



Ankyrins and Spectrins in Cardiovascular Biology and Disease

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Ankyrins are adaptor proteins critical for the expression and targeting of cardiac membrane proteins, signaling molecules, and cytoskeletal elements. Findings in humans and animal models have highlighted the *in vivo* roles for ankyrins in normal physiology and in cardiovascular disease, most notably in cardiac arrhythmia. For example, human *ANK2* loss-of-function variants are associated with a complex array of electrical and structural phenotypes now termed “ankyrin-B syndrome,” whereas alterations in the ankyrin-G pathway for Na_v channel targeting are associated with human Brugada syndrome. Further, both ankyrin-G and -B are now linked with acquired forms of cardiovascular disease including myocardial infarction and atrial fibrillation. Spectrins are ankyrin-associated proteins and recent studies support the critical role of ankyrin-spectrin interactions in normal cardiac physiology as well as regulation of key ion channel and signaling complexes. This review will highlight the roles of ankyrins and spectrins in cardiovascular physiology as well as illustrate the link between the dysfunction in ankyrin- and spectrin-based pathways and disease.

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INTRODUCTION

Cardiovascular disease is the leading cause of death in the United States (Rosamond et al., 2008). In 2013, the overall prevalence of death attributable to cardiovascular diseases (CVD) was 222.9 per 100,000 Americans. On the basis of 2013 data, more than 2,200 Americans die of CVD each day, representing an average of 1 death every 40 s. Arrhythmia is the direct cause of death in many cases. Nearly 80% of cases of sudden cardiac death are the result of ventricular arrhythmias (Mehra, 2007). Thus, understanding the molecular mechanisms underlying human arrhythmias has been an active area of cardiovascular research (Cunha and Mohler, 2006), with a major focus on ion channels, transporters and receptors that regulate the action potential at baseline and in disease. Ankyrins and spectrins are adaptor proteins now linked with both electrical and structural forms of heart disease. Here, we will review the roles of ankyrin and spectrin polypeptides as critical components of cardiac myocytes using findings from human and animal models.

ANKYRIN GENES

Ankyrins are a family of intracellular adaptor proteins that organize and anchor integral membrane protein complexes to the spectrin- and actin-based cytoskeleton (Bennett and Baines, 2001). In vertebrates, three genes (*ANK1*, *ANK2*, *ANK3*), encode ankyrin polypeptides. *ANK1* is located on

human chromosome 8p11 and encodes ankyrin-R polypeptides (Lambert et al., 1990). *ANK2* is located on human chromosome 4q25-27 and encodes ankyrin-B polypeptides (Otto et al., 1991). *ANK3* is located on human chromosome 10q21 and encodes ankyrin-G polypeptides (Kordeli et al., 1995). Alternative splicing produces a series of ankyrin gene products with unique subcellular distributions and functional properties (Bennett and Baines, 2001; Cunha and Mohler, 2006; Cunha et al., 2008). For example, alternative splicing of *ANK2* results in 440 kDa and 220 kDa ankyrin-B (Kunimoto, 1995; Hashemi et al., 2009). Ankyrins R, B, and G polypeptides have been identified in ventricular myocytes (Li et al., 1993; Mohler et al., 2002, 2004a,b). However, the full extent of alternative splicing, expression, localization, and function is still under investigation.

ANKYRIN DOMAINS AND BINDING PARTNERS

Canonical ankyrins have four domains: a membrane-binding domain (MBD), a spectrin-binding domain (SBD), a death domain (DD) and a C-terminal domain (CTD) (Mohler, 2006). Together, DD and CTD encompass the “regulatory domain” (RD). Ankyrin-B and ankyrin-G are closely related in amino acid sequence with 67% amino acid identity between the MBD, SBD, and DD. Despite this homology and shared expression in cardiac myocytes, ankyrin-B and ankyrin-G maintain differential distributions and non-overlapping functions. For example, while ankyrin-B is required for the localization of the Na/K-ATPase and Na/Ca exchanger (NCX) (Figure 1) to transverse-tubule membranes, ankyrin-G is required for targeting $\text{Na}_v1.5$ to the intercalated disc (Mohler et al., 2004a; Makara et al., 2014; Wu et al., 2015).

The MBD is comprised of 24 consecutive *ANK* repeats. In heart, this domain is essential for the interaction with ion channels and transporters (Figure 2) including the Na/K-ATPase (Koob et al., 1988), NCX (Li et al., 1993), voltage-gated Na_v channel (Mohler et al., 2004a), the inward rectifier subunit (Kir6.2) (Li et al., 2010), voltage-gated Ca^{2+} channels ($\text{Ca}_v1.3$) (Cunha et al., 2011), and inositol trisphosphate receptor (InsP₃ receptor) (Bourguignon et al., 1993; Hortsch et al., 2009). MBD binds to a variety of cell adhesion molecules including members of the L1 family (Davis et al., 1993). The impact of these interactions in the heart is not well studied, but an important area for future research.

The SBD associates with high affinity to β spectrin polypeptides (Bennett and Stenbuck, 1979) via the N-terminal ZU5 (Zu5N) domain (Ipsaro and Mondragon, 2010). This interaction is critical for a number of physiological functions including maintenance of normal erythrocyte cell membrane

stability (Bodine et al., 1984). As described later, human *ANK2* variants that alter spectrin-binding are now linked with potentially fatal forms of cardiac arrhythmia (Smith et al., 2015). The SBD also interacts with signaling molecules such as $\text{B56}\alpha$, the regulatory subunit of protein phosphatase 2A (PP2A) (Bhasin et al., 2007; Little et al., 2015).

The ankyrin regulatory domain (RD) interacts with proteins including obscurin. Obscurin is a giant scaffolding protein with key roles in muscle myosin incorporation, A-band formation and lateral alignment of M-lines in adjacent myofibrils (Benian et al., 1996; Borisov et al., 2006; Cunha and Mohler, 2008). Cunha and colleagues demonstrated that ankyrin-B/obscurin interaction recruits PP2A to the cardiac M-line (Cunha and Mohler, 2008) (Figure 1). In addition to the intermolecular interactions, the regulatory domain regulates the intramolecular interactions with the ankyrin MBD (Abdi et al., 2006).

SPECTRIN POLYPEPTIDES IN CARDIAC PHYSIOLOGY

Spectrins are actin-associated proteins that provide structural support to the plasma membrane and play roles in the localization of membrane and signaling proteins in the heart (Hund and Mohler, 2010). Spectrin alpha (α) and beta (β) subunits are assembled laterally to form anti-parallel heterodimers, and these heterodimers are assembled to form a flexible hetero-tetramic molecule (Bennett and Baines, 2001). There are two α spectrin genes and five β spectrin genes that encode a diverse group of alternatively-spliced spectrin isoforms with specific functions (Bennett and Healy, 2009). In ventricular myocytes, αII and βII spectrin are found at transverse-tubule and sarcoplasmic reticulum (SR) membranes (Baines and Pinder, 2005), whereas βIV spectrin is located at the intercalated disc (Hund et al., 2010).

In heart, βII spectrin directly interacts with ankyrin-B at transverse-tubule membranes (Mohler et al., 2004c; Smith et al., 2015). In fact, βII spectrin is a critical element of the larger ankyrin-B complex that directly links membrane and signaling proteins including NCX, Na/K ATPase, and $\beta\text{56}\alpha$ with the underlying actin-based cytoskeleton. Loss of βII spectrin in adult ventricular myocytes directly impacts the expression and localization of ankyrin-B as well as ankyrin-B-associated proteins (Smith et al., 2015). Beyond loss of ankyrin-binding proteins, βII spectrin null myocytes unexpectedly showed abnormal localization of the cardiac ryanodine receptor. The relationship of βII spectrin and ryanodine receptor is currently unknown. At the animal level, loss of βII spectrin in mice is lethal in mid-gestation due to incomplete development of the heart (Tang et al., 2003). Conditional deletion of βII spectrin in ventricular myocytes results in sinus node dysfunction and ventricular arrhythmia. These mice also display cytoskeletal remodeling and accelerated heart failure following transverse aortic constriction. As described later, human *ANK2* variants that alter the interaction with βII spectrin are linked with congenital arrhythmia (Smith et al., 2015). Further, βII spectrin levels are altered in acquired forms of human heart failure and arrhythmia,

Abbreviations: MBD, Membrane-binding domain; CTD, C-terminal domain; SBD, Spectrin-binding domain; DD, Death domain; LQTS, Long QT syndrome; NCX, Sodium-Calcium exchanger; Na/K ATPase, Sodium-potassium ATPase; IP3R, Inositol trisphosphate receptor; cKO, Conditional knock-out; INa_L , Late component of Nav current; CaMKII, Calcium/calmodulin-dependent kinase; KATP, ATP-sensitive K⁺ channel; $\text{Ca}_v1.3$, Voltage gated L-type Ca^{2+} channel; PP2A, Protein phosphatase 2A; $\text{Nav}1.5$, Voltage-gated sodium channel; TREK-1, TWIK-related potassium channel-1.

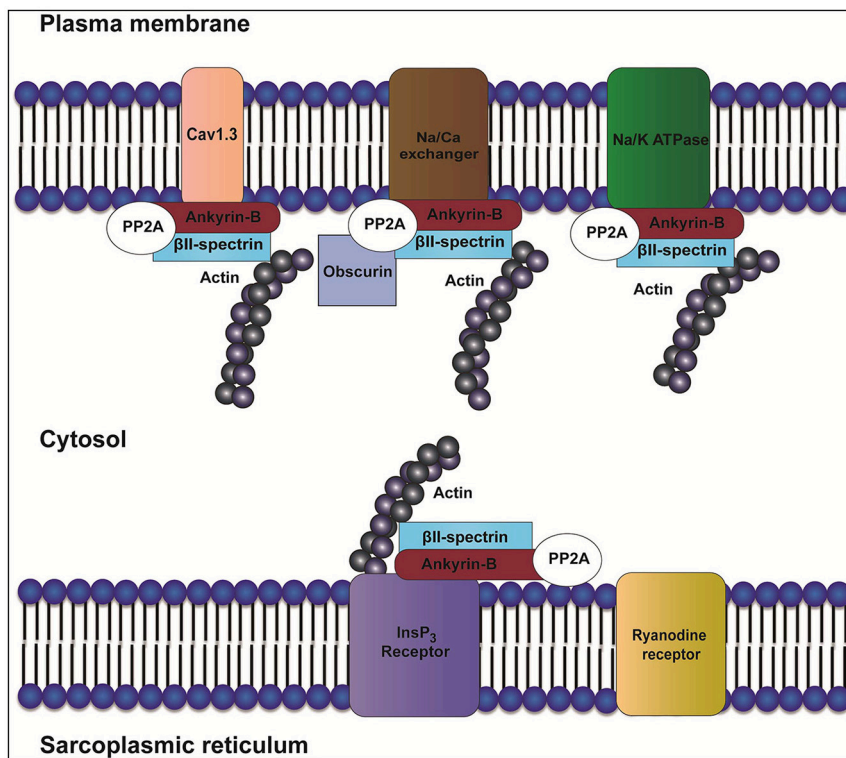


FIGURE 1 | Role of ankyrin-B in localization of the InsP_3 receptor, Na/K-ATPase, $\text{Ca}_v1.3$, and NCX. In heart, ankyrin-B targets and localizes ion channels and transporters such as inositol trisphosphate receptor (InsP_3R), sodium/potassium ATPase (Na/K-ATPase), $\text{Ca}_v1.3$, and Na/Ca exchanger (NCX). Ankyrin-B also targets protein phosphatase type 2A (PP2A) through its regulatory subunit B56 α . Interaction between ankyrin-B and β II spectrin forms a complex that is important for the localization and stability of ion channels and transporters such as Na/K-ATPase, $\text{Ca}_v1.3$, InsP_3R , and NCX.

as well as in animal models of cardiovascular disease (Smith et al., 2016). Finally, individuals with Beckwith-Wiedemann syndrome (BWS) with a reduction in β II spectrin expression (Yao et al., 2010) show congenital heart failure and ventricular septal defects (Drut et al., 2006).

In heart, β IV spectrin directly associates with ankyrin-G and is required for the targeting of calcium/calmodulin-dependent kinase II (CaMKII) to the intercalated disc (Hund et al., 2010). Notably, this interaction is critical for the regulation of I_{Na} by modulating CaMKII-dependent phosphorylation of $\text{Na}_v1.5$ (Hund et al., 2010; Koval et al., 2012). Beyond, ankyrin-G and CaMKII, β IV spectrin regulates TREK-1, a mechano-sensitive $\text{K}_{2\text{P}}$ channel (Heurteaux et al., 2004). More specifically, β IV spectrin and TREK-1 associate, and β IV spectrin is required for TREK-1 localization and function in ventricular myocytes (Hund et al., 2014). Mice lacking TREK-1 binding display delayed action potential repolarization and arrhythmia. These data support a broader role for β IV spectrin in cardiovascular electrophysiology by regulation of TREK-1 membrane localization and function. Like β II spectrin, β IV spectrin levels are altered in human heart failure as well as in animal models of cardiovascular disease (Hund et al., 2014).

Finally, α II spectrin-deficient embryos are embryonic lethal with abnormal cardiac shape, cardiac dilatation and changes in myocardium (Stankewich et al., 2011). Beyond ankyrins,

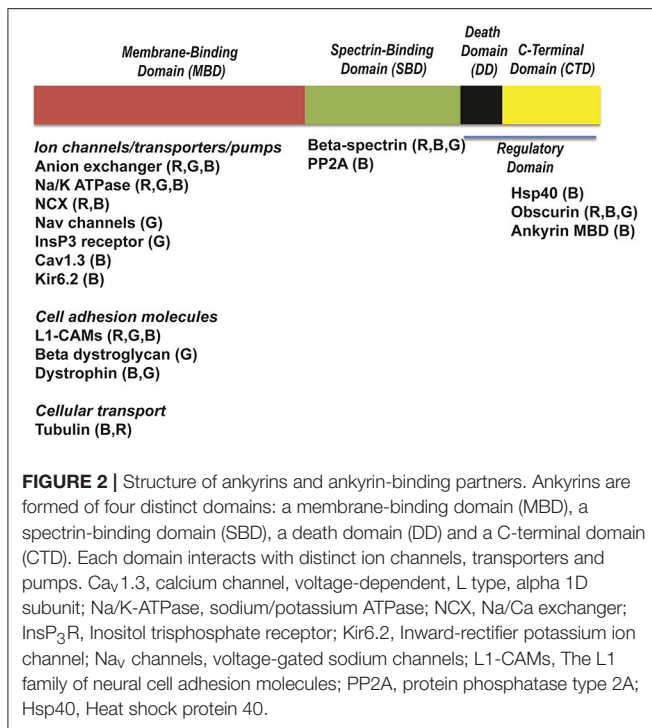
Mena and VASP, important regulators of actin dynamics form a complex with α II spectrin in adult cardiomyocytes (Benz et al., 2013). The *in vivo* role(s) of α II spectrin in adult heart is currently unknown but an important area for future research.

IN VIVO ROLES FOR CARDIAC ANKYRINS

Ankyrin-G is Required for Targeting $\text{Na}_v1.5$

In neurons, the link between ankyrin-G and Na_v channel targeting is well established (Jenkins and Bennett, 2001; Saifetiarova et al., 2017). In heart, rat ventricular myocytes lacking ankyrin-G display a reduction in $\text{Na}_v1.5$ expression, localization, and I_{Na} (Lowe et al., 2008). In fact, in myocytes with reduced ankyrin-G expression, $\text{Na}_v1.5$ localization is limited in the perinuclear region of rat ventricular myocytes. Notably, this role is specific for ankyrin-G vs. ankyrin-B as ankyrin-B-null neonatal cardiomyocytes show no difference in $\text{Na}_v1.5$ expression or localization. Further, loss of ankyrin-G is specific for I_{Na} vs. other cardiac currents (e.g., $I_{\text{Ca,L}}$) (Lowe et al., 2008).

In animals, ankyrin-G expression is required for the targeting of $\text{Na}_v1.5$ and CaMKII to the intercalated disc (Makara et al., 2014). $\text{Na}_v1.5$ expression and I_{Na} is reduced in myocytes derived from mice with selective loss of cardiomyocyte ankyrin-G (cKO) (Makara et al., 2014). Further, β IV spectrin



is absent in the intercalated disc of myocytes of ankyrin cKO mice. Electrocardiographic studies of these mice show significant reduction in heart rate, impaired atrioventricular conduction, and an increase in PR interval. Moreover, cKO mice display a significant increase in the QRS interval and delayed intraventricular conduction. Administration of flecainide or catecholamines (elevated adrenergic stimulation) induces arrhythmia in these mice.

Beyond animals, work in humans supports the link between ankyrin-G and Na_v channel targeting. Priori et al. (2000) identified an individual with Brugada syndrome harboring an SCN5A variant in the Na_v1.5 motif that interacts with ankyrin-G (Mohler et al., 2004a). The group identified the SCN5A variant in a 47-year-old female with a history of palpitations and an unexplained syncope at rest. Using *in vitro* binding assays, the p.E1053K variant was found to disrupt the interaction between Na_v1.5 and ankyrin-G resulting in abnormal targeting in myocytes.

A canine myocardial infarction (MI) model was used to study the role of ankyrin-G in acquired arrhythmic disease. At 5 days, there was a 42% reduction in Na_v1.5 protein expression and a 50% increase in ankyrin-G expression in the epicardial border zone. These data suggest that ankyrin-G levels increase to regulate Na_v1.5 recruitment to sarcolemmal membrane (Dun et al., 2013).

Ankyrin-B

First identified in brain, ankyrin-B has now been directly linked with a host of functions in the heart. Further, ankyrin-B dysfunction is now well documented in multiple forms of congenital and acquired cardiovascular disease. While ankyrin-B

was known to directly associate with critical cardiac ion channels and transporters, an *in vivo* role for ankyrin-B in cardiac function was first illustrated using mice heterozygous for an ankyrin-B null mutation. While ankyrin-B null mice die shortly after birth, ankyrin-B^{+/-} mice live to adulthood but display bradycardia, heart rate variability, prolonged QT intervals, and sudden cardiac death in response to exercise combined with catecholaminergic stimulation (Mohler et al., 2003). While adult ventricular myocytes from ankyrin-B^{+/-} mice display normal action potential duration, these myocytes display early and delayed after-depolarizations in response to catecholamines. At the molecular level, ankyrin-B^{+/-} ventricular myocytes display reduced expression and abnormal localization of ankyrin-B-binding partners including Na/K ATPase and NCX. As illustrated in computational models (Wolf et al., 2010), loss of Na/K ATPase and NCX results in increased SR calcium stores that result in an arrhythmogenic substrate in the ankyrin-B^{+/-} animals (Mohler et al., 2004b). Subsequent work from Despa and colleagues revealed significant alterations in local [Ca²⁺] as well as hyper-phosphorylation of ryanodine receptors in ankyrin-B^{+/-} ventricular myocytes (Camors et al., 2012; Popescu et al., 2016) potentially due to altered PP2A regulation (DeGrande et al., 2012). Further characterization of ankyrin-B^{+/-} mice showed premature senescence and reduced longevity with mice showing loss of adiposity starting at 6 months of age (Mohler et al., 2007a).

Ankyrin-B dysfunction is directly linked with human cardiovascular disease. Over two decades ago, Schott and colleagues identified type 4 long QT syndrome in a large French kindred with sinus node dysfunction and episodes of atrial fibrillation (Schott et al., 1995). Prolonged QTc was observed among the 25 affected patients (21 adults and 4 children). Individuals in the family also displayed catecholaminergic polymorphic ventricular arrhythmia and sudden death. Sequencing of the gene encoding ankyrin-B (ANK2) revealed a positional transition (A to G) mutation at position 4,274 in exon 36, leading to the substitution of a glycine for a glutamic acid at amino acid residue 1,425 (p.E1425G) near the regulatory domain of ankyrin-B in forty-five family members (24 carriers and 21 non-carriers). The p.E1425G variant, subsequently identified as a loss-of-function variant in myocytes, showed prevalence in 22 out of 24 individuals with the LQT4 phenotype and was absent in non-affected individuals (Mohler et al., 2003). Since this initial discovery, a number of new ankyrin-B loss-of-function variants have been identified in individuals with ventricular arrhythmias. However, as discussed below, due to the diversity of non-ventricular phenotypes associated with the disease (including many lacking QTc prolongation), the term “ankylin-B syndrome” has been adopted to describe individuals harboring ANK2 loss-of-function variants (Mohler et al., 2004b, 2007b). As in the case of many cardiovascular disease genes, the penetrance of phenotypes in ANK2 variant carriers is variable. As ankyrin-B polypeptide stability and function is directly impacted by physiological and pathological stress (e.g., inflammation, elevated reactive oxygen species), we predict that a “second hit” may be necessary for full disease penetrance.

Active areas of research in this field are currently focused on identifying the mechanisms underlying both congenital and acquired ankyrin-B loss in human disease as well as the discovery of new variants. For example, while loss-of-function variants were limited to the RD, new variants in the ankyrin-B MBD have shed light on new phenotypes associated with “ankyrin-B syndrome.” Recently, a novel MBD variant in the *ANK2* gene (p.S646F) was identified in two Gitxan families with LQTS phenotypes by the Swayne and Arbour groups (Swayne et al., 2017). The first person identified was a 28-year old man with a clinical diagnosis of LQTS and recurrent syncope. Five of this patient’s relatives were confirmed to harbor this variant. This variant was also identified in a second family with a clinical diagnosis of LQTS. Within these two families, a total of 13 adults and 5 children have been identified with this novel *ANK2* variant. *In vitro* evaluation illustrated that the expression of ankyrin MBD with p.S646F variant in bacteria was normal and MBD structural properties were intact. However, expression of ankyrin-B p.S646F variant in neonatal cardiomyocytes showed a reduction in ankyrin-B expression and alteration of expression of NCX (Swayne et al., 2017). Finally, beyond LQT and catecholaminergic polymorphic ventricular tachycardia, *ANK2* variants have been associated with other forms of ventricular arrhythmia including early repolarization syndrome (Krogh Broendberg et al., 2015).

ANK2 variants are not limited to MBD or RD. Smith et al. identified an *ANK2* arrhythmia variant (c.2969G>A) caused by the substitution of Arg to Gln at position 990 (p. R990Q). This variant is rare with a minor allele frequency of 0.007% and resides near the central ZU5 binding surface for β II spectrin. Using myocytes from ankyrin-B conditional knockout mice (cKO), exogenous expression of WT ankyrin-B, but not ankyrin-B p.R990Q was able to rescue the localization of NCX (Smith et al., 2015).

Ankyrin-B dysfunction is also linked with non-ventricular forms of human arrhythmia. Individuals with select *ANK2* loss-of-function variants display atrial fibrillation and/or sinus node dysfunction (Le Scouarnec et al., 2008; Cunha et al., 2011). Consistent with these data, ankyrin-B^{+/-} mice also display both atrial and sinus node phenotypes. At the cellular level, primary atrial and sinus node myocytes from ankyrin-B^{+/-} mice display shortened action potentials and loss of L-type Ca²⁺ current. In ankyrin-B^{+/-} atrial myocytes, voltage-gated L-type Ca²⁺ channel Ca_v1.3 (but not Ca_v1.2) expression and function were reduced.

Until recently, *ANK2*-based disease was linked only to individuals with single “point mutations” (Mohler et al., 2004b, 2007b; Sherman et al., 2005; Krogh Broendberg et al., 2015; Swayne et al., 2017). However, recently a family was identified with an *ANK2* transection in chromosome 4 (Huq et al., 2017). The proband had a balanced translocation between the long arms of chromosomes 4 and 9. Five other family members were carriers of the same balanced translocation. The 16-week-old fetus of the proband from a terminated pregnancy was found to have multiple cardiac abnormalities. The proband showed normal echocardiography findings and only Holter monitor recorded few episodes of bradycardia at night. Isolated lymphoblasts from the proband demonstrated a significant

reduction in ankyrin-B and binding partners, confirming a model of ankyrin-B haploinsufficiency. The mother of the proband was also a carrier of the translocation and displayed frequent ventricular ectopy and periods of bigeminy.

Beyond congenital disease, dysfunction in ankyrin-B-based pathways has been linked with acquired forms of cardiovascular disease. In a canine model of myocardial infarction, ankyrin-B mRNA levels were increased and protein levels were decreased in day 5-post infarction in the border zone (Hund et al., 2009). At the myocyte level, abnormal distribution of ankyrin-B was noted. The authors further showed changes in expression and/or distribution of downstream ankyrin-associated proteins Na/K ATPase, NCX, InsP₃ receptor and PP2A (Hund et al., 2009). In addition to its role in myocardial infarction, Li et al. identified the association of ATP-sensitive K⁺ channel (K_{ATP}) with ankyrin-B as a cardio-protective mechanism against acute ischemia (Li et al., 2010). Ankyrin-B levels are now also known to be reduced in patients with atrial fibrillation (Cunha et al., 2011).

Ankyrin-R

To date, human variants in *ANK1* have not been directly linked with cardiac arrhythmia phenotypes. While not the focus on this review, it is of note that variants in *ANK1* are a primary cause for hereditary spherocytosis (Eber et al., 1996; Tse and Lux, 1999; Perrotta et al., 2008; Satchwell et al., 2016).

SUMMARY

Cardiac ankyrins and spectrins are key multifunctional molecules in the heart. In myocytes, ankyrins, and spectrins serve essential roles in targeting ion channels and transporters to critical membrane domains. However, beyond membrane proteins, these molecules serve as crucial cellular nodes to integrate membrane proteins with critical signaling and structural proteins in diverse cardiac cell types. While animals have served as important models to define ankyrin and spectrin function *in vivo*, more recent findings in patients have both solidified the importance of ankyrins and spectrin in human physiology and disease, and raised important questions related to unexpected roles of these polypeptides. Key future questions in the field remain the impact of environment or “second hit” gene variants on disease susceptibility/penetrance in *ANK2* variant carriers, as well as potential non-myocyte/non-cardiac phenotypes in patients with ankyrin gene variants.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Conflict of Interest Statement: The authors declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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