**ABSTRACT**

**BACKGROUND** Antiarythmic drugs are widely used to treat patients with atrial fibrillation (AF), but the mechanisms conveying their variable effectiveness are not known. Recent data suggested that paired like homeodomain-2 transcription factor (PITX2) might play an important role in regulating gene expression and electrical function of the adult left atrium (LA).

**OBJECTIVES** After determining LA PITX2 expression in AF patients requiring rhythm control therapy, the authors assessed the effects of Pitx2c on LA electrophysiology and the effect of antiarythmic drugs.

**METHODS** LA PITX2 messenger ribonucleic acid (mRNA) levels were measured in 95 patients undergoing thoracoscopic AF ablation. The effects of flecainide, a sodium (Na⁺)-channel blocker, and d,l-sotalol, a potassium channel blocker, were studied in littermate mice with normal and reduced Pitx2c mRNA by electrophysiological study, optical mapping, and patch clamp studies. PITX2-dependent mechanisms of antiarythmic drug action were studied in human embryonic kidney (HEK) cells expressing human Na channels and by modeling human action potentials.

**RESULTS** Flecainide 1 μmol/l was more effective in suppressing atrial arrhythmias in atria with reduced Pitx2c mRNA levels (Pitx2c⁺/–). Resting membrane potential was more depolarized in Pitx2c⁺/– atria, and TWIK-related acid-sensitive K⁺ channel 2 (TASK-2) gene and protein expression were decreased. This resulted in enhanced post-repolarization refractoriness and more effective Na-channel inhibition. Defined holding potentials eliminated differences in flecainide’s effects between wild-type and Pitx2c⁺/– atrial cardiomyocytes. More positive holding potentials replicated the increased effectiveness of flecainide in blocking human Na1.5 channels in HEK293 cells. Computer modeling reproduced an enhanced effectiveness of Na-channel block when resting membrane potential was slightly depolarized.

**CONCLUSIONS** PITX2 mRNA modulates atrial resting membrane potential and thereby alters the effectiveness of Na-channel blockers. PITX2 and ion channels regulating the resting membrane potential may provide novel targets for antiarythmic drug development and companion therapeutics in AF. (J Am Coll Cardiol 2016;68:1881–94) © 2016 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
Atrial fibrillation (AF) causes cardiovascular death, frequent hospitalization, and cognitive decline even in patients treated according to guidelines (1–3). Antiarrhythmic drug (AAD) therapy remains the most commonly used treatment to maintain sinus rhythm in AF patients, but AAD effectiveness remains limited (3). Unfortunately, we lack a basic understanding of why AADs prevent AF over long periods in some patients but not in others (4,5). Identifying factors that modify the effects of AADs would allow the selection of responsive patients and could help guide development of novel AADs (6).

Paired like homeodomain-2 transcription factor (PITX2) is a transcription factor that regulates the development of the left atrium (LA) and thoracic organs. Its c isoform is expressed in the adult LA and regulates the expression of LA ion channels (7–9). Low atrial Pitx2 expression renders mice susceptible to AF and shortens the LA action potential (8,10,11). In this study, we investigated how atrial PITX2 modifies the effects of AADs.

**METHODS**

All experiments were conducted under the Animals (Scientific Procedures) Act 1986, and approved by the home office (PPL number 30/2967) and the institutional review board at the University of Birmingham. Analyses of human atrial tissue were approved by the institutional review board of Academic Medical Center, Amsterdam, the Netherlands. All patients provided written informed consent.

Left atrial appendages (LAAs) were excised from 95 patients undergoing bilateral thoracoscopic AF ablation either in the AFACT (Atrial Fibrillation Ablation and Autonomic Modulation via Thoracoscopic Surgery) trial (12) or undergoing similar procedures in the same centers using an endoscopic stapling device, snap frozen in liquid nitrogen and stored at −80°C (13). Deoxyribonucleic acid and ribonucleic acid were extracted using DNeasy kits (Qiagen Ltd., Manchester, United Kingdom), respectively. PITX2 mRNA content was quantified by quantitative polymerase chain reaction. Single nucleotide polymorphisms (SNPs) rs2200773, rs6838973, and rs1448818 (14) were identified using TaqMan assays (Thermo Fisher Scientific Inc., Waltham, Massachusetts).

Adult mice (age 12 to 16 weeks) on an MF1 background with normal or reduced (Pitx2c−/−) atrial Pitx2c expression were studied (8). LA epicardial monophasic action potentials were recorded from Langendorff-perfused murine hearts (8,15). Programmed stimulation was performed at baseline and with flecainide 1 µmol/l or d,l-sotalol 10 µmol/l. Arrhythmia inducibility and effective refractory period (ERP) were measured by using single right atrial extrastimuli after steady-state pacing in 1-ms decrements (15–18). Transmembrane action potentials were recorded using borosilicate glass microelectrodes from superfused murine LAs (17), RMP, action potential duration (APD), upstroke velocity, and activation times were analyzed (15,17,18).

The human atrial cell model of Courtemanche et al. (19) was used. Pitx2c−/− deficiency was modeled by reducing I_{Kr} conductance by 25% and doubling I_{Ks} conductance. Simulations were run in strands of 100 atrial cells (cell length 100 μm). The 5 leftmost cells of the strand were paced (S1) for 2 min at 1,000- and 500-ms basic cycle lengths. Premature stimulation (S2) was applied to determine the ERP and conduction velocity as measured from cells 25 to 75. Values for all other parameters were measured from the 50th cell. For the modeling, post-repolarization...
refractoriness (PRR) was calculated as the difference between APD at -60 mV repolarization and ERP.

LA cell isolation was performed as previously reported (20). Standard $I_{Na}$ and $I_{Kr}$ currents were recorded as previously published (18–20). Background K⁺ (TASK-like) currents sensitive to high Ba²⁺ (10 mM) were measured (21–23). Human embryonic kidney (HEK) 293 cells stably expressing the human Na₉,1.5 channel were obtained (SB Ion Channels, Glasgow, UK).

Ribonucleic acid and complementary deoxyribonucleic acid were synthesized from murine LA, (SuperScript VIILO, Thermo Fisher Scientific Inc.) to quantify expression of 20 atrial ion channels and genes with suspected PITX2-dependent regulation (9) using custom-designed Taqman low density array plates (Thermo Fisher Scientific Inc.). Western immunoblotting was performed on murine LA tissue lysates with antibodies detecting TASK-2, K₁,1.6, Na/K ATPase alpha-1, Na/K ATPase alpha-2, Na/Ca exchanger 1, Serca2a, Na₉,1.5, or calnexin, using standard methods.

Optical action potentials and calcium ion (Ca²⁺) transients were recorded in murine LA and analyzed using custom-made MATLAB algorithms (MathWorks, Natick, Massachusetts) as previously described (17).

**STATISTICAL ANALYSIS.** All experiments were performed and analyzed in a blinded fashion. Murine studies were performed and analyzed blinded to genotype in littermate pairs. Categorical data were compared using the Fisher exact test. Numerical data were compared by 2-sided paired parametric Student $t$ tests (e.g., measurements before and after perfusion of flecainide or sotalol) and Wilcoxon signed rank tests. Multiple measurements were assessed by repeated measures of analysis of variance followed by correction for multiple comparison (Bonferroni test) if the overall test was significant. Two-sided $p < 0.05$ were considered significant. Box plots depict individual measurements (points), mean, and SEM. Statistics and figures were created using Prism 5 (GraphPad Software, San Diego, California).

**RESULTS**

PITX2 mRNA varied markedly in human LAA (Central Illustration) harvested from AF patients (Table 1) (13), suggesting that a 50% lowered PITX2 expression defines a large, potentially clinically relevant group of AF patients. This did not directly correlate with SNP haplotype (Table 2), although we found numerically lower PITX2c levels in patients with 5 risk alleles.

Flecainide suppressed atrial arrhythmias in murine Pitx2c⁻/⁻ hearts. Flecainide abolished induced atrial arrhythmias in hearts with reduced Pitx2c expression (0 of 17 hearts with atrial arrhythmias) but not in hearts with normal Pitx2c expression (atrial arrhythmias remained in 3 of 12 hearts) (Figures 1A to 1C).

**TABLE 1** Baseline Characteristics (N = 101) *

| Age, yrs | 59.7 ± 8.4 (40–76) |
| Male     | 79 |
| Congestive heart failure | 6 |
| Hypertension | 34 |
| Age ≥75 yrs | 1 |
| Diabetes | 9 |
| Stroke/transient ischemic attack/embolus | 10 |
| Vascular disease | 10 |
| Female | 22 |
| Age ≥65 yrs | 31 |
| CHA₂DS₂-VASc score | 6.0 (1–35) |
| Previous catheter ablation for AF | 20 |
| Type of AF | |
| Paroxysmal | 44 |
| Persistent | 56 |
| Longstanding persistent | 1 |
| AF duration, yrs | 6.0 (1–35) |
| Antiarhythmic drugs and rate control agents | |
| Quinidine or disopyramide | 4 |
| Flecainide or propafenone | 33 |
| Amiodarone, dronedarone, or sotalol | 41 |
| Beta blockers | 53 |
| Verapamil or diltiazem | 17 |
| Digoxin | 15 |
| Anticoagulant agents (before PVI procedure) | |
| Vitamin K antagonists | 89 |
| Antiplatelets | 6 |

Values are mean ± SD (range), n, or mean (range). *Left atrial appendages were collected from these patients with atrial fibrillation (AF). PVI = pulmonary vein isolation.

**TABLE 2** PITX2 mRNA Expression in Left Atrial Appendages From AF Ablation Patients *

| Risk Alleles | 25% IQR | Median | 75% IQR | Mean | SEM | No. of Patients |
|--------------|---------|--------|---------|------|-----|----------------|
| 0            | 3.22    | 3.69   | 5.22    | 4.04 | 0.6 | 3              |
| 1            | 2.96    | 4.25   | 6.25    | 4.54 | 0.5 | 13             |
| 2            | 2.65    | 3.78   | 4.75    | 3.94 | 0.3 | 22             |
| 3            | 2.74    | 3.72   | 4.92    | 3.83 | 0.4 | 17             |
| 4            | 3.00    | 4.29   | 5.41    | 4.39 | 0.5 | 10             |
| 5            | 1.96    | 2.66   | 4.66    | 3.10 | 0.7 | 4              |
| 6            | 4.95    | 4.95   | 4.95    | 4.95 | 0.0 | 1              |

*This dataset was grouped according to the number of risk single nucleotide polymorphism (SNP) alleles for AF on chromosome 4q25 (rs2200733, SNP2 rs6838973, rs1448818) [13]. Although PITX2 mRNA is numerically lower in patients with 5 or 6 risk alleles, we did not find a PITX2 mRNA gradient according to AF risk. IQR = interquartile range; LA = left atrium; other abbreviations as in Table 1.
Figure 1: Atrial Arrhythmia Inducibility in Pitx2c⁺/– Murine Whole Hearts

(A) Image and schematic representation of the Langendorff-perfused heart. (B) Atrial arrhythmia inducibility in isolated, beating hearts from wild-type (WT) and reduced paired like homeodomain 2 messenger ribonucleic acid (Pitx2c⁺/–) mice. Flecainide abolished atrial arrhythmia inducibility in Pitx2c⁺/– hearts only. *p < 0.05 flecainide versus baseline. (C) Representative trace of atrial fibrillation (AF) induced during programmed stimulation at baseline, showing reduced severity of arrhythmias with 1 µmol/l flecainide in Pitx2c⁺/– atria. (D) Effects of flecainide on atrial effective refractory period (ERP) in wild-type and Pitx2c⁺/– isolated, beating hearts. Shown is the difference in atrial ERP between baseline and 1 µmol/l flecainide at 80- to 120-ms paced cycle length following a single extrastimulus (S2) in WT and Pitx2c⁺/– isolated, beating hearts. *p < 0.05 between genotypes across all cycle lengths. (E) Whereas flecainide prolonged ERP in both genotypes, this effect was more pronounced in Pitx2c⁺/– atria. Flecainide caused post-repolarization refractoriness (PRR), the difference between ERP (orange and grey lines) and APD₉₀ (blue lines), in WT and Pitx2c⁺/– atria. Flecainide-induced PRR in Pitx2c⁺/– is almost 3 times that of WT atria. *p < 0.05 WT versus Pitx2c⁺/–. *p < 0.05 baseline versus 1 µmol/l flecainide. APD = action potential duration; EG = intracardiac electrogram; EP = electrophysiology; LA = left atrium; LV = left ventricle; MAP = monophasic action potential; RA = right atrium; RV = right ventricle.
### TABLE 3: Effect of Flecainide on Refractoriness and Repolarization in Mouse Hearts

| Paced CL, ms | Wild-Type | Pitx2c<sup>−/−</sup> |
|--------------|-----------|---------------------|
|              | Baseline | Flecainide | Baseline | Flecainide | Baseline | Flecainide | Baseline | Flecainide |
| LA ERP, ms   |          |           |          |           |          |           |          |           |
| 120          | 23.5 ± 2.3 (11) | 29.8 ± 3.0 (11) | 22.2 ± 2.1 (11) | 29.6 ± 3.3 (11) | 21.9 ± 2.4 (10) | 28.7 ± 3.5* (10) | 30.5 ± 2.4 (11) | 38.5 ± 3.3* (11) |
| 100          | 28.0 ± 2.3 (13) | 40.2 ± 2.8* (13) | 27.5 ± 2.5 (13) | 41.2 ± 3.0† (13) |
| LA monophasic APD, ms |          |           |          |           |          |           |          |           |
| APD<sub>50</sub> | 10.2 ± 1.3 (8) | 14.5 ± 1.7 (8) | 10.8 ± 1.8 (8) | 11.9 ± 1.6 (8) | 10.4 ± 0.7 (7) | 12.0 ± 1.1 (7) | 12.4 ± 1.1 (15) | 14.4 ± 1.3 (15) |
| APD<sub>70</sub> | 17.8 ± 2.2 (9) | 23 ± 2.1 (9) | 18.4 ± 1.6 (9) | 18.7 ± 2.2 (9) | 18.1 ± 1.2 (8) | 18.1 ± 1.9 (8) | 18.0 ± 1.6 (15) | 19.2 ± 1.8 (15) |
| APD<sub>90</sub> | 31.3 ± 3.0 (8) | 37.4 ± 2.8 (8) | 31.5 ± 2.5 (9) | 29.9 ± 2.9 (9) | 31.0 ± 1.4 (8) | 28.4 ± 2.7 (8) | 28.3 ± 2.2 (13) | 29.9 ± 2.2 (13) |

Values are mean ± SEM (number of atria). *p < 0.05 vs. baseline. †p < 0.05 vs. wild-type.

### TABLE 4: Electrophysiological Effects of Sotalol

| Paced CL, ms | Wild-Type | Pitx2c<sup>−/−</sup> |
|--------------|-----------|---------------------|
|              | Baseline | Sotalol | Baseline | Sotalol | Baseline | Sotalol | Baseline | Sotalol |
| LA ERP, ms   |          |           |          |           |          |           |          |           |
| 120          | 38.7 ± 7.8 (7) | 33.9 ± 6.3 (7) | 32.2 ± 6.1 (6) | 29.2 ± 5.3 (6) | 39.3 ± 4.0 (4) | 26.8 ± 3.5 (4) | 37.0 ± 5.7 (4) | 24.0 ± 3.7 (4) |
| 100          |          |           |          |           |          |           |          |           |
| LA APD, ms   |          |           |          |           |          |           |          |           |
| APD<sub>50</sub> | 11.5 ± 1.2 (9) | 13.4 ± 1.2 (9) | 10.9 ± 2.0 (7) | 12.2 ± 1.3 (7) | 10.8 ± 1.1 (7) | 11.2 ± 1.0 (7) | 8.3 ± 0.9 (4) | 11.1 ± 1.7 (4) |
| APD<sub>70</sub> | 17.6 ± 2.2 (9) | 20.0 ± 1.9 (9) | 16.0 ± 1.3 (7) | 18.2 ± 2.3 (7) | 16.5 ± 1.6 (7) | 17.3 ± 1.2 (7) | 13.0 ± 1.5 (4) | 17.0 ± 1.9 (4) |
| APD<sub>90</sub> | 30.7 ± 3.2 (9) | 33.5 ± 2.7 (9) | 29.0 ± 1.9 (7) | 30.9 ± 3.2 (7) | 29.7 ± 2.7 (7) | 31.2 ± 2.0 (7) | 23.8 ± 2.8 (4) | 29.6 ± 2.9 (4) |

Values are mean ± SEM (number of atria). Abbreviations as in Tables 2 and 3.
Flecainide prolonged ERPs and refractoriness beyond the end of repolarization (PRR) calculated as the difference between ERP and APD90 (ms). Flecainide prolonged PRR more in hearts with reduced Pitx2c expression (Figures 1D and 1E, Table 3). PITX2c+/– hearts had shorter atrial action potentials (8). Flecainide abolished APD differences between Pitx2c+/– and wild-type LA by prolonging early repolarization (APD30, APD50, and APD70) (Table 3). Murine atrial PITX2 expression did not modulate the effects of sotalol on atrial APD or ERP (Table 4).
RMP was slightly depolarized in LA murine cells with reduced Pitx2c expression (range of mean depolarization 1.2 to 2.4 mV over 5 cycle lengths; all \( p < 0.05 \) (Figures 2A and 2B). Atrial Pitx2c levels did not significantly affect \( \frac{dV}{dt_{\text{max}}} \) (100-ms paced cycle length: wild-type: 104.4 ± 4.3 V/s; Pitx2c\(+/-\): 93.7 ± 4.5 V/s) (Figure 2C). Flecainide did not modify atrial RMP (Figure 2B) but reduced action potential amplitude consistent with its Na-channel blocking effect, specifically at 100-ms cycle length: wild-type baseline: 77.5 ± 1.2 mV (n = 30); wild-type flecainide: 71.3 ± 1.2 mV (n = 31); Pitx2c\(+/-\) baseline: 73.4 ± 1.3 mV (n = 22); and Pitx2c\(+/-\) flecainide: 65.1 ± 1.45 mV (n = 24).

Because the Courtemanche-Ramirez-Nattel model does not incorporate background K⁺ currents (19), we simulated a depolarized RMP in this model by a 25% reduction in \( I_{K1} \). This reduced the RMP at 500-ms paced cycle length by 2 mV from 79.9 mV (“normal PITX2”) to -77.9 mV (“low PITX2”). NA channels recovered from inactivation more slowly upon partial \( I_{Na} \) block (50% or 60%) (Figure 3A). Furthermore, PRR was enhanced in the PITX2 deficiency model (Figure 3B and Table 5). Inhibition of \( I_{Na} \) reduced upstroke velocity (\( \frac{dV}{dt_{\text{max}}} \)) and conduction velocity in both models, and reproduced the prolongation of PRR (Figure 3B).

Kcnq6 and Kcnk5 mRNA expression were reduced in Pitx2c\(+/-\) murine LA (Figure 4A, Online Table 1), whereas mRNA concentrations of 20 other ion channels or related genes were not altered. Kv1.6 protein concentration was unaltered, whereas TASK-2 protein concentration was reduced in murine atria with reduced Pitx2c expression (Figure 4B). Na\(_{\text{v}}\)1.5 mRNA...
and protein expression were not changed (Figures 4A and 4B).

Atrial Pitx2c expression did not modify peak Na\(^+\) currents (\(I_{\text{Na}}\)) recorded from isolated murine cardiomyocytes at holding potentials ranging from -100 to -65 mV (Figures 5A to 5C). Peak \(I_{\text{Na}}\) was reduced at more depolarized holding potentials (Figure 5). Flecainide inhibited \(I_{\text{Na}}\) better at more positive holding potentials (inhibition at -70 mV: 68 ± 5%; inhibition at -65 mV: 75 ± 5%; \(n = 86\) cells from \(n = 17\) atria) in cells from murine atria with normal or reduced Pitx2c expression, suggesting that the greater efficiency of flecainide in atria with reduced Pitx2c expression is secondary to RMP depolarization (Figure 5C). Consistent with this, flecainide inhibited human Na\(_{\text{v}1.5}\) channels expressed in HEK cells more potently at more depolarized test potentials (-65 to -75 mV) (Figures 5D and 5E).

Background K\(^+\) currents, which include TASK currents, were reduced in Pitx2c\(^{-/-}\) murine atria, whereas \(I_{\text{Kl}}\) did not differ between genotypes (Figure 6).

Reduced Pitx2c expression did not alter atrial conduction velocities or activation patterns (Online Figures 1A to 1C, Table 6), consistent with published data (8). We found that 1 \(\mu\)mol/l flecainide decreased atrial conduction velocities without differences between wild-type and Pitx2c\(^{-/-}\) mice (Online Figures 1B and 1C). Calcium transient relaxation times at 50% relaxation were not different between wild-type and Pitx2c\(^{-/-}\) (Online Figures 1D and 1E). Flecainide 1 \(\mu\)mol/l shortened 50% Ca\(^{2+}\) relaxation times by approximately 10% and decreased Ca\(^{2+}\) transient amplitude by approximately 50% in murine atria with normal and reduced Pitx2c expression (Online Figures 1E and 1F). Additionally, expression of the Na/Ca exchanger Serca2a and Na/K ATPase alpha-1 and alpha-2 subunit protein did not differ between wild-type and Pitx2c\(^{-/-}\) atria (Online Figure 2).

### DISCUSSION

This study demonstrated that LA Pitx2 mRNA concentrations vary in patients with AF requiring rhythm control therapy (Central Illustration). Furthermore, flecainide increases PRR and suppresses arrhythmias more effectively in atria with halved Pitx2c expression, mediated by a more depolarized RMP (Central Illustration). Drug-induced PRR is thought to prevent arrhythmias, as reactivation can then occur only after full recovery of excitability, avoiding slow propagation during the vulnerable period (16,24,25). We found similar effects in cells expressing human Na channels and in the Courtemanche–Ramirez–Nattel model of human atrial action potentials.

Thus, this study highlighted modulation of the atrial RMP by Pitx2, possibly mediated by background currents such as TASK-2, as a target for AAD therapy, including atrial-selective therapy. Furthermore, the results suggested that markers for atrial Pitx2 expression may identify AF patients who benefit from Na-channel blocker therapy (Central Illustration).

Low atrial Pitx2 expression was identified as an important determinant of the antiarrhythmic effects of Na channel blockers. Low LA Pitx2c mRNA depolarized atrial RMP (Figure 2), consistent with a previous report (11). A depolarized RMP increased flecainide-induced PRR (Figure 1) (26–30). The conduction-slowing effect of flecainide was not modulated by reduced atrial Pitx2c (Online Figure 1), an important surrogate for drug safety. Both the modeling experiments (Figure 3) and the experiments in HEK cells expressing human Na channels (Figure 5)
confirmed that small changes in RMP can markedly modulate Na-channel inhibition.

RESTING MEMBRANE POTENTIAL. Open-state Na-channel blockers such as flecainide and propafenone bind preferentially to Na channels integrated in membranes with slightly depolarized resting potentials, where more channels are in the open or inactivated state (31,32). Our data can be interpreted as suggesting that AAD combinations that include a Na-channel blocker with a membrane potential modifying substance, such as amiodarone (16,33) or the combination of dronedarone and ranolazine (29,34,35), may have synergistic antiarrhythmic effects because they modulate atrial RMP and thereby enhance the effect of Na-channel blockade. Further studies of such drug combinations and the relationship between their effectiveness and the patient’s atrial PITX2 mRNA levels are warranted. Our data also suggested that such combined effects may be of special relevance in patients who have a depolarized RMP, such as secondary to low LA PITX2. Because PITX2 expression is confined to the LA in the heart, AAD therapy that leverages modifications in RMP may achieve “atrial-specific” AAD therapy.

RMP is maintained by an intricate balance of different transmembrane currents and is closely related to the potassium equilibrium potential. We identified that PITX2 modifies expression of the genes encoding K_v1.6 and TASK-2 (Figure 4). Complete deletion of PITX2 regulates other potassium and Na channels such as Kcnj2 (8,36), which alter the RMP, but these were not responsible for the depolarized RMP observed in our study. Two-pore domain potassium channels, such as TASK-2, contribute to RMP in various cells, including skeletal and cardiac muscle (37,38). To date, an altered function of the TASK-1 channel and of I_K1 has been implicated in atrial remodeling and AF (39,40). This study demonstrated that TASK-2 is expressed in atrial myocardium (Figure 4B), suggesting that a reduced function of TASK-2 could depolarize RMP (Figures 1 and 5) (8,11), analogous to the effect of TASK-2 in neuronal and cartilage tissue (41,42).

DEVELOPING CLINICAL MARKERS FOR PATIENTS WITH DEPOLARIZED RMP. It will be challenging to directly assess LA RMP in AF patients, but our data suggested that differences in atrial RMP could explain the effectiveness of Na-channel blockers in carriers of
common gene variants on chromosome 4q25 (43), although LA PITX2 levels are modulated by factors other than SNP status (Table 2) (44). It seems desirable to develop and validate drivers that modify RMP and clinical markers for patients prone to a depolarized atrial RMP to select appropriate AADs for individual patients in the future, thus enabling personalized AAD selection (6,45).

**STUDY LIMITATIONS.** This study provided robust evidence that LA PITX2 expression varies in AF patients and that reduced PITX2c expression enhances the antiarrhythmic effects of Na-channel blockers by modulating atrial RMP. The study was partly motivated by the assumption that gene variants on chromosome 4q25 modify PITX2 expression, an assumption that has not been definitively proven (9,11,44,46). Our analysis (Table 2) and that of others indicate that SNP status does not always correlate with PITX2 levels (47,48). Our findings are relevant to AAD therapy even if the presumed link between PITX2 expression and genetic variants on
chromosome 4q25 proves elusive. The mechanisms by which reduced PITX2 mRNA concentrations shorten the LA action potential at high heart rates remain to be fully elucidated (8,20). Validating our findings in patients is desirable but will be challenging because access to fresh LA cardiomyocytes and LA tissue is limited.

Due to the novelty of our findings, we could not perform a priori power calculations for our mechanistic experiments, and we analyzed several functional parameters to identify potential mechanisms conveying the antiarrhythmic effects of flecainide in atria with low Pitx2c concentrations. Our findings thus require independent validation.

### TABLE 6 Electrical Activation Time and Conduction Velocity in Isolated Atria in the Presence of Flecainide (1 μmol/l)

| Wild-Type | Ptx2c+/− |
|-----------|----------|
| Paced CL, ms | 1,000 | 300 | 120 | 100 | 80 | 1,000 | 300 | 120 | 100 | 80 |
| Activation time (isolated left atrium), ms | 6 ± 0.3 (22) | 6 ± 0.3 (22) | 9 ± 0.5 (22) | 12 ± 1.0 (22) | 16 ± 1.4 (22) | 6 ± 0.2 (24) | 7 ± 0.3 (24) | 12 ± 0.9 (24) | 13 ± 0.9 (24) | 18 ± 1.2 (24) |
| Conduction velocity (optical mapping), cm/s | — | 30 ± 1.8 (8) | 25 ± 2.4 (8) | 25 ± 1.9 (8) | 23 ± 1.9 (8) | — | 29 ± 1.5 (8) | 26 ± 1.6 (8) | 25 ± 1.6 (8) | 23 ± 1.6 (8) |

Values are mean ± SEM (number of atria).  
— = not applicable; other abbreviations as in Table 3.
CONCLUSIONS

This study shows that low LA PITX2 mRNA levels increase atrial RMP and thereby increase the effectiveness of flecainide (Central Illustration). This finding calls for appropriately designed clinical studies to assess whether AF patients with low atrial PITX2 levels respond favorably to Na-channel blockade. Further studies exploring the relevance of TASK channels to atrial RMP also are warranted.

ACKNOWLEDGMENTS The authors thank Sian Marie O’Brien, Sarah Hopkins, Syeeda Nashitha Kabir, Pushpa Patel, and Charles Carey for technical support; Marta Coric for help with HEK cells; and Ilaria Piccini for advice on TLDA.

REPRINT REQUESTS AND CORRESPONDENCE: Prof. Paulus Kirchhof, Institute of Cardiovascular Sciences, University of Birmingham, Wolfson Drive, Birmingham B15 2TT, United Kingdom. E-mail: p.kirchhof@bham.ac.uk.

REFERENCES

1. Chugh SS, Havmoeller R, Narayan K, et al. Worldwide epidemiology of atrial fibrillation: a Global Burden of Disease 2010 Study. Circulation 2014;129:837-47.

2. January CT, Wann LS, Alpert JS, et al. 2014 AHA/ACC/HRS guideline for the management of
patients with atrial fibrillation: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines and the Heart Rhythm Society. J Am Coll Cardiol 2014; 64:e1-76.

2. Kirchhof P, Beruschi S, Kotecha D, et al. 2016 ESC Guidelines for the management of atrial fibrillation developed in collaboration with EACTS: The Task Force for the management of atrial fibrillation of the European Society of Cardiology (ESC) developed with the special contribution of the European Heart Rhythm Association (EHRA) of the ESC Endorsed by the European Stroke Orga- nisation (ESO). Eur Heart J 2016 Aug 27 [E-pub ahead of print].

3. Kirchhof P, Andresen D, Bosch R, et al. Short-term versus long-term antiarrhythmic drug treatment after cardioversion of atrial fibrillation (Flec-SL): a prospective, randomised, open-label, blinded endpoint assessment trial. Lancet 2012; 380:238-46.

4. Schotten U, Verheule S, Kirchhof P, Goette A. Pathophysiological mechanisms of atrial fibrillation: a translational appraisal. Physiol Rev 2011;91: 265-325.

5. Fabritzi L, Guasch E, Antoniades C, et al. Expert consensus document: defining the major health modifiers causing atrial fibrillation: a roadmap to underpin personalized prevention and treatment. Nat Rev Cardiol 2015;13:230-7.

6. Li N, Dobrev D, Wehrens XH. PITX2: a master regulator of cardiac channellopathy in atrial fibrillation? Cardiovasc Res 2016;109:345-7.

7. Li N, Dobrev D, Wehrens XH. PITX2: a master regulator of cardiac channellopathy in atrial fibrillation? Cardiovasc Res 2016;109:345-7.

8. Arfnoff V, Kindler CH, Paul M, Zou H, et al. Amide local anaesthesia suppresses induction of ventricular fibrillation. J Pharmacol Exp Ther 2003;305:257-63.

9. Yu TY, Syeda F, Holmes AP, et al. An automated system using spatial oversampling for optical mapping in murine atria. Development and validation with monophasic and transmembrane action potentials. Prog Biophys Mol Biol 2014;115: 340-8.

10. Lemoine MD, Duverger JE, Naud P, et al. Arrhythmogenic left atrial cellular electrophysi- ology in a murine genetic long QT syndrome model. Cardiovasc Res 2011;92:67-74.

11. Courtemanche M, Ramirez RJ, Nattel S. Ionic mechanisms underlying human atrial action po- tential properties: insights from a mathematical model. Am J Physiol 1998;275:H301-21.

12. Holmes AP, Yu TY, Tuli S, et al. A regional reduction in Ito and IKACH in the murine posterior left atrial myocardium is associated with action potential prolongation and increased ectopic activity. PLoS One 2016;11:e0154077.

13. Barbuti A, Ishii S, Shimizu T, Robinson RB, Feinmark SJ. Block of the background K(þ) channel TASK-1 contributes to arrhythmogenic effects of platelet-activating factor. Am J Physiol Heart Circ Physiol 2002;282:H2024-30.

14. Buckler KJ. A novel oxygen-sensitive potas- sium current in rat carotid body type I cells. J Physiol 1997;498 Pt 3:649-62.

15. Limberg SN, Netter MF, Rolfes C, et al. TASK-1 channels may modulate action potential duration of human atrial cardiomyocytes. Cell Physiol Biochem 2011;28:613-24.

16. Kirchhof P, Fabritzi L, Franz MR. Post- repolarization refractoriness versus conduction slowing caused by class I antiarrhythmic drugs: antiarrhythmic and proarrhythmic effects. Circulation 1998;97:2567-74.

17. Millberg P, Frommeyer G, Uphaus T, et al. Sodium channel block by ranolazine in an experi- mental model of stretch-related atrial fibrillation: prolongation of interstitial conduction time and increase in post-repolarization refractoriness. Europace 2013;15:761-9.

18. Fukuda K, Watanabe J, Yagi T, et al. A sodium channel blocker, pilsicainide, produces atrial post-repolarization refractoriness through the reduction of sodium channel availability. Tohoku J Exp Med 2011;225:35-42.

19. Burashnikov A, Sicouri S, Di Diego JM, Belardinelli L, Antzelevitch C. Synergistic effect of the combination of ranolazine and dronedarone to suppress atrial fibrillation. J Am Coll Cardiol 2010; 56:1216-24.

20. Kirchhof P, Engelen M, Franz MR, et al. Electro- physiologic effects of flecainide and sotalol in the human atrium during persistent atrial fibrilla- tion. Basic Res Cardiol 2005;100:112-21.

21. Anno T, Hondeghem LM. Interactions of fle- cainide with guinea pig cardiac sodium channels. Importance of activation unblocking to the voltage dependence of recovery. Circ Res 1990; 66:789-803.

22. Nitta J, Sunami A, Marumo F, Hiraoka M. States and sites of actions of flecainide on guinea-pig cardiac sodium channels. Eur J Pharmacol 1992; 214:191-7.

23. Singh BN, Singh SN, Reda DJ, et al. Amiodar- one versus sotalol for atrial fibrillation. N Engl J Med 2005;352:1861-72.

24. Burashnikov A, Di Diego JM, Zygmunt AC, et al. Atrial-selective sodium channel block as a strategy for suppression of atrial fibrillation. Ann N Y Acad Sci 2008;1123:105-12.

25. Reiffel JA, Camm AJ, Belardinelli L, et al. The HARMONY trial: combined ranolazine and dronedarone in the management of paroxysmal atrial fibrillation: mechanistic and therapeutic syner- gism. Circ Arrhythm Electrophysiol 2015;8: 1048-56.

26. Tao Y, Zhang M, Li L, et al. Pitx2, an atrial fibrillation predisposition gene directly regulates ion transport and intercalated disc genes. Circ Cardiovasc Genet 2014;7:23-32.

27. Reyes R, Duprat F, Lesage F, et al. Cloning and expression of a novel pH-sensitive two pore domain K+ channel from human kidney. J Biol Chem 1998;273:30863-9.

28. Enyedi P, Czirjak G. Molecular background of leak K+ currents: two-pore domain potassium channels. Physiol Rev 2010;90:559-605.

29. Harleton E, Besana A, Chandra P, et al. TASK-1 current is inhibited by phosphorylation during human and canine chronic atrial fibrilla- tion. Am J Physiol Heart Circ Physiol 2015;308: H126-34.

30. Liang B, Soka M, Christensen AH, et al. Ge- netic variation in the two-pore domain potassium channel, TASK-1, may contribute to an atrial sub- strate for arrhythmogenesis. J Mol Cell Cardiol 2014;67:69-76.

31. Clark RR, Kondo C, Belke DD, Giles WR. Two- pore domain K(þ) channels regulate membrane potential of isolated human articular chon- drocytes. J Physiol 2011;589:5071-89.

32. Kindler CH, Paul M, Zou H, et al. Amide local anesthetics potently inhibit the human tandem pore domain background K+ channel TASK-2 (KCNK5). J Pharmacol Exp Ther 2003;306: 84-92.

33. Ellinor PT, Lunetta KL, Albert CM, et al. Meta-analysis identifies six new susceptibility
44. Gore-Panter SR, Hsu J, Barnard J, et al. PANCR, the PITX2 Adjacent noncoding RNA, is expressed in human left atria and regulates PITX2c expression. Circ Arrhythm Electrophysiol 2016;9:e003197.

45. Kirchhof P, Sipido KR, Cowie MR, et al. The continuum of personalized cardiovascular medicine: a position paper of the European Society of Cardiology. Eur Heart J 2014;35:3250-7.

46. Holmes AP, Kirchhof P. Pitx2 adjacent non-coding RNA: a new, long, noncoding kid on the 4q25 Block. Circ Arrhythm Electrophysiol 2016;9:e003808.

47. Martin RI, Babaei MS, Choy MK, et al. Genetic variants associated with risk of atrial fibrillation regulate expression of PITX2, CAV1, MYOZ1, C9orf3 and FANCC. J Mol Cell Cardiol 2015;85:207-14.

48. Gore-Panter SR, Hsu J, Hanna P, et al. Atrial fibrillation associated chromosome 4q25 variants are not associated with PITX2c expression in human adult left atrial appendages. PLoS One 2014;9:e86245.

KEY WORDS antiarrhythmic drugs, atrial fibrillation, drug targets, electrophysiology, personalized medicine, rhythm control

APPENDIX For an expanded Methods section as well as supplemental figures and a table, please see the online version of this article.
Supplementary Figure 1

A Experimental setup of the high density optical activation mapping system. This system was also used to measure Ca^{2+} transients.

B Representative electrical activation maps of wildtype (WT) and Pitx2c+/− LA before and after flecainide, recorded at 100ms cycle length pacing. Flecainide causes conduction slowing in both genotypes to the same degree.

C Conduction velocity determined by optical activation mapping in superfused Pitx2c+/− and wildtype LA, measured at 80-300ms paced CL. Baseline (black): wildtype n=5; Pitx2c+/− n=11. Flecainide (red): wildtype: n=4; Pitx2c+/− n= 8. Individual values are displayed. Data points for each genotype have been offset at each cycle length for clarity.

D Representative calcium transients recorded during 100ms cycle length pacing at baseline (black) and with 1µmol/L flecainide (red) from isolated superfused wildtype and Pitx2c+/− LA. Calcium transients did not differ between genotypes with or without flecainide. Horizontal line= 40 ms.

E Time to 50% calcium relaxation at baseline and after 1µmol/L flecainide in LA. Calcium relaxation time did not differ between genotypes with or without flecainide. Baseline (black): wildtype n=5; Pitx2c+/− n=7. Flecainide (red): wildtype: n=5; Pitx2c+/− n = 7.

F Calcium signal amplitude at baseline and after 1 µmol/L flecainide in LA. AU= arbitrary units; fold-change in fluorescence. Baseline (black): wildtype n=5; Pitx2c+/− n=7. Flecainide (red): wildtype: n=5; Pitx2c+/− n = 7. Data points for each genotype have been offset at each cycle length for clarity.
Supplementary Figure 2

Protein concentrations of Na/Ca exchanger, Serca2a, Na/K ATPase alpha-1 and alpha-2 proteins, relative to calnexin (arbitrary units) in left atrial homogenates of wild type (WT) and heterozygous Pitx2c+/− mice. Representative immunoblots are displayed below the corresponding dot plot. WT = 7, Pitx2c+/− = 7.
**Supplementary Table:** Quantification of mRNA of ion channels in left atria from Pitx2c+/‐ and wild-type mice. Expression levels were measured relative to wildtype sample 1. * and boldface indicate p<0.05

| Gene   | Protein | WT Rq<sub>control</sub> ±SEM | Pitx2c+/‐ Rq<sub>control</sub> ±SEM | Mean CT ±SEM |
|--------|---------|-----------------------------|-------------------------------------|--------------|
| Pitx2  | PITX2   | 1 ± 0.13                     | 0.77 ± 0.09                         | 26.4* ± 0.12 |
| Kcna6  | K<sub>v</sub>1.6 | 1 ± 0.15                     | 0.65 ± 0.09                         | 27.2* ± 0.17 |
| Kcnk5  | TASK-2  | 1 ± 0.1                      | 0.71 ± 0.12                         | 28.6* ± 0.13 |
| Actb   | ACTB    | 1 ± 0.19                     | 0.84 ± 0.09                         | 19.7 ± 0.06  |
| Cacna1c| Cav1.2   | 1 ± 0.08                     | 1.03 ± 0.08                         | 22.7 ± 0.11  |
| Cacna2d2| CACNA2D | 1 ± 0.11                     | 1.27 ± 0.15                         | 25.1 ± 0.17  |
| Casq2  | CSQ2    | 1 ± 0.06                     | 0.98 ± 0.06                         | 19.9 ± 0.18  |
| Cul7   | P185    | 1 ± 0.07                     | 0.96 ± 0.08                         | 25.5 ± 0.1   |
| Itpr2  | Al649341 | 1 ± 0.14                    | 0.87 ± 0.07                         | 25.3 ± 0.11  |
| Kcna3  | K<sub>v</sub>1.3 | 1 ± 0.8                      | 0.16 ± 0.03                         | 31.6 ± 0.17  |
| Kcna4  | K<sub>v</sub>1.4 | 1 ± 0.12                     | 0.94 ± 0.08                         | 27.7 ± 0.16  |
| Kcnk4  | K<sub>v</sub>3.4 | 1 ± 0.43                     | 0.85 ± 0.39                         | 32.3 ± 0.43  |
| Kcnj2  | IRK1    | 1 ± 0.07                     | 0.91 ± 0.07                         | 23.9 ± 0.12  |
| Kcnj3  | GIRK-1  | 1 ± 0.05                     | 0.98 ± 0.03                         | 19.5 ± 0.13  |
| Kcnj5  | GIRK4   | 1 ± 0.08                     | 0.95 ± 0.07                         | 21.5 ± 0.11  |
| Ryr2   | RYR2    | 1 ± 0.09                     | 0.88 ± 0.06                         | 18.4 ± 0.11  |
| Scn1b  | SCN1B   | 1 ± 0.08                     | 0.88 ± 0.06                         | 23.6 ± 0.1   |
| Scn4a  | Na<sub>v</sub>1.4 | 1 ± 0.1                     | 1.25 ± 0.12                         | 24.4 ± 0.11  |
| Scn5a  | Na<sub>v</sub>1.5 | 1 ± 0.07                     | 0.98 ± 0.1                          | 21.2 ± 0.1   |
| Scn7a  | Na<sub>v</sub>2   | 1 ± 0.13                     | 0.8 ± 0.04                          | 24 ± 0.09    |
| Trpc1  | TRP1    | 1 ± 0.07                     | 0.94 ± 0.06                         | 26.5 ± 0.1   |
| Trpc6  | TRP-6   | 1 ± 0.13                     | 0.88 ± 0.14                         | 31.1 ± 0.15  |
| Trpm7  | TRPM7   | 1 ± 0.12                     | 0.84 ± 0.04                         | 23.7 ± 0.12  |
Supplementary methods

Electrophysiological study in the isolated heart.

Mice were terminally anaesthetized with 400mg/kg pentobarbital sodium and heparinized. Their hearts were rapidly excised, mounted on a Langendorff apparatus (Hugo Sachs, Germany) and retrogradely perfused with 37°C Krebs-Henseleit (KH) containing (in mmol/l): NaCl 118; NaHCO₃ 24.88; KH₂PO₄ 1.18; Glucose 5.55; Na-Pyruvate 5; MgSO₄ 0.83; CaCl₂ 1.8; KCl 3.52 (95% O₂–5% CO₂, pH 7.4) at constant perfusion pressure (100 ± 5 mmHg) and coronary flow (4 ± 0.5ml/min).

A 2.0 French octapolar mouse electrophysiological catheter (0.5 mm electrode spacing, CIB’ER MOUSE, NuMED, USA) was inserted into the right atrium and right ventricle for pacing and recording intracardiac electrograms. Left atrial epicardial monophasic action potentials (MAP) were recorded using custom-made electrodes, preamplified using a DC-coupled pre amplifier (Model 2000, EP Technologies, USA), recorded at 2 kHz sampling frequency, and analyzed offline (EMKA technologies, France). Inducibility of atrial arrhythmias (triplets, salvos and AF) and effective refractory periods, with and without flecainide acetate (Sigma, 1µmol/L) and sotalol hydrochloride (Sigma, 10µmol/L), were determined using 120 to 80 ms paced cycle lengths and premature single RA extrastimulus. Activation times and action potential durations at different repolarization levels were analyzed.

Transmembrane action potential recordings in isolated, superfused left atria.

Transmembrane action potentials were recorded using borosilicate glass microelectrodes, filled with 3 M KCl (tip resistance 15–30 MΩ), from intact
superfused left atria isolated from mice under inhalation anaesthesia (2.5-4% isoflurane in O₂, 1.5L/min). Voltage signals were amplified (Axoclamp 2B; Molecular Devices, USA), digitized, displayed and analyzed using spike2 software (Cambridge Electronic Design, UK) at 20 kHz sampling frequency. LA were paced at 2× diastolic threshold through bipolar platinum electrodes. After 15 min pacing for equilibration, preparations were paced successively at 1000ms to 80ms. Action potentials were analyzed following 200 beats at each frequency to ensure steady state prior to recording.

**Na⁺ current recordings in murine left atrial cardiomyocytes and HEK293 cells expressing the human Na₁.5 channel**

Murine hearts were removed under terminal anaesthesia and cell isolation was performed as published. For Iₙa recordings, murine left atrial cardiomyocytes or HEK293 cells stably expressing the human Na₁.5 channel (SB Ion Channels, Glasgow, UK) were superfused at 5ml.min⁻¹, 22±0.5 °C with a solution containing in mM: NaCl 130, CsCl 5, HEPES 10, CaCl₂ 1.8, MgCl₂ 1.2 and glucose 10, pH 7.4 (CsOH). 100μM CdCl₂ was added to block L-type Ca²⁺ currents. Whole cell patch clamp recordings were obtained in voltage clamp mode using borosilicate glass pipettes (tip resistance 1–3 MΩ, pipette solution CsCl 115, NaCl 5, HEPES 10, EGTA 10, MgATP 5, MgCl₂ 0.5 and TEA 10, pH 7.2). Na⁺ currents were elicited at 100ms steps to -10mV from holding potentials of -100 to -65mV using an Axopatch 1D amplifier (Molecular Devices, USA) and a CED micro1401 driven by Signal v6 (CED, UK).
Standard $I_{K1}$ currents were isolated using 50μM BaCl2 and applying 10mV step depolarisations (500ms) from -120mV to +50mV. For background K+ current measurements, the external solution contained in mM: NaCl 130, KCl 5.4, CaCl2 1, MgCl2 1, HEPES 10, TEA 10, 4-AP 5, glibenclamide (2μM), BaCl2 (100μM), NiCl2 2 and glucose 5.5 (pH 7.4 with NaOH), to block voltage-gated K+ and Ca2+ channels and $I_{K1}$ and $I_{KATP}$. Currents were measured in response to a 5s voltage ramp from -80 to 0mV. Background TASK-like K+ current was isolated with 10mM BaCl2.

**Quantitative immunoblotting of atrial tissue.** Frozen LA were homogenized in 30 ml of homogenization buffer per gram of tissue (100 mM Tris, pH 7.4, 2 mM sodium vanadate, 5 mM sodium fluoride, 1× protease inhibitor cocktail tablet/50 mL, 4°C). Samples were separated by electrophoresis on a 10% or 15% SDS polyacrylamide gel. Proteins were transferred to PVDF membranes (0.45μm; GE Healthcare, UK) using semidry blotting (Trans-Blot® Turbo™ Transfer System BioRad). Immunoblots were blocked with 5% non-fat milk in PBS-Tween overnight at 4°C. Blots were incubated for 1-1.5 hours at room temperature with primary antibodies raised against: TASK-2 (1:200; Alomone), K_1.6 (1:200; Alomone), Na/K ATPase alpha-1 (1:10000; Milipore, MA, USA), Na/K ATPase alpha-2 (1:5000; Milipore, MA, USA), Na/Ca exchanger 1 (1:1000; Swant, Switzerland), Serca2a (1:5000; Badrilla, UK) and calnexin (1:2000, ab22595 Abcam). Blots were incubated with Na_1.5 antibody (1:200, Alomone) overnight at 4°C. After incubation with HRP-labeled secondary antibodies, blots were developed using enhanced chemiluminescence (Amersham Pharmacia Biotech) on.
Odyssey Fc imager (Li-Cor). Calnexin was used as a loading control. Signals from

\textit{Pitx2c}^{+/-} LA samples were normalized to wildtype samples signals on the same gels.