeks of age. Cardiomyocytes with normal, speckle and cluster PLN distribution were counted within an area and are presented as a percentage of the total amount of cardiomyocytes counted (n=3-5 mice per group and about 50 cells per mouse were counted), corresponding to representative IF staining from figure S11.

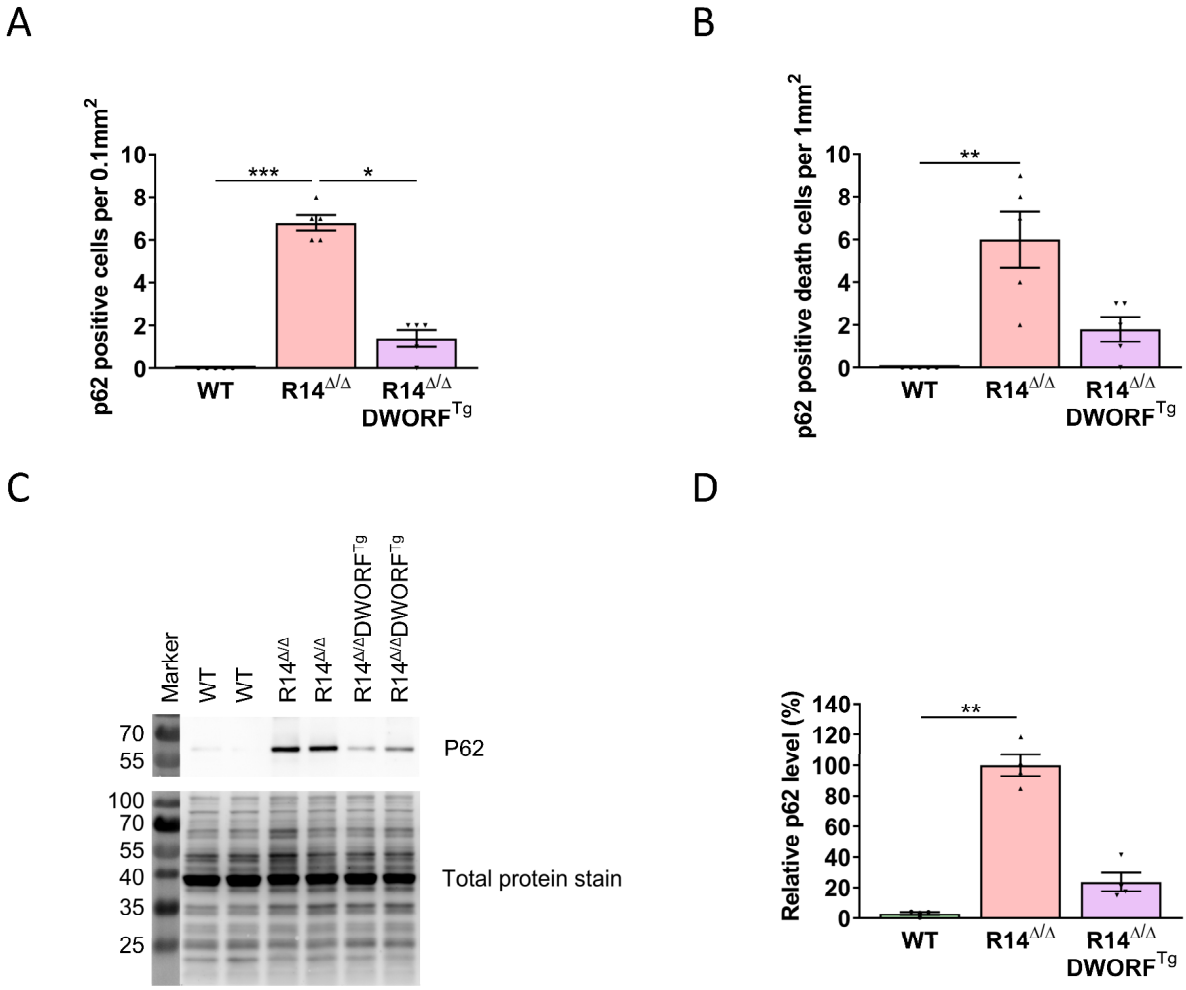

Figure S13 Cardiomyocyte death quantified in R14$^{\Delta/\Delta}$ and R14$^{\Delta/\Delta}$DWORF^{Tg} mice at age 6 weeks. **A,** P62 positive cardiomyocyte count per 0.1mm^2 at 6 weeks of age (n=5 per group), corresponding to representative IF staining from figure 7A. **B,** P62 positive and troponin I negative cardiomyocyte count per 1mm^2 at 6 weeks of age, indicative of cardiomyocyte death occurrence (n=5 per group). corresponding to representative IF staining from figure 7B. **C,** Representative (average-based) Western blot images of p62 protein levels in LVs of WT, R14$^{\Delta/\Delta}$ and R14$^{\Delta/\Delta}$DWORF^{Tg} mice at 7 weeks of age. **D,** P62 level quantified and shown as a percentage with mean R14$^{\Delta/\Delta}$ levels set at 100% (n=4 per group). * P<0.05, ** P<0.01, *** P<0.001. Significance was examined by Kruskal-Wallis with Dunn's multiple comparisons test.

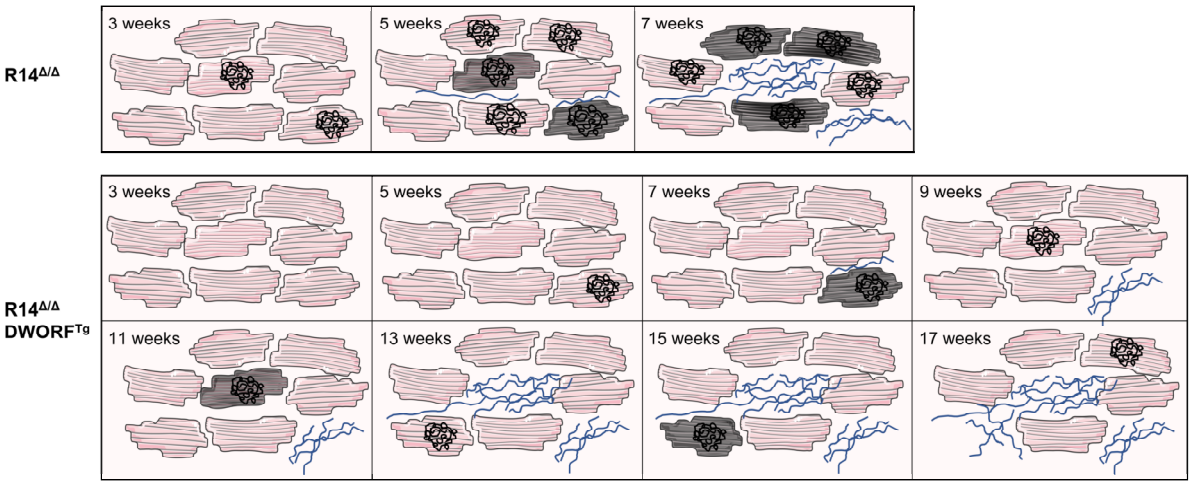

Figure S14 Simplified schematic representation of the balance between PLN/SR cluster formation and cardiomyocyte death, accompanied by the formation of replacement fibrosis. PLN/SR clusters are illustrated by the black cluster structure, grey cardiomyocytes depict dying cells that will be cleared afterwards and replacement fibrosis is visualized by blue strands. This simplified figure explains the disease progression occurring in $R14^{\Delta/\Delta}DWORF^{Tg}$ mice, despite the low number of cluster positive cardiomyocytes at any given time point and shows the comparison to the accelerated disease progression in $R14^{\Delta/\Delta}$ mice. It also depicts both $R14^{\Delta/\Delta}$ and $R14^{\Delta/\Delta}DWORF^{Tg}$ mice having an equal amount of cardiac damage at end stage disease, but with a significant extension of life span by DWORF overexpression.

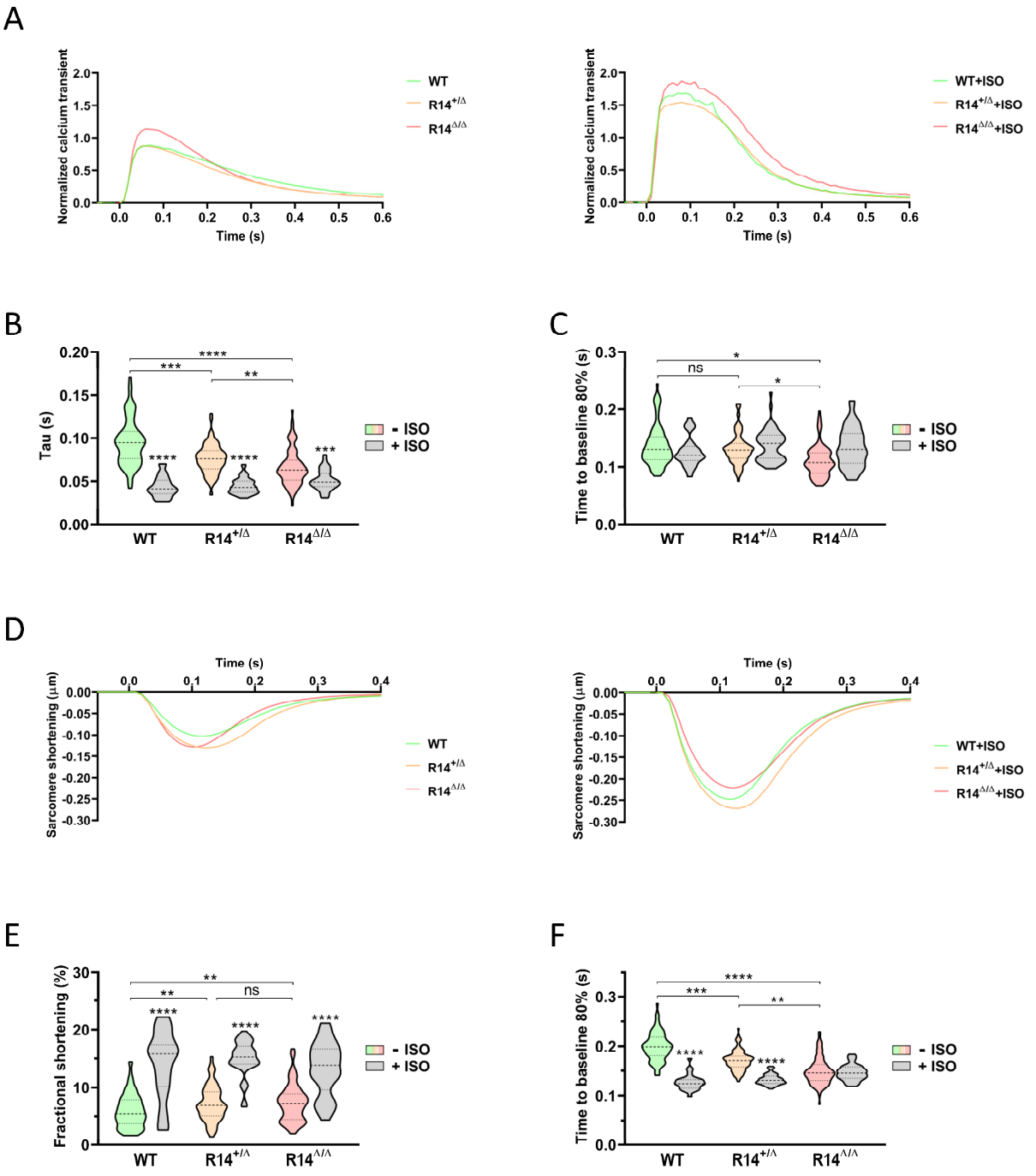

Figure S15 Calcium handling and sarcomere function parameters in WT, R14$^{+/\Delta}$ and R14$^{\Delta/\Delta}$ cardiomyocytes. A, Normalized calcium transients under pacing-conditions with and without ISO stimulation, with quantification of (**B**) decay time constant (Tau) and (**C**) time to reduce systolic calcium level by 80%. **D,** Normalized pacing-induced sarcomere contraction-relaxation curves with and without ISO stimulation, with quantification of (**E**) fractional shortening and (**F**) time to reach 80% of the diastolic sarcomere length after systole. All experiments were performed in isolated adult mouse cardiomyocytes from 3-week-old WT, R14$^{+/\Delta}$ and R14$^{\Delta/\Delta}$ mice (-ISO: n = 80–100 cells from 4 mice per group, +ISO: n = 30–40 cells from 4 mice per group). ISO= isoproterenol, ISO condition was compared with the untreated matched genotype. * P<0.05,** P<0.01, *** P<0.001, **** P<0.0001. Significance was examined by Hierarchical cluster analysis using ANOVA with Bonferroni correction.

Supplementary tables

Table S1. Antibodies that were used for IF-staining

Antibody	Catalogue number	Host	Type
Anti-PLN	MA3-922 ThermoFisher	Mouse	Primary
Anti-SERCA2	MA3-919 ThermoFisher	Mouse	Primary
Anti-calnexin	Ab 22595 Abcam	Rabbit	Primary
Anti-HRC	HPA004833 Merck	Rabbit	Primary
Anti-ATL3	16921-1-AP-s Proteintech	Rabbit	Primary
Anti-cardiac troponin I	ab47003	Rabbit	Primary
Anti-Tom20	sc-11415 Santa Cruz	Rabbit	Primary
Anti-p62	ab56416, Abcam	Mouse	Primary
Donkey anti mouse	A21202 Invitrogen	Donkey	Secondary
Donkey anti rabbit	A31572 Invitrogen	Donkey	Secondary
Alexa 555 streptavidin	S32355 Invitrogen	-	Secondary

Table S2. Primer sequences used for qPCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Rplp0 (36B4)	AAGCGCGTCCTGGCATTGTC	GCAGCCGCAAATGCAGATGG
Col1a1	AGAGCATGACCGATGGATTC	CGCTGTTCTTGCAGTGATAG
Lgals3	CAGTGAAACCCAACGCAAAC	AGGCAACATCATTCCCTCTC
Nppa	GCTTCCAGGCCATATTGGAG	GGTGGTCTAGCAGGTTCTTG
Myh6	AGCTCATGGCTACACTCTTC	GTGGGTGGTCTTCAGGTTTG
Myh7	GAGCATTCTCCTGCTGTTTC	GAGCCTTGGATTCTCAAACG
Dworf	TTCTTCTCCTGGTTGGATGG	TCTTCTAAATGGTGTCAGATTGAAGT

Table S3. Antibodies that were used for Western blot

Antibody	Catalogue number	Host	Type	Note
Anti-DWORF	Custom made	Rabbit	Primary	Nelson, Science 2016
Anti-PLN	14562 Cell Signaling	Rabbit	Primary	
Goat anti rabbit	P044801 Dako	Goat	Secondary	
Rabbit anti mouse	P0260 Dako	Rabbit	Secondary	

Table S4. Isolated cardiomyocyte calcium transient amplitudes at 3 weeks of age

Genotype	Ca^{2+} amplitude -ISO	Ca^{2+} amplitude +ISO
WT	0.95 ± 0.04	1.87 ± 0.20 **
$R14^{\Delta/\Delta}$	1.15 ± 0.04	1.87 ± 0.10 *
$R14^{\Delta/\Delta}DWORF^{Tg}$	1.27 ± 0.04	1.81 ± 0.13

Data are represented as means ± SEM. ISO= isoproterenol. -ISO: n = 80–100 cells from 4 mice per group, +ISO: n = 30–40 cells from 4 mice per group. -ISO conditions were compared to WT control and +ISO conditions were compared to the corresponding -ISO genotype-matching control. * $P<0.05$, ** $P<0.01$. Significance was examined by Hierarchical cluster analysis using ANOVA with Bonferroni correction.