LQT2 nonsense mutations generate trafficking defective NH$_2$-terminally truncated channels by the reinitiation of translation

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Stump MR, Gong Q, Zhou Z. LQT2 nonsense mutations generate trafficking defective NH$_2$-terminally truncated channels by the reinitiation of translation. Am J Physiol Heart Circ Physiol 305: H1397–H1404, 2013. First published August 30, 2013; doi:10.1152/ajpheart.00304.2013.—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 have previously shown 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 Met$^{124}$, resulting in the generation of NH$_2$-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 Met$^{60}$. Deletion of the first 59 residues of the channel truncated nearly one-third of the highly structured Per-Arnt-Sim domain and resulted in the generation of trafficking-defective proteins and a complete loss of hERG current. Partial deletion of the Per-Arnt-Sim domain also resulted in the accelerated degradation of the mutant channel proteins. The coexpression 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.

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 nonfunctional 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 to 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 (28) have recently reported on an NMD-resistant LQT2 nonsense mutation, Q81X, that generated NH$_2$-terminally truncated channels with abnormal functional properties. Analysis of the hERG sequence reveals one in-frame ATG codon upstream of Met$^{124}$. We hypothesized that LQT2 PTC mutations occurring upstream of Met$^{60}$ will be resistant to NMD by translation reinitiation and that the deletion of 59 residues from the NH$_2$-terminus of the channel will have significant functional consequences.

In this study, we characterized two LQT2 nonsense mutations, C39X and C44X, previously reported by Sławiński et al. (27) and Fodstad et al. (5). We found that both mutations were resistant to NMD and were expressed as NH$_2$-terminally truncated channel proteins. After premature termination, translation of the mutant channels was reinitiated at Met$^{60}$, 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 with wild-type hERG, and the coexpression 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 showing 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). LQT2 nonsense mutations C39X and C44X and valine substitutions at Met$^{60}$, Met$^{124}$, Met$^{133}$, and Met$^{137}$ were generated using the pAlter mutagenesis system (Promega, Madison, WI). Wild-type and mutant minigenes were stably transfected into Flp-In human embryonic kidney (HEK)-

THE RAPIDLY ACTIVATING delayed rectifier K$^+$ current ($I_{K_C}$) in the heart functions in the repolarization of the cardiac action potential (22). $I_{K_C}$ is generated by the human ether-a-go-go-related gene (hERG) K$^+$ channel encoded by 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 NH$_2$- and COOH-termini. The NH$_2$-terminus of the channel is responsible for maintaining the slow deactivation of the channel (24, 32). The crystal structure of highly conserved residues 26–135 revealed a eukaryotic Per-Arnt-Sim (PAS) domain that directs protein-protein interactions between the hERG NH$_2$-terminus and other regions of the channel (9, 15). The COOH-terminus of the channel contains a cyclic nucleotide-binding domain tethered to the transmembrane domain by a helical COOH-linker region.

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293 cells (Life Technologies, Grand Island, NY) using the Effectene method (Qiagen, Valencia, CA) as previously described (30). Flip-In HEK-293 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. C39X and C44X mutations were introduced into hERG cDNA constructs with an in-frame hemagglutinin (HA) epitope (YPYDVPDYA) inserted at the COOH-terminus. Wild-type hERG cDNA constructs contained an in-frame Flag epitope (DYKDDDDK) at the COOH-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 cotransfected into Flp-Cre cells. Flip-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 Flip-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. HA-tagged mutant constructs were then introduced into the FRT target site by Flp recombinase-mediated recombination. Flip-In HEK-293 and Flip-Cre cells were cultured in DMEM supplemented with 10% FBS at 37°C in 5% CO₂. Stable cell lines were cultured in the presence of 10 μM 4-031 for 24 h to test the drug-induced rescue of hERG trafficking.

**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 Flip-In HEK-293 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 with wild-type RNA (P < 0.01). Wild-type and mutant RNA expression levels were normalized and quantified and revealed no significant differences between wild-type, C39X, and C44X minigenes (P > 0.05; Fig. 1C). As previously described, treatment with CHX resulted in increased expression of P141fs+2X RNA (P < 0.05). These results indicated that hERG transcripts containing the C39X and C44X mutations are not degraded by NMD.

![Image](http://alpahjphysiology.org/10.21033/en/2017/612.0x792.0)
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 holding potential followed by 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 after 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 the 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 analysis (Fig. 3). hERG protein was detected using an antibody directed to the COOH-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 with the wild-type minigene. Quantitative analysis revealed that total proteins 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, that 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 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 Met\textsuperscript{124}, Met\textsuperscript{133}, and Met\textsuperscript{137} 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.

**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 cotransfected with either wild-type + empty vector, wild-type + C39X, or wild-type + C44X cDNA constructs. hERG current was recorded using the voltage-clamp protocol shown in Fig. 2. Representative current traces are shown in Fig. 5A, and a plot of the averaged peak tail current densities is shown in Fig. 5B. Coexpression of the mutant and wild-type channels did not significantly decrease hERG current levels. The averaged peak tail current densities recorded after depolarizing potentials to +30 mV from cells coexpressing 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).

![Fig. 5. Analysis of hERG current after coexpression of WT and mutant channels.](image)

**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 cotransfected 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 band to total hERG protein. Data are plotted as means ± SE; n = 3.

**C44X channels are rapidly degraded and trafficking is not rescued by E-4031.** The immunoblot analyses 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 analysis after 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 with 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 coexpressed from the Flp-Cre cell lines (Fig. 8). Coexpression 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) compared with coexpression 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 coexpressed 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 (2, 36) have shown that the trafficking defects of mutant hERG channels can be restored by the pore-blocking drug E-4031. 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 NH₂-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 nonfunctional NH2-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 the expression of trafficking-defective channels. This study establishes translation reinitiation as a pathogenic mechanism of LQT2 in patients with 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 COOH-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 after splicing and is normally displaced by the ribosome during the pioneer round of translation (12, 14). The EJC is displaced by the translating ribosome after 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 amphiphilic helix that links the domain to 12 disordered residues at the channel NH2-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). A fluorescence resonance energy transfer study (9) has revealed that the functional effects of the PAS domain are mediated by physical interactions between the domain and the core of the channel. Functional studies (15, 32) of NH2-terminally truncated channels have shown that deletions up to residue 26 generate functional channels with accelerated deactivation rates. Increased deactivation rates were also observed in channels in which the PAS domain (residues 1–135) is deleted and in channels lacking the entire NH2-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 NH2-terminally truncated, trafficking-defective hERG channels. A recent study by Harley et al. (10) reported that hERG trafficking defects were correlated with the misfolding and decreased stability of isolated PAS domains containing LQT2 missense mutations. 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 COOH-terminus of the channel (11). A cyclic nucleotide-binding domain, resulting in trafficking-defective channel proteins (29). It is likely that the partial deletion of the hERG NH2-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 with 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 coexpression experiments.

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 regurgitant 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 after 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 residues 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 Met\textsuperscript{37} 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 transcriptional sequence that separates NMD-resistant and NMD-sensitive LQT2 PTC mutations.

**REFERENCES**

**AUTHOR CONTRIBUTIONS**

Author contributions: M.R.S., Q.G., and Z.Z. conception and design of experiments; M.R.S. drafted manuscript; M.R.S. and Z.Z. edited and revised manuscript.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

