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Kv1.5 channelopathy due to *KCNA5* loss-of-function mutation causes human atrial fibrillation

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Atrial fibrillation is a rhythm disorder characterized by chaotic electrical activity of cardiac atria. Predisposing to stroke and heart failure, this common condition is increasingly recognized as a heritable disorder. To identify genetic defects conferring disease susceptibility, patients with idiopathic atrial fibrillation, lacking traditional risk factors, were evaluated. Genomic DNA scanning revealed a nonsense mutation in *KCNA5* that encodes Kv1.5, a voltage-gated potassium channel expressed in human atria. The heterozygous E375X mutation, present in a familial case of atrial fibrillation and absent in 540 unrelated control individuals, introduced a premature stop codon disrupting the Kv1.5 channel protein. The truncation eliminated the S4–S6 voltage sensor, pore region and C-terminus, preserving the N-terminus and S1–S3 transmembrane domains that secure tetrameric subunit assembly. Heterologously expressed recombinant E375X mutant failed to generate the ultrarapid delayed rectifier current I_{Kur} vital for atrial repolarization and exerted a dominant-negative effect on wild-type current. Loss of channel function translated into action potential prolongation and early after-depolarization in human atrial myocytes, increasing vulnerability to stress-provoked triggered activity. The pathogenic link between compromised Kv1.5 function and susceptibility to atrial fibrillation was verified, at the organism level, in a murine model. Rescue of the genetic defect was achieved by aminoglycoside-induced translational read-through of the E375X premature stop codon, restoring channel function. This first report of Kv1.5 loss-of-function channelopathy establishes *KCNA5* mutation as a novel risk factor for repolarization deficiency and atrial fibrillation.

INTRODUCTION

Synchronized activation of atria underlies proper heart function, with normal excitation–contraction coupling reliant upon a balance of depolarizing and repolarizing ionic currents. During lifetime, there is a 25% risk for the development of atrial fibrillation, a condition of electrical chaos within the cardiac atria and a growing public health epidemic (1–3). This rhythm disorder increases stroke risk 5-fold, exacerbates heart failure and doubles mortality (4). Prevalent in the aging population, atrial fibrillation is typically viewed as an acquired disorder attributable to structural heart disease in patients with co-morbidities including coronary artery and mitral valve diseases, hypertension, or thyroid dysfunction (4). However, recent recognition of familial aggregation has increasingly implicated a heritable basis for atrial fibrillation (5–13). A primary genetic defect for this disorder is particularly likely in familial cases of early-onset lone atrial fibrillation. These patients with apparently normal hearts and without traditional risk factors (14–16) provide an opportunity to define molecular mechanisms for disease in the absence of confounding variables.

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The prevailing conceptual model for atrial fibrillation describes reduced electrical refractoriness as a substrate for re-entrant arrhythmia (17). This paradigm is supported by reports of gain-of-function mutations in genes encoding cardiac I_{Ks} (*KCNQ1*/*KCNE2*) and I_{K1} (*KCNJ2*) channels predicted to decrease action potential duration (10–12). Such understanding provides a therapeutic rationale for prolonging the atrial refractory period; yet this approach is not universally effective and can lead to pro-arrhythmia in patients with atrial fibrillation (2,4,17). Atrial fibrillation may in fact occur without action potential shortening, implicating alternative pathogenic etiologies and treatment strategies (18,19).

Here, we report a previously unrecognized genetic risk factor for disease in a case of early-onset lone atrial fibrillation refractory to conventional therapy. The mutation was identified in *KCNA5*, a conserved gene that encodes the voltage-gated Kv1.5 potassium channel (20–22). The rationale for targeting *KCNA5* as a candidate gene was based on its tissue-specific functional expression in human atrium, where Kv1.5 underlies the ultrarapid delayed rectifier I_{Kur} current vital for atrial repolarization (23). Moreover, Kv1.5 contributes to electrical homeostasis under adrenergic stress and is down-regulated in atrial fibrillation (24,25). The *KCNA5* mutation identified herein disrupted the structure and function of Kv1.5 conferring susceptibility to atrial action potential prolongation and early after-depolarizations, defining thereby a novel molecular mechanism for human atrial fibrillation.

RESULTS

KCNA5 mutation in lone atrial fibrillation

In a cohort of individuals with idiopathic atrial fibrillation, scans for *KCNA5* mutations in genomic DNA identified a unique sequence variant in a familial case. The proband developed symptoms of recurrent palpitation in childhood and was diagnosed with lone atrial fibrillation at age 35 (Fig. 1A). Paroxysms of atrial fibrillation worsened over time, increasing in frequency to multiple daily occurrences. Rhythm control was refractory to the conventional anti-arrhythmics, flecainide, sotalol and atenolol with sodium channel, potassium channel and beta-adrenoceptor blocking properties. Optimal medication dosing was limited by bradycardia, necessitating pacemaker implantation. Breakthrough episodes of atrial fibrillation occurred despite further treatment with amiodarone, prompting radiofrequency ablation to eliminate arrhythmogenic triggers. However, symptomatic paroxysmal atrial fibrillation recurred shortly after the procedure and remained refractory to pharmacotherapy including addition of dofetilide, a new generation potassium channel blocker. Family history was positive for early-onset atrial fibrillation (Fig. 1B). Sequencing of the genetic variant identified in the proband (Fig. 1C) revealed a heterozygous nonsense mutation (1123G $\rightarrow$ T) in exon 4 of *KCNA5*, causing a premature stop codon at residue 375 (E375X) (Fig. 1D). The E375X mutation was inherited by each of the proband's siblings who provided a sample for genetic analysis, one with documented atrial fibrillation and the other with paroxysms of rapid palpitations consistent with the paroxysmal nature of the disease (Fig. 1B). The E375X mutation was not found in 540 unrelated control DNA samples, comprising 1080 chromosomes from a cross-section of the population.

E375X disrupts Kv1.5 channel structure and function

The E375X mutation truncated the *KCNA5*-encoded Kv1.5 channel protein, retaining the N-terminus and S1–S3 transmembrane domains that secure the specificity and stability of interaction between channel subunits (26), but eliminating the S4–S6 voltage sensor and pore regions as well as the C-terminus (Fig. 1E). Heterologous expression of wild-type (WT) *KCNA5* yielded a full-length protein, with mutant construct generating a truncated form, both detected with the N-terminal Kv1.5 antibody (Fig. 1F). WT Kv1.5 channels, reconstituted in human HEK293 cells, displayed vigorous and instantaneous generation of outward K^+-driven current, with a tail typical of the ultrarapid delayed rectifier I_{Kur} current (Fig. 2A). Recombinant E375X mutant, despite active biogenesis and membrane trafficking (Fig. 1F), failed to generate current (Fig. 2A). At +60 mV of membrane potential, 0.26 ± 0.09 nA/pF was recorded from cells expressing WT Kv1.5 (0.4 μg/ml, $n = 8$), in contrast to 0.03 ± 0.01 nA/pF in E375X-expressing cells (0.4 μg/ml, $n = 9$; $P < 0.01$) indistinguishable from current density of untransfected cells (0.02 ± 0.01 nA/pF, $n = 5$; $P =$ NS). Thus, the expression of the identified nonsense *KCNA5* mutation translated into Kv1.5 loss-of-function.

E375X exerts a dominant-negative effect on Kv1.5 current

Functional Kv1.5 channels are formed by the tetramerization of individual subunits (26), a requirement for the quaternary pore structure as modeled (Fig. 2B) on the crystal structure of the prototypic voltage-gated KvAP channel (27). Within the multimeric complex, the E375X mutation disrupted the conduit and regulator of ion permeation, yet preserved S1–S3 transmembrane domains sufficient for subunit assembly (26). Indeed, the co-expression ($n = 9$) of mutant E375X (0.4 μg/ml) with WT Kv1.5 (0.4 μg/ml) significantly reduced current density at each positive voltage, leading to an overall 62% reduction in net outward current when compared with the equivalent expression of WT alone (0.4 μg/ml) (Fig. 2C). These data indicate that the mutant protein would co-assemble with WT subunits, exerting a dominant-negative effect on Kv1.5 current in the heterozygous state.

Kv1.5 deficit predisposes to atrial fibrillation

Deficit in Kv1.5 was simulated by channel blockade with 4-aminopyridine (Fig. 3A), which at 50 μM concentration is a selective probe of native I_{Kur} current (21). Human atrial myocytes, serving as their own controls, responded with prolongation of action potential duration. Measured at 60% repolarization, action potential duration significantly increased from 44 ± 9 to 79 ± 15 ms ($P < 0.01$, $n = 6$) (Fig. 3B). The observed action potential prolongation was associated with a propensity for early after-depolarizations, episodic oscillations in membrane potential that disrupted normal repolarization

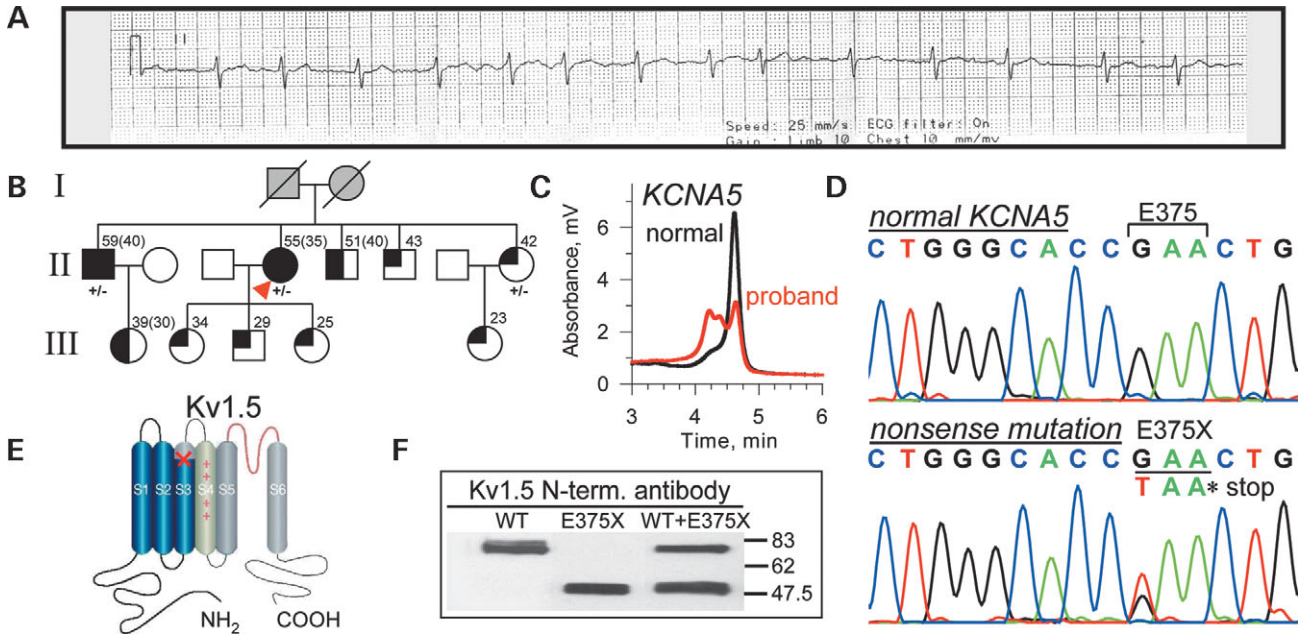

Figure 1. *KCNA5* mutation identified in human atrial fibrillation truncates the Kv1.5 channel protein. (**A**) Electrocardiographic tracing from the proband showed irregular heart rhythm with rapid low-amplitude oscillations between ventricular beats reflecting chaotic atrial electrical activity. (**B**) Family history suggested a hereditary basis for arrhythmia. Besides the proband (arrowhead), lone atrial fibrillation was also diagnosed in the older brother (full-shading) and, by history, in a younger brother and the oldest niece (half-shading). Palpitations consistent with paroxysmal disease were present in two younger siblings, all three children and the youngest niece (quarter-shading). Current ages are shown, with the age at diagnosis in parentheses. Heterozygosity for the E375X mutation (+/−) was identified in the three family members who provided a DNA sample. Other family members did not provide DNA. (**C**) Mutation scans of *KCNA5* by heteroduplex analysis revealed an anomalous chromatographic tracing in the proband indicative of an abnormal allele. (**D**) DNA sequencing in the proband and her two siblings identified a heterozygous nonsense mutation (1123G → T) causing a premature stop codon at residue 375 (E375X). (**E**) E375X (red X) mapped to the S3 transmembrane domain of Kv1.5. The voltage sensor (S4), S5, pore and S6 domains (semi-transparent) remained untranslated. (**F**) Western blot of membranes purified from HEK293 cells transfected with WT or mutant (E375X) Kv1.5 cDNA, alone or in combination (WT + E375X), showed the full-length (∼72 kDa) and truncated (∼48 kDa) protein distinguished by an N-terminal antibody.

(Fig. 3B and C). Under adrenergic challenge to stress electrical stability, Kv1.5 current deficit rendered human atrial myocytes further susceptible to pathological excitability, resulting in an 80% incidence of after-depolarizations and a 50% frequency of abnormally generated action potentials, i.e. triggered activity (Fig. 3B and C), absent in atrial cells with operational Kv1.5 channels (Fig. 3C). The pathogenic link between compromised Kv1.5 function and susceptibility to atrial fibrillation was verified at the whole organism level, in a murine model. Disrupting normal baseline sinus rhythm, 4-aminopyridine provoked premature atrial complexes, providing a substrate for atrial fibrillation following isoproterenol challenge (Fig. 3D), a pro-arrhythmic outcome not observed in the absence of Kv1.5 blockade (data not shown). These experimental findings validate the vulnerability to adrenergic stress observed in the E375X mutation-carrying patient, in whom isoproterenol infusion induced irregular atrial discharges degenerating into overt atrial fibrillation (Fig. 3E). Defective Kv1.5 channels thus render atria prone to electrical instability and arrhythmogenesis both *in vitro* and *in vivo*.

E375X rescue restores Kv1.5 current

The E375X mutation prevented the translation of the full-length Kv1.5 protein by a premature stop codon interrupting the reading frame of *KCNA5*. Rejection of functional pairing of the stop codon with a near-cognate anticodon can however be overridden by the aminoglycoside antibiotic-mediated alteration of the proofreading process, allowing translational read-through (28) (Fig. 4A). In HEK293 cells expressing E375X, the prototype aminoglycoside gentamicin induced a protein with a molecular mass identical to WT Kv1.5, detected by the immunoprobing of purified plasma membranes with a C-terminal Kv1.5 antibody incapable of recognizing the truncated form (Fig. 4B). Conversion of the E375X Kv1.5 mutant into a full-length protein, following gentamicin treatment, generated an increased density of current with properties similar to those of the WT (Fig. 4B), rescuing the underlying defect.

DISCUSSION

Diseases resulting from impaired ion channel function, channelopathies, are increasingly recognized in human pathology and cardiovascular medicine (29). Here, we identified Kv1.5 channelopathy as a novel genetic basis for idiopathic atrial fibrillation. The demonstrated *KCNA5* nonsense mutation disrupted the atrial-specific Kv1.5 channel providing a novel molecular substrate for electrical instability and susceptibility to atrial fibrillation.

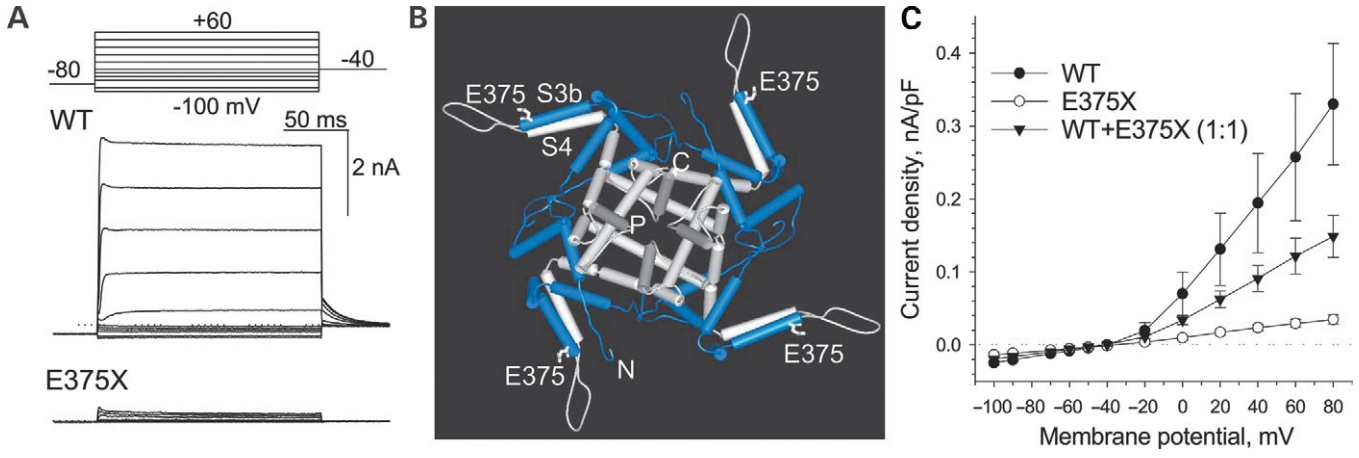

Figure 2. Loss-of-function E375X mutation exerts a dominant-negative effect on Kv1.5 function. (**A**) WT but not mutant E375X Kv1.5 demonstrated outward-rectifying potassium current at multiple membrane potentials upon expression in HEK293 cells. Clamped at −80 mV, cells were stimulated with a rectangular stepwise protocol (top) from −100 to +60 mV to induce voltage-dependent current and back to −40 mV to elicit current inactivation. (**B**) Atomic model of the Kv1.5 tetramer shows in gray the S4–S6 transmembrane/C-terminal domains eliminated by E375X. S3b–S4, voltage-sensor paddle unit; P, pore; C/N, carboxy/amino-terminus. (**C**) Voltage–current relationship demonstrated that co-expression of E375X with WT significantly reduced the Kv1.5-mediated outward current density when compared with WT alone. WT and E375X cDNA, alone or in combination, were transfected at 0.4 μg/ml of culture media.

The *KCNA5* mutation was absent in 540 unrelated control individuals, in line with the reported limited variability in the coding region of this gene (22) and ruling out this nonsense mutation as a common polymorphism in the population at large. Association with disease susceptibility was underscored *in vitro* and *in vivo*. First, the E375X truncation mutation disrupted Kv1.5 structure, cleaving the S4–S6 pore-forming domains from the protein. Secondly, functional hemizygosity, a potentially silent condition, was excluded by demonstrating active membrane trafficking and a dominant-negative effect of the mutant protein. Thirdly, deficit in Kv1.5, underlying the atrial repolarizing I_{Kur} current (23), increased susceptibility to arrhythmogenic after-depolarization and stress-provoked triggered activity. Finally, the electrophysiological substrate for atrial fibrillation in carriers of the E375X mutation was recapitulated at the cellular and whole organism levels by pharmacological channel blockade simulating the Kv1.5 loss-of-function, underscoring thereby the requirement of operational Kv1.5 channels for adequate repolarization reserve. Moreover, the molecular defect was corrected by aminoglycoside-induced translational read-through suppressing the dominant-negative effect of the mutant protein on channel function, a therapeutic approach recently translated into targeted treatment of disease conditions caused by nonsense mutations (30).

To date, atrial fibrillation has been linked to gain-of-function ion channel mutations favoring excessive potassium current generation and action potential shortening (10–12), albeit mechanistic heterogeneity has been proposed (18,19). The Kv1.5 loss-of-function mutation discovered herein identifies atrial action potential prolongation as a predisposing factor for disease. Analogous to the long QT syndrome substrate for chaotic polymorphic ventricular tachyarrhythmia, torsades de pointes (29,31), the Kv1.5 channelopathy resulted in atrial repolarization deficit and after-depolarizations triggering 'atrial torsades', a novel mechanism for atrial fibrillation. The recent identification of non-synonymous genetic variants in *KCNA5* in the population (22,32), in fact, raises the possibility of a broader role for Kv1.5 in arrhythmia susceptibility.

MATERIALS AND METHODS

Mutational analysis

With approval from the Mayo Clinic Institutional Review Board and following informed consent, a cohort of 154 unrelated individuals predominantly of European descent with idiopathic atrial fibrillation, including the proband, and a population-based cohort of 540 control individuals were scanned for mutations in *KCNA5*. Primers for PCR amplification were designed using the OLIGO v6.51 Primer Analysis Software (National Biosciences) and the WAVEMAKER 4.0.32 Software (Transgenomic) (13,33). Sequence variants in PCR-amplified DNA fragments were identified by denaturing high-performance liquid chromatography heteroduplex analysis (WAVE DNA Fragment Analysis System, Transgenomic). Fragments that formed heteroduplexes were amplified by PCR and sequenced.

Structural modeling

The crystal structure of the voltage-gated potassium channel, KvAP (PDB code 1ORQ) (27) served as a template to construct an atomic model of Kv1.5 as a monomer using the homology modeling program Swiss Model (www.expasy.org). Assembled as a tetramer, the 3D Kv1.5 model was refined using the DAS transmembrane prediction server (www.sbc.su.se/~miklos/DAS/maindas.html) to accommodate a cytosolic α-helix domain between the S1 and S2 transmembrane domains.

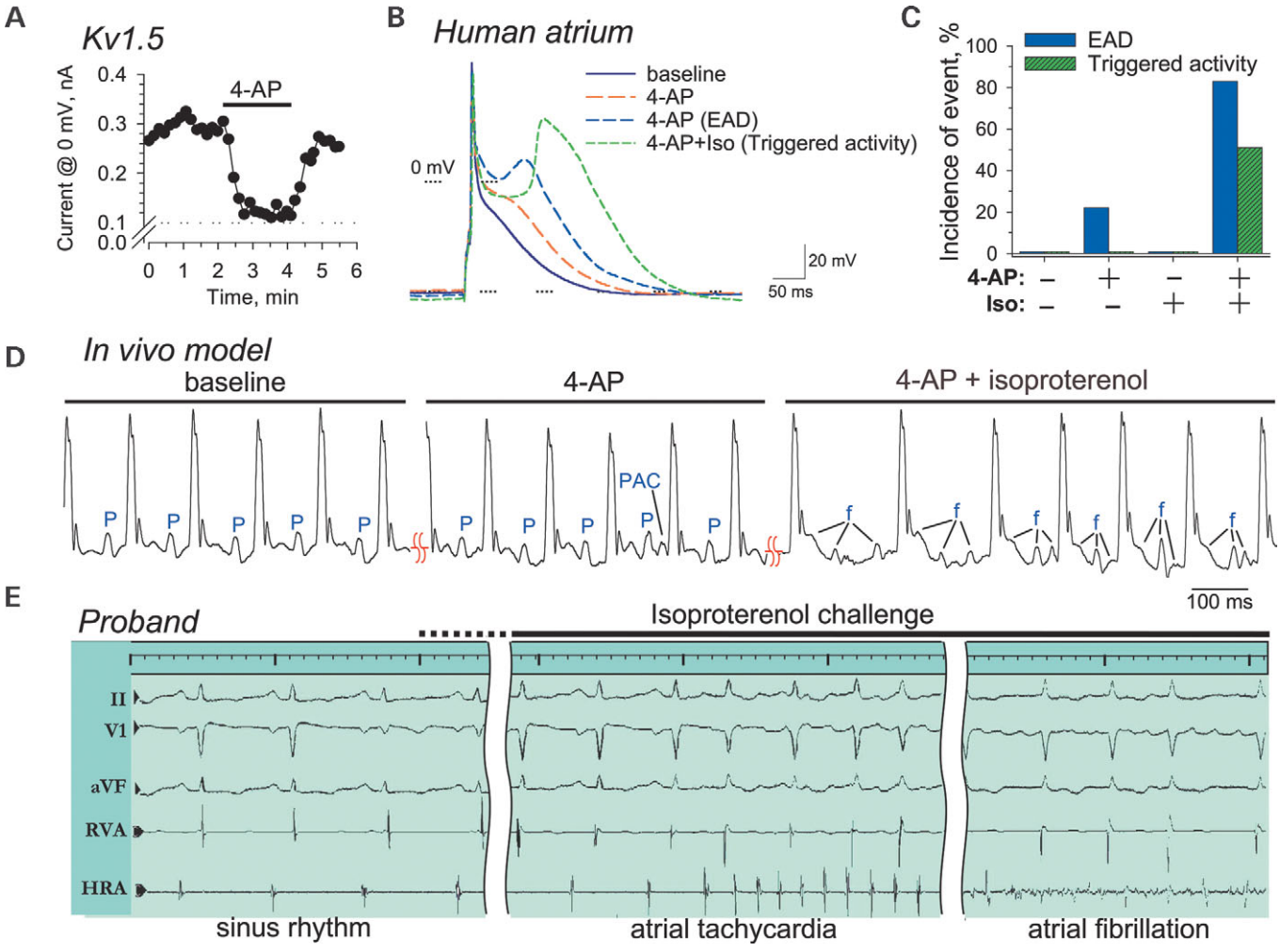

Figure 3. Compromised Kv1.5 function predisposes to repolarization deficit and atrial fibrillation. (**A**) Kv1.5 current was reversibly inhibited by 4-aminopyridine (4-AP, 50 μM) in voltage-clamped HEK293 cells. (**B**) Under current-clamp, in response to a 30 mV/10 ms pulse, 4-AP (50 μM) prolonged action potential duration in human atrial myocytes precipitating early after-depolarization (EAD) and triggered activity upon isoproterenol (Iso; 1 μM) challenge. (**C**) 4-AP (50 μM) and/or isoproterenol (1 μM) effect on EAD and triggered activity ($n = 9$). (**D**) Normal sinus rhythm with a 1:1 ratio between P waves (P) and QRS complexes, recorded by telemetry in ambulatory mice ($n = 3$), was interrupted by premature atrial complexes (PAC) following intraperitoneal (IP) administration of 4-AP (18 μg). Upon isoproterenol challenge (4 μg IP), rhythm degenerated into atrial fibrillation characterized by rapid and irregular atrial discharges (f) with loss of atrio-ventricular synchrony and variable ventricular rates. (**E**) Electrophysiological evaluation of the proband harboring the E375X mutation demonstrated isoproterenol-induced atrial tachycardia degenerating into atrial fibrillation. Sinus rhythm was lost with disruption of the normal 1:1 relationship between atrial and ventricular beats. II, V1, aVF, surface electrocardiographic recordings; HRA/RVA, intra-atrial/intra-ventricular recordings.

Channel reconstitution

Human HEK293 cells, cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum plus 2 mM glutamine, were transfected with WT or mutant human *KCNA5* cDNA subcloned into the expression vector pCDNA3.1/Zeo (Invitrogen). Plasmid DNA was applied at 0.4 μg/ml, using Fugene (Roche; 5.2 μl/ml), along with 0.2 μg/ml of the reporter green fluorescent protein.

Immunoprobing

Transfected cells were harvested in PBS containing a protease inhibitor cocktail (Complete, Roche). The pellet, obtained on centrifugation, was lysed in a hypotonic buffer (in mM) 1 $MgCl_2$, 0.5 EGTA, 4 DTT, 10 Tris–HCl; pH 7.5), homogenized, supplemented with (in mM) 4 DTT, 500 sucrose, 300 KCl, 10 Tris–HCl (pH 7.5) and re-homogenized. The lysate was re-centrifuged and the supernatant ultracentrifuged with the pellet resuspended and homogenized in (in mM) 10 Tris–HCl, 2 DTT, 250 sucrose, 150 KCl and 0.02 $CaCl_2$. The isolated membrane fraction was separated on 10% SDS–PAGE and proteins transferred by electrophoresis onto the nitrocellulose membrane. The N-terminal (Santa Cruz Biotechnology) Kv1.5 antibody was used for western blotting.

Patch-clamp recording

Recombinant Kv1.5 channel activity in transfected HEK293 cells was recorded using the whole-cell configuration of the patch-clamp technique in the voltage-clamp mode. Patch electrodes, with 5–7 MΩ resistance, were filled with (in mM) 120

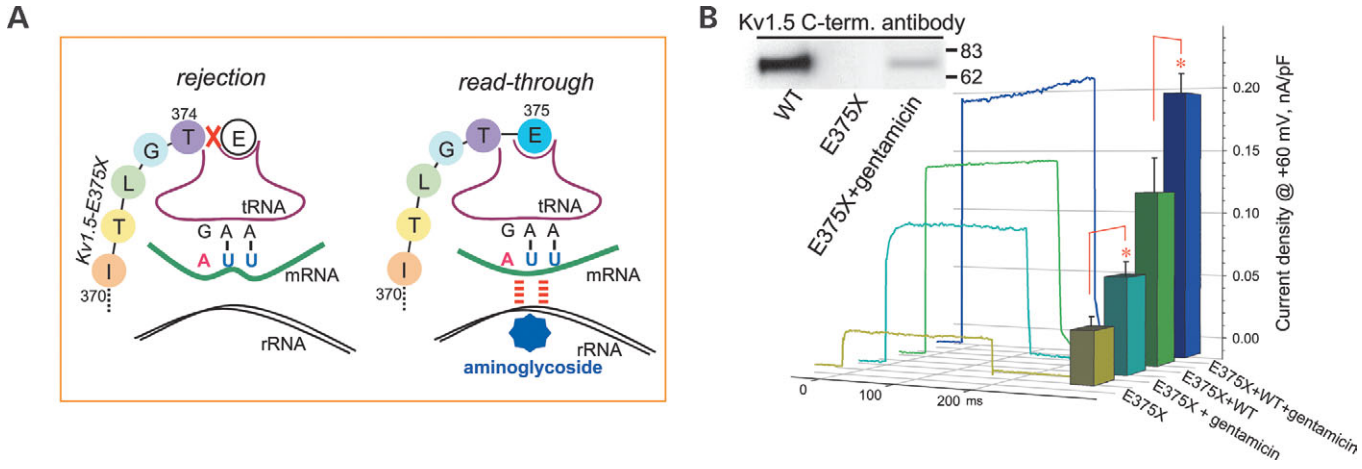

Figure 4. E375X repair achieved by translational read-through of the premature stop codon. (**A**) Mismatch between tRNA carrying glutamic acid (E), normally present at position 375, and mutant *KCNA5* mRNA is introduced by the stop codon, prematurely terminating the translation of Kv1.5. Aminoglycosides bind to rRNA, where they override the high-fidelity proofreading process allowing translational read-through. (**B**) Gentamicin treatment (1 mg/ml) induced full-length Kv1.5 demonstrated by western blot of membranes purified from transfected HEK293 cells using a C-terminal antibody. Whole-cell patch-clamp currents, obtained from the holding potential of −80 mV by depolarizing pulses to +60 mV and back to −40 mV, in cells expressing E375X and E375X plus WT Kv1.5 in the absence ($n = 9$ and 9, respectively) and presence ($n = 8$ and 6, respectively) of gentamicin. Currents were recorded 48 h following transfection in the continuous presence of 1 mg/ml gentamicin. Aminoglycoside treatment partially restored current density in E375X or E375X plus WT Kv1.5. Stars indicate significant current increase in gentamicin-treated *versus* untreated cells. Gentamicin had no effect on WT current (data not shown).

KCl, 1 $MgCl_2$, 5 EGTA and 10 HEPES supplemented with 5 mM of ATP (pH 7.3), and cells were superfused with (in mM) 136.5 NaCl, 5.4 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$ and 5.5 HEPES plus glucose 1 g/l (pH 7.3). Current density was obtained by normalizing whole-cell currents based on individual cell capacitance. Cell capacitance (C_m) was derived as $C_m = \tau I_o^2/E/(I_o - I_{ss})$, where I_o is the maximal current measured at the onset of the membrane depolarization step from −80 mV ($E = 30$ mV), I_{ss} the steady state current at the end of the 10–20 ms depolarization pulse and τ the characteristic time of cellular capacitance recharging derived from fitting the capacitance current decay time-course with the exponential function $I = (I_o - I_{ss})(\exp(-t/\tau) + I_{ss}$. Cells exhibiting multiexponential capacitance current were excluded from the analysis. Command pulses were specified by the Bioquest software (34). Experiments were performed at 33 ± 1°C.

Human atrial myocyte electrophysiology

Specimens of human right atrial appendage were obtained from patients who underwent cardiac surgery and who provided informed consent under a protocol approved by the Mayo Clinic Institutional Review Board. Patients with a history of atrial arrhythmia were excluded. Harvested samples were immersed in nominally Ca^{2+}-free solution (in mM: 136 NaCl, 5.4 KCl, 1 $MgCl_2$, 0.33 NaH_2PO_4, 10 dextrose, 10 HEPES; pH 7.4, 100% O_2, 37°C), cut into cubic pieces, and atrial cardiomyocytes were enzymatically dissociated using 200 U/ml collagenase (CLS II; Worthington Biochemicals) and 4 U/ml protease (type XXIV; Sigma). Atrial myocytes were incubated in (in mM) 130 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 glucose (pH 7.35) and attached to coverslips pre-coated with laminin. Action potential profiles were recorded in the current-clamp mode of the whole-cell patch-clamp technique using pipettes filled with (in mM) 100 KCl, 10 HEPES, 5 K_4BAPTA, 5 K_2ATP and 1 $MgCl_2$ (pH 7.2). Experiments were performed at 34 ± 1°C.

Telemetry

With approval of the Mayo Clinic Institutional Animal Care and Use Committee, continuous heart rate and electrocardiographic monitoring was carried out in conscious 8- to 12-week-old C57BL/6 mice implanted with telemetry devices (Data Sciences International) and leads tunneled subcutaneously under isoflurane anesthesia (35). Following a 2-week recovery from surgery, serial signals were acquired at 2 kHz.

Clinical electrophysiology

Light sedation was administered in the fasting state to the proband. Following heparinization, femoral and internal jugular venous sheaths were introduced for the placement of intra-cardiac monitoring catheters, positioned in the atria, coronary sinus and right ventricle. Electrical activation was recorded from intracardiac and surface leads, both at baseline and under stress provocation. Adrenergic stimulation was carried out by intravenous infusion of isoproterenol at 2 μg/min.

Kv1.5 rescue

HEK293 cells transfected with mutant *KCNA5* cDNA were treated with gentamicin (1 mg/ml), maintaining a constant aminoglycoside concentration for 48–72 h. Rescue of the full-length protein expression was detected by a C-terminal (Chemicon) Kv1.5 antibody using western blotting. Restoration of current density was measured by patch-clamp electrophysiology.

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Conflict of Interest statement. None declared.

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