

# Novel Variant in the ANK2 Membrane-Binding Domain Is Associated With Ankyrin-B Syndrome and Structural Heart Disease in a First Nations Population With a High Rate of Long QT Syndrome

Leigh Anne Swayne, PhD; Nathaniel P. Murphy, BSc; Sirisha Asuri, PhD; Lena Chen, BSc; Xiaoxue Xu, BSc; Sarah McIntosh, MSc; Chao Wang, PhD; Peter J. Lancione, BS; Jason D. Roberts, MD; Charles Kerr, MD; Shubhayan Sanatani, MD; Elizabeth Sherwin, MD; Crystal F. Kline, PhD; Mingjie Zhang, PhD; Peter J. Mohler, PhD; Laura T. Arbour, MD

**Background**—Long QT syndrome confers susceptibility to ventricular arrhythmia, predisposing to syncope, seizures, and sudden death. While rare globally, long QT syndrome is  $\approx 15\times$  more common in First Nations of Northern British Columbia largely because of a known mutation in *KCNQ1*. However, 2 large multigenerational families were affected, but negative for the known mutation.

**Methods and Results**—Long QT syndrome panel testing was carried out in the index case of each family, and clinical information was collected. Cascade genotyping was performed. Biochemical and myocyte-based assays were performed to evaluate the identified gene variant for loss-of-function activity. Index cases in these 2 families harbored a novel *ANK2* c.1937C>T variant (p.S646F). An additional 16 carriers were identified, including 2 with structural heart disease: one with cardiomyopathy resulting in sudden death and the other with congenital heart disease. For all carriers of this variant, the average QTc was 475 ms ( $\pm 40$ ). Although ankyrin-B p.S646F is appropriately folded and expressed in bacteria, the mutant polypeptide displays reduced expression in cultured H9c2 cells and aberrant localization in primary cardiomyocytes. Furthermore, myocytes expressing ankyrin-B p.S646F lack normal membrane targeting of the ankyrin-binding partner, the Na/Ca exchanger. Thus, ankyrin-B p.S646F is a loss-of-function variant.

**Conclusions**—We identify the first disease-causing *ANK2* variant localized to the membrane-binding domain resulting in reduced ankyrin-B expression and abnormal localization. Further study is warranted on the potential association of this variant with structural heart disease given the role of *ANK2* in targeting and stabilization of key structural and signaling molecules in cardiac cells. (*Circ Cardiovasc Genet.* 2017;10:e001537. DOI: 10.1161/CIRCGENETICS.116.001537.)

**Key words:** arrhythmia ■ genetic variation ■ heart rate ■ long QT syndrome ■ Wolff–Parkinson–White syndrome

A prolonged QT interval on ECG, corrected for heart rate (QTc), is a marker for increased risk for arrhythmia and sudden cardiac death<sup>1</sup> and may be a result of genetic and/or nongenetic factors. Congenital long QT syndrome (LQTS types 1–13) is caused by variants in at least 13 known genes that encode for or affect the stability of critical ion channel proteins.<sup>2</sup> LQTS is characterized by a QTc  $>450$  ms for men and  $>460$  ms for women<sup>1,3,4</sup> and can be associated with ventricular arrhythmia, syncope, and seizures. Notably, sudden

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death can be the first manifestation.<sup>5</sup> Certain LQTS-associated variants also contribute to broader phenotypes, such as heart failure, cardiomyopathy, congenital structural heart disease, and non-LQTS arrhythmia.<sup>6</sup> Although relatively rare (1:2000 people world-wide<sup>7</sup>), LQTS is at least 15 times more common ( $\approx 1:125$ )<sup>8</sup> in the Gitksan First Nation, a remote community in

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From the Division of Medical Sciences, University of Victoria, BC, Canada (L.A.S., L.C., X.X., L.T.A.); University of British Columbia Island Medical Program, Victoria, BC, Canada (L.A.S., L.T.A.); Department of Medical Genetics (S.A., S.M., L.T.A.), Division of Cardiology (C.K.), and Division of Cardiology, Department of Pediatrics, BC Children's Hospital (S.S., E.S.), University of British Columbia, Vancouver, BC, Canada; Division of Cardiovascular Medicine, Department of Internal Medicine, Dorothy M. Davis Heart and Lung Research Institute (N.P.M., P.J.L., C.F.K., P.J.M.) and Department of Physiology and Cell Biology (N.P.M., P.J.L., C.F.K., P.J.M.), The Ohio State University Wexner Medical Center, Columbus, OH; Division of Life Science, State Key Laboratory of Molecular Neuroscience, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China (C.W., M.Z.); and Section of Cardiac Electrophysiology, Division of Cardiology, Department of Medicine, Western University, London, ON, Canada (J.D.R.).

Correspondence to Laura Arbour, MD, Department of Medical Genetics, University of British Columbia, C/o UBC Island Medical Program, Medical Sciences Bldg-Rm 104, PO Box 1700 STN CSC, Victoria, BC V8W 2Y2, Canada. E-mail larbour@uvic.ca

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Northern British Columbia whose ancestors have resided in the region for thousands of years.<sup>8</sup> The frequency in this community is consistent with one or more “founder effects,” whereby rare autosomal-dominant conditions not affecting fecundity may become common as rare variants are passed from generation to generation. We have previously identified a pathogenic variant (*KCNQ1* p.V205M), which could be traced back at least 7 generations, explaining LQTS type 1 in the majority of cases of LQTS<sup>9,10</sup> in that region; however, until recently we were unable to identify a potentially causal variant in a second group of kindreds with LQTS, but lacking the *KCNQ1* variant.

Here, we report the identification of a novel *ANK2* variant (*ANK2* c.1937C>T resulting in p.Ser646Phe) associated with LQTS and cardiac structural abnormalities in the Gitksan population. This loss-of-function variant is the first identified human “ankyrin-B syndrome” variant reported in the ankyrin membrane-binding domain (MBD).

## Methods

As part of a larger study,<sup>9</sup> participants were invited to enroll if they had clinical features of LQTS or were related to an individual with a diagnosis of LQTS. Community approval and individual consents were obtained, following University of British Columbia (H05-70330) and Northern Health Authority (RRC-2007-0038) ethics approval. Referrals for participation were through affected family members and healthcare providers. Health information was recorded upon enrollment through questionnaire and medical records review to confirm clinical diagnosis or suspicion of LQTS. Multigenerational family histories were obtained.

## Index Case Identification

Two apparently unrelated persons from the community, each with a clinical electrophysiology diagnosis of LQTS, were negative for the *KCNQ1* p.V205M variant; therefore, 12-gene clinical LQTS sequencing panels were carried out revealing the *ANK2* c.1937C>T (p.Ser646Phe) variant in each of the cases.

## Expanded Clinical Studies

Twenty-one additional close family members (first- and second-degree relatives) were tested for the *ANK2* c.1937C>T (p.S646F)

variant. The majority had been previously ascertained through the original LQTS study. All ECGs on file were reviewed by cardiac electrophysiologists blinded to mutation and clinical status. The QT interval was measured manually using the tangent method.<sup>11</sup> The reported QT interval was the longest identified within all 12 leads and corrected for heart rate using the Bazett formula. Echocardiograms were performed in carriers when possible.

## Animals

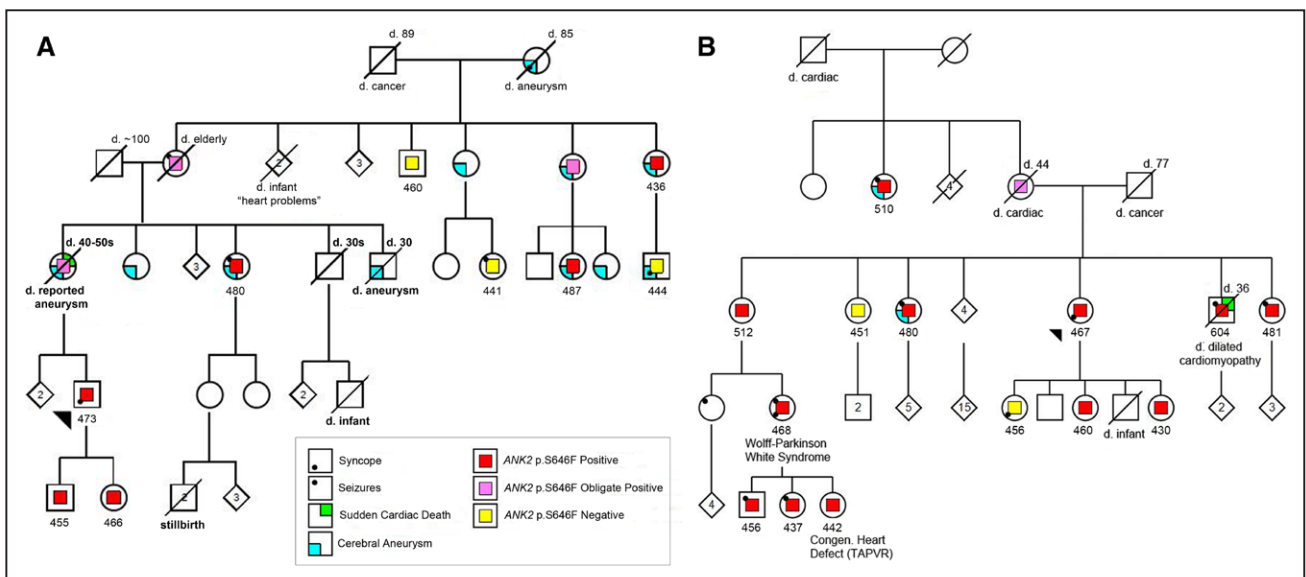
All animal studies were performed in accordance with the American Physiological Society *Guiding Principles for Research Involving Animals and Human Beings*, and approved by the Ohio State University IACUC (2011A0000034-R2). The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health. Mice utilized were postnatal day 1 ankyrin-B<sup>+/-</sup> mice.<sup>12</sup>

## Neonatal Myocyte Experiments

Cardiomyocytes from ankyrin-B<sup>+/-</sup> neonatal mice were isolated and transfected as described.<sup>13</sup> Neonatal myocytes were transfected with wild-type (WT) green fluorescent protein (GFP)-ankyrin-B DNA, p.S646F GFP-ankyrin-B, and GFP 24 hours post isolation. Constructs were generated from cDNA for canonical 220-kDa ankyrin-B (NP\_066187.2) and variants introduced by standard site-directed mutagenesis. Constructs were fully sequenced to ensure no additional variants were present. Cells were allowed to recover from transfection for 48 hours in complete media. Cells were fixed for 15 minutes in 2% PFA and immunolabeled utilizing primary antibodies specific for Na/Ca exchanger (NCX; Swant, 1:50 and GFP, 1:100).<sup>14</sup> Myocytes were colabeled to mark the nuclei (DAPI, 1.5 µg/mL). Secondary antibodies included Alexa-conjugated donkey antimouse 568 and donkey antirabbit 488 (Invitrogen). Neonatal cardiomyocytes were mounted on Mat-Tek plates and imaged on an LSM780 confocal microscope.

## Protein Expression and Purification

The coding sequences of ankyrin-B MBD (residues 28–873) were polymerase chain reaction amplified using the full-length 220-kDa ankyrin-B as the template. The point mutation was created using the Quick Change site-directed mutagenesis kit and confirmed by DNA sequencing. These coding sequences were cloned into a home-modified pET32a vector for protein expression. The N-terminal



**Figure 1.** Pedigrees of 2 Gitksan families with the p.S646F variant. **A**, Family 1; **B**, Family 2. Legend denotes variant status and clinical features. Number below each individual represents manual QTc (ms) read blinded to mutation and clinical status.

**Table 1. Summary of Clinical Features for 2 Gitxsan Families With the Ankyrin-B p.S646F Variant**

Family	Indv No.	Sex	p.S646F Variant Status	Age, y	Highest QTc, ms*	ECG Findings	Echocardiogram	Clinical Features/ Diagnoses	Seizures	Cerebral Aneurysms (Age, y)
1	1	M	Positive	34	473	U-wave, Tachycardia	Mild mitral and tricuspid insufficiency	Recurrent presyncope, 2X syncope with exercise while on $\beta$ -blockers (before ICD implant)	-	-
	2†	F	Positive	2	466		NA		-	-
	3†	M	Positive	12	455	Notching in V2 and V3	NA	Pre-syncope with exercise	-	-
	4	F	Positive	54	480		NA		+	++(30-40)
	5	F	Positive	64	436		Mild diastolic dysfunction	Recurrent presyncope	-	++(60)
	6	F	Positive	40	487		Trace mitral and tricuspid regurgitation		-	+(39)
	N1	M	Negative	44	444		NA	Syncope prior to aneurysm diagnosis	-	+(33)
	N2	F	Negative	51	441		NA		+	-
	N3	M	Negative	67	460		NA		-	-
	2	7	F	Positive	43	467	Mild U-wave, incomplete Right Bundle Branch Block	Normal	Implantable loop recorder revealed SVT, recurrent syncope	-
8		F	Positive	35	481		NA	Recurrent presyncope	+	-
9		F	Positive	48	480	Borderline broad-based T-waves in V2 and V3	NA		++	+(40)
10		M	Positive	d.36	604	Possible preexcitation, possible nodal accessory pathway	Severe dilated left ventricle with ejection fraction 20% to 25%, diastolic dysfunction, valve regurgitation, pulmonary hypertension, inferior vena cava dilation	Severe idiopathic dilated cardiomyopathy on autopsy (sudden cardiac death at 36 y)	++	-
11		F	Positive	51	512	Early precordial transition (R>S in V1), U pattern in V2	Mild mitral, tricuspid, and aortic regurgitation. Ascending aorta upper limits of normal (36 mm)		-	-
12		F	Positive	23	460		Mild mitral and tricuspid regurgitation		-	-
13		F	Positive	24	430	U-wave, sinus bradycardia	NA		-	-

(Continued)

Table 1. Continued

Family	Indv No.	Sex	p.S646F Variant Status	Age, y	Highest QTc, ms*	ECG Findings	Echocardiogram	Clinical Features/ Diagnoses	Seizures	Cerebral Aneurysms (Age, y)
	14	F	Positive	69	510	Right bundle branch block, notching in V2	NA		++	+ (47)
	15	F	Positive	23	468	Pre-excitation, AVRT	Trivial mitral and tricuspid regurgitation	Wolff–Parkinson–White syndrome, recurrent syncope	+	–
	16†	M	Positive	8	456	Borderline U-wave, notching in V2	Normal		+	–
	17†	F	Positive	6	437	Abnormal T-waves in V1 and V2, T-wave inversion in V1, notching in V2	Normal		+	–
	18†	F	Positive	1	442		Congenital heart defect: TAPVR	TAPVR, surgical repair at 2 mo of age	–	–
	N4	F	Negative	49	451		NA		–	–
	N5†	F	Negative	17	456	Notching in V2	NA	Reflex-mediated syncope	–	–

N1–N5 are negative for the variant, all others are positive. ++ indicates multiple; F, female; M, male; NA, not available; and TAPVR, total anomalous pulmonary venous return.

\*Manually read, blinded to clinical and mutation status.

†Age <18 years.

thioredoxin-His<sub>6</sub>-tagged proteins were expressed in *Escherichia coli* BL21 (DE3) and purified as previously described.<sup>15</sup> The thioredoxin-His<sub>6</sub>-tag was removed by incubation with HRV 3C protease and separated by size exclusion columns.

### Fast Protein Liquid Chromatography Coupled With Static Light Scattering

Protein samples (100  $\mu$ L at a concentration of 50  $\mu$ mol/L, pre-equilibrated with corresponding column buffer) were injected into an AKTA fast protein liquid chromatography system with a Superose 12 10/300 GL column (GE Healthcare) with the column buffer of 50 mmol/L Tris–HCl, 100 mmol/L NaCl, and 1 mmol/L Dithiothreitol, pH 7.8. The chromatography system was coupled to a static light-scattering detector (MiniDawn, Wyatt) and differential refractive index detector (Optilab, Wyatt). Data were analyzed with ASTRA 6 (Wyatt).

### Circular Dichroism

Circular Dichroism (CD) spectra of WT ankyrin-B MBD and the p.S646F variant were measured on a JASCO J-815 CD spectropolarimeter at room temperature using a cell path length of 1 mm. Each spectrum was collected with 3 scans spanning a spectral window of 200 to 260 nm. The samples were dissolved in 25 mmol/L Tris buffer containing 50 mmol/L NaCl, 0.5 mmol/L EDTA, and 0.5 mmol/L Dithiothreitol at pH 7.8 with the increasing concentrations of urea in the same buffer. The protein concentration used in the CD experiment was 10  $\mu$ mol/L.

### Structural Modeling

Analysis of the location of p.S646 residue and proposed binding groove of the ANK repeat 17 to 21 was performed using the high-resolution crystal structure of ankyrin-B MBD.<sup>16</sup> The structural figures were prepared using PyMOL ([www.pymol.org](http://www.pymol.org)).

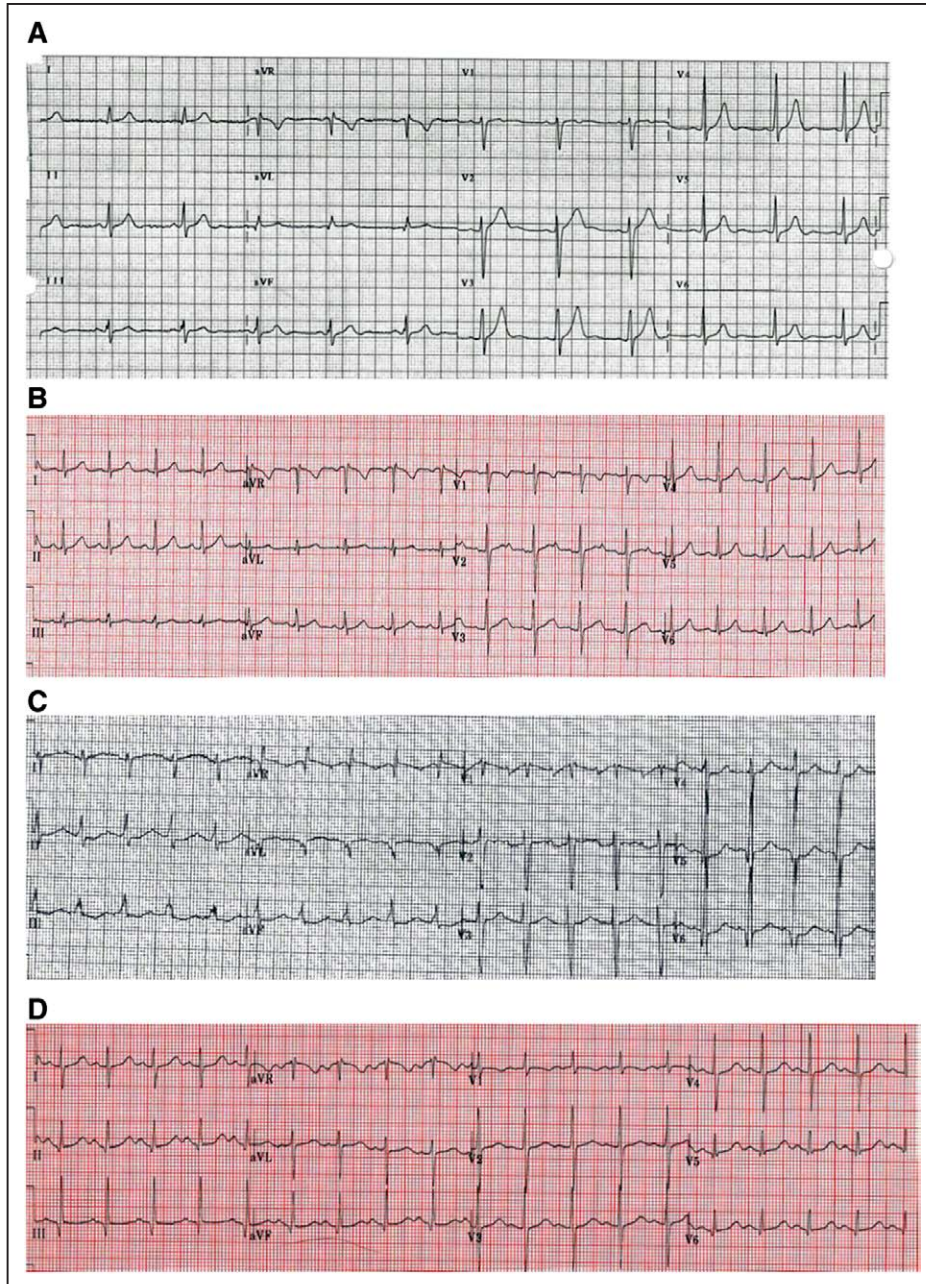
### H9c2-Cell Studies

H9c2 rat ventricular-derived cardiomyoblasts were purchased from American Type Culture Collection (ATCC CRL-1446). Cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin (37°C, 5% CO<sub>2</sub>) and passaged at 70% to 80% confluence. Transfections were performed using jetPEI (Polyplus/VWR) according to the manufacturer's protocol. The 220-kDa ankyrin-B isoform 2 (NP\_066187.2) was commercially synthesized (Bio Basic Inc., Markham, ON) and subcloned into pACGFP1-n1. Ankyrin-B p.S646F-pACGFP1-n1 was constructed using a QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Mississauga, ON) following the manufacturer's protocol. All primers were purchased from Integrated DNA Technologies (Coralville, IA). All plasmids were verified by sequencing before use. Cell lysis was performed as previously described.<sup>17–19</sup> Lysates were heated for 5 minutes (95°C) under reducing conditions, separated by SDS–PAGE and transferred to 0.2- $\mu$ m pore size polyvinylidene fluoride membrane for 3 hours at 200 mA. Primary antibodies used were anti-GFP polyclonal (1:2000; Life Technologies) and anti- $\beta$ -actin monoclonal (1:1000; Sigma-Aldrich). Secondary antibody used was horseradish peroxidase–conjugated AffiniPure donkey anti-rabbit IgG (1:2000; Jackson ImmunoResearch) and horseradish peroxidase–conjugated AffiniPure donkey antimouse IgG (1:2000; Jackson ImmunoResearch). Proteins were detected using Clarity enhanced chemiluminescence reagent (BioRad) and were quantified using Image J. Statistical analyses were performed using Prism for Mac OS X v5.0d software (<http://graphpad.com>; GraphPad Software). All variances were reported as SEM.

## Results

### Identification of Novel LQTS Variant in the Gitxsan First Nations Community

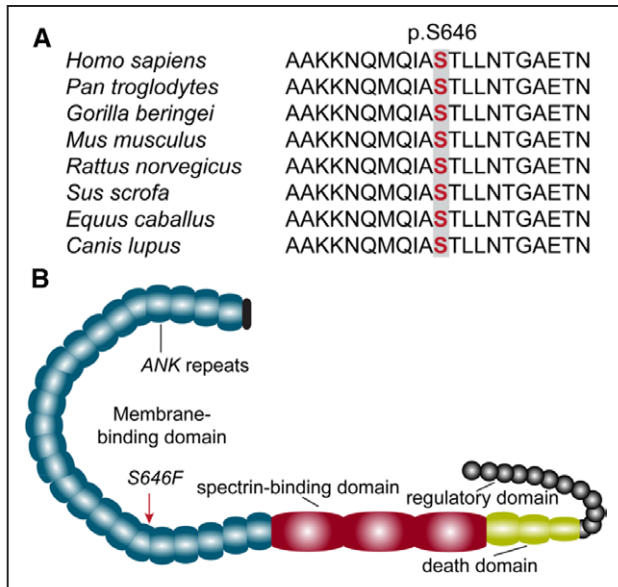
We identified a novel c.1937 C>T variant in exon 18 (p.S646F) in the ANK2 gene in 2 Gitxsan multigenerational families



**Figure 2.** Representative ECGs from 4 carriers of the p.S646F variant. **A**, Family 1, Indv 1: note U waves; **(B)** Family 1, Indv 3: note abnormal notching in V2; **(C)** Family 2, Indv 10: note prolonged QTc; **(D)** Family 2, Indv 11: note prolonged QTc.

with LQTS featured in Figure 1A and 1B. We first identified ankyrin-B p.S646F in a 28-year-old man with a clinical diagnosis of LQTS who experienced recurrent syncope, despite treatment with  $\beta$ -blockers necessitating the insertion of an implantable cardioverter–defibrillator. The *KCNQ1* p.V205M mutation was absent, and no other potential disease causing mutations were detected on a standard 12 gene LQTS sequencing panel.<sup>20</sup> Five of his relatives, including 2 with a previous diagnosis of LQTS, were subsequently confirmed to harbor the ankyrin-B p.S646F variant (Figure 1A; Table 1). The ankyrin-B p.S646F variant was also identified in a second “unrelated” family with LQTS (Figure 1B). The proband for this family had a clinical diagnosis of LQTS provided by her electrophysiologist, prompting the genetic testing. LQTS segregated with the

variant in an additional 5 members of the family, including one who died suddenly with a diagnosis of dilated cardiomyopathy. An additional variant-positive family member had a borderline QTc of 468 ms, but has been noted on repeated ECGs to have Wolff–Parkinson–White syndrome. Her infant daughter inherited the variant and notably was born with a congenital structural heart defect (total anomalous pulmonary venous return). To date, within these 2 families, a total of 13 adults and 5 children have been identified with ankyrin-B p.S646F (Table 1). The LQTS phenotype (Figure 1) segregates with the variant (average QTc in adults =475 ms $\pm$ 40; range 430–604 ms), and noncarriers do not have the LQTS phenotype. Of note, there is only minimal evidence of a prolonged QTc interval in the age group <25 years. Of 9 individuals reporting a



**Figure 3.** Ankyrin-B p.S646 is located within membrane-binding domain and highly conserved across species. **A**, Conservation of p.S646 across vertebrates. **B**, Ankyrin-B domain organization and location of p.S646F variant on membrane-binding domain.

history of seizures, 8 were carriers of the p.S646F variant (44% of carriers). In summary, LQTS, dilated cardiomyopathy with associated sudden death, congenital heart malformation total anomalous pulmonary venous return, Wolff–Parkinson–White syndrome, and seizures have been identified in the ankyrin-B p.S646F carriers (Figure 1; Table 1). See also 4 representative ECGs from carriers shown in Figure 2.

The ankyrin-B p.S646 residue is highly conserved across evolution, and this variant results in a nonconservative amino acid substitution of a polar amino acid (serine) for a nonpolar amino acid (phenylalanine) in the large aminoterminal ankyrin-B membrane-binding domain (Figure 3A and 3B). In silico analyses using MutationTaster,<sup>21</sup> Polyphen,<sup>22</sup> and PROVEAN<sup>23</sup> predict the p.S646F variant as disease causing/possibly damaging/deleterious. This variant was absent in >6500 control population samples in the National Heart, Lung, and Blood Institute Exome Sequencing Project. Furthermore, to date, this variant in *ANK2* is not reported in >120,000 alleles (minor allele frequency=0.0000 from Exome Aggregation Consortium<sup>24</sup>; National Heart, Lung, and Blood Institute<sup>25</sup>; 1000 genomes<sup>26</sup>; Table 2).

### Ankyrin-B p.S646F Membrane-Binding Domain Is Appropriately Expressed and Folded

To test the impact of the p.S646F variant on the ankyrin-B polypeptide, we performed biochemical analysis of purified

**Table 2. Minor Allele Frequency Data for *ANK2* c.1937 C>T**

Database*	Variant Allele Count	Total Allele Count
ExAC	0	117,264
NHLBI	0	13,004
1000 Genomes	0	5008

\*For Exome Aggregation Consortium (ExAC), National Heart, Lung, and Blood Institute (NHLBI), and 1000 genomes.<sup>24–26</sup>

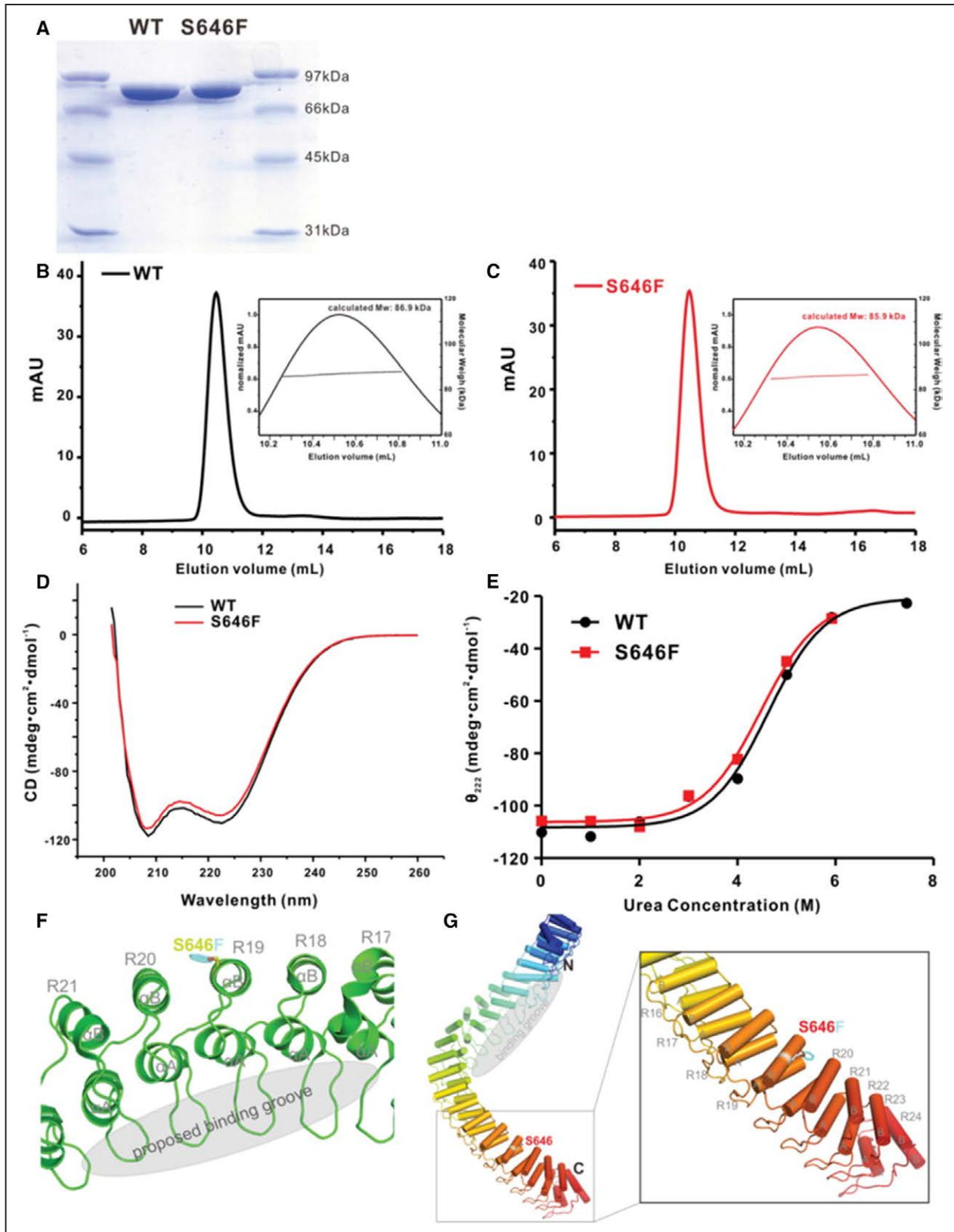
ankyrin-B MBD (residues 28–873) harboring the p.S646F variant in parallel with WT ankyrin-B MBD. Following the expression of the recombinant proteins in bacteria, polypeptides were purified and subjected to fast protein liquid chromatography analytic gel filtration coupled with static light-scattering to detect column behavior and molecular weight, CD to evaluate secondary structure, and CD-based denaturation to analyze protein stability (Figure 4). We observed no significant difference in protein expression between ankyrin-B MBD and ankyrin-B MBD p.S646F (Figure 4A). Specifically, protein quantities and column behavior were nearly identical for the 2 polypeptides, with appropriate molecular weight mobility (Figure 4B- and 4C). We did, however, observe that the ankyrin-B p.S646F polypeptide was unable to be concentrated to the same extent as WT ankyrin-B MBD, causing protein precipitation at high concentrations. CD spectra showed that both WT ankyrin-B MBD and ankyrin-B p.S646F MBD are enriched in helical structures with no significant structural differences (Figure 4D). Finally, using CD-based urea denaturation experiments, we observed no differences in protein stability between WT ankyrin-B MBD and ankyrin-B p.S646F MBD (Figure 4E). It is worth noting that these biochemical data are in line with previous structural analysis of the ankyrin-B MBD. Ankyrin-B p.S646F resides on the  $\alpha$ B of the 19th ANK repeat on the outer surface of the ANK repeat solenoid (Figure 4F and 4G). While increasing local hydrophobicity, this site is not predicted to directly interfere with binding sites for ankyrin-B membrane protein targets (sites located on inner groove of ANK repeats; Figure 4F and 4G). In summary, p.S646F does not significantly alter ankyrin-B MBD expression or structural properties.

### Ankyrin-B p.S646F Alters Polypeptide Stability in a Model Cardiomyocyte Cell Line

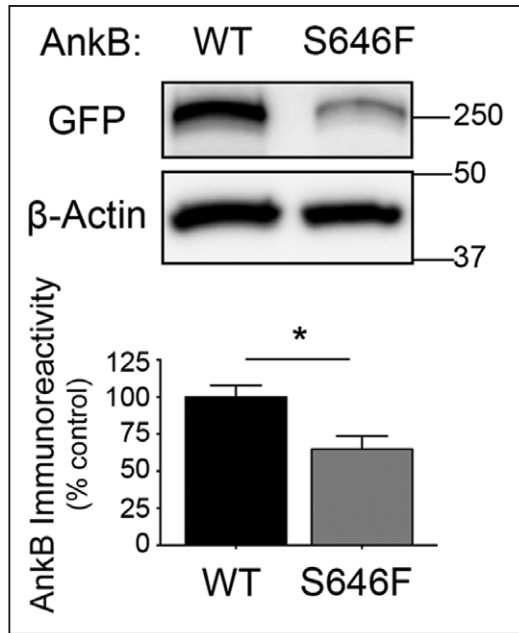
Ankyrin-B p.S646F does not alter the expression or folding of the bacterially expressed purified MBD (Figure 4); however, to interrogate the stability of the full-length ankyrin-B p.S646F in living cells, we transfected H9c2 rat ventricular cardiomyoblasts, a commonly used model cardiomyocyte cell line, with GFP-tagged WT ankyrin-B and ankyrin-B harboring the p.S646F variant. Although cells transfected at similar efficiencies, subsequent immunoblotting revealed that 2 days post transfection, steady-state ankyrin-B p.S646F levels were significantly lower than WT ankyrin-B (Figure 5). Together, these data support that while the purified MBD harboring the p.S646F variant displayed normal expression, folding and stability, the full-length ankyrin-B p.S646F variant was unstable when expressed in model cardiomyocytes.

### Ankyrin-B p.S646F Alters Ankyrin-B and NCX Targeting in Primary Cardiomyocytes

We tested the expression and function of the ankyrin-B p.S646F variant in the primary cardiomyocytes. Specifically, we introduced GFP, GFP-ankyrin-B, and GFP-ankyrin-B p.S646F into myocytes derived from mice heterozygous for a null mutation in ankyrin-B (*ankyrin-B<sup>+/-</sup>*) to mimic the autosomal-dominant background in human carriers. Unlike WT GFP-ankyrin-B that localized to the plasma membrane and rescued localization of the NCX, ankyrin-B p.S646F was unable to rescue abnormal targeting of NCX (Figure 6).

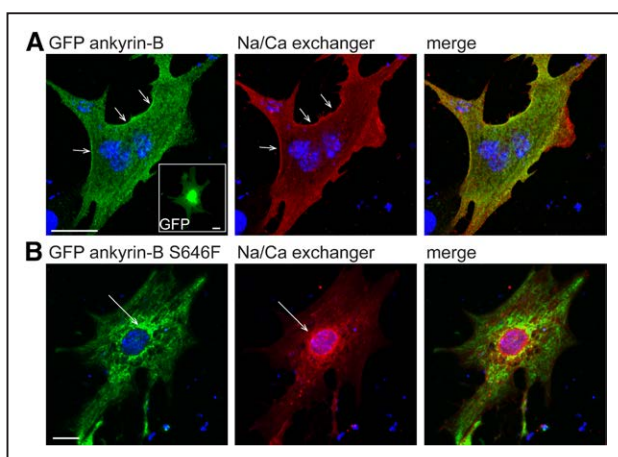


**Figure 4.** Ankyrin-B membrane-binding domain (MBD) p.S646F protein is appropriately expressed and folded in vitro. **A**, SDS-PAGE shows the purified protein qualities of wild-type (WT) ankyrin-B MBD and p.S646F mutant. The molecular markers are shown on both sides. **B** and **C**, FPLC analytic gel filtration coupled with static light scattering profiles of WT and p.S646F show symmetrical column behavior and nearly identical molecular weight. **D**, Circular Dichroism (CD) spectra of WT ankyrin-B MBD and p.S646F show the enrichment of helical structures. **E**, Urea denaturation-based assay of the stabilities of the WT ankyrin-B MBD and p.S646F mutant shows no obvious difference. **F** and **G**, Structural model shows that the p.S646F residue locates on the outer surface of the ANK repeat solenoid (Protein Data bank ID code: 4 RLV code: 4 RLV).



**Figure 5.** Ankyrin-B p.S646F exhibits decreased post-translational stability in H9c2 cardiomyoblasts. **A**, Immunoblot of green fluorescent protein (GFP)-tagged ankyrin-B (AnkB; wild-type [WT] or p.S646F)-expressing H9c2 cell lysates 48 hours post-transfection probed with anti-GFP (top) and anti- $\beta$ -actin (bottom). **B**, Quantification of GFP immunoreactivity revealed that ankyrin-B p.S646F expression is significantly reduced in comparison to the WT protein ( $n=3$ ;  $*P=0.0408$  by unpaired  $t$  test). These data are representative of 3 separate independent experiments, each with  $n \geq 3$  independent biological replicates.

In fact, compared with membrane-associated GFP-ankyrin-B, GFP-ankyrin-B p.S646F was concentrated intracellularly in large cytoplasmic puncta resembling the endosome/lysosome network (Figure 6B). In summary, our data support that ankyrin-B p.S646F displays loss-of-function activity in



**Figure 6.** Ankyrin-B p.S646F is a loss-of-function variant in myocytes. **A**, Green fluorescent protein (GFP)-ankyrin-B localizes to the membrane (arrows) of ankyrin-B<sup>-/-</sup> neonatal cardiomyocytes. We observe similar localization of Na/Ca exchanger at the membrane of immature primary neonatal cardiomyocytes. Note: GFP localized primarily to nucleus in control experiments as expected (inset). **B**, GFP-ankyrin-B p.S646F is enriched in the perinuclear region of ankyrin-B<sup>-/-</sup> neonatal cardiomyocytes. We observed similar localization of the Na/Ca exchanger in these myocytes (arrows). Scale bar, 20  $\mu$ m.

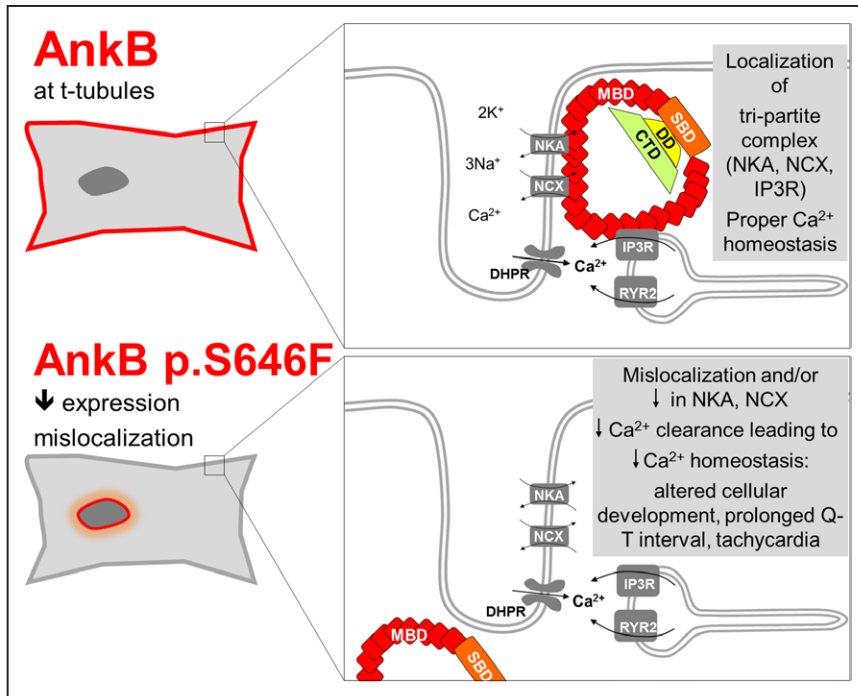
myocytes resulting in reduced expression and abnormal localization, ultimately affecting both expression and localization of downstream-binding partners, including NCX.

## Discussion

LQTS type 4, caused by mutations in *ANK2*, accounts for <1% of all cases of LQTS.<sup>2</sup> The clinical presentation is well known for its variable phenotype and association with non-LQTS cardiac phenotypes,<sup>27</sup> necessitating the broader term named for the protein responsible, “the ankyrin-B syndrome.” Consistent with previously published reports,<sup>12,28–30</sup> the QTc range in p.S646F variant carriers was broad (430–604 ms) and only 8 of 18 carriers had a QTc >470 ms. Of note, and also in keeping with previous reports, there was minimal evidence of a prolonged QTc interval in the age group <25 years. Other cardiac phenotypes, however, were present in Table 1.

Ankyrin-B dysfunction is associated with acquired cardiovascular disease in humans and cardiovascular disease modeled in animals.<sup>30–33</sup> Loss-of-function ankyrin-B variants underlie a complex cardiovascular human phenotype. Previously reported cases have displayed ventricular arrhythmias (with or without history of prolonged QTc), sinus node dysfunction and atrial fibrillation<sup>12,27,28,30,34–37</sup> depending on the location and severity of the specific variant. Ankyrin-B is composed of 4 domains: the MBD required for association with key membrane protein targets and autoinhibition,<sup>38,39</sup> a spectrin-binding domain, a death domain, and a C-terminal regulatory domain. Although human arrhythmia disease variants have previously been localized to both the spectrin-binding domain and C-terminal regulatory domains, ankyrin-B p.S646F is the first identified loss-of-function variant located within the MBD. On the basis of our findings along with our previous work, we propose that the “ankyrin-B syndrome” phenotype is broader than previously reported, and our study adds to that spectrum. We propose the phenotype (LQTS4, Wolff–Parkinson–White syndrome/tachycardia, congenital heart malformation, and cardiomyopathy with associated sudden death) observed in individuals expressing ankyrin-B p.S646F is caused at the cellular level by impaired Ca<sup>2+</sup> clearance (Figure 7), with consequences for the regulation of cardiomyocyte excitability similar to previous ankyrin-B mutations.<sup>40</sup> Ankyrin-B is responsible for the proper anchoring of the tripartite complex (NCX, Na/K ATPase, and inositol 1,4,5-trisphosphate receptor; Figure 7). The NCX normally extrudes Ca<sup>2+</sup>, in exchange for 3 Na<sup>+</sup> ions inward, using the Na<sup>+</sup> gradient from the Na/K ATPase. On the basis of this study together with previous reports on ankyrin-B variants, it is reasonable to postulate that the unchecked accumulation of cytosolic Ca<sup>2+</sup> due to the loss of NCX localization produces Ca<sup>2+</sup> overload in the sarcoplasmic reticulum resulting in uncontrolled leakage and afterdepolarizations, and a prolonged QT interval (Figure 7). Similarly, the occurrence of Wolff–Parkinson–White syndrome (essentially an extra electric pathway between the atria and the ventricles that causes tachycardia) in the ankyrin-B p.S646F population could also be linked to the impaired localization of NCX, with recent work in genetic models demonstrating that NCX plays a critical role in cardiac pacemaking.<sup>41,42</sup> Furthermore, [Ca<sup>2+</sup>]<sub>i</sub> is a critical





**Figure 7.** Model for novel disease-causing variant identified in the membrane-binding domain of the Ankyrin B protein (ANK2 c. 1937C>T p.Ser646Phe). DHPR indicates dihydropyridine receptor; IP3R, inositol 1,4,5-trisphosphate receptor; NCX indicates Na/Ca exchanger; NKA, Na/K ATPase.

determinant of cardiomyocyte development<sup>43</sup> regulating the transcription of several pivotal developmental genes.

In addition to the variable phenotype observed, another notable characteristic of the affected individuals is that the clinical presentation of LQTS4 may be proportional to age.<sup>27</sup> We suspect this could be due to age-related decreased expression of molecular chaperones,<sup>44</sup> which normally act to buffer the cardiomyocyte from stress associated with genetic mutations.<sup>45</sup> For example, with a high level of molecular chaperone coexpression, the catalytic activity of enzymes harboring mutations is preserved to a significantly greater extent.<sup>46</sup> Therefore, it is reasonable to speculate that the relatively small difference in protein expression and partial mislocalization of AnkB p.S646F with concomitant effects on NCX observed in the *in vitro* system (Figure 6) during a relatively short duration (days), would likely be exacerbated with age in the context of the living organism, consistent with what is observed in our patient population. Furthermore, heterogeneity in these cellular systems that buffer against genetic mutations and associated cellular dysfunction likely also affects the phenotypic variability that manifests with the mutation. Future *in vitro* work will examine the interplay between AnkB p.S646F and cellular chaperone systems to further explore these ideas and potentially to develop novel therapeutic strategies targeting molecular chaperones.

As noted above, ankyrin-B p.S646F is localized to the MBD, a large protein domain composed of 24 consecutive ANK repeats. Computational algorithms predict the effect of this nonconservative substitution of a polar residue (serine) for a nonpolar amino acid (phenylalanine) is deleterious. Because of the critical role of the ankyrin-B MBD, it was generally viewed that significant variation in this domain would be incompatible with postnatal life. Because the localization of this variant to the 19th ANK repeat is not predicted to directly interfere with the binding site for known ankyrin-B-interacting

proteins (Figure 3) and the overall structure of the MBD was indistinguishable from WT, the variant likely modulates tertiary structure in such a way that determinants of its own localization and stability are disrupted. In fact, we have learned that the ankyrin MBD plays a critical role in regulating binding to interacting proteins through a complex “auto-regulatory” mechanism that involves MBD association with the spectrin-binding domain.<sup>38</sup> Furthermore, mutations within this region, highly conserved across evolution, may interfere with a critical trafficking molecule required for the localization of ankyrin-B to the myocyte membrane after biosynthesis. The aberrant immunolocalization of GFP-ankyrin-B p.S646F to intracellular clusters supports this hypothesis (Figure 6). Finally, it is worth noting that beyond the fact that this variant was not present in 6500 control samples at the time of sequencing, this variant has not been identified in any global cohorts regardless of ancestry (minor allele frequency of 0.000 in >120,000 alleles; Table 2). Consistent with the category of “likely pathogenic,” predicting at least a 90% chance of pathogenicity as per the American College of Medical Genetics and Genomics *Standards and Guidelines for Interpretation of Sequence variants*,<sup>47</sup> our studies support that this variant contributes to inherited heart disease. It will be important in future experiments to further define the role of this variant, and this specific ANK repeat in ankyrin-B biosynthesis and regulation in myocytes, both *in vitro* and *in vivo*.

An unexpected and intriguing finding here was the presence of 7 p.S646F carriers (5 established with genetic testing and 2 obligate carriers), between the 2 families confirmed to be affected with cerebral aneurysm. One family, in particular (Figure 1A), has multiple additional relatives with cerebral aneurysms who have not yet undergone p.S646F testing. Familial cerebral aneurysm is a rare event with only 0.2% of cases reporting >1 affected first-degree relative.<sup>48</sup> It is premature, however, to postulate any role of the variant on the cerebral vasculature, especially

given a paucity of evidence for ankyrin-B expression in endothelial cells.<sup>49,50</sup> Furthermore, of note, one individual without the p.S646F variant developed a cerebral aneurysm (Figure 1A). Of possible relevance, given the high rate of seizures in p.S646F carriers with and without cerebral aneurysms (Table 1), ankyrin-B has previously been shown to play a critical role in neurite outgrowth and perhaps may contribute to the genesis of abnormal brain activity, such as that leading to seizures. This hypothesis remains to be tested in the laboratory.

Given its critical roles in targeting and stabilization of key structural and signaling molecules in cardiac cells and other organ systems,<sup>13,51–53</sup> it is not surprising that phenotypes beyond ventricular arrhythmia were observed in the 2 p.S646F kindreds. The presence of other arrhythmias, as well as structural heart disease and possibly cerebral involvement, underscores the importance of future studies investigating the broad impact of the p.S646F variant as a model for other variants localized to the membrane-binding domain.

### Limitations

This study featured a sub population of a cohort being investigated for a high rate of LQTS in Northern British Columbia leading to ascertainment of these families. Most clinical data collection was by medical record review. Because of the nature of the original study, the delineation of the possible neurological phenotype deserves further and detailed study. Despite these limitations, this study represents an important advance in our understanding of the role of ankyrin-B in cardiac health and disease and lays the groundwork for additional studies.

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### Disclosures

None.

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### CLINICAL PERSPECTIVE

Human ANK2 loss-of-function variants are linked with type 4 long QT syndrome. ANK2 encodes ankyrin-B, a critical multifunctional cardiac regulatory “scaffold” protein with key roles in ion channel and transporter membrane targeting and modulation. It is not surprising, therefore, that ANK2 variants contribute to cardiac arrhythmia phenotypes beyond long QT syndrome (sinus node dysfunction and atrial fibrillation), necessitating the broader term, “ankyrin-B syndrome.” Here, we report the identification of a novel ANK2 variant (ANK2 c.1937C>T resulting in p.Ser646Phe) and describe the clinical characteristics in 18 carriers, introducing the notion that ANK2 dysfunction causes both cardiac electric and structural phenotypes. The variant we describe, present in a remote Indigenous community, is uniquely placed in the membrane-binding domain of ankyrin-B, supporting that altered protein expression and function, along with the specific location of the variant may influence the clinical presentation. We show that the p.S646F variant alters both ankyrin-B expression and activity in in vitro cell lines and primary cardiomyocytes. We propose that the described phenotype in our cohort, (long QT syndrome type 4, Wolff–Parkinson–White syndrome/tachycardia, congenital heart malformation, and cardiomyopathy) arises at the cellular level resulting from dysregulation of Ca<sup>2+</sup> clearance affecting cardiomyocyte excitability and further affects the localization of ankyrin-B to the myocyte membrane, potentially leading to congenital and later life structural heart disease. In summary, our findings represent a critical advance in our understanding of the complexities of heart disease within a defined population and further underscore the importance of ankyrin-B in cardiac health.

## Novel Variant in the ANK2 Membrane-Binding Domain Is Associated With Ankyrin-B Syndrome and Structural Heart Disease in a First Nations Population With a High Rate of Long QT Syndrome

Leigh Anne Swayne, Nathaniel P. Murphy, Sirisha Asuri, Lena Chen, Xiaoxue Xu, Sarah McIntosh, Chao Wang, Peter J. Lancione, Jason D. Roberts, Charles Kerr, Shubhayan Sanatani, Elizabeth Sherwin, Crystal F. Kline, Mingjie Zhang, Peter J. Mohler and Laura T. Arbour

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