An In Vivo Cardiac Assay to Determine the Functional Consequences of Putative Long QT Syndrome Mutations

Chuan Chau Jou, Spencer M. Barnett, Jian-Tao Bian, H. Cindy Weng, Xiaoming Sheng, Martin Tristani-Firouzi

Rationale: Genetic testing for Long QT Syndrome is now a standard and integral component of clinical cardiology. A major obstacle to the interpretation of genetic findings is the lack of robust functional assays to determine the pathogenicity of identified gene variants in a high-throughput manner.

Objective: The goal of this study was to design and test a high-throughput in vivo cardiac assay to distinguish between disease-causing and benign KCNH2 (hERG1) variants, using the zebrafish as a model organism.

Methods and Results: We tested the ability of previously characterized Long QT Syndrome hERG1 mutations and polymorphisms to restore normal repolarization in the kcnh2-knockdown embryonic zebrafish. The cardiac assay correctly identified a benign variant in 9 of 10 cases (negative predictive value 90%), whereas correctly identifying a disease-causing variant in 39/39 cases (positive predictive value 100%).

Conclusions: The in vivo zebrafish cardiac assay approaches the accuracy of the current benchmark in vitro assay for the detection of disease-causing mutations, and is far superior in terms of throughput rate. Together with emerging algorithms for interpreting a positive long QT syndrome genetic test, the zebrafish cardiac assay provides an additional tool for the final determination of pathogenicity of gene variants identified in long QT syndrome genetic screening. (Circ Res. 2013;112:826-830.)

Key Words: arrhythmia □ channelopathy □ gene mutation □ genetic testing □ genetics polymorphism □ ion channels □ long QT syndrome □ model organism □ sudden death

Mutations in KCNH2 (human ether-a-go-go related gene [hERG1]), the gene encoding the rapidly activating, delayed rectifier K+ current (IKr), account for 30% to 45% of mutation-positive Long QT Syndrome (LQTS).1,2 Commercial genetic testing for the known LQTS disease-causing genes is now the standard of care in the work-up of a new patient with clinical LQTS. Policy experts agree that a major obstacle to the interpretation of genetic findings is the lack of robust functional assays to determine the pathogenicity of identified gene variants.3,4 The current gold-standard assays for functional characterization of LQTS mutants (noncardiac mammalian cell expression systems, such as HEK293, CHO cells) are laborious and unable to meet the demands of LQTS genetic testing. Thus, there is an urgent need to develop the tools to characterize the functional consequences of LQTS gene variants in a high-throughput fashion to improve counseling and clinical care.

The zebrafish is a powerful vertebrate genetic model to explore the molecular basis of cardiac development, disease, and arrhythmia. Although the zebrafish heart is 2-chambered, fundamental electric properties are similar to higher mammals, including embryonic/adult heart rates,5,6 action potential parameters,7 QT interval,6,7 and the relationship between QT interval and heart rate.7 We previously validated a zebrafish model of human LQT-2 and showed that the zebrafish orthologue of hERG1, kcnh2 is essential for cardiac repolarization in embryonic zebrafish ventricle. Homozygous kcnh2 mutations cause ventricular asystole as a consequence of membrane depolarization. Submaximal IKr blockade prolongs ventricular action potential duration and causes 2:1 atrioventricular block (AVB), whereas maximal IKr blockade causes membrane depolarization leading to cessation of action potential generation.7 We sought to take advantage of the observation that kcnh2 channels provide the principal repolarizing current in embryonic ventricle to design an in vivo system to discriminate deleterious mutations in hERG1 variants.

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Methods

*Kcnh2* antisense morpholino (MO) was injected alone or coinjected with wild-type (WT) or mutant *hERG1* cRNA in WT zebrafish embryos at 1- to 2-cell stage. Cardiac repolarization phenotypes (normal 1:1 atrioventricular [AV] conduction, 2:1 AVB, or ventricular asystole) were screened under light microscopy at 48-hours postfertilization. Statistical analyses were performed as described in the online-only Data Supplement.

Results

**Morpholino kcnh2 Knockdown Disrupts Cardiac Repolarization**

*Kcnh2* is expressed very early in cardiac development in the bilateral cardiac primordia before the onset of cardiac contraction and specifically localized to the cardiac chambers at 48-hours postfertilization (Figure 1A). MO-knockdown of *kcnh2* caused a spectrum of repolarization defects, similar to those observed in the zebrafish *knch2* mutants, *breakdance*, and *silent ventricle*.7,8 Ninety-one percent of MO-injected embryos (n=210) displayed a repolarization-deficient phenotype (2:1 AVB or ventricular asystole), confirming the robust nature of the MO-induced *kcnh2*-knockdown. WT *hERG1* RNA injection restored normal repolarization in 55% of MO-injected embryos, and shifted the severity of the repolarization defect from ventricular asystole to 2:1 AVB (Figure 1B). To define the cellular correlates of our observed repolarization phenotypes, we directly measured *V*_m* in explanted embryonic ventricle. Spontaneous ventricular action potentials recorded from embryos manifesting 2:1 AVB were significantly prolonged compared with embryos that displayed normal repolarization (1:1 AV conduction; Figure 1C and the online-only Data Supplement Table I). The resting ventricular *V*_m recorded from embryos displaying a ventricular asystole phenotype was markedly depolarized and spontaneous action potentials were not observed (Figure 1C), similar to that recorded in the complete loss-of-function *knch2* mutant, *silent ventricle*.7 MO-injected embryos displaying a normal repolarization phenotype captured the pacing stimulus in a 1:1 fashion, whereas those displaying a 2:1 AVB phenotype were unable to pace in a 1:1 manner as a consequence of action potential duration prolongation (Figure 1D). Taken together, these data demonstrate that *kcnh2* MO-knockdown produces repolarization-deficient phenotypes that can be rescued by injection of WT *hERG1* RNA.

**An In Vivo Cardiac Assay to Determine the Functional Consequences of hERG1 Variants**

Next, we tested the ability of previously described LQTS *hERG1* mutations (n=40) and *hERG1* polymorphisms (n=10)
to restore normal repolarization in the kcnh2-knockdown embryonic zebrafish (Figure 2). A spectrum of normal repolarization rescue was observed for the 40 putative LQTS hERG1 mutants, but the degree of rescue was statistically lower than WT hERG1 rescue (the online-only Data Supplemental Table II). All 10 kcnh2 MO-knockdown embryos injected with WT, N470D, A614V, and A1116V hERG1 (the online-only Data Supplemental Figure I). These data confirm that 2:1 AVB is the result of marked action potential duration prolongation, whereas ventricular asystole is because of marked depolarization.

Forty-nine of 50 hERG1 variants have been functionally characterized using the current gold-standard in vitro heterologous expression system. When compared with the benchmark assay, the cardiac assay correctly identified a nondisease-causing variant in 9 of 10 cases (negative predictive value 90%), whereas correctly identifying a disease-causing variant in 39/39 cases (positive predictive value 100%). The only variant incorrectly assigned by the in vivo assay was T436M, which was predicted to be a pathogenic variant.

As an alternative comparison between the 2 assays, we normalized the values obtained using the in vivo zebrafish cardiac assay to the gold-standard in vitro assay as follows. For each hERG1 variant, the fraction of embryos with normal repolarization was normalized to WT value and plotted against the fraction of WT current measured in vitro, using published values (Figure 3). We used hierarchical cluster analysis to group the values into 2 or more unique subsets that represent concordance between the 2 assays. The data were best fit by 2 distinct clusters that distinguish pathogenic from nonpathogenic variants (the online-only Data Supplemental Figure II). For example, variants with current magnitude <0.5 of WT clustered tightly with variants, whose repolarization rescue was <0.5 of WT. Variants with current magnitude >0.7 of WT clustered with a broader range of rescued repolarization values, but were generally concordant with rescue values >0.67 of WT. One variant fell outside of the 95% confidence interval ellipse of either cluster (T474I). Although T474I generates functional channels in vitro, it was considered a pathogenic mutation based on altered kinetic properties and was correctly identified as disease-causing by the in vivo cardiac assay.

Finally, we tested the ability of the in vivo cardiac assay to corroborate the clinical observation that inheriting a common polymorphism (K897T) and an LQT-2 variant (A490T) in cis orientation reduces the severity of the LQTS phenotype compared with subjects with the A490T mutation alone. The A490T single mutant behaved as a pathogenic variant in the in vivo assay, whereas the double mutant A490T-K897T rescued repolarization similar to WT hERG1 (the online-only Data Supplemental Figure III). Taken together, our results suggest that replacing the endogenous kcnh2 gene with the human orthologue has predictive value for testing putative disease-causing LQT-2 variants, and corroborates an interesting clinical observation that a common polymorphism modifies the severity of a specific LQTS phenotype.

**Discussion**

Although genetic testing for channelopathies is now a standard and integral component of clinical cardiology, a major obstacle to the interpretation of genetic findings is the lack of robust functional assays. Here, we describe the utility of an in vivo cardiac assay to distinguish between disease-causing and benign hERG1 variants, and demonstrate the proof-of-principle concept that replacing an endogenous gene with the human orthologue has predictive value for testing genetic variants. As compared with the current benchmark in vitro assay, the in vivo cardiac assay correctly distinguished a pathogenic from a benign variant with high accuracy (positive predictive value 100%, negative predictive value 90%). T436M represented the only discrepant variant between the 2 assays.
5400 participants of a whole-exome sequencing project, 13 im-
ly-associated LQTS genetic variants were identified in 3% of
Moreover, the importance of functionally characterizing LQTS
cod-
hERG
tification and functionality. The Inherited Arrhythmia Database
is less laborious and high-throughput than the in vitro assays,
and thus, can bridge the knowledge gap between variant iden-
tifications. These results imply that, in addition to temperature,
there may be cell-specific mechanisms that influence traffick-
ing in vivo.
There are several limitations inherent in this study. The ze-
brafish is not a mammalian organism, and it remains unclear
that the behavior of the hERG1 mutants in zebrafish heart
fully recapitulates that in humans. Moreover, the functional
readouts from the zebrafish cardiac assay do not provide a
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The zebrafish system does not provide fine details of channel
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listed, the in vivo zebrafish cardiac assay does provide a yes/no
screen for the high-throughput evaluation of novel hERG1
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preting a positive LQTS genetic test, 14, 15 the zebrafish cardiac
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Sources of Funding
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Disclosures
None.

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Figure 3. Comparison of in vivo zebrafish cardiac assay
with in vitro mammalian cell assay. For each human ether-a-
go-go related gene [hERG] variant, the fraction of embryos with
normal repolarization is normalized to wild-type (WT) value and
plotted against fraction of WT current assayed in mammalian
cells, using published values. Cluster analysis identified 2 distinct
subsets that represent concordance between the assays to
distinguish pathogenic and benign variants. T474I fell outside
the 95% confidence interval (CI) ellipses (dotted gray lines) of the
2 clusters. Although T474I generates functional channels in vitro,
it was considered a pathogenic mutation based on altered kinetic
properties9 and was correctly identified as disease-causing by
the in vivo cardiac assay.

T436M was originally reported in a single LQTS individual
as a de novo variant11 and has not been subsequently reported
in the literature as either a rare polymorphism or an LQTS
mutation. Until additional genetic information is available, the
pathogenicity of this rare variant and the superiority of the
individual assay remain to be determined.

The in vivo cardiac assay provides several practical advan-
tages over the current gold-standard in vitro assays. Although
the in vitro assays reveal important biophysical characteristics
of the mutant channel, in silico modeling is required to infer
the consequences of altered channel gating/conductance on
cardiac action potential duration. By contrast, the in vivo ass-
say provides a functional readout in the form of normal/abnor-
mal repolarization. For example, although the abnormal gating
properties of the T474I variant were predicted to cause the
LQTS phenotype,12 the in vivo assay confirmed the abnormal
nature of cardiac repolarization. In addition, the in vivo assay
is less laborious and high-throughput than the in vitro assays,
and thus, can bridge the knowledge gap between variant iden-
tification and functionality. The Inherited Arrhythmia Database
(www.fsm.it/cardmod/) lists 335 nonsynonymous hERG cod-
ing variants, the vast majority of whose function is not known.
Moreover, the importance of functionally characterizing LQTS
variants is further underscored by recent report that previously-
related LQTS genetic variants were identified in 3% of
5400 participants of a whole-exome sequencing project,13 im-
plying a high false-positive designation as disease-causing.

Temperature is known to play an important role in protein
processing/trafficking in heterologous expression systems.
Lowering the incubation temperature rescues the cell surface
expression of several trafficking-deficient hERG1 mutations.
Zebrafish larvae were raised and repolarization was assayed
at 25°C to 28°C (whereas mammalian cells are incubated at
36°C). We were initially concerned that these temperature-
sensitive mutants might traffick and function normally in the
zebrafish assay. However, the temperature-sensitive mutants,
N470D and R752W, did not restore repolarization in the ze-
brafish cardiac assay, as expected for disease-causing
mutations. These results imply that, in addition to temperature,
there may be cell-specific mechanisms that influence traffick-
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Disclosures
None.

Novelty and Significance

What Is Known?
- A major obstacle to the interpretation of Long QT Syndrome (LQTS)—associated genetic variants is the lack of robust functional assays that determine the pathogenicity of the identified variants.
- Current functional assays using noncardiac heterologous expression systems are costly and labor-intensive.

What New Information Does This Article Contribute?
- The current study addresses a significant and increasing clinical problem with a straightforward assay, using the zebrafish as a model organism.
- The zebrafish cardiac assay approaches the accuracy of the current functional assays, and is far superior in terms of throughput rate.
- The zebrafish model provides practical advantages over noncardiac expression systems, including a functional readout of repolarization that is based on specific electrophysiological correlates.

Next-generation sequencing has revolutionized our ability to study the contribution of genetic variation to human disease. To keep pace with these advances, there is an urgent need for practical, functional assays that distinguish pathogenic from benign variants. The present work describes and validates an in vivo cardiac assay to determine the functional consequences of putative LQTS mutations, using the zebrafish model organism. Our findings support the proof-of-principle concept that replacing an endogenous gene with its human orthologue has predictive value for testing disease-causing gene variants. Together with emerging algorithms for interpreting a positive LQTS genetic test, our work provides a framework to bridge the gap between variant identification and disease risk.
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Supplemental Methods

Construction of hERG1 mutations, in vitro transcription of cRNA and morpholino design
WT hERG1 cDNA was previously subcloned into the pSP64 (Promega) plasmid expression vector. LQTS disease-causing mutations and known polymorphisms were introduced into WT hERG1 cDNA using QuickChange II Site-Directed Mutagenesis kit (Agilent Technologies, Santa Ana, CA). cRNA was prepared with SP6 Capscribe (Boehringer Mannheim) after linearization with BamHI. RNA concentrations were quantified by RiboGreen fluorescence assay and RNA quality was confirmed by gel electrophoresis. We used a MO oligonucleotide targeted against the translational start site of kcnh2 (National Center for Biotechnology Information sequence no. NM_212837) with the following sequence: 5'-CGCGTGGACAGATTCAAGAGCCCTC-3' (Gene Tools LLC, Philomath, OR). Note that the MO targets only the endogenous kcnh2 initiation site; injected hERG1 RNA is resistant to the MO as the construct contains a 5'-UTR beta-globin sequence.

hERG1 cRNA and morpholino injections
Kcnh2 ATG MO (0.8 ng) was injected alone or co-injected with WT or mutant hERG1 cRNA (350-400 pg, total volume 1 nl) in WT (AB strain) zebrafish embryos at 1-2 cell stage. In preliminary studies, we optimized the basic conditions of the MO-knockdown and hERG1 rescue to give us the greatest dynamic range to study the functional consequences of putative LQT2 mutations. Injections were repeated on 3 or more different days. Zebrafish embryos were raised at 28°C. Cardiac repolarization phenotypes of kcnh2-morphants were screened under light microscopy at 48 hour-post-fertilization (hpf) at 25°C. A minimum of 150 embryos was screened for each variant.

Action potential recordings from embryonic heart
Four-eight hpf larvae were dechorionated and anesthetized with 0.02% tricaine before dissecting the heart en bloc as described. Only spontaneously beating whole hearts were studied. All experiments were performed at 22°C to 24°C. The recording chamber was superfused with external control solution containing (in mmol/L) NaCl 140, KCl 4, CaCl2 1.8, MgCl2 1, glucose 10, and HEPES 10 (pH 7.4). Suction pipettes were made from borosilicate capillary tubes (8250 glass; A-M Systems) and fire-polished to obtain resistances of 6-9 MΩ when filled with solution containing (in mmol/L) KCl 120, EGTA 5, K2ATP 5, MgCl2 5, and HEPES 10 (pH 7.2). Vm was measured by using an AxoClamp 2A amplifier (Molecular Devices) in the bridge mode with the disrupted patch technique as described. Vm was filtered at 10 kHz and digitized at a sampling frequency of 20 kHz with a 12-bit analog-to-digital converter (Digidata 1322A Interface, Molecular Devices). Action potential duration (APD) from a series of five APs was calculated as the time interval between the peak maximum upstroke velocity (phase 0) and the time at 10% (APD10), 50% (APD50) and 90% of repolarization (APD90).

Statistical analysis
A Generalized Linear Mixed Model was performed to estimate the likelihood of a normal repolarization phenotype for the hERG1 variant, compared to the reference normal phenotype for WT hERG1. A random intercept was specified in the Generalized Linear Mixed Model to account for the variability of experiments administered at each unique day. Response variable was a binary outcome dichotomized as normal (1:1 AV conduction) vs abnormal (2:1 AV block or ventricular asystole) repolarization phenotype. The percentage of normal phenotype for each variant and 95% confidence interval were calculated using the binominal proportion method with the normal approximation assumption. Positive and negative predictive values were calculated to
assess the concordance between putative disease-causing hERG1 variants as determined using an in vitro assay (HEK 293 or CHO cells) and the in vivo zebrafish cardiac assay.

The purpose of cluster analysis is to place objects into groups such that objects in a given cluster tend to be similar to each other in some sense, and objects in different clusters tend to be dissimilar. The CLUSTER procedure in SAS hierarchically clusters the observations in a SAS data set by using one of 11 clustering methods. We used average linkage method in this dataset analysis. For a set of observations containing one or more quantitative variables and a classification variable defining groups of observations, the discriminant analysis develops a discriminant criterion to classify each observation into one of the groups. Because of the relatively small sample size, we used nonparametric normal kernel methods to estimate the group-specific densities. With the estimated group-specific densities and their associated prior probabilities, the posterior probability estimates of group membership for each class can be evaluated. Peak cubic clustering criterion (CCC) values ≥ 3 were considered to represent statistically relevant clusters.

The level of statistical significant was set at P <0.05. All analyses were performed using SAS 9.2 (SAS institution Inc., NC, USA).
### Online Supplemental Tables

**Online Table I.** Ventricular action potential parameters recorded from MO-injected embryos displaying normal (1:1 AV conduction) or abnormal (2:1 AVB, ventricular asystole) repolarization phenotypes.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal (n=4)</th>
<th>2:1 AVB (n=3, <em>p</em>-value)</th>
<th>Ventricular asystole (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CL</strong></td>
<td>1235 ± 459</td>
<td>856 ± 60.5 (0.47)</td>
<td>NA</td>
</tr>
<tr>
<td><strong>MDP</strong></td>
<td>-68.0 ± 1.0</td>
<td>-68.4 ± 1.7 (0.90)</td>
<td>-2.7 ± 4.1</td>
</tr>
<tr>
<td><strong>P_max</strong></td>
<td>5.9 ± 1.1</td>
<td>7.1 ± 1.2 (0.51)</td>
<td>NA</td>
</tr>
<tr>
<td><strong>APD_{10}</strong></td>
<td>149.7 ± 8.6</td>
<td>256.3 ± 8.6 (&lt;0.001)</td>
<td>NA</td>
</tr>
<tr>
<td><strong>APD_{50}</strong></td>
<td>241.8 ± 10.3</td>
<td>482.5 ± 14.9 (&lt;0.001)</td>
<td>NA</td>
</tr>
<tr>
<td><strong>APD_{90}</strong></td>
<td>275.0 ± 9.7</td>
<td>543.6 ± 14.7 (&lt;0.001)</td>
<td>NA</td>
</tr>
</tbody>
</table>

AVB: Atroventricular block; CL: cycle length (msec); MDP: maximum diastolic potential (mV); P_max: maximum upstroke velocity (mV/msec); AP_{10}, AP_{50} or AP_{90}: action potential duration to 10%, 50% or 90% of repolarization (msec). Values are mean ± SEM.
### Online Table II. Generalized linear mixed effect statistical model comparing repolarization rescue of hERG1 variants against that of WT hERG1 in kcnh2 MO-knockdown embryos.

<table>
<thead>
<tr>
<th>hERG1 variant</th>
<th>% normal repolarization</th>
<th>95%CI (+/-)</th>
<th>N</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MO alone</td>
<td>10.0</td>
<td>9.7</td>
<td>210</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>WT hERG</td>
<td>55.5</td>
<td>14.5</td>
<td>431</td>
<td>----</td>
</tr>
<tr>
<td>K28E</td>
<td>5.9</td>
<td>8.9</td>
<td>204</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>F29L</td>
<td>20.1</td>
<td>16.8</td>
<td>199</td>
<td>0.0042</td>
</tr>
<tr>
<td>I31S</td>
<td>15.3</td>
<td>15.9</td>
<td>294</td>
<td>0.0008</td>
</tr>
<tr>
<td>N33T</td>
<td>18.8</td>
<td>17.5</td>
<td>197</td>
<td>0.0066</td>
</tr>
<tr>
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Online Figure I. Electrophysiological effects of WT, polymorphism and LQT-2 hERG1 variants in kcnh2 MO-knockdown embryonic ventricle. A, APD90 measured in explanted embryonic ventricle is plotted against repolarization phenotype for kcnh2-MO knockdown embryos injected with WT, A1116V, A614V, N470D hERG1 RNA. Values for individual measurements and the mean ± SEM for each phenotype are plotted. Action potentials were absent in ventricles that displayed a ventricular asystole phenotype and are plotted above the dotted line as individual samples. B, Examples of $V_m$ recordings from explanted embryonic ventricle of MO-injected embryos that displayed normal 1:1 AV conduction (solid black), 2:1 AVB (dashed black), or ventricular asystole phenotypes (solid grey). These data support that notion that 2:1 AVB phenotype occurs as the result of APD prolongation, while ventricular asystole results from $V_m$ depolarization.
Online Figure II. Results from hierarchal cluster analysis of *in vivo* cardiac and *in vitro* assays.

Data from Figure 3 was subjected to hierarchal cluster analysis to group the values into 0-8 unique subsets to identify concordance between the 2 assays. Plotted are the cubic clustering criterion (CCC), Pseudo F and Pseudo T-squared values along the range of 0-8 clusters. The data was best fit by 2 distinct clusters as noted in the peak in the CCC plot. CCC values > 3 indicate the best fit. There is a local peak of the CCC (4.5) when the number of clusters is 2. The large pseudo F-statistic value suggests that the data is well fit into 2 clusters. In the context of the data presented in Figure 3, the 2 clusters represent concordance between the 2 assays to detect disease-causing and benign variants.
Online Figure III. The double mutant A490T-K897T rescues normal repolarization, while the single mutant LQT-2 mutant A490T is pathogenic. % of embryos manifesting normal repolarization (1:1 AV conduction) plotted for anti-\textit{kcnh2} MO-injected embryos alone (MO) and in combination with WT, K897T, A490T and A490T-K897T \textit{hERG1}. The common polymorphism K897T rescues repolarization even when combined with the LQT-2 mutant A490T (A490T-K897T). These data corroborate the clinical observation that patients who inherit A490T and K897T in cis orientation exhibit a less severe LQTS phenotype than subjects with A490T alone \textsuperscript{7}. Note that error bars represent +95% confidence intervals.