Defects in Ankyrin-Based Membrane Protein Targeting Pathways Underlie Atrial Fibrillation

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Background—Atrial fibrillation (AF) is the most common cardiac arrhythmia, affecting >2 million patients in the United States alone. Despite decades of research, surprisingly little is known regarding the molecular pathways underlying the pathogenesis of AF. ANK2 encodes ankyrin-B, a multifunctional adapter molecule implicated in membrane targeting of ion channels, transporters, and signaling molecules in excitable cells.

Methods and Results—In the present study, we report early-onset AF in patients harboring loss-of-function mutations in ANK2. In mice, we show that ankyrin-B deficiency results in atrial electrophysiological dysfunction and increased susceptibility to AF. Moreover, ankyrin-B+/− atrial myocytes display shortened action potentials, consistent with human AF. Ankyrin-B is expressed in atrial myocytes, and we demonstrate its requirement for the membrane targeting and function of a subgroup of voltage-gated Ca2+ channels (Ca1.3) responsible for low voltage-activated L-type Ca2+ current. Ankyrin-B is associated directly with Ca1.3, and this interaction is regulated by a short, highly conserved motif specific to Ca1.3. Moreover, loss of ankyrin-B in atrial myocytes results in decreased Ca1.3 expression, membrane localization, and function sufficient to produce shortened atrial action potentials and arrhythmias. Finally, we demonstrate reduced ankyrin-B expression in atrial samples of patients with documented AF, further supporting an association between ankyrin-B and AF.

Conclusions—These findings support that reduced ankyrin-B expression or mutations in ANK2 are associated with AF. Additionally, our data demonstrate a novel pathway for ankyrin-B–dependent regulation of Ca1.3 channel membrane targeting and regulation in atrial myocytes. (Circulation. 2011;124:1212-1222.)

Key Words: arrhythmia (mechanisms) ■ cytoskeleton ■ ion channel ■ protein trafficking

Atrial fibrillation (AF), which is characterized by rapid and irregular activation of the atrium, is the most common sustained arrhythmia found in clinical practice, affecting ≈2.3 million people in the United States alone.1 The prevalence of AF increases with age, with ≈2% to 6% of people aged >65 years exhibiting AF.2,3 Given the demographics of the United States, it is predicted that there will be a 5-fold increase in the prevalence of AF by 2050.4 Although AF is frequently associated with other cardiac disorders including coronary artery disease, mitral valve disease, congenital heart disease, and congestive heart failure, ≈30% of all AF cases are described as lone AF, in which patients exhibit no previous cardiac pathology.5 The majority of monogenic AF cases described to date are linked to mutations that affect potassium channels, although these cases represent only a small fraction of total monogenic AF cases.6

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Ankyrin-B (encoded by ANK2) is an adapter protein expressed in a number of excitable cells including neurons,
cardiomyocytes, and pancreatic β cells. Given that ankyrin-B targets select ion channels, transporters, and signaling molecules to specific membrane domains, it is not surprising that dysfunction in the ankyrin-B cellular pathway has been linked to neuronal defects, ventricular arrhythmia, and neonatal diabetes. 

In the present study, we demonstrate a critical role for ankyrin-B in atrial function. Individuals harboring loss-of-function mutations in ANK2 developed early-onset AF. Ankyrin-B–deficient mice phenocopied ANK2-based human disease and displayed striking susceptibility to AF. Primary atrial myocytes from ankyrin-B–deficient mice displayed shortened action potentials, a hallmark of AF, and this decrease was associated with loss of L-type Ca2+ current ($I_{Ca,L}$). Our data revealed that Ca1.3 is a novel ankyrin-B–binding partner, and the structural requirements for ankyrin-B/Ca1.3 association were mapped. Specifically, a unique C-terminal motif in Ca1.3 was found to be necessary and sufficient for ankyrin binding. In ankyrin-B+/− atrial myocytes, Ca1.3 protein expression and membrane function were reduced. Moreover, we demonstrated that ankyrin-B levels are reduced in human AF, providing an association between reduced. Moreover, we demonstrated that ankyrin-B levels are reduced in human AF, providing an association between reduced

### Methods

#### Immunofluorescence

Myocytes were washed with phosphate-buffered saline (pH 7.4) and fixed in warm 2% paraformaldehyde (37°C). Cells were blocked/permeabilized in phosphate-buffered saline containing 0.075% Triton X-100 and 3% fish oil gelatin (Sigma) and incubated in primary antibody overnight at 4°C. After washes (phosphate-buffered saline plus 0.1% Triton X-100), cells were incubated in secondary antibody (Alexa 488 and 568 and ToPro-3AM 633) for 8 hours at 4°C and mounted with the use of Vectashield (Vector). Images were collected using a Zeiss 510 Meta confocal microscope (40-power oil, 1.40 numerical aperture, pinhole equals 1.0 Airy Disc) with Zeiss Imaging software. Images were imported into Adobe Photoshop for cropping and linear contrast adjustment. Imaging experiments were performed at least 3 times for each experimental protocol, and >20 myocytes were examined from each experimental set.

#### ImmunobLOTS

Protein lysates were isolated from atria and ventricles of wild-type (WT) and heterozygous ankyrin-B mice as described. Antibodies and tissues are described in the online-only Data Supplement.

#### Cellular Electrophysiology

Membrane currents and action potentials were measured with an Axon 200B patch-clamp amplifier controlled by a personal computer with the use of a Digidata 1320A acquisition board driven by pClamp 8.0 software (Axon Instruments). Electrophysiological recordings were obtained only from Ca2+-tolerant, rod-shaped atrial cells. We used a perforated (amphotericin B) patch for calcium current and action potential studies. All experiments were conducted at room temperature, unless otherwise noted. Other currents were measured as described. Recording pipettes, fabricated from borosilicate glass, had resistance of 2 to 4 mol/LΩ when filled with recording solution. All solutions were adjusted to 275 to 295 mOsm.

### Statistical Analysis

For human data, differences between group means were compared by unpaired Student t test. Frequency data were analyzed with the Fisher exact test. The Kolmogorov-Smirnov test was used to test whether data followed a normal distribution. Assumption of equal variance per group was confirmed with the use of Bartlett’s test. Data are mean±SE. P<0.05 was considered statistically significant. For nonhuman data, all values are presented as mean±SE. P values were assessed with a paired Student t test (2-tailed) or ANOVA, as appropriate, for continuous data. The Bonferroni test was used for post hoc testing. The null hypothesis was rejected for P<0.05.

### Human Tissue Samples

Human tissue experimental protocols were approved by the ethics committee of the Medical Faculty Mannheim, University of Heidelberg (No. 2011–216N-MA). Each patient gave written informed consent. During routine cannulation procedures in patients undergoing open-heart surgery for cardiac bypass grafting and valve replacement, the tip of the right atrial appendage was removed and immediately snap-frozen in liquid nitrogen. Appendages were obtained from 12 sinus rhythm and 10 paroxysmal AF (Table II in the on-line only Data Supplement) patients. The AF group included patients in sinus rhythm at surgery with a history of at least 1 episode of self-terminating AF lasting <7 days (paroxysmal AF patients).

Additional methods are presented in the online-only Data Supplement.

### Results

**Ankyrin-B Dysfunction Is Associated With Human AF**

We identified a high incidence of AF in ANK2 mutation–positive probands. Probands harboring ANK2 loss-of-function alleles displayed early-onset AF, commonly progressing to permanent AF. In 1 large kindred (74 members), 13 of 25 ANK2 loss-of-function variant carriers (phenotypes previously mapped to ANK2; $Z_{max}=7.05^o$) displayed AF (mean onset, 40±18 years; 5 paroxysmal, 8 permanent), whereas noncarriers were asymptomatic. Only 2 individuals were nonpenetrant for atrial phenotypes. The others had AF, AF with sinus node dysfunction, or sinus node dysfunction alone. In an unrelated large family, 20 of 36 individuals were positive for the ANK2 disease allele (maximal logarithm of odds score was for marker D4S1616; linkage $Z_{max}=5.9$, θ=0), and 3 of 20 disease allele carriers displayed AF (mean onset, 48±12 years; 2 paroxysmal, 1 permanent), whereas 13 displayed sinus node disease requiring pacemaker implantation (mean age for implantation, 30±18 years). Only 4 individuals were nonpenetrant for atrial phenotypes. In addition to AF, prior work demonstrates that ANK2 variant carriers may display prolonged ventricular QT intervals, which predisposes individuals to ventricular arrhythmias (examples of cardiac phenotypes are presented in Table I in the online-only Data Supplement). Because AF and long-QT syndrome are often associated with opposing action potential characteristics (ie, AF with shortened atrial action potentials and long-QT syndrome with prolonged ventricular action potentials), we hypothesized that ankyrin-B might uniquely affect atrial ion channels, resulting in an increased susceptibility to AF.
Mice Deficient for Ankyrin-B Expression Display Atrial Dysfunction

Previous studies have demonstrated that human ANK2 gene mutations linked with arrhythmia behave as loss of function when analyzed in primary cardiomyocytes. Therefore, we assessed the role of ankyrin-B deficiency in atria of mice heterozygous for a null mutation in ankyrin-B (ankyrin-B<sup>+/−</sup> mice, because ankyrin-B<sup>−/−</sup> mice die at birth). Continuous telemetry ECG recordings from conscious ankyrin-B<sup>−/−</sup> mice revealed atrial arrhythmias also present in human carriers of ANK2 variants, including spontaneous bradycardia with erratic atrial activity, lack of discrete P waves, and variable ventricular response (Figure 1A through 1D).

Figure 1. Ankyrin-deficient mice display atrial dysfunction and atrial fibrillation (AF) inducibility. A to D, Conscious ECGs of wild-type (WT) (A and B) and ankyrin B (AnkB)<sup>−/−</sup> mice (C and D) recorded by telemetry. WT mice show normal sinus rhythm, whereas AnkB<sup>+/−</sup> mice display spontaneous bradycardia with erratic atrial activity, including absence of discreet P waves and variable ventricular responses indicative of spontaneous AF. B and D represent red magnified areas of A and C. E, Simultaneous surface ECG (top) and atrioventricular electrogram recording (bottom) in WT (left) and AnkB<sup>−/−</sup> mice (right) are shown. Top, Lead I ECG from WT and AnkB<sup>+/−</sup> mice shows rapid atrial electric activity and the variability in RR interval in AnkB<sup>+/−</sup> mouse compared with WT mouse. F, Incidence of AF in WT and AnkB<sup>+/−</sup> mice during burst pacing. Whereas WT mice displayed low incidence of AF (2 of 9 mice), AnkB<sup>+/−</sup> mice were highly susceptible to AF induction (11 of 15 mice; *P<0.05 for AF susceptibility in WT vs AnkB<sup>+/−</sup> mice).
We next tested the susceptibility to atrial arrhythmias in ankyrin-B<sup>-/-</sup> mice after atrial burst pacing. Intracardiac electrograms were recorded simultaneously with the surface ECG to confirm the nature of abnormal atrial electric activities. Periods of pacing-induced AF were observed in the majority (>75%) of ankyrin-B<sup>-/-</sup> mice (11 of 15 mice; Figure 1E and 1F). In contrast, a significantly lower fraction of WT mice demonstrated AF inducibility with the use of this protocol (2 of 9 WT mice; *P* <0.05 versus ankyrin-B<sup>-/-</sup>; Figure 1F). Ankyrin-B<sup>-/-</sup> atrial episodes showed characteristic absence of P waves and RR-interval variability. Moreover, AF/atrial tachycardia was confirmed by the presence of rapid and irregular A waves on the atrioventricular electrogram (Figure 1E). Together, the electrophysiological data in human ANK2 mutation carriers and ankyrin-B<sup>-/-</sup> mice clearly demonstrate that ankyrin-B dysfunction is detrimental for normal atrial electric activity.

**Ankyrin-B Is Expressed in Atria**

We used immunoblot to identify ankyrin-B expression patterns in human atria. Ankyrin-B was expressed in all human heart chambers at similar levels (Figure 2A). As expected, voltage-gated calcium channel Ca<sub>1.2</sub> was most abundant in the right (RA) and left atria (LA) and in right (RV) and left ventricles (LV). Ca<sub>1.3</sub> expression was greater in ventricles than atria, whereas Ca<sub>1.3</sub> expression is greater in atria than ventricles. Na-H exchange regulatory factor-1 (NHERF1) expression demonstrates equal loading between the samples. B, Localization of AnkB in a single mouse atrial myocyte. Note that AnkB is expressed at both the sarcolemma (yellow arrows) and M-line domains (white arrows). Bar =10 µm. C and D, AnkB<sup>+/−</sup> atrial myocytes display reduced action potential duration (APD90 at 22°C; n=7 WT, n=8 AnkB<sup>+/−</sup>; *P* <0.05). E and F, AnkB<sup>+/−</sup> atrial myocytes display reduced I<sub>Ca,L</sub> compared with WT atrial myocytes (n=7 WT, n=8 AnkB<sup>+/−</sup>; *P* <0.05). G and H, WT and AnkB<sup>+/−</sup> atrial myocytes display no difference in I<sub>Na</sub> (n=7 WT, n=8 AnkB<sup>+/−</sup>; *P* <0.05).
Ankyrin-B^{+/−} Atrial Myocytes Display Aberrant Atrial Electric Activity

To define the role of ankyrin-B in atria, we evaluated action potentials from isolated WT and ankyrin-B^{+/−} atrial cardiomyocytes. Notably, ankyrin-B^{+/−} atrial myocytes displayed significantly reduced action potential duration (APD) (measured at 90% of repolarization [APD_{90}]) compared with WT atrial cardiomyocytes (Figure 2C and 2D). In fact, APD_{90} was reduced ~25% in ankyrin-B^{+/−} atrial cardiomyocytes at both room (Figure 2C and 2D; n=7 WT, n=8 ankyrin-B^{+/−}; P<0.05) and physiological temperatures (Figure I in the online-only Data Supplement; n=6 WT, n=5 ankyrin-B^{+/−}; P<0.05), a cellular phenotype consistent with that observed in AF patients. In contrast, prior studies revealed that ankyrin-B^{+/−} ventricular cardiomyocytes do not display significant differences in APD_{90} compared with WT.

Atrial myocyte depolarization and repolarization are regulated by the synchronized activities of membrane-associated ion channels, transporters, and pumps. To define the molecular basis of action potential shortening in ankyrin-B^{+/−} atrial myocytes, we investigated the activities of a number of critical atrial myocyte currents. We observed a significantly reduced I_{Ca,L} in ankyrin-B^{+/−} atrial myocytes (Figure 2E and 2F; n=7 WT, n=8 ankyrin-B^{+/−}; P<0.05) in contrast to ankyrin-B^{+/−} ventricular myocytes that display normal I_{Ca,L} and APD. We observed no difference in I_{Ks} (Figure 2G and 2H; n=7 WT, n=8 ankyrin-B^{+/−}; P=NS), total I_{Ks} or I_{Ko} (transient, outward) between WT and ankyrin-B^{+/−} atrial myocytes (n=7 WT, n=8 ankyrin-B^{+/−}; P=NS; not shown).

However, we observed a significant decrease in Na^{+}-Ca^{2+} exchange current (I_{NCX}), consistent with previous findings in ankyrin-B–deficient ventricular myocytes (Figure II in the online-only Data Supplement; n=8 WT, n=8 ankyrin-B^{+/−}; P<0.05). Thus, atrial ankyrin-B^{+/−} myocytes display decreased APD and reduced I_{Ca,L}. Both phenotypes are unique to ankyrin-B^{+/−} atrial cells compared with ventricular myocytes.

Reduced Ca_{1.3} Expression in Ankyrin-B^{+/−} Atria

In atrial myocytes, I_{Ca,L} is composed of the combined activities of Ca_{1.2} (α1C) and Ca_{1.3} (α1D). On the basis of reduced I_{Ca,L} in ankyrin-B^{+/−} atrial cardiomyocytes, we examined the expression and localization of both Ca_{1.2} and Ca_{1.3} in WT and ankyrin-B^{+/−} mouse atria. Consistent with electrophysiological data, we observed a significant decrease in Ca_{1.3} expression in ankyrin-B^{+/−} atria (reduced ~30%; n=3; P<0.05; Figure 3A and 3B), whereas Ca_{1.2} expression was unchanged between genotypes (Figure 3A and 3B; P=NS). Furthermore, in agreement with our earlier studies in ventricle, Na_{1.5} expression levels were not altered in ankyrin-B^{+/−} atrial cells (Figure 3A). Immunofluorescence of individual atrial myocytes with the use of channel-specific antibodies demonstrated that Ca_{1.3} but not Ca_{1.2}, Ca_{3.1}, or Na_{1.5} membrane expression was reduced (Figure 3C through 3F). In fact, we observed a ~40% decrease in membrane staining intensity for Ca_{1.3} in ankyrin-B^{+/−} atrial cardiomyocytes compared with WT cells (Figure 3E; P<0.05; n=5 WT, n=6 ankyrin-B^{+/−}).

As a final test to assess ankyrin-B function in Ca_{1.3} membrane targeting, we evaluated Ca_{1.3} membrane activity in primary cardiac fibroblasts from WT and ankyrin-B^{−/−} (homozygous null) mice (harvested before death of ankyrin-B^{−/−} neonatal mice). Primary fibroblasts were chosen to allow recording of I_{Ca,L} from exogenously expressed Ca_{1.3} without confounding native current from endogenous Ca^{2+} channels (note that WT and ankyrin-B^{−/−} primary fibroblasts display little endogenous I_{Ca}; open circles in Figure 3G). As expected, WT fibroblasts expressing Ca_{1.3} displayed significantly greater I_{Ca,L} compared with nontransfected cells (Figure 3G; n=9 WT, n=7 ankyrin-B^{−/−} cells; P<0.05). In contrast, ankyrin-B^{−/−} primary cells expressing Ca_{1.3} showed ~50% reduction in I_{Ca,L} (Figure 3G; n=9 WT, n=7 ankyrin-B^{−/−} cells; P<0.05) and reduced Ca_{1.3} membrane immunofluorescence (Figure 3H) compared with WT cells despite similar Ca_{1.3} protein expression in both cell types (based on immunoblot). These phenotypes were directly related to ankyrin-B expression because exogenous ankyrin-B expression restored I_{Ca,L} levels in ankyrin-B^{−/−} cells (Figure 3I and 3J; n=8; P<0.05). Notably, in contrast to Ca_{1.3}, we observed no functional difference in Ca_{1.2} membrane expression between WT and ankyrin-B–deficient cells (Figure IIIA and IIIB in the online-only Data Supplement; n=5 WT, n=6 ankyrin-B^{+/−}; P=NS).

Ankyrin-B Is Directly Associated With Ca_{1.3}

On the basis of electrophysiological data from primary myocytes and fibroblasts showing reduced Ca_{1.3} in ankyrin-B–deficient atrial myocytes, we tested for a direct interaction between ankyrin-B and Ca_{1.3}. Full-length Ca_{1.3} has 5 major cytoplasmic domains including an amino terminal domain, 3 cytoplasmic segments that connect the 4 major transmembrane regions (DI through DIV), and a large C-terminal domain (Figure 4A). We generated radiolabeled purified protein of each Ca_{1.3} cytoplasmic region (N1, L1 through L3, C1 through C4). Because of the extensive length of the C-terminal domain, this region was further subdivided into 4 segments for direct binding experiments (Figure 4A, C1 through C4). We observed significant association between glutathione-S-transferase-labeled ankyrin-B membrane-binding domain and only 1 intracellular region of Ca_{1.3}, the C4 region of the C-terminus (residues 2014 to 2203; Figure 4B). This interaction was specific to ankyrin-B because it was not observed for the similar ankyrin-G membrane-binding domain or glutathione-S-transferase alone (Figure 4B). These data support a direct and specific interaction between ankyrin-B membrane-binding domain and the C-terminal domain of Ca_{1.3}.

Although our functional data suggested a specific interaction of ankyrin-B with Ca_{1.3}, Ca_{1.2} and Ca_{1.3} share significant sequence similarity in the distal C-terminal regions (residues 2014 to 2203 of Ca_{1.3}; Figure 4C). We therefore tested for the interaction of the ankyrin-B membrane-binding domain with both Ca_{1.2} and Ca_{1.3} C-terminal domains (residues 1958 to 2159 of Ca_{1.2}). Consistent with our previous imaging, biochemical, and functional data, we observed no interaction of ankyrin-B or ankyrin-G with the Ca_{1.2} C-terminal domain (Figure 4D).
Figure 3. Reduced Ca\textsubscript{1.3} expression and targeting in ankyrin-B (AnkB) \textsuperscript{+/−} atria. A, Immunoblots to evaluate expression of AnkB, Nav1.5, Na-H exchange regulatory factor-1 (NHERF1), Cav1.2, and Ca\textsubscript{1.3} protein in wild-type (WT) and AnkB \textsuperscript{+/−} mouse atria. Note reduced protein expression of AnkB and Ca\textsubscript{1.3} in AnkB \textsuperscript{+/−} atria compared with WT atria (n=3; P<0.05). No change was detected in the protein expression of Cav1.2, Na\textsubscript{1.5}, and NHERF1 between WT and AnkB \textsuperscript{+/−} atria (n=3; P=NS). B, Coomassie blue stain demonstrating equal loading of protein lysates from WT and AnkB \textsuperscript{+/−} atria. C through F, Immunolocalization of AnkB (C), Cav1.2 (D), Ca\textsubscript{1.3} (E), and Cav3.1 (F) in WT (left) and AnkB \textsuperscript{−/−} atrial myocytes (right). Note decreased membrane targeting of Ca\textsubscript{1.3} but not other membrane proteins (Cav1.2, Cav3.1) in AnkB \textsuperscript{−/−} atrial myocytes (n=5 WT, n=6 AnkB \textsuperscript{−/−}; P<0.05 for Cav1.3). Bar=10 μm. G though J, AnkB expression is required for normal atrial myocyte \textit{I}_{\text{Ca,L}}. G, \textit{I}_{\text{Ca,L}} in WT and AnkB \textsuperscript{−/−} primary cells with and without exogenous Ca\textsubscript{1.3}. Note that AnkB \textsuperscript{−/−} cardiac fibroblasts expressing Ca\textsubscript{1.3} display significant reductions in \textit{I}_{\text{Ca,L}} compared with WT fibroblasts expressing identical Ca\textsubscript{1.3} cDNAs (n=9 WT, n=7 AnkB \textsuperscript{−/−}). *P<0.05 for black vs red symbols; #P<0.05 for red symbols vs nontransfected cells. H, Decreased membrane expression of Ca\textsubscript{1.3} (red) in WT (left) vs AnkB-deficient (right) primary cells (bar=10 μm; blue staining represents nuclei). I and J, Reduced \textit{I}_{\text{Ca,L}} in AnkB-null fibroblasts is rescued to normal by coexpression of green fluorescent protein (GFP)-tagged AnkB. *P<0.05 for black and green vs red symbols; #P<0.05 for red symbols vs nontransfected cells. Δ represents P<0.05 between black and green symbols (n=8 per group).
Ankyrin-B (AnkB) is directly associated with Cav1.3.

A, Schematic representation of rat Cav1.3 and intracellular domains for in vitro binding assays. Intracellular domains correspond to amino acids N1: 1 to 127; L1: 406 to 583; L2: 811 to 946; L3: 1207 to 1263; C1: 1504 to 1670; C2: 1671 to 1853; C3: 1854 to 2019; and C4: 2014 to 2202.

B, In vitro binding assays between Cav1.3 intracellular domains and ankyrin membrane-binding domains. Note that the membrane-binding domain of AnkB, but not ankyrin-G (AnkG), binds to Cav1.3 C4, representing the terminal 200 amino acids.

C, Amino acid alignment of the terminal 200 amino acids of Cav1.3 and Cav1.2. Identical amino acids are boxed in light gray, and similar amino acids are boxed in dark gray.

D, In vitro binding assays of AnkB membrane-binding domain with the corresponding C4 regions of Cav1.3 and Cav1.2. Note that AnkB selectively interacts with Cav1.3 C4.

E, Amino acid alignment of known ankyrin-binding domains in Nav1.5 and Kir6.2 with the ankyrin-binding domain in the terminal 26 amino acids of Cav1.3.

F, High amino acid conservation is observed in Cav1.3 ankyrin-binding domains across species.

G, Amino acid sequences of full-length Cav1.3-C4 and Cav1.3-C4 lacking the terminal 29 amino acids including the ankyrin-binding domain (Cav1.3-C4 ABD).

H, In vitro binding assays of AnkB membrane-binding domain with Cav1.3-C4 and Cav1.3-C4 ABD. Note that AnkB does not bind to Cav1.3-C4 ABD (experiments were done in parallel with those in D, thus top control blots are the same).

I, Amino acid sequences of biotinylated peptides of the terminal 25 residues of Cav1.3 and Cav1.2.

J, Immunoprecipitation of AnkB with the biotinylated Cav1.3 peptide but not with the corresponding Cav1.2 peptide. SA indicates streptavidin agarose.

K, Compared with primary cardiac fibroblasts expressing wild-type Cav1.3, primary cardiac fibroblasts expressing Cav1.3 lacking the AnkB-binding motif display reduced $I_{\text{Ca,L}}$. *$P<0.05$ compared with red symbols; #$P<0.05$, triangles compared with squares ($n=7$ per group).
The Cav1.3 but not Cav1.2 C-terminal domain contains a short motif resembling the ankyrin-binding sequence found in voltage-gated Na+ channels (ie, Na1.2, Na1.5) and inwardly rectifying K+ channels (ie, Kir6.2; Figure 4E).8,20,21 Importantly, this sequence is conserved across Cav1.3 orthologs (Figure 4F). We tested the requirement of this motif for ankyrin-B binding using a mutant C-terminal domain lacking these residues (Figure 4G). Although Cav1.3 C-terminal domain was associated with ankyrin-B (but not ankyrin-G), Cav1.3 C4 ankyrin-binding domain lacking the putative ankyrin-binding domain failed to associate with ankyrin-B (Figure 4H). On the basis of these results, we tested whether residues 2175 to 2198 were sufficient for ankyrin-binding using biotinylated peptides. We observed an association of full-length endogenous ankyrin-B from human atrial lysates with an immobilized peptide containing the ankyrin-B-binding domain in Cav1.3 (Figure 4I and 4J). We observed no interaction with a peptide of the corresponding region in Cav1.2 or with streptavidin beads alone (Figure 4J). Together, our data demonstrate that Cav1.3 residues 2175 to 2198 are necessary and sufficient for direct ankyrin-B association.

To test whether Cav1.3 membrane expression requires a direct interaction with ankyrin-B, we measured Ica,L in primary cardiac fibroblasts expressing WT Cav1.3 and a mutant Cav1.3 lacking the ankyrin-binding motif. As expected, WT Cav1.3 expression resulted in robust Ica,L in primary cells (Figure 4K; n=7; P<0.05). In contrast, Cav1.3Δ2174 lacking the ankyrin-B-binding motif displayed >50% reduction in membrane activity compared with the WT channel (Figure 4K; n=7; P<0.05). These data support the requirement of a direct ankyrin-B interaction for Cav1.3 membrane targeting. However, these data also suggest that an ankyrin-independent pathway(s) also plays a critical role in Cav1.3 membrane delivery because Cav1.3 lacking ankyrin binding still displayed a greatly reduced but detectable current.

**Loss of Ica,L Alters APD90 in Ankyrin-B+/- Atrial Myocytes**

Our results identify a direct association of ankyrin-B with Cav1.3 and a striking reduction of Cav1.3 and Ica,L in primary ankyrin-B+/- atrial cardiomyocytes. To test whether observed differences in Ica,L in ankyrin-B+/- atrial myocytes are sufficient to account for dramatic action potential shortening and AF susceptibility, we performed computational modeling of WT and ankyrin-B+/- atrial action potentials (Figure 5). We first incorporated experimentally measured changes in Ica,L (Figure 2) and INa,CX (Figure II in the online-only Data Supplement) and reduction in Na+,K+-ATPase expression12,22 into a physiological and well-validated model of the atrial action potential from Nattel and colleagues.23 Decreasing all 3 currents was sufficient to decrease the action potential nearly 25%, similar to the observed differences in APDs between ankyrin-B+/- and WT atrial myocytes (Figure 5B and 5G). We next simulated action potentials (B) and calcium transients (C) from wild-type (WT) and AnkB+/- atrial myocytes are shown. Consistent with experimental data, the AnkB+/- model shows decreased Ica,L (D, H), INa,CX(E, I), and INa,K (F, J) and action potential duration (APD) at 90% of repolarization (AnkB+/- APD 75% of WT in model compared with 75±10% in experiment) (G). K, Ica,L,INa,CX, and INa,K were altered independently to determine the mechanism for APD shortening in AnkB+/- atrial myocytes. Decreasing Ica,L alone shortened APD by nearly the same amount as observed in the AnkB+/- model. LTCC indicates L-type calcium channels; JSR, junctional sarcoplasmic reticulum; NSR, network sarcoplasmic reticulum.
I_{Ca,L}, alone shortened APD by nearly the same amount as observed in the ankyrin-B--/- model, indicating that loss of I_{Ca,L} is the dominant mechanism for APD shortening in ankyrin-B+/- myocytes (Figure 5K).

**Patients With Common Atrial Fibrillation Display Reduced Ankyrin-B Expression**

Finally, as a first step to further investigate the linkage of ankyrin-B with human AF in the general population, we examined the protein expression of ankyrin-B in the right atria of patients with normal sinus rhythm versus patients with paroxysmal AF. Patients with documented paroxysmal AF displayed striking reductions in ankyrin-B expression compared with individuals in normal sinus rhythm (Figure 6A and 6B; n=12 patients in sinus rhythm; n=10 patients with paroxysmal AF; P<0.05). Notably, we also observed a significant reduction in Ca_{1.3} protein expression levels in paroxysmal AF samples (Figure 6C and 6D; n=12 patients in sinus rhythm; n=10 patients with paroxysmal AF; P<0.05). Together, these findings provide additional data that associates reduced ankyrin-B function with human atrial disease.

**Discussion**

Ankyrin-B, required for the targeting and stability of critical membrane and submembrane molecules, is important for normal membrane excitability in multiple cardiac cell types. The multifunctional capacity of ankyrin-B is evident in the strong link between ankyrin dysfunction and a spectrum of cardiac disorders collectively referred to as ankyrin-B syndrome. In this study, we identify the L-type calcium channel 1.3 (α1D) as a novel ankyrin-B-binding partner and provide an association between decreased ankyrin-B function and AF. Specifically, we demonstrate early-onset familial AF in human patients with ANK2 missense mutation. Ankyrin-B--/- mice display similar atrial dysfunction including increased incidence of spontaneous AF episodes and enhanced susceptibility to burst pacing–induced AF. At the cellular level, ankyrin-B--/- atrial myocytes exhibit decreased I_{Ca,L} and shortened APD, both hallmarks of clinical AF. Underlying the reduced I_{Ca,L} in ankyrin-B--/- atrial myocytes, there is a selective loss of both protein expression and membrane targeting of Ca_{1.3} but not Ca_{1.2}. This selective interaction is mediated by a unique motif in the C-terminal domain of Ca_{1.3} that is both necessary and sufficient for ankyrin binding. Thus, ankyrin-B–dependent membrane targeting of Ca_{1.3} is required for normal atrial I_{Ca,L} and atrial function. Although ankyrin-B haploinsufficiency in atrial myocytes reduces NCX and Na+/K+-ATPase currents, ankyrin-B–dependent reduction in I_{Ca,L} appears primarily responsible for shortened APDs, as suggested by computational modeling.

There is a strong association between reduced I_{Ca,L} and AF. Although it is unclear whether I_{Ca,L} dysfunction is a cause or effect of AF, there are numerous examples of AF linked to reduced I_{Ca,L}. Atrial tissues from human AF patients display reduced mRNA expression of L-type Ca^{2+}+ channels Ca_{1.2} and Ca_{1.3} in addition to an overall decrease in I_{Ca,L}. In addition, patients with loss-of-function mutations in L-type Ca^{2+}+ channel Ca_{1.2} manifest complex cardiac phenotypes including AF. Similarly, atrial myocytes from Ca_{1.3}--/- mice exhibit reduced I_{Ca,L}, a depolarizing shift in voltage-dependent I_{Ca,L} activation (Ca_{1.3} activates at more negative potentials than Ca_{1.2}), and increased AF susceptibility on burst-pacing stimulation. Given the increased susceptibility to AF in Ca_{1.3}--/- mice and our findings that Ca_{1.3} membrane targeting is regulated by a direct interaction with ankyrin-B, it will be critical in future experiments to determine the direct role of Ca_{1.3} dysfunction in human ANK2-related atrial disease.

An intriguing finding from this study is that despite overlapping expression patterns of the L-type calcium channel α-subunits in atrial cells, ankyrin-B preferentially interacts with Ca_{1.3} and not Ca_{1.2} in atrial myocytes. A second interesting observation is that Ca_{1.3} is expressed, albeit to a lesser extent, in membranes of ankyrin-B--/- cells, suggesting that ankyrin-independent mechanisms also contribute to Ca_{1.3} membrane expression. These observations are consistent with L-type calcium channels interacting with a variety of signaling and scaffolding proteins that influence membrane expression. Some of the molecular mechanisms that regulate L-type calcium channel membrane expression include channel auxiliary subunits (β-subunits), signaling molecules (calmodulin), and scaffolding/adapter proteins (α-actinin2, A-kinase anchoring protein 79).
Conclusions

We propose that ankyrin-B dysfunction is associated with AF on the basis of our findings that (1) a loss-of-function ANK2 mutation is associated with highly penetrant AF; (2) ankyrin-B haploinsufficient mice exhibit increased susceptibility to AF; (3) ankyrin-B–deficient atrial myocytes display shortened action potentials, a hallmark of clinical AF; (4) ankyrin-B−/− atrial myocytes exhibit reduced expression and membrane targeting of Ca$_2$1.3, an L-type calcium channel subunit previously associated with increased AF susceptibility; and (5) patients with paroxysmal AF demonstrate reduced ankyrin-B. Collectively, these findings suggest that ankyrin-B dysfunction may account for cases of monogenic AF in the general human population and that ANK2 should be considered for familial AF screening.

Acknowledgments

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Disclosures

None.

References

Atrial fibrillation (AF) is the most prevalent sustained arrhythmia in clinical practice. In fact, in the United States alone, AF is present in >2 million individuals. Despite the high incidence of AF in the population, surprisingly little is known regarding the molecular mechanisms underlying this complex disease. Ankyrin proteins target and stabilize proteins at specialized membrane domains. Notably, dysfunction in ankyrin- and ankyrin-associated pathways has been linked with disorders including spherocytosis, spinocerebellar ataxia, diabetes mellitus, neurological deficits, and cardiac arrhythmias. Nearly a decade ago, ankyrin-B (ANK2) was discovered as a critical component of heart, and work in humans and mice has implicated ankyrin-B as critical for cardiac function. In fact, human ANK2 loss-of-function variants are associated with potentially fatal ventricular arrhythmias. In the present study, we demonstrate the importance of ankyrin-B for atrial function and identify an association between ankyrin-B dysfunction and AF. Individuals harboring ANK2 variants display AF, and these phenotypes are reproduced in mice deficient in ankyrin-B. Ankyrin-B is expressed in the atria, and ankyrin-B−/− myocytes display shortened action potentials, a hallmark of AF, and decreased L-type calcium channel current. We show that Cα,1.3, responsible for 1 component of L-type calcium channel current in atria, is a novel ankyrin-binding partner and that Cα,1.3 expression/activity is reduced in ankyrin-deficient atrial myocytes. Finally, ankyrin-B is reduced in atrial samples from human AF patients, further supporting the role of ankyrin-B in normal atrial function. Together, our work implicates ankyrin-B as a surprising yet critical component of atrial excitability and supports the role of atypical myocyte proteins in disease pathogenesis.
Defects in Ankyrin-Based Membrane Protein Targeting Pathways Underlie Atrial Fibrillation

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Supplemental Methods

In vivo electrophysiology in mice. *In vivo* electrophysiology studies were performed as previously described.\(^1\) Briefly, atrial and ventricular intracardiac electrograms were recorded using a 1.1F octapolar catheter (EPR-800, Millar Instruments, Houston, Texas) inserted via the right jugular vein. Surface and intracardiac electrophysiology parameters were assessed at baseline. Right atrial pacing was performed using 2-ms current pulses delivered by an external stimulator (STG-3008, Multi Channel Systems, Reutlingen, Germany). Atrial fibrillation (AF) was determined using the protocol described\(^2\), and defined as the occurrence of rapid and fragmented atrial electrograms with irregular AV-nodal conduction and ventricular rhythm for at least 1 second. Inducibility of AF was considered positive if at least 2 of 3 pacing trials induced AF.\(^3\)

Calcium current measurements. Atrial myocytes: For formation of gigaohm seals and initial perforation- cells were perfused with normal Tyrode solution containing (mM): 138 NaCl, 4 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 0.33 NaH\(_2\)PO\(_4\), 10 HEPES, and 10 glucose (pH 7.4, adjusted with NaOH). Following successful perforation the perfusing medium was switched to an external recording solution containing (mM): 137 N-methyl-D-glucamine aspartate (NMG-Asp), 10 Glucose, 10 HEPES, 1.8 CaCl\(_2\), 0.5 MgCl\(_2\), 25 CsCl (pH 7.4, adjusted with NMG). Signals were filtered at 2 kHz. Data traces were acquired at a repetition interval of 2-s from -70 to +60 with 10 mV increment at -80 holding potential.\(^4\) Fibroblasts: Recordings were acquired at RT. For whole-cell \(I_{\text{Ca,L}}\) recordings, electrodes were filled with an internal solution containing (mM): 150 Cs-MeSO\(_3\), 5 CsCl, 10 HEPES, 10 EGTA, 1 MgCl\(_2\), 4 MgATP (pH 7.2, adjusted with CsOH). The bath solution contained (in mM): 135 Choline Chloride, 10 HEPES, 5 (or 10) CaCl\(_2\), 1 MgCl\(_2\), 25 CsCl (pH 7.4, adjusted with TEA-OH). Like in case with atrial myocytes signals were filtered at
Atrial myocyte computational modeling. Electrical activity of the atrial myocyte was simulated using a well-validated mathematical model of the mammalian atrial action potential.\textsuperscript{6,7} Numerical integration of model equations was performed with a modified Euler method and a fixed time step of 0.005 ms.\textsuperscript{6,8,9}

\textit{Ca}_{v}1.3 and \textit{Ca}_{v}1.2 cDNA constructs.} cDNA constructs of rat \textit{Ca}_{v}1.3 intracellular domains were generated by PCR amplification of \textit{Cacna1d} (NM_017298.1). PCR products were subcloned into pEGFP-N3 (Clontech) to append a C-terminal GFP and this product was subcloned into pcDNA3.1\textsuperscript{+} (Invitrogen) for \textit{in vitro} translation (Promega) with \textsuperscript{35}S L-methionine (PerkinElmer). Amino acids of the \textit{Ca}_{v}1.3 intracellular domains are N1 (1-127); L1 (406-583), L2 (811-946), L3 (1207-1263), C1 (1504-1670), C2 (1671-1853), C3 (1854-2019), and C4 (2014-2202). A similar procedure was performed to generate a protein construct of the terminal ~200 amino acids of rat \textit{Ca}_{v}1.2 (\textit{Cacna1c}: NM_012517.2).

\textit{Immunoblots.} Protein lysates were isolated from atria and ventricles of WT and heterozygous ankyrin-B mice as described.\textsuperscript{10} Equal amounts of protein lysates were separated by SDS-PAGE and immunoblots were performed to detect the protein levels of \textit{AnkB} (1:500\textsuperscript{11}), \textit{Ca}_{v}1.2 (1:250; Alomone Labs), \textit{Ca}_{v}1.3 (1:250; Alomone Labs); \textit{Na}_{v}1.5 (1:1000\textsuperscript{12}), and \textit{NHERF1} (1:2000; Sigma) (see Supplemental Figure 4 for expanded view of representative immunoblots). All antibodies utilized have been tested for specificity using either gene-specific knock-out mice or gene-specific/deficient cell lines. For human atrial samples, primary antibodies against ankyrin-B (1:1000; raised in
mouse) were used to quantify corresponding proteins in atrial-tissue homogenates. Peroxidase-conjugated goat anti-mouse (1:5000) and goat anti-rabbit (1:5000), respectively, were used as secondary antibodies and visualized by chemi-fluorescence (GE-Healthcare, Chalfont St Giles, UK). Quantity-One-Software (Bio-Rad, Hercules, USA) was used for quantification. Calsequestrin expression (1:2500 anti-CSQ, Dianova, Hamburg, Germany; 1:30,000 goat anti-rabbit) was quantified as loading control.

**Action Potentials.** Action potentials (APs) were evoked by brief current pulses 1.5–4 pA, 0.5–1 ms. AP duration (APD) was assessed as the time from the AP upstroke to 90% repolarization to baseline (APD$_{90}$). APs were recorded using the perforated (amphotericin B) patch-clamp technique in Tyrode’s solution (bath) with the pipette filled with (mmol/L): 130 potassium aspartate, 10 NaCl, 10 HEPES, 0.04 CaCl$_2$, 2.0 MgATP, 7.0 phosphocreatine, 0.1 NaGTP, and amphotericin B 240 μg/mL, with the pH adjusted to 7.2 with KOH. APDs were measured at both 22° C and 37° C.

**In vitro binding assays.** In vitro binding assays were performed as previously described using GST-fusion proteins of ankyrin-B or ankyrin-G membrane-binding domains and $^{35}$S-labelled calcium channel protein products. Reactions were performed at 4°C for 3 hours in a high stringency binding buffer (50 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 500 mM NaCl, 0.1% Triton X-100), washed 5 times in a high stringency wash buffer (1 M NaCl binding buffer), separated by SDS-PAGE, and visualized by autoradiography. In vitro binding assays with biotinylated-peptides were performed in 150 mM NaCl binding buffer overnight at 4°C, washed 5 times in 250 mM NaCl wash buffer, separated by SDS-PAGE, and visualized by autoradiography.
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Unsequenced affected patients. AF, atrial fibrillation; HR, heart rate; CSR, coronary sinus rhythm; JER, junctional escape rhythm; LBBB, left bundle branch block; SD, sudden death. Data as of 2008. Table created using cardiac phenotype data from two large families harboring variant ANK2 alleles (LeScouarnec et al., PNAS, 2008).
Supplemental Table 2. Characteristics of patients for expression studies (Figure 6)

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CAD, coronary artery disease; MVD/AVD, mitral/aortic valve disease; LVEF, left ventricular ejection fraction; LVEDD, left ventricular end-diastolic diameter; LAD, left atrial diameter; IVS, interventricular septum thickness; LVPW, left ventricular posterior wall thickness; ACE, angiotensin-converting enzyme; AT, angiotensin receptor.
**Supplemental Figure 1**

(A) Action potentials, and (B) summary data on APD90 from WT (black) and AnkB+/− (red) atrial myocytes. Action potentials were measured using perforated patch as described in Methods at 37°C. Consistent with measurements at room temperature, AnkB+/− atrial myocytes display reduced APD at physiological temperature (n=6 for WT, n=5 for AnkB+/−, *p<0.05).

**Supplemental Figure 2**

Figure 2. AnkB+/− atrial myocytes display reduced $I_{NCX}$. Levels in this graph are compared at 0 mV (n=8, p<0.05).
Figure 3. Cav1.3, but not Cav1.2 membrane function is affected by ankyrin-B deficiency. Ca^{2+} currents were measured from (A) WT fibroblasts and (B) AnkB-deficient fibroblasts transfected with Cav1.2 + beta2 subunit (black) or Cav1.3 + beta2 subunit (red). Cav1.3 peak current is similar to Cav1.2 in WT cells but significantly reduced in AnkB^{+/−} fibroblasts (n=5 for WT, n=6 AnkB^{+/−}, *p<0.05).

Supplemental Figure 4

Figure 4. Expanded view of blots for immunoblot data presented in Figure 2. All antibodies utilized have been tested for specificity using either gene-specific knock-out mice or gene-specific/deficient cell lines.
Supplemental References

5. Wu Y, Dzhura I, Colbran RJ, Anderson ME. Calmodulin kinase and a calmodulin-binding 'iq' domain facilitate l-type ca2+ current in rabbit ventricular myocytes by a common mechanism. *J Physiol*. 2001;535:679-687
11. Mohler PJ, Gramolini AO, Bennett V. The ankyrin-b c-terminal domain determines activity of ankyrin-b/g chimeras in rescue of abnormal inositol 1,4,5-trisphosphate and ryanodine receptor distribution in ankyrin-b (-/-) neonatal cardiomyocytes. *J Biol Chem*. 2002;277:10599-10607
Ankyrin의 이상이 심방세동 발생에 관여한다

오 세 일 교수 서울대학교병원 순환기내과

Summary

배경
심방세동은 가장 흔한 부정맥으로, 미국에서만 200만 명 이상의 환자가 있다. 수십 년 간의 연구에도 불구하고, 심방세동의 병태생리에서의 분자적 경로에 대해서는 알려진 바가 거의 없다. ANK2 유전자에 의해 발현되는 ankyrin-B는 다기능을 가진 연결 분자로 세포막의 이온 통로, 전달체, 신호전달 물질 등에 관여한다.

방법 및 결과
본 연구에서 연구자들은 심방세동이 조기에 발생한 환자에서 ANK2의 기능이 없어지는 변이를 보고하였다. 쥐에서 ankyrin-B 결핍은 심방의 전기생리학적인 기능이상을 초래하였으며, 심방세동에 대한 취약성을 증가시켰다. 게다가 ankyrin-B는 Ca1.3에 특이한 짧고 매우 보존된 모티프에 의해 조절된다. 게다가 심방에서 ankyrin-B의 상실은 Ca1.3의 발현, 세포막 국소화 및 기능 감소를 유발하여 심방의 활동전 위 단축과 부정맥 유발에 충분하였다. 마지막으로, 연구 자들은 심방세동 환자들의 심방조직에서 ankyrin-B의 발 현이 감소되어 있음을 증명하여 ankyrin-B와 심방세동의 연관성을 입증하였다.

결론
이들 소견은 ankyrin-B의 발현 감소 또는 변이가 심방세 동과 연관 있음을 의미한다. 또한, 연구자들은 심방에서 Ca1.3 통로의 세포막 표적화 및 조절이 ankyrin-B 의존 적임을 새로이 규명하였다.

Reference
Defects in Ankyrin-Based Membrane Protein Targeting Pathways Underlie Atrial Fibrillation

Shane R. Cunha, PhD; Thomas J. Hund, PhD; Seyed Hashemi, MD; Niel V oigt, MD; Na Li, MD; Patrick Wright, BS; Olha Koval, PhD; Jingdong Li, MD; Hjalene Gumundsson, MD; Richard J. Gumina, MD, PhD; Matthias Karck, MD; Jean-Jacques Schott, PhD; Vincent Probst, MD, PhD; Herve Le Marec, MD, PhD; Mark E. Anderson, MD, PhD; Dobromir Dobrev, MD; Xander H.T. Wehrens, MD, PhD; Peter J. Mohler, PhD

Background—Atrial fibrillation (AF) is the most common cardiac arrhythmia, affecting >2 million patients in the United States alone. Despite decades of research, surprisingly little is known regarding the molecular pathways underlying the pathogenesis of AF. ANK2 encodes ankyrin-B, a multifunctional adapter molecule implicated in membrane targeting of ion channels, transporters, and signaling molecules in excitable cells.

Methods and Results—In the present study, we report early-onset AF in patients harboring loss-of-function mutations in ANK2. In mice, we show that ankyrin-B deficiency results in atrial electrophysiological dysfunction and increased susceptibility to AF. Moreover, ankyrin-B+/− atrial myocytes display shortened action potentials, consistent with human AF. Ankyrin-B is expressed in atrial myocytes, and we demonstrate its requirement for the membrane targeting and function of a subgroup of voltage-gated Ca2+ channels (Ca1.3) responsible for low voltage-activated L-type Ca2+ current. Ankyrin-B is associated directly with Ca1.3, and this interaction is regulated by a short, highly conserved motif specific to Ca1.3. Moreover, loss of ankyrin-B in atrial myocytes results in decreased Ca1.3 expression, membrane localization, and function sufficient to produce shortened atrial action potentials and arrhythmias. Finally, we demonstrate reduced ankyrin-B expression in atrial samples of patients with documented AF, further supporting an association between ankyrin-B and AF.

Conclusions—These findings support that reduced ankyrin-B expression or mutations in ANK2 are associated with AF. Additionally, our data demonstrate a novel pathway for ankyrin-B-dependent regulation of Ca1.3 channel membrane targeting and regulation in atrial myocytes. (Circulation. 2011;124:1212-1222.)

Key Words: arrhythmia (mechanisms) ☐ cytoskeleton ☐ ion channel ☐ protein trafficking

Atrial fibrillation (AF), which is characterized by rapid and irregular activation of the atrium, is the most common sustained arrhythmia found in clinical practice, affecting ~2.3 million people in the United States alone.1 The prevalence of AF increases with age, with ~2% to 6% of people aged >65 years exhibiting AF.2,4 Given the demographics of the United States, it is predicted that there will be a 5-fold increase in the prevalence of AF by 2050.4 Although AF is frequently associated with other cardiac disorders including coronary artery disease, mitral valve disease, congenital heart disease, and congestive heart failure, ~30% of all AF cases are described as lone AF, in which patients exhibit no previous cardiac pathology.4 The majority of monogenic AF cases described to date are linked to mutations that affect potassium channels, although these cases represent only a small fraction of total monogenic AF cases.6

Clinical Perspective on p 47

Ankyrin-B (encoded by ANK2) is an adapter protein expressed in a number of excitable cells including neurons,
cardiomyocytes, and pancreatic β cells.7 Given that ankyrin-B targets select ion channels, transporters, and signaling molecules to specific membrane domains, it is not surprising that dysfunction in the ankyrin-B cellular pathway has been linked to neuronal defects, ventricular arrhythmia, and neonatal diabetes.8–10

In the present study, we demonstrate a critical role for ankyrin-B in atrial function. Individuals harboring loss-of-function mutations in ANK2 developed early-onset AF. Ankyrin-B-deficient mice phenocopied ANK2-based human disease and displayed striking susceptibility to AF. Primary atrial myocytes from ankyrin-B-deficient mice displayed shortened action potentials, a hallmark of AF, and this decrease was associated with loss of L-type Ca2+ current (Ica,L). Our data revealed that Ca2,1,3 is a novel ankyrin-B-binding partner, and the structural requirements for ankyrin-B/Ca2,1,3 association were mapped. Specifically, a unique C-terminal motif in Ca2,1,3 was found to be necessary and sufficient for ankyrin binding. In ankyrin-B−/− atrial myocytes, Ca2,1,3 protein expression and membrane function were reduced. Moreover, we demonstrated that ankyrin-B levels are reduced in human AF, providing an association between ankyrin-B and the pathogenesis of AF. Together, our data describe a novel mechanism for Ca2,1,3 membrane targeting in primary atrial cardiomyocytes and reveal a potential molecular mechanism underlying ankyrin-associated AF.

**Methods**

**Immunofluorescence**

Myocytes were washed with phosphate-buffered saline (pH 7.4) and fixed in warm 2% paraformaldehyde (37°C). Cells were blocked/permeabilized in phosphate-buffered saline containing 0.075% Triton X-100 and 3% fish oil gelatin (Sigma) and incubated in primary antibody overnight at 4°C. After washes (phosphate-buffered saline plus 0.1% Triton X-100) cells were incubated in secondary antibody (Alexa 488 and 568 and ToPro-3AM 633) for 8 hours at 4°C and mounted with the use of Vectashield (Vector). Images were collected on a Zeiss 510 Meta confocal microscope (40-power oil, 1.40 numerical aperture, pinhole equals 1.0 Airy Disc) with Zeiss Imaging software. Images were imported into Adobe Photoshop for cropping and linear contrast adjustment. Imaging experiments were performed at least 3 times for each experimental protocol, and >20 myocytes were examined from each experimental set.

**Immunoblots**

Protein lysates were isolated from atria and ventricles of wild-type (WT) and heterozygous ankyrin-B mice as described.11 Antibodies and tissues are described in the online-only Data Supplement.

**Cellular Electrophysiology**

Membrane currents and action potentials were measured with an Axon 200B patch-clamp amplifier controlled by a personal computer with the use of a Digidata 1320A acquisition board driven by pClamp 8.0 software (Axon Instruments). Electrophysiological recordings were obtained only from Ca2+−tolerant, rod-shaped atrial cells. We used a perforated (amphotericin B) patch for calcium current and action potential studies. All experiments were conducted at room temperature, unless otherwise noted. Other currents were measured as described.12 Recording pipettes, fabricated from borosilicate glass, had resistance of 2 to 4 MΩ when filled with recording solution. All solutions were adjusted to 275 to 295 mOsm.

**Statistical Analysis**

For human data, differences between group means were compared by unpaired Student t test. Frequency data were analyzed with the Fisher exact test. The Kolmogorov-Smirnov test was used to test whether data followed a normal distribution. Assumption of equal variance per group was confirmed with the use of Bartlett’s test. Data are mean ± SEM. P < 0.05 was considered statistically significant. For nonhuman data, all values are presented as mean ± SEM. P values were assessed with a paired Student t test (2-tailed) or ANOVA, as appropriate, for continuous data. The Bonferroni test was used for post hoc testing. The null hypothesis was rejected for P < 0.05.

**Human Tissue Samples**

Human tissue experimental protocols were approved by the ethics committee of the Medical Faculty Mannheim, University of Heidelberg (No. 2011–216N-MA). Each patient gave written informed consent. During routine cannulation procedures in patients undergoing open-heart surgery for cardiac bypass grafting and valve replacement, the tip of the right atrial appendage was removed and immediately snap-frozen in liquid nitrogen. Appendages were obtained from 12 sinus rhythm and 10 paroxysmal AF (Table II in the online-only Data Supplement) patients. The AF group included patients in sinus rhythm at surgery with a history of at least 1 episode of self-terminating AF lasting <7 days (paroxysmal AF patients). Additional methods are presented in the online-only Data Supplement.

**Results**

Ankyrin-B Dysfunction Is Associated With Human AF

We identified a high incidence of AF in ANK2 mutation-positive probands. Probands harboring ANK2 loss-of-function alleles displayed early-onset AF, commonly progressing to permanent AF.9,13,14 In 1 large kindred (74 members), 13 of 25 ANK2 loss-of-function variant carriers (phenotypes previously mapped to ANK2; Zmax = 7.059) displayed AF (mean onset, 40 ± 18 years; 5 paroxysmal, 8 permanent), whereas noncarriers were asymptomatic. Only 2 individuals were nonpenetrant for atrial phenotypes. The others had AF, AF with sinus node dysfunction, or sinus node dysfunction alone. In an unrelated large family, 20 of 36 individuals were positive for the ANK2 disease allele (maximal logarithm of odds score was for marker D4S1616; linkage Zmax = 5.9, θ = 0), and 3 of 20 disease allele carriers displayed AF (mean onset, 48 ± 12 years; 2 paroxysmal, 1 permanent), whereas 13 displayed sinus node disease requiring pacemaker implantation (mean age for implantation, 30 ± 18 years). Only 4 individuals were nonpenetrant for atrial phenotypes. In addition to AF, prior work demonstrates that ANK2 variant carriers may display prolonged ventricular QT intervals, which predisposes individuals to ventricular arrhythmias (examples of cardiac phenotypes are presented in Table I in the online-only Data Supplement).9,13 Because AF and long-QT syndrome are often associated with opposing action potential characteristics (ie, AF with shortened atrial action potentials and long-QT syndrome with prolonged ventricular action potentials), we hypothesized that ankyrin-B might uniquely affect atrial ion channels, resulting in an increased susceptibility to AF.
C-terminal motif in Cav1.3 was found to be necessary and describe a novel mechanism for Cav1.3 membrane targeting reduced. Moreover, we demonstrated that ankyrin-B levels measured as described.12 Recording pipettes, fabricated from boro-
at room temperature, unless otherwise noted. Other currents were
cells. We used a perforated (amphotericin B) patch for calcium
function mutations in 
shortened action potentials, a hallmark of AF, and this

demonstrated that ankyrin-B might

- develop early-onset AF.
- ANK2
- based human
- atrial myo-
- ANK2
- loss-of-function
- ANOVA, as appropriate, for continuous data. The Bonferroni test
- Fisher exact test. The Kolmogorov-Smirnov test was used to test
- ECG (top) and atrioventricular electrogram recording (bottom) in WT (left) and AnkB
- Mice Deficient for Ankyrin-B Expression Display Atrial Dysfunction

Previous studies have demonstrated that human ANK2

gene mutations linked with arrhythmia behave as loss of

function when analyzed in primary cardiomyocytes.9,12-14 Therefore, we assessed the role of ankyrin-B deficiency in
atria of mice heterozygous for a null mutation in ankyrin-B

(ankyrin-B−/− mice, because ankyrin-B−/− mice die at birth). Continuous telemetry ECG recordings from conscious ankyrin-B−/− mice revealed atrial arrhythmias also

present in human carriers of ANK2 variants, including spontaneous bradycardia with erratic atrial activity, lack of
discrete P waves, and variable ventricular response (Figure

1A through 1D).

Figure 1. Ankyrin-deficient mice display atrial dysfunction and atrial fibrillation (AF) inducibility. A to D, Conscious ECGs of wild-
type (WT) (A and B) and ankyrin B (AnkB)−/− mice (C and D) recorded by telemetry. WT mice show normal sinus rhythm, whereas
AnkB−/− mice display spontaneous bradycardia with erratic atrial activity, including absence of discreet P waves and variable
ventricular responses indicative of spontaneous AF. B and D represent red magnified areas of A and C. E, Simultaneous surface
EGG (top) and atrioventricular electrogram recording (bottom) in WT (left) and AnkB−/− mice (right) are shown. Top, Lead I EGG
from WT and AnkB−/− mice shows rapid atrial electric activity and the variability in RR interval in AnkB−/− mouse compared with
WT mouse. F, Incidence of AF in WT and AnkB−/− mice during burst pacing. Whereas WT mice displayed low incidence of AF (2
of 9 mice), AnkB−/− mice were highly susceptible to AF induction (11 of 15 mice; *P<0.05 for AF susceptibility in WT vs AnkB−/−
mice).
We next tested the susceptibility to atrial arrhythmias in ankyrin-B \(-/-\) mice after atrial burst pacing. Intracardiac electrograms were recorded simultaneously with the surface ECG to confirm the nature of abnormal atrial electric activities. Periods of pacing-induced AF were observed in the majority (\(\geq 75\%\)) of ankyrin-B \(-/-\) mice (11 of 15 mice; Figure 1E and 1F). In contrast, a significantly lower fraction of WT mice demonstrated AF inducibility with the use of this protocol (2 of 9 WT mice; \(P < 0.05\) versus ankyrin-B \(-/-\); Figure 1F). Ankyrin-B \(-/-\) atrial episodes showed characteristic absence of P waves and RR-interval variability. Moreover, AF/atrial tachycardia was confirmed by the presence of rapid and irregular A waves on the atrioventricular electrogram (Figure 1E). Together, the electrophysiological data in human ANK2 mutation carriers and ankyrin-B \(-/-\) mice clearly demonstrate that ankyrin-B dysfunction is detrimental for normal atrial electric activity.

**Ankyrin-B Is Expressed in Atria**

We used immunoblot to identify ankyrin-B expression patterns in human atria. Ankyrin-B was expressed in all human heart chambers at similar levels (Figure 2A). Cav1.2 expression is greater in ventricles than atria, whereas Cav1.3 expression is greater in atria than ventricles. NHERF1 expression demonstrates equal loading between the samples. Localization of ankyrin-B in a single mouse atrial myocyte. Note that ankyrin-B is expressed at both the sarcolemma (yellow arrows) and M-line domains (white arrows). Bar=10 \(\mu\)m. C and D, Ankyrin-B \(-/-\) atrial myocytes display reduced action potential duration (APD) at 22°C (WT, \(n = 7\); ankyrin-B \(-/-\), \(n = 8\); \(P < 0.05\)). E and F, Ankyrin-B \(-/-\) atrial myocytes display reduced \(I_{Ca,L}\) compared with WT atrial myocytes (WT, \(n = 7\); ankyrin-B \(-/-\), \(n = 8\); \(P < 0.05\)). G and H, WT and ankyrin-B \(-/-\) atrial myocytes display no difference in \(I_{Na}\) (WT, \(n = 7\); ankyrin-B \(-/-\), \(n = 8\); \(P < 0.05\)).
Ankyrin-B \( ^{\pm/ -} \) Atrial Myocytes Display Aberrant Atrial Electric Activity

To define the role of ankyrin-B in atria, we evaluated action potentials from isolated WT and ankyrin-B \( ^{\pm/ -} \) atrial cardiomyocytes. Notably, ankyrin-B \( ^{\pm/ -} \) atrial myocytes displayed significantly reduced action potential duration (APD) (measured at 90% of repolarization [APD\(_{90} \)] compared with WT atrial cardiomyocytes (Figure 2C and 2D). In fact, APD\(_{90} \) was reduced \( \sim 25\% \) in ankyrin-B \( ^{\pm/ -} \) atrial cardiomyocytes at both room (Figure 2C and 2D; \# = 7 WT, \# = 8 ankyrin-B \( ^{\pm/ -} \); \( P < 0.05 \)) and physiological temperatures (Figure I in the online-only Data Supplement; \# = 6 WT, \# = 5 ankyrin-B \( ^{\pm/ -} \); \( P < 0.05 \)), a cellular phenotype consistent with that observed in AF patients.\(^9\) In contrast, prior studies revealed that ankyrin-B \( ^{\pm/ -} \) ventricular cardiomyocytes do not display significant differences in APD\(_{90} \) compared with WT.\(^9\)

Atrial myocyte depolarization and repolarization are regulated by the synchronized activities of membrane-associated ion channels, transporters, and pumps. To define the molecular basis of action potential shortening in ankyrin-B \( ^{\pm/ -} \) atrial myocytes, we investigated the activities of a number of critical atrial myocyte currents. We observed a significantly reduced I\(_{\text{Ca,L}} \) in ankyrin-B \( ^{\pm/ -} \) atrial myocytes (Figure 2E and 2F; \# = 7 WT, \# = 8 ankyrin-B \( ^{\pm/ -} \); \( P < 0.05 \)) in contrast to ankyrin-B \( ^{\pm/ -} \) ventricular cardiomyocytes that display normal I\(_{\text{Ca,L}} \) and APD.\(^9\) We observed no difference in I\(_{\text{Na}} \) (Figure 2G and 2H; \# = 7 WT, \# = 8 ankyrin-B \( ^{\pm/ -} \); \( P = \) NS), total I\(_{Kv} \), or I\(_{\text{to}} \) (transient, outward) between WT and ankyrin-B \( ^{\pm/ -} \) atrial myocytes (\# = 7 WT, \# = 8 ankyrin-B \( ^{\pm/ -} \); \( P = \) NS; not shown). However, we observed a significant decrease in Na\(^+\)/Ca\(^{2+}\) exchange current (I\(_{\text{Na,Ca}} \)) consistent with previous findings in ankyrin-B\(-/-\) deficient ventricular myocytes (Figure II in the online-only Data Supplement; \# = 8 WT, \# = 8 ankyrin-B \( ^{\pm/ -} \); \( P < 0.05 \)).\(^9\) Thus, atrial ankyrin-B \( ^{\pm/ -} \) myocytes display decreased APD and reduced I\(_{\text{Ca,L}} \). Both phenotypes are unique to ankyrin-B \( ^{\pm/ -} \) atrial cells compared with ventricular myocytes.

Reduced Ca\(_{1.3} \) Expression in Ankyrin-B \( ^{\pm/ -} \) Atria

In atrial myocytes, I\(_{\text{Ca,L}} \) is composed of the combined activities of Ca\(_{1.2} \) (\( \alpha 1C \)) and Ca\(_{1.3} \) (\( \alpha 1D \)). On the basis of reduced I\(_{\text{Ca,L}} \) in ankyrin-B \( ^{\pm/ -} \) atrial cardiomyocytes, we examined the expression and localization of both Ca\(_{1.2} \) and Ca\(_{1.3} \) in WT and ankyrin-B \( ^{\pm/ -} \) mouse atria. Consistent with electrophysiological data, we observed a significant decrease in Ca\(_{1.3} \) expression in ankyrin-B \( ^{\pm/ -} \) atria (reduced \( \sim 30\% \); \# = 3; \( P < 0.05 \); Figure 3A and 3B), whereas Ca\(_{1.2} \) expression was unchanged between genotypes (Figure 3A and 3B; \( P = \) NS). Furthermore, in agreement with our earlier studies in ventricle,\(^9,19\) Na\(_{1.5} \) expression levels were not altered in ankyrin-B \( ^{\pm/ -} \) atrial cells (Figure 3A). Immunofluorescence of individual atrial myocytes with the use of channel-specific antibodies demonstrated that Ca\(_{1.3} \) but not Ca\(_{1.2} \), Ca\(_{3.1} \), or Na\(_{1.5} \) membrane expression was reduced (Figure 3C through 3F). In fact, we observed a \( \sim 40\% \) decrease in membrane staining intensity for Ca\(_{1.3} \) in ankyrin-B \( ^{\pm/ -} \) atrial cardiomyocytes compared with WT cells (Figure 3E; \( P < 0.05 \); \# = 5 WT, \# = 6 ankyrin-B \( ^{\pm/ -} \)).

As a final test to assess ankyrin-B function in Ca\(_{1.3} \) membrane targeting, we evaluated Ca\(_{1.3} \) membrane activity in primary cardiac fibroblasts from WT and ankyrin-B \( ^{\pm/ -} \) (homozygous null) mice (harvested before death of ankyrin-B \( ^{\pm/ -} \) neonatal mice). Primary fibroblasts were chosen to allow recording of I\(_{\text{Ca,L}} \) from exogenously expressed Ca\(_{1.3} \) without confounding native current from endogenous Ca\(_{2+} \) channels (note that WT and ankyrin-B \( ^{\pm/ -} \) primary fibroblasts display little endogenous I\(_{\text{Ca,L}} \) open circles in Figure 3G). As expected, WT fibroblasts expressing Ca\(_{1.3} \) displayed significantly greater I\(_{\text{Ca,L}} \) compared with nontransfected cells (Figure 3G; \# = 9 WT, \# = 7 ankyrin-B \( ^{\pm/ -} \) cells; \( P < 0.05 \)). In contrast, ankyrin-B \( ^{\pm/ -} \) primary cells expressing Ca\(_{1.3} \) showed \( > 50\% \) reduction in I\(_{\text{Ca,L}} \) (Figure 3G; \# = 9 WT, \# = 7 ankyrin-B \( ^{\pm/ -} \) cells; \( P < 0.05 \)) and reduced Ca\(_{1.3} \) membrane immunofluorescence (Figure 3H) compared with WT cells despite similar Ca\(_{1.3} \) protein expression in both cell types (based on immunoblot). These phenotypes were directly related to ankyrin-B expression because exogenous ankyrin-B expression restored I\(_{\text{Ca,L}} \) levels in ankyrin-B \( ^{\pm/ -} \) cells (Figure 3I and 3J; \# = 8; \( P < 0.05 \)). Notably, in contrast to Ca\(_{1.3} \), we observed no functional difference in Ca\(_{1.2} \) membrane expression between WT and ankyrin-B\(-/-\) deficient cells (Figure IIIA and IIIB in the online-only Data Supplement; \# = 5 WT, \# = 6 ankyrin-B \( ^{\pm/ -} \); \( P = \) NS).

Ankyrin-B Is Directly Associated With Ca\(_{1.3} \)

On the basis of electrophysiological data from primary myocytes and fibroblasts showing reduced Ca\(_{1.3} \) in ankyrin-B\(-/-\) atrial myocytes, we tested for a direct interaction between ankyrin-B and Ca\(_{1.3} \). Full-length Ca\(_{1.3} \) has 5 major cytoplasmic domains including an amino terminal domain, 3 cytoplasmic segments that connect the 4 major transmembrane regions (D1 through D4), and a large C-terminal domain (Figure 4A). We generated radiolabeled purified protein of each Ca\(_{1.3} \) cytoplasmic region (N1, L1 through L3, C1 through C4). Because of the extensive length of the C-terminal domain, this region was further subdivided into 4 segments for direct binding experiments (Figure 4A, C1 through C4). We observed significant association between glutathione-S-transferase-labeled ankyrin-B membrane-binding domain and only 1 intracellular region of Ca\(_{1.3} \), the C4 region of the C-terminus (residues 2014 to 2203; Figure 4B). This interaction was specific to ankyrin-B because it was not observed for the similar ankyrin-G membrane-binding domain or glutathione-S-transferase alone (Figure 4B). These data support a direct and specific interaction between ankyrin-B membrane-binding domain and the C-terminal domain of Ca\(_{1.3} \).

Although our functional data suggested a specific interaction of ankyrin-B with Ca\(_{1.3} \), Ca\(_{1.2} \) and Ca\(_{1.3} \) share significant sequence similarity in the distal C-terminal regions (residues 2014 to 2203 of Ca\(_{1.3} \); Figure 4C). We therefore tested for the interaction of the ankyrin-B membrane-binding domain with both Ca\(_{1.2} \) and Ca\(_{1.3} \) C-terminal domains (residues 1958 to 2159 of Ca\(_{1.2} \)). Consistent with our previous imaging, biochemical, and functional data, we observed no interaction of ankyrin-B or ankyrin-G with the Ca\(_{1.2} \) C-terminal domain (Figure 4D).
Figure 3. Reduced Ca,1,3 expression and targeting in ankyrin-B (AnkB)\textsuperscript{−/−} atria. A, Immunoblots to evaluate expression of AnkB, Na,1.5, Na-H exchange regulatory factor-1 (NHERF1), Ca,1.2, and Ca,1.3 protein in wild-type (WT) and AnkB\textsuperscript{−/−} mouse atria. Note reduced protein expression of AnkB and Ca,1.3 in AnkB\textsuperscript{−/−} atria compared with WT atria (n=3; P<0.05). No change was detected in the protein expression of Ca,1.2, Na,1.5, and NHERF1 between WT and AnkB\textsuperscript{−/−} atria (n=3; P=NS). B, Coomassie blue stain demonstrating equal loading of protein lysates from WT and AnkB\textsuperscript{−/−} atria. C through F, Immunolocalization of AnkB (C), Ca,1.2 (D), Ca,1.3 (E), and Ca,3.1 (F) in WT (left) and AnkB\textsuperscript{−/−} atrial myocytes (right). Note decreased membrane targeting of Ca,1.3 but not other membrane proteins (Ca,1.2, Ca,3.1) in AnkB\textsuperscript{−/−} atrial myocytes (n=5 WT, n=6 AnkB\textsuperscript{−/−}; P<0.05 for Ca,1.3). Bar=10 μm. G through J, AnkB expression is required for normal atrial myocyte \(I_{\text{Ca,L}}\) in WT and AnkB\textsuperscript{−/−} primary cells with and without exogenous Ca,1.3. Note that AnkB\textsuperscript{−/−} cardiac fibroblasts expressing Ca,1.3 display significant reductions in \(I_{\text{Ca,L}}\) compared with WT fibroblasts expressing identical Ca,1.3 cDNAs (n=9 WT, n=7 AnkB\textsuperscript{−/−}). \#P<0.05 for black symbols vs nontransfected cells.

*P<0.05 for red symbols vs nontransfected cells. ∆represents P<0.05 between black and green symbols (n=8 per group).
Figure 4. Ankyrin-B (AnkB) is directly associated with Ca_v1.3. **A**, Schematic representation of rat Ca_v1.3 and intracellular domains for in vitro binding assays. Intracellular domains correspond to amino acids N1: 1 to 127; L1: 406 to 583; L2: 811 to 946; L3: 1207 to 1263; C1: 1504 to 1670; C2: 1671 to 1853; C3: 1854 to 2019; and C4: 2014 to 2202. **B**, In vitro binding assays between Ca_v1.3 intracellular domains and ankyrin membrane-binding domains. Note that the membrane-binding domain of AnkB, but not ankyrin-G (AnkG), binds to Ca_v1.3 C4, representing the terminal ~200 amino acids. **C**, Amino acid alignment of the terminal ~200 amino acids of Ca_v1.3 and Ca_v1.2. Identical amino acids are boxed in light gray, and similar amino acids are boxed in dark gray. **D**, In vitro binding assays of AnkB membrane-binding domain with the corresponding C4 regions of Ca_v1.3 and Ca_v1.2. Note that AnkB selectively interacts with Ca_v1.3 C4. **E**, Amino acid alignment of known ankyrin-binding domains in Na_v1.5 and Kir6.2 with the ankyrin-binding domain in the terminal 26 amino acids of Ca_v1.3. **F**, High amino acid conservation is observed in Ca_v1.3 ankyrin-binding domains across species. **G**, Amino acid sequences of full-length Ca_v1.3-C4 and Ca_v1.3-C4 lacking the terminal 29 amino acids including the ankyrin-binding domain (Ca_v1.3-C4 ΔABD). **H**, In vitro binding assays of AnkB membrane-binding domain with Ca_v1.3-C4 and Ca_v1.3-C4 ΔABD. Note that AnkB does not bind to Ca_v1.3-C4 ΔABD (experiments were done in parallel with those in **D**, thus top control blots are the same). **I**, Amino acid sequences of biotinylated peptides of the terminal ~25 residues of Ca_v1.3 and Ca_v1.2. **J**, Immunoprecipitation of AnkB with the biotinylated Ca_v1.3 peptide but not with the corresponding Ca_v1.2 peptide. SA indicates streptavidin agarose. **K**, Compared with primary cardiac fibroblasts expressing wild-type Ca_v1.3, primary cardiac fibroblasts expressing Ca_v1.3 lacking the AnkB-binding motif display reduced I_{Ca,L}. *P<0.05 compared with red symbols; #P<0.05, triangles compared with squares (n=7 per group).
The Ca$_{\alpha}$.1,3 but not Ca$_{\alpha}$.1,2 C-terminal domain contains a short motif resembling the ankyrin-binding sequence found in voltage-gated Na$_{\alpha}$ channels (ie, Na$_{\alpha}$.1,2, Na$_{\alpha}$.1,5) and inwardly rectifying K$^+$ channels (ie, Kir6.2; Figure 4E).$^8$,$^{20}$,$^{21}$ Importantly, this sequence is conserved across Ca$_{\alpha}$.1,3 orthologs (Figure 4F). We tested the requirement of this motif for ankyrin-B binding using a mutant C-terminal domain lacking these residues (Figure 4G). Although Ca$_{\alpha}$.1,3 C-terminal domain was associated with ankyrin-B (but not ankyrin-G), Ca$_{\alpha}$.1,3 C4 ankyrin-binding domain lacking the putative ankyrin-binding domain failed to associate with ankyrin-B (Figure 4H). On the basis of these results, we tested whether residues 2175 to 2198 were sufficient for ankyrin-binding using biotinylated peptides. We observed an association of full-length endogenous ankyrin-B from human atrial lysates with an immobilized peptide containing the ankyrin-B-binding domain in Ca$_{\alpha}$.1,3 (Figure 4I and 4J). We observed no interaction with a peptide of the corresponding region in Ca$_{\alpha}$.1,2 or with streptavidin beads alone (Figure 4J). Together, our data demonstrate that Ca$_{\alpha}$.1,3 residues 2175 to 2198 are necessary and sufficient for direct ankyrin-B association.

To test whether Ca$_{\alpha}$.1,3 membrane expression requires a direct interaction with ankyrin-B, we measured I$_{\text{Ca,L}}$ in primary cardiac fibroblasts expressing WT Ca$_{\alpha}$.1,3 and a mutant Ca$_{\alpha}$.1,3 lacking the ankyrin-binding motif. As expected, WT Ca$_{\alpha}$.1,3 expression resulted in robust I$_{\text{Ca,L}}$ in primary cells (Figure 4K; n=7; P<0.05). In contrast, Ca$_{\alpha}$.1,3A32174 lacking the ankyrin-B binding motif displayed >50% reduction in membrane activity compared with the WT channel (Figure 4K; n=7; P<0.05). These data support the requirement of a direct ankyrin-B interaction for Ca$_{\alpha}$.1,3 membrane targeting. However, these data also suggest that an ankyrin-independent pathway(s) also plays a critical role in Ca$_{\alpha}$.1,3 membrane delivery because Ca$_{\alpha}$.1,3 lacking ankyrin binding still displayed a greatly reduced but detectable current.

**Loss of I$_{\text{Ca,L}}$ Alters APD$_{90}$ in Ankyrin-B$^{-/-}$ Atrial Myocytes**

Our results identify a direct association of ankyrin-B with Ca$_{\alpha}$.1,3 and a striking reduction of Ca$_{\alpha}$.1,3 and I$_{\text{Ca,L}}$ in primary ankyrin-B$^{-/-}$ atrial cardiomyocytes. To test whether observed differences in I$_{\text{Ca,L}}$ in ankyrin-B$^{-/-}$ atrial myocytes are sufficient to account for dramatic action potential shortening and AF susceptibility, we performed computational modeling of WT and ankyrin-B$^{-/-}$ atrial action potentials (Figure 5). We first incorporated experimentally measured changes in I$_{\text{Ca,L}}$ (Figure 2) and I$_{\text{NCX}}$ (Figure II in the online-only Data Supplement) and reduction in Na$^+$,K$^+$-ATPase expression$^{12,22}$ into a physiological and well-validated model of the atrial action potential from Nattel and colleagues.$^{23}$ Decreasing all 3 currents was sufficient to reduce the action potential nearly 25%, similar to the observed differences in APDs between ankyrin-B$^{-/-}$ and WT atrial myocytes (Figure 5B and 5G). We next simulated action potentials in atrial myocytes deficient in Na$^+$,Ca$^{2+}$ exchange (NCX), Na$^+$,K$^+$-ATPase, or I$_{\text{Ca,L}}$ and compared APDs with those in the WT model (Figure 5K). Reduction of
Ankyrin-B, required for the targeting and stability of critical membrane and submembrane molecules, is important for normal membrane excitability in multiple cardiac cell types. The multifunctional capacity of ankyrin-B is evident in the strong link between ankyrin dysfunction and a spectrum of cardiac disorders collectively referred to as ankyrin-B syndrome. In this study, we identify the L-type calcium channel 1.3 (α1D) as a novel ankyrin-B binding partner and provide an association between decreased ankyrin-B function and AF. Specifically, we demonstrate early-onset familial AF in human patients with an ANK2 missense mutation. A nkyrin-B α1D−/− mouse display similar atrial dysfunction including increased incidence of spontaneous AF episodes and enhanced susceptibility to burst pacing-induced AF. At the cellular level, ankyrin-B α1D−/− atrial myocytes exhibit decreased I_Ca,L and shortened APD, both hallmarks of clinical AF. Underlying the reduced I_Ca,L in ankyrin-B α1D−/− atrial myocytes, there is a selective loss of both protein expression and membrane targeting of Ca,1.3 but not Ca,1.2. This selective interaction is mediated by a unique motif in the C-terminal domain of Ca,1.3 that is both necessary and sufficient for ankyrin binding. Thus, ankyrin-B dependent membrane targeting of Ca,1.3 is required for normal atrial I_Ca,L and atrial function. Although ankyrin-B haploinsufficiency in atrial myocytes reduces NCX and Na+/K+-ATPase currents, ankyrin-B dependent reduction in I_Ca,L appears primarily responsible for shortened APDs, as suggested by computational modeling.

There is a strong association between reduced I_Ca,L and AF. Although it is unclear whether I_Ca,L dysfunction is a cause or effect of AF, there are numerous examples of AF linked to reduced I_Ca,L. Atrial tissues from human AF patients display reduced mRNA expression of L-type Ca2+ channels Ca,1.2 and Ca,1.3 in addition to an overall decrease in I_Ca,L. In addition, patients with loss-of-function mutations in L-type Ca2+ channel Ca,1.2 manifest complex cardiac phenotypes including AF. Similarly, atrial myocytes from Ca,1.3−/− mice exhibit reduced I_Ca,L, a depolarizing shift in voltage-dependent I_Ca,L activation (Ca,1.3 activates at more negative potentials than Ca,1.2), and increased AF susceptibility on burst-pacing stimulation. Given the increased susceptibility to AF in Ca,1.3−/− mice and our findings that Ca,1.3 membrane targeting is regulated by a direct interaction with ankyrin-B, it will be critical in future experiments to determine the direct role of Ca,1.3 dysfunction in human ANK2-related atrial disease.

A intriguing finding from this study is that despite overlapping expression patterns of the L-type calcium channel α-subunits in atrial cells, ankyrin-B preferentially interacts with Ca,1.3 and not Ca,1.2 in atrial myocytes. A second interesting observation is that Ca,1.3 is expressed, albeit to a lesser extent, in membranes of ankyrin-B α1D−/− cells, suggesting that ankyrin-independent mechanisms also contribute to Ca,1.3 membrane expression. These observations are consistent with L-type calcium channels interacting with a variety of signaling and scaffolding proteins that influence membrane expression. Some of the molecular mechanisms that regulate L-type calcium channel membrane expression include channel auxiliary subunits (β-subunits), signaling molecules (calmodulin), and scaffolding/adaptor proteins (α-actinin2, A-kinase anchoring protein 79).
C Conclusions

We propose that ankyrin-B dysfunction is associated with AF on the basis of our findings that (1) a loss-of-function ANK2 mutation is associated with highly penetrant AF; (2) ankyrin-B haploinsufficient mice exhibit increased susceptibility to AF; (3) ankyrin-B deficient atrial myocytes display shortened action potentials, a hallmark of clinical AF; (4) ankyrin-B -/- atrial myocytes exhibit reduced expression and membrane targeting of Cas,1,3, an L-type calcium channel subunit previously associated with increased AF susceptibility; and (5) patients with paroxysmal AF demonstrate reduced ankyrin-B. Collectively, these findings suggest that ankyrin-B dysfunction may account for cases of monogenic AF in the general human population and that ANK2 should be considered for familial AF screening.

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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

Atrial fibrillation (AF) is the most prevalent sustained arrhythmia in clinical practice. In fact, in the United States alone, AF is present in >2 million individuals. Despite the high incidence of AF in the population, surprisingly little is known regarding the molecular mechanisms underlying this complex disease. Ankyrin proteins target and stabilize proteins at specialized membrane domains. Notably, dysfunction in ankyrin- and ankyrin-associated pathways has been linked with disorders including spherocytosis, spinocerebellar ataxia, diabetes mellitus, neurological deficits, and cardiac arrhythmias. Nearly a decade ago, ankyrin-B (ANK2) was discovered as a critical component of heart, and work in humans and mice has implicated ankyrin-B as critical for cardiac function. In fact, human ANK2 loss-of-function variants are associated with potentially fatal ventricular arrhythmias. In the present study, we demonstrate the importance of ankyrin-B for atrial function and identify an association between ankyrin-B dysfunction and AF. Individuals harboring ANK2 variants display AF, and these phenotypes are reproduced in mice deficient in ankyrin-B. Ankyrin-B is expressed in the atria, and ankyrin-B+/− myocytes display shortened action potentials, a hallmark of AF, and decreased L-type calcium channel current. We show that Ca_{1.3}, responsible for 1 component of L-type calcium channel current in atria, is a novel ankyrin-binding partner and that Ca_{1.3} expression/activity is reduced in ankyrin-deficient atrial myocytes. Finally, ankyrin-B is reduced in atrial samples from human AF patients, further supporting the role of ankyrin-B in normal atrial function. Together, our work implicates ankyrin-B as a surprising yet critical component of atrial excitability and supports the role of atypical myocyte proteins in disease pathogenesis.