A cardiac arrhythmia syndrome caused by loss of ankyrin-B function

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220-kDa ankyrin-B is required for coordinated assembly of Na/Ca exchanger, Na/K ATPase, and inositol trisphosphate (InsP_3) receptor at transverse-tubule/sarcoplasmic reticulum sites in cardiomyocytes. A loss-of-function mutation of ankyrin-B identified in an extended kindred causes a dominantly inherited cardiac arrhythmia, initially described as type 4 long QT syndrome. Here we report the identification of eight unrelated probands harboring ankyrin-B loss-of-function mutations, including four previously undescribed mutations, whose clinical features distinguish the cardiac phenotype associated with loss of ankyrin-B activity from classic long QT syndromes. Humans with ankyrin-B mutations display varying degrees of cardiac dysfunction including bradycardia, sinus arrhythmia, idiopathic ventricular fibrillation, catecholaminergic polymorphic ventricular tachycardia, and risk of sudden death. However, a prolonged rate-corrected QT interval was not a consistent feature, indicating that ankyrin-B dysfunction represents a clinical entity distinct from classic long QT syndromes. The mutations are localized in the ankyrin-B regulatory domain, which distinguishes function of ankyrin-B from ankyrin-G in cardiomyocytes. All mutations abolish ability of ankyrin-B to restore abnormal Ca^{2+} dynamics and abnormal localization and expression of Na/Ca exchanger, Na/K ATPase, and InsP_3R in ankyrin-B$^{+/-}$ cardiomyocytes. This study, considered together with the first description of ankyrin-B mutation associated with cardiac dysfunction, supports a previously undescribed paradigm for human disease due to abnormal coordination of multiple functionally related ion channels and transporters, in this case the Na/K ATPase, Na/Ca exchanger, and InsP_3 receptor.

Primary arrhythmogenic disorders of the heart are a major cause of sudden cardiac death in otherwise healthy, and frequently young individuals. These arrhythmias are likely to be caused by aberrant function of ion channels leading to abnormal electrical properties of the heart. Mutations in genes encoding ion channels have emerged in the last decade as the basis for a variety of inherited arrhythmias, including long QT syndrome types 1–3 and 5–7, Brugada syndrome, and catecholaminergic polymorphic ventricular tachycardia (1). However, 30–45% of cases with arrhythmia cannot be explained by mutation of currently identified genes (2–4, ‖).

Physiological function of ion channels in the context of the heart requires precise cellular targeting in addition to activity at a single-channel level. This consideration suggests the possibility of a class of arrhythmias caused by defective cellular processing of channels. In support of this idea, mutation of the gene encoding the membrane adaptor protein ankyrin-B (*Ank2*, E1425G) was identified as the cause of type 4 long QT syndrome (5). Type 4 long QT syndrome has been characterized in a single large French kindred (5, 6). Affected family members display many atypical features compared to other long QT syndromes, including pronounced sinus bradycardia, polyphasic T waves, and atrial fibrillation (5). Moreover, cardiac repolarization defects in this family (heart-rate-corrected QT interval, or QTc) are not as severe as in other patients with long QT syndromes (4–7). Nonetheless, humans with ankyrin-B mutations experience polymorphic ventricular arrhyth-

mia, syncope, and sudden death in response to exercise or emotional stress (5, 6).

Mice heterozygous for a null mutation in ankyrin-B (ankyrin-B$^{+/-}$ mice) display a cardiac phenotype similar to humans with the ankyrin-B E1425G mutation (5). Adult cardiomyocytes from ankyrin-B$^{+/-}$ mice have elevated Ca^{2+} transients and catecholamine-induced afterdepolarizations and extrasystoles (5). Adult ankyrin-B$^{+/-}$ cardiomyocytes also exhibit loss of the subpopulation of ankyrin-B localized at transverse (T)-tubule/sarcoplasmic reticulum (SR) membrane sites (5). Moreover, the Na/K ATPase, Na/Ca exchanger, and inositol 1,4,5 trisphosphate (InsP_3) receptor, all ankyrin-binding proteins, also are coordinately reduced at T-tubule/SR membrane sites (5). Elevated calcium transients in ankyrin-B$^{+/-}$ cardiomyocytes can be rationalized by reduction of the Na/K ATPase, which would mimic the action of cardiac glycosides in elevating intracellular Na$^+$, and thus inhibit exchange of extracellular Na$^+$ for intracellular Ca^{2+} by the Na/Ca exchanger.

Ankyrin family polypeptides (ankyrin-R, -B, and -G) are closely related and often are coexpressed in the same cell types (reviewed in ref. 8). However, unlike many protein families, ankyrin gene products have distinct functions (5, 9–12). Specific function of ankyrin-B in cardiomyocytes is determined by its divergent regulatory domain (13).

We report here four previously undescribed ankyrin-B mutations, all located in the regulatory domain, in humans with cardiac dysfunction including sudden death. We identified eight unrelated probands harboring ankyrin-B mutations whose clinical features distinguish the cardiac phenotype associated with loss of ankyrin-B activity from classic long QT syndromes. All four of these mutations cause loss of function of ankyrin-B in restoring normal Ca^{2+} dynamics and normal expression of Na/K ATPase, Na/Ca exchanger, and InsP_3 receptor in neonatal cardiomyocytes.

Materials and Methods

Genotyping and DNA Analysis. Genomic DNA was prepared from peripheral blood lymphocytes by using standard procedures. All exons of ankyrin-B (*Ank2*), with exception of brain-specific exon 38, were screened by single-strand conformation polymorphism (SSCP) or by denaturing HPLC (see *Supporting Methods*, which is published as supporting information on the PNAS web site). Of the 664 probands tested, 370 displayed long QT, 226 displayed idiopathic ventricular fibrillation, 21 displayed Andersen syndrome, 12 displayed LQT plus syndactyly, 27 displayed sick sinus syndrome,
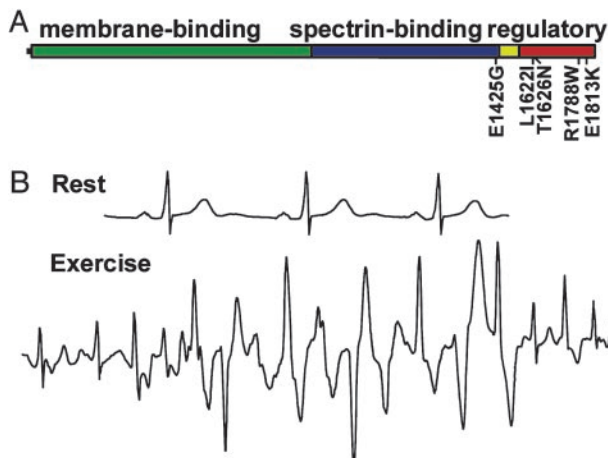
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Fig. 1. Identification of ankyrin-B mutations in human probands with cardiac arrhythmia. (*A*) Domain organization of 220-kDa ankyrin-B with previously identified E1425G mutation and L1622I, T1626N, R1788W, and E1813K mutations denoted. Yellow box denotes death domain, red box denotes C-terminal domain. (*B*) ECG recordings from European proband with ankyrin-B R1788W mutation. ECG recordings show normal resting ECG and polymorphic ventricular tachycardia induced by exercise.

Fig. 2. Pedigrees of probands with *Ank2* mutations. Females are denoted by circles, and males are denoted by squares. Hatched symbols represent humans with uncertain or unknown LQT phenotype. Open symbols represent individuals without LQT phenotype at rest. Filled symbols represent individuals diagnosed with LQT phenotype. Mutation carriers are denoted by an asterisk. Probands are marked by a star. Sudden death is denoted by SD, and death is indicated by a diagonal slash. Roman numerals indicate generations in the respective families.

three displayed atrial fibrillation, and five displayed unexpected sudden death. Phenotypic criteria for long QT syndrome were as described (ref. 14; also see *Supporting Methods*).
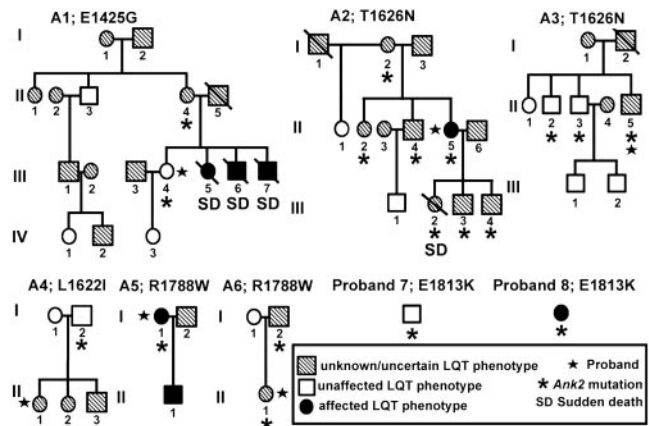
Isolated Cardiomyocytes. Animal care was in accordance with Duke University Medical Center institutional guidelines. Neonatal cardiomyocytes were isolated from postnatal day 1 (P1) or P2 mice and transfected as described (5, 13). After transfection, isolated cardiomyocyte spontaneous contraction rates and/or Ca^{2+} dynamics (Fluo-3 AM) were monitored as described (5, 13). Immunolocalization experiments were performed as described (5, 13). Expression of GFP–220-kDa ankyrin-B constructs could not be visualized in transfected cardiomyocytes without the use of GFP antibody due to low expression levels. We observed no difference in the localization between endogenous ankyrin-B and any of the GFP–220-kDa ankyrin-B constructs. $Na_v1.5$ polyclonal antibody was a generous gift of W. Catterall (University of Washington, Seattle).

Ankyrin-B Mutagenesis. Single amino acid mutations were introduced into human GFP–220-kDa ankyrin-B by using QuikChange mutagenesis (Stratagene). Mutated regions were sequenced and then subcloned into original vectors to ensure that no additional mutations were introduced into the vector.

Results

Cardiac Dysfunction in Humans Associated with *Ank2* Mutations. We screened a large group of patients (664) for mutations to ankyrin-B (*Ank2*). These patients were selected based on presence of cardiac arrhythmias (long QT syndrome, catecholaminergic polymorphic ventricular tachycardia, idiopathic ventricular fibrillation, Andersen's syndrome, sick sinus syndrome, and unexplained sudden death) and lack of mutation in *KCNQ1*, *HERG*, *SCN5A*, *KCNE1*, *KCNE2*, and *KCNJ2*. None of the patients had structural heart disease as assessed by MRI and echocardiography. All exons of ankyrin-B (*Ank2*), with the exception of neuron-specific exon 38, were screened by SSCP in 344 probands. Denaturing HPLC was used to screen an additional 325 probands for mutations in *Ank2* exons 36 and 42–45. No abnormal variants in the probands were detected within exons encoding the ankyrin-B membrane-binding domain (exons 1–23; Fig. 1*A*).

Only one SSCP variant was identified within the ankyrin-B spectrin-binding domain (exons 24–36; Fig. 1*A*), and this proband was heterozygous for the previously identified A4274G (E1425G) mutation. This Caucasian female is clinically unaffected, and, in contrast to previously identified E1425G patients (5), had a normal QTc of 410 msec with a heart rate of 60 beats per min. The proband's 67-year-old mother is a carrier of the E1425G variant with slightly elevated QTc (430–450 msec) and moderately low heart rate (63 beats per min; see Fig. 2 and Table 1, Kindred A1). Three siblings of the proband died young of sudden death at 25, 17, and 15 years of age. The 25-year-old died while winning a prize. The 17-year-old died in the shower and had previously experienced syncopal events associated with athletics. The 15-year-old died getting out of a pool after swimming. DNA and QTc data were unattainable from the siblings. The E1425G mutation was not observed in 550 control individuals.

SSCP/denaturing HPLC and DNA sequence analyses identified four potential ankyrin-B loss-of-function mutations in probands with varied arrhythmia phenotypes. All four mutations are localized to the ankyrin-B C-terminal regulatory domain previously demonstrated to confer ankyrin-B-specific activity in cardiomyocytes (Fig. 1*A*; ref. 13). We identified two unrelated probands with identical C-to-A mutations in exon 42 (C4877A) leading to a threonine-to-asparagine substitution at amino acid 1626 (T1626N, Fig. 1*A*). Both Caucasian probands were from the United States and had marginally elevated QTc (450 and 440–450 msec). One 46-year-old female proband (Fig. 2 and Table 1, Kindred A2) with a QTc of 450 msec had experienced syncope, but had a normal resting heart rate of 72 beats per min. The daughter of this proband was also heterozygous for the T1626N mutation and died of sudden death at age 19 with no previous cardiac symptoms (QTc and heart rate data are unavailable). Two siblings, two sons, and the mother of the same proband are also carriers of the mutation and have resting QTc in the normal range (430, 440, 440, 430, and 440 msec, respectively). To date, these carriers are asymptomatic. The second T1626N proband is a 51-year-old Caucasian male from the United States. This patient displays mildly elevated QTc (450 msec) with sinus arrhythmia (heart rate 50–110 beats per min). Two of the proband's siblings with normal QTc (420 and 400 msec) are heterozygous for the ankyrin-B T1626N mutation (Fig. 2 and Table 1, Kindred A3) but are asymptomatic to date. This mutation was not observed in 550 control individuals.

Table 1. Cardiac phenotypes of humans with *Ank2* mutations

Individual	Age	HR	QTc	Mutation	Phenotype
A1-II4	67	63	430	E1425G	Asymptomatic
A1-III4	28	60	410	E1425G	Asymptomatic
A1-III5	25	NA	NA	Untested	Sudden death after stress
A1-III6	17	NA	NA	Untested	History of syncope with athletics, sudden death
A1-III7	15	NA	NA	Untested	Sudden death after exercise
A2-I2	70	74	430	T1626N	Asymptomatic
A2-II2	48	67	430	T1626N	Asymptomatic
A2-II4	44	86	440	T1626N	Asymptomatic
A2-II5	46	72	450	T1626N	Long QT, syncope
A2-III2	19	NA	NA	T1626N	Sudden death, previously asymptomatic
A2-III3	24	83	430	T1626N	Asymptomatic
A2-III4	18	66	430	T1626N	Asymptomatic
A3-II2	58	63	420	T1626N	Asymptomatic
A3-II3	53	65	440	T1626N	Asymptomatic
A3-II5	51	50–110	450	T1626N	Sinus arrhythmia
A4-I2	43	71	440	L1622I	Asymptomatic
A4-II1	23	63	445	L1622I	Long QT
A4-II2	20	71	435	L1622I	Ventricular tachycardia, ventricular fibrillation
A4-II3	17	66	430	L1622I	Asymptomatic
A5-I1	37	60	530	R1788W	Syncope during sleep Long QT, bradycardia
A5-II1	NA	NA	NA	Untested	Long QT
A6-I2	52	67	425	R1788W	Asymptomatic
A6-II1	35	62	430	R1788W	Syncope with exercise; supraventricular and ventricular arrhythmia
Proband 7	61	64	395	E1813K	Recurring arrhythmia; ventricular tachycardia
Proband 8	24	62	490	E1813K	Idiopathic ventricular fibrillation

HR, heart rate in beats per min; NA, not available.

We identified a C-to-A mutation (C4864A) in exon 42 resulting in a leucine-to-isoleucine substitution at amino acid 1622 (L1622I) in one European female proband with a normal QTc (445 msec) (Fig. 1*A*). This Caucasian proband displayed polymorphic ventricular tachycardia, ventricular fibrillation, and a resting heart rate of 63 beats per min. The proband's mother, sister, and brother are carriers of the mutation (Fig. 2 and Table 1, Kindred A4), but to date all are asymptomatic with normal resting QTc (440 msec, 435 msec, and 430 msec, respectively) and normal heart rates (71, 71, and 66 beats per min, respectively). The L1622I mutation was not observed in control individuals.

We observed two unrelated probands with identical C-to-T mutations (C5336T) in exon 45 of *Ank2* leading to arginine-to-tryptophan substitution at amino acid 1788 (R1788W; Fig. 1*A* and Fig. 7, which is published as supporting information on the PNAS web site). One proband was a 37-year old-Caucasian female from the United States (Fig. 2 and Table 1, Kindred A5). She presented with syncope (originally treated as a seizure) at age 12. This woman subsequently had multiple episodes of syncope associated with sleep, and torsades de pointes ventricular tachycardia was documented. β-Blocker therapy failed to eliminate symptoms, and she was treated with an implantable cardiac defibrillator. ECGs revealed a heart rate of 60 beats per min, prominent T–U waves, and prolongation of the QT interval with a QTc of 530 msec. The proband's son has also been diagnosed with long QT syndrome, although DNA from this subject is unattainable.

The second proband, heterozygous for a R1788W mutation, is a Caucasian female from Europe with multiple episodes of exercise-associated syncope. She presented with supraventricular and ventricular tachycardias that were reproducibly elicited by exercise tests. The patient has normal QTc at rest (430 msec), but prolongation of 470 msec was observed after a syncopal event. This woman was successfully treated with β-blockers. However, exercise-induced nonsustained supraventricular and ventricular arrhythmias persisted and were recorded in a clinical setting (see ECGs in Fig. 1*B*). The father of the proband is a carrier of the mutation (Fig. 2 and Table 1, Kindred A6) with QTc of 430 msec and a heart rate of 67 beats per min. The R1788W mutation was not identified in 280 DNA samples obtained from individuals with normal ECG.

Finally, we identified two unrelated probands with a fourth ankyrin-B mutation, a G-to-A mutation (G5437A) in exon 45 leading to a glutamate-to-lysine substitution at amino acid 1813 (E1813K, Fig. 1*A*). The first proband was a 24-year-old Caucasian female from Europe diagnosed with recurring arrhythmia and documented torsades de pointes ventricular tachycardia (Fig. 2 and Table 1, Proband 7). The proband presented with an elevated resting QTc of 490 msec and mild bradycardia (62 beats per min). We were unable to obtain DNA samples from family members. The second proband is a 60-year-old Caucasian male from the United States (Fig. 2 and Table 1, Proband 8). This proband displayed idiopathic ventricular fibrillation (15) with a normal QTc (395 msec), a normal resting ECG, and a heart rate of 64 beats per min. No DNA was available from close relatives (Fig. 2; Kindred G). The E1813K mutation was found in five DNA samples obtained from individuals with normal ECGs.

In summary, we have identified four ankyrin-B mutations (T1626N, L1622I, R1788W, and E1813K) in humans with cardiac dysfunction. We have also identified an additional proband with an ankyrin-B E1425G mutation. Probands with ankyrin-B mutations display varying degrees of cardiac dysfunction including bradycardia, sinus arrhythmia, idiopathic ventricular fibrillation, catecholaminergic polymorphic ventricular tachycardia, and sudden death. In contrast to the original E1425G kindred, ankyrin-B mutation carriers do not necessarily display a prolonged QTc or significant bradycardia at rest.

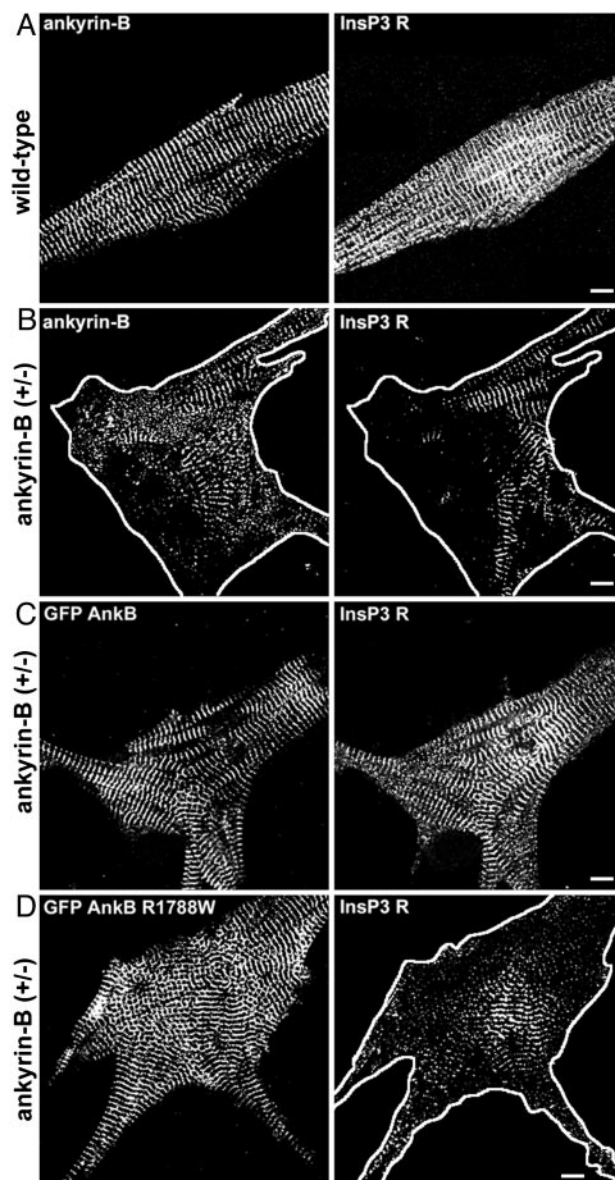**Human Mutations Abolish Function of Ankyrin-B in Cardiomyocytes.** Functional consequences of the four human ankyrin-B mutations were evaluated by using a neonatal cardiomyocyte rescue assay (13). Compared with neonatal cardiomyocytes derived from wild-type mice, ankyrin-B$^{+/-}$ neonatal cardiomyocytes display reduced frequency of spontaneous contractions and abnormal Ca^{2+} dynam-
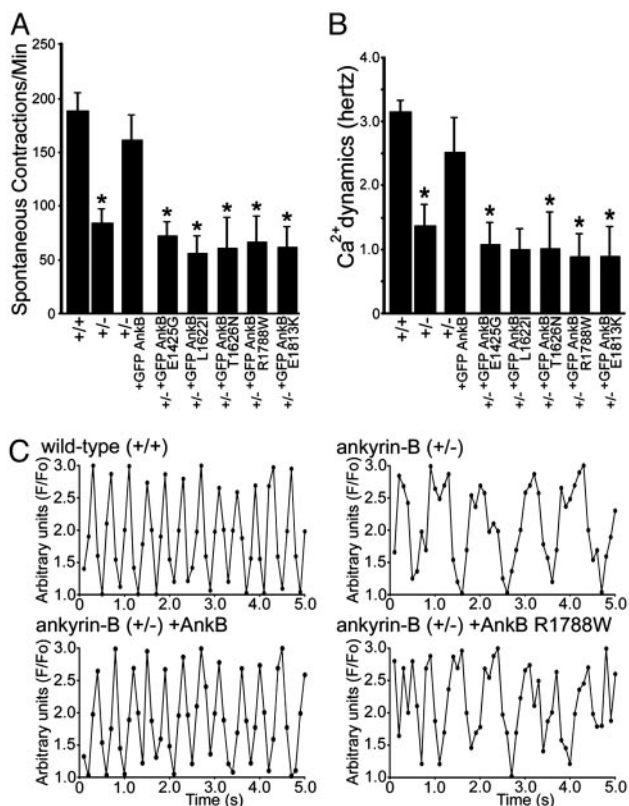
Fig. 3. Human ankyrin-B mutations do not restore abnormal spontaneous contractions or abnormal Ca^{2+} dynamics in ankyrin-B$^{+/-}$ cardiomyocytes. Compared to wild-type cardiomyocytes, ankyrin-B$^{+/-}$ cardiomyocytes display decreased spontaneous contraction rates (*A*) and abnormal calcium dynamics (*B*). Abnormal ankyrin-B$^{+/-}$ cardiomyocyte contraction rates and calcium dynamics are rescued by transfection of GFP–220-kDa ankyrin-B, but not GFP–220-kDa ankyrin-B mutants E1425G, L1622I, T1626N, R1788W, or E1813K (*n* > 200 cells; *P* < 0.05). In *B*, calcium dynamics (expressed in Hz) were measured by using Fluo-3 AM. (*C*) Raw data of Ca^{2+} levels in cardiomyocytes (F/F$_o$). Graphs represent untransfected wild-type and ankyrin-B$^{+/-}$ neonatal cardiomyocytes and GFP–220-kDa ankyrin-B and GFP–220-kDa ankyrin-B R1788W transfected ankyrin-B$^{+/-}$ myocytes.

ics (Fig. 3 and ref. 13). Additionally, ankyrin-B$^{+/-}$ cardiomyocytes display abnormal localization and expression of InsP$_3$ receptor, Na/K ATPase, and Na/Ca exchanger (Figs. 4 and 5, and Figs. 8–10, which are published as supporting information on the PNAS web site). In contrast, ankyrin-B$^{+/-}$ cardiomyocytes display normal localization and expression of endoplasmic and sarcoplasmic reticulum proteins (SERCA2, calreticulin, calsequestrin, ryanodine receptor 2), plasma membrane Ca^{2+} channels (DHPR, PMCA2), ion channels and associated subunits (Na$_v$1.5, Na$_v$1.6, KCNQ1, MinK, Kir1.2, Kir2.3, ERG1), structural proteins (α-actinin, dystrophin), and connexin 43 (5). Abnormal ankyrin-B$^{+/-}$ phenotypes can be restored to normal by transfection of GFP–220-kDa ankyrin-B human cDNA into ankyrin-B$^{+/-}$ cardiomyocytes (Figs. 3–5, refs. 5 and 13). In contrast, GFP–220-kDa ankyrin-B containing the human E1425G mutation is unable to rescue spontaneous contractions, Ca^{2+} dynamics, or abnormal ion channel and transporter localization when expressed in ankyrin-B$^{+/-}$ cardiomyocytes (ref. 5, see Figs. 8 and 9). Therefore, this cardiomyocyte rescue assay can be used to test potential functional consequences of ankyrin-B mutations.

Individual GFP–220-kDa ankyrin-B mutants L1622I, T1626N, R1788W, and E1813K were created by using site-directed mutagenesis. After each plasmid was sequenced to ensure that no additional mutations were introduced, the four human mutants were trans-

Fig. 4. Human mutations are ankyrin-B loss-of-function mutations in mouse cardiomyocytes. (*A*) Immunolocalization of endogenous ankyrin-B (*Left*) and InsP$_3$ receptor (*Right*) in wild-type neonatal cardiomyocytes. (Scale bar, 10 μm.) (*B*) Immunolocalization of ankyrin-B (*Left*) and InsP$_3$ receptor (*Right*) in ankyrin-B$^{+/-}$ neonatal mouse cardiomyocytes. Reduction in ankyrin-B levels leads to loss of InsP$_3$ receptor expression and organization (compare InsP$_3$ receptor staining in *A* and *B*). (Scale bar, 10 μm.) (*C*) Abnormal localization of InsP$_3$ receptor in ankyrin-B$^{+/-}$ cardiomyocytes is rescued by transfection of GFP–220-kDa ankyrin-B. GFP–220-kDa ankyrin-B was immunolocalized by using affinity-purified GFP antibody. (Scale bar, 10 μm.) (*D*) R1788W is an ankyrin-B loss-of-function mutation. Expression of GFP–220-kDa ankyrin-B R1788W does not rescue abnormal expression or localization of InsP$_3$ receptor in ankyrin-B$^{+/-}$ neonatal cardiomyocytes. GFP–220-kDa ankyrin-B was immunolocalized by using affinity-purified GFP antibody. (Scale bar, 10 μm.) GFP–220-kDa ankyrin-B, GFP–220-kDa ankyrin-B R1788W, and endogenous ankyrin-B (in +/+ cell) are localized in the same striated pattern. The perimeter of the cell has been outlined in *B* and *D*.

fected into ankyrin-B$^{+/-}$ neonatal cardiomyocytes. All of the GFP–220-kDa ankyrin-B mutants were localized in the same striated pattern as endogenous ankyrin-B and GFP–220-kDa wild-type ankyrin-B (assessed by using affinity-purified GFP antibody), and were expressed at similar levels to GFP–220-kDa ankyrin-B when transfected into ankyrin-B$^{+/-}$ neonatal cardiomyocytes (Figs.
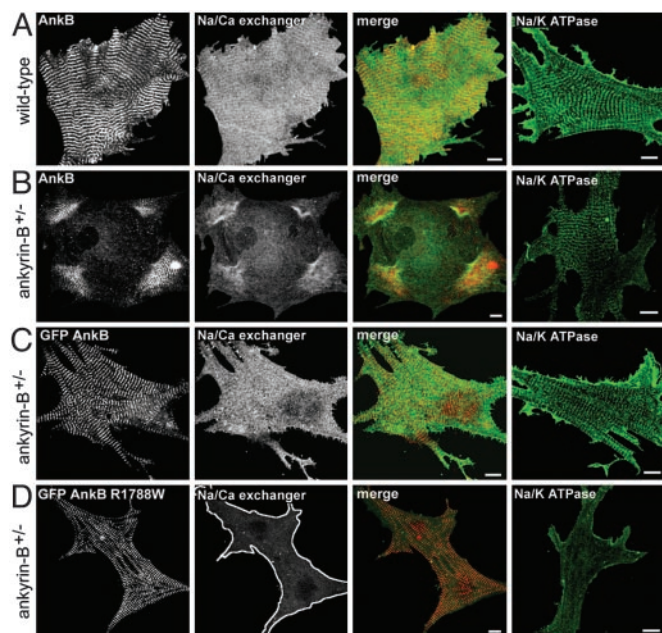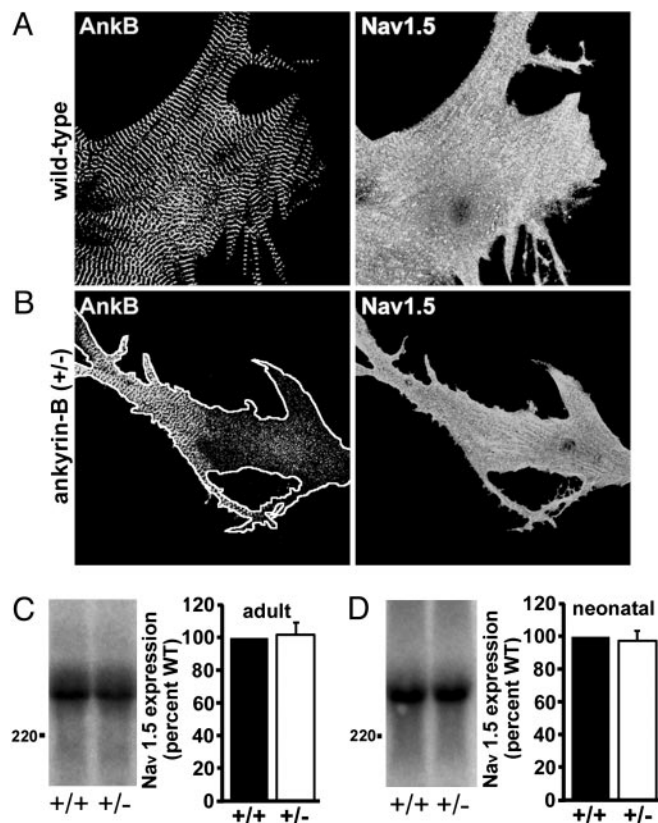
Fig. 5. GFP–220-kDa ankyrin-B, but not GFP–220-kDa ankyrin-B R1788W, rescues abnormal Na/Ca exchanger and Na/K ATPase localization in ankyrin-B$^{+/-}$ cardiomyocytes. (*A*) Immunolocalization of endogenous ankyrin-B with Na/Ca exchanger and Na/K ATPase in wild-type neonatal cardiomyocytes. (Scale bar, 10 μm.) (*B*) Immunolocalization of endogenous ankyrin-B with Na/Ca exchanger and Na/K ATPase in ankyrin-B$^{+/-}$ neonatal mouse cardiomyocytes. Reduction in ankyrin-B levels leads to loss of Na/Ca exchanger and Na/K ATPase expression and organization (compare Na/Ca exchanger and Na/K ATPase staining in *A* and *B*). (Scale bar, 10 μm.) (*C*) Abnormal expression and localization of Na/Ca exchanger and Na/K ATPase in ankyrin-B$^{+/-}$ neonatal cardiomyocytes is rescued by transfection of GFP–220-kDa ankyrin-B. GFP–220-kDa ankyrin-B was immunolocalized by using affinity-purified GFP antibody. (Scale bar, 10 μm.) (*D*) R1788W is an ankyrin-B loss-of-function mutation. Expression of GFP–220-kDa ankyrin-B R1788W does not rescue abnormal expression or localization of Na/Ca exchanger or Na/K ATPase in ankyrin-B$^{+/-}$ neonatal cardiomyocytes. (Scale bar, 10 μm.) GFP–220-kDa ankyrin-B, GFP–220-kDa ankyrin-B R1788W, and endogenous ankyrin-B (in +/+ cell) are localized in the same striated pattern. The perimeter of the cardiomyocyte in *D* is outlined.

Voltage-Gated Sodium Channel (Na$_v$1.5) Is Normally Expressed and Localized in Ankyrin-B$^{+/-}$ Cardiomyocytes. Ankyrin-G associates with the voltage-gated Na$^+$ channel at axon initial segments (16,

4, 5, 8, and 9). However, in contrast to GFP–220-kDa ankyrin-B, GFP–220-kDa ankyrin-B L1622I, T1626N, R1788W, or E1813K mutants were unable to rescue abnormal spontaneous contraction rates (Fig. 3*A*) or abnormal Ca^{2+} dynamics (Fig. 3 *B* and *C*) in ankyrin-B$^{+/-}$ neonatal cardiomyocytes.

We next analyzed the localization and expression of InsP$_3$ receptor, Na/Ca exchanger, and Na/K ATPase in ankyrin-B$^{+/-}$ cardiomyocytes expressing individual human ankyrin-B mutants. As noted above, all GFP–220-kDa ankyrin-B mutants were expressed at levels similar to GFP–220-kDa ankyrin-B, and were normally localized in a striated pattern (Figs. 4, 5, 8, and 9). Similar to our previous results with the E1425G mutant, human ankyrin-B mutants L1622I, T1626N, R1788W, and E1813K were unable to rescue the abnormal patterns of staining for InsP$_3$ receptor, Na/Ca exchanger, and Na/K ATPase (Figs. 4, 5, 8, and 9). In fact, in the presence of GFP–220-kDa ankyrin-B human mutants, expression of Na/Ca exchanger and Na/K ATPase is decreased to below levels observed in nontransfected ankyrin-$^{+/-}$ cardiomyocytes (Figs. 5, 9, and 10). These combined results demonstrate that the four human ankyrin-B mutations (L1622I, T1626N, R1788W, E1813K) abolish function of ankyrin-B in neonatal cardiomyocytes.

Fig. 6. Reduction of ankyrin-B does not affect Na$_v$1.5 expression or localization in ankyrin-B$^{+/-}$ cardiomyocytes. Immunolocalization of endogenous ankyrin-B and Na$_v$1.5 in wild-type (*A*) and ankyrin-B$^{+/-}$ (*B*) neonatal cardiomyocytes using affinity-purified antibodies. We observed no difference in Na$_v$1.5 expression or localization between wild-type and ankyrin-B$^{+/-}$ cardiomyocytes. The perimeter of the cell is outlined in *B*. Distance between ankyrin-B striations equals 1.8 μm. (*C* and *D*) Quantitative Western blot analysis of Na$_v$1.5 expression in wild-type and ankyrin-B$^{+/-}$ adult (*C*) and neonatal (*D*) cardiomyocytes using ^{125}I-labeled protein A. Protein levels were quantitated by PhosphorImaging. Reduction of ankyrin-B levels in ankyrin-B$^{+/-}$ cardiomyocytes has no affect on Na$_v$1.5 expression (n = 3, P > 0.05; not significant).

17). Therefore, we determined whether loss of ankyrin-B expression in ankyrin-B$^{+/-}$ mice altered the distribution and/or expression of the cardiac voltage-gated Na$^+$ channel (Na$_v$1.5) in isolated neonatal or adult mouse cardiomyocytes. In contrast to the striated ankyrin-B staining pattern, Na$_v$1.5 in wild-type neonatal cardiomyocytes is localized at the peripheral sarcolemma as assessed by using an affinity-purified polyclonal antibody (Fig. 6*A*). The localization of Na$_v$1.5 in ankyrin-B$^{+/-}$ neonatal (Fig. 6*B*) and adult cardiomyocytes (data not shown) is identical by immunofluorescence. Additionally, neonatal and adult cardiomyocytes derived from wild-type or ankyrin-B$^{+/-}$ mice have identical levels of Na$_v$1.5 protein expression by Western blot analysis (Fig. 6 *C* and *D*; P > 0.05).

A previous study reported prolonged action potentials and minor differences in Na$^+$ channel kinetics in primary cultures of ankyrin-B-null neonatal cardiomyocytes (18). However, the I$_{Na}$ density was only modestly affected, implying no major difference in numbers of Na$^+$ channels. Moreover, no significant differences in the upstroke or action potential duration (including repolarization) were detected between isolated adult ventricular cardiomyocytes of wild-type and ankyrin-B heterozygous mice when two different pacing protocols were used (5). The two studies cannot be directly compared, because one study described ankyrin-B null neonatal cardiomyocytes, and the other characterized ankyrin-B heterozygous

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adult cardiomyocytes. However, we can conclude that Na$_v$1.5 expression or localization is not altered in adult ankyrin-B$^{+/-}$ cardiomyocytes and does not contribute to elevated Ca^{2+} transients noted in these cells.

Discussion

This study reports clinical features of eight unrelated probands harboring loss-of-function mutations in the *Ank2* gene, including four previously undescribed mutations, that define a cardiac arrhythmia syndrome associated with loss of ankyrin-B activity. All four of the mutations are localized to the ankyrin-B regulatory domain, and cause loss-of-function of ankyrin-B in neonatal cardiomyocytes. Humans with ankyrin-B mutations display varying degrees of cardiac dysfunction including bradycardia, sinus arrhythmia, idiopathic ventricular fibrillation, catecholaminergic polymorphic ventricular tachycardia, and risk of sudden death. However, prolonged QTc intervals were not a consistent feature, indicating that ankyrin-B dysfunction represents a distinct clinical entity from classic long QT syndrome.

The finding that ankyrin-B mutations are associated with complex cardiac defects is consistent with the expression of ankyrin-B in multiple cell-types of the heart. For example, ankyrin-B is localized in both atrial and ventricular cardiomyocytes, as well as cardiac Purkinje conduction fibers (data not shown). Moreover, a 440-kDa alternatively spliced variant of ankyrin-B is present in unmyelinated axons of neurons innervating the heart (not shown). Loss-of-function mutation in ankyrin-B thus is likely to affect multiple aspects of heart function with consequences for conduction, atrial and ventricular rhythm, and function of sinus and atrioventricular nodes. In addition, ankyrin-B is expressed in multiple cell types of multiple organs, including beta cells of the pancreas, the nervous system, and the retina. Therefore, humans with ankyrin-B syndrome may display phenotypes in addition to cardiac arrhythmia.

This study provides evidence for facets of ankyrin-B function that go beyond the simple role of a scaffolding protein predicted from the role of ankyrin in the erythrocyte membrane skeleton. Four different mutations in the regulatory domain of ankyrin-B, distant from binding sites in the membrane-binding domain, cause the same level of loss of expression and mis-localization of the Na/K ATPase, Na/Ca exchanger and InsP_3 receptor. Thus, ankyrin-B domains are not independent modules, but work together, either through intramolecular interactions between membrane-binding and regulatory domains, and/or through binding to other proteins. One critical role for the regulatory domain is in restricting interactions of the membrane-binding domain. For example, ankyrin-G is required for targeting of voltage-gated Na$^+$ channels at axon initial segments, whereas ankyrin-B, which has a closely related membrane-binding domain, is not required for expression of voltage-gated Na$^+$ channels in cardiomyocytes (Fig. 6).

All of the ankyrin-B mutations are located in or near the C-terminal regulatory domain. The regulatory domain of ankyrins can modulate the activities of the N-terminal membrane- and spectrin-binding domains (19, 20). Therefore, these mutations could affect intramolecular association of the regulatory domain with membrane- or spectrin-binding domains, or interfere with intermolecular interactions. One of the ankyrin-B mutations (R1788W) directly abolishes a recently described interaction between ankyrin-B and the molecular cochaperone Hsp40/Hdj1 (21). The R1788W mutation is located on the surface of an amphipathic alpha-helix structure required for ankyrin-B function and for association with Hsp40/Hdj1 (21). R1788W mutation blocks Hsp40/Hdj1 binding with ankyrin-B in solution (Fig. 11*A*, which is published as supporting information on the PNAS web site) and in yeast two-hybrid assays (data not shown). Moreover, immobilized GFP–220-kDa ankyrin-B, but not GFP–220-kDa ankyrin-B R1788W, associates with Hsp40/Hdj1 from cardiac lysates (Fig. 11*B*). Therefore, the R1788W mutation abolishes ankyrin-B binding to Hsp40/Hdj1 and suggests that disruption of the ankyrin-B–Hsp40/Hdj1 interaction may lead to human cardiac dysfunction.

In summary, this study, considered together with the description of ankyrin-B mutation associated with cardiac dysfunction (5), supports a previously undescribed paradigm for human disease due to abnormal coordination of multiple functionally related ion channels and transporters, in this case the Na/K ATPase, Na/Ca exchanger, and InsP_3 receptor. Identification of the structural basis for ankyrin-B interactions with Na/K ATPase, Na/Ca exchanger, and InsP_3 receptor and dissection of the pathway for assembly of these proteins promises to yield common elements used more generally in targeting membrane proteins to specialized membrane domains in other physiological systems.

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