Published in final edited form as:
Heart Rhythm. 2014 June ; 11(6): 1055–1062. doi:10.1016/j.hrthm.2014.03.002.

A Novel Trafficking-defective *HCN4* Mutation is Associated with Early-Onset Atrial Fibrillation

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Author Contributions V.M. performed site-directed mutagenesis, tissue culture, DNA transfection, cellular electrophysiology experiments, data analysis, created figures and wrote the paper. S.N.M., M.L.Z., M.F.S., M.M., and M.A.S. collected AF and referent samples, carried out HRM screening and sequencing. E.V.D. made the tagged constructs and performed immunocytochemistry. N.R.T. imaged constructs using confocal microscopy. K.L.N. and E.J.B. for statistical and sequencing analysis. D.J.M., E.J.B. and P.T.E. contributed resources. P.T.E. supervised and wrote the paper. All authors edited, reviewed and approved the paper.

Disclosures None.

Conflict of interests: None.

Abstract

Background—Atrial fibrillation (AF) is the most common arrhythmia, and a recent genome-wide association study identified *HCN4* as a novel AF susceptibility locus. *HCN4* encodes for the cardiac pacemaker channel and *HCN4* mutations are associated with familial sinus bradycardia and AF.

Objective—To determine whether novel variants in the coding region of *HCN4* contribute to the susceptibility for AF.

Methods—We sequenced the coding region of *HCN4* for novel variants from 527 cases with early-onset AF from the Massachusetts General Hospital AF Study and 443 referents from the Framingham Heart Study. We used site-directed mutagenesis, cellular electrophysiology, immunocytochemistry and confocal microscopy to functionally characterize novel variants.

Results—We found the frequency of novel coding *HCN4* variants was 2-fold greater for individuals with AF (seven variants) compared to the referents (three variants). We determined that one, (p.Pro257Ser, located in the amino-terminus adjacent to the first transmembrane spanning domain) of the seven novel *HCN4* variants in our AF cases did not traffick to cell membrane while the remaining six were not functionally different from wild type. Also, the three novel variants in our referents did not alter function compared to wild type. Co-expression studies showed that the p.Pro257Ser mutant channel failed to co-localize with the wild type HCN4 channel on the cell membrane.

Conclusion—Our findings are consistent with *HCN4* haploinsufficiency as the likely mechanism for early-onset AF in the p.Pro257Ser carrier.

Keywords

HCN4; mutation; atrial fibrillation; electrophysiology

Introduction

In recent years, compelling evidence has emerged from epidemiological studies to suggest that atrial fibrillation (AF) is a heritable disease[1]. The advent of genome wide association studies (GWAS) has considerably enhanced our ability to identify common genetic variants underlying complex traits such as AF. In a recent meta-analysis of GWAS, we identified six novel genetic loci for AF[2]. At one of the loci, on chromosome 15q24, the risk variants are intronic to the hyperpolarization-activated cyclic nucleotide-gated 4 (*HCN4*) gene. *HCN4* encodes for the cardiac HCN4 channel and represents a compelling candidate gene for AF.

The HCN4 channel is abundantly expressed in the sinoatrial (SA) node and cardiac conduction tissue, and underlies the funny current (I_f). I_f is an inward current that contributes to spontaneous pacemaking during the diastolic depolarization phase of the SA node action potential[3]. Several previous studies have reported an association between loss of function mutations in the *HCN4* channel and familial sinus node dysfunction and sinus bradycardia[4-8]. The association between sinus node dysfunction and AF is well recognized[9,10]. Interestingly, dominant negative mutations in the *HCN4* channel have been previously associated with familial bradycardia and AF[5,7,11].

Therefore, we sought to determine if genetic variation in the coding region of the *HCN4* gene is associated with AF. We identified seven novel HCN4 variants in our cohort with early-onset AF and three novel HCN4 variants in our referent population. The variants in the referents had no functional effect. One (p.Pro257Ser) of the novel AF variants had defective trafficking to the cell membrane. Co-expression of the p.Pro257Ser mutant did not alter the activity of the wild type HCN4 channel, consistent with haploinsuffiency as the likely mechanism of disease.

Methods

Study Samples

Cases included unrelated individuals with early-onset AF from the Massachusetts General Hospital (MGH) AF Study. Early-onset AF was defined as a first occurrence of AF at less than 66 years of age, with no history of myocardial infarction, heart failure or structural heart disease as assessed by echocardiography. Referent individuals were drawn from the Framingham Heart Study (FHS) and had no history or electrocardiographic evidence of AF on serial examinations. The baseline characteristics of both study populations are shown in Table 1. Written informed consent for genetic research was obtained from each individual and the Institutional Review Boards at MGH and FHS approved the study.

Screening and Mutagenesis

The coding region of *HCN4*, which consists of 8 exons and 3612 base pairs, was screened using a combination of high resolution melting and Sanger sequencing. Oligonucleotide primers were designed using the genomic sequence from the University of California, Santa Cruz (UCSC) Genome Browser (hg19 assembly).

High resolution melting was performed using the Light Scanner technology (Idaho Technologies) following the manufacturer's recommendations. All variants identified by high resolution melting were confirmed using standard Sanger sequencing. Exons 2-6 of the *HCN4* gene were screened using high resolution melting whereas the remaining exons (1, 7 and 8) were sequenced directly using Sanger sequencing.

Nonsynonymous variants that had not been reported previously in publically available databases (dbSNP, 1000 Genomes Project, exome variant server), and hence were deemed to be novel, were prioritized for further analysis. Common nonsynonymous and synonymous variants with a mean allele frequency >1% are reported in the Supplementary Information. Each novel rare variant was introduced into the human *HCN4* clone (OpenBiosystems) using the QuikChange mutagenesis kit (Stratagene, Inc.) with appropriate mutagenic primers. For immunocytochemisty and confocal microscopy studies of co-expressed wild type and p.Pro257Ser mutant channel, the constructs were tagged with C-terminal epitopes for myc and V5 using pcDNA4/myc-His and pcDNA4/V5-His (Invitrogen). All constructs were followed by confirmatory sequencing of the entire clone.

Cell Culture and Electrophysiology

Chinese Hamster Ovary (CHO) cells were transfected with 2 μg of wild type or variant *HCN4* with 0.6 μg of GFP using Fugene (Promega) according to the manufacturer's instructions. For co-expression experiments, 1 μg of HCN4 and 1 μg of p.Pro257Ser with 0.6 μg GFP were used. GFP positive cells were used for whole-cell patch clamp experiments 1-2 days post transfection. The extracellular solution used contained, 135 mM KCl, 5 mM NaCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 5 mM HEPES, pH 7.4 with KOH; intracellular solution: 130 mM potassium aspartate, 10 mM NaCl, 0.5 mM $MgCl_2$, 1 mM EGTA, and 5 mM HEPES, pH 7.4 with KOH. Signals were amplified using an Axon 200B amplifier, digitized with a Digidata 1322A A/D converter and analyzed with Clampex 9.2, Clampfit 9.2 (Molecular Devices, Inc.) and SigmaPlot 9.0 (Systat Software Inc.). Normalized current-voltage (I-V) relationships were determined from tail currents elicited at -35 mV for 2 s following 4 to 20 s test pulses ranging from 150 to +10 mV in 20 mV steps. Single tail current test pulses were followed by a 0.5 s pulse to +5 mV to ensure complete channel closure. The normalized I-V relationships were fitted with a Boltzmann, $I/I_{max} = I_{min} + I_{max}$-$I_{min}/(1 + \exp\text{-}(V\text{-}V_{1/2}/k))$, to determine the midpoint of activation ($V_{1/2}$) and slope (k). Current density was determined by dividing the fully-activated current at -150 mV at the end of the 4 second pulse by cell capacitance. Significant differences in $V_{1/2}$, k and current densities between the wild type and variant channels were determined using a *t*-test ($p < 0.05$).

Immunocytochemistry and Confocal Microscopy

CHO cells were grown on 12 mm coverslips and transfected with 2 μg of wild type or variant *HCN4*. For co-expression studies 1 μg was used for wild type and variant HCN4 channels. Thirty-six hours after transfection, the cells were rinsed with PBS, fixed in 4% cold paraformaldehyde, blocked in 10% horse serum and immunolabeled with primary rabbit anti-HCN4 (Alomone labs) and Alexa546-conjugated secondary antibody (Invitrogen). For co-expression studies with C-terminal tagged wild HCN4 channels and p.Pro257Ser mutant channel, primary rabbit anti-V5 (SigmaAldrich) and Alexa488-conjugated secondary goat antibody (invitrogen) were used or primary mouse anti-myc (Origene) and Alexa546-conjugated secondary goat antibody were used. Coverslips were mounted onto glass slides using Vectashield containing DAPI. The confocal micrographs were acquired on a Zeiss LSM510 system (Carl Zeiss MicroImaging, Inc.). Results were representative of those obtained from three independent preparations.

Results

Clinical Characteristics of Study Subjects

We screened for genetic variation in *HCN4* among 527 unrelated early-onset AF cases from the MGH AF Study and 443 AF-free referents from FHS. In the MGH AF cases the mean age of onset of AF was 47.2 ± 10.9 years and the mean age at enrollment was 54.2 ± 10.5 years. As has been observed previously, the majority of early-onset AF cases were men with a 4:1 ratio of men to women. Among early-onset AF cases, 40.9% had a positive family history for AF. In the referent participants from FHS, the mean age was 66.0 ± 9.2 years and 51.2% were female. Detailed baseline characteristics are presented in Table 1.

Identification of Novel HCN4 Variants

A combination of high resolution melting and direct sequencing was used to screen the coding region of *HCN4* for variants (see methods). We identified seven novel variants in the AF cases. Two of the variants were located in the N-terminus (p.Asp89Arg and p.Pro257Ser) and five in the distal C-terminus (p.Thr822Met, p.Gly885Arg, p.Pro945Ser, p.Gly1077Ser and p.Glu1193Gln) beyond the cyclic nucleotide-binding domain (CNBD) (Figure 1A, red circles). Clinical characteristics for AF variant carriers are presented in Supplementary Table 1. In FHS referents, we identified three novel variants, all of which were located in the carboxyl-terminus of *HCN4*. Specifically, p.Asn688Ser was located in the distal end of the B-helix of the CNBD and p.Ala1045Val and p.Arg1068His were located beyond the CNBD (Figure 1B, blue circles). There was no significant difference in the frequency of common variants (with a minor allele frequency >1%) between the MGH AF cases and FHS referents (Supplementary table 2).

Conservation of Novel HCN4 Variants

In order to determine evolutionary conservation of amino acid identity for each of the novel variants we performed a multiple alignment using ClustalX2.0.10 (Supplementary Figure 1; species: human, mouse, rabbit and zebrafish). Of the seven variants identified in the AF cases, two residues, p.Lys189Arg, p.Pro257Ser (N-terminus) shared complete identity. Of the three variants identified in the FHS referents, one residue (p.Asn688Ser), which is located in the B-helix of the CNBD, shared complete identity. We also calculated phyloP and SIFT scores for all the variants.[12,13] The phyloP analysis did not observe significant differences in the scores between the variants from MGH-AF and FHS; however, the SIFT prediction analysis identified the p.Pro257Ser MGH-AF variant as a “not tolerated substitution” whereas it identified the other MGH-AF and FHS variants as “tolerated substitutions” (Supplementary Table 3).

Electrophysiological Properties of the HCN4 Variants in AF cases and controls

To assess the functional effects of the eight novel variants identified in the AF cases, we individually over-expressed wild type and variant HCN4 channels in CHO cells and performed whole-cell patch clamp electrophysiology. Example current recordings of wild type HCN4 and two variants, p.Lys189Arg and p.Gly1077Ser, channels are shown in Figure 2A. Activation curves for wild type HCN4, p.Lys189Arg and p.Gly1077Ser are shown in Figure 2B. The curves through the data points were fitted with a Boltzmann function to determine the midpoint ($V_{1/2}$) and slope (k). The $V_{1/2}$ and slope for p.Lys189Arg and p.Gly1077Ser were not significantly different from wild type HCN4 (Fig. 2B, C and D, Table 2). We performed the same analysis with the other variants that expressed robust currents and also found no difference in $V_{1/2}$ and k compared to wild type HCN4. The current densities of the six variants that produced currents also were not significantly different from wild type HCN4 (Table 2). The p.Pro257Ser variant channel resulted in the lack of measurable current at the fully-activated potential of -150 mV (see Figure 3A). We saw no significant differences in HCN4 channel function in the three variants identified in the FHS referents (Figure 4 and Table 2).

The p.Pro257Ser disrupts trafficking to the cell membrane

The absence of a measurable current at the fully-activated potential of -150 mV for the p.Pro257Ser mutant channel (Figure 3A) could be explained by either an inhibition on channel opening or current flow, or by the disruption of normal channel trafficking to the cell membrane. Using immunocytochemistry and confocal microscopy, we demonstrated that, unlike wild type HCN4, the p.Pro257Ser mutant channel was undetectable at the plasma membrane, and was instead retained in the cytoplasm (Figure 3B). These findings suggest that the lack of current is due to the failure of the p.Pro257Ser mutant channel to traffick to the cell membrane.

Co-Expression Studies are Consistent with Haploinsufficiency as a Potential Mechanism for AF

To recapitulate the heterozygous condition with the p.Pro257Ser loss of function mutation, we co-expressed wild type HCN4 channel with the p.Pro257Ser mutant channel, and performed the identical whole-cell electrophysiology experiments as for single-copy recordings described above. Representative current recordings are shown in Figure 5A for wild type HCN4 alone (2 μg); wild type HCN4 (1 μg) and p.Pro257Ser (1 μg). Current density measurements at -150 mV for wild type HCN4+p.Pro257Ser were not significantly different from wild type HCN4 alone (Figure 5B, Table 2). The activation properties ($V_{1/2}$ and k) for wild type HCN4+p.Pro257Ser were also not significantly different from wild type HCN4 alone (Fig. 5C, Table 2). These findings suggest that the p.Pro257Ser mutant channel may lead to AF due to haploinsufficiency with only the single wild type allele contributing to the production of the HCN4 channel protein and current.

To further delineate this hypothesis, we made wild type HCN4 and p.Pro257Ser constructs which had unique C-terminal tagged epitopes in order to visualize the distribution of co-expressed wild type and mutant HCN4 channels in CHO cells using immunocytochemistry and confocal microscopy. Here we show that co-expression of 1 μg wild type HCN4-myc (green) and 1 μg wild type HCN4-V5 (red) were distributed together within the cytoplasm and on the cell membrane (Fig. 5Di). The wild type HCN4-myc and wild type HCN4-V5 expressed currents with similar functional properties as the untagged wild type HCN4 currents (Supplementary Figure 2). However, co-expression of 1 μg wild type HCN4-myc (green) with 1 μg p.Pro257Ser-V5 (red) showed differential cellular distribution. The wild type HCN4-myc was found within the cytoplasm and on the cell membrane while the p.Pro257Ser-V5 was only localized within the cytoplasm and not on the cell membrane (Fig. 5Dii). These data demonstrate that the wild type HCN4 channel does not co-localize with the p.Pro257Ser mutant channel at the cell membrane.

Clinical Features of the p.Pro257Ser Mutation Carrier

Individual AF-22 with the p.Pro257Ser mutation is currently 65 years of age. He was diagnosed with paroxysmal AF at age 29 years that became permanent AF at 43 years of age. His father had AF diagnosed at 63 years of age and he died of a myocardial infarction at 85 years of age; there is no other family history of AF or conduction system disease. Since enrollment twelve years ago he has remained in asymptomatic AF for which he has been rate controlled and anticoagulated. Recent twenty-four hour Holter monitoring showed

baseline atrial fibrillation with a mean ventricular response of 71 bpm (range 52-115). There were 1167 premature ventricular contractions (PVCs) versus aberrantly conducted beats along with 73 pauses of >2.0 seconds, the longest of which was 2.4 seconds (Figure 6).

Discussion

In the present study, we compared the frequency of rare variants in *HCN4* between 527 early-onset AF cases and 443 referent individuals. We found a variant in the early-onset AF cases (p.Pro257Ser) that resulted in a loss of channel function as a result of defective trafficking to the cell membrane.

The loss of function mutation, p.Pro257Ser, resulted in the absence of measurable current at fully-activated potentials because the mutant channels did not express on the cell membrane. The p.Pro257Ser mutation is located at the boundary of the N-terminus and S1 of the HCN4 channel. The proximal end of the N-terminus is highly conserved among the four mammalian HCN channel isoforms and has been previously shown to be necessary for co-assembly and trafficking to the cell membrane in HCN2 channels [14,15]. Our results further highlight and give insight into the functional and physiological importance of this conserved region of the N-terminus in HCN channels, since we discovered that a single point mutation in the HCN4 channel in an individual with idiopathic atrial fibrillation completely disrupted trafficking to the cell membrane.

Interestingly, all previous reports on HCN4 channel loss of function mutations in patients with sinus node dysfunction, bradycardia, chronotropic incompetence, and AF produced dominant negative effects on HCN4 channel function[4,5,7,11]. In contrast, our results support haploinsufficiency as a potential mechanism of disease. We showed that co-expression of equal amounts of the p.Pro257Ser mutant channel with the wild type HCN4 channel did not reduce current density or change the activation properties in CHO cells. Furthermore, confocal microscopy showed that p.Pro257Ser mutant channels did not co-localize on the cell membrane with wild type HCN4 channels. Our findings suggest the lack of heteromeric alpha-subunit interaction between the wild type and trafficking-defective p.Pro257Ser mutant channel at the cell membrane. This most likely occurred because the p.Pro257Ser mutation prevented co-assembly and/or disrupted folding of heteromeric wild type/mutant channels. Haploinsufficiency as a mechanism of disease due to loss of function mutations have been previously reported for other cardiac and neuronal ion channels[16-18]. Whereas haploinsufficiency appears as a plausible mechanism, we acknowledge that we did not have access to cardiac tissue to directly demonstrate a reduction in overall HCN4 protein and current in the affected individual.

AF is associated with sinus node dysfunction and prolongation of atrial conduction time[19]. HCN4 is the most abundant HCN isoform expressed in the human SA node and the atria[20,21]. A reduction in the HCN4 current could potentially lead to diminished action potential firing frequency. A possible consequence of suppressed electrical activity may be the development of arrhythmogenic delayed afterdepolarizations which could trigger AF. In isolated atrial myocytes from AF patients, delayed afterdepolarizations have been shown to be caused by an enhanced Ca^{2+} leak out of the sarcoplasmic reticulum through the

ryanodine receptor channel producing a large inward current carried by the Na^{+}-Ca^{2+} exchanger[22]. Therefore, it is plausible that a reduction of HCN4 current and enhanced Ca^{2+} leak together could produce a substrate for AF in the p.Pro257Ser mutation carrier.

It would be interesting to perform an electrophysiologic study to investigate sinoatrial nodal function in our p.Pro257Ser mutation carrier, but such a study is currently not clinically indicated. Furthermore, it would have been informative to determine if the p.Pro257Ser mutation segregated with AF. Unfortunately the proband's father was deceased and there were no other relatives available with AF in the family.

In recent years we have come to appreciate that there is a background rate of genetic variation or apparent "mutations" in genes that may not contribute to disease. This challenge has been exemplified by the natural variation identified in genes associated with arrhythmogenic right ventricular cardiomyopathy and long QT syndrome[23,24]. Our results for AF are in keeping with these prior observations as we identified apparent mutations in our control populations without AF and apparent mutations in our cases that resulted in normal channel function. Strengths of our study include the sequencing of a large control population and the electrophysiological characterization of all novel variants identified in cases and controls. As large-scale exome or genome sequencing becomes increasingly common, our results highlight the continued need for the functional evaluation of apparently pathological variants.

Conclusion

We have identified a novel trafficking-defective mutation in the amino-terminus of the HCN4 channel in a large cohort of individuals with early-onset AF. Our findings provide support that novel loss of function mutations in the HCN4 channel may increase susceptibility and have a role in the pathogenesis of AF. Future research will be necessary to further understand the mechanistic link between altered HCN4 channel function and the development of a proarrhythmic substrate in the atrium.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The current work was funded by NIH grants to Dr. Ellinor (1RO1HL104156, 1K24HL105780), Drs. Benjamin and Ellinor (1RO1HL092577). Dr. Ellinor is supported by an Established Investigator Award from the American Heart Association (13EIA14220013). Dr. Tucker was supported by award number T32HL007208 from the National Heart, Lung, And Blood Institute. Dr. Macri is supported by the Heart Rhythm Society (The Max Schaldach Fellowship in Cardiac Pacing and Electrophysiology). Thanks to Dr. Alexander A. Shiskin for help with sequencing reactions.

Abbreviations

AF	Atrial fibrillation
CNBD	Cyclic nucleotide binding domain

FHS	Framingham Heart Study
GWAS	Genome-wide association study
HCN4	Hyperpolarization-activated cyclic nucleotide-gated channel 4
k	Slope
MGH	Massachusetts General Hospital
SA	Sinoatrial
$V_{1/2}$	Midpoint of activation

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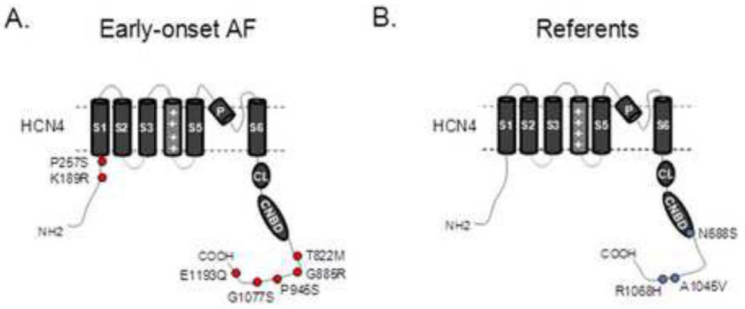

Figure 1. Location of novel HCN4 coding variants in early-onset AF cases and referents

A & B, illustration of a HCN4 -subunit with the cytoplasmic NH2- and COOH-terminus, six transmembrane segments (S1–S6), including the S4 voltage sensor ('+' sign denotes amino acid residues with positive charge), the pore loop between S5 and S6, and the C-linker (CL) with the cyclic-nucleotide binding domain (CNBD). The red (seven) and blue (three) circles denote the location of the novel variants identified in the early-onset AF cases and referents, respectively.

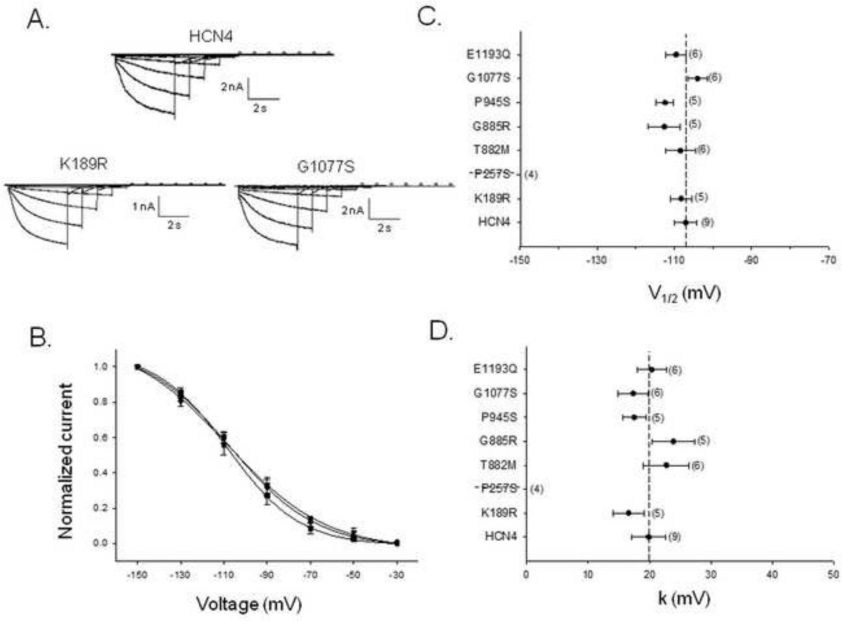

Figure 2. Functional characterization of the seven novel HCN4 variants identified in early-onset AF cases

A, current recordings of wild type HCN4, p.Lys189Arg and p.Gly1077Ser channels (see methods). B, plots of activation curves for wild type HCN4, p.Lys189Arg and p.Gly1077Ser. C & D, plots of $V_{1/2}$ and k for wild type HCN4 and the seven variants. The dashed black line through p.Pro257Ser denotes the lack of measureable current (see figure 3). The $V_{1/2}$ and k for the six variants expressing current were not significantly different from wild type (t-test, $p > 0.05$). The numbers in the parentheses represent the number of cells.

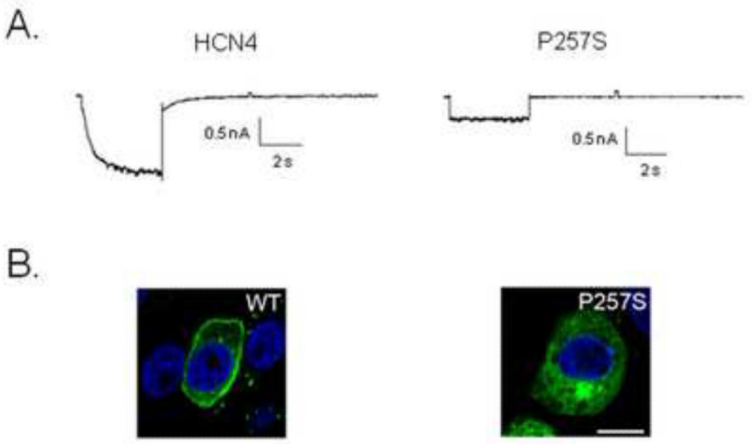

Figure 3. The p.Pro257Ser mutant channel does not produce a measurable current and does not express on the cell membrane

A, Currents were elicited from a holding current of -35 mV to a test pulse of -150 mV (fully-activated voltage) for 4 seconds and returned back to the holding current. The wild type HCN4 channel produced a current and the p.Pro257Ser mutant did not. B, confocal micrographs of wild type HCN4 and p.Pro257Ser channels expressed in CHO cells. The cells were stained with rabbit anti-HCN4 antibody (green) and DAPI (blue). The wild type HCN4 channel is expressed on the cell membrane and in the cytoplasm, whereas the p.Pro257Ser mutant channel is restricted to the cytoplasm. The scale bar denotes 50μm.

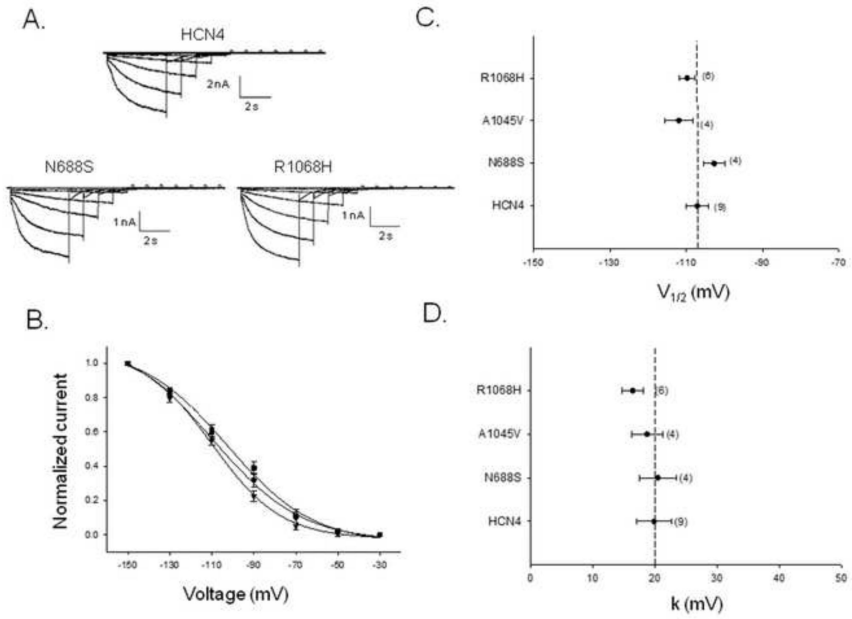

Figure 4. The three novel HCN4 variants identified in the referents do not alter function compared to the wild type HCN4 channel

A, current recordings of wild type HCN4, p.Asn688Ser and p.Arg1068His channels. B, plots of activation curves for wild type HCN4, p.Asn688Ser and p.Arg1068His channels. C & D, plots of $V_{1/2}$ and k for wild type HCN4 and the three mutants. The $V_{1/2}$ and k for the three mutants were not significantly different from wild type (t-test, $p > 0.05$). The numbers in parentheses represent the number of analyzed cells.

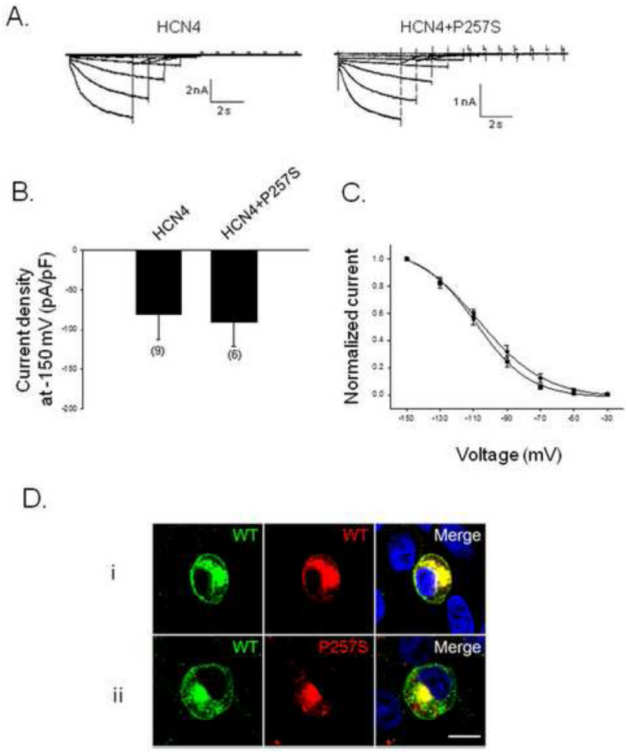

Figure 5. Co-expression of the wild type HCN4 channel the p.Pro257Ser mutant channel produce currents that are not functionally different from wild type

A, current recordings of wild type HCN4 (2 μg) and wild type HCN4 (1 μg)+p.Pro257Ser (1 μg). B, plot of current density (pA/pF) measured at -150 mV for wild type HCN4 and wild type HCN4+p.Pro257Ser. C, plots of activation curves for wild type HCN4 and wild type HCN4+p.Pro257Ser. The number in parentheses represents the number of cells. D, confocal micrographs of co-expressed wild type HCN4 and p.Pro257Ser constructs tagged with unique C-terminal epitopes in CHO cells (see methods); i) wild type HCN4-myc (green)+ wild type HCN4-V5 (red) and ii) wild type HCN4-myc (green)+p.Pro257Ser-V5(red). Co-expressed wild type HCN4-myc and wild type HCN4-V5 channels both traffick and are distributed together on cell membrane. ii) wild type HCN4-myc +p.Pro257Ser-V5 images show the wild type HCN4-myc channel expressed on the cell membrane and the p.Pro257Ser-V5 channel distributed in the cytoplasm and not on the cell membrane. Cells were also stained with DAPI (blue) to visualize the nucleus which is shown in the merged images. The scale bar denotes 50μm.

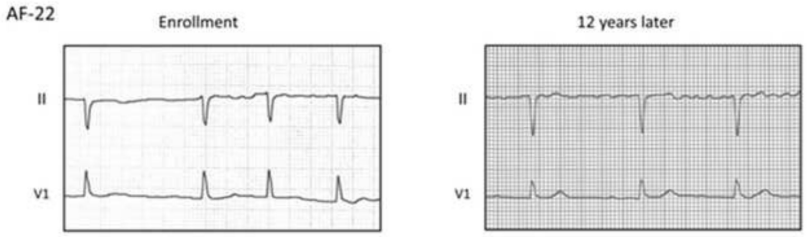

Figure 6. ECG recordings from AF-22 who carries the HCN4 trafficking-defective p.Pro257Ser mutation

Surface ECG of leads II and V1 from AF-22. AF-22 presents with the absence of p-waves indicative of AF and conduction pauses at the time of enrollment and remains in AF 12 years later.

Table 1

Baseline characteristics of Early-onset AF cases and referents.

Study	MGH AF cases	FHS referents
Affection status	Early-onset AF	No AF
Number	527	443
Female	103 (19.5)	227 (51.2)
Age, years	54.2 ± 10.5	66.0 ± 9.2
Age, onset AF	47.2 ± 10.9	NA
FH of AF	211 (40.9)	176 (39.7)
HTN	163 (30.9)	259 (58.6)
CHF	16 (3.2)	14(3.2)
Diabetes	26 (5.0)	57(13.0)
BMI	28.2 ± 5.3	28.3 ± 5.1

Values are n (%) or mean ± SD. Abbreviations: BMI, body mass index; CHF, congestive heart failure; FH, family history; HTN, hypertension; NA, not applicable.

Table 2

Electrophysiology properties of wild type and variant HCN4 channels.

		$V_{1/2}$ (mV)	k (mV)	Cells, n	pA/pF at −150 mV	Cells, n
Wild type	HCN4	−107.1 ± 2.9	19.9 ± 2.8	9	−81.5 ± 31.5	9
AF cases	p.Lys189Arg	−108.2 ± 2.8	16.6 ± 2.6	5	−111.7 ± 39.5	5
	p.Pro257Ser	n.e.	n.e.	4	n.e.	4
	p.Thr822Met	−108.5 ± 3.9	22.7 ± 3.7	6	−119.0 ± 46.6	6
	p.Gly885Arg	−112.6 ± 4.1	23.9 ± 3.5	5	−132.6 ± 35.6	5
	p.Pro945Ser	−112.4 ± 2.3	17.5 ± 1.9	5	−84.1 ± 24.8	5
	p.Gly1077Ser	−104.0 ± 2.5	17.3 ± 2.4	6	−167.8 ± 45.9	6
	p.Glu1193Gln	−109.6 ± 2.6	20.3 ± 2.4	6	−82.8 ± 19.2	6
Referents	p.Asn688Ser	−102.7 ± 2.8	20.5 ± 2.9	4	−64.0 ± 24.6	4
	p.Ala1045Val	−111.9 ± 3.8	18.7 ± 2.5	4	−102.9 ± 64.2	5
	p.Arg1068His	−109.8 ± 2.0	16.4 ± 1.8	6	−191.3 ± 60.6	6
Co-expression	HCN4+p.Pro257 Ser	−108.5 ± 2.3	16.1 ± 2.1	6	−91.2 ± 29.7	6

n.e.,no cell membrane expression.