CARDIAC HYPERTROPHY AND ARRHYTHMIA IN MICE INDUCED BY A MUTATION IN RYANODINE RECEPTOR 2

Alvarado FJ et al.

DATA SUPPLEMENT

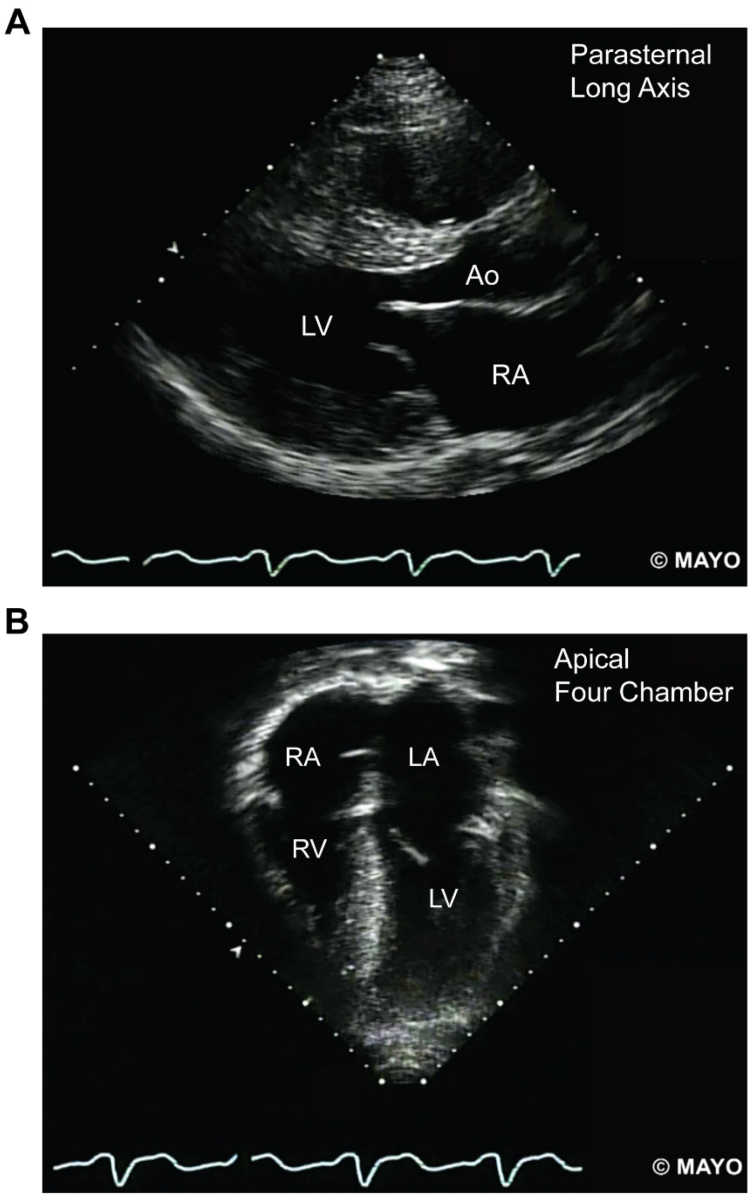

Figure S1. Echocardiographic recordings from the patient harboring the P1124L mutation.

Parasternal long axis (**A**) and apical four-chamber (**B**) echocardiographic window demonstrating hypertrophic, obstructive cardiomyopathy.

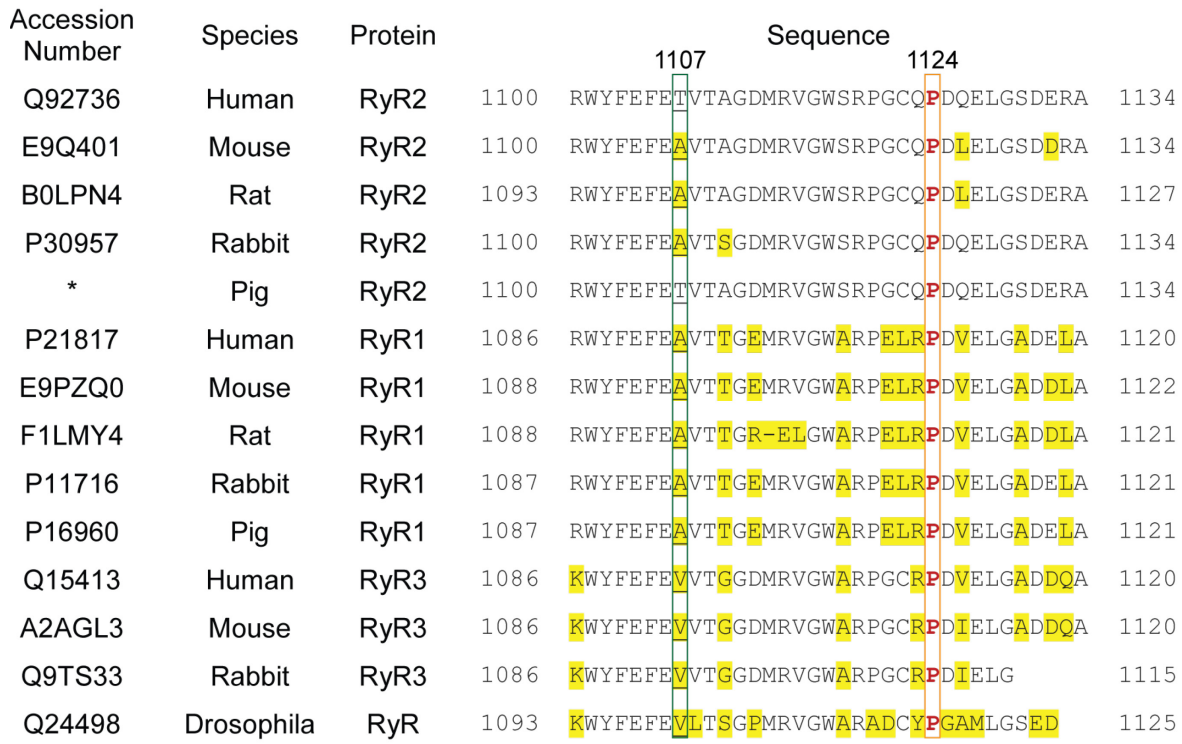

Figure S2. Amino acid sequence alignment of RyR isoforms from various species.

Sequence alignment of residues 1100–1134 of the human RyR2 with RyR isoforms from other species. Shaded residues are different from the human template. P1124 is conserved in all RyR isoforms from all the species compared. Accession numbers and protein sequences from UniProt (www.uniprot.org). *: Pig RyR2 sequence reconstructed from Peng et al. (1).

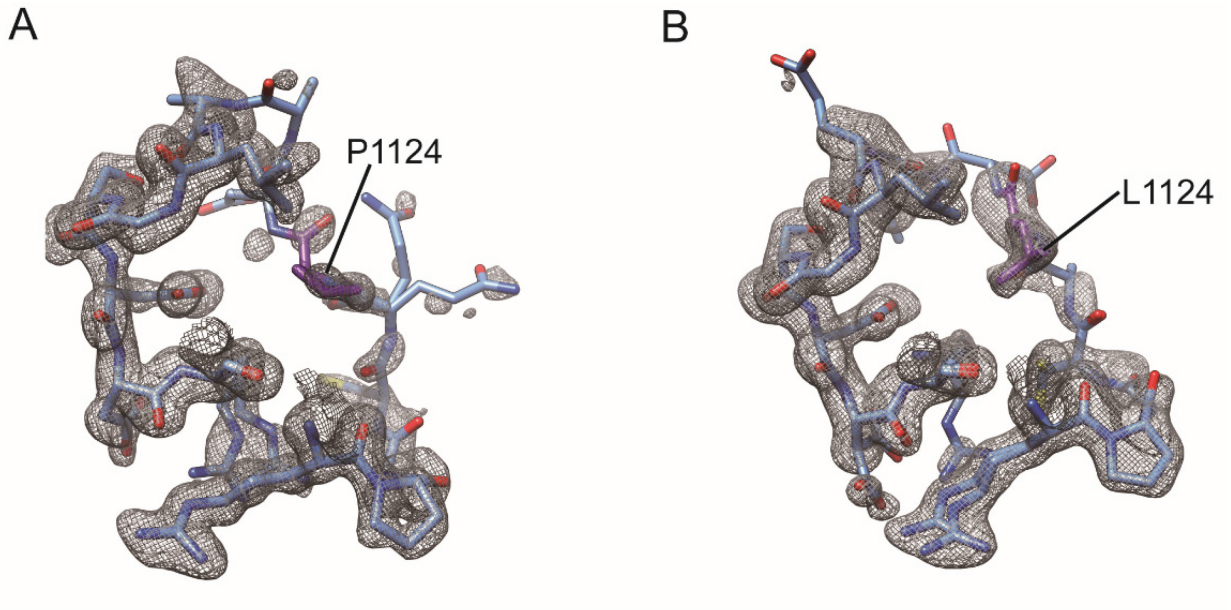

Figure S3. Omit maps of the loops containing P1124L obtained by refinement of x-ray crystallography data.

Omit maps of the loops containing the mutation P1124L are presented at 1 σ for the 2mFo-dFc maps. WT and mutant loops are shown in panel A and B, respectively, with the mutation site colored in purple.

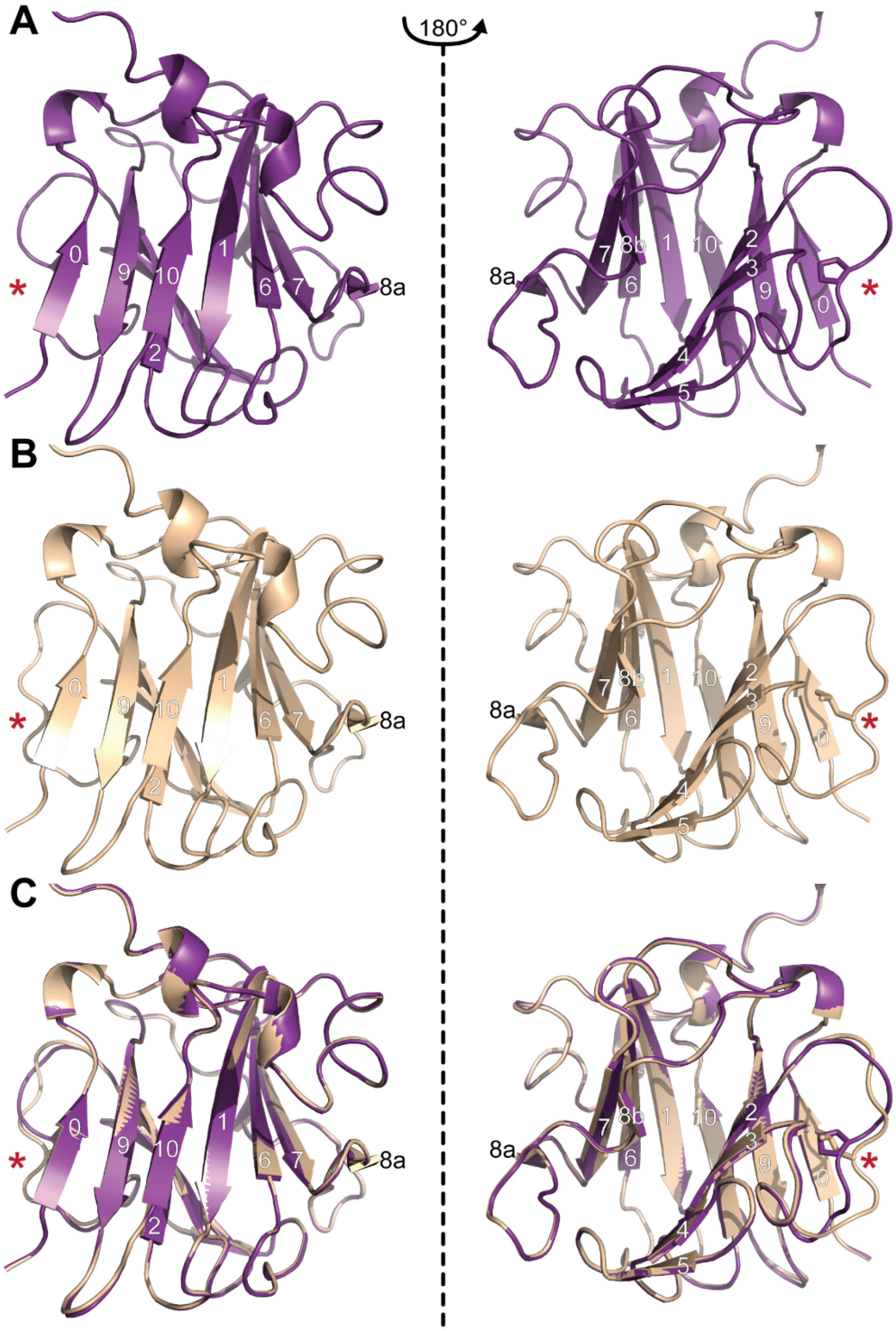

Figure S4. Structure of the SPRY2 domain at 1.44 Å resolution modeled using the PyMol software.

A. Crystal structures of the WT SPRY2 domain. **B.** Crystal structure of the P1124L SPRY2. **C**. Superposition of the structures from panels A and B. Numbers indicate β-strands as defined by Lau et al. Asterisk indicates the loop containing residue 1124.

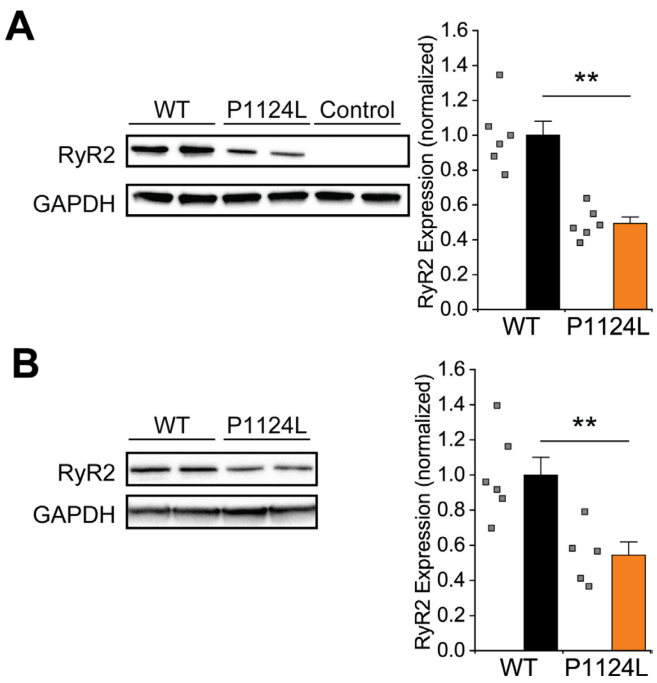

Figure S5. RyR2 expression in transiently transfected and stable HEK293 Cells.

A. *Left*: Representative Western blot of P1124L and WT RyR2 expression in HEK293 cells transiently transfected with the mouse *Ryr2*. *Right*: Quantification of band intensity. P1124L expression is lower than WT expression (n = 6 transfections. ** $p < 0.01$, t-test). This blot is representative for samples run in three different gels. **B**. *Left*: Representative Western blot of P1124L and WT RyR2 expression in stable HEK293 cells inducible expression of RyR2, induced with tetracycline 1 µg/mL to the culture medium. *Right*: Quantification of band intensity. P1124L expression is lower than WT expression (n = 6 WT, PL 5 independent samples. ** $p < 0.01$, t-test)

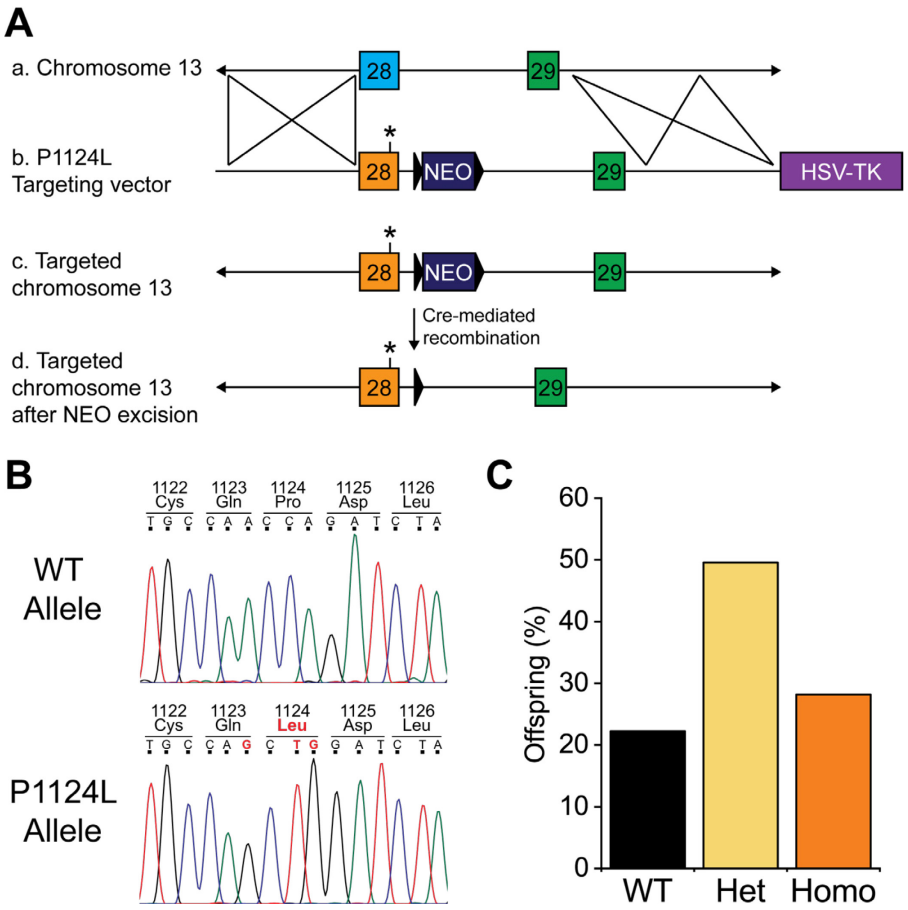

Figure S6. Generation of the P1124L Mouse Model

A. Strategy for the generation of the mouse model by homologous recombination. Numbers indicate *Ryr2* exons. **B**. Sanger sequencing of the WT and mutant alleles in a heterozygous mouse. Numbers indicate the codon number and the encoded amino acid. **C**. Offspring proportions obtained from mating heterozygous mice (n = 243 pups; N = 41 litters. No significant difference, chi-square).

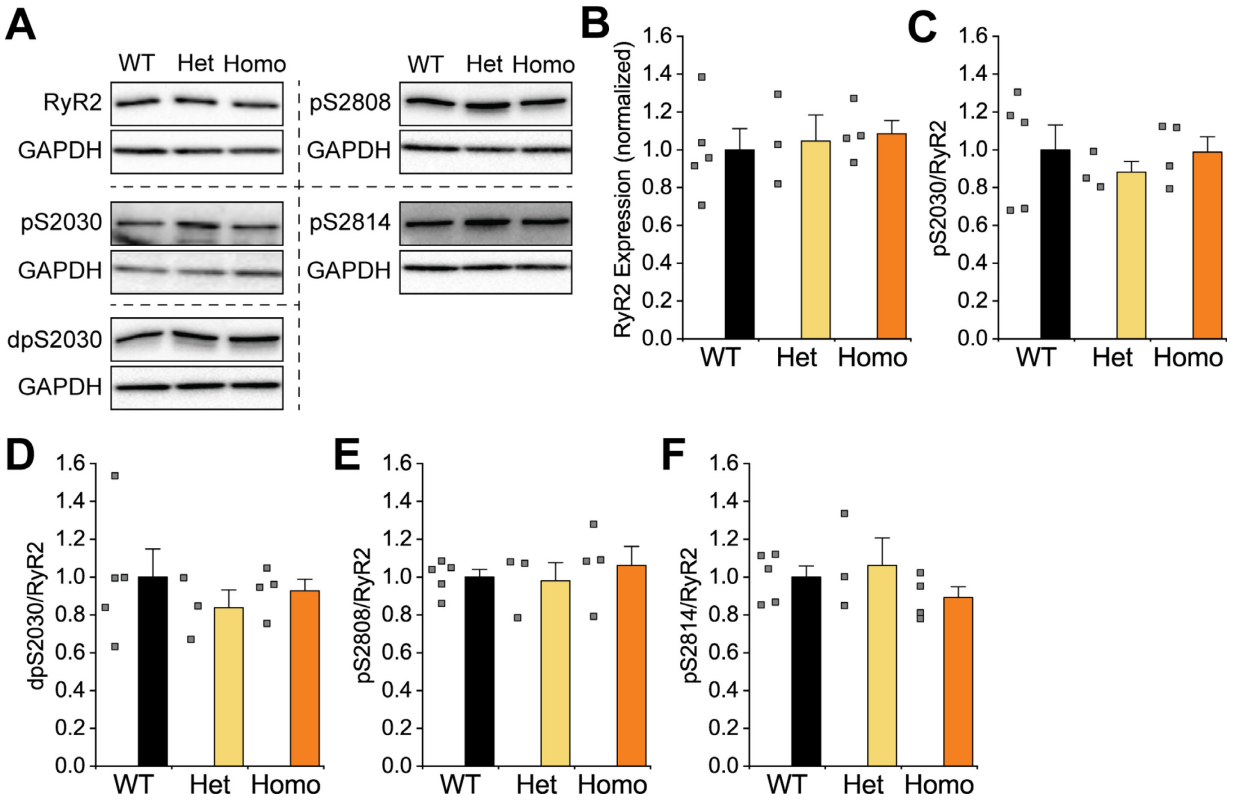

Figure S7. RyR2 Expression and Phosphorylation in 1 yr-old Mice.

A. Representative Western blots of RyR2 expression and phosphorylation. **B**. Quantification of RyR2 expression. **C-F**. Phosphorylation levels of the three major RyR2 phospho-sites (dp: dephospho; p: phospho): S2031 (**C,D**), S2808 (**E**), and S2814 (**F**) (*n* = 5 WT, 3 Het, 5 Homo hearts. No significant differences, one-way ANOVA). This is a subset of the samples used in Figure 6 for the group over 1 yr. Some blots come from the same gels as blots in Figure S7 and Figure S8; therefore, the loading control for the following proteins appears in more than one figure: pS2030, NCX and Casq2; dpS2030 and SERCA2.

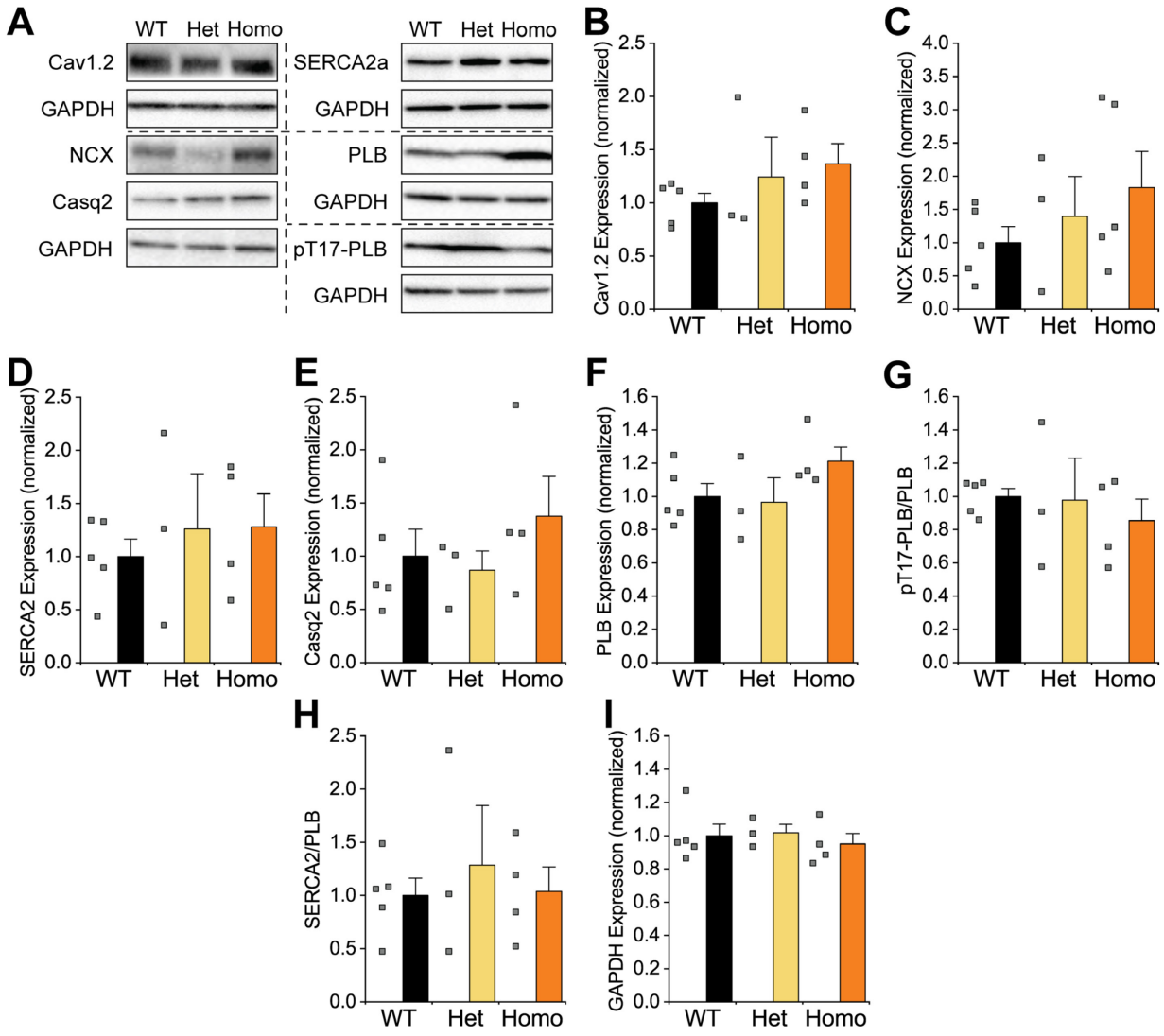

Figure S8. Expression of E-C Coupling Proteins in 1 yr-old Mice.

A. Representative Western blots of e-c coupling protein expression and phosphorylation. **B-F**. Quantification of expression of Cav1.2 (catalytic subunit of LTCC, **B**), NCX (**C**), SERCA2 (**D**), Casq2 (**E**) and PLB (**F**). **G**. Phosphorylation level of PLB at the CaMKII site, T17. **H**. Ratio between the expression of SERCA2 and PLB. **I**. Quantification of GAPDH expression as a percentage of WT level (*n* = 5 WT, 3 Het, 4 [panels C, D-I] and 5 [panel C] Homo hearts. No significant differences, one-way ANOVA [panels B, C, E, H, I), ANOVA on Ranks [panel D, F, G]. This is a subset of the samples used in Figure 6 for the group over 1 yr. Some blots come from the same gels as blots in Figure S7, Figure S8 and Figure S9; therefore, the loading control for the following proteins appears in more than one figure: pS2030, NCX and Casq2; dpS2030 and SERCA2; and ERK1/2 and pT17-PLB. Quantification of GAPDH (panel I) is an average of data from six gels containing the same set of samples.

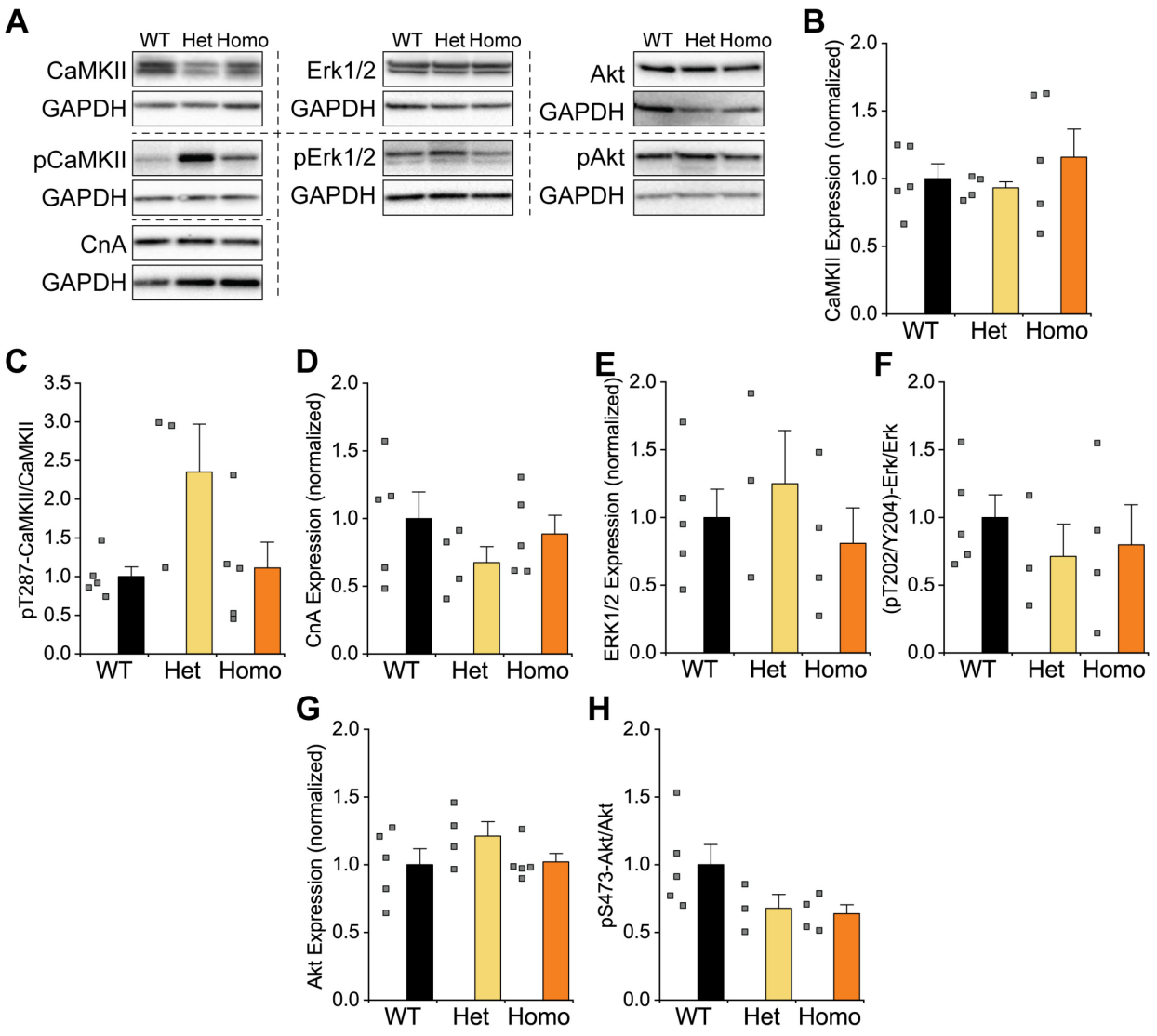

Figure S9. Expression of Hypertrophic Signaling Pathways in P1124L hearts at 1 yr of age.

A. Representative Western blots. **B-D**. Quantification of the expression and phosphorylation of proteins involved in three hypertrophic signaling pathways: CaMKII (**B**), pT287-CaMKII (**C**) and CnA, the catalytic subunit of CaN (**D**); Erk1/2 (**E**) and (pT202/Y204)-Erk1/2 (**F**); and Akt (**G**) and pS473-Akt (**H**). (*n* = 5 WT, 3-4 Het, 4-5 Homo hearts. No significant differences, ANOVA on Ranks [panel B], one-way ANOVA [panels C-H]). This is a subset of the samples used in Figure 6 for the group over 1 yr. Some blots come from the same gels as blots in Figure S8 and Figure S9; therefore, the loading control for the following proteins appears in more than one figure: ERK1/2 and pT17-PLB.

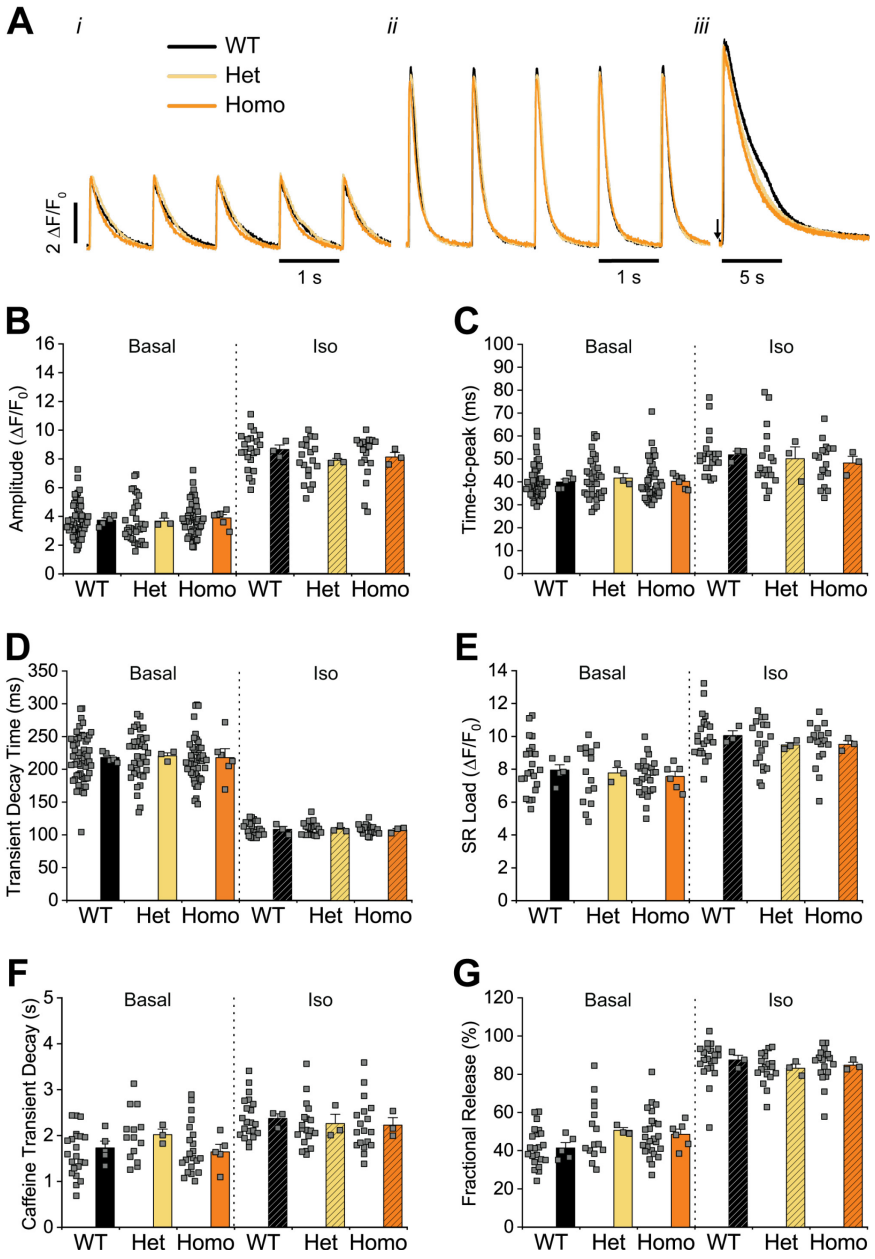

Figure S10. Ca^{2+} Transients and SR Load.

A. Representative traces of Ca^{2+} fluorescence produced by fluo-4, at control conditions (*i*), in the presence of 300 nM isoproterenol (*ii*) and upon rapid perfusion of 20 mM caffeine (*iii*, perfusion indicated by arrow). **B-D.** Quantification of Ca^{2+} transient amplitude (**B**), time-to-peak (**C**) and 50% transient decay time (**D**) during 1 Hz field stimulation (*left*: individual measurements from *n* = 56 WT, 15 Het, 47 Homo basal cells and 22 WT, 19 Het and 18 Homo Iso-stimulated cells; *right*: *right*: mean values for *N* = 5 WT, 3 Het, 6 Homo hearts [basal] and 3 hearts per genotype [Iso]). **E-G.** Quantification of SR load (**E**), 50% caffeine transient decay time (**F**) and fractional release of Ca^{2+} (**G**) (*left*: individual measurements from *n* = 22 WT, 15 Het, 25 Homo basal cells and 21 WT, 19 Het, 18 Homo Iso-stimulated cells; *right*: mean values for *N* = 5 WT, 3 Het, 6 Homo hearts [basal] and 3 hearts per genotype [Iso]) (For all panels: no significant differences, one-way ANOVA [panels B,C,E-G], ANOVA on Ranks [panel D]).

Table S1. X-ray crystallography data collected and refinement statistics.

Parameters	P1124L
Wavelength (Å)	1.00
Resolution range (Å)	23.55 - 1.439 (1.491 - 1.439)
Space group	P 2 21 21
Unit cell	36.29 66.23 66.98 90 90 90
Total reflections	29741
Unique reflections	29697 (2662)
Multiplicity	6.7 (5.2)
Completeness (%)	98.88 (89.81)
Mean I/sigma(I)	12.97 (1.96)
Wilson B-factor	21.94
R-merge (%)	0.109 (0.830)
R-meas	0.118 (0.918)
R-pim (%)	0.045 (0.384)
Reflections used in refinement	29665 (2643)
Reflections used for R-free	1480 (129)
R-work	0.1736 (0.2979)
R-free	0.1926 (0.2986)
Number of non-hydrogen atoms	1518
Macromolecules	1400
Ligands	7
Solvent	111
Protein residues	169
RMS(bonds)	0.005
RMS(angles)	0.76
Ramachandran favored (%)	97.01
Ramachandran allowed (%)	2.99
Ramachandran outliers (%)	0.00
Rotamer outliers (%)	0.00
Clash score	2.94
Average B-factor	31.74
Macromolecules	30.96
Ligands	49.03
Solvent	40.45

Statistics for the highest-resolution shell are shown in parentheses.

Table S2. Echocardiographic Parameters in 8 mo-old P1124L Mice.

	WT	Het	Homo	ANOVA	t-test WT vs Homo
n	6 (3 F, 3 M)	6 (3 F, 3 M)	7 (3 F, 4 M)	-	-
BW (g)	26.00±1.88	25.83±1.66	27.43±1.76	NS	NS
HR (bpm)	534±13	552±11	495±11	Het vs Homo $p = 0.008$	$p = 0.040$
IVSd (mm)	0.78±0.02	0.81±0.03	0.82±0.02	NS	NS
IVSs (mm)	1.07±0.03	1.20±0.08	1.29±0.03	WT vs Homo $p = 0.023$	$p < 0.001$
IVSth (%)	37.88±5.94	47.35±7.00	58.2±3.75	NS	$p = 0.019$
PWd (mm)	0.78±0.02	0.8±0.04	0.86±0.03	NS	$p = 0.062$
PWs (mm)	1.06±0.03	1.05±0.07	1.22±0.05	NS	$p = 0.016$
PWth (%)	35.56±5.01	31.83±5.04	42.08±2.81	NS	NS
LVDd (mm)	3.99±0.13	3.97±0.14	4.25±0.11	NS	NS
LVDs (mm)	2.96±0.15	2.98±0.19	3.03±0.13	NS	NS
FS (%)	26.09±1.63	25.04±2.63	28.73±2.06	NS	NS
LVVd (µL)	70.3±5.52	69.58±6.12	81.48±4.88	NS	NS
LVVs (µL)	34.54±4.52	35.45±5.6	36.57±3.81	NS	NS
EF (%)	51.53±2.84	50.06±4.1	55.33±3.13	NS	NS
SV (µL)	35.76±2.49	34.13±2.7	44.9±3.47	NS	*NS*
CO (ml/min)	19.13±1.5	18.83±1.49	22.04±1.38	NS	NS
LVMass (% of BW)	0.44±0.02	0.45±0.02	0.51±0.03	NS	NS

HR: heart rate; IVSd: interventricular septum thickness in diastole; IVSs: interventricular septum thickness in systole; IVSth: percent change in IVS between systole and diastole; PWd: posterior wall thickness in diastole; PWs: posterior wall thickness in systole; PWth: percent change in PW between systole and diastole; LVDd: left ventricle diameter in diastole; LVDs: left ventricle diameter in systole; FS: fractional shortening; LVVd: left ventricle volume in diastole; LVVs: left ventricle volume in systole; EF: ejection fraction; SV: stroke volume; CO: cardiac output; LVmass: left ventricle mass; NS: not significant.

Table S3. Echocardiographic Parameters in 1 yr-old P1124L Mice.

	WT	Het	Homo	ANOVA	t-test WT vs Homo
n	8 (4 F, 4 M)	7 (3 F, 4 M)	8 (4 F, 4 M)	-	-
BW (g)	28.25±1.31	31.14±1.78	30.75±1.80	NS	NS
HR (bpm)	539±13	553±14	566±19	NS	NS
IVSd (mm)	0.75±0.03	0.9±0.06	0.88±0.02	WT vs Het $p = 0.056$ WT vs Homo $p = 0.040$	$p = 0.005$
IVSs (mm)	1.11±0.04	1.28±0.08	1.28±0.06	NS	$p = 0.041$
IVSth (%)	48.34±4.89	43.49±3.37	44.13±4.30	NS	NS
PWd (mm)	0.78±0.02	0.94±0.05	0.91±0.03	WT vs Het $p = 0.014$ WT vs Homo $p = 0.041$	$p = 0.004$
PWs (mm)	1.14±0.05	1.32±0.07	1.27±0.05	NS	$p = 0.079$
PWth (%)	46.22±5.54	41.79±4.13	39.8±4.04	NS	NS
LVDd (mm)	3.93±0.1	3.99±0.12	4.00±0.10	NS	NS
LVDs (mm)	2.89±0.12	2.84±0.11	2.84±0.11	NS	NS
FS (%)	26.56±1.42	28.8±1.3	29.12±1.53	NS	NS
LVVd (µL)	67.91±4.36	70.19±5.13	70.70±4.36	NS	NS
LVVs (µL)	32.73±3.25	31.17±2.99	31.17±2.98	NS	NS
EF (%)	52.38±2.34	55.87±2.05	56.24±2.37	NS	NS
SV (µL)	35.18±1.87	39.02±2.74	39.53±2.38	NS	NS
CO (ml/min)	18.93±1.05	21.77±2	22.3±1.36	NS	NS
LVMass (% of BW)	0.38±0.02	0.47±0.05	0.45±0.01	NS	$p = 0.003$

HR: heart rate; IVSd: interventricular septum thickness in diastole; IVSs: interventricular septum thickness in systole; IVSth: percent change in IVS between systole and diastole; PWd: posterior wall thickness in diastole; PWs: posterior wall thickness in systole; PWth: percent change in PW between systole and diastole; LVDd: left ventricle diameter in diastole; LVDs: left ventricle diameter in systole; FS: fractional shortening; LVVd: left ventricle volume in diastole; LVVs: left ventricle volume in systole; EF: ejection fraction; SV: stroke volume; CO: cardiac output; LVmass: left ventricle mass; NS: not significant.

Table S4. Ca^{2+} Handling Parameters in Isolated P1124L Myocytes.

Ca^{2+} transient amplitude and SR Ca^{2+} load measured in basal conditions and with 300 nM Iso stimulation.

	WT	Het	Homo
Cells (n)	21–56	15–36	18–47
Mice (N)	3–5	3	3–6
$[Ca^{2+}]_i$ transient amplitude basal ($\Delta F/F_0$)	3.72±0.16	3.64±0.24	3.82±0.17
SR Ca^{2+} Load basal ($\Delta F/F_0$)	8.17±0.34	7.70±0.44	7.52±0.22
Fractional release basal (%)	41.02±2.14	50.68±3.99	48.29±2.42
$[Ca^{2+}]_i$ transient amplitude Iso ($\Delta F/F_0$)	8.57±0.28	7.91±0.32	8.13±0.38
SR Ca^{2+} Load Iso ($\Delta F/F_0$)	10.02±0.30	9.50±0.33	9.52±0.31
Fractional release Iso (%)	86.95±2.25	83.21±1.79	84.82±2.23

References

1. Peng W, et al. Structural basis for the gating mechanism of the type 2 ryanodine receptor RyR2. *Science.* 2016;354(6310):ahh5324.