Hypertrophic cardiomyopathy (HCM) is triggered mainly by mutations in genes encoding sarcomeric proteins, but a significant proportion of patients lack a genetic diagnosis. We identified a novel mutation in the ryanodine receptor 2, RyR2-P1124L, in a patient from a genotype-negative HCM cohort. The aim of this study was to determine whether RyR2-P1124L triggers functional and structural alterations in isolated RyR2 channels and whole hearts. We found that P1124L induces significant conformational changes in the SPRY2 domain of RyR2. Recombinant RyR2-P1124L channels displayed a cytosolic loss-of-function phenotype, which contrasted with a higher sensitivity to luminal $[Ca^{2+}]$, indicating a luminal gain-of-function. Homozygous mice for RyR2-P1124L showed mild cardiac hypertrophy, similar to the human patient. This phenotype, evident at 1 yr of age, was accompanied by an increase in the expression of calmodulin (CaM). P1124L mice also showed higher susceptibility to arrhythmia at 8 mo of age, before the onset of hypertrophy. RyR2-P1124L has a distinct cytosolic loss-of-function and a luminal gain-of-function phenotype. This bifunctionally-divergent behavior triggers arrhythmias and structural cardiac remodeling, and involves overexpression of calmodulin as a potential hypertrophic mediator. This study is relevant to continue elucidating the possible causes of genotype-negative HCM and the role of RyR2 in cardiac hypertrophy.
CARDIAC HYPERTROPHY AND ARRHYTHMIA IN MICE INDUCED BY A MUTATION IN RYANODINE RECEPTOR 2

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Conflict of Interest Statement

FJA, ZY, CRV, Y-TZ, DSH, YC, JJH, TRB, JMB, CAM, HHV, FVP: declare no conflict of interest.

MJA: is a consultant for Audentes Therapeutics, Boston Scientific, Gilead Sciences, Invitae, Medtronic, MyoKardia, and St. Jude Medical. MJA and Mayo Clinic have an equity/royalty relationship with Alive Cor, Blue Ox Health, and StemoneX but without remuneration thus far. None of these entities contributed to this study in any manner.
Abstract

Hypertrophic cardiomyopathy (HCM) is triggered mainly by mutations in genes encoding sarcomeric proteins, but a significant proportion of patients lack a genetic diagnosis. We identified a novel mutation in ryanodine receptor 2, RyR2-P1124L, in a patient from a genotype-negative HCM cohort. The aim of this study was to determine whether RyR2-P1124L triggers functional and structural alterations in isolated RyR2 channels and whole hearts.

We found that P1124L induces significant conformational changes in the SPRY2 domain of RyR2. Recombinant RyR2-P1124L channels displayed a cytosolic loss-of-function phenotype, which contrasted with a higher sensitivity to luminal [Ca²⁺], indicating a luminal gain-of-function. Homozygous mice for RyR2-P1124L showed mild cardiac hypertrophy, similar to the human patient. This phenotype, evident at 1 yr of age, was accompanied by an increase in the expression of calmodulin (CaM). P1124L mice also showed higher susceptibility to arrhythmia at 8 mo of age, before the onset of hypertrophy.

RyR2-P1124L has a distinct cytosolic loss-of-function and a luminal gain-of-function phenotype. This bifunctionally-divergent behavior triggers arrhythmias and structural cardiac remodeling, and involves overexpression of calmodulin as a potential hypertrophic mediator. This study is relevant to continue elucidating the possible causes of genotype-negative HCM and the role of RyR2 in cardiac hypertrophy.
Introduction

Hypertrophic cardiomyopathy (HCM) is one of the most common congenital cardiac diseases, affecting approximately one out of every 500 individuals (0.2% of the general population) (1). HCM is characterized at the tissue level by myocyte hypertrophy and disarray, and interstitial fibrosis. Clinical features involve thickening of the left ventricle (LV) wall, diastolic dysfunction, heart failure, and increased risk of life-threatening arrhythmias that may lead to sudden cardiac death (SCD) (2, 3). Mutations affecting over 24 genes have been implicated in the pathogenesis of HCM (4, 5), most commonly encoding sarcomeric proteins. For example, mutations in the genes MYH7 and MYPBC3, encoding myosin heavy chain 7 and cardiac myosin-binding protein C (MyBP-C), respectively, account for approximately 90% of patients with HCM who have a positive genetic test result (6). However, approximately half of the patients who meet the diagnostic criteria of HCM and undergo genetic testing are negative for mutations in the genes included in HCM panels (4, 7). As genetic testing has become more common and gene panels expand, new HCM-associated mutations have been identified in several genes encoding the myofilaments and beyond. While rare, there are reports suggesting that mutations in genes encoding Ca²⁺- handling proteins contribute to HCM pathogenesis, including genes such as SRI (sorcin), CASQ2 (calsequestrin 2), TNNC1 (troponin C1), and RYR2 (ryanodine receptor 2, RyR2) (8). Except for a limited number of TNNC1 mutations (9), HCM associated to perturbations in Ca²⁺-handling proteins has not been characterized thoroughly.

RyR2 is the major Ca²⁺ release channel in the heart and localizes on the sarcoplasmic reticulum (SR). During an action potential, a small inward Ca²⁺ current through L-type Ca²⁺ channels activates RyR2, triggering more Ca²⁺ release from the SR. RyR2 provides most of the Ca²⁺ required for this process, called excitation-contraction (e-c) coupling. Tight regulation of Ca²⁺ release in the heart, and thus of RyR2 function, is essential because the same Ca²⁺ used for e-c coupling can induce Ca²⁺-dependent arrhythmias (10) or modulate hypertrophic signaling pathways (11). Over the last 20 years, nearly 200 RyR2 mutations have been identified in patients...
with catecholaminergic polymorphic ventricular tachycardia (CPVT) (12), a disorder involving severe ventricular arrhythmias triggered by stress, but without structural alterations of the myocardium. The mechanisms underlying RyR2-mediated CPVT (CPVT1) have been the subject of extensive research, uncovering a variety of molecular and cellular mechanisms for Ca\textsuperscript{2+} mishandling and, ultimately, arrhythmia. More recently, a RyR2 mutation, T1107M, was reported in a family with HCM (13). This mutation generated considerable interest in the field, as it was the first RyR2 variant potentially contributing to a structural disorder (HCM) rather than an entirely functional disorder (CPVT). Recombinant A1107M, the mouse analog, behaved considerably different in a heterologous expression system when compared to other CPVT1 mutations, suggesting a different pathogenic mechanism (14). T1107M has also appeared in cohorts of whole-exome sequencing (WES) (15) and of CPVT patients (16), weakening its potential as an HCM-associated mutation. Altogether, these data indicated that RyR2 can produce a wide range of cardiac dysfunctions, but the link between RyR2 mutations and structural cardiomyopathy remains elusive.

In this study, we describe the comprehensive characterization of a novel RyR2 variant (P1124L), identified in a patient with sarcomere mutation-negative HCM. While the P1124L variant is in the same domain as T1107M, herein we present defining characteristics of P1124L that bolster its potential association with the patient's phenotype, including a) satisfying a genetic criterion (identification of a rare variant in \textit{RYR2} in a patient with HCM), b) \textit{in silico} tools suggesting pathogenicity, c) molecular and functional criteria (validation assays demonstrating that this variant is disruptive functionally), and d) whole organism/animal model criterion (mice harboring P1124L develop arrhythmia and hypertrophy, although not as dramatically as the patient). Hearts from P1124L mice showed an increase in the expression of calmodulin (CaM), a classical inhibitor of RyR2 and signaling molecule, but had no significant changes in the expression of downstream signaling molecules involved in hypertrophy. Thus, although the precise molecular mechanism(s) underlying hypertrophy in P1124L mice could not be fully discerned and need further study, here
we offer evidence that links RyR2 dysfunction with cardiac hypertrophic remodeling, in addition to the demonstrated role of this protein in the pathogenicity in CPVT.
Results

Identification of RyR2-P1124L in a Human Patient with HCM

Among the 36 patients with genotype negative/phenotype positive HCM who underwent WES, in one of them we identified a novel, candidate HCM-associated variant in RYR2, P1124L, without any other candidate disease-associated mutations. This RyR2-P1124L-positive patient was a male diagnosed with HCM at 17 years of age following a syncopal episode and was placed on β-blocker therapy. Four months later, because of persistent chest tightness and paroxysmal nocturnal dyspnea, he received a dual chamber pacemaker elsewhere. He was evaluated at Mayo Clinic 1 yr later, exhibiting moderate concentric hypertrophy (21 mm) in the absence of hypertension (Figure S1). The ejection fraction (EF) was 75% with decreased left ventricle (LV) chamber size and hyperdynamic function. Obstruction of the left ventricle outflow tract (LVOT) was dynamic measuring 46 mmHg at rest, increasing to 88 mmHg after provocation. There was also evidence of systolic anterior motion of the mitral valve with grade III/IV mitral regurgitation. ECG monitoring showed artifacts consistent with a rhythm maintained by firing of the pacemaker, but without signs of ventricular arrhythmia. Because the symptoms remained refractory to pharmacotherapy and pacing, the patient underwent an extensive surgical myectomy at Mayo Clinic. An ECG performed four days post-surgery showed normal sinus rhythm, signs of left atrial enlargement, left ventricular hypertrophy and QRS widening. Pathological examination of the surgically removed myocardium showed mild endocardial thickening, severe myocyte hypertrophy, mild interstitial fibrosis, and myocyte disarray.

No HCM-causative mutations were noted in any of the established HCM-susceptibility genes, including all the canonical genes implicated in sarcomeric HCM: MYBPC3, MYH7, TNNT2, TNNI3, TNNC1, TPM1, ACTC, MYL2, and MYL3. Instead, a base change was identified following WES in RYR2, generating the missense variant RyR2-P1124L. To our knowledge, this variant has never been associated with CPVT1 and is extremely rare, being found in five out of 246,156 alleles in the Genome Aggregation Database (gnomAD) giving it a minor allele frequency of
0.00002 (http://gnomad.broadinstitute.org/). Additionally, consensus results from the most commonly used in silico tool for variant prediction suggested P1124 is (likely) pathogenic or damaging (7 out of 7 tools used; CADD-score: 32; PolyPhen-2: probably damaging; SIFT/PROVEAN: deleterious; PANTHER: probably damaging; AlignGVD: most likely damaging [C65]; MutationTaster: probably damaging; Grantham score: 98). At the time of this evaluation at Mayo Clinic, the patient’s mother had no evidence for HCM, while the father and three siblings were not screened; nonetheless, the patient reported a positive family history of HCM in two maternal cousins. Secondary to familial non-participation, additional evidence of co-segregation could not be determined. Given that there are not multiple unrelated cases with putative RyR2-mediated HCM, without compelling functional data, RYR2 in general and RyR2-P1124L in particular would be classified as a gene of uncertain significance (GUS) and variant of uncertain significance (VUS; RyR2-P1124L-ACMG criteria: PP3, PP4), respectively, in terms of an association with HCM in accordance with the 2015 ACMG criteria for variant interpretation (17). To determine the potential association between P1124L and HCM, we studied this mutation on multiple levels.

**Localization of P1124L in the Three-Dimensional Structure of RyR2**

CPVT1-associated RyR2 mutations mainly cluster in four “hot spots”, which coincide with functional domains of the channel, called CPVT-I to IV (Figure 1A). P1124, a highly conserved residue among species (Figure 1B) and RyR isoforms (Figure S2), is located outside these hot spots in SPRY2, one of the three SPRY domains of RyR2 (Figure 1C). The crystal structures of SPRY1 and SPRY2 were solved recently to a resolution below 1.5 Å (18, 19), while high-resolution structures of SPRY3 are not available. P1124 is part of a flexible linker connecting strands β2 and β3 that was not included in the initial SPRY2 model due to poor electron density; yet, recent cryo-EM structures of RyR1 and RyR2 suggest that this loop is at the SPRY2-SPRY3 interface (Figure 1D) (20, 21).
Since all RyR cryo-electron microscopy (cryo-EM) structures originate from native material, analyzing RyR disease mutants is currently not feasible. We therefore crystallized the SPRY2 domain containing the P1124L mutation and solved its structure at an average 1.44 Å resolution. The loop containing the mutation is well-ordered, in contrast to the WT, which shows less defined density (19). The WT loop could still be traced, and thus we further refined this structure to allow a direct comparison (Figure S3 and Figure S4, Table S1). Introducing the proline to leucine substitution produces an extensive conformational change that affects neighboring residues, notably C1122 and D1125, with D1125 swinging outside and facing SPRY3 (Figure 1E,F and Figure S4). Also, a new salt bridge forms between R1119 and D1132, located in opposite ends of the β2-β3 linker. Overall, these changes provide stability to the β2-β3 linker, explaining why the density for this loop is better defined in the P1124L mutant. Details of the SPRY2-SPRY3 interactions cannot yet be discerned in the cryo-EM reconstructions, because the local resolution in those areas is low. However, just like mutations analyzed in the N-terminal disease hot spot, such changes at interdomain interfaces are likely to cause relative domain orientations that indirectly affect channel gating (22, 23). For example, the altered D1125 side chain may affect interactions with R1599 in SPRY3, although this remains to be tested.

Molecular Phenotype of P1124L-Containing RyR2 Channels

To determine the possible molecular alterations induced by P1124L in RyR2, we introduced the mutation into the mouse Ryr2 cDNA, and expressed the WT or P1124L protein in HEK293 cells. The approach is justified because P1124 and the neighboring region of SPRY2 are highly conserved among species (Figure 1B, Figure S2). Indeed, within the entire SPRY2 domain, mouse and human RyR2 protein only differ in the three residues highlighted in Figure 1B. 48 hr post-transfection, cell lysates were used for functional studies. We found that expression of P1124L-containing RyR2 channels was significantly reduced compared to the WT RyR2 (Figure S5A). This is an uncommon observation (reported only for one CPVT-associated mutation
(24)), as mutation-harboring RyR2 channels are often expressed to the same level as WT channels in this heterologous system (14). Next, we performed [³H]ryanodine binding assays to assess RyR2 channel activity. Ryanodine only binds to the open state of the channel; hence, [³H]ryanodine binding is suitable to evaluate RyR2 activity in a population of channels at varying cytosolic [Ca²⁺]. To account for the different RyR2 density between groups, we performed Western blots to determine the expression level in each transfection as a percentage of the WT samples (Figure S5A), [³H]ryanodine binding was then normalized to this value. We found that P1124L behaves as a loss-of-function mutation, with lower maximum [³H]ryanodine binding than WT (Figure 2A,B) and requiring higher [Ca²⁺] for activation (Figure 2C,D).

**Single Channel Recordings**

Recombinant RyR2 channels from heterologous systems are expressed in the absence of other important regulatory co-factors. Therefore, we prepared SR-enriched microsomal fractions from pooled WT or homozygous P1124L mouse hearts to assess RyR2 activity from native channels reconstituted in planar lipid bilayers (the strategy used to create mice harboring P1124L is described in the following sections). Figure 2E,F show representative current traces and histograms obtained from single RyR2 channels from SR microsomes. P1124L channels showed lower open probability than WT channels (Figure 2G) at nominally-free [Ca²⁺] (3-5 μM), while average unitary current at 0 mV in a Cs⁺ gradient was not altered (trans/cis 300/50 mM; Figure 2H). These data agree with the [³H]ryanodine binding assays and support the idea that P1124L is a loss-of-function mutation, at least at physiologically-relevant cytosolic [Ca²⁺].

**Spontaneous Ca²⁺ Oscillations in HEK293 Cells Expressing RyR2**

Using heterologous expression of RyR2 in HEK293 cells and monitoring of intra-ER [Ca²⁺], Tang et al. (14) determined that some RyR2 mutations induce abnormal termination of spontaneous Ca²⁺ release (SCR) events triggered by ER Ca²⁺ overload. We used a similar
approach to gain a better understanding of the possible mechanisms of dysfunction associated with P1124L. Inducible HEK293 cells stably expressing RyR2 were transfected with the FRET-based ER Ca\(^{2+}\) sensor D1ER (25). Then, cells were perfused with 2 mM Ca\(^{2+}\) to fill the ER and induce luminal [Ca\(^{2+}\)] oscillations due to spontaneous RyR2 activation. Under these conditions, oscillations are observed as a decrease in the FRET ratio. Figure 3A shows representative FRET traces measured in cells expressing WT and P1124L channels. From these recordings, we determined the FRET level at which Ca\(^{2+}\) oscillations activate (F\(_{Act}\)) and terminate (F\(_{Term}\)). 1 mM tetracaine was perfused to inhibit RyR2 and determine the FRET level at maximum store capacity (F\(_{Max}\)), followed by 20 mM caffeine to empty the ER and determine the residual FRET signal (F\(_{Min}\)). The store capacity was determined as F\(_{Max}\) – F\(_{min}\). In both groups, the activation threshold was ~80% of the maximum ER store capacity (Figure 3B); however, P1124L showed a significant reduction in the termination threshold (Figure 3C), resulting in a larger fractional release (Figure 3D). These observations did not involve a change in the maximum capacity of the ER store (Figure 3E). Interestingly, while the percentage of HEK293 cells showing oscillations of intra-ER [Ca\(^{2+}\)] was not different (Figure 3F), cells expressing P1124L channel showed an increased number of oscillations over the monitoring period (Figure 3G). Similar to HEK293 cells with transient transfection of P1124L, cells with stable expression also showed lower RyR2 expression (Figure S5B). The lower expression of P1124L channels may suggest reduced stability of the channel protein in heterologous systems, but this remains to be tested. Altogether, these data suggest that P1124L channels have impaired luminal regulation and, upon activation, may release more Ca\(^{2+}\) than WT channels.

**Generation and Cardiac Structural Phenotype of P1124L Knock-In Mice**

Knock-in RyR2-P1124L mice were generated following the strategy summarized in Figure S6A. Using homologous recombination, we introduced three base substitutions in exon 28 of Ryr2 (Figure S6B): a silent A to G substitution in codon 1123, creating a PvuII restriction site; and two
substitutions in codon 1124, accounting for the proline (P) to leucine (L) change. P1124L mice show Mendelian propagation (Figure S6C). To determine the physiological effect of the molecular dysfunction observed in RyR2-P1124L channels, we performed a thorough characterization of the mice harboring the mutation. At 8 mo of age, both homozygous (Homo) and heterozygous (Het) P1124L mice showed comparable cardiac structure and function with WT littermates, although Homo mice also showed significantly lower hear rate (HR) than Het mice (Table S2). At 1 yr of age, this difference in HR was no longer evident and RyR2-P1124L mice showed mild but significant hypertrophy (Figure 4A, Table S3). This phenotype is evident in both Homo and Het mice, and involves thickening of the posterior wall (Figure 4B) and septum (Figure 4C), without deterioration of the ejection fraction (Figure 4D). In both Het and Homo mice, we also observed a significant increase in heart weight as percentage of BW (Figure 4E), without significant differences in BW (Table S3). We did not observe microscopic alterations of the cardiac muscle or fibrotic infiltrations (fibrosis quantification; WT: 4.54±0.57%, Het: 5.21±0.74%, Homo: 4.19±0.57%; p > 0.05, t-test. Figure 4A).

As an independent test of hypertrophy, we measured isolated ventricular myocytes from 1 yr-old P1124L mice. To avoid bias, fields of view were selected randomly to observe myocytes plated on 35 mm glass-bottom dishes. Myocytes within the field of view were centered and focused prior to collecting transmitted-light images using a confocal microscope; measurements were performed offline. All the cells in an image were measured unless they were partially out of frame or out of focus (Figure 5A). While we did not detect a difference in cell width (Figure 5B), Homo myocytes were longer than WT and Het myocytes (Figure 5C), resulting in Homo myocytes having a significantly larger surface area than WT (Figure 5D). Finally, we measured the cardiac expression of three hypertrophic gene markers using qPCR: natriuretic peptide A (Nppa), natriuretic peptide B (Nppb), and myosin heavy chain 7 (Myh7). Consistent with the mild hypertrophy, we only measured a significant increase in Nppa expression in Homo mice (Figure 5E).
Taken together, these data suggest that P1124L induces mild cardiac hypertrophy without overt hemodynamic dysfunction in mice, similar to the patient's clinical presentation. This structural phenotype is detectable in both Homo and Het mice, but the number of significant differences in indexes of hypertrophy were greater in Homo mice. We have followed P1124L mice for ~70 weeks of age (~1.3 yrs) without observing evident differences in sudden death or overall mortality.

**Signaling Pathways Associated with Cardiac Hypertrophy**

To determine the mechanisms by which P1124L might induce hypertrophy, we used Western blots to measure the expression and phosphorylation of relevant signaling molecules. Unlike our previous observation in HEK293 cells, we did not detect differences in the expression of RyR2 in mice under 8 mo or over 1 year of age (Figure 6B). This suggests that the mechanisms reducing the expression of P1124L channels in heterologous systems are compensated for in the mouse heart. Additionally, we did not observe differences in the phosphorylation level of RyR2 (Figure S6) or other e-c coupling proteins (Figure S7) in hearts of 1 yr-old mice. We observed evident variability within samples of the same genotype, and tendencies, such as an increase in NCX expression (Figure S7C), were not significant. Nevertheless, GAPDH expression showed little variation, suggesting that the variability in the e-c coupling proteins is not due to inadequate normalization (Figure S7I).

We then assessed the expression of relevant molecules from two Ca^{2+}-dependent signaling pathways associated with cardiac hypertrophy: CaM-CaMKII-HDAC and CaM-CaN-NFAT. In both cases, CaM acts as a Ca^{2+} sensor that activates either the Ca^{2+}/CaM-dependent kinase II (CaMKII) or the phosphatase calcineurin (CaN). Downstream, each pathway follows a different signaling cascade that ultimately activates hypertrophic genes (26). At 1 yr of age, we did not detect a difference in the expression of CaMKII between genotypes (Figure S9B), and the auto-phosphorylated form of the enzyme was not detectable in any of the samples (not shown).
We also measured the expression of CnA, the catalytic subunit of CaN (Figure S9D). Again, there was no significant difference among genotypes. We therefore moved upstream in the signaling cascade and measured the expression of CaM. Remarkably, CaM was increased in hearts from mice over 1 yr of age but not in mice younger than 8 mo (Figure 6C). This difference was only significant in Homo mice, consistent with the more prominent phenotype discussed in the previous section. Transgenic overexpression of CaM (27, 28) and increased levels of free CaM dissociated from RyR2 channels (29) have been linked to cardiac hypertrophy due to activation of downstream signaling. Other hypertrophic signaling pathways reported in a mouse model of severe cardiac hypertrophy due to inability of RyR2 to bind CaM (30) were not altered in P1124L mice (Figure S9C,D).

**Cellular Ca\(^{2+}\) Handling in Ventricular Myocytes**

The cytosolic loss-of-function phenotype displayed by P1124L channels could lead to reduced Ca\(^{2+}\) release, contractility and compensatory hypertrophy. Hence, we measured the kinetics of Ca\(^{2+}\) handling in isolated myocytes from 1 yr-old mice to determine whether P1124L induces alterations of intracellular Ca\(^{2+}\) homeostasis. We first paced the cells at 1 Hz to quantify the properties of the cellular Ca\(^{2+}\) transient at basal conditions and in the presence of Isoproterenol (Iso). We observed no significant differences in Ca\(^{2+}\) release, Ca\(^{2+}\) removal, or SR content between WT and P1124L myocytes (Figure S10, Table S4). Next, we studied the propensity for spontaneous Ca\(^{2+}\) release during a 30 s resting period, following a train of 3 Hz stimulation (Figure 7A). The propensity for SCR was higher in Homo myocytes at basal conditions, compared to WT and Het (Figure 7B). This difference was reduced by Iso; however, the frequency of SCR was still significantly higher in Homo myocytes treated with Iso than in WT (Figure 7C). Het myocytes displayed an intermediate phenotype, with a non-significant tendency for higher SCR frequency under Iso compared to WT. Interestingly, SCR frequency was not statistically different between Het and Homo under Iso.
Arrhythmia Susceptibility of P1124L Mice

The high incidence of diastolic Ca\textsuperscript{2+} waves in RyR2-P1124L cardiomyocytes suggest that mice harboring the mutation may be more susceptible to develop cardiac arrhythmia. We used a challenge protocol (i.p. injection of epinephrine 2 mg/kg and caffeine 120 mg/kg) (31) during surface ECG recording, to determine the susceptibility of P1124L mice to develop ventricular arrhythmia. We performed this test in 8 mo-old mice because at this age they do not show signs of cardiac hypertrophy (Table S2). Figure 8A shows representative 2 s lead-II ECG traces. Most of the arrhythmia episodes in P1124L mice involved ventricular bigeminy (alternated premature and sinus beats) and bidirectional ventricular tachycardia (BDVT). The effect of the treatment was minimal in WT mice, which only showed sporadic premature ventricular beats (PVBs, Figure 8A,B) and non-sustained arrhythmias (Figure 8C). P1124L mice exhibited a non-significant tendency to develop more PVBs than WT (Figure 8B), which coalesced into significantly more episodes of arrhythmia in Homo mice, including BDVT (Figure 8C-E), the pathognomonic sign of RyR2-induced arrhythmia. Again, Het mice had an intermediate phenotype between WT and Homo. While the difference in the number of episodes of sustained arrhythmia and incidence of BDVT was not statistically different between WT and Het mice, WT animals did not show these types of events, consistent with the literature (Figure 8D,E). This suggests that the phenotype of Het mice is noteworthy.

In summary, RyR2-P1124L predisposes 8 mo-old mice to develop cardiac arrhythmia before the onset of hypertrophy at 1 yr of age. This suggests that the arrhythmia is a result of RyR2 dysfunction rather than cardiac structural remodeling. Furthermore, these data suggest that the cellular mechanism for the ventricular arrhythmias in P1124L mice is diastolic SCR.
Discussion

In 2006, Fujino et al. reported for the first time a RyR2 mutation in a family with HCM, T1107M (13). Two subsequent, separate studies showed that this mutation alters the structure of the SPRY2 domain (19) and produces a loss-of-function phenotype (14). Medeiros-Domingo et al. later identified this mutation in patients with CPVT, the inherited arrhythmia already strongly associated with RyR2 dysfunction (16), weakening its exclusive association with HCM. T1107M is not a conserved residue among species and two studies questioned its pathogenicity altogether because it appears with relatively high frequency in WES cohorts (seen in 118 of 277,072 alleles, frequency of 0.0004) (15, 32, 33), higher than the prevalence of CPVT. In this report, we thoroughly characterized the novel RyR2-P1124L mutation, detected in a patient with obstructive HCM. P1124L also appears in publicly available exome databases, although with a ~20-fold lower frequency than T1107M and we propose its association with a disease with much higher prevalence than CPVT. Hence, to our knowledge this is the first comprehensive study of a RyR2 mutation strongly associated with HCM, a structural disorder of the heart. We provide multiple layers of evidence, from single molecule to whole animal, suggesting a potential association between P1124L and HCM. Additionally, our data show that P1124L underlies an arrhythmogenic phenotype, suggesting that some RyR2 mutations may lead to presentations that cross the boundaries from purely functional (CPVT) to structural (HCM) alterations.

First, we show that P1124L involves a highly conserved residue among RyR isoforms and species and is located within SPRY2, one of the peripheral domains of RyR2, where it produces structural disturbances in arrangement of a flexible linker at the SPRY2-SPRY3 interface. P1124L is one of a handful of mutations that fall outside of the canonical CPVT1-associated mutation “hotspots” of RyR2 (Figure 1A), which contain ~93% of the disease-causing mutations. Our model of the SPRY2-SPRY3 interface suggests that P1124L promotes intradomain rearrangement and alters interdomain interactions that could affect allostERIC coupling mechanisms in the channel. The large size of RyR2 makes it difficult to model single amino acid mutations using the whole
channel protein; hence, the long-range effects of P1124L are not known. Even then, it is conceivable that the peripheral domains of RyR2 allosterically regulate channel gating or its association with protein partners. This idea is supported by the model suggesting that phosphorylation of S2814, also located in a peripheral domain of RyR2, may turn the channel “leaky” in some forms of heart disease (34). Ultimately, the consequence of the structural alterations to the SPRY region produced by P1124L appears to be a cytosolic loss-of-function, as we determined using two complementary assays, [$^3$H]ryanodine binding with recombinant protein and single channel recordings with mouse SR microsomes, and a SR luminal gain-of-function, which we measured in the SCR assay in HEK293 cells. This is a rather distinct phenotype that, to our knowledge, has only been identified in one other mutation of variable penetrance linked to CPVT1 (24, 35). While our experiments were carried out using homozygous P1124L channels, in the heterozygous condition, as in the patient, any phenotype will be the aggregate result the absolute expression of each allele and the resulting heterogeneous population of channels containing none to four mutant subunits. We have previously addressed this issue for two other CPVT1-related RyR2 mutations (31, 36).

Second, we show that mice expressing RyR2-P1124L channels develop cardiac hypertrophy at 1 yr of age. This phenotype, albeit mild, involves a significant increase in LV wall thickness, myocyte size and ANP expression in homozygous mice. Het hearts (resembling the genotype of the human patient), on the other hand, show only a tendency for hypertrophy. As there are significant differences between mouse and human cardiac physiology that can modify the effect of specific mutations, it is not entirely surprising that mice may show a milder phenotypic penetrance. For example, mutations that truncate MyBP-C are overtly hypertrophic in heterozygous humans (37), but produce cardiomyopathy only in homozygous mice (38-40). Additionally, the penetrance and expressivity of a specific mutation may be affected by the genetic background of the mouse model, as has been previously recognized (41). Indeed, we have...
observed phenotypic differences between P1124L in the C57Bl/6 genetic background used in this study and a strain with a mixed Sv129/C57Bl6 background (FJA, unpublished observations).

To our knowledge, this report is the first instance in which a clinically relevant RyR2 mutation produces cardiac hypertrophy of any degree in an animal model. In our search for the possible signaling pathways implicated in the hypertrophic phenotype shown by P1124L mice, we found that CaM expression is increased in Homo mice at 1 yr of age. CaM is a ubiquitously-expressed signaling molecule with multiple partners in cardiac cells. Among its many functions, CaM works as the Ca\(^{2+}\) sensor of CaMKII and CaN, two signaling molecules implicated in hypertrophic signaling (26). Indeed, both overexpression of CaM (27, 28) and its dissociation from membrane-bound partners (29) are linked with cardiac hypertrophy. Our data suggest that an increase in CaM expression may be implicated in the development of hypertrophy in P1124L mice. A hypertrophic phenotype could occur compensatory to a lower cellular/cardiac contractility, resulting from a decreased systolic Ca\(^{2+}\) release. This could occur in the P1124L mouse model as a result of the cytosolic loss-of-function we uncovered at the molecular level. However, the Ca\(^{2+}\) transient data (Figure S1 and Table S4) suggest there are no differences in the force of contraction, provided there are no changes in the sensitivity of the myofilaments to Ca\(^{2+}\). At this point we have no evidence of such alterations. The link between the unique biophysical properties uncovered for P1124L, the increase in CaM expression and the hypertrophic phenotype observed in P1124L remains to be elucidated. We surmise that subjecting P1124L mice to chronic stress through drug treatments or a surgical approach to induce heart failure may provide additional insight into the pathogenicity of the mutation. Together with the possible influence of the genetic background over the phenotype of the mouse model, as discussed previously, this notion will require further studies.

Finally, we observed cardiac arrhythmia, particularly BDVT, in 8 mo-old P1124L mice undergoing arrhythmia challenge. In HCM, ventricular arrhythmias often develop because of the extensive structural and electrophysiological remodeling (42). Interestingly, P1124L mice show
both structural and functional phenotypes independently (arrhythmia at 8 mo and hypertrophy at 1 yr, respectively). Unlike hypertrophy, it is not unexpected to link RyR2 mutations with cardiac arrhythmias since many have been documented to cause CPVT1. From the molecular phenotypes we uncovered for P1124L channels, cytosolic loss-of-function and luminal gain-of-function, the latter may be more prone to drive the arrhythmia process: spontaneous activation of RyR2-P1124L during diastole may produce larger Ca\(^{2+}\) releases that can diffuse to neighboring RyR2 clusters and propagate as arrhythmogenic Ca\(^{2+}\) waves. These events may activate NCX during diastole and produce triggered activity due to delayed afterdepolarizations. Indeed, we observed a high incidence of diastolic SCR in myocytes from P1124L hearts even under basal conditions; however, it was Iso treatment which uncovered an evident increase in the frequency of such events. These observations are consistent with the mechanisms broadly assigned to gain-of-function RyR2 mutations (10). In contrast, mutations that depress channel function favor a mechanism involving sustained bursts of Ca\(^{2+}\) leak after the peak of a Ca\(^{2+}\) transient, leading to early afterdepolarizations (36). In both cases, however, a form of stress (e.g. arrhythmia challenge or Iso) seems necessary to create the substrate required to promote aberrant Ca\(^{2+}\) release and arrhythmia.

As expected from the literature, WT mice did not show BDVT. However, both Het and Homo mice did show this type of arrhythmia, although the incidence was not statistically significant between Het and WT mice. In Het mice, permutation analysis predicts that most RyR2 channels should contain at least one P1124L subunit, and ~6% of the channels should contain four P1124L subunits (31, 36). This distribution may be skewed if one allele is expressed more predominantly; nonetheless, in the cellular mechanism discussed above, homozygous channels are likely driving the arrhythmogenic phenotype.
Clinical Relevance

Following its discovery in a patient with HCM, P1124L was classified as a VUS based on ACMG guidelines (17). Our characterization of P1124L in different model systems adds an important layer of evidence to classify P1124L as 'likely pathogenic (III)' in the context of HCM, with one strong (PS3) and two moderate (PP3, PP4) supporting criteria. The presence of both hypertrophy and increased susceptibility to arrhythmia in P1124L mice suggest that certain RyR2 variants could lead to both phenotypes, as discussed next. This is a tantalizing idea that requires further research, but the significant differences in mouse and human cardiac physiology and Ca\textsuperscript{2+} handling could make the mouse a less-than-ideal model to address these questions.

Since the identification of the first mutation associated with HCM affecting myosin heavy chain 7 in 1990 (MYH7-R403Q) (43), the number of genes and variants associated with HCM continues to grow. Even as HCM is overwhelmingly recognized as a monogenic disease caused primarily by mutations in sarcomeric genes, the fact that nearly half of the patients lack a genetic diagnosis cannot be overlooked. This large proportion of mutation-negative cases suggests HCM has unknown genetic causes if we continue to consider HCM predominantly a monogenic disease; alternatively, it suggests that HCM may also appear as a complex disorder in which genetic modifiers and factors such as age, gender, comorbidities and lifestyle intervene in the phenotypic outcome (44). This idea is increasingly recognized, and the variable inheritance of non-sarcomeric HCM may favor a multifactorial model (42, 45).

As we propose to consider a novel association between RyR2 mutations and non-sarcomeric HCM, and due to the mild hypertrophic phenotype present only in homozygous mice, it is important to consider the possibilities outlined above. The role of RYR2 as a CPVT gene is robust, therefore the increased susceptibility of P1124L mice to arrhythmia is not surprising. However, some mutations, such as P1124L, might in fact present not only as pro-arrhythmic variants but also as HCM modifiers. In this scenario, P1124L may generate a pro-hypertrophic substrate in association with other variables, fitting within a multifactorial HCM model.
Accordingly, the rare finding of P1124L in WES cohorts with variant-positive subjects displaying a sub-clinical phenotype or no phenotype at all could be explained by the absence of those yet-to-be-determined factors. Variable phenotypic penetrance is already recognized, but incompletely understood, even for well-established HCM genes: previous studies have described families carrying MyBP-C variants where some individuals show the characteristic early onset of hypertrophy while others remain asymptomatic even as adults (46-48). Multiple studies have shown that even among mutations in known HCM-associated genes, patients with >1 variant generally have a more severe phenotype compared to those with a single identified variant (49, 50). We speculate this type of variation may occur in the patient’s family, since the positive family history of HCM on the maternal side suggest the mother may be a carrier of the mutation with decreased or subclinical disease expressivity. Further screening of heterozygous individuals carrying P1124L may show a similar phenomenon and perhaps a mixed HCM/CPVT phenotype, as seen in the P1124L mouse model. Unfortunately, an expanded genotypic and phenotypic familial assessment was not possible in this study and we cannot test the possibility that the patient acquired a de novo mutation or shows genetic mosaicism.

In conclusion, although our data does not provide unequivocal evidence to classify RYR2 as an HCM-causing gene, it presents a compelling argument for the importance of studying and understanding the possible implication of rare genes in different forms of HCM. Ultimately, this is an important effort to elucidate the variable disease penetrance and determine the causes of genotype-negative HCM.
Methods

Identification of RyR2-P1124L in a Human Patient with Clinically Diagnosed HCM

Following previous studies, 36 patients (systematically evaluated in Mayo Clinic’s HCM Clinic) with clinically diagnosed HCM and a clinical HCM phenotype score predicting a ≥ 60% probability of having a positive HCM genetic test, remained genotype negative for the established HCM-susceptibility genes (7). Whole exome sequencing (WES) and variant filtering under an IRB-approved research protocol was performed on this cohort of genotype negative/phenotype positive HCM. Following identification of the RyR2-P1124L variant in one of the patients, variant adjudication regarding its potential pathogenicity followed the 2015 guidelines on variant interpretation of the American College of Medical Genetics (ACMG) (17).

Crystallization of SPRY2

Mouse RyR2 SPRY2 domain (residues 1080–1253) including the P1124L substitution was cloned, expressed and purified according to a similar strategy previously used for WT RyR2 SPRY2 domain (19). Protein crystals were grown using hanging drop method at 20°C. P1124L (5 mg/mL) was crystallized in 0.1 M potassium thiocyanate, 25-35% PEG2000MME with seeding of WT crystals. Crystals were harvested and flash frozen in the original growth condition supplemented with 25-30% glycerol. Diffraction data were collected at the Advanced Photon Source (APS) beamline 23ID-D. Data were processed with HKL2000. The WT RyR2 SPRY2 structure (PDB ID 4P9I) was used as a search model for molecular replacement to solve the RyR2 SPRY2 P1124L mutant structure. The structure was refined using successive rounds of manual building in COOT (51) and automated refinement using Phenix (52). The statistics for data collection and refinement is shown in Table S1. Coordinate and structure factor for P1124L is available in the Protein Data Bank (www.rcsb.org) with accession code 5VSN.
**Transient Expression of RyR2 in HEK293 Cells**

HEK293 cells were grown in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin on 100 mm tissue culture dishes. Cells were transfected with a plasmid containing the mouse Ry2 cDNA using the Xtreme-DNA reagent (Roche Laboratories), following the instructions of the manufacturer. Cell lysates were prepared from transfected cells as previously described (36). 48 h after transfection, cells were washed twice with PBS, then scraped from the plate and solubilized in 200 μL of lysis buffer, containing 25 mM Tris/50 mM HEPES (pH 7.4), 137 mM NaCl, 1% CHAPS, 0.5% soybean phosphatidylcholine, 2.5 mM DTT, and protease inhibitors (2 μM leupeptin, 100 μM phenylmethylsulphonyl fluoride, 500 μM benzamidine, 100 nM aprotinin). The cells were incubated on ice for 1 h and lysates were obtained by centrifuging at 16,000 × g at 4 °C for 10 min to remove insoluble material. Protein concentrations were determined using the Bradford method (Bio-Rad).

**[^3]H]Ryanodine Binding Assays**

Binding assays were carried out following a modified version of a protocol previously described (53). Binding mixtures were prepared containing 50–100 μg of cell lysate, 0.2 M KCl, 20 mM Na-HEPES pH 7.4, 6.5 nM[^3]H]ryanodine (NET950, PerkinElmer), and enough CaCl₂ to set free [Ca²⁺] between 100 nM to 100 μM. 1 mM EGTA was used to buffer Ca²⁺. The Ca²⁺/EGTA ratio for these solutions was determined using MaxChelator (WEBMAXCLITE v1.15, http://maxchelator.stanford.edu/). The binding reactions were incubated for 2 h at 37°C, then filtered through Whatman GF/B filters pre-soaked in 5% polyethyleneimine to maximize protein retention and washed three times with 5 mL of distilled water in a Brandel M24-R Harvester. Non-specific binding was determined in the presence of 20 μM unlabeled ryanodine (2153770, MP Biomedicals).[^3]H]ryanodine binding was measured by liquid scintillation using Bio-Safe II counting cocktail (RPI Research). Corrections for RyR2 expression were determined by dividing the total[^3]H]ryanodine binding by the intensity from Western blots, expressed as a percentage of
the WT. Hill’s equation was used to determine the maximum [³H]ryanodine binding and the EC₅₀ in Origin 2018b (Origin Lab).

**Spontaneous Ca²⁺ Release in HEK293 Cells**

Stable inducible HEK293 cells lines expressing the WT (53) or P1124L-RyR2 were cultured as described above. Spontaneous Ca²⁺ release was measured using the ER-targeted FRET Ca²⁺ sensor D1ER (25, 54). After 18-24 h of subculture on 35 mm glass-bottom dishes, pcDNA-D1ER (36325, Addgene) was transfected as described in the previous section. 18–24 h post-transfection, RyR2 expression was induced by adding tetracycline 1 μg/mL to the culture medium. For imaging (18–24 h after induction), cells were continually perfused with Krebs-Ringer-HEPES buffer containing (mM): 125 NaCl, 5 KCl, 1.2 KH₂PO₄, 1.2 MgCl₂, 6 glucose, 25 HEPES, pH 7.4. 1 mM and 2 mM CaCl₂, 1 mM tetracaine and/or 20 mM caffeine were added as indicated. Images were captured at room temperature using a LSM800 confocal microscope (Carl Zeiss) with a Plan-Apochromat 20X/0.8 M27 objective at 405 nm excitation, and 400-500 nm (CFP) and 500-700 (YFP) emission. Time-lapse images were collected every 1.5 s with a scanning time of 1.29 s/frame. FRET was calculated as the YFP/CFP ratio. The maximum store capacity, fractional release, and activation and termination thresholds were calculated using the following equations, where Fₜₐₜ and Fₜₑₐₙ are the FRET levels when spontaneous Ca²⁺ oscillations activate and terminate, Fₘₐₓ is the maximum FRET level with tetracaine perfusion, and Fₘᵢₗ is the residual FRET level after the ER is emptied with caffeine.

\[
\text{Store Capacity} = (F_{\text{Max}} - F_{\text{Min}})
\]

\[
\text{Activation Threshold} = \frac{F_{\text{Act}} - F_{\text{Min}}}{\text{Store Capacity}} \times 100\%
\]

\[
\text{Termination Threshold} = \frac{F_{\text{Term}} - F_{\text{Min}}}{\text{Store Capacity}} \times 100\%
\]

\[
\text{Fractional Release} = \frac{F_{\text{Act}} - F_{\text{Term}}}{\text{Store Capacity}} \times 100\%
\]
Generation of the RyR2-P1124L Targeted Mice

The RyR2-P1124L-KI targeting vector was constructed using a combination of traditional cloning techniques and “recombineering”, a highly efficient phage-based *Escherichia coli* homologous recombination system (55, 56). The RyR2-P1124L-KI targeting vector was linearized with *NotI* and electroporated into murine SV/129 AB2.2 embryonic stem cells (57). ES cells that integrated the targeting vector either by homologous or random integration were selected by growth on G418. Gancyclovir (GANC) selected against clones that contained the HSV-TK cassette, thus enriching for clones that integrated the Neo cassette by homologous recombination. 472 Neo⁺, GANC⁺ colonies were selected, replicated and expanded. DNA was isolated from 192 replica ES clones, digested with *BamH1*, electrophoresed on agarose gels, transferred to charged nylon membranes and hybridized to radiolabeled 3’ probe. 36 correctly targeted clones were identified by a 17.2 kb band in addition to the 21.7 kb native band. DNA from 19 clones that appeared to be correctly targeted on the 3’ side were digested with *Nco1* electrophoresed on agarose gels, transferred to charged nylon membranes and hybridized to radiolabeled 5’ probe. Nineteen positive clones were identified. Six clones (2B2, 2C3, 2E5, 2B9, 2C9 and 2G9 1F2) were thawed from the master plate and fully expanded. The remaining 13 clones were partially expanded and cryopreserved. The expanded clones were genotyped using both the 5’ and 3’ probes. Correctly targeted clones were identified by the presence of a 21.7 kb native band and a 17.2 kb altered band when digested with *BamH1*, transferred to a charged nylon membrane and hybridized to the 3’ probe, and the presence of a 15.2 kb native band and an 8.7 kb altered band when digested with *Nco1* and hybridized with the 5’ probe. DNA sequence analysis was used to identify the correctly targeted clones and to confirm the presence of the floxed Neo cassette and the P to L substitution at position 1124. The chromosomes of 2 Ryr2-P1124L clones were counted and both clones were found to be euploid. Clones RyR2-P1124L 2E5 and RyR2-P1124L 2B2 were microinjected into C57Bl/6 blastocyst to produce chimeric founders. Highly chimeric males were crossed with C57Bl/6J females (000664, Jackson
Laboratory). Agouti pups carrying the P1124L were identified by PCR. The floxed Neo cassette was excised by mating heterozygous RyR2-P1124L mice with Ella-Cre transgenic mice (003724, Jackson Laboratory). Pups with complete Neo excision were identified by PCR and then backcrossed with C57Bl/6J mice. RyR2-P1124L pups negative for the Cre recombinase gene were further backcrossed for 7 additional generations with C57Bl/6J mice to obtain a 99%+ congenic strain (58). To confirm the presence of P1124L, the region of Ryr2 containing the mutation was amplified with PCR and sequenced using the following primers: 5’- CGCATCTTCAGGGCAGAG-3’ and 5’-GAGTGAAGCGGGAATGTC-3’. The sequences obtained were compared with the targeting vector, Ryr2 (chromosome 13, accession number NT_039578.8), as well as the corresponding mRNA (accession number NM_023868.2) using Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The translated protein was compared to the mouse RyR2 (accession number E9Q401).

**Mouse Phenotyping**

Transthoracic echocardiography (Echo) was performed by the Echocardiography Service of the Frankel Cardiovascular Center Animal Phenotyping Core at the University of Michigan using a Vevo 2100 system with a 22–55 MHz transducer (MS550D; Visual Sonics), as described previously (59). Mice were anesthetized with 1–2% isoflurane and maintained on a heated platform. Two-dimensionally guided M-mode images of the left ventricle were acquired at the tip of the papillary muscles. Left ventricle diameter, septum wall and posterior wall thickness were recorded to determine the fractional shortening, LV volume, ejection fraction, and heart rate. All parameters were measured over at least three consecutive cycles.

Surface electrocardiograms were recorded as described previously (31). Mice were anesthetized with isoflurane (2–5%) and maintained on a heated pad. Needle electrodes were placed under the skin to record in Lead-I and Lead-II configurations, using a PowerLab system (ADInstruments). After 5–10 minutes of stable baseline recording, epinephrine (2 mg/kg) and
caffeine (120 mg/kg) were applied i.p. Animals were monitored for 30 minutes after injection in search of arrhythmic events (typically ventricular bigeminy and ventricular tachycardia) defined as 3 or more ventricular ectopic beats, consecutive or alternated with normal beats. These events were further classified as non-sustained (lasting less than 5 seconds) or sustained (lasting 5 seconds or longer).

For histological staining, FFPE hearts were sectioned, stained and imaged by the Unit of Laboratory Animal Medicine In-Vivo Animal Core at the University of Michigan. An expert pathologist performed a detailed analysis of the slides and quantified the level of fibrosis.

The experimenter was blinded in all procedures performed at institutional cores. Age- and gender-matched WT littermates and C57Bl/6 mice were used as controls. All sets include males and females. Numbers and age of the mice are indicated in each figure.

**Tissue Homogenization**

Whole heart homogenates were prepared as previously described (60). Briefly, frozen hearts were pulverized in liquid nitrogen, suspended in homogenization buffer (0.9% NaCl, Tris-HCl 10 mM pH 6.8, 20 mM NaF, 2 µM leupeptin, 100 µM phenylmethylsulphonyl fluoride, 500 µM benzamidine, 100 nM aprotinin), homogenized using a Teflon pestle, and centrifuged at 1000 x g for 10 minutes at 4 °C. Supernatants were aliquoted and stored at -80 °C until used. Protein concentrations were determined using the Bradford method (Bio-Rad).

**Western Blotting**

50 µg of tissue homogenate were suspended in Laemmliii buffer and separated by SDS-PAGE in 4-20% TGX or AnyKD precast gels (Bio-Rad). Proteins were then transferred to PVDF membranes using the iblot2 transfer system (ThermoFisher) or overnight wet transfer. Membranes were probed with the following primary antibodies: anti-RyR [clone 34C] (1:2000; MA3-925, Thermo), pS2808-RyR (1:5000; A010-30, Badrilla), pS2030 (1:1000, custom-made)
(60), pS2814 (1:1000, custom-made) (60), dpS2030 (1:1000, custom-made) (60), SERCA2 [clone 2A7-1] (1:1000; ab2861, Abcam), NCX1 [clone 6H2] (1:200; ab6495, Abcam), PLB [clone A1] (1:5000; A010-14, Badrilla), pS16-PLB (1:5000; A010-12, Badrilla), pT17-PLB (1:5000; A010-13, Badrilla), Cav1.2 (1:200; ACC-003, Alomone), CaMKII [clone D11A10] (1:1000; 4436, Cell Signaling), pT287-CaMKII [clone D21E4] (1:1000; 12716, Cell Signaling), p44/42 MAPK (Erk1/2) (1:1000; 9102, Cell Signaling), pT202/Y204-p44/42 MAPK (Erk1/2) (1:1000; 9101, Cell Signaling), Akt (1:1000; 9272, Cell Signaling), pS473-Akt (1:1000; 9271, Cell Signaling) and GAPDH [clone 6C5] (1:10000; MAB374, Millipore). Secondary antibodies, used as appropriate, were goat anti-mouse-HRP (1:1000 or 1:5000; 31437, Thermo) or goat anti-rabbit-HRP (1:2000 or 1:10000; 31463, Thermo). Membranes were developed using SuperSignal Femto ECL reagent (Thermo) and imaged with a ChemiDoc MP apparatus (Bio-Rad). Band intensity was quantified with the ImageLab software (Bio-Rad). Custom-made antibodies against the phosphorylated forms of RyR2 are described elsewhere (60).

**Single Channel Recordings**

Cardiac SR-enriched microsomes for single channel recordings were prepared from mouse hearts using differential centrifugation, as previously described (36). 10–15 pooled mouse hearts were homogenized as described above. Homogenates were centrifuged a second time at 8000 x g for 20 minutes at 4˚C. Supernatants were further centrifuged at 100,000 x g for 35 minutes at 4˚C. The pellets containing SR-enriched microsomes were resuspended in homogenization buffer supplemented with 0.3 M sucrose.

RyR2 channels from SR microsomes were incorporated into planar lipid bilayers to record single channel activity as previously reported (36). The trans (1000 µl) and the cis (1000 µl) chambers (corresponding to the luminal and cytoplasmic side of the channel) contained 300 and 50 mM CsCH₃SO₃, respectively, 20 mM MOPS (pH 7.2), and nominally free [Ca²⁺] (~3-5 µM). A phospholipid bilayer of phosphatidylethanolamine:phosphatidylserine:phosphatidylycholine
(1.0:0.8:0.2 ratio dissolved in n-decane to 20 mg/ml) was "painted" with a glass rod across an aperture of ~200 μm diameter in a Delrin cup. The trans chamber was the voltage control side connected to the head stage of a 200-A Axopatch amplifier (Molecular Devices), while the cis side was held at virtual ground. Channel activity was recorded after filtration with an 8-pole low pass Bessel filter set at 1.5 kHz, and digitized at a rate of 4 kHz using a Digidata 1200 AD/DA interface (Molecular Devices). Data acquisition and analysis was performed with Axon Instruments hardware and software (pClamp 10, Molecular Devices).

**Gene Expression Analysis**

Tissue was homogenized in TRIzol reagent (ThermoFisher) following the manufacturer’s instructions. After addition of an appropriate amount of chloroform, mixing, incubation, and centrifugation, the RNA-containing aqueous phase was collected and treated with DNase I (Qiagen) for one hour. RNA was subsequently purified using the RNeasy mini kit (Qiagen), following the manufacturer’s protocol. First strand cDNA synthesis was performed using the iScript Reverse Transcription Kit (Bio-Rad). To facilitate full length first strand cDNA synthesis, samples were incubated for 90 minutes at 50 °C prior to heat inactivation of the reverse transcriptase at 70 °C for 15 minutes. Expression levels of Nppa (Mm01255748_g1, ThermoFisher), Nppb (Mm01255770_g1, ThermoFisher), Myh7 (Mm00600555_m1, ThermoFisher), and Actb (4352933E, ThermoFisher) were assessed using TaqMan gene expression probes.

**Isolation of Ventricular Myocytes**

Ventricular myocytes were isolated as previously described (59, 61). Briefly, mice were heparinized (0.5 U/g IP, Sagent Pharmaceuticals) and anesthetized with urethane (1mg/kg IP, Sigma-Aldrich). The heart was quickly excised, mounted on a Langendorff apparatus, and perfused with Perfusion Buffer (PB) containing (mM) 113 NaCl, 4.7 KCl, 1.2 MgSO₄-7H₂O, 10
HEPES, 0.6 Na$_2$HPO$_4$, 12 NaHCO$_3$, 0.6 KH$_2$PO$_4$, 10 KHCO$_3$, 30 Taurine, 10 2,3-Butanedione monoxime, 5.5 glucose, pH 7.46 at 37 °C and 3 mL/min. The heart was then perfused with PB supplemented with 773.48 U/ml Collagenase Type II (Worthington), 0.14 mg/ml Trypsin (Gibco) and 12.5 μM CaCl$_2$. Once fully digested (5–7 min), the ventricles were minced in PB buffer containing with 10% FBS and 12.5 μM CaCl$_2$. Tissue pieces were gently pipetted to dissociate cells. Ca$^{2+}$ was reintroduced to 1.8 mM in five steps and cells were kept in Tyrode’s solution containing (mM) 135 NaCl, 4 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, 1.2 NaH$_2$PO$_4$, and 10 glucose, pH 7.40, until used.

**Confocal Ca$^{2+}$ Imaging.**

Ca$^{2+}$ transients triggered by field stimulation were recorded from cardiac myocytes as previously described (59). Cells were incubated in 10 μM fluo-4 AM (ThermoFisher) with 0.4% Pluronic F-127 (ThermoFisher) at 37 °C for 5 min. Cells were then washed and kept in fresh Tyrode’s solution. Longitudinal line-scan images were recorded using a LSM510 Meta confocal microscope (Carl Zeiss) with a 40×/1.2 water immersion objective at 3.072 ms/line. Fluorescence was excited at 488 nm and recorded at >505 nm. Myocytes were first perfused with Tyrode’s solution and paced at 1 Hz using a Grass Stimulator (2 ms field-stimulation pulses, 60-70 V). When a stable response was established, cells were perfused with Tyrode’s solution containing 300 nM Iso for 2 min. Finally, 20 mM caffeine was applied to measure the SR Ca$^{2+}$ load. The last five Ca$^{2+}$ transients under basal conditions and with Iso were averaged for each cell. The fractional release was calculated as a percentage of the SR Ca$^{2+}$ load released during field stimulation. To determine the susceptibility to develop spontaneous Ca$^{2+}$ release events, cells were paced at 3 Hz for 30 s at basal conditions and in the presence of 300 nM Iso. Following this train of stimulation, cells were monitored for 30 seconds for Ca$^{2+}$ waves and triggered activity.
**Statistics**

Data are presented as mean ± SEM with individual data points indicated. Measurements performed in isolated cardiac myocytes are presented as scatter plots (on the left), while the average value for each heart is presented over a bar graph showing mean ± SEM (on the right). Data shown in box plots are presented with whiskers indicating the most extreme data points within the 1.5 inter-quartile range. Statistical significance was determined at $p < 0.05$ for comparisons between mice/hearts using z-test, two-tailed t-test, Rank Sum Test, one-way ANOVA with Holm-Sidak’s post-hoc or ANOVA on Ranks with Dunn’s post-hoc. All analyses and assessments of whether data met assumptions were carried out in SigmaPlot 13 (Systat Software). Data were plotted in Origin 2018b (Origin Lab).

**Study Approval**

Human studies were performed under IRB approval number 811-98 from Mayo Clinic. Patients provided a written informed consent prior to the inclusion in this study. Animal use was approved by the University of Michigan IACUC (PRO6075) and the University of Wisconsin-Madison School of Medicine and Public Health IACUC (M5944), both institutions run animal programs accredited by AAALAC. Animal husbandry was performed by specialized personnel following each institution’s standard procedures.
Author Contributions

FJA designed experiments, performed arrhythmia studies, expression and analysis of recombinant RyR2, confocal imaging, Western blots and most data analysis. He also wrote the manuscript. JMB, CAM and MJA conducted the human studies and identified P1124L patient. ZY and FVP crystallized and solved the atomic structure of SPRY2. CRV collected and analyzed the single channel recordings. JJH performed and analyzed the gene expression assays. Y-TZ participated in the isolation of ventricular myocytes and analysis of confocal images. DSH prepared recombinant protein and maintained cell cultures. YC maintained and genotyped the mouse colony. TRB performed some Western blot measurements. HHV conceived the project and designed experiments, supervised the work and wrote the manuscript. All authors participated in the editing of the manuscript.
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Figure Legends

Figure 1. Position of P1124L in RyR2 and crystal structure of the SPRY2 domain.

A. Linear representation of a single ryanodine receptor 2 (RyR2) subunit, indicating the regions where CPVT mutations are clustered (CPVT-I through -IV). The three SPRY domains are indicated and color-coded in all panels. B. Amino acid sequence of a region of SPRY2 RyR2 from five relevant species. Residues corresponding to the human 1107 and 1124 are highlighted. Accession numbers and other species and isoforms are indicated in Figure S2. C. Electron density map of RyR2 from the literature (20). Subunits are delimited by black lines. D. Modeled interface between the three SPRY domains of RyR2. The position of the two HCM-associated mutations, A1107 and P1124, is indicated. E-F. Superimposed crystal structures of the WT and P1124L SPRY2 domains obtained from the literature (19) and this work, respectively. Asterisk indicates positions of residue 1124 within the β2-β3 linker. Blue arrows indicate movement of four residues between WT and P1124L structures. Red dashed lines indicate salt bridges.
Figure 2. Molecular phenotype of RyR2-P1124L.

A. Ca$^{2+}$-dependent $[^{3}H]$ryanodine binding curve corrected for ryanodine receptor 2 (RyR2) expression. The mouse Ryr2 cDNA was transiently transfected into HEK293 cells. Cells were lysed 48 hr after transfection. Recombinant RyR2 was then incubated with $[^{3}H]$ryanodine in the presence of increasing concentrations of [Ca$^{2+}$] to activate the channel. $[^{3}H]$ryanodine was determined by liquid scintillation. B. Maximum binding ($B_{\text{Max}}$) calculated from the curves in A using Hill's equation. C. Ca$^{2+}$-dependent $[^{3}H]$ryanodine binding curves (same as in A) normalized to 10 µM [Ca$^{2+}$]. D. EC$_{50}$ for [Ca$^{2+}$]-dependent $[^{3}H]$ryanodine binding (Panels A-D: $n = 7$ curves from at least four independent transfections. * $p < 0.05$, ** $p < 0.01$, Rank Sum Test). E. Representative single RyR2 channel recordings from cardiac microsomes prepared WT and homozygous (Homo) P1124L hearts. Channels were fused into artificial planar lipid bilayers. Single channel current and open probability ($P_{o}$) were recorded at nominally free [Ca$^{2+}$] in a 300/50 mM Cs$^{+}$ gradient. F. Overlapped histograms of normalized current calculated from two representative channels. G. Average $P_{o}$ of WT and P1124L channels. H. Average single channel current calculated from recordings in a 300/50 mM Cs$^{+}$ gradient (Panels G-H: $n = 3$ WT, 5-6 P1124L channels. * $p < 0.05$, or no significant difference, t-test).
Figure 3. Abnormal termination of spontaneous Ca\textsuperscript{2+} release in HEK293 cells expressing RyR2.

A. Representative traces of the intra-ER FRET signal using the intra-ER Ca\textsuperscript{2+} sensor D1ER in cells expressing RyR2. Stable HEK293 cells with inducible expression of the mouse RyR2 were transfected with D1ER. RyR2 expression was induced with 1 µg/mL tetracycline 24 hr after transfection. Time-lapse images of D1ER FRET were recorded 24 hr RyR2 induction for 3 min at 0 [Ca\textsuperscript{2+}], 3 min at 1 mM [Ca\textsuperscript{2+}], 5 min at 2 mM [Ca\textsuperscript{2+}], 5 min in the presence of 1 mM tetracycline to inhibit RyR2 and measure maximum ER Ca\textsuperscript{2+} capacity (F\textsubscript{Max}), and ~3 min in the presence of 20 mM caffeine to empty the ER and measure the residual FRET signal (F\textsubscript{Min}).

B-D. Activation (B) and termination (C) thresholds and fraction of the ER Ca\textsuperscript{2+} content released (D) during spontaneous oscillations (* p < 0.05, ** p < 0.01, Rank Sum Test [panels B], t-test [panel C-D]).

E. ER store capacity determined as (F\textsubscript{Max}–F\textsubscript{Min}) and expressed as percentage of WT (No significant difference, Rank Sum Test).

F. Percentage of cells showing oscillations in the store [Ca\textsuperscript{2+}] (No significant difference, z-test).

G. Average number of [Ca\textsuperscript{2+}] oscillations per cell (**) p < 0.01, Rank Sum Test) (n = 40 [panels B-D], 49 [panel E], 87 [panel F] and 39 [panel G] WT cells; 44 [panels B-D], 61 [panel E], 104 [panel F] and 43 [panel G] P1124L cells from four independent D1ER transfections and inductions of RyR2 expression).
Figure 4. Cardiac hypertrophy in 1 yr-old P1124L mice.

A. Representative images of formalin-fixed hearts (first row, scale bar: 5 mm), H&E-stained coronal sections at low magnification of FFPE tissue (second row, scale bar: 5 mm), micrographs of H&E-stained tissue sections (third row, scale bar: 200 µm) and micrographs of Masson's Trichrome-stained tissue sections (fourth row; scale bar: 200 µm). Images are representative of n = 3 hearts per genotype. B-D. M-Mode echocardiography measurements of posterior wall (B) and septum thickness (C), ejection fraction (D). Mice were lightly anesthetized with isoflurane during the recordings. E. Heart weight as a percentage of total BW (n = 8 WT, 7 Het, 8 Homo mice. * p < 0.05, ANOVA on Ranks [panel B], one-way ANOVA [panel C-E]).
Figure 5. Cellular hypertrophy in cardiac myocytes from 1 yr-old P1124L mice.

A. Representative transmitted light images of ventricular myocytes (scale bar 50 µm). Cardiac myocytes were enzymatically isolated from freshly-explanted hearts. Images were obtained with a confocal microscope. Cells are delineated and colored green (measured cells) or purple (out of focus or out of frame). B-D. Cell width (B), length (C) and surface area (D) measured from transmitted light images (left: individual measurements from n = 75 WT, 81 Het, 84 Homo cells; right: mean values for N = 3 hearts per genotype. * p < 0.05, one-way ANOVA). E. Box plots showing transcript expression level of three hypertrophic genes: natriuretic peptide A (Nppa), natriuretic peptide B (Nppb), and myosin heavy chain 7 (Myh7). Data normalized to actin (Actb) expression (n = 5 WT, 4 Het, 5 Homo hearts per genotype. ** p < 0.01, * p < 0.05, one-way ANOVA).
Figure 6. Expression of RyR2 and calmodulin in P1124L hearts.

A. Representative Western blots of ryanodine receptor 2 (RyR2) and calmodulin (CaM) expression in heart homogenates from mice under at 3-6 mo of age and over 1 yr of age. Band intensities were normalized to the GAPDH signal from the same gel. A separate loading control (GAPDH) is shown for proteins run in different gels. B. Quantification of RyR2 expression (No significant difference, one-way ANOVA [3-6 mo] or ANOVA on Ranks [1 yr]). C. Quantification of CaM expression (n = 10 WT, 8 [3-6 mo] and 9 [1 yr] Het, 10 Homo hearts for each age group. * p < 0.05, ANOVA on Ranks).
Figure 7. Susceptibility to spontaneous Ca²⁺ release in ventricular myocytes from 1 yr-old mice.

A. Representative traces of WT and Homo ventricular myocytes stimulated with 300 nM Isoproterenol (Iso), paced at 1 Hz for 30 s and monitored at rest for 30 s. Enzymatically isolated cardiac myocytes were loaded with the fluorescent Ca²⁺ indicator fluo-4 AM and field-stimulated as indicated. Line-scan images were recorded using a confocal microscope. B. Percentage of cells showing spontaneous Ca²⁺ release events (SCR, defined as Ca²⁺ waves) during the monitoring period. SCR incidence is increased in Homo mice in basal conditions (n = 3 hearts per genotype. * p < 0.05, one-way ANOVA). C. Average number of SCR events in 30 seconds of monitoring after a 3 Hz train of stimulation (left: individual measurements from n = 17 WT, 16 Het, 16 Homo basal cells and 12 WT, 14 Het, 23 Homo Iso-stimulated cells; right: mean values for N = 3 hearts per genotype. * p < 0.05, one-way ANOVA).
Figure 8. Susceptibility to ventricular arrhythmia in P1124L mice challenged with epinephrine/caffeine at 8 mo of age.

A. 2-second representative lead-II ECG traces from mice undergoing arrhythmia challenge. Mice were anesthetized using isoflurane and the basal ECG was monitored for 10-15 min. The ECG was further monitored for 30 min following an i.p. injection of epinephrine (2 mg/kg) and caffeine (120 mg/kg) (Epi/Caff). From top to bottom, the traces indicate: sinus rhythm; premature ventricular beats; non-sustained ventricular bigeminy; bidirectional ventricular tachycardia (BDVT).

B-D. Average number of premature ventricular beats, and episodes of non-sustained (lasting ≤ 5 s) and sustained arrhythmias (lasting > 5 s) in mice undergoing arrhythmia challenge.

E. Incidence of sustained BDVT, defined as ectopic beats with alternating axes of depolarization (n = 7 mice per genotype. * p < 0.05 vs WT, ANOVA on Ranks [panel B-C], one-way ANOVA [panels D], z-test [panel E]).