LQT2 Nonsense Mutations Generate Trafficking Defective N-Terminally

Truncated Channels by the Reinitiation of Translation

Matthew R. Stump, Qiuming Gong and Zhengfeng Zhou*

Knight Cardiovascular Institute, Oregon Health & Science University, Portland, Oregon 97239

Running Title: Truncation of hERG PAS domain associated with LQT2 mutations

*Corresponding Author: Dr. Zhengfeng Zhou

Knight Cardiovascular Institute
Oregon Health & Science University
3181 SW Sam Jackson Park Road
Portland, OR 97239
Phone: (503) 494-2713
Fax: (503) 418-9381
Email: zhouzh@ohsu.edu
The human ether-a-go-go related gene (hERG) encodes a voltage-activated K⁺ channel that contributes to the repolarization of the cardiac action potential. Long QT syndrome type 2 (LQT2) is an autosomal dominant disorder caused by mutations in hERG and patients with LQT2 are susceptible to severe ventricular arrhythmias. We previously showed that nonsense and frameshift LQT2 mutations caused a decrease in mutant mRNA by the nonsense-mediated mRNA decay (NMD) pathway. The Q81X nonsense mutation was recently found to be resistant to NMD. Translation of Q81X is reinitiated at Met124 resulting in the generation of N-terminally truncated hERG channels with altered gating properties. In the present study we identified two additional NMD-resistant LQT2 nonsense mutations, C39X and C44X, in which translation is reinitiated at Met60. The deletion of the first 59 residues of the channel truncated nearly one-third of the highly structured Per–Arnt–Sim (PAS) domain and resulted in the generation of trafficking defective proteins and a complete loss of hERG current. The partial deletion of the PAS domain also resulted in the accelerated degradation of the mutant channel proteins. The co-expression of mutant and wild-type channels did not significantly disrupt the function and trafficking properties of wild-type hERG. Our present findings indicate that translation reinitiation may generate trafficking defective as well as dysfunctional channels in patients with LQT2 premature termination codon mutations that occur early in the coding sequence.

Keywords: hERG, long QT syndrome, PAS domain, protein trafficking, potassium channels
INTRODUCTION

The rapidly activating delayed rectifier current ($I_{Kr}$) in the heart functions in the repolarization of the cardiac action potential (22). $I_{Kr}$ is generated by the hERG K$^+$ channel encoded by the human ether-a-go-go related gene (hERG) (21, 31). Each pore-forming subunit of the tetrameric channel is composed of six membrane-spanning subdomains, which include the voltage-sensing domain and channel pore, that are flanked by highly structured, functionally important domains in the cytosolic N- and C-termini. The N-terminus of the channel is responsible for maintaining the slow deactivation of the channel (24, 32). The crystal structure of the highly conserved residues 26–135 revealed a eukaryotic Per–Arnt–Sim (PAS) domain that directs protein–protein interactions between the hERG N-terminus and other regions of the channel (9, 15). The C-terminus of the channel contains a cyclic nucleotide binding domain tethered to the transmembrane domain by a helical C-linker region.

Mutations in hERG cause long QT syndrome type 2 (LQT2), an autosomal dominant disorder, characterized by prolonged action potential durations that may trigger severe arrhythmias and lead to syncope or sudden death (13, 23). Over 500 mutations have been identified to date in patients with LQT2. Missense mutations may give rise to non-functional channels, channels with trafficking defects and channels with abnormal gating properties (3, 20, 35). hERG transcripts containing premature termination codons (PTCs) introduced by LQT2 nonsense and frameshift mutations have been shown be eliminated by the nonsense-mediated mRNA decay (NMD) pathway (8, 33). NMD is protective against severe forms of LQT2 by preventing the translation of truncated proteins that may dominantly-suppress hERG current. Approximately one-third of the known LQT2 mutations are predicted to be targets of NMD. We recently reported on an NMD-resistant LQT2 nonsense mutation Q81X that generated N-
terminally truncated channels with abnormal functional properties (28). Analysis of the hERG sequence reveals one in-frame ATG codon upstream of Met124. We hypothesized that LQT2 PTC mutations occurring upstream of Met60 will be resistant to NMD by translation reinitiation and that the deletion of 59 residues from the N-terminus of the channel will have significant functional consequences.

In this study we characterized two LQT2 nonsense mutations, C39X and C44X, previously reported by Splawski et al. and Fodstad et al. (5, 27). We found that both mutations were resistant to NMD and were expressed as N-terminally truncated channel proteins. Following premature termination, translation of the mutant channels was reinitiated at Met60 resulting in the deletion of nearly one-third of the PAS domain. The truncated channels were trafficking defective and did not express hERG current. The mutant channels were rapidly degraded compared to wild-type hERG and the co-expression of mutant and wild-type channels did not prevent the trafficking or disrupt the functional properties of the wild-type channels. The present results provide evidence that translation reinitiation of hERG transcripts containing LQT2 PTC mutations generates trafficking defective as well as dysfunctional channel proteins. This study further defines the positional requirements of NMD susceptibility in PTC-containing hERG transcripts.

MATERIALS AND METHODS

hERG minigene and cDNA constructs. The hERG minigene is composed of cDNA from exon 1 to exon 10 and genomic DNA from intron 10 to the polyadenylation signal and was subcloned into the pcDNA5 vector as previously described (7). The LQT2 nonsense mutations C39X and C44X and valine substitutions at Met60, Met124, Met133 and Met137 were generated
using the pAlter mutagenesis system (Promega, Madison, WI). Wild-type and mutant minigenes were stably transfected into Flp-In HEK293 cells (Life Technologies, Grand Island, NY) using the Effectene method (Qiagen, Valencia, CA) as previously described (30). Flp-In HEK293 cells contain the Flp recombination target (FRT) site at a single genomic locus allowing the stable integration of the gene of interest at a single, specific location by Flp recombinase-mediated recombination. The C39X and C44X mutations were introduced into hERG cDNA constructs with an in-frame hemagglutinin (HA) epitope (YPYDVPDYA) inserted at the C-terminus. The wild-type hERG cDNA constructs contained an in-frame Flag epitope (DYKDDDDK) at the C-terminus. The design of the HA- and Flag-tagged hERG cDNA constructs has been previously described (6, 29). Wild-type and mutant cDNA constructs were stably co-transfected into Flp-Cre cells. Flp-Cre cells contain the FRT site and a single copy of the loxP2272/loxP target site. The design of the Flp-Cre cell line and the generation of double-stable Flp-Cre cell lines have been previously described (30). Briefly, Flag-tagged wild-type constructs were introduced into the loxP2272/loxP target site using Cre recombinase-mediated cassette exchange. The HA-tagged mutant constructs were then introduced into FRT target site by Flp recombinase-mediated recombination. Flp-In HEK293 and Flp-Cre cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37 °C in 5% CO₂. Stable cell lines were cultured in the presence of 10 μM E-4031 for 24 h to test the drug-induced rescue of hERG trafficking.

**RNase protection assay.** The isolation of RNA expressed from hERG minigenes and the RNase protection assay have been previously described (8). Briefly, a 409 nt probe was used to detect hERG RNA. The probe contained 277 nt specific to exons 12 and 13 and was flanked by sequences specific to the pCRII vector. A second 228 nt probe was used to detect RNA from the hygromycin B resistance gene and contained sequences from the pGEM vector at both ends. The
expression of RNA from the hygromycin B resistance gene was used as a loading control. The
probes were completely digested in the presence of yeast RNA. Relative quantitation of hERG
RNA was determined by densitometry using ImageJ (1). Cells were cultured in the presence or
absence of 100 µg/ml cycloheximide (CHX) for 3 h to inhibit protein synthesis and thus abrogate
NMD.

Electrophysiology. The recording of membrane currents in whole-cell configuration has
been previously described (35). Cells were superfused with a 10 mM HEPES-buffered solution
(pH 7.4) containing 1.8 mM CaCl₂, 4 mM KCl, 1 mM MgCl₂, 137 mM NaCl and 10 mM
glucose. A 10 mM HEPES-buffered solution (pH 7.2) containing 130 mM KCl, 1 mM MgCl₂, 5
mM MgATP and 5 mM EGTA was used as the pipette solution. Experiments were performed at
room temperature. Data was recorded using an Axopatch-200B patch clamp amplifier and
analyzed with pCLAMP10 software (Molecular Devices, Sunnyvale, CA).

Immunoblot. hERG channel proteins from whole cell lysates were detected using the anti-
hERG, anti-HA and anti-Flag antibody and visualized with Plus-ECL (PerkinElmer, Waltham,
MA) as previously described (29, 35). The expression levels of β-tubulin and hygromycin B
phosphotransferase (HPH) were used as loading controls as previously described (7).

Cycloheximide-chase assay. Wild-type and mutant cell lines were treated with 100 µg/ml
CHX and harvested after 0, 2, 4 and 8 h. hERG channel proteins from whole cell lysates were
detected by immunoblot using anti-hERG antibody. The relative intensity of the total hERG
protein was quantified by densitometry using ImageJ and normalized to the amount present at 0
h. The time at which a 50% decrease in the total amount of hERG protein occurred was reported
as the channel half-life. The expression of β-tubulin, which has a long half-life compared with
hERG, was used as a control.
Data analysis. Data were analyzed and presented as mean ± standard error of the mean (SEM) using SigmaPlot (San Jose, CA). Student’s t-test was used for statistical comparison involving two experimental groups. ANOVA with the Bonferroni correction was used for statistical comparison in tests containing more than two experimental groups. $P<0.05$ was considered statistically significant.

RESULTS

LQT2 nonsense mutations C39X and C44X are resistant to NMD. To test the susceptibility of LQT2 PTC mutations upstream of Met60 to NMD we introduced the C39X and C44X nonsense mutations into full-length hERG minigenes (Fig. 1A). Wild-type and mutant minigenes were stably transfected into Flp-In HEK293 cells. A stable cell line expressing the NMD-sensitive LQT2 mutation P141fs+2X was included as a control. Cells were cultured in the absence or presence of the protein synthesis inhibitor CHX, which has been shown to reverse NMD (11). The RNase protection assay was used to quantify the expression of hERG mRNA from wild-type and mutant minigenes. In the absence of CHX, wild-type, C39X and C44X RNA were expressed at similar levels (Fig. 1B). In contrast, the amount of RNA expressed from minigenes containing the P141fs+2X mutation was significantly reduced compared to wild-type ($P<0.01$). Wild-type and mutant RNA expression levels were normalized and quantified and revealed no significant differences between the wild-type, C39X and C44X minigenes ($P>0.05$, Fig. 1C). As previously described, treatment with CHX resulted in the increased expression of P141fs+2X RNA ($P<0.05$). These results indicated that hERG transcripts containing the C39X and the C44X mutations are not degraded by NMD.
C39X and C44X channels do not express hERG current. To test the functional properties of the mutant hERG channels we performed whole-cell patch clamp analysis. hERG current was recorded by activating the channels with test potentials ranging between −70 and +60 mV from a −80 mV resting potential followed with a −50 mV repolarizing pulse. Representative current traces are shown in Fig. 2A and the current-voltage relationship of tail current recorded at the onset of repolarization, is shown in Fig. 2B. Wild-type channels exhibited an averaged current density of 19.4 ± 1.3 pA/pF following depolarization to +30 mV (n = 12). In contrast, hERG current was not detected from cells stably transfected with C39X (n = 6) and C44X (n = 8) minigenes.

C39X and C44X are translated as truncated, trafficking deficient channels. To identify biochemical basis for the functional defects associated with the C39X and C44X mutations we analyzed the proteins expressed from wild-type and mutant minigenes by immunoblot (Fig. 3). hERG protein was detected using an antibody directed to the C-terminus of the channel. As previously shown, wild-type hERG was detected as two bands representing the immature, core-glycosylated channel located in the endoplasmic reticulum (135 kDa) and the fully-glycosylated mature form (155 kDa) expressed at the cell surface (35). In contrast, C39X and C44X channel proteins were detected as a single band at a slightly lower molecular weight than the immature wild-type channel protein (~130 kDa). This result suggested that the nonsense mutations resulted in the expression of truncated, trafficking defective channel proteins. Immunoblot analysis also revealed that the mutant minigenes expressed significantly less total protein compared to the wild-type minigene. Quantitative analysis revealed that total protein expressed from C39X and C44X minigenes were 28.5 ± 6.0% (n = 3) and 35.0 ± 9.0% (n = 3) of the total protein expressed from wild-type hERG (n = 3, P<0.05).
Translation of C44X channels is reinitiated at Met60. Translation reinitiation has been implicated in the NMD resistance of PTC mutations that occur early in the coding sequence of several genes including hERG (4, 18, 28). Analysis of the hERG sequence downstream of the C39X and C44X mutations revealed several in-frame start codons Met60, Met124, Met133 and Met137, which could potentially serve as the site of reinitiation. As shown in Fig. 4, mutating all four downstream methionine codons to valine resulted in the complete loss of C44X protein expression. This confirmed that the reinitiation of translation is associated with the NMD resistance of the PTC-containing transcripts. When Met124, Met133 and Met137 were mutated to valine we observed a single band at the same molecular weight as found in the C44X minigenes. This result strongly suggested that reinitiation of translation occurs at Met60 in C44X transcripts. Interestingly, we observed a faint band with a lower molecular weight expressed from minigenes containing the C44X and M60V mutations. This result indicated that in the absence of Met60, the downstream methionine codons Met124, Met133 and Met137 are able to reinitiate translation but with a significantly reduced efficiency, likely due to the increased distance between the C44X mutation and the downstream reinitiation site.

The C39X and C44X mutations do not significantly disrupt the function or trafficking of wild-type hERG. To determine whether C39X and C44X channels were able to dominantly disrupt the function and trafficking of wild-type channels Flp-Cre cells were stably co-transfected with either wild-type + empty vector, wild-type + C39X or wild-type + C44X cDNA constructs. hERG current was recorded using the voltage clamp protocol described in Figure 2. Representative current traces are shown in Fig. 5A and the plot of the averaged peak tail current densities is shown in Fig. 5B. The co-expression of the mutant and wild-type channels did not significantly decrease hERG current levels. The averaged peak tail current densities recorded
following depolarizing potentials to +30 mV from cells co-expressing wild-type + empty vector, wild-type + C39X and wild-type + C44X were 19.8 ± 2.3 pA/pF (n = 10), 16.6 ± 1.4 pA/pF (n = 11) and 19.0 ± 1.6 pA/pF (n = 10), respectively (P>0.05).

To determine the effect of the mutant channels on the trafficking of wild-type hERG we performed immunoblot analysis of proteins expressed from Flp-Cre cell lines co-transfected with Flag-tagged wild-type hERG and either empty vector, HA-tagged C39X or HA-tagged C44X. The expression of wild-type and mutant channels is shown in Fig. 6A. The trafficking efficiency of hERG was determined by the ratio of the upper, mature form of the band to the total hERG protein as detected by the anti-Flag antibody. As shown in Fig. 6B the mutant channels did not significantly decrease the trafficking efficiency of wild-type hERG. The trafficking efficiencies from cells co-expressing wild-type + empty vector, wild-type + C39X and wild-type + C44X were 73.1 ± 3.7% (n = 4), 62.7 ± 5.6% (n = 4) and 63.1 ± 4.0% (n = 4), respectively (P>0.05).

C44X channels are rapidly degraded and trafficking is not rescued by E-4031. The immunoblot studies revealed that the mutant channels were expressed at significantly lower levels than wild-type hERG. To determine whether the mutant channels exhibited increased rates of degradation we analyzed the expression of wild-type and mutant proteins by immunoblot following treatment with the protein synthesis inhibitor CHX for 0, 2, 4 and 8 h (Fig. 7A). Immunoblots were quantified by densitometry and the relative intensity of wild-type and C44X was plotted as a function of the total protein at 0 h (Fig. 7B). The housekeeping gene β-tubulin served as a relatively stable control protein. As shown in Fig. 7, the mutant channels exhibited accelerated rates of degradation compared to wild-type hERG. The half-lives of the wild-type and C44X channels were 5.3 ± 1.7 h (n = 4) and 1.3 ± 0.1 h (n = 4, P<0.05). We also compared the degradation rates of the differentially tagged wild-type and mutant channels co-expressed
from the Flp-Cre cell lines (Fig. 8). Co-expression with the HA-tagged mutant channel did not significantly reduce the half-life of Flag-tagged wild-type hERG (7.2 ± 2.4 h, n = 3) as compared to the co-expression of wild-type channels with empty vector (8.0 ± 1.0 h, n = 3, P>0.05). The half-life of the HA-tagged C44X channels co-expressed with Flag-tagged wild-type hERG (1.7 ± 0.7 h, n = 3) was similar to that found from the cell lines stably transfected with the C44X minigene shown in Fig. 7.

To further characterize the rapidly degraded mutant channels we determined whether or not the trafficking defects associated with the partial deletion of the PAS domain could be reversed. Studies have shown that the trafficking defects of mutant hERG channels can be restored by the pore-blocking drug E-4031 (2, 36). We cultured wild-type and mutant stable cell lines in media containing E-4031 for 24 h and found that the drug was unable to rescue the trafficking-defective phenotype of the mutant channels (Fig. 9). The rapid degradation of the mutant channel and the failure of E-4031 to rescue trafficking strongly suggested that the N-terminus of the C39X and C44X channels is significantly destabilized.

DISCUSSION

In the present study we identified two new NMD-resistant LQT2 nonsense mutations that were translated by reinitiation and generated non-functional N-terminally truncated channels. hERG channel dysfunction associated with the C39X and C44X nonsense mutations was characterized at the RNA, protein and functional levels. We identified Met60 as the site of translation reinitiation. The deletion of the first 59 residues of the channel resulted in the truncation of the PAS domain and in the expression of trafficking defective channels. This study establishes translation reinitiation as a pathogenic mechanism of LQT2 in patients PTC.
mutations that occur near the translation start site and that early nonsense and frameshift
mutations in hERG may give rise trafficking as well as functional defects.

NMD is an evolutionary conserved surveillance mechanism that eliminates PTC-containing transcripts and prevents the translation of potentially harmful C-terminally truncated proteins. According to the proposed rule of NMD in mammalian cells, PTCs occurring 50–55 nt upstream of the last exon-exon junction target the transcript for degradation (17). The activation of NMD is dependent on the interaction between the translation termination complex, formed at the site of the PTC, and NMD associated components of the exon-junction complex (EJC) (25). The EJC is a protein complex deposited by the spliceosome 20–24 nt upstream of the exon-exon junction following splicing and is normally displaced by the ribosome during the pioneer round of translation (12, 14). The EJC is displaced by the translating ribosome following reinitiation resulting in the NMD resistance of early PTC mutations. The elimination of putative reinitiation codons by mutagenesis is an established method to confirm NMD resistance by translation reinitiation (34). The dependence of NMD on protein translation and splicing necessitates the use of hERG minigenes in the biochemical and functional characterization of LQT2 nonsense and frameshift mutations. Our findings that translation of C39X and C44X is reinitiated at Met60 is in agreement with an in silico analysis of translation initiation sites using the NetStart 1.0 artificial neural network server (19). NetStart 1.0 predicts the likelihood of translation in the context of local and global sequence information, and scores between 0.5 and 1.0 are strong predictors for translation initiation. Met60 received a score of 0.78, which indicated that it is a strong candidate site for translation reinitiation.

The structures of the hERG PAS domain obtained by X-ray crystallography and NMR are well described by a helix-loop-helix architecture (15, 16). The PAS domain encompasses
residues 26–135 of the channel and is capped by a short amphipathic helix that linking the
domain to 12 disordered residues at the channel N-terminus (16). A well-conserved hydrophobic
patch on the surface of the PAS domain is proposed to mediate protein–protein interactions with
other regions of the channel (15). FRET studies have revealed the functional effects of the PAS
domain are mediated by physical interactions between the domain and the core of the channel
(9). Functional studies of N-terminally truncated channels have shown that deletions up to
residue 26 generate functional channels with accelerated deactivation rates (15, 32). Increased
deactivation rates were also observed in channels in which PAS domain (residues 1-135) is
deleted and in channels lacking the entire N-terminus (residues 1-354) (15, 26, 32). This is the
first description of a naturally occurring mutation that partially deletes the PAS domain and
generates N-terminally truncated, trafficking defective hERG channels. A recently study by
Harley et al. reported that hERG trafficking defects were correlated with the misfolding and
decreased stability of the isolated PAS domains containing LQT2 missense mutations (10). We
have previously reported trafficking defects associated with large deletions from highly
structured cytosolic channel domains. The LQT2 splice site mutation G2592+1G>A mutation
activates a cryptic splice site deleting 24 residues from the C-terminal cyclic nucleotide binding
domain resulting in trafficking defective channel proteins (29). It is likely that the partial deletion
of the hERG N-terminus in C39X and C44X channels disrupted the proper folding of the PAS
domain resulting in trafficking defective channels. The accelerated degradation rates and the
failure of the pore-binding drug E-4031 to rescue trafficking provide further evidence of the
significant misfolding the mutant channel proteins. The significantly decreased expression of the
C39X and C44X mutants compared to the wild-type channels and the rapid degradation of the
mutant channel proteins are also likely to be responsible for the minimal dominant-negative
effects observed in the co-expression studies.

We recently identified a NMD-resistant LQT2 mutation, Q81X, which was translated by
reinitiation at Met124 (28). In contrast to reinitiation at Met60, the deletion of the first 123
residues of the channel did not result in a trafficking defective phenotype. Reinitiation at Met124
deleted nearly all of the PAS domain residues and resulted in the expression of channels that
exhibited accelerated deactivation rates and decreased resurgent outward current during the late
stages of ventricular action potential repolarization. The previous study also found that Met133
and Met137 were competent to reinitiate translation following premature termination at the
Q81X PTC. Based on our previous study and our present findings we propose that the
reinitiation of LQT2 PTC mutations upstream of residue 60 will result in the translation of
trafficking defective channels and that the reinitiation of PTC mutations occurring between
residue 60 the methionine codons at residues 124, 133 and 137 will generate abnormal channels
with altered gating properties that are expressed at the plasma membrane. Because the
P141fs+2X mutation is sensitive to NMD it appears that Met137 represents a boundary in the 5’
region of the hERG coding sequence that separates NMD-resistant and NMD-sensitive LQT2
PTC mutations.

In summary, the identification of two new LQT2 mutations C39X and C44X that are
resistant to NMD establishes translation reinitiation as an important mechanism of disease in
patients with early PTC mutations in hERG. This study highlights the importance of the folding
and stability of the PAS domain in channel trafficking and contributes to the collective
understanding of mechanisms by which hERG mutations cause LQT2.
ACKNOWLEDGMENTS

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Fig. 1. Analysis of hERG mRNA by the RNase protection assay. (A) Schematic of the full-length hERG minigene. The position of the C39X, C44X and P141fs+2X mutations, the wild-type termination codon (TER) and the polyadenylation signal (pA) are indicated. (B) RNase protection assay. hERG mRNA was harvested from cells stably transfected with wild-type and mutant minigenes. A probe specific to a 277 nt sequences spanning exons 12 and 13 was used to hybridize to hERG mRNA. The stable cell lines were treated with CHX for three h prior to RNA isolation (+) or left untreated (−). RNA expressed from the hygromycin B resistance gene (Hygro) served as a loading control. (C) RPA signals were normalized to the hygromycin B resistance gene and plotted as the percentage of untreated wild-type, mean ± SEM (n = 3). **, $P<0.01$; *, $P<0.05$.

Fig. 2. Functional properties of wild-type and mutant hERG channels. (A) Representative currents from cells stably transfected with wild-type and mutant minigenes. hERG current was activated from a $-80$ mV holding potential with 4 s depolarizing test potentials between $-70$ and $+60$ mV and deactivated with a repolarizing pulse to $-50$ mV. (B) Current−voltage plot of tail current of wild-type (circles, n = 12), C39X (squares, n = 6) and C44X (diamonds, n = 8) current. Tail current was recorded at $-50$ mV following each depolarizing pulse. Data are plotted as mean ± SEM.

Fig. 3. Immunoblot of wild-type, C39X and C44X channels. Proteins from whole cell lysates of cells expressing wild-type and mutant minigenes were detected using anti-hERG and anti-HPH
antibody. HPH levels served as a loading control. The mature, fully-glycosylated wild-type channel is detected at 155 kDa and the immature, core-glycosylated channel is detected at 135 kDa. Results shown are representative of three independent experiments.

**Fig. 4.** Translation of C44X channels is reinitiated at Met60. hERG channel proteins from whole-cell lysates from cells stably transfected with wild-type, C44X and C44X plus methionine to valine mutations. Proteins were detected with anti-hERG and anti-HPH antibody. HPH expression levels served as a loading control. Results shown are representative of three independent experiments.

**Fig. 5.** Analysis of hERG current following co-expression of wild-type and mutant channels. 

(A) Representative currents from Flp-Cre cells stably co-transfected with WT + empty vector, WT + C39X, or WT + C44X. Current was recorded using the protocol described in the legend of Fig. 2.

(B) Plot of the averaged tail current amplitudes measured at −50 mV following depolarizing voltages to +30 mV. Data are plotted as mean ± SEM. The number of cells is shown in parentheses.

**Fig. 6.** Co-expression with mutant channels does not alter the trafficking properties of wild-type hERG. (A) Flp-Cre cells were stably co-transfected with WT-Flag + empty vector, WT-Flag + C39X-HA or WT-Flag + C44X-HA. hERG channels were detected using anti-Flag and anti-HA antibody. β-tubulin served as a loading control. (B) The trafficking efficiency of the Flag-tagged wild-type channels was plotted as the ratio of upper, mature band to the total hERG protein. The data are plotted as mean ± SEM (n=3).
Fig. 7. Characterization of the stability of wild-type and mutant channels. (A) Immunoblots of proteins harvested from cells stably transfected with wild-type and C44X minigenes. Protein synthesis was inhibited by treating the cells with 100 µg/ml cycloheximide (CHX). Cells were harvested at 0, 2, 4 and 8 h following CHX treatment. Proteins were detected with hERG and β-tubulin antibody. (B) The amount of total hERG protein was quantified using ImageJ and plotted as the percentage of the total protein at 0 h. Data are plotted as mean ± SEM, wild-type (circles, n = 4), C44X (diamonds, n = 4).

Fig. 8. Effect of co-expression of mutant and wild-type channels on the stability of hERG channel proteins. (A) Immunoblots of proteins harvested from Flp-Cre cells stably co-transfected with Flag-tagged wild-type + empty vector or Flag-tagged wild-type + HA-tagged C44X. Cells were treated with 100 µg/ml CHX and harvested after 0, 2, 4 and 8 h. hERG channels were detected using anti-Flag and anti-HA antibody. β-tubulin served as a loading control. (B) The amount of total Flag-tagged or HA-tagged protein detected was quantified using ImageJ and plotted as the percentage of the total protein at 0 h. Data are plotted as mean ± SEM. WT-Flag + Vector, detected by anti-Flag (circles, n = 3), WT-Flag + C44X-HA, detected by anti-Flag (diamonds, n = 3); WT-Flag + C44X-HA, detected by anti-HA (squares, n = 3).

Fig. 9. Trafficking of C39X and C44X channels is not rescued by the hERG channel-blocking drug E-4031. Representative immunoblot of proteins harvested from cells stably transfected with wild-type, C39X and C44X minigenes and detected using anti-hERG C-terminal and anti-HPH
antibody. Cells were incubated in the absence or presence of 10 µM E-4031 for 24 h prior to harvest. Results shown are representative of three independent experiments.
Figure 1.
Figure 2.

A

WT

C39X

C44X

B

Tail Current (pA/pF)

Membrane Potential (mV)

-80 -60 -40 -20 0 20 40 60

0 5 10 15 20

WT

C39X

C44X
Figure 3.
Figure 4.

[Image of a gel electrophoresis with bands at 155 kDa and 135 kDa labeled with hERG and HPH]
Figure 5.

A

WT + Vector

WT + C39X

WT + C44X

B

Tail Current (pA/pF)

WT + Vector

WT + C39X

WT + C44X

(10) (11) (10)
Figure 6.

A

Flag-Tagged
HA-Tagged

WT
C39X
C44X

Flag

155 kDa
135 kDa

HA

130 kDa

β-tubulin

B

Trafficcking Efficiency

0
20
40
60
80
100

WT +
Vector
WT +
C39X
WT +
C44X
Figure 7.

A

WT

0  2  4  8

h, CHX

hERG

155 kDa

135 kDa

β-tubulin

C44X

0  2  4  8

h, CHX

hERG

130 kDa

β-tubulin

B

Relative Density (% at 0 h)

0  2  4  6  8

Time (h)

WT

C44X

0  2  4  6  8

Relative Density (% at 0 h)
Figure 8.

A

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h, CHX
Flag

155 kDa
135 kDa

β-tubulin

155 kDa
135 kDa

HA

130 kDa

β-tubulin

B

- WT-Flag + Vector, Flag
- WT-Flag + C44X-HA, Flag
- WT-Flag + C44X-HA, HA

Relative Density (% at 0 h)

0 20 40 60 80 100

Time (h)

0 2 4 6 8
Figure 9.

WT | C39X | C44X | E-4031
---|------|------|------
-  | -    | -    | -    | hERG
+  | +    | +    | +    |

155 kDa
135 kDa

HPH