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The Evolving Role of Ankyrin-B in Cardiovascular Disease

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Abstract

Over the last decade, ankyrin-B has been identified as a prominent player in cardiac physiology. Ankyrin-B has a multitude of functions, with roles in expression, localization, and regulation of proteins critical for cardiac excitability, cytoskeletal integrity, and signaling. Further, human *ANK2* variants that result in ankyrin-B loss-of-function are associated with 'Ankyrin-B syndrome', a complex cardiac phenotype that may include bradycardia and heart rate variability, conduction block, atrial fibrillation, QT interval prolongation, and potentially fatal catecholaminergic polymorphic ventricular tachycardia. However, our understanding of the molecular mechanisms underlying ankyrin-B function at baseline and in disease is still not fully resolved due to the complexity of ankyrin-B gene regulation, number of ankyrin-B-associated molecules, multiple roles of ankyrin-B in the heart and other organs that modulate cardiac function, and a host of unexpected clinical phenotypes. Here, we summarize known roles of ankyrin-B in the heart and the impact of ankyrin-B dysfunction in animal models and in human disease, as well as highlight important new findings illustrating the complexity of ankyrin-B signaling.

Keywords

ankyrin-B; *ANK2*; arrhythmias; ion channel; channelopathy

Introduction

Ankyrin-B is a member of the adapter protein family of ankyrins that includes ankyrin-R (*ANK1*), ankyrin-B (*ANK2*), and ankyrin-G (*ANK3*). These proteins display significant homology, yet maintain distinct functions and spatiotemporal dynamics. Within the heart, ankyrin-B (AnkB) and ankyrin-G (AnkG) are the major ankyrin gene products, although ankyrin-R (AnkR) isoforms have also been identified.[1, 2] AnkG functions in the targeting of voltage-gated sodium channel $Na_v1.5$ to the intercalated disc. Further, human variants in

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$Na_v1.5$ that block interaction with AnkG are associated with Brugada syndrome, an arrhythmia syndrome associated with ST segment elevation in precordial leads V1–V3 and susceptibility to sudden cardiac death.[3, 4] On the other hand, AnkB is primarily localized at the myocyte M-line and transverse-tubule (T-tubule) membranes where it associates with select membrane and signaling proteins to regulate excitation-contraction (EC) coupling. Human *ANK2* loss-of-function variants are associated with a variety of arrhythmia phenotypes, including sinus node disease, atrial fibrillation, ventricular arrhythmia, and risk of sudden cardiac death.

To understand the phenotypic effects of loss-of-function *ANK2* variants, it is first critical to understand excitation-contraction (EC) coupling. EC coupling is the process by which electrical stimuli ultimately lead to cardiac contraction. The initiation of cardiac contraction begins at pacemaker cells in the sinoatrial node with generation of an action potential (AP). The cardiac impulse spreads rapidly to the atrioventricular node, and then depolarization moves quickly though the ventricles, originating at the His Bundle before bifurcating to the left and right bundles. Purkinje fibers then rapidly propagate the AP into the ventricles. As the cardiac impulse spreads from myocyte to myocyte through gap junctions, action potentials are initiated by the activation of voltage-gated sodium channels (Na_v). Subsequently, calcium channels (Ca_v) in the sarcolemma membrane at the T-tubules open. The influx of Ca^{2+} activates adjacent ryanodine receptors (RyR2) to release stored Ca^{2+} from the sarcoplasmic reticulum (SR). Free Ca^{2+} then binds cardiac Troponin-C, inducing a conformational change of Troponin that releases Tropomyosin from myosin. Actin then binds myosin to initiate contraction. Finally, the myosin head binds ATP to pull the actin filament towards the sarcomere. For relaxation to occur, Ca^{2+} is removed from the cytoplasm by the SR Ca^{2+} ATPase (SERCA2) and the Na^+/Ca^{2+} exchanger (NCX1). Cytosolic Ca^{2+} moves into the extracellular space via NCX and is sequestered into the SR by SERCA2 until it is required for the next contraction. As Ca^{2+} is depleted from the cytosol, Tropomyosin and Troponin return to myosin to inhibit actin binding and relax the actin filament. This sequence repeats with the next AP.[5–8] As described below, AnkB plays a critical role in the expression and localization of key proteins required throughout the cardiac excitation pathway. Thus, it is not surprising that deficiency or altered AnkB function has significant impact on cardiac function.

Ankyrin-B domains and binding partners

Canonical AnkB is 220 kDa and consists of four primary domains – a membrane- binding domain (MBD), a spectrin-binding domain (SBD), a death domain (DD), and a C-terminal domain (Figure 1). The MBD consists of 24 consecutive *ANK* repeats, and it is responsible for AnkB-dependent interactions with ion channels, transporters, and cell adhesion molecules. While ankyrin-binding partners are often organ- and cell-selective, the AnkB MBD in vertebrate cardiomyocytes directly associates with membrane Na/Ca exchanger (NCX1), Na/K ATPase (NKA),[9, 10] the alpha subunit of the ATP-sensitive potassium channel (Kir6.2),[11] and in atria, the L-type calcium channel $Ca_v1.3$.[12] Of note, AnkB also regulates $Ca_v2.1$ expression and localization in the brain.[13]. While AnkB has been suggested to regulate $Na_v1.5$ in heart, adult mice deficient in AnkB display no alteration in $Na_v1.5$ expression, localization, or I_{Na}.[14] Beyond the plasma membrane, the MBD associates with

the inositol 1,4,5-trisphosphate receptor (IP_3R) at the sarcoplasmic reticulum.[11, 15, 16] The SBD interacts with β-spectrin at the N-terminal ZU5 domain to provide structural continuity between proteins associated with the MBD and the cytoskeleton of the cell.[17] Through a direct interaction with the B56α subunit, the SBD is also responsible for localization of protein phosphatase 2A (PP2A), a regulator of NKA, NCX, RyR2, and IP_3R that has a significant role in Ca^{2+} modulation via phosphorylation of these key players in the EC machinery.[18] Finally, the regulatory domain (RD), so called because of its influence on MBD and SBD interactions, is comprised of the DD and the C-terminal domain. Interestingly, while highly unstructured, the C-terminal domain has been shown to bind the AnkB MBD to regulate localization of its binding partners.[19] It is in this region where the highest number of pathogenic variants in *ANK2* have been identified, yet this is also an area of innate amino acid variability between species and across human populations.[20] This variability contributes to the difficulty of determining variant pathogenicity within the RD.

Role of ankyrin-B in cardiac excitability

Mouse models of AnkB deficiency have been critical to dissect the role of AnkB in vertebrate physiology. Mice homozygous for an AnkB null mutation suffer neonatal lethality.[21] However, mice heterozygous for an AnkB null mutation ($AnkB^{+/-}$) have been indispensable in elucidating the mechanisms of AnkB in cardiomyocytes. $AnkB^{+/-}$ mice display bradycardia and heart rate variability, atrial arrhythmias, minor defects in QT interval, catecholamine-induced ventricular arrhythmias, and sudden death.[22] At the cellular level, consistent with the roles of AnkB in binding NKA, NCX, and IP_3R, loss of AnkB has been shown to decrease the expression and normal localization of NKA, NCX, and IP_3R (Figure 2).[23] Decreased expression of AnkB also causes an attenuated response to ouabain, a cardiac glycoside that inhibits NKA, resulting in altered calcium oscillations.[23] $AnkB^{+/-}$ cardiomyocytes have unaltered diastolic [Ca^{2+}], yet they display larger Ca^{2+} transients, SR Ca^{2+} content, and fractional SR Ca^{2+} release, causing an increased frequency in Ca^{2+} sparks. Furthermore, $Ca_v1.2$ expression and function is reduced in a mouse model of cardiac-specific deficiency of EHD3, an endosomal protein that binds and traffics AnkB. [24, 25] Alterations in calcium signaling due to dysregulation of the RyR2 are attributed to enhanced coupling of RyR2 openings in $AnkB^{+/-}$ cardiomyocytes.[26] RyR2 pS1814 phosphorylation by calcium/calmodulin-dependent kinase (CaMKII) may also be amplified in $AnkB^{+/-}$ mice, potentially via disruption of the AnkB-dependent B56α targeting mechanism.[27] Interestingly, hyperphosphorylation of RyR2 through CaMKII has also been identified in cardiomyocytes from patients with paroxysmal and chronic atrial fibrillation.[28, 29] By an independent mechanism, activity of CaMKII may be activated due to increased levels of Ca^{2+} in the junctional cleft that are associated with decreased NCX activity in $AnkB^{+/-}$ mice.[30] These Ca^{2+} sparks can lead to aberrant release of Ca^{2+} that compromises the specificity of EC signaling, ultimately increasing the propensity for arrhythmias and electrical dysfunction [31]

Beyond the ventricle, AnkB directly binds a subgroup of voltage-gated Ca^{2+} channels ($Ca_v1.3$) that is predominately expressed in the atria and is responsible for voltage-activated L-type Ca^{2+} current. Heterozygosity of AnkB in mouse sinoatrial node and atrial myocytes is associated with reduced expression of [12, 32] Additionally, $Ca_v1.3$.cardiac-specific deletion

of EHD3 results in dysregulated expression and localization of $Ca_v3.1$ and $Ca_v3.2$ and reduced T-type mediated Ca^{2+} current.[33] $AnkB^{+/-}$ mice display sinus node and atrial electrophysiological dysfunction, abnormal SAN electrical activity (including altered diastolic depolarization), shortened atrial action potentials, atrial fibrosis, and increased susceptibility to atrial fibrillation.[12, 32, 34] Additionally, Glukhov et al found that isoproterenol-treated $AnkB^{+/-}$ mice exhibited competing multiple pacemakers and beat-to-beat variability within the leading pacemaker.[35] As with ventricular myocytes, $AnkB^{+/-}$ atrial and sinus node myocytes also display loss of NCX and NKA.[12]

Human *ANK2* variants

Loss-of-function variants in *ANK2* have been associated with a variety of cardiovascular phenotypes, and most notably present as Ankyrin-B Syndrome (Table 1). Formerly known as Type 4 Long QT Syndrome, Ankyrin-B Syndrome presents with an autosomal-dominant pattern of inheritance and displays a wide spectrum of phenotypes, including sinus node bradycardia, conduction block, prolonged rate corrected QT interval, and catecholaminergic polymorphic ventricular tachycardia.[14, 36] Mice heterozygous for a null mutation in *Ank2* ($AnkB^{+/-}$ mice) display similar arrhythmogenic disease to humans, exhibiting altered Ca^{2+} signaling and decreased expression and localization of the AnkB binding proteins that are necessary to maintain normal EC coupling.[22] AnkB is required for T-tubule/SR localization of NCX, NKA and IP_3R, and this interaction is lost in both $AnkB^{+/-}$ mice and in the human arrhythmia causing p.E1425G variant. Normal localization of NCX and AnkB was also found to be disrupted in the MBD variant p.S646F, indicating that NCX localization is regulated by two AnkB domains – MBD and RD.[38] *ANK2* variants have also been found to cause sinus node disease (SND), and AnkB heterozygous ($AnkB^{+/-}$) mice phenocopy human SND patients, presumably due to loss of AnkB-mediated organization and signaling in sinoatrial node (SAN) cells.[34] Another variant in *ANK2* that disrupts the AnkB/βII-spectrin interaction (AnkB p.R990Q) causes severe arrhythmia phenotypes. By studying this variant, Smith et al discovered that βII-spectrin regulates the localization of NCX, AnkB, and RyR2. Furthermore, βII-spectrin-deficient mice display lethal arrhythmias and an accelerated heart failure phenotype.[39]

Although the function of the four protein domains of AnkB are well described, the heterogeneous nature of *ANK2* variants remains unclear. To gain insight into the various clinical phenotypes observed with *ANK2* variants, Mohler et al. characterized nine loss-of-function variants in vitro by monitoring contraction rates, calcium release, and channel/transporter expression and localization. They found that the phenotypes presented by these variants fell into three categories – negligible, minor, and severe loss-of-function, and that they were localized to one of two domains, the SBD or the RD.[40] To date, over 2,500 *ANK2* variants are reported in the Exome Aggregation Consortium (ExAC) at the Broad Institute and over 350 *ANK2* variants have been logged in ClinVar at the National Institutes of Health. Despite the abundance of identified variants, few patterns have emerged to identify probable deleterious variants. Although the membrane-binding domain (MBD) comprises the majority of the genomic DNA and is directly responsible for interactions with membrane-bound ion channels and transporters, it is the least likely domain to harbor a variant (Figure 3). Only recently was the first account of a familial disease-causing, loss-of-

function, *ANK2* variant in the MBD reported. Segregation of the AnkB p.S646F variant was found in affected individuals from two multi-generational families within the Gitxsan First Nation with Long QT syndrome. The loss-of-function variant was found to prohibit normal membrane targeting of NCX, presumably due to the altered localization of AnkB p.S646F.[38] Ichikawa et al also recently found possibly damaging variants in the MBD in isolated cases of arrhythmia. All other reported disease causing loss-of-function variants occur in the SBD, DD, or C-terminal domain.[14, 22, 34, 42] Many loss-of-function variants in the SBD do not disrupt the interaction between AnkB and spectrin. Interestingly, Wang et al discovered that loss-of-function *ANK2* variants within the SBD that did not affect spectrin-binding were located within a structural supramodule at the $ZU5^{N}$-$ZU5^{C}$-UPA domain, providing evidence of an additional function for the SBD.[43–45]

Common variants in *ANK2* may also be relevant, as they have been shown to influence the QT interval length in the general population. In large general population surveys, Cooperative Health Research in the Region of Augsburg (KORA S3 and KORA S4), Sedlacek et al. found that common genetic variants in the distant 5′ region of *ANK2* significantly influenced rate-corrected QT intervals. Additionally, *ANK2* p.L1622I is associated with mild loss-of-function and is disproportionately identified in individuals of African descent, a population that also experiences increased incidence of ventricular arrhythmias and sudden cardiac death.[47] Similar common *ANK2* variants may introduce compound phenotypes with other unrelated cardiac diseases. Recently, evidence has emerged of a non-arrhythmogenic role for AnkB in heart disease, as *ANK2* variants are associated with greater maximum left ventricular wall thickness in patients with hypertrophic cardiomyopathy.[48] AnkB dysregulation is also associated with ischemic heart disease. Five days after canine myocardial infarction, AnkB protein expression is downregulated in the border zone, which is accompanied by dysregulation of AnkB-associated ion channels and transporters. However, upregulation of *Ank2* gene expression at this time results in recovery of AnkB levels at 14 days post-myocardial infarction.[49] Furthermore, Kashef et al showed decreased expression of AnkB and its downstream targets in human ischemic and non-ischemic failing heart tissue, and calpain, a calcium-dependent protease, was identified as a regulator of AnkB in ischemic disease.[50]

Mechanisms of functional specificity

There is a striking amount of homology amongst the ankyrin proteins, yet they have completely unique, non-redundant functions. In 2002, Mohler et al explored the non-redundant functions of AnkB and AnkG through the use of ankyrin-B/G chimeras. They found that AnkG was unable to rescue aberrant expression of AnkB-binding partners in $AnkB^{-/-}$ cardiomyocytes and attributed the functional specificity of AnkB to its regulatory domain.[20, 51] It is also fascinating that AnkB has numerous functions within cardiomyocytes. Alternative splicing of the *ANK2* mRNA transcript is one mechanism that has been explored to explain this characteristic. Cunha et al were the first to identify alternative splicing in *ANK2* and show a heterogeneous population of AnkB polypeptides in the heart, likely explaining the functional specificity observed in AnkB. Recently, two novel AnkB isoforms have been identified, AnkB-188 and AnkB-212 that also display unique functions. AnkB-188 functions in the localization and expression of NCX at the T-tubules,

while AnkB-212 interacts with obscurin at the M-line.[53, 54] The role of these variants in vivo is not yet known. Another mechanism of functional specificity is inter-domain interactions within AnkB. The C-terminal portion of the regulatory domain binds the membrane binding domain, and this binding is necessary for proper localization of IP_3R.[19] While studying the localization of AnkB in human bronchial epithelial (HBE) cells, He et al identified a linker peptide that interacts with the *ANK* repeat domains and the $ZU5_2$-UPA module and regulates the binding activity of AnkB, preventing membrane binding in HBE cells. This B-linker peptide is highly conserved in AnkB, and offers an additional mechanism by which AnkB and AnkG maintain divergent functions.[55]

Future investigations

Human disease-causing variants in *ANK2* present with incomplete penetrance and variable phenotypes (Table 1), as is commonly observed in familial human genetics.[56, 57] Phenotypic variability is likely due to the presence of genetic modifiers, a concept that dates back to 1941. Genetic modifiers may have a significant influence over the expressed phenotype that is a result of monogenic disease. Additional genetic variants as well as epigenetic modifications due to environmental influences may act as genetic modifiers. Chemical mutagenesis was recently used in zebrafish to identify genetic modifiers of cardiomyopathy, and a similar approach in mice was employed to identify genetic mechanisms of congenital heart disease.[59, 60] Future efforts must be placed on identifying genes and elucidating mechanisms that contribute to the phenotypic variability associated with *ANK2* variants.

Additional research to understand in the molecular roles of cardiac AnkB will also benefit several other areas of disease. To date, functional roles for AnkB have been implicated in neuronal disease, diabetes, and cancer.[61–63] AnkB, along with many other cardiac ion channel-related proteins, is also expressed in neurons. $AnkB^{-/-}$ mice are born with severe neurological defects including brain and optical nerve malformation.[64] Recently, AnkB and AnkG were shown to be decreased in human neurons with deletion of *L1CAM*, a gene that has been associated with hydrocephalus and intellectual disability.[65] Furthermore, seizures have been shown to correlate with AnkB-associated cardiac channelopathies.[38, 66, 67] Initially discovered in 1993 and 1995, the 440kDa AnkB (AnkB-440) and 480kDa AnkG (AnkG-480) isoforms, also known as giant ankyrins, are a result of the addition of a single large exon.[68, 69] The role of giant ankyrins in patterning the axon initial segment was demonstrated in Drosophila and was shown to be conserved across Bilatera. These giant ankyrins have specific functions in neurobiology[71–73], yet nothing is known about the role of giant ankyrins in the heart. The re-emergence of giant ankyrins in recent literature warrants investigation into potential localization and functions specific to these giant ankyrins in the heart.

Swayne et al recently reported the first account of a loss-of-function disease-causing *ANK2* variant in the membrane-binding domain in families with Long QT syndrome. While this finding is significant in itself, this group also reported the presence of congenital malformations associated with the AnkB p.S646F variant.[38] Interestingly, $AnkB^{-/-}$ mice suffer neonatal lethality[21], which could be associated with a congenital heart defect. Additionally, these families displayed an incidence of cerebral aneurysms, suggesting a

novel vascular role for AnkB. Further investigation and characterization of neonatal hearts and adult vasculature from AnkB deficient mice may provide insight into potentially novel roles for AnkB in cardiovascular development and disease.

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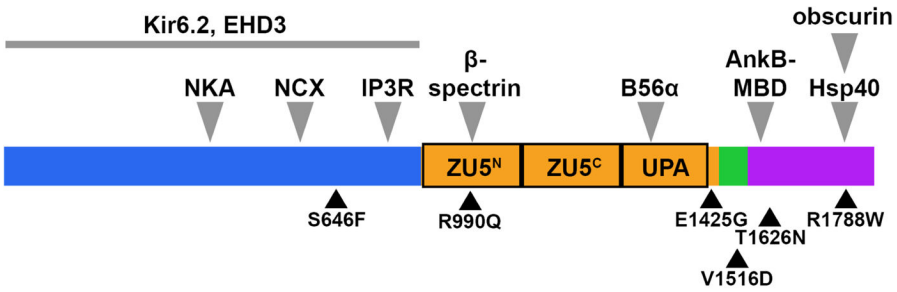

Figure 1.

Schematic of Ankyrin-B depicting protein domains and binding sites. Kir6.2 and EHD1–4 bind an undetermined location within the membrane-binding domain. Disease-causing variants in familial arrhythmogenic disease are shown. Blue, membrane-binding domain; orange, spectrin-binding domain; green, death domain; purple, C-terminal domain.

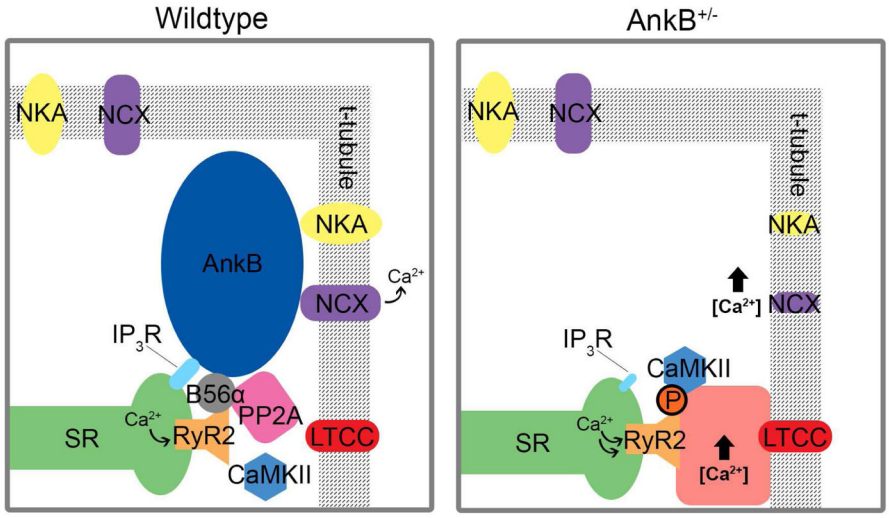

Figure 2.

Schematic of cardiomyocyte signaling changes with loss of AnkB. Deficiency of AnkB has been shown to decrease the expression and normal localization of Na/K ATPase (NKA) and Na/Ca exchanger (NCX), ultimately resulting in altered SR calcium load. Secondarily, altered ankyrin-B dependent-targeting of PP2A subunit B56α alters the PP2A/CaMKII balance favoring RyR2 hyperphosphorylation. Together, at the tissue level, these cellular events support arrhythmia, particularly in response to catecholamines.

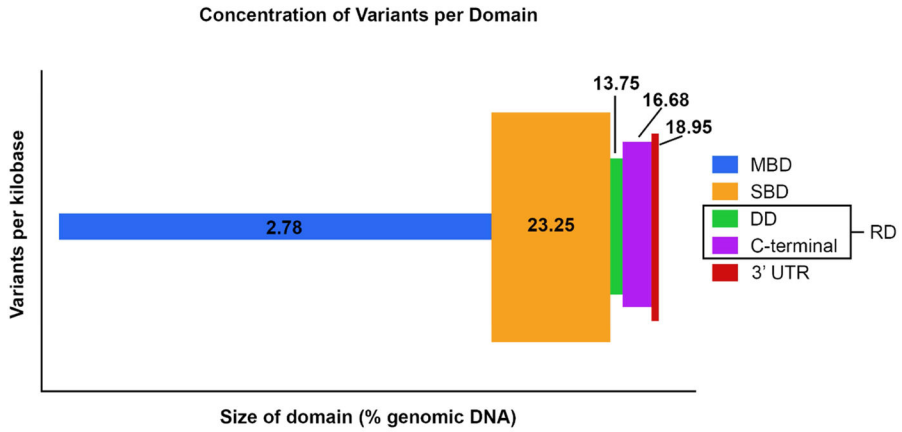

Figure 3.

Over 2,500 *ANK2* variants listed in the Exome Aggregation Consortium at the Broad Institute were mapped to a protein domain. The length of the domain along the x-axis is based on percentage of genomic DNA that makes up that domain. Variants per kilobase on the y-axis depicts the average number of variants/Kb/domain. MBD, membrane-binding domain; SBD, spectrin-binding domain; DD, death domain; RD, regulatory domain; UTR, untranslated region.

Table 1

Human *ANK2* variants linked to in vitro loss-of-function

Variant	Domain	Phenotype	Reference
S646F	MBD	Long QT, syncope, seizures, dilated cardiomyopathy with SCD, congenital heart defect, Wolff-Parkinson-White syndrome	38
R990Q	SBD	Long QT, syncope, ventricular fibrillation, sudden cardiac arrest	39
E1425G	SBD	Long QT, sinus node bradycardia, CPVT, atrial fibrillation, polyphasic t-waves, SCD	22
V1516D	DD	Atrial fibrillation, exercise-induced VT, drug-induced long QT, syncope, Brugada syndrome, bradycardia, CPVT	40
T1626N	CTD	Long QT, sinus arrhythmia, syncope, SCD	14
R1788W	CTD	Long QT, bradycardia, syncope, supraventricular and ventricular arrhythmias	14

MBD, membrane-binding domain; SBD, spectrin-binding domain; DD, death domain; CTD, C-terminal domain; SCD, sudden cardiac death; CPVT, catecholaminergic polymorphic ventricular tachycardia