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Ankyrin protein networks in membrane formation and stabilization

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Abstract

In eukaryotic cells, ankyrins serve as adaptor proteins that link membrane proteins to the underlying cytoskeleton. These adaptor proteins form protein complexes consisting of integral membrane proteins, signalling molecules and cytoskeletal components. With their modular architecture and ability to interact with many proteins, ankyrins organize and stabilize these protein networks, thereby establishing the infrastructure of membrane domains with specialized functions. To this end, ankyrin collaborates with a number of proteins including cytoskeletal proteins, cell adhesion molecules and large structural proteins. This review addresses the targeting and stabilization of protein networks related to ankyrin interactions with the cytoskeletal protein β-spectrin, L1-cell adhesion molecules and the large myofibrillar protein obscurin. The significance of these interactions for differential targeting of cardiac proteins and neuronal membrane formation is also presented. Finally, this review concludes with a discussion about ankyrin dysfunction in human diseases such as haemolytic anaemia, cardiac arrhythmia and neurological disorders.

Keywords: ankyrin • β-spectrin • obscurin • L1-CAM • cardiomyocyte • axon initial segment • node of Ranvier

Introduction

Unique cellular functions are the result of cell-type specific proteins and the distinctive arrangement of these proteins in the context of organelles, the cytoskeleton and the plasma membrane. In eukaryotic cells, there are a number of adaptor proteins that serve as the interface between the plasma membrane and cytoskeleton including 4.1 proteins, proteins from the ezrin–radixin–moesin family and ankyrins. These adaptor proteins organize protein networks with particular structural, signalling and electrogenic properties. Accordingly, these adaptor proteins are integral for the formation of subcellular domains with specialized functions such as ionic movement across the plasma membrane or providing adherence between cell membranes. The prevalence of human disease associated with dysfunction in these adaptor proteins and associated molecules attests to their significance for normal cellular physiology. This review focuses on the adaptor protein ankyrin and its role in forming protein networks that constitute specialized membrane domains in cardiomyocytes and neurons. Specifically, the first section provides a general overview of ankyrins covering topics such as functional domains, genes and alternative isoforms. The second section is focused on proteins that contribute to ankyrin targeting and stabilization at specialized cardiac and neuronal membrane domains. Such proteins include the cytoskeletal protein β-spectrin, the L1-family of cell adhesion molecules and the large structural protein obscurin. The final section describes ankyrin dysfunction in human diseases including haemolytic anaemia, cardiac arrhythmias and neurological disorders.

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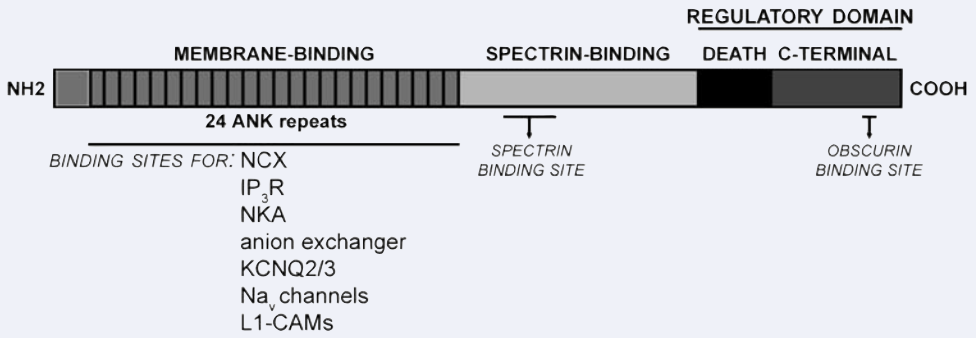

Fig. 1 Ankyrin functional domains. The membrane binding domain consists of 24 *ANK* repeats that mediate interactions with a variety of ion channels, transporters and cell adhesion molecules. Ankyrin interacts with the cytoskeleton *via* β-spectrin binding to the spectrin binding domain. The death and C-terminal domains comprise the C-terminal regulatory domain that governs ankyrin inter- and intra-molecular interactions. NCX: sodium/calcium exchanger, NKA: sodium/potassium ATPase, IP_3R: inositol$_{(1,4,5)}$ triphosphate receptor.

Ankyrins

Ankyrins are a family of adaptor proteins that link integral membrane proteins with the submembranous actin/β-spectrin cytoskeleton. The first ankyrin was characterized over 30 years ago as an adaptor protein that tethered the anion exchanger to β-spectrin in red blood cells [1]. Ankyrins are now regarded as pivotal choreographers in the formation of protein complexes consisting of ion channels and transporters, cell adhesion molecules, signalling proteins and cytoskeletal elements. These ankyrin-associated protein complexes comprise specialized membrane domains with distinct electrogenic and/or structure properties in eukaryotic cells. In addition, new functions now ascribed to ankyrin include membrane biogenesis and the formation of diffusion barriers that maintain the subcellular polarity of migrating proteins.

Ankyrin functional domains

The prototypical ankyrin consists of three functional domains (Fig. 1). The membrane-binding domain, which mediates ankyrin binding to integral membrane proteins, contains 24 *ANK* repeats assembled as a superhelical spiral. An *ANK* repeat consists of 33 amino acids arranged as two anti-parallel α-helices followed by a long loop [2]. Adjacent *ANK* repeats are connected by a β-hairpin loop and these solvent-exposed domains mediate protein interactions. The binding sites are often spread across adjacent β-hairpin loop tips. For example, ankyrin interacts with the sodium/calcium exchanger (NCX) *via ANK* repeats 16–18 [3], the inositol$_{(1,4,5)}$-triphosphate receptor (IP_3 receptor) *via ANK* repeats 22–24 [4] and the voltage-gated sodium channel *via ANK* repeats 14 and 15 [5]. Additional integral membrane proteins that interact with the ankyrin membrane-binding domain include the anion exchanger [6–8], sodium/potassium ATPase (NKA) [9, 10], voltage-gated potassium channel subunits (KCNQ2 and KCNQ3) [11–14] and the L1 family of cell adhesion molecules (Fig. 1) [15, 16].

The ankyrin associated protein complex are tethered to the actin/spectrin cytoskeleton *via* its spectrin-binding domain. This domain is relatively large with a molecular weight of 62 kD, but the minimal spectrin-binding domain is contained within a 160 amino acid ZU-5 motif [17]. This motif has two conserved sites that are critical for spectrin-binding activity (ankyrin-B: DAR976, A1000 and ankyrin-G: DAR999, A1024) [17, 18]. For β-spectrin, the minimal ankyrin-binding domain is contained in spectrin repeats 14 and 15 [19–21]. The complementary electrostatic charges of ankyrin's ZU-5 motif (positive) and β-spectrin's repeat 14 (negative) suggest that the protein interactions are partially mediated by their oppositely charged domains [20]. With the exception of identifying the minimal ankyrin and spectrin binding sites, very little is known about the regulatory mechanisms of ankyrin/spectrin interactions. For example, it is not known whether different ankyrins preferentially associate with particular spectrins.

The C-terminal regulatory domain is comprised of a death domain and an unstructured stretch of 300 amino acids. This domain regulates protein interactions with ankyrin's membrane-binding and spectrin-binding domains. For example, an alternative isoform of ankyrin-R lacking a portion of the C-terminal domain exhibits increased binding affinity for the anion exchanger and spectrin [22, 23]. This increase in binding affinity is reversed by co-expression of the C-terminal fragment, validating the auto-inhibitory functions of the C-terminal regulatory domain [22, 23]. The regulatory activity appears to be mediated by an intra-molecular interaction between this domain and the first *ANK* repeat of the membrane-binding domain [24]. Additional evidence for an intra-molecular interaction comes from studies of ankyrin-B mutations in cardiac disorders. In particular, the ankyrin-B loss-of-function mutation E1425G impairs ankyrin-B binding to NCX, NKA and the IP_3 receptor [9]. Interestingly, this mutation is not found in the membrane-binding or the C-terminal regulatory domain, but at the junction of the spectrin-binding and C-terminal

Table 1 Ankyrin isoforms and tissue expression

Tissue	Ankyrin-G	Ankyrin-B	Ankyrin-R
Brain	270, 480 kD	220, 440 kD	186, 215 kD
Heart	190 kD	160, 220 kD	210 kD
Skeletal muscle	107–130, 119 kD	220 kD	20–30 kD
Lung	190, 200–215 kD	220 kD	
Kidney	119, 190, 200--215 kD	220 kD	
Erythrocyte			186, 215 kD

domains. This mutation may reside in a 'hinge' domain that allows the C-terminal domain to pivot over the membrane-binding domain. Currently, the mechanisms regulating these intra-molecular interactions are unknown. Ankyrin function is also indirectly regulated by the C-terminal domain through targeting motifs present in this domain that govern ankyrin recruitment and retention to different subcellular domains. As will be elaborated on in a subsequent section, a subpopulation of ankyrin-B is targeted to the M-line of ventricular cardiomyocytes through an interaction between the C-terminal regulatory domain of ankyrin-B and the large Rho-GEF obscurin (Fig. 1).

Ankyrin genes, alternative splicing and the diversity of ankyrin polypeptides

The diversity of ankyrin polypeptides is the product of unexpectedly complex alternative splicing of three genes (see Table 1). Located on human chromosome 8p11, *ANK1* contains 42 exons that are alternatively spliced to encode a variety of ankyrin-R isoforms expressed in erythrocytes, cardiac and skeletal muscle, and neurons [23, 25–33]. Ankyrin-B isoforms are encoded by *ANK2*, which is located on human chromosome 4q25–27 and contains 53 exons spanning approximately 560 kb [34, 35]. Ankyrin-B isoforms have been found in a variety of tissues including brain, heart, skeletal muscle and thymus [36–40]. Finally, the gene for ankyrin-G (*ANK3*) located on human chromosome 10q21 encodes numerous isoforms broadly expressed in epithelial tissue, kidney, skeletal and cardiac muscle, and brain [41–49].

Alternative splicing of the ankyrin genes enables ankyrin polypeptides to associate with many different proteins and display differential subcellular localization. For example, the targeting of ankyrin-G to the axon initial segments (AIS) of peripheral neurons is partly dependent on a serine/threonine rich domain that is only present in neuronal isoforms of ankyrin-G [49]. In addition, alternative transcription of *ANK1* results in small ankyrin-R isoforms that integrate into the sarcoplasmic reticulum (SR) of skeletal muscle *via* unique N-terminal transmembrane domains [25, 33, 50, 51]. With all ankyrin genes, the exons encoding the C-terminal regulatory domains are often extensively spliced. As mentioned previously, this domain regulates ankyrin's association with integral membrane proteins and cytoskeletal elements. Altering the composition of this domain would partially explain both the functional diversity and differential localization of alternative ankyrin isoforms. Alternative splicing of exons encoding the membrane-binding domain would also contribute to ankyrin functional diversity. In heart, alternative splicing of the *ANK2* gene removes key exons that encode known binding sites for ankyrin-B associated proteins NCX and IP_3 receptor (reviewed in Van Oort, 2008) [34, 52]. Taken together, alternative splicing alters binding sites and targeting motifs in ankyrin, thereby enabling its selective association with particular proteins that ultimately culminate in the distinct subcellular localization of ankyrin-associated protein complexes. By some accounts, these mechanisms may also underlie a role for ankyrin in the formation of specialized membrane domains. The following section will discuss three proteins that contribute to the targeting and retention of ankyrin-associated protein complexes to specific membrane domains.

Mechanisms that target and stabilize ankyrin

β-spectrin

Spectrins are a family of filamentous proteins that assemble as heterotetramers of two α and two β subunits. In conjunction with actin filaments, these heterotetramers form the underlying cytoskeleton of plasma membranes in all metazoan cell types including erythrocytes, cardiomyocytes and neurons. While two genes encode the various isoforms of α-spectrin, five genes encode numerous isoforms of β-spectrin [53]. The prototypical β-spectrin contains an N-terminal actin-binding domain, 16 triple-helical spectrin repeats and a C-terminal pleckstrin homology domain. In addition to acting as molecular springs that ensure membrane resilience to mechanical stress, the spectrin repeats also mediate protein interactions. For example, ankyrin interacts with β-spectrin repeats 14 and 15 [19–21]. β-spectrin associates with the plasma membrane through adaptor proteins such as ankyrin and protein 4.1 [54], integral membrane proteins including the neuronal glutamate transporter [55] and phosphatidylinositol lipids [56]. In human, mutations to β-spectrin have been linked to hereditary elliptocytosis, a type of haemolytic anaemia, and spinocerebellar ataxia type 5, a progressive neurodegenerative disorder characterized by slurred speech and loss of coordination [57–59]. The association of spectrin mutations with these two seemingly disparate diseases underscores spectrin's role as a multifunctional protein that not only insures membrane integrity in erythrocytes but also contributes to the stabilization of membrane proteins in the cerebellum.

Previous studies have focused on the relative significance of ankyrin and β-spectrin in the formation of specialized membrane

domains. This analysis includes examining the respective roles for ankyrin and β-spectrin in the targeting and stabilization of each other. Some studies have concluded that β-spectrin is targeted and stabilized by ankyrin. For example, in ventricular cardiomyocytes the M-line localization of β_2-spectrin is dependent on ankyrin-B [17]. Likewise, ankyrin-B is necessary for β_2-spectrin localization at the inner segments of rod photoreceptors [60]. In cerebellar-specific ankyrin-G knockout mice, the loss of ankyrin-G disrupts β_4-spectrin localization at AIS in Purkinje cells [61]. Contrary to these findings, some studies have concluded that ankyrin targeting and stabilization is dependent on β-spectrin. In *Drosophila melanogaster*, ankyrin is localized to the basolateral domains of midgut copper cells through its interaction with β-spectrin [62].

Recently, an emerging theme is that ankyrin and β-spectrin are interdependent and are mutually involved in the formation and maintenance of membrane domains. For example, evidence from co-localization experiments demonstrate that expression of ankyrin-G or β_4-spectrin at AIS of Purkinje neurons is dependent on each other [61, 63]. In bronchial epithelial cells, both ankyrin-G and β_2-spectrin cooperate in the formation of lateral membrane domains. Specifically, loss of lateral membrane domains following AnkG-siRNA treatment is not rescued by exogenous expression of an ankyrin-G construct that lacks β-spectrin binding activity [64].

The relative prominence of ankyrin or spectrin in membrane biogenesis most likely depends on additional factors in the microdomain including cell adhesion molecules, other cytoskeletal elements and regional phospholipids. In support of this hypothesis, it was shown that different domains are necessary for β-spectrin targeting in various *Drosophila* tissues. For example, β-spectrin targeting in midgut copper cells is dependent on the C-terminal PH-domain, while β-spectrin targeting to neuronal plasma membranes requires both the PH and ankyrin-binding domains [65]. Interestingly, neither the PH nor the ankyrin-binding domains are required for β-spectrin localization in salivary epithelial cells [65]. In fact, the novel targeting motif has yet to be identified. Ankyrin-independent β-spectrin targeting mechanisms may include phospholipids, integral membrane proteins (α-catenin) [66] and additional adaptor proteins (4.1 protein) [53]. Similar to β-spectrin, multiple ankyrin-G domains contribute to its retention at specific membrane domains. For example, axolemmal targeting information is contained in the ankyrin-G spectrin-binding and C-terminal regulatory domains [49]. Furthermore, additional targeting motifs that restrict ankyrin-G expression to AIS are present in the serine/threonine rich and C-terminal regulatory domains [49]. These findings suggest that ankyrin-G targeting to AIS is dependent on multiple protein interactions with different ankyrin-G domains. Apparent discrepancies in the targeting and retention of ankyrin/spectrin complexes to different membrane domains may be explained by the involvement of multiple proteins. For example, as will be described in greater detail below, the cell adhesion molecule neurofascin recruits ankyrin-G to the nodes of Ranvier, but not to AIS of peripheral neurons [67–69].

L1-cell adhesion molecules

Ankyrins bind to a variety of cell adhesion molecules including CD44 [70], E-cadherin [71], β-dystroglycan [72] and members of the L1 family [15, 16]. This association is important for the proper subcellular targeting of these cell adhesion molecules. For example, ankyrin-G recruits E-cadherin to the lateral membrane domains of bronchial epithelial cells where in concert with β_2-spectrin it facilitates membrane biogenesis [18, 64, 71]. Recently, it was shown that ankyrin-B and ankyrin-G are involved in the targeting and retention of β-dystroglycan to the sarcolemma, neuromuscular junction and costameres of skeletal muscle [72].

Many studies have characterized the association of ankyrins with members of the L1 family of cell adhesion molecules that includes L1, close homologue of L1 (CHL1), neuron glia related CAM (NrCAM) and neurofascin. These proteins are predominantly expressed in the nervous system where they have been implicated in many aspects of neural differentiation including neurite outgrowth, axonal guidance and synaptogenesis [73]. The prototypical extracellular domain of L1 cell adhesion molecules has six immunoglobulin domains followed by four to five fibronectin type III domains. The cytoplasmic domain is relatively small ranging in size from 85 to 148 amino acids and contains the ankyrin-binding motif FIGQY that is highly conserved in L1 proteins [74–76]. Homophilic interactions between the extracellular domains of these proteins are stabilized by ankyrin binding to the FIGQY motif. Moreover, phosphorylation of the tyrosine residue in this motif disrupts ankyrin interaction with the C-terminal domain [74, 75]. For example, ankyrin/L1 interactions cause neuroblastoma cells to aggregate *via* homophilic interactions of the L1 extracellular domains [75]. These aggregates disperse following treatment with nerve growth factor because tyrosine phosphorylation of the FIGQY motif disrupts ankyrin binding to L1.

The phosphorylation-dependent regulation of ankyrin/L1 interaction allows L1 to function in two different capacities during neural development. In developmental events that favour static adhesion such as axon fasciculation or the formation of AIS, the tyrosine residue is not phosphorylated and ankyrin interacts with the L1 protein [73]. In contrast, for dynamic developmental processes such as neurite outgrowth and migration, L1 proteins are phosphorylated thereby inhibiting ankyrin interactions. Consistent with this hypothesis, neurite outgrowth is increased by 50% in cerebellar granular neurons following inhibition of L1/ankyrin interactions [77]. Interestingly, phosphorylated FIGQY preferentially interacts with the microtuble associated protein doublecortin that is linked to the neuronal migration disorder lissencephaly, which is characterized by thickening of the neocortex and reduced cortical gyrations [78, 79].

The relative contribution of ankyrin or L1 proteins to the targeting and retention of the other depends on the membrane domain. For example, ankyrin-G recruits neuronal isoforms of neurofascin (neurofascin-186) to AIS of cerebellar Purkinje neurons [80]. Similarly, in hippocampal neurons ankyrin-G depletion by RNA interference extinguishes AIS targeting of neurofascin-186, Na_V 1.6, and β_4-spectrin [81]. As in the central nervous system,

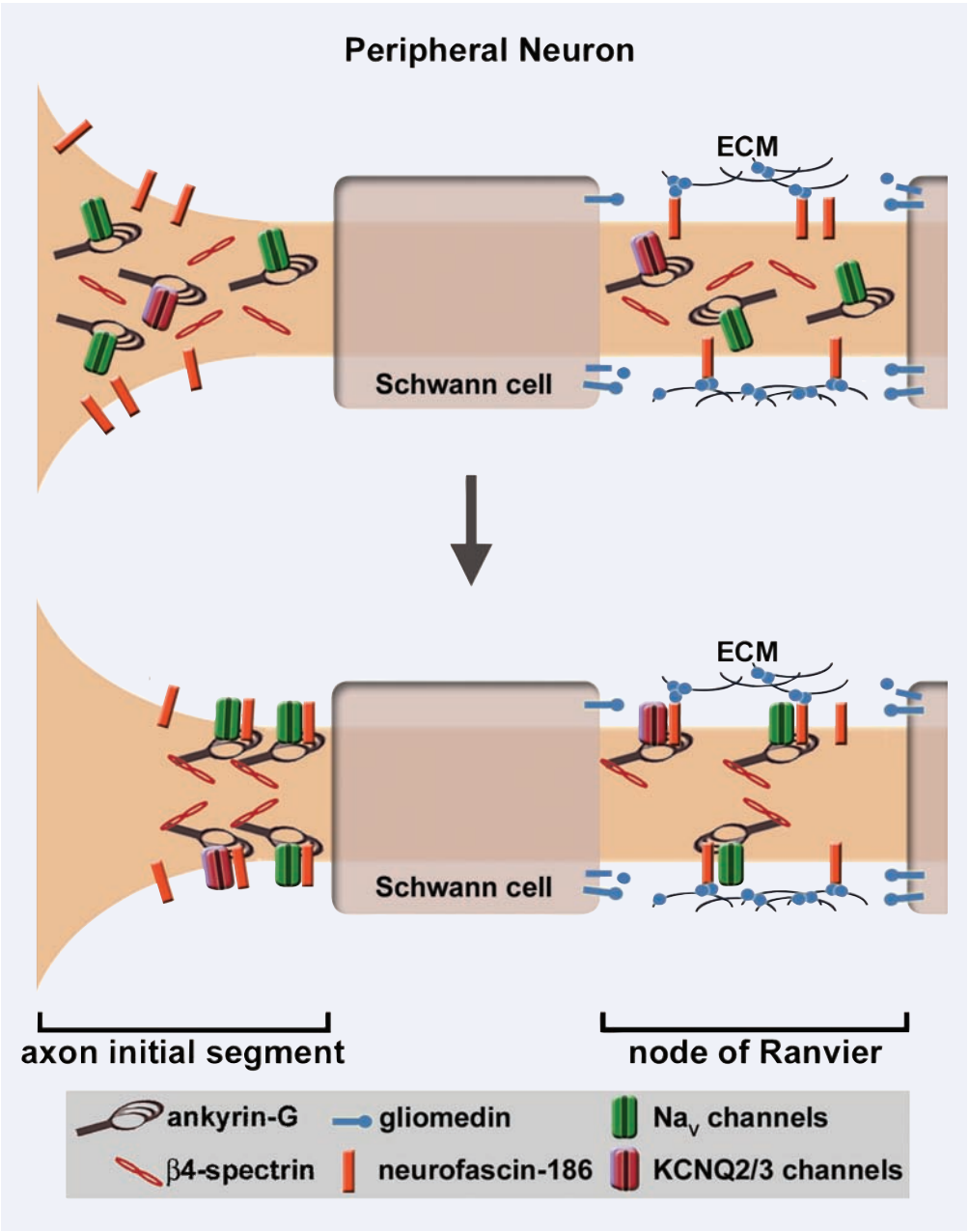

Fig. 2 Ankyrin-G targeting to membrane domains in the peripheral neuron. Ankyrin-G is recruited to the nodes of Ranvier by gliomedin, which is produced by Schwann cells and accumulates in the perinodal extracellular matrix. As a ligand for neurofascin-186, gliomedin causes the nodal clustering of this cell adhesion molecule, which in turn recruits to the nodal plasma membrane an ankyrin-G protein network consisting of voltage-gated sodium or potassium channels (KCNQ2/3) and β_4-spectrin. In contrast, ankyrin-G localization to the AIS is not dependent on an extracellular cue or neurofascin-186. The AIS targeting of ankyrin-G appears to be mediated by an intrinsic mechanism that has yet to be discovered, but AIS targeting of ankyrin-G associated proteins (*i.e.* neurofascin-186, sodium and potassium channels, β_4-spectrin) is dependent on ankyrin-G.

ankyrin-G recruits neurofascin-186 to AIS of peripheral neurons, but interestingly their roles are reversed at the nodes of Ranvier (Fig. 2). Specifically, neurofascin-186 recruits ankyrin-G to the nodes of peripheral neurons [67–69]. Consistent with this finding, neurofascin-186 clusters at the nodes before ankyrin-G or voltage-gated sodium channels [82, 83]. The nodal clustering of neurofascin-186 is mediated by direct interactions between its extracellular domain and gliomedin, an extracellular matrix protein that accumulates in the perinodal region after being cleaved from the membrane surface of Schwann cell microvilli [84]. Decreasing gliomedin expression through RNA interference reduces the nodal expression of both neurofascin and Na_V channels [84]. In summary, ankyrin interactions with L1 proteins demonstrate the relative contribution of intracellular and/or extracellular cues to the formation of specialized membrane domains. Namely, ankyrin-G clustering and the ensuing formation of nodes of Ranvier are dependent on extracellular cues from myelinating Schwann cells that are relayed *via* neurofascin-186.

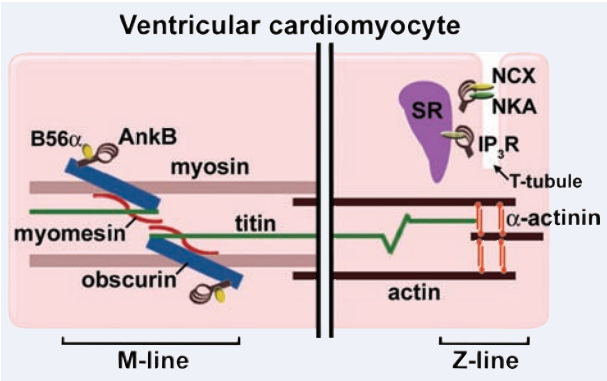

Fig. 3 Ankyrin-B localization in ventricular cardiomyocytes. Ankyrin-B is targeted to the M-line *via* its interaction with the C-terminal domain of the large sarcomeric protein obscurin. Obscurin is targeted to the M-line *via* its N-terminal interactions with myomesin and titin. This population of ankyrin-B recruits B56α, a regulatory subunit of protein phosphatase 2A, to the M-line where the phosphatase may regulate the phosphorylation status of contractile and signalling proteins. Another population of ankyrin-B is located at the Z-lines where they interact with ion channels and transporters that regulate calcium efflux from the SR/T-tubule junction. The protein network associated with this ankyrin-B subpopulation includes NCX, NKA and the inositol$_{(1,4,5)}$ triphosphate receptor (IP_3R).

Obscurin

In skeletal and cardiac tissue, large sarcomeric proteins coordinate the proper alignment of protein complexes to facilitate the integration of membrane structures including the SR and transverse tubules (T-tubules) with myofibrils. Obscurin is an 800 kD protein that regulates the formation and alignment of myofibrils. In skeletal muscle, obscurin is expressed early at the M-lines of developing sarcomeres in nascent myofibrils [85, 86]. Loss of obscurin activity causes the misalignment of M-lines in adjacent myofibrils and reduced myosin incorporation into maturing thick filaments [85, 87]. These observations are contradicted by the recent finding that there is no significant difference in skeletal muscle M-line architecture between obscurin knockout and wild-type mice [88]. It is possible that obscurin like 1 protein compensates for the loss of obscurin to preserve M-line architecture. Nevertheless, this knockout model demonstrates that obscurin plays a key role in establishing/maintaining the longitudinal SR that extends the length of a sarcomere and connects neighbouring junctional SR [88]. In zebrafish, obscurin deficiency due to antisense morpholino treatment results in ventricular hypoplasia and a reduced heart rate [89]. Obscurin mutations have been also associated with human cardiomyopathy [90].

While many models of obscurin deficiency demonstrate that this protein predominantly functions at the M-line, obscurin isoforms have also been detected at the M-line, Z-line, Z/I junctions and A/I junctions of skeletal muscle [91]. Multiple obscurin isoforms are produced by alternative splicing of the 117 exons that make up the obscurin gene *OBSCN* that is located on human chromosome 1q42.13 [92]. These isoforms contain variable combinations of modular domains including immunoglobulin (Ig) and fibronectin (Fn) repeats, a calcium/calmodulin binding domain, a Rho-GEF domain, and two serine/threonine kinase domains. Only the 800 kD obscurin isoform contains an ankyrin-binding site following the Rho-GEF domain in a unique C-terminus [92]. This isoform is predominantly localized to the M-line *via* the association of its N-terminal Ig domains with the M-line resident proteins myomesin and M-line titin (Fig. 3) [93]. Interestingly, titin mutations within the region of the obscurin-binding site are associated with tibial muscular dystrophy and limb girdle muscular dystrophy [94–96].

In ventricular cardiomyocytes, ankyrin-B is localized at two distinct domains: overlying the structural and myofibrillar proteins of the M-line and at the SR/T-tubule junctions (Fig. 3). Recently, it was demonstrated that a subpopulation of ankyrin-B is targeted to the M-line *via* its interaction with the C-terminal domain of 800 kD obscurin [97]. This interaction is regulated by alternative splicing of an exon in the C-terminal regulatory domain that dramatically increases obscurin-binding activity of ankyrin. Moreover, it was demonstrated that a regulatory subunit of protein phosphatase 2A, a signalling molecule that controls the phosphorylation status of proteins such as myosin-binding protein C and troponin-I, is brought to the M-line *via* ankyrin-B/obscurin interactions [97].

In addition to ankyrin-B, small ankyrin-R isoforms 1.5 and 1.9 interact with the carboxyl-terminus of 800 kD obscurin [50, 51, 98]. These small ankyrins are unique because they lack many of the domains found in a prototypical ankyrin. In fact, they only retain a small portion of the C-terminal regulatory domain of ankyrin-R. The amino terminal domain contains a novel stretch of amino acids that forms a transmembrane domain that integrates into the membrane of the SR. Based on their interactions with obscurin, these small isoforms are targeted to the M-line where it is thought that they position the developing SR in reference to myofibrils [50, 51, 98].

Ankyrins and disease

As the field of ankyrin biology continues to advance, the clinical manifestations of ankyrin dysfunction will be recognized as complex and multi-systemic for a number of reasons. First, ankyrins (R, G, B) and their alternative isoforms are expressed in many tissues (see Table 1). Often they display overlapping expression patterns in the same tissues. For example, isoforms of all three ankyrins are expressed in brain, heart and skeletal muscle. Findings from ankyrin knockout mouse models and cell-based structure/function analysis suggest that ankyrins are not functionally redundant. For example, ankyrin-G does not compensate for ankyrin-B loss in ventricular cardiomyocytes [99]. Nevertheless,

nothing is known about the relationship between ankyrins co-expressed in the same tissue, so the extent of functional redundancy between ankyrins has yet to be fully explored. Finally, owing to the structural and functional diversity of ankyrin isoforms across different tissues, an amino acid variation/mutation in ankyrin may be tolerated in a particular tissue background, all the while causing significant dysfunction in another tissue background. Moreover, the location of these variable residues may have a significant impact on ankyrin function. For example, 8 out of 9 loss-of-function mutations associated with 'ankyrin-B syndrome', which encompasses a wide range of cardiac disorders, reside within the C-terminal regulatory domain (see Table 2) [100–102]. The following sections will present diseases associated with ankyrin dysfunction in the erythrocyte, heart and brain.

Table 2 Clinical phenotypes of ankyrin-B missense mutations

ANK2 exon	Residue change	Protein domain	Clinical phenotype
Exon 37	T1404I	Spectrin binding	Ankyrin-B syndrome
Exon 38	E1425G	Spectrin binding	Ankyrin-B syndrome
Exon 43	V1516D	Death	Ankyrin-B syndrome
Exon 43	T1552N	Death	Ankyrin-B syndrome
Exon 44	L1622I	C-terminal	Ankyrin-B syndrome
Exon 44	T1626N	C-terminal	Ankyrin-B syndrome
Exon 47	V1777M	C-terminal	Ankyrin-B syndrome
Exon 48	R1788W	C-terminal	Ankyrin-B syndrome
Exon 48	E1813K	C-terminal	Ankyrin-B syndrome

Ankyrin-B syndrome may include a number of clinical phenotypes including sinus node dysfunction (bradycardia and heart rate variability), atrial fibrillation, polymorphic ventricular arrhythmia and risk of sudden cardiac death.

Ankyrin-R

While ankyrin-R is expressed in red blood cells, muscle tissue and neurons, the most prominent phenotype associated with ankyrin-R mutations is hereditary sphereocytosis. This haemolytic anaemia is characterized by increased haemolysis due to altered red blood cell morphology from the normal biconcave disk to a spherical conformation. Although it is rare, some neurological problems have been associated with hereditary sphereocytosis [103]. As a model of ankyrin-R deficiency, *normoblastosis* (*nb/nb*) mice display motor ataxia in addition to severe haemolytic anaemia [32, 104]. These deficiencies are the result of a hypomorphic mutation in ankyrin-R [105] and the movement disorder is consistent with the predominant expression of ankyrin-R in the cell bodies and dendrites of Purkinje and granule cells of the cerebellum [32, 104]. While multiple ankyrin-R isoforms have been characterized in skeletal muscle, no muscular dystrophies in human have been associated with ankyrin-R dysfunction.

Ankyrin-G

Recently, multiple genome wide association studies have linked single nucleotide polymorphisms in the *ANK3* locus to bipolar disorder, a mental illness characterized by recurring episodes of mania and depression [106–108]. Considering neurotransmitters commonly associated with mood disorders (*i.e.* serotonergic, dopaminergic and cholinergic neurotransmitters) are not preferentially affected in bipolar disorder, the molecular basis for bipolar disorder may reflect wholesale changes to synaptic connectivity and neural circuitry. This view is consistent with the loss of synaptic connectivity between basket interneurons and Purkinje neurons in ankyrin-G deficient cerebellums [109]. To regulate neuronal excitability, basket interneurons form GABAergic synapses with AIS of Purkinje neurons, a developmental process guided by an ankyrin-G dependent subcellular gradient of neurofascin in Purkinje neurons [109]. Additional support for ankyrin function in synaptogenesis comes from genetic screens that identified the large isoform of *Drosophila* ankyrin 2 as a critical regulator of synaptic stability and maintenance at the *Drosophila* neuromuscular junction [110, 111]. Specifically, the loss of this ankyrin precipitates the disassembly and retraction of presynaptic boutons, caused by cytoskeletal destabilization following the loss of an interaction between synaptic microtubules and the extended C-terminal domain of this ankyrin [110, 111].

Another mechanism by which ankyrin-G impacts overall neural circuitry is through its targeting of voltage-gated sodium and potassium channels to AIS and nodes of Ranvier in neurons of the central nervous system. For example, ankyrin-G directs the targeting of voltage-gated sodium channels to AIS of granule cells and Purkinje neurons [80]. In the absence of ankyrin-G, Purkinje neurons display impaired ability to initiate action potentials and the mice demonstrate neuronal degeneration of the cerebellum and progressive motor ataxia [80].

Many disease-related mutations have been characterized in ankyrin-G associated proteins. For example, a mutation in cardiac voltage-gated sodium channel $Na_V1.5$ was shown to disrupt the channel's association with ankyrin-G and to cause Brugada syndrome, a cardiac disorder characterized by precordial ST segment elevation, right bundle branch block, and fatal arrhythmias [5, 45]. The Brugada syndrome mutation (E1053K) resides within a 9 amino acid ankyrin-binding motif that is highly conserved in the DII-DIII loops of voltage-gated sodium channels [45, 112, 113]. By disrupting the association of ankyrin-G and $Na_V1.5$, this mutation impairs ankyrin-G dependent targeting of the channel to the intercalated disc resulting in decreased sodium channel current density (I_{Na}) [5, 45]. Similar ankyrin-binding motifs are also present in the voltage-gated potassium channel subunits KCNQ2 and KCNQ3. These motifs are required for ankyrin-dependent targeting of these channel subunits to AIS where they control overall neuronal excitability [11, 13, 14].

Mutations in these subunits associated with benign familial neonatal convulsions (BFNC) cause epilepsy and myokymia [114–116]. Moreover, BFNC mutations in the KCNQ2 subunit that remove the ankyrin-binding site either through a frameshift or premature stop codon cause reduced axonal surface expression of potassium channels in hippocampal neurons [11]. Recently, the β_1 subunit of cyclic nucleotide-gated channel (CNG-β_1) was identified as a binding partner for ankyrin-G in the retina [117]. This interaction is necessary for ankyrin-dependent targeting of CNG-β_1 to rod outer segments and domain formation [117]. Interestingly, the ankyrin-G binding site resides in the last twenty amino acids of CNG-β_1 and a mutation that abolishes this domain is associated with retinitis pigmentosa, a progressive retinal dystrophy that causes gradual vision loss due to abnormalities in the photoreceptors or retinal pigment epithelium [117, 118]. Finally, both ankyrin-B and ankyrin-G were recently shown to interact with dystrophin, an essential component of the dystrophin glycoprotein complex that provides membrane stability by linking the actin-based cytoskeleton to integral membrane proteins connected to the extracellular matrix [72]. A dystrophin mutation linked to Becker muscular dystrophy was shown to reduce ankyrin binding thereby resulting in decreased sarcolemmal localization of dystrophin [72].

Ankyrin-B

While ankyrin-B is expressed in a variety of tissues, human mutations to ankyrin-B have almost exclusively been associated with cardiac disorders (see Table 2). The uniform expression of ankyrin-B polypeptides in different cardiac regions (*i.e.* ventricles, atria, sinoatrial node) accounts for the diversity of ankyrin-B associated cardiac disorders including bradycardia, sinus arrhythmia, delayed conduction/conduction block, idiopathic ventricular fibrillation and catecholaminergic polymorphic ventricular tachycardia [100–102]. In the ventricles, ankyrin-B is responsible for the proper targeting and retention of ion channels and transporters that regulate local calcium dynamics at the SR/T-tubule junction (Fig. 3). In ankyrin-B haploinsufficient mice, ventricular cardiomyocytes display reduced expression and proper SR/T-tubule localization of NCX, NKA and the IP_3 receptor [9, 101, 102]. Furthermore, when given a catecholamine bolus during exercise, these mice exhibit ventricular tachycardia and often sudden death, thereby mimicking human cases of catecholaminergic polymorphic ventricular tachycardia that result from ankyrin-B dysfunction [101].

Recently, the molecular basis for ankyrin-B dysfunction was characterized in 'sick sinus syndrome' including bradycardia and variable heart rate. Specifically, ankyrin-B is necessary for the proper targeting of ion channels and transporters associated with calcium influx and efflux in the cardiac pacemaker, or sinoatrial node [119]. In addition to reduced expression of NCX, NKA and IP_3 receptor, sinoatrial cells from ankyrin-B heterozygous mice display inappropriate targeting of $Ca_V1.3$, an important regulator of extracellular calcium entry that is necessary for normal cardiac pacemaker function [119]. Future research efforts to assess ankyrin-B activity in other cardiac regions (*i.e.* the atrium and other components of the conduction system) will advance our understanding of ankyrin-B function in normal cardiac physiology.

Studies of the ankyrin-B knockout mouse provide insight into potential disorders linked to human mutations in ankyrin-B. For example, ankyrin-B deficiency in mouse has been reported to be associated with mild skeletal muscular phenotypes including elevated creatine kinase levels and sporadically disorganized sarcomeres [40]. As mentioned above, ankyrin-B was recently found to interact with dystrophin consistent with its role in skeletal muscle organization and integrity. To date, no human skeletal muscle disorder has been associated with an ankyrin-B mutation, although co-expression of ankyrin-G and ankyrin-R isoforms may compensate for ankyrin-B dysfunction.

Ankyrin-B deficient mice also exhibit significant neurological pathology including hypoplasia of the corpus callosum and pyramidal tracts, dilated ventricles and severe postnatal degeneration of the optic nerve [39]. At the cellular level, many of these abnormalities are the result of ankyrin-B functions in neurite outgrowth, axonal guidance and axon fasciculation. At the molecular level, these functions are fulfilled by ankyrin-B interactions with the cell adhesion molecule L1. In fact, many of the neurological defects manifested in ankyrin-B deficient mice are mimicked in L1-deficient mice including axon guidance errors in pyramidal tracts and corpus callosum, degeneration of sensory axons and enlarged lateral ventricles [120–123]. The neuropathology manifested in ankyrin-B deficient mice may also arise in part from a loss of ankyrin-B dependent targeting of β-dystroglycan to astrocyte endfeet, which ensures the compact formation of the glia limitans that serves as an interface between cerebral spinal fluid and the brain [72].

In many animal models, both ankyrin and L1 are involved in axonal guidance and fasciculation. For example, mutations to the ankyrin-related gene *unc-44* in *C. elegans* result in abnormal axonal guidance and fasciculation [124, 125]. Likewise, mutations to the *Drosophila* homologue of L1 neuroglian cause abnormal pathfinding of motorneuron projections and impaired contralateral projections of olfactory receptor neurons [126, 127]. Two different mechanisms have been shown to regulate L1-mediated axonal guidance and neurite outgrowth. First, L1 interacts with neuropilin-1, which is a receptor for the semaphorin family of chemoattractants and repellents [128–130]. Second, the homophilic interactions between L1 proteins activate receptor tyrosine kinases (*i.e.* the epidermal and fibroblast growth factor receptors) and second messenger pathways (*i.e.* MAP kinase and phospholipase C) [131]. Tyrosine phosphorylation of the FIGQY motif abolishes ankyrin interaction with L1, and neurite outgrowth is positively regulated by untethered L1 molecules. Interestingly, mutations in the L1 FIGQY motif have been linked to human cases of CRASH syndrome that is characterized by corpus callosum hypoplasia, retardation, adducted thumbs, spastic paraplegia and hydrocephalus [132, 133].

Conclusions

While ankyrins were initially characterized as anchors tethering membrane proteins to the underlying cytoskeleton, they are now thought to play a crucial role in the formation of specialized membrane domains. With their modular architecture and ability to interact with many proteins, ankyrins organize and stabilize protein networks in collaboration with other proteins including cytoskeletal proteins, cell adhesion molecules and large structural proteins. Cytoskeletal proteins such as β-spectrin contribute stability to the ankyrin protein network. This stability underlies the structural integrity of the plasma membrane such that cells remain resilient to mechanical stress associated with development and normal cellular functions. Furthermore, incorporation of β-spectrin allows the ankyrin protein network to remain static in the fluid lipid bilayer thereby accommodating the initiation, development and maintenance of membrane domains. Cell adhesion molecules enable extracellular signals from neighbouring cells or the extracellular matrix to interact with the ankyrin protein complex and to guide the formation of membrane domains such as the nodes of Ranvier in peripheral neurons. Ankyrin interactions with cell adhesion molecules are important for connecting adjacent cells and anchoring cellular projections that migrate through the extracellular matrix. Finally, ankyrin interactions with large structural proteins such as obscurin allow for the proper integration of ankyrin protein complexes into the larger cellular scheme, ensuring its correct placement in the context of other protein networks and neighbouring cellular structures. While the targeting and stabilization of ankyrin protein networks are facilitated by each of the aforementioned proteins, it is certain that many additional proteins will contribute to ankyrin-dependent membrane formation. The tailoring of ankyrin polypeptides through alternative splicing enables modified ankyrins to adapt to the variable conditions presented during the formation of different specialized domains. This versatility accounts for ankyrin's multifunctional capabilities in diverse cellular backgrounds (*i.e.* erythrocytes *versus* cardiomyocytes) and their apt intercellular functioning within different organelles (*i.e.* lysosome, trans Golgi network and SR) [41, 47, 134, 135]. As the field of ankyrin biology continues to develop, so too will our appreciation of ankyrin's pivotal role in normal and diseased cellular conditions.

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