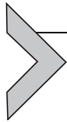

CHAPTER ONE

Spectrin- and Ankyrin-Based Membrane Domains and the Evolution of Vertebrates

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Abstract

Spectrin and ankyrin are membrane skeletal proteins that contribute to mechanical support of plasma membranes and micron-scale organization of diverse membrane-spanning proteins. This chapter provides a plausible scenario for the evolution of ankyrin- and spectrin-based membrane domains with a focus on vertebrates. The analysis integrates recent phylogenetic information with functional analyses of spectrin and ankyrin in erythrocytes, axon initial segments and nodes of Ranvier in neurons, T-tubules and intercalated disks of cardiomyocytes, lateral membrane domains of epithelial cells, and costameres of striated muscle. A core spectrin–ankyrin mechanism for coordinating membrane-spanning proteins and mechanically stabilizing membrane bilayers was expanded in vertebrates by gene duplication events, insertion of giant alternately spliced exons of axonal ankyrins, and a versatile peptide-binding fold of ANK repeats that facilitated acquisition of new protein partners. Cell adhesion molecules (CAM), including dystroglycan, L1 CAM family members, and cadherins, are the earliest examples of membrane-spanning proteins with ankyrin-binding motifs and were all present in urochordates. In contrast, ion channels have continued to evolve ankyrin-binding sites in vertebrates. These considerations suggest a model where proto-domains formed through interaction of ankyrin and spectrin with CAMs. These proto-domains then became populated with ion channels that developed ankyrin-binding

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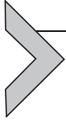
activity with selective pressure provided by optimization of physiological function. The best example is the axon initial segment where ankyrin-binding activity evolved sequentially and independently first in L1 CAMs, then in voltage-gated sodium channels, and finally in KCNQ2/3 channels, with the selective advantage of fast and precisely regulated signaling.

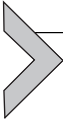


1. INTRODUCTION

First year medical students learn that plasma membranes of cells in human tissues are beautifully organized into functional micron-scale domains that are the basis for much of our physiology. What is less appreciated is the molecular novelty and evolutionary origin of these structures, especially those related to fast signaling in the heart and nervous system that exist only in vertebrates. Following the emergence of polarized epithelial cells in early metazoans beginning around 650 million years before present (mybp), animal cells rapidly diversified to include neurons and other sensory cells, as well as muscle cells. The first neurons and striated muscle in cnidarians (jellyfish, hydra, and corals) appeared by 580 mybp and were followed by multiple cell types organized with bilateral symmetry in the bilaterian lineage (nematodes, arthropods, flatworms, mollusks, etc.) around 550 mybp. A modern myelinated nervous system and closed cardiovascular system were likely present in the first jawed vertebrates by 420 mybp. Soon after these evolutionary developments, tetrapods invaded terrestrial environments, which required many adaptations, including new approaches to respiration and excretion. By 170 mybp, early eutherian mammals had developed homeothermy and high cardiac output, with cellular specializations including cardiomyocyte T-tubules and enucleated erythrocytes. Following the emergence of the first bilaterian, mammals had acquired a diverse set of newly configured assemblies of ion transporters and cell adhesion molecules (CAMs), each requiring new protein interactions and mechanisms for precise spatial patterning in the plasma membranes of multiple cell types.

Spectrin and ankyrin are membrane skeletal proteins present in their modern forms in bilaterians that contribute to mechanical support of plasma membranes and micron-scale organization of membrane-spanning proteins in many tissues (Bennett & Baines, 2001; Bennett & Healy, 2009). This chapter will consider the role of ankyrins and spectrins in the evolution of diverse vertebrate membrane domains. We will develop the thesis that members of the ankyrin and spectrin families were key substrates in adaptive

evolution of diverse plasma membrane domains, including excitable membranes in neurons and heart, costameres of striated muscle, and lateral membrane domains of epithelial cells. We will review intrinsic features of spectrin and ankyrin that made these interacting proteins excellent starting points for establishing long-range order in an otherwise fluid phospholipid bilayer. We will consider the basis for diversity within the basic theme of a core spectrin–ankyrin mechanism for coordinating membrane-spanning proteins. We also will discuss gene duplication events in early vertebrates, vertebrate-specific alternately spliced isoforms of axonal ankyrins, and a versatile peptide-binding fold of ankyrins that facilitated acquisition of new protein partners. Lastly, we will review a parallel and perhaps ancient function for spectrins and ankyrins in directed intracellular trafficking of membrane organelles.



2. AN ANCIENT SPECTRIN–ANKYRIN PARTNERSHIP FOR COORDINATING MEMBRANE-SPANNING PROTEINS

Ankyrin and spectrin family members cooperate to provide a widely utilized mechanism for coordinating membrane-spanning proteins in the plane of the plasma membrane and coupling these proteins to an extended mechanically resilient submembrane network. The logic of this system is straightforward: membrane-spanning proteins, including cell adhesion proteins capable of interaction with the extracellular matrix and other cell surfaces as well as membrane transporters, are "anchored" by ankyrin to an extended spectrin network tightly associated with the plasma membrane (Fig. 1.1). Spectrin polymerizes into membrane-associated networks through association with specialized actin filaments, which interact with multiple spectrins and also are independently coupled to the membrane bilayer. Spectrin association with actin filaments is promoted by accessory proteins, including adducin, protein 4.1, and p55/MPP1 (described in more detail in the succeeding text) (Fig. 1.1). Ankyrin through its ANK repeats (Lee et al., 2006) and spectrin through its triple-helical repeats (Rief, Pascual, Saraste, & Gaub, 1999) both behave as elastic elements in single molecule atomic force microscopy measurements. Ankyrin and spectrin both experience stretching in erythrocytes under shear stress (Krieger et al., 2011). Moreover, deficiency of ankyrin and spectrin lead to fragile erythrocyte membranes (Eber & Lux, 2004) and axons (Hammarlund, Jorgensen, & Bastiani, 2007). The ankyrin–spectrin assembly thus provides mechanical stability to the lipid bilayer in addition to organization of protein assemblies.

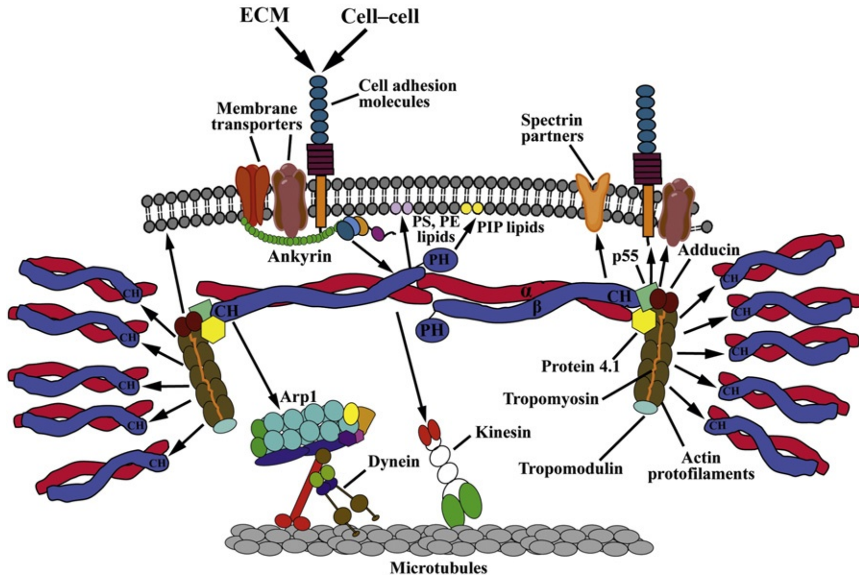


Figure 1.1 A conserved spectrin–ankyrin partnership coordinates membrane-spanning proteins within micron-scale plasma membrane domains responsive to extracellular cues. Membrane-spanning proteins, including cell adhesion proteins and membrane transporters, are "anchored" by ankyrin through its ANK repeats to an extended spectrin–actin network tightly associated with the plasma membrane. Spectrin polymerizes into membrane-associated networks through association with specialized actin filaments, which interact with multiple spectrins and also are independently coupled to the membrane bilayer. Spectrin association with actin filaments is promoted by accessory proteins, including adducin, protein 4.1, and p55/MPP1. The ankyrin–spectrin assembly provides mechanical stability to the lipid bilayer in addition to organization of membrane proteins. Parallel functions of spectrin and ankyrin in intracellular organelle transport are mediated by interactions with dynactin (shown for CH domains of beta-spectrin) and kinesin.

The human erythrocyte provided the prototype where the elements of a spectrin–ankyrin-based assembly were first elucidated (see Chapter 2; Bennett & Baines, 2001). Ankyrin and/or spectrin has subsequently been implicated in organization and/or stabilization of multiple membrane domains, including axon initial segments and nodes of Ranvier (Dzhashiashvili et al., 2007; Galiano et al., 2012; Hedstrom, Ogawa, & Rasband, 2008; Hedstrom et al., 2007, Jenkins & Bennett, 2001; Sobotzik et al., 2009; Susuki et al., 2013; Zhou et al., 1998), unmyelinated axons (Scotland, Zhou, Benveniste, & Bennett, 1998), cardiomyocyte T-tubules and intercalated disks (Hund et al., 2010; Lowe et al., 2008;

Mohler, Davis, & Bennett, 2005; Mohler, Rivolta, et al., 2004), epithelial lateral membranes (Kizhatil & Bennett, 2004; Kizhatil, Davis, et al., 2007; Kizhatil, Yoon, et al., 2007), costameres, which are mechano-domains in heart and skeletal muscles (Ayalon, Davis, Scotland, & Bennett, 2008, Ayalon et al., 2011), and photoreceptor inner and outer segments (Kizhatil, Baker, Arshavsky, & Bennett, 2009, Kizhatil, Sandhu, Peachy, & Bennett, 2009; reviewed by Bennett & Healy, 2009) (see chapters 3–5; 7 in this volume).

We will next consider the molecular properties of spectrin and ankyrin that provide the basis for their function as membrane domain coordinators (Figs. 1.2 and 1.3). Spectrin is a flexible elongated tetramer nearly 200 nm in length that is comprised of alpha- and beta-subunits assembled side-to-side in an antiparallel orientation and head to head through association of alpha-spectrin with beta-spectrin (Figs. 1.1 and 1.2) (Shotton, Burke, & Branton, 1979). Alpha- and beta-spectrin subunits are both related to alpha-actinin (Djinovic-Carugo, Young, Gautel, & Saraste, 1999; Viel, 1999) but are extended in length from 40 to nearly 100 nm by multiple copies of a triple-helical repeat (Grum et al., 1999; Speicher & Marchesi, 1984; Yan et al., 1993). End-to-end association between alpha- and beta-spectrins results from noncovalent assembly of partial triple-helical repeats of each spectrin subunit (Ipsaro et al., 2010; Mehboob et al., 2010; Tse et al., 1990).

Beta-spectrin contributes to the principal interactions with other proteins and mediates interactions with F-actin (or Arp1 of the dynactin complex; see succeeding text) through N-terminal tandem calponin homology (CH) domains (Banuelos, Saraste, & Carugo, 1998; Carugo, Banuelos, & Saraste, 1997), with ankyrin through the 14th and 15th triple-helical repeats (Davis et al., 2009; Ipsaro, Huang, & Mondragón, 2009; Ipsaro & Mondragón, 2010; Kennedy, Warren, Forget, & Morrow, 1991; Stabach et al., 2009) and with $PI4,5P_2$ phosphatidylinositol lipids through a C-terminal pleckstrin homology (PH) domain (Hyvönen et al., 1995; Macias et al., 1994; Fig. 1.2). The beta-spectrin PH domain is absent as a result of alternative splicing in mammalian erythrocyte spectrin as well as some other spectrin isoforms (Hayes et al., 2000, Winkelmann, Chang, et al., 1990; Winkelmann, Costa, Linzie, & Forget, 1990).

Spectrin tetramers in erythrocyte membranes form a membrane-coupled polygonal network through association of five to seven spectrins at 50–70° angles with short 40 nm actin protofilaments (Byers & Branton, 1985). These actin protofilaments are capped on their fast-growing ends by adducin, which also promotes association with spectrin (Gardner & Bennett, 1987; Kuhlman,

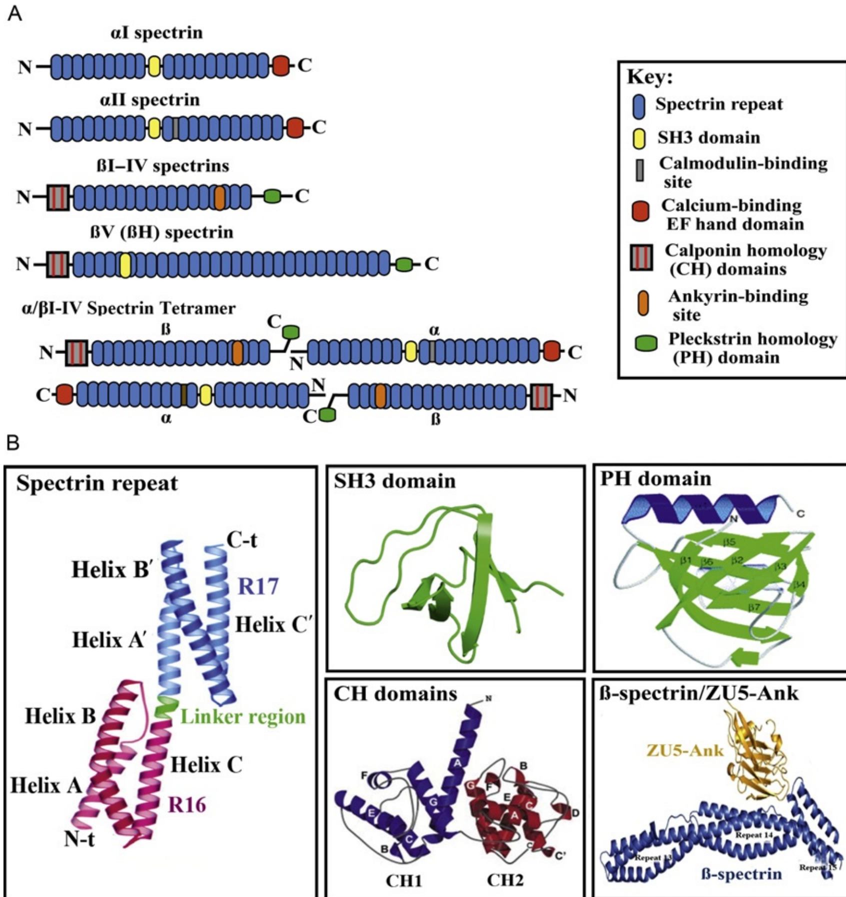


Figure 1.2 Domain structure of spectrins. (A) The domain organization of two α- and five β-spectrins is shown. Spectrins are comprised of modular spectrin repeat units (blue). Other functional domains include Src-homology domain (SH3, yellow), calcium-binding EF hand domain (red), and calmodulin-binding domain (gray). β-Spectrin proteins also have two in tandem calponin homology domains (CH, gray and red) and a pleckstrin homology domain (PH, green), and with the exception of β_V-spectrin, they contain an ankyrin-binding site (orange). The spectrin tetramer is the fundamental unit of the spectrin-based membrane skeleton. The N-terminus of each α-spectrin subunit associates with the C-terminal portion of β-spectrin to form a dimer. Tetramer formation depends on the lateral and antiparallel head-to-head association between two α/β-spectrin heterodimers. (B) Ribbon diagram representation of the crystal structure of two spectrin repeats (Grum, MacDonald, & Mondragón, 1999), SH3 domain (Musacchio, Noble, Pauptit, Wierenga, & Saraste, 1992), in tandem CH domains (Sjöblom, Ylänne, & Djinović-Carugo, 2008), PH domain (Lemmon, Ferguson, & Abrams, 2002), and the spectrin–ankyrin interaction binding domains (Ipsaro & Mondragón, 2010).

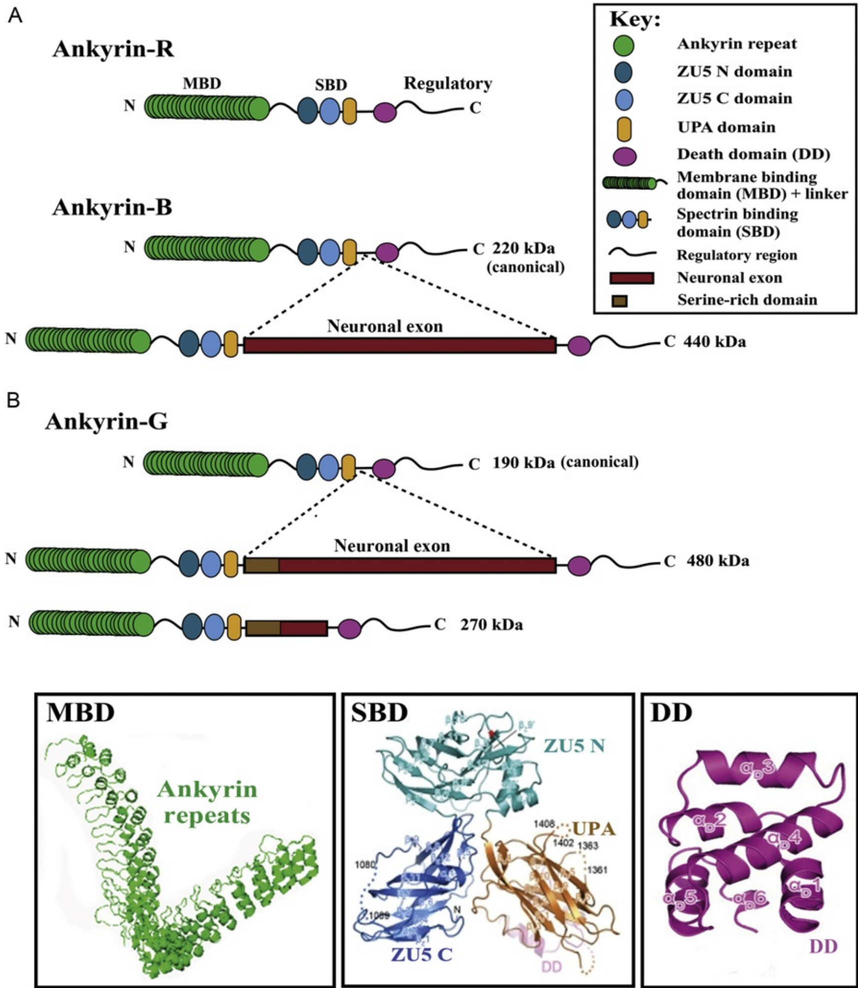


Figure 1.3 Domain structure of ankyrins. (A) The domain organization of canonical and neuronal giant ankyrins is shown. The membrane-binding domain of ankyrin (green) is comprised of 24 ankyrin repeats. The spectrin-binding domain supermodule contains two ZU5 domains (teal and blue) and a UPA domain (orange). Other functional domains include a death domain (pink) and a C-terminal unstructured regulatory domain (black line). The giant ankyrin isoforms have an insertion of a single exon (red) after the UPA domain. Neuronal giant 480 kDa and 270 kDa ankyrin-G proteins also contain a serine-rich domain at the beginning of the inserted region (black bar). (B) Ribbon representation of the crystal structure of the deduced 24 ANK repeat ankyrin membrane-binding domain (Michaely, Tomchick, Machius, & Anderson, 2002), ZU5n–ZU5c–UPA supermodule (Wang, Yu, Ye, Wei, & Zhang, 2012), and death domain (Wang et al., 2009).

Hughes, Bennett, & Fowler, 1996; Li, Matsuoka, & Bennett, 1998), and on the slow-growing ends by tropomodulin (Weber, Pennise, Babcock, & Fowler, 1994). Spectrin interaction with actin in erythrocytes also is promoted by protein 4.1, a member of the FERM family (Baines, Lu, & Bennett, 2013; Pearson, Reczek, Bretscher, & Karplus, 2000; Ungewickell, Bennett, Calvert, Ohanian, & Gratzer, 1979). The spectrin–actin network is attached to erythrocyte membranes through a high-affinity association between beta-spectrin and ankyrin near the midregion of the spectrin tetramers (Bennett, 1978; Bennett & Stenbuck, 1979a; Ipsaro & Mondragón, 2010; Kennedy et al., 1991; Tyler, Hargreaves, & Branton, 1979). Additional protein-dependent membrane contacts at the spectrin–actin junction are provided by association of the MAGUK protein p55/MPP1 with protein 4.1 and the membrane-spanning protein glycophorin C (Marfatia, Leu, Branton, & Chishti, 1995; Marfatia, Lue, Branton, & Chishti, 1994, Ruff, Speicher, & Husain-Chishti, 1991) and of adducin with the anion exchanger (Anong et al., 2009). In addition to protein-based interactions, spectrin associates with membrane phosphatidylinositol lipids in nonerythroid cells through its PH domain (Das, Base, Dhulipala, & Dubreuil, 2006; Das, Base, Manna, Cho, & Dubreuil, 2008, Wang, Miller, Shaw, & Shaw, 1996). Spectrin also interacts with other membrane phospholipids such as phosphatidylserine at multiple sites identified in both alpha- and beta-subunits (An et al., 2004). Palmitoylation of ankyrin (He, Jenkins, & Bennett, 2012; Staufenbiel, 1987) and p55/MPP1 (membrane-palmitoylated protein-1) (Ruff et al., 1991) provides yet another mechanism for membrane attachment of the ankyrin–spectrin network.

Plasma membrane-associated spectrin–actin–adducin assemblies have been resolved in axons of cultured neurons using super-resolution light microscopy, although they exhibit a different organization from the polygonal erythrocyte configuration (Xu, Zhong, & Zhuang, 2013). Axonal actin and adducin are organized into periodic membrane-associated rings separated by 190 nm, which is precisely the length of brain spectrin tetramers (Bennett, Davis, & Fowler, 1982). Spectrin antibody labels between the actin–adducin rings suggesting that these structures are interconnected by spectrin tetramers attached at a 90° angle (Xu et al., 2013). Spectrin–actin–adducin networks, now resolved in both erythrocytes and axons, are likely to be a general feature of spectrin organization in epithelial lateral membranes as well as other membrane domains (Abdi & Bennett, 2008), although their precise geometry likely will depend on the cellular context.

Ankyrin provides a mechanism for utilizing extracellular cues to form plasma membrane domains through its ability to associate with CAMs as well as membrane transporters and to couple these membrane-spanning proteins to membrane-associated spectrin–actin networks. Ankyrin is a monomer with an N-terminal membrane-binding domain containing 24 tandem ANK repeats folded as a solenoid (Michaely et al., 2002), followed by a supermodule comprised of two ZU5 domains and a UPA domain (Wang et al., 2012), a death domain (Wang et al., 2009), and an unstructured regulatory domain (Fig. 1.3). The binding site for beta-spectrin is located in the first ZU5 domain (Ipsaro & Mondragón, 2010; Mohler, Yoon, & Bennett, 2004). The anion exchanger (band 3 in early literature) of erythrocytes provided the first example of an ankyrin-linked integral membrane protein (Bennett & Stenbuck, 1979a,1979b, 1980a). Membrane-spanning ankyrin-binding partners now include over 14 families of CAMs and membrane transporters (Bennett & Healy, 2009; Table 1.1).

Ankyrins interact with their membrane protein partners through ANK repeats that are folded into an extended solenoid with a 240-Å groove running along its length (Michaely et al., 2002). Interestingly, ankyrin can bind to more than one partner at a time and thus can form homo- and heterocomplexes (Michaely & Bennett, 1995a,1995b). ANK repeats in general perform a wide range of functions related to protein recognition and occur in tandem arrays throughout nature including viruses, bacteria, archaea, fungi, plants, and animals (Al-Khodor, Price, Kalia, & Abu Kwaik, 2010; Mosavi, Cammett, Desrosiers, & Peng, 2004). The versatility of ANK repeats in macromolecular recognition has been exploited using designed ankyrin repeats (DARPINS) expressed in bacteria, which provide an alternative to antibodies with biomedical applications, including diagnostics and drug delivery (Stumpp & Amstutz, 2007).

The ankyrin-binding motifs identified so far are relatively short peptide sequences (10–20 residues) that are distinct in their primary sequence but share a lack of secondary structure. For example, the ankyrin-binding activity of the erythrocyte anion exchanger is due to two loops evident in its crystal structure (Chang & Low, 2003; Grey et al., 2012), the cytoplasmic domains of E-cadherin and L1 CAMs are established to be natively unstructured by biophysical methods (Huber, Stewart, Laurents, Nelson, & Weis, 2001; Zhang et al., 1998), and sites of Nav channels, KCNQ2/3 channels, RhBG ammonium transporter, and beta-dystroglycan are predicted to be unstructured (Bennett & Healy, 2009). The ANK repeat groove can bind peptides based on an atomic structure of erythrocyte ankyrin where the

Table 1.1 Representative independently evolved ankyrin-binding sites of membrane-spanning proteins

Family	*Ankyrin Partner*	*Ankyrin Binding Motif*	*Present*	*~ mybp*
Cell adhesion molecules	Dystroglycan	GVPIIFADELDDSK	Cnidarian	580
	NF/L1CAMs	QFNEDGSFIGQY	Bilaterian	555
	E-cadherin	KEPLLPPEDDTRDNVYYYDEE	Urochordate	520
Membrane Transporters	NaV	VPIAVGESDFE	Cephalochordate	500
	KCNQ2	PYIAEGESDTDSD	Jawed fish	440
	Kir6.2	VPIVAEED	Jawed fish	440
	CNG beta	PEPGEQILSVKMPE	Jawed fish	440
	AE1	ELVMDEKNQEL...PAVLTRSGDPS	Mammals	200
	RhBG	KLPFLDSPP	Mammals	200

Ankyrin-binding sites in cell adhesion molecules and membrane transporters: dystroglycan (Ayalon et al., 2008); L1 CAMS (Zhang, Davis, Carpenter, & Bennett, 1998), E-cadherin (Jenkins et al., 2013; Kizhatil, Davis, et al., 2007), voltage-gated sodium channels (Garrido et al., 2003; Lemaillet, Walker, & Lambert, 2003), KCNQ2/3 channels (Pan et al., 2006), Kir6.2 (Kline et al., 2009), cyclic nucleotide-gated channel beta-subunit (Kizhatil, Baker, et al., 2009), anion exchanger 1 (Grey, Kodippili, Simon, & Low, 2012), and RhB/G ammonium transporter (Lopez et al., 2005). Residues that are critical for ankyrin binding are depicted in red. Residues required for interacting with other partners are illustrated in green.

linker following the ANK repeats was resolved lying in the groove (Michaely et al., 2002). However, a structure of ANK repeats in a complex with a membrane-spanning protein is not yet available.

Natively unstructured motifs in general are widely utilized in protein recognition and are rapidly evolving in eukaryotic genomes (Dyson & Wright, 2005). Such a code offers multiple advantages, including ease of evolution of new partners as well as capacity for integrating interacting pathways through multiple partners. It is of interest with respect to multitasking that the ankyrin-binding motif of E-cadherin contains dileucine residues that are required for clathrin-dependent endocytosis, but do not participate in ankyrin binding (Jenkins et al., 2013). The E-cadherin ankyrin-binding motif thus is better described as a polarity motif that utilizes both ankyrin binding for retention and clathrin for editing to maintain E-cadherin apical–lateral polarity (Jenkins et al., 2013).

Efforts to determine epistatic relationships between spectrin and ankyrin have had mixed results. In *Drosophila* epithelial development, spectrin acts either upstream or independently from ankyrin (Das et al., 2006, 2008; Dubreuil, Wang, Dahl, Lee, & Goldstein, 2000). However, ankyrin-G recruits beta-4 spectrin to axon initial segments and nodes of Ranvier (Jenkins & Bennett, 2001; Komada & Soriano, 2002; Yang, Ogawa, Hedstrom, & Rasband, 2007), and ankyrin-B directs beta-2 spectrin to an intracellular compartment in cardiomyocytes (Mohler, Yoon, et al., 2004). Both ankyrin-G and beta-2 spectrin are required for biogenesis of epithelial lateral membranes, although ankyrin-G lacking beta-spectrin-binding activity still associates with lateral membranes (Kizhatil, Yoon, et al., 2007). These observations suggest that ankyrin and spectrins should be viewed as obligatory partners in an interactive network rather than individual components of a linear pathway.

Which came first, spectrin or ankyrin? The answer to this question is clearly spectrin and is facilitated by mapping binding sites of spectrin for ankyrin (Davis et al., 2009; Ipsaro et al., 2009; Ipsaro & Mondragón, 2010) and of ankyrin for spectrin (Ipsaro & Mondragón, 2010; Mohler, Yoon, et al., 2004) (Fig. 1.4). The most ancient beta-spectrins are evident in sponge and Placozoa genomes. These spectrins were of the same length as vertebrate spectrins, had tandem calponin homology domains and a PH domain, but lacked a recognizable ankyrin-binding site, which includes a critical tyrosine at position 1874 (Davis et al., 2009; Ipsaro & Mondragón, 2010). In addition, these organisms also express a much larger protein termed beta-H spectrin, with homologues in *Drosophila* (Dubreuil,

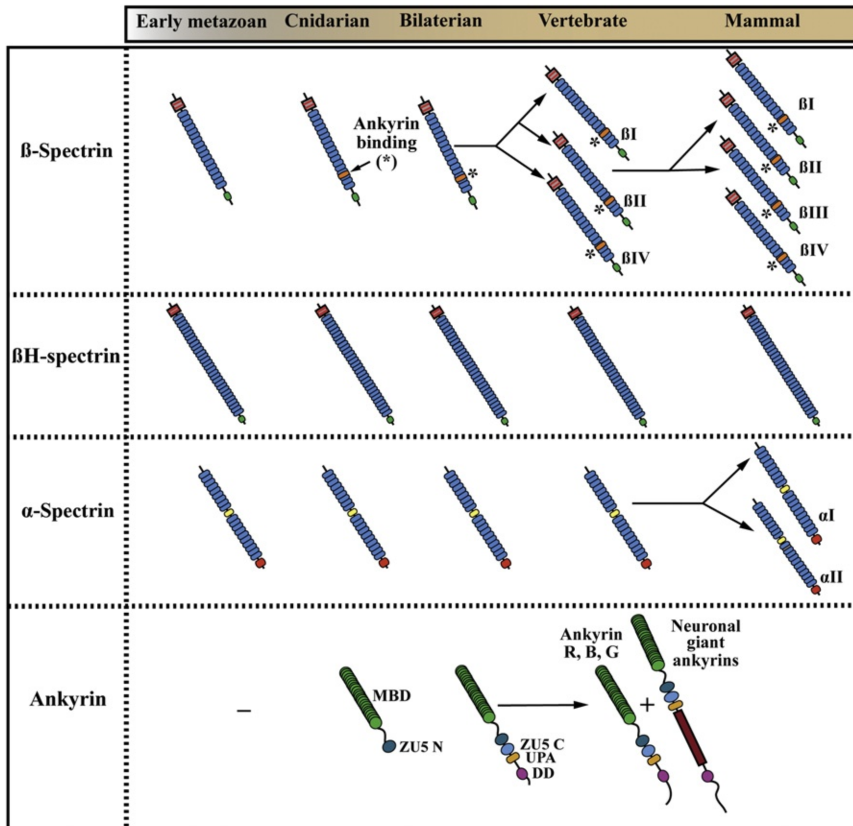
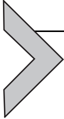


Figure 1.4 Evolution of ankyrin and spectrin protein diversity in vertebrates. Vertebrate ankyrin and spectrin proteins have diversified through evolution as a result of gene duplication, insertion of new sequences, and alternative splicing events.

Byers, Stewart, & Kiehart, 1990), *C. elegans* (McKeown, Praitis, & Austin, 1998) and vertebrates (*SPTBN5* or beta-5 spectrin) (Stabach & Morrow, 2000). Beta-H spectrins lack ankyrin-binding activity and their functions include intracellular trafficking in mammalian photoreceptors (Papal et al., 2013) and *Drosophila* epithelial cells (Phillips & Thomas, 2006) as well as participation in apical domains of epithelial cells (Médina et al., 2002).

Cnidarians have a beta-spectrin with a potential ankyrin-binding site, which has not been evaluated experimentally (Fig. 1.4). In addition, cnidarian genomes also contain ankyrin-like sequences, including a ZU5 domain with a potential spectrin-binding site containing a characteristic DARGG motif. Ankyrins with all modern folded domains (ANK repeats, tandem

ZU5 domains, UPA domain, and death domain) are present throughout bilaterians (Fig. 1.4). Thus, a fully functional spectrin and ankyrin system with potential for lateral organization of membrane-spanning proteins and capable of cell–cell and cell–matrix interactions into micron-scale mechanically resilient domains likely was in place in the precambrian, by the time of kimberella, the first bilaterian fossil dated to 555 million years ago (Martin et al., 2000).



3. DIVERSIFICATION OF VERTEBRATE ANKYRINS AND SPECTRINS

The basic bilaterian repertoire of a single copy of alpha-spectrin, one beta-spectrin with ankyrin-binding activity and a single ankyrin, has been markedly expanded in vertebrates (Figs. 1.4 and 1.5). Ankyrins have provided a major source of diversification due to gene duplications resulting in three ankyrin genes, insertion of new nervous system-specific exons, divergence of regulatory exons, and alternative splicing. These events have resulted in the acquisition of new functions as well as the partition of old functions between the duplicated genes. An additional driver of diversity has resulted from ANK repeats and their ability to bind to intrinsically unstructured peptides, as discussed in the preceding text. The number of spectrin genes also has expanded to two alpha-spectrins (one expressed only in mammalian erythrocytes) and four beta-spectrins with ankyrin-binding activity. Together, these mechanisms have fueled a dramatic expansion of the roles of the ankyrin–spectrin partnership in vertebrate physiology.

Ohno's conjecture that vertebrates experienced two rounds of whole genome duplication has been validated based on genomic data (Dehal & Boore, 2005; Kasahara, 2013; Ohno, 1970, 1999). Many duplicated genes were lost and only a minority has persisted in modern genomes. Ankyrins and spectrins are among those gene families that expanded by whole genome duplications and are candidates to play special roles in vertebrate adaptations. In addition, genome duplications also expanded ankyrin-binding partners, including L1 CAMs and cadherins, that had evolved binding activity prior to duplication events, but these will not be discussed further.

The three vertebrate ankyrins originated from a single ankyrin gene present in urochordates (Cai & Zhang, 2006) and include *ANK1*, encoding ankyrin-R, first characterized in red blood cells (Bennett & Stenbuck, 1980b; Lux, John, & Bennett, 1990); *ANK2*, encoding ankyrin-B, first

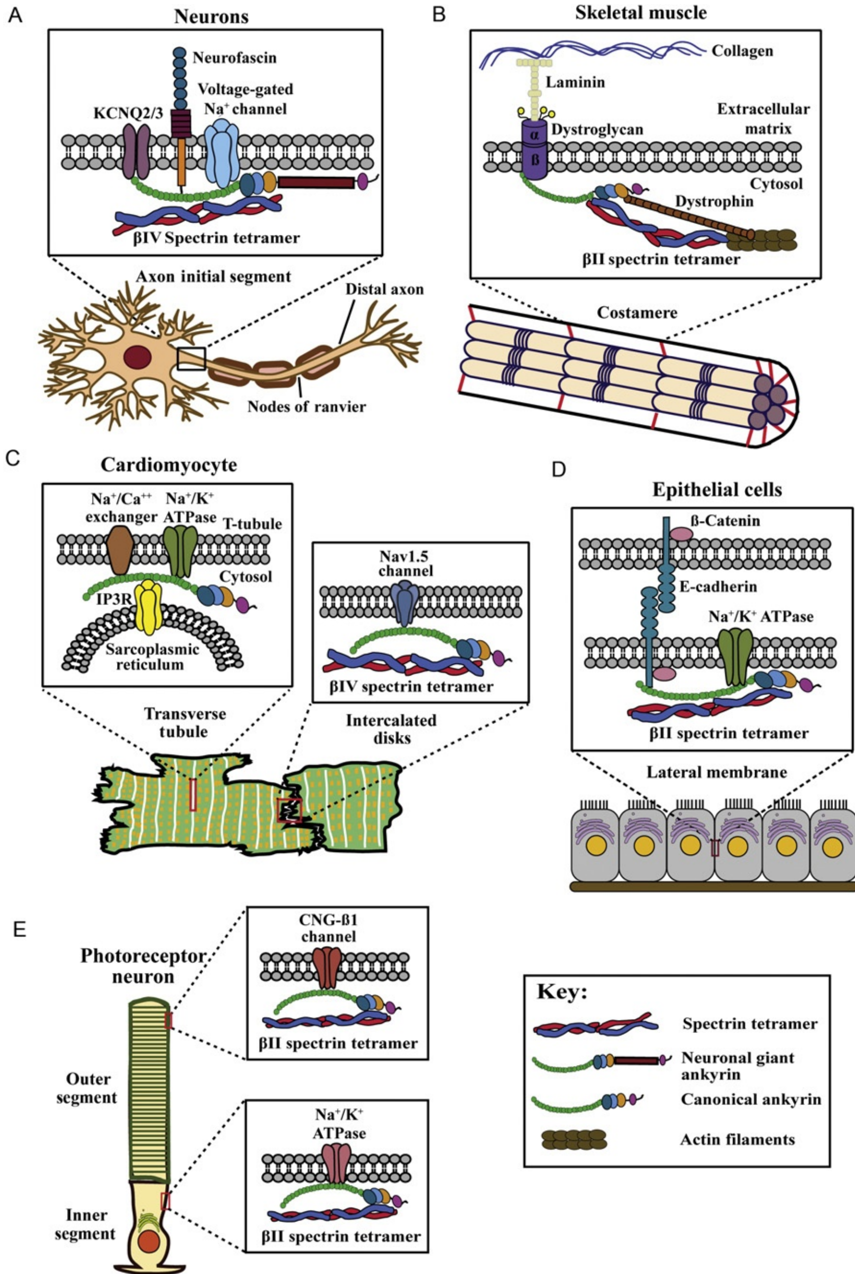


Figure 1.5 Vertebrate variations employing a core ankyrin–spectrin mechanism for coordinating membrane-spanning proteins into membrane domains. The ankyrin–spectrin network targets and stabilizes membrane transporters, cell adhesion molecules, and the dystrophin/dystroglycan complex to specialized membranes, including the axon initial segment of neurons (A), costameres of skeletal muscle (B), transverse tubules and intercalated disks of cardiomyocyte (C), lateral membranes of epithelial cells (D), and the outer and inner segments of photoreceptor neurons (E).

characterized in the brain (Davis & Bennett, 1984; Otto, Kunimoto, McLaughlin, & Bennett, 1991); and *ANK 3,* encoding ankyrin-G, initially characterized in the brain (Kordeli, Lambert, & Bennett, 1995) as well as epithelial tissues (Peters et al., 1995; Thevananther, Kolli, & Devarajan, 1998). Phylogenetic tree analysis indicates that the first duplication event resulted in *ANK1* and the precursor of *ANK2* and *ANK3*, while the second event resulted in *ANK2* and *ANK3* but loss of the duplicate of *ANK1* (Cai & Zhang, 2006).

Vertebrate ankyrins retain extensive sequence similarity in their core folded domains but are divergent in intrinsically unstructured regulatory sequences. A major site of variation is in the unstructured C-terminal domain that modulates interactions with membrane proteins and spectrin through direct interactions with ANK repeats and the spectrin-binding domain (Abdi, Mohler, Davis, & Bennett, 2006; Davis, Davis, & Bennett, 1992; Hall & Bennett, 1987; Mohler, Gramolini, & Bennett, 2002). Another site of regulation and sequence divergence between ankyrin-G (encoded by *ANK3*) and ankyrin-B (encoded by *ANK2)* is located in the linker peptide connecting ANK repeats with the first ZU5 domain (He, Tseng, & Bennett, 2013). This peptide associates with ANK repeats and prevents binding of ankyrin-B with neurofascin and E-cadherin as well as association of ankyrin-B with the plasma membrane (He et al., 2013).

Alternative splicing adds a major source of functional diversity for vertebrate ankyrins (Cunha, Le Scouarnec, Schott, & Mohler, 2008; Cunha & Mohler, 2008; Hall & Bennett, 1987; Hopitzan, Baines, & Kordeli, 2006; Hopitzan, Baines, Ludosky, Recouvreur, & Kordeli, 2005, Lux et al., 1990; Otto et al., 1991; Peters et al., 1995). For example, an in-frame splice in the regulatory domain of ankyrin-R (band 2.2 in erythrocyte membranes) results in elimination of an acidic 186-residue segment and increased affinity for the anion exchanger as well as for spectrin (Davis et al., 1992; Hall & Bennett, 1987; Lux et al., 1990). Splicing within the regulatory domain of ankyrin-B regulates association with obscurin and the cochaperone Hsp40 (Cunha & Mohler, 2008). Ankyrin-G (and likely ankyrin-B) polypeptides include spliced variants lacking ANK repeats altogether (Hopitzan et al., 2005; Peters et al., 1995). These truncated polypeptides retain spectrin-binding domains and associate with intracellular organelles (Hoock, Peters, & Lux, 1997), although their functions are not known. The most extreme example of alternative splicing is small ankyrin1 that has lost both ANK repeats, as well as ZU5 and UPA

domains, and retains only a C-terminal domain (Ackermann et al., 2011; Zhou et al., 1997).

Interestingly, vertebrate ankyrins share the unique feature that their ANK repeats are encoded by exons that begin and end at the same amino acid residue within the repeat sequence (Cai & Zhang, 2006; Cunha et al., 2008). In principle, it is thus possible, to generate transcripts encoding different numbers and linear combinations of ANK repeats that would still fold into extended although shorter solenoids. These variants would be predicted to both lose interactions and potentially to gain new partners due to juxtaposition of otherwise separated ANK repeats. Alternatively spliced variants lacking internal ANK repeats have been reported for ankyrin-B (Cunha et al., 2008), although the functional properties of the predicted polypeptides remain to be evaluated. A challenge in understanding the full scope of ankyrin diversity due to alternative splicing will be to determine actual exon usage in full-length transcripts. New sequencing technologies may be required to achieve this goal, given the diversity, low abundance, and large sizes of transcripts (4–13 kB), combined with incomplete annotation (Cunha et al., 2008; Otto et al., 1991; Peters et al., 1995).

Both *ANK2* and *ANK*3 have a vertebrate-specific giant exon inserted at identical sites between their UPA and death domains that is subject to alternative splicing and is selectively expressed in the cells of the neuronal lineage (Fig. 1.3; Chan, Kordeli, & Bennett, 1993; Kordeli et al., 1995; Kunimoto, 1995; Kunimoto, Otto, & Bennett, 1991). This giant exon encodes 2085 amino acids in ankyrin-B and 2608 residues in ankyrin-G. The inserted exon of ankyrin-G also includes an N-terminal 40 kDa region absent from ankyrin-B that is enriched in serine and threonine residues and is modified by O-GlucNac monosaccharide residues (Vosseller et al., 2006; Zhang & Bennett, 1996). Ankyrin-G isoforms also include a 270 kDa polypeptide missing the C-terminal portion of this exon due to in-frame splicing (Kordeli et al., 1995). Acquisition of the giant exon likely occurred in the precursor to *ANK2* and *ANK3* genes prior to the second whole genome duplication event based on sequence similarity of inserted domains of ankyrin-B and ankyrin-G and precise maintenance of the insertion sites in the two genes.

480/270 kDa ankyrin-G and 440 kDa ankyrin-B polypeptides bearing giant exons are localized in axons (Chan et al., 1993; Kordeli et al., 1995; Kunimoto, 1995; Kunimoto et al., 1991). 440 kDa ankyrin-B is the predominant isoform in the neonatal brain prior to myelination but is largely

replaced by 220 kDa ankyrin-B in adult rodents (Chan et al., 1993; Kunimoto, 1995). 480 and 270 kDa ankyrin-G variants containing the axonal exon are targeted to axon initial segments as well as nodes of Ranvier, although their specialized functions remain to be established (Kordeli et al., 1995; Zhang & Bennett, 1998). Interestingly, the closest mammalian matches to the giant axonal exons in Blast searches are proteins related to Titin, which have multiple Ig- and Fn3-like domains and perform mechanical roles in stabilizing sarcomeres of striated muscle. The functional importance of this domain is underscored by a report of a truncating mutation within the axonal exon of ankyrin-G that results in cognitive and behavioral deficits in humans (Iqbal et al., 2013).

Phenotypes of mice with knockout or deficiency of *ANK1*, *2*, and *3* indicate that the three vertebrate ankyrin genes have acquired distinct and nonoverlapping functions (Abdi et al., 2006; He et al., 2013; Mohler et al., 2002). For example, mice lacking ankyrin-R due to an ENU-induced null mutation in the *ANK1* gene die perinatally due to profound anemia and loss of the spectrin-based membrane skeleton (Rank et al., 2009). Ankyrin-G and ankyrin-B are coexpressed in neurons and striated muscle, where they have nonredundant functions, but also can collaborate in activities such as maintenance of costamere structure and organization of the axonal spectrin–actin skeleton (Ayalon et al., 2008; Galiano et al., 2012).

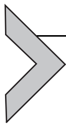
Alpha- and beta-spectrin genes have duplicated in parallel with the ankyrins (Fig. 1.4). Vertebrates, with the exception of mammals, have a single alpha-spectrin gene, *SPTAN1*, which is the generally expressed partner of beta-spectrins (Bennett et al., 1982, Davis & Bennett, 1983; Wasenius et al., 1989). In addition, mammals express a second alpha-spectrin gene, *SPTA1*, that is expressed primarily in erythrocytes and has reduced ability to assemble into tetramers (Mehboob et al., 2010; Salomao et al., 2006). Vertebrates, except for mammals, have three beta-spectrins genes: *SPTB*, encoding beta-1 spectrin, first characterized in red blood cells (Winkelmann, Chang, et al., 1990); *SPTBN1*, encoding beta-2 spectrin, first characterized in the brain (Bennett et al., 1982; Hu, Watanabe, & Bennett, 1992); and *SPTBN4,* encoding beta-4 spectrin, that is localized with 480 kDa ankyrin-G at nodes of Ranvier and axon initial segments (Berghs et al., 2000), as well as at intercalated disks of cardiomyocytes (Hund et al., 2010).

Mammals have an additional beta-spectrin gene, *SPTBN2*, encoding beta-3 spectrin, that was first identified in the brain (Ohara, Ohara, Yamakawa, Nakajima, & Nakayama, 1998; Stankewich et al., 1998) where

it is most highly expressed in the cerebellum and is mutated in human spinocerebellar ataxia type 5 (Ikeda et al., 2006). Knockout of beta-3 spectrin in mice recapitulates human spinocerebellar ataxia symptoms and results in abnormal development of Purkinje cerebellar neurons (Gao et al., 2011; Perkins et al., 2010). In contrast, knockout of the generally expressed beta-2 spectrin markedly impairs development and is embryonic lethal (Tang et al., 2003).

While beta-1–3 spectrins are overall similar in sequence and domain organization, beta-4 spectrin has additional sequence between the final spectrin repeat and the PH domain (Berghs et al., 2000). Beta-4 spectrin also associates with calmodulin-dependent protein kinase 2 (CAM kinase 2) through this sequence and recruits CAM kinase 2 to cardiac intercalated disks and axon initial segments (Hund et al., 2010).

Alternative splicing increases the diversity of both alpha- (Zhang et al., 2010) and beta-spectrins (Berghs et al., 2000; Hayes et al., 2000; Winkelmann, Costa, et al., 1990). Variations include deletion of PH domains in beta-1 and beta-2 spectrins (Hayes et al., 2000; Winkelmann, Costa, et al., 1990) and deletion of either N-terminal or C-terminal portions of beta-4 spectrin (Berghs et al., 2000; Tse et al., 2001; Uemoto et al., 2007).



4. EVOLUTION OF SPECTRIN–ANKYRIN-BASED DOMAINS: LESSONS FROM THE AXON INITIAL SEGMENT

Axon initial segments with their dense clusters of ion channels, CAMs, and synaptic endings are responsible for both generation and modulation of action potentials (Chang & Rasband, 2013). This membrane domain and the closely related nodes of Ranvier of myelinated axons underlie the evolution of fast signaling in vertebrates and are the best understood ankyrin–spectrin-based membrane structures. Ankyrin-G is required for action potential initiation and for localization of all of the known initial segment components (Hedstrom et al., 2007; Jenkins & Bennett, 2001; Kordeli et al., 1995; Zhang & Bennett, 1998; Zhou et al., 1998). These ankyrin-G-dependent initial segment proteins include voltage-gated sodium channels (Hedstrom et al., 2007; Jenkins & Bennett, 2001), KCNQ2/3 channels that modulate sodium channel activity (Chung, Jan, & Jan, 2006; Cooper, 2011; Pan et al., 2006), 186 kDa neurofascin, a L1 CAM that directs GABAergic synapses to the initial segment (Ango et al., 2004; Jenkins & Bennett, 2001),

and beta-4 spectrin, which stabilizes initial segments (Komada & Soriano, 2002; Lacas-Gervais et al., 2004; Yang et al., 2007). Moreover, ankyrin-G is also required to form microtubule bundles at the initial segment (Sobotzik et al., 2009). Consistent with the findings that multiple initial segment proteins depend on ankyrin-G, ankyrin-G-null axons acquire properties of dendrites both in cultured neurons and in mice (Hedstrom et al., 2008; Sobotzik et al., 2009).

The phylogenetic record of evolution of ankyrin-binding motifs in L1 CAMs and in ion channels provides a series of molecular “snapshots” of the events that ultimately resulted in emergence of axon initial segments and fast signaling in vertebrates. The L1 family of CAMs was the first among initial segment components to acquire ankyrin-binding activity. L1 CAM family members are expressed throughout modern bilaterian organisms and likely had evolved over 550 mybp in the Ediacaran era proceeding the Cambrian period. L1 CAMs are represented in *C. elegans* by LAD1, encoded by the *Sax*-7 gene, and in *Drosophila* by neuroglian (Chen, Ong, & Bennett, 2001; Hortsch, Nagaraj, & Godenschwege, 2009). L1 CAMs all have a highly conserved ankyrin-binding motif, including the residues FIGQY (Chen et al., 2001; Garver, Ren, Tuvia, & Bennett, 1997; Zhang et al., 1998). Phosphorylation of the FIGQY tyrosine eliminates ankyrin binding, which is promoted in *C. elegans* by FGF receptor signaling (Chen et al., 2001; Garver et al., 1997). The *C. elegans* L1 CAM is localized with ankyrin (Unc44) at sites of cell–cell contact in multiple cell types (Chen et al., 2001). The adhesive functions of the *C. elegans* L1 CAM include correct positioning of neuronal cell bodies and axons, and the FIGQY ankyrin-binding motif is both necessary and sufficient for these activities (Pocock, Bénard, Shapiro, & Hobert, 2008). The *Drosophila* L1 CAM, neuroglian, has roles in coordinating synaptic connections and again these functions require ankyrin-binding activity (Enneking et al., 2013; Godenschwege, Kristiansen, Uthaman, Hortsch, & Murphey, 2006; Hortsch et al., 2009).

Voltage-gated sodium channels acquired an ankyrin-binding motif following evolution of the L1 CAM site, and this occurred early in chordate evolution, likely in the Cambrian period between 550 and 500 mybp (Hill et al., 2008). KCNQ2/3 channels were the next initial segment channel to gain ankyrin-binding activity, which occurred in jawed fish at the end of the Ordovician period around 450 mybp (Hill et al., 2008). Cooper and colleagues have reported an extensive phylogenetic analysis of ankyrin-binding motifs of both the voltage-gated sodium

channels and KCNQ2/3 channels (Hill et al., 2008). The most distant organism with a sodium channel containing a recognizable ankyrin-binding motif was Amphioxus, which is a cephalochordate (Hill et al., 2008). Interestingly, even though the ankyrin-binding motifs of KCNQ2/3 and voltage-gated sodium channels are similar, these sequences most likely evolved independently (Hill et al., 2008).

The sequential and independent evolution of ankyrin-binding activities beginning with the L1 CAMs suggests a scenario where a proto-axon initial segment containing ankyrin and an L1 CAM evolved first and then was populated by ion channels, first the voltage-gated sodium channel and later by KCNQ2/3 channels. The selective pressure presumably was the advantages of faster signaling and its regulation. Higher concentration of sodium channels would have resulted in increased current density and greater amplitudes of depolarization that eventually became self-renewing action potentials. Evolutionary selection likely acted on ankyrin in parallel with its binding partners and thus development of the axon initial segment was an iterative as well as a sequential process. For example, urochordates have sodium channels with an ankyrin-binding motif but a single ankyrin gene that lacked the axonal-specific exons acquired later in vertebrate giant ankyrins. The giant exon of vertebrate 480 kDa ankyrin-G thus evolved in the context of axonal expression and clustering of sodium channels and now likely has axon initial segment-specific capabilities.

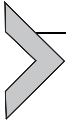
The axon initial segment with its clustered sodium channels likely was present in jawless fish, now represented by lampreys, and thus predated myelination and development of nodes of Ranvier, which appeared only in jawed fish (Hill et al., 2008). Interestingly, even though there are many parallels between the composition of nodes of Ranvier and initial segments (Davis, Lambert, & Bennett, 1996), these domains exhibit distinct mechanisms of assembly (Dzhashiashvili et al., 2007; Susuki et al., 2013). Thus, the basic ankyrin interactome of the axon initial segment was co-opted and modified during the process of myelination, resulting in closely related excitatory domains at nodes of Ranvier. A similar process of co-option likely resulted in utilization of ankyrin voltage-gated sodium channel interaction in intercalated disks and T-tubules in mammalian cardiomyocytes, where Nav1.5 requires ankyrin-binding activity for cell surface expression (Lowe et al., 2008; Mohler, Rivolta, et al., 2004). In this example of the heart, an ankyrin–sodium channel coupling occurs in T-tubules that lack cell–cell interactions and are present only in mammals. Thus, the initial connection of ankyrin with L1 CAMs that occurred in ancient axons and may

have facilitated interaction with sodium channels no longer is required for ankyrin partners in the heart and possibly other tissues.

More generally, CAMs are the earliest examples of membrane-spanning proteins with ankyrin-binding motifs (Table 1.1). Dystroglycan has an ankyrin-binding site that retains critical residues in cnidarians, while the cadherin ankyrin-binding site is present in the urochordate *Ciona intestinalis* (Jenkins et al., 2013). In contrast, ion channel binding sites have continued to evolve in terrestrial vertebrates, where the RhB/G ammonium transporter has an ankyrin-binding motif present in mammals but is not conserved at critical residues in chickens (Lopez et al., 2005; Table 1.1). These considerations suggest a model for ankyrin-based membrane domains where the initiating event was the formation of a proto-domain through interaction of membrane-associated ankyrin and spectrin with membrane-spanning CAMs that in turn engaged in transcellular and cell–matrix interactions. These proto-domains then became populated with ion channels and likely other molecules that developed ankyrin-binding activity at sites determined by extracellular signals with selective pressure provided by optimization of physiological function.

We have focused on axon initial segments where the physiological adaptation would be efficient generation of action potentials and fast signaling. Ankyrin, along with L1 CAMs and cadherins, also is localized at sites of cell–cell contact in other cell types including epithelial tissues (Chen et al., 2001). Thus, it is conceivable that epithelial lateral membranes served as an "incubator" similar to the axon initial segment, where membrane transporters could have acquired ankyrin-binding activity with the advantage of increased efficiency through polarized fluxes of ions and nutrients. An apparent exception to the initiating role of CAMs in this scheme is the circulating erythrocyte, which contains only ion transporters as ankyrin-binding partners and does not engage in interactions with other cells. However, it is possible that an ankyrin–anion exchanger interaction evolved first in an epithelial cell type and later was co-opted in erythrocytes.

Ankyrins, with exception of the giant axonal variants, have remained relatively unchanged while their partners have evolved ankyrin-binding activity. An essential feature of this model is the ease of evolution of ankyrin-binding motifs, which require no folded structure and only 10–20 amino acids. It will be of interest in the future to understand the structural basis for ANK repeat association with peptides and to use this information to systematically identify potential partners through bioinformatics.



5. FUNCTIONS OF SPECTRIN AND ANKYRIN IN POLARIZED ORGANELLE TRANSPORT

An implication of the proposed role of CAMs in recruitment of a preexisting ankyrin–spectrin skeleton is that ankyrin and spectrin may have other functions that occur in parallel and/or predated their association with the plasma membrane. In fact, multiple lines of evidence support roles of ankyrin and spectrin in polarized organelle transport. Brain spectrin was initially characterized by Willard and colleagues based on its association with multiple classes of axonally transported organelles (Levine & Willard, 1981). The kinesin KIF3 associates with alpha-spectrin through its light chain Kap3, and this interaction was implicated in organelle transport required for neurite extension (Takeda et al., 2000). Actin-related protein 1 (Arp1) of the dynactin complex, which interacts with both dynein and kinesin motors, associates with beta-spectrin through its calponin homology domains (Holleran et al., 2001, Holleran, Tokito, Karki, & Holzbaur, 1996). Arp1 shares sequence and folding similarity to actin, and calponin homology domains of beta-spectrin likely interact with residues conserved between these proteins. Interestingly, overexpression of beta-3 spectrin bearing mutations that cause spinocerebellar ataxia type 5 impairs axonal transport in *Drosophila* larvae (Lorenzo et al., 2010). Moreover, beta-spectrin together with dynactin and dynein is sufficient to reconstitute motility of liposomes lacking membrane proteins (Muresan et al., 2001). In addition to axonal transport, *Drosophila* spectrin, ankyrin, adducin, and tropomodulin are all components of an intracellular microtubule-based structure termed the fusome, which delivers organelles and RNAs from nurse cells to oocytes (de Cuevas, Lee, & Spradling, 1996; Lighthouse, Buszczak, & Spradling, 2008; Lin, Yue, & Spradling, 1994).

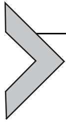
Ankyrin in *C. elegans* and *Drosophila* determines polarized organelle transport in dendrites and axons through organization of microtubules (Koch et al., 2008; Maniar et al., 2011; Pielage et al., 2008). A dual role of invertebrate ankyrins in both cell surface and intracellular functions appears to have largely been subdivided between ankyrin-B and ankyrin-G vertebrates. Ankyrin-G primarily is localized to plasma membrane domains, while ankyrin-B associates with intracellular membranes despite a high level of sequence similarity (He et al., 2013). This behavior results from loss of plasma membrane activity of ankyrin-B due to an ankyrin-B-specific linker peptide connecting the ankyrin repeat domain to the ZU52–UPA module

that inhibits binding of ankyrin-B to membrane protein partners E-cadherin and neurofascin and prevents association of ankyrin-B with epithelial lateral membranes as well as the axon initial segment. The residues of the ankyrin-B linker required for autoinhibition are encoded by a small exon that is highly divergent between ankyrin family members but conserved in the ankyrin-B lineage. These considerations argue against neofunctionalization of ankyrin-B and rather support partition of the dual functions of the ancestral single ankyrin following gene duplication in the vertebrate lineage (He et al., 2013).

Further support for partition of plasma membrane and intracellular functions between ankyrin-B and ankyrin-G comes from the coordinated but distinct role of these proteins in organization of dystrophin and dystroglycan at costameres of striated muscle (Ayalon et al., 2008, 2011). Ankyrin-G binds directly to dystroglycan and dystrophin and stabilizes these proteins at costameres, but is not required for their accumulation at the plasma membrane. Ankyrin-B, in contrast, does not bind directly to dystroglycan, but is required for transport of dystroglycan from an intracellular compartment to the plasma membrane, perhaps through association with dystrophin. Ankyrin-B also directs a specialized population of microtubules to costameres through binding to p62/dynactin-4 subunit of the dynactin complex (Ayalon et al., 2008, 2011). Interestingly, p62/dynactin-4 and associated proteins at the minus end of the dynactin protofilament are proposed to couple the dynactin complex to membrane cargo (Yeh, Quintyne, Scipioni, Eckley, & Schroer, 2012). Dynactin engages both kinesin and dynein motors in transport of membrane cargo in axons and likely other examples of directed organelle movement (Schroer, 2004). It will be of interest to determine if ankyrin-B plays a general role in linking organelles to the dynactin complex and the evolutionary origins of this function.

Ankyrin-B also interacts with members of the family of Eps15 homology (EH) domain containing (EHD)/receptor-mediated endocytosis (Rme) proteins, which are ATPases related to dynamin that are involved in endosomal recycling (Daumke et al., 2007; Gudmundsson et al., 2010). EHD/Rme1–4 proteins exhibit increased cardiac expression in ankyrin-B haploinsufficient mice and in acquired ankyrin-B-deficiency that occurs in heart failure (Gudmundsson et al., 2010, 2012). These observations suggest that ankyrin-B, which has been viewed as a T-tubule-associated structural protein that stabilizes the Na/Ca exchanger and Na/K ATPase (Mohler et al., 2005), may also have a dynamic role in directing intracellular trafficking of these transporters. Live cell imaging of ankyrin-B, ankyrin-B

membrane partners, and EHD/Rme proteins has not yet been reported but could give insight into the nature or their interaction.



6. SUMMARY AND PERSPECTIVES

This chapter provides a plausible scenario for evolution of ankyrin- and spectrin-based plasma membrane domains in vertebrates. The analysis integrates recent phylogenetic information with extensive functional analyses of spectrin and ankyrin beginning in human erythrocytes and more recently in excitable membranes in neurons and heart, lateral membrane domains of epithelial cells, and costameres of striated muscle. A membrane-associated spectrin–actin network likely was the starting point in early metazoans. Alpha-spectrin, and a beta-spectrin with the same length as modern beta-spectrins with calponin homology domains, and a PH domain are present in sponges and Placozoa. These spectrin subunits presumably are capable of forming tetramers, binding to phosphoinositide lipids, and of forming a network through association with actin, but lack an ankyrin-binding site, and precede appearance of ankyrin. In addition, a large beta-related spectrin lacking ankyrin-binding activity, termed beta-H, also was present and has a homologue in vertebrates. Both beta- and beta-H-spectrins also likely functioned in polarized organelle transport and this activity either preceded or has evolved in parallel with their association with plasma membranes.

The potential of spectrin–actin networks to recruit and coordinate membrane-spanning proteins was greatly amplified in cnidarians by the emergence of a partnership with ankyrins with their highly versatile ANK repeat domain configured as a solenoid with an extended peptide-binding groove (Fig. 1.5). The most ancient currently known membrane proteins with ankyrin-binding peptide motifs engage in cell–cell and cell–matrix interactions. These ankyrin partners include dystroglycan, present in cnidarians; the L1 family of CAMs, present in bilaterians; and cadherins, with an ankyrin-binding motif, evident in early chordates. Functions of these early plasma membrane-associated ankyrin–spectrin–actin networks likely included mechanical support for the membrane bilayer, an activity still evident in mammalian erythrocytes (Eber & Lux, 2004) and in *C. elegans* axons (Hammarlund et al., 2007).

The next phase in evolution of functional membrane domains was the independent acquisition of ankyrin-binding activity by diverse membrane transporters. This process was greatly facilitated by the ability of ankyrin

to associate with unstructured peptides. The vertebrate axon initial segment with its ankyrin-G-dependent characteristics and clear physiological benefit of fast signaling with small diameter axons provides an instructive case history. Ankyrin is associated with L1 CAMs in axons of *C. elegans* and *Drosophila*, which either lack voltage-gated sodium channels altogether (*C. elegans*) or have sodium channels lacking an ankyrin-binding motif (*Drosophila*) (Hill et al., 2008). Sodium channels developed a recognizable ankyrin-binding motif in early chordates and were followed by KCNQ2/3 channels, which independently acquired their ankyrin-binding motif nearly 100 million years later, during the emergence of jawed fish (Hill et al., 2008). The principal ion channels clustered at vertebrate axon initial segments thus evolved ankyrin-binding activity sequentially and independently through rather modest changes over a short stretch of protein sequence. This adaptive process presumably was favored by selective advantages of clustering sodium channels to generate action potentials and modulating their excitability by KCNQ2/3 channels. We speculate that other proto-domains based on spectrin–ankyrin–CAM assemblies served a similar role as "incubators," where ion transporters and likely other yet to be identified membrane-spanning proteins acquired ankyrin-binding activity through convergent evolution. One candidate incubator is the lateral membrane domain of epithelial cells, where ankyrin and L1 CAMs are colocalized in *C. elegans* and *Drosophila*. Here, a selective advantage favoring gain of ankyrin-binding activity could have arisen from increased efficiency due to vectorial transport of ions and nutrients.

Two whole genome duplication events in the vertebrate lineage have markedly expanded the repertoire of ankyrin–spectrin assemblies and their membrane-spanning partners (Figs. 1.4 and 1.5). Vertebrate spectrins and ankyrins have retained core features including ANK repeats that are highly conserved among bilaterians. However, ankyrins in particular have diversified in other respects due to gain of protein interactions of regulatory domains (both intramolecular and intermolecular), gain of large neuronal-specific exons, and through alternative splicing. The scope of ankyrin partners and their membrane domains in vertebrates as well as other organisms thus likely is quite broad and only partially appreciated at this point.

A recurrent theme in this discussion is the acquisition of new protein function through mutation for ankyrin partners and ankyrin regulatory domains or insertion of a new exon in the case of the giant axonal ankyrins. The role of adaptive evolution of protein structure has been the subject of active debate among evolutionary biologists. The evolutionary

developmental (evo devo) school favors the idea that the principal origin of variation lies in cis elements of regulatory DNA that determine levels of protein expression through regulatory networks, while the proteins themselves are essentially unchanged (Carroll, 2008). This view has been countered by arguments citing examples of positive functional consequences of variation in protein sequences (Hoekstra & Coyne, 2007; Linnen et al., 2013; Nery, González, & Opazo, 2013). A major concern has been that variation in protein sequence may lead to misfolding and is highly selected against due to pleiotropic expression of most genes. However, intrinsically unstructured protein sequences, which provide ankyrin-binding sites for membrane-spanning proteins as well as interactions of ankyrin regulatory domains, are relatively tolerant of mutation since they have no folded structure. Another general concern is the challenge in establishing a direct causal connection between evolutionary variation in either protein or cis-regulatory DNA sequences and actual morphological phenotype. However, a connection between protein and phenotype is clear in the example of the vertebrate axon initial segment, which depends on ankyrin-G for its defining characteristics both in cultured neurons and in animals.

Finally, it is important to not underestimate the creativity of adaptive evolution in response to selective pressures. Striking examples of this are the morphologically similar versions of striated muscle that evolved independently in cnidarians and bilaterians (Steinmetz et al., 2012). Cnidarian and bilaterian muscles both were based on the same ancient motility proteins and solved similar problems of generating coordinated contractile force but arrived at solutions with distinct molecular organization and composition. Extrapolation of this lesson to plasma membrane domains suggests that it is likely that there also are multiple independently evolved approaches, many utilizing spectrin and ankyrin, to address the core functional problem of establishing long-range organization and mechanical stability in a fluid membrane bilayer.

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