Homozygous Missense N629D hERG (KCNH2) Potassium Channel Mutation Causes Developmental Defects in the Right Ventricle and Its Outflow Tract and Embryonic Lethality

Guo Qi Teng,* Xian Zhao,* James P. Lees-Miller, F. Russell Quinn, Pin Li, Derrick E. Rancourt, Barry London, James C. Cross, Henry J. Duff

Abstract—Loss-of-function mutations in the human ERG1 potassium channel (hERG1) frequently underlie the long QT2 (LQT2) syndrome. The role of the ERG potassium channel in cardiac development was elaborated in an in vivo model of a homozygous, loss-of-function LQT2 syndrome mutation. The hERG N629D mutation was introduced into the orthologous mouse gene, mERG, by homologous recombination in mouse embryonic stem cells. Intact homozygous embryos showed abrupt cessation of the heart beat. N629D/N629D embryos die in utero by embryonic day 11.5. Their developmental defects include altered looping architecture, poorly developed bulbous cordis, and distorted aortic sac and branchial arches. N629D/N629D myocytes from embryonic day 9.5 embryos manifested complete loss of $I_{Ks}$ function, depolarized resting potential, prolonged action potential duration (LQT), failure to repolarize, and propensity to oscillatory arrhythmias. N629D/N629D myocytes manifest calcium oscillations and increased sarcoplasmic reticulum Ca$^{2+}$ content. Although the N629D/N629D protein is synthesized, it is mainly located intracellularly, whereas +/- mERG protein is mainly in plasmalemma. N629D/N629D embryos show robust apoptosis in craniofacial regions, particularly in the first branchial arch and, to a lesser extent, in the cardiac outflow tract. Because deletion of Hand2 produces apoptosis, in similar regions and with a similar final developmental phenotype, Hand2 expression was evaluated. Robust decrease in Hand2 expression was observed in the secondary heart field in N629D/N629D embryos. In conclusion, loss of $I_{Ks}$ function in N629D/N629D cardiovascular system leads to defects in cardiac ontogeny in the first branchial arch, outflow tract, and the right ventricle. (Circ Res. 2008;103:1483-1491.)

Key Words: KCNH2 (hERG) ■ knock-in mouse ■ embryo developmental defect

The human ERG gene (hERG/KCNH2) encodes a potassium channel that is important in the late stage of action potential repolarization in heart. Mutations in this gene, which generally reduce plasmalemmal expression of hERG, lead to the long QT2 (LQT2) syndrome in humans.1,2 Patients with the LQT2 syndrome have a delay in cardiac repolarization that predisposes them to cardiac arrhythmias that can be lethal.1,2 Mutations in hERG are associated with embryonic lethality and the sudden infant death syndrome.3-4 Although the LQT2 syndrome generally occurs in individuals heterozygous for the mutant allele, individuals homozygous for the exon 4 duplication manifest embryonic lethality or are rescued in the neonatal period by pacing.5 Although not widely recognized, mutations of hERG appear to be associated with structural congenital cardiovascular anomalies including: tetralogy of Fallot, atrial-septal defects, ventricular-septal defects, and patent ductus arteriosus.6-9 Mouse ERG (mERG) is the dominant repolarizing current in the mouse embryonic heart.10 A channel analogous to hERG is expressed in differentiating quail neural crest cells11 early in development. These data imply a potential role of the ERG potassium channel in cardiovascular development. We created, by homologous recombination in embryonic mouse stem cells, mice bearing the human LQT2 N629D hERG mutation12-16 inserted in situ in the mouse gene homolog (mERG). The cardiovascular developmental consequences were evaluated.

Materials and Methods

The methods used to create the N629D mice are detailed in the online data supplement, available at http://circres.ahajournals.org. All animals were housed in the Animal Resource Centre of the

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From the Libin Cardiovascular Institute (G.Q.T., J.P.L.-M., F.R.Q., P.L.), the Faculty of Veterinarian Medicine (X.Z., J.C.C.), and Biochemistry and Molecular Biology (D.E.R.), University of Calgary, Canada; and the Cardiovascular Institute (B.L.), University of Pittsburgh, Pa.

*Both authors contributed equally to this work.

Correspondence to Henry J. Duff, MD, Libin Cardiovascular Institute, University of Calgary, 3330 Hospital Dr, NW, Calgary, Canada, T2N 4N1. E-mail hduff@ucalgary.ca

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Faculty of Medicine, University of Calgary, Alberta, Canada, using protocols in accordance with animal care guidelines established by the Canadian Council on Animal Care.

**Immunohistochemistry and Immunofluorescence**

For immunohistochemistry, cryosection was made from 4% paraformaldehyde-fixed embryos, sections were cut at 10 μm, and incubated in blocking solution (PBS containing 0.1% Triton X-100, 0.05% Tween 20, 2% donkey serum, and 1% BSA) for 2 hours, followed by overnight incubation in the anti-HERG antibodies 1:300 (Alomone Labs, Israel) in 1% BSA PBS solution at 4°C. As negative control, 10 μg/ml the mERG peptide was added together with the first antibody. After washing, sections were incubated in blocking solution 2 hours, then incubated with donkey anti-rabbit horseradish peroxidase conjugate (Amersham) 1:300. For immunofluorescence, cardiomyocytes or cryosections from OCT-embedded embryos were fixed with −20°C methanol for 10 minutes and then blocked and labeled with mERG antibody as described above, followed by incubation with cy3-conjugated goat anti-rabbit secondary antibody (Jackson Immunoresearch Laboratories, West Grove, Pa).

**In Situ Hybridization**

Whole-mount in situ hybridization was performed on embryonic day (E)9.5+/+ and N629D/N629D embryos. An EcoRI/Xho1 fragment at the 3' end of the coding sequence was subcloned into pGEN-T easy vector to produce the mERG1 specific probe. The Hand2 probe consisted of a 1.2-kb fragment from the 3' end of Hand2 cDNA. In situ hybridization studies were also performed with a mERG1 probe.

**Apoptosis Assay**

TUNEL assays were carried out using the Apoptag peroxidase in situ apoptosis detection kit (Chemicon).

**Isolated Murine Embryonic Cardiomyocytes and Electrophysiological Recording**

Isolation and dispersion of E9.5 cardiomyocytes were performed by the method similar to that described by Burton et al. The extracellular solution (36±1°C) contained 140 mM NaCl, 5.4 mM CaCl2, 1 mM MgCl2, 1 mM CaCl2, 5 mM L HEPES, and 5.5 mM glucose (pH 7.4 with NaOH). The pipette solution contained 110 mM KCl/K-aspartate, 10 mM KCl, 5 mM MgCl2, 5 mM L ATP-Na2, 10 mM L EGTA, 10 mM L HEPES, and 1 mM CaCl2 (pH 7.2 with KOH). A liquid junction potential of −10 mV was corrected. The holding potential was −70 mV.

**Intracellular Calcium Measurement**

Cells were loaded with the membrane-permeable acetoxyxymethyl ester of Fluo-4 (Fluo-4-AM) (Molecular Probes, Invitrogen Inc), and intracellular Ca2+ transients were measured at 37°C (see the expanded Materials and Methods section in the online data supplement). Membrane potential was simultaneously measured in current-clamp mode. Sarcoplasmic reticulum Ca2+ content was estimated from the amplitude of the Ca2+ transient induced by rapid application of 10 mM L caffeine.

**Video Microscopy**

Pregnant females at 9.5 days postcoitus were anesthetized (isoflurane) and the embryos placed in DMEM at 37°C. Video microscopy was performed at ×10 using a digital video camera.

**In Vivo Embryonic Echocardiography**

An echocardiographic machine (Model VS40, VisualSonics, Toronto, Canada; nominal center frequency of 40 MHz and a focal length of 6 mm) was used11 to observe the characteristics of the intrauterine heart beating.

**Results**

**Homozygous N629D Results in Embryonic Lethality**

The N629D mutation was inserted into the mouse ERG gene by homologous recombination in mouse embryonic stem cells (see Figure I in the online data supplement). To remove the neomycin cassette, N629D mice were mated to Mox2-Cre mice.

Heterozygous N629D mutant offspring were viable and fertile. However, no viable N629D/N629D homozygous offspring were obtained in litters from N629D heterozygote intercrosses (supplemental Figure I, B). To further explore when N629D/N629D embryos die, embryos were dissected at various times during gestation. Their genotypes and morphologies were related (Figure 1 and supplemental Figure I). Before E9.0, no obvious difference in body size at matched stages of development (as judged by somite pairs) was observed (Figure 1A and 1H). After 20 somites, the N629D/N629D embryos showed significantly smaller body size. By E11.5, no live N629D/N629D embryos were found.

**Developmental Cardiac Defects in N629D/N629D Embryos**

At E9.5, the morphology of the +/+ hearts shows the expected bulboventricular groove and a U-shaped cardiac loop (Figure 1B; see arrow). In contrast, the N629D/N629D embryos (Figure 1D) did not show this normal bulboventricular groove (n=14), and, instead, the silhouette of the heart showed an L-shaped heart tube. These data suggested abnormalities in loop architecture. N629D/N629D embryos were observed to have a massive pericardial effusion, likely a consequence of heart failure. Defective development was observed in the bulbus cordis right ventricle and the outflow tract. Figure 1C through 1E compares representative hematoxylin/eosin coronal sections of the embryos at E9.5 in +/+ versus N629D/N629D embryos (Figure 1J through 1L). In N629D/N629D embryos, the right ventricle is not normally developed; the outflow tract is distorted and is continuous with a dilated aortic sac and first branchial arch artery. Hypoplasia of first branchial arch is shown in Figure 1I. Heterozygote embryos have neither developmental phenotypes nor pericardial effusion.

To assess whether the N629D/N629D genotype altered the overall expression levels of wild-type (WT) or mutant mERG protein, immunohistology studies were performed in +/+ (Figure 1F and 1G [higher power magnification of boxed portion of Figure 1F]) and in N629D/N629D (Figure 1M and 1N [higher power]) hearts. Overall mERG protein expression is similar in +/+ (Figure 1F and 1G) and N629D/N629D hearts (Figure 1M and 1N).

The +/+ hearts consistently beat regularly without arrhythmias. In contrast, N629D/N629D hearts beat irregularly with abrupt pauses (attached video supplement). The +/+ embryos show flow both into and out of the heart. The N629D/N629D embryos have arrhythmias, and there is no flow into or out of the heart even during normal beats. Fetal echocardiograms confirm irregular beating and abrupt asystolic episodes in N629D/N629D mice but never in +/+ embryos.
I_{Kr}, Action Potential, and Arrhythmic Phenotypes in N629D/N629D Myocytes

At E9.5, WT myocytes show typical hERG currents (Figure 2A), whereas complete loss of I_{Kr} tail current was observed in the N629D/N629D myocytes (Figure 2B). Whereas most of the +/N629D myocytes from 10 hearts (29/38) have an I_{Kr} whose character and density is similar to +/+ (Figure 2C), a minority of myocytes (6/38) showed an I_{Kr} with a much smaller ratio of tail current to time-dependent current magnitude (Figure 2D). This characteristic is the same as the “intermediate” phenotype previously reported. In addition, a small proportion of +/N629D cells (3/38) showed an N629D-like phenotype, absent tail currents (Figure 2E). Mean current–voltage relationships of the peak tail currents are shown in Figure 2F.

For each cell, after recording I_{Kr} in voltage-clamp mode, spontaneous paired action potentials were recorded in current-clamp mode. In +/+ myocytes, a spectrum of action potential shapes were observed, some with features of sinus node cells, some atrial-like, and some ventricular-like (Figure 3A). Even so, +/+ myocytes manifest relatively hyperpolarized resting membrane potentials (mean data in supplemental Figure II). In comparison to +/+ , the action potential in N629D/N629D myocytes (Figure 3B) show more depolarized resting membrane potentials and prolonged action potential durations (APDs). Moreover, failure to repolarize and spontaneous oscillatory triggered activity were commonly observed in N629D/N629D myocytes (Figure 3B). Most +/N629D cells had a WT-like I_{Kr} phenotype (Figure 3C) and had action potentials similar to +/+ . In contrast, +/N629D myocytes which manifest the intermediate I_{Kr} phenotype (Figure 3D) had resting potentials that were significantly more depolarized with prolonged action potentials. In addition, cells with an N629D-like phenotype had marked depolarization of resting potential and spontaneous arrhythmias occurred frequently (Figure 3E). The +/+ myocytes manifest a resting membrane potential of −64±2 mV compared to −38±2 mV for N629D/N629D myocytes (P<0.001). The +/N629D cells with a WT-like I_{Kr} phenotype (Figure 3C) had a resting potential (−67±1 mV) similar to +/+ . In contrast, +/N629D myocytes that manifest the intermediate and N629D-like I_{Kr} phenotype (Figure 3D) had depolarized resting potentials of −50±4 and −39±3 mV, respectively; both P<0.01. Mean APDs (in ms) for +/+, N629D/N629D, +/N629D with WT-like phenotype, +/N629D with intermediate I_{Kr} phenotype, and +/N629D with N629D-like phenotypes were 65±6, 143±25 (P<0.01), 59±12 (P=NS), 139±30 (P<0.01), and 145±29 (P<0.01), respectively.

Distribution of N629D Protein Expression in Myocytes

To assess the cellular distribution of the N629D protein, immunocytochemical studies were performed on cardiac myocytes isolated from E9.5 heart tubes. The distributions of the mERG protein are shown in Figure 3 in the right column. Typically, the +/+ mERG protein is localized dominantly in the surface plasmalemma, where it is expressed as continuous linear sheaths of staining. In contrast, the N629D/N629D protein was expressed intracellularly, in a pattern of punctate concentric rings of staining that do not generally touch the plasmalemma. In contrast, a spectrum of expression patterns are observed in heterozygous +/N629D myocytes. The majority of the cells (108/133 cells) had a pattern of distribution of mERG protein similar to WT. A minority of cells (14/133 cells) had a pattern of staining indistinguishable from N629D/ N629D myocytes. The remaining cells had an intermediate
pattern, wherein the mERG protein was expressed in punctuate dots in a subplasmalemmal location, with some expression likely in the plasmalemma as well. The proportions of +/N629D cells showing a WT-like phenotype by electrophysiology (29/38 cells; 76% of cells) were roughly equivalent to the proportion of cells showing a WT-like phenotype on immunocytochemistry (108/133; 81%). The same is true in terms of the proportions of cells showing intermediate and N629D-like electrophysiology and immunocytochemical phenotype. Lower-power images of the cellular distribution of mERG proteins are shown in supplemental Figure III.

**N629D/N629D Myocytes Manifest Calcium Oscillations and Increased Sarcoplasmic Reticulum Ca\(^{2+}\) Content**

Because involution of the fetus and failure of the heart to develop are characteristics of the N629D/N629D embryos and because abnormalities of intracellular calcium are a common denominator of cell death, we compared intracellular calcium homeostasis in N629D/N629D versus +/+ E9.5 fetal myocytes (Figure 4). The N629D/N629D cells were depolarized and they displayed abnormal Ca\(^{2+}\) oscillations, with elevated peak F/F\(_{0}\) (Figure 4A and 4B). In these circumstances, injection of negative current (to give a resting membrane potential comparable to that of WT cells) lowered diastolic F/F\(_{0}\) and produced abnormal prolonged action potentials with marked early afterdepolarizations (Figure 4A and 4B). The sarcoplasmic reticulum Ca\(^{2+}\) content (estimated from the amplitude of the caffeine-induced Ca\(^{2+}\) transient) was significantly higher in N629D cells (F/F\(_{0}\): N629D versus WT, 4.02±0.24 versus 3.40±0.17, P=0.04; Figure 4C and 4D).

**Potential Mechanisms by Which mERG K\(^{+}\) Channel Plays a Role in Development**

mERG expression was abundant in the craniofacial region and first branchial arch (Figure 5A). mERG expression was also compared in right ventricle, outflow tract, and left ventricle in +/+ E10.5 embryonic heart (Figure 5B). Because the developmental defects seen in the N629D/N629D fetuses are quite similar to that seen in Hand2-deficient embryos, we assessed Hand2 expression in the N629D/N629D embryonic hearts versus +/+ (Figure 6). Figure 6 shows that in +/+ embryos Hand2 is abundantly expressed in the right ventricle, bulbus cordis, and the branchial arches, compared to the left ventricle. In contrast, Hand2 is substantially downregulated in N629D/N629D E9.5 embryos (Figure 6).
Apoptosis is also seen in the first branchial arch in hand2-deleted mice at E9.5. TUNEL immunocytochemical assays were also performed on N629D/N629D embryos. Figure 7 shows robust TUNEL-positive staining at E9.5 in N629D/N629D fetuses in the craniofacial region. Apoptotic cells were also observed in the outflow tract of the N629D/N629D hearts, albeit sporadically, but were rare in these areas in the +/+ embryos. Our data indicate that the earliest signs of apoptosis develop in the first branchial arches (Figure 1J and Figure 7C) and craniofacial regions. To assess the time course of the onset of the apoptosis, we assessed the presence of apoptosis at earlier developmental times. Even earlier, at E9 (somite 17 to 19), robust apoptosis was present in the craniofacial maxillary regions and the first branchial arches in N629D/N629D embryos. At that time, there was no witnessed apoptosis in the heart (data not shown). No overt...
difference in phospho-histone H3 staining (M-phase marker) was observed comparing E9.5 N629D/N629D and +/+ embryos (data not shown).

Discussion
The N629D/N629D embryos show the following novel features. (1) All embryos die by E11.5. (2) An I_{Kr}-null phenotype is associated with defects in cardiac looping and development of the right ventricle, bulbus cordis, and pharyngeal arches. These gross histopathologic phenotypes are very similar to that reported in abstract form by London et al.24 (3) N629D/N629D myocytes show prolonged APD and depolarization of the resting potential and propensity to oscillatory cardiac arrhythmias and recurrent asystolic episodes in intact embryos. (4) The depolarization of the resting potential is associated with abnormalities of intracellular calcium homeostasis. Intracellular calcium overload is a common denominator to cell death in excitable tissues.22 (5) Apoptosis is abundant at E 9.5 in the craniofacial region and the first branchial arch and this occurs before apoptosis in the outflow tract.

Because a large number of medications inadvertently block the hERG potassium channel, these novel findings have substantial clinical relevance.

Causes of Embryonic Lethality
There are a number of reasons to believe that the cardiovascular defects are causative of the embryonic lethality: (1) all propulsive flow into and out of the fetal heart is lost in the N629D/N629D embryos; and (2) embryonic N629D/N629D embryos manifest arrhythmias and bradycardias. Previous studies report that embryonic hearts respond to pharmacological hERG blockers with bradycardia and cardiac arrest resulting in ischemia,25–28 ventriculoseptal defects, vascular defects, and death. One limitation of those studies is that the drugs used are not at all specific for I_{Kr}. Secondly, blockade of I_{Kr} in embryonic cardiac myocytes was not established.

Potential Mechanisms by Which mERG K+ Channel Plays a Role in Development
In the present study, we observed that mERG protein expression in the developing embryonic heart is not homogeneous.
Protein expression is exaggerated in the right ventricle and in the outflow tract. Previous studies by Franco et al.\(^2^9\) also reported inhomogeneous expression of \(\beta\) subunits of the \(I_{Kr}\) channel complex in embryonic heart: expression of \(mIRP\) and \(mink\) are also exaggerated in the bulbus cordis.\(^2^9\) An exaggerated phenotype of the functionally knockout might be expected in tissue with endogenous exaggerated mERG expression.

Recent studies in chicken embryos by Tirosh-Finkel et al.\(^3^0\) indicate that cells from the cranial paraxial mesoderm migrate to the branchial arches and subsequently give rise to the facial structures and populate the outflow tract of the heart. Other studies by Ko et al.\(^3^1\) propose that cranial neural crest cells appear to contribute to cardiac outflow tract. N629D/N629D embryos manifest extensive apoptosis, particularly in the first branchial arch and the facial region. Given that cells from the branchial arch populate the outflow tract,\(^3^0\) we propose that early apoptosis in the branchial arch and facial region would prevent those cells from contributing to the development of the outflow tract in N629D/N629D hearts.\(^3^0,3^1\) This is our working model. Thus, we propose that \(Hand2\) expression is downregulated in N629D/N629D embryonic right ventricle and outflow tract because progenitor cells that populate the outflow tract undergo apoptosis while in the facial region and branchial arch. Thus, tissues that would be expected to express \(Hand2\) are absent, simply because those structures fail to develop.

Previous studies indicate that \(Hand2\) deletion results in craniofacial apoptosis with defects in the development of the right ventricle, outflow tract, and bulbus cord. Thus, deletion of \(Hand2\) produces a final developmental phenotype\(^3^2\) similar to N629D/N629D embryos. The molecular mechanism underlying the defect in the pharyngeal arches in \(Hand2\)-null mice has been recently explored showing apoptosis in an Apaf-1–dependent fashion; Apaf-1 is a central downstream mediator of mitochondrial damage-induced apoptosis.\(^3^3\) Our data indicate that the deficiency of mERG functional expression in N629D/N629D embryos results in depolarization of the resting membrane potential. We propose a working mechanistic model in which protracted depolarization of the resting membrane potential triggers apoptosis attributable to intracellular calcium overload. Dysfunction of calcium homeostasis is a common trigger for apoptosis in a wide range of cellular systems, including heart.\(^2^2\) Other studies confirm that pharmacological block of hERG results in apoptosis. The antihypertensive agent doxazosin pharmacologically blocks the hERG channel.\(^3^4\) Specifically, doxazosin induced apoptosis in hERG-overexpressing HEK cells but did not produce apoptosis in untransfected control cells. Pharmacological blockade of hERG also leads to apoptosis in wide range of native tumor cells that endogenously overexpress the hERG current.\(^3^5\)

An alternative working model for the apoptosis and mortality in N629D/N629D mice is that the proven arrhythmias and asystolic episodes and abnormalities of flow lead to hemodynamic insufficiency, which can secondarily increase...
apoptosis, change Ca$^{2+}$ handling, and induce heart defects. Nevertheless, this does not readily explain why the apoptosis begins in the branchial arch.

**Limitations**

Our study provides a working hypotheses to explain the developmental defects in N629D/N629D mice. Although our model is based on and is consistent with our data, we cannot unambiguously prove that all of the phenotypes observed in N629D/N629D embryos are caused by a loss of function of $I_{Kr}$ and subsequent depolarization-mediated calcium overload, resulting in apoptosis. It remains a possibility that the N629D mutation results in a gain of function in noncardiac cells. Further studies will be necessary to address these issues.

**Conclusion**

Loss of $I_{Kr}$ function in the N629D/N629D cardiovascular system leads to defects in cardiac ontogeny mainly in the first branchial arch, outflow tract, and the right ventricle.

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**Disclosures**

None.

**References**


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SUPPLEMENT MATERIAL

Methods:

Creation of the N629D mice.

mERG genomic DNA spanning intron 5 to exon 15 [1] was used to construct a targeting vector containing a pol II-driven neomycin resistance cassette flanked by loxP sites which was cloned into intron 7 and the N629 residue in exon 7 was mutated to D (Online Figure I A). The targeting construct was assembled in lambda2TK [2]. The arms of the phage-targeting vector were removed via NotI digestion. The targeting vector was introduced into 5 -7 x 10^6 mouse R1 embryonic stem (ES) cells by electroporation (Bio-Rad Gene Pulser). Positive and negative selections were carried out using 300 U of Geneticin (G418)/ml and 2X10^{-7} M 1-(2-deoxy-2-fluoro-D-arabinofuranosyl)-5-iodouracil, respectively. ES cell clones containing the neomycin cassette were identified by Southern blot analysis with a 3' probe external to the ERG genomic DNA present in the vector and was confirmed by a 5' probe. The karyotype of targeted ES cell clones was determined to verify diploid status. Three targeted ES cell clones were injected into 3.5-day-postcoitum (dpc) C57BL/6 blastocysts. One male chimera with 60% agouti pigmentation was bred to C57BL/6 females, and agouti pups were genotyped to confirm germ line transmission. The genotypes from the F1 and F2 generation were determined by Southern blotting, while genotypes of all subsequent mating were determined by PCR.

For PCR analysis, oligonucleotides for common forward (5'-CATCGGCTGGCTGACAAC-3'), wild type reverse (5'-GCCAACCTTCACCATTTCCT-3') and mutant (neo cassette) reverse (5'-GGAATGGGTTGGTAGAGCGA-3') were used in a 3 primer assay. PCR conditions were as follows: denaturation at 95°C for 1 min followed by 30 cycles of 1 min at 94°C, 1 min at 60°C, and
1 min at 72°C. Analysis thus far has been carried out on the hybrid C57BL/6–129-SV/J background. To remove the neomycin cassette, N629D mice were mated to Mox2-Cre mice.

To identify the neomycin cassette -removed mice, we synthesized a reverse primer (5’-ACGCCAACCTTCACCATCGA-3’) that incorporated an intron7 Cla1 site created for neo cassette insertion. This was used for PCR in conjunction with the above common forward primer. The product was sequenced to confirm the existence of the N629D mutation and removal of the neo-cassette.

**Intracellular calcium measurement**

Fluo-4 AM (Molecular Probes, Invitrogen Inc.) was dissolved at a concentration of 1 mmol/L in 20% Pluronic F127 in dimethylsulfoxide (Molecular Probes, Invitrogen Inc.). 1-1.5 μL of this solution was added to 1 mL of cell culture solution containing a glass coverslip with attached cells. Myocytes were loaded for 30 min at 37°C then the coverslip was placed on the chamber of an inverted microscope (Axiovert 200, Carl Zeiss Inc.) and perfused with Tyrode’s solution at 37°C. At least 10 min were allowed for de-esterification of the dye. Light from a 100 W mercury arc lamp (HBO 100, Carl Zeiss Inc.) was filtered to give excitation at 488 nm (10 nm bandwidth). Emission light was passed through a 500 nm long-pass beamsplitter and a 525 nm bandpass filter (50 nm bandwidth) before being collected by a photomultiplier tube (PTI Inc.). The voltage output from the PMT was digitized and recorded using pCLAMP 9 software (Axon Instruments, Molecular Devices) to allow simultaneous measurement of membrane potential and calcium signals.
For each cell under study, an adjustable rectangular diaphragm in the emission lightpath was used to mask off light from any other cells or debris in the optical field. Background fluorescence ($F_B$) was measured by moving the cell of interest outside this aperture. Cellular autofluorescence ($F_{Auto}$) was estimated to be $1.04 \times F_B$ in separate experiments using unloaded cells. Minimum fluorescence ($F_0$) was obtained for each cell by perfusing with Ca-free Tyrode’s solution containing 1 mM EGTA. Fluorescent signals were expressed as $(F - F_{Auto})/(F_0 - F_{Auto})$. If it is assumed that the intracellular Ca concentration in the presence of 1 mM EGTA is the same for WT and N629D cells, then valid comparisons can be made between these cell types in terms of their Ca transient characteristics, without further conversion into absolute $[\text{Ca}^{2+}]_i$ (which is difficult for non-ratiometric dyes such as Fluo-4). $F_0-F_{Auto}$ was no different between the two cell types (WT $0.84 \pm 0.04$ V vs N629D $0.89 \pm 0.06$ V, $p=0.5$).

Rapid application of 10 mM caffeine was achieved using a custom-built heated solution switcher whose tip was placed 2-3 mm from the cell.

**Results:**

**Online Figure I - Creation of mERG- N629D Mice. Panel A.** The targeting vector and the strategy to create the recombinant mouse is shown. A pol II-driven neomycin cassette flanked by loxP sites was cloned into intron 7 and the N629 residue in exon 7 was mutated to D. Subsequent mating to Mox2-Cre mice deleted the neo-cassette insert. **Panel B.** The genotypes at different stage of development are shown. The frequency of the genotypes at E8.5 to 11 versus post natal day 1 (P1) embryos were compared. Note that no N629D/N629D mice (-/-) are observed at P1.
Online Figure II - **Mean action potential characteristics** are related to the characteristics of the paired recordings of $I_{Kr}$ in each cell. Resting membrane potential (RMP) data, on the left, and action potential duration ($APD_{90}$) data on the right are related to genotype and to the $I_{Kr}$ phenotype. * indicates a statistically significant difference $p<0.01$ compared to +/+.

Online Figure III. **Representative examples of the cellular distribution of the mERG proteins** were evaluated in isolated cardiac myocytes show at lower power magnification. The left hand panel shows $+/+$ protein which is dominantly expressed in the plasma-lemma. The right hand panel shows that the N629D/N629D protein is dominantly expressed intracellularly. The middle panel shows the mERG proteins in heterozygous cells manifesting a spectrum of patterns of protein expression.

**Online Videos**

1) Shows *in vitro* microscopy of the $+/+$ embryo with normal heart beating.

2) Shows *in vitro* microscopy of the N629D/N629D embryo showing abrupt asystolic episodes, irregular beating patterns and no propulsive flow into or out of the N629D/N629D embryos.

3) Echocardiographic assessment of *in situ* embryos at E9.5 in $+/+$ embryos showing normal and regular heart beat without pauses.

4) Echocardiographic assessment of *in situ* embryos at E9.5 in N629D/N629D embryos showing abrupt pauses.
References


Online Figure I

A

1A 1A' 2 3 4 5 1B 6 7 8 9 10 11 12 13 14 15

+/+

N629D

+/- -/-

E8.5-11.5 8 48 11 26 11

Genotype of frequency of embryos from N629D heterozygote intercrosses

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<th>Stage</th>
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<th>+/-</th>
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Online Figure II

Fig. 5
Online Figure III

+/- +/N629D N629D/N629D

Bar indicates 20uM