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DEFECTS IN ANKYRIN-BASED CELLULAR PATHWAYS IN METAZOAN PHYSIOLOGY

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TABLE OF CONTENTS

1. ABSTRACT

Ankyrins are a ubiquitously expressed family of membrane-adaptor proteins found in most vertebrate tissues. Since the first ankyrin polypeptide was identified over 25 years ago (1), studies in humans, mice, and lower organisms have implicated critical roles for ankyrins in normal metazoan physiology. This review will provide an overview of the ankyrin family and highlight seminal findings in the field which have linked dysfunction in ankyrin-based pathways with defects in metazoan physiology and human disease.

2. ANKYRINS

Mammalian ankyrin polypeptides are derived from three ankyrin genes including ankyrin-R (R for restricted, *ANK1*, human chromosome 8p11), ankyrin-B (B for broad, *ANK2*, human chromosome 4q25-27), and ankyrin-G (G for giant or general, *ANK3*, human chromosome 10q21). Ankyrins are also found within other metazoan genomes including one ankyrin in *Caenorhabditis elegans* (*unc-44*) and two ankyrin genes in *Drosophila* (*Dank1, Dank2*). Ankyrin genes are not present in genomes of lower organisms including yeast (*Saccharomyces cerevisiae, Schizosaccharomyces pombe*), *Arabidopsis thaliana,* and *Zea Mays*. These observations indicate that ankyrins have evolved to perform specialized functions in multi-cellular organisms.

2.1. Ankyrin Domains

Canonical ankyrins have four major domains including an NH_2-terminal membrane-binding domain, a 62 kDa spectrin-binding domain, a death domain, and a C-terminal regulatory domain (Figure 1). The NH_2-terminal membrane-binding domain (MBD) is comprised of twenty-four consecutive *ANK* repeats. *ANK* repeats are 33 amino acid protein interaction motifs found in hundreds of human proteins including p53BP2, TRP calcium channels, cardiac *ANK* repeat protein (CARP), and the ARF GTPase-

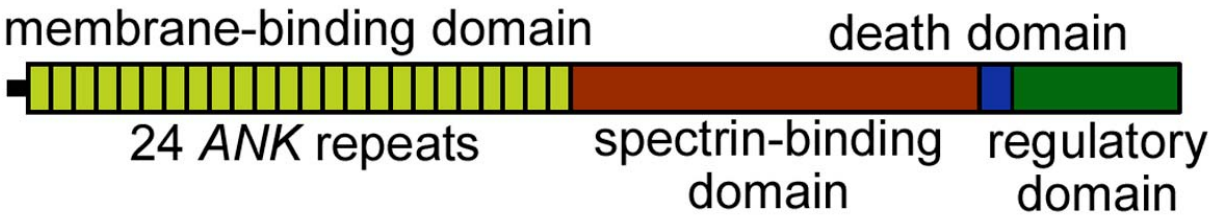

Figure 1. Canonical ankyrin domain organization. Ankyrins have a large membrane-binding domain comprised of 24 *ANK* repeats (yellow boxes), a spectrin-binding domain (red), a death domain of unknown function (blue), and a C-terminal regulatory domain (green).

ctivating protein GIT1. *ANK* repeats form stacks of anti-parallel alpha-helices linked by loop motifs arranged perpendicular to the alpha-helices (2).

The membrane-binding domain is the site for ankyrin interactions with multiple membrane proteins (*discussed in Section 2.3*). The ankyrin membrane-binding domain is multivalent with respect to protein partners first evidenced by the finding of two distinct binding sites on the ankyrin-R membrane-binding domain for the anion exchanger (3, 4). Ankyrins may also form hetero-complexes with binding partners as evidenced by the ability of ankyrin-R membrane-binding domain to simultaneously associate with the L1 cell adhesion molecule (L1CAM) neurofascin and the anion exchanger (3-6). The multivalent properties of the ankyrin membrane-binding domain give ankyrins the ability to form large homomeric or heteromeric protein complexes *in vivo*.

The large 62 kDa central domain (spectrin-binding domain) of ankyrins is critical for spectrin-binding activity *in vitro* and *in vivo* (7, 8). Ankyrin spectrin-binding activity is localized near the NH_2-terminal region of the 62 kDa domain, and we recently mapped the critical surface residues required for ankyrin-B/beta 2-spectrin interactions *in vivo* (9). Additional roles for the spectrin-binding domain are an important future area of interest as recent findings indicate a critical role for the C-terminal residues of this domain for ankyrin function *in vivo* (see Section 3.4).

Canonical ankyrins express a ~90 amino acid death domain. This domain in other proteins is hypothesized to play an important role in the cell death pathway and is found in a number of proteins in the apoptotic pathway including the p75 neurotrophin receptor and Fas (Apo-1). The role of the ankyrin death domain is currently unknown. However, the death domain of ankyrin-G was recently shown to interact with the death domain of Fas to promote renal tubule cell death (10). Death domains have also been implicated in low affinity homo- or heteromeric interactions with other death domains (11). Therefore, in an appropriate cellular context (high ankyrin concentrations), ankyrin death domains may mediate the formation of ankyrin homo- or hetero-dimers.

Ankyrin polypeptides have a C-terminal regulatory domain. This domain is the most divergent domain between ankyrins-R, -B, and –G, and has been shown to play important roles for regulating specific ankyrin gene product functions in cells. For example, an ankyrin-R variant (protein 2.2) lacks 161 amino acids in the regulatory domain and displays increased binding activity for ankyrin-partners spectrin and the anion exchanger (12, 13). These 161 residues associate with ankyrin-R, but not with purified ankyrin-R membrane-binding or spectrin-binding domains alone. Additionally, the 161 residues reverse the increased binding affinity of protein 2.2 for the anion exchanger (13). These data provide evidence for potential intramolecular interactions of the ankyrin-R C-terminus with sites spanning the membrane-binding domain and spectrin-binding domains to allosterically repress binding of ankyrin to protein partners. Additional evidence for a role for the C-terminal regulatory domain in ankyrin-specific function comes from studies using ankyrin-B/-G chimeras to rescue abnormal localization of $InsP_3$ receptor in neonatal cardiomyocytes from ankyrin-$B^{-/-}$ mice (14). Expression of GFP 220 kDa-ankyrin-B, but not GFP-190 kDa ankyrin-G, rescues abnormal ankyrin-$B^{-/-}$ cardiomyocyte phenotypes (14, 15). However, an ankyrin-B mutant lacking the regulatory domain, or an ankyrin chimera of the ankyrin-B membrane- and spectrin-binding domains fused with the ankyrin-G C-terminal domain is ineffective in restoring normal cardiomyocyte phenotypes (14). These results in ankyrin-$B^{-/-}$ cardiomyocytes strongly support a key role for the ankyrin C-terminal regulatory domain in providing specificity for ankyrin function *in vivo*. Further evidence for the role of the C-terminal regulatory domain in ankyrin-B function will be discussed in section 3.4.

2.2. Ankyrin polypeptides

Canonical ankyrins are approximately 190-220 kDa in size with standard membrane-binding, spectrin-binding, death, and regulatory domains (see above). However, numerous gene splicing events within the membrane-binding, spectrin-binding, and C-terminal regulatory domains produce a diverse set of functionally unique ankyrin polypeptides ranging from 26 kDa to 480 kDa that are expressed in tissue- and developmental-specific fashion. For example, 440 kDa ankyrin-B and 480 kDa ankyrin-G polypeptides are produced by insertion of a

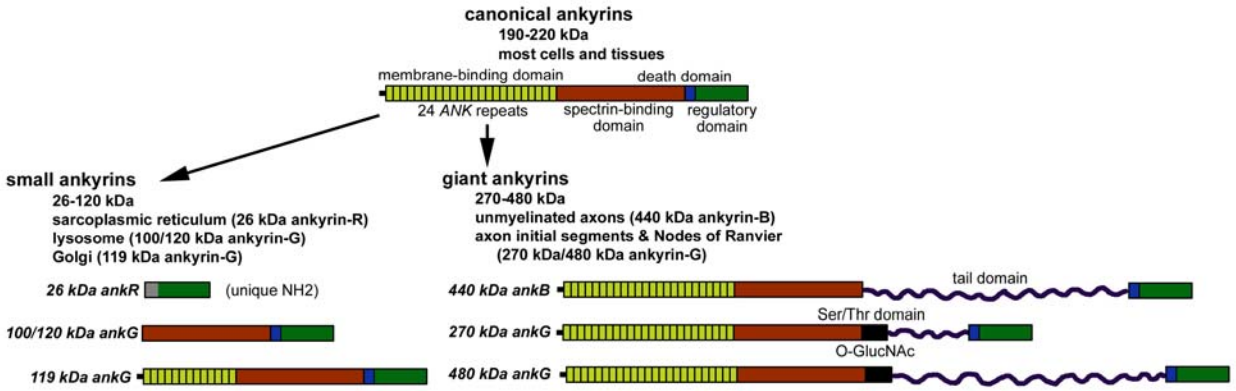

Figure 2. Alternative splicing of ankyrin genes produce a number of ankyrin polypeptides with diverse localizations and functions.

single exon encoding a 220 kDa random coil between the spectrin-binding domain and the death domain (Figure 2, (5)). Moreover, 480 kDa and 270 kDa ankyrin-G also express a 40 kDa serine/threonine-rich domain. These large ankyrins have specialized functions at specific sites in the nervous system including targeting of proteins to nodes of Ranvier and axon initial segments (ankyrin-G polypeptides) and in unmyelinated axons (440 kDa ankyrin-B) (5, 6). Additionally, alternative splicing of ankyrin genes may produce small ankyrin polypeptides including 26 kDa ankyrin-R (16), 119 kDa ankyrin-G (17), and 100/120 kDa ankyrin-G (18) that are localized to a variety of intracellular membrane compartments (Figure 2).

2.3. Ankyrin-associated proteins

Ankyrins interact with beta-spectrin isoforms and directly bind and co-localize with plasma membrane ion channels and transporters, intracellular calcium-release channels, cell adhesion molecules, as well as a number of cytosolic proteins (Table 1). Ankyrin polypeptides interact with structurally diverse plasma membrane ion channels and transporters including anion exchanger (7, 19-21), Na/K ATPase (22-24), Na/Ca exchanger (25), voltage-gated Na^+ channels (26, 27), the ammonium transporter (RhBG, (28)), and H^+/K^+ ATPase (29). Ankyrins also interact with calcium-induced calcium-release channels including the inositol 1,4,5 trisphosphate ($InsP_3$) receptor and ryanodine receptor (RyR) (30) (31, 32). Two families of cell adhesion molecules bind ankyrins including CD44 polypeptides (33, 34) and L1CAMs (35-38). Most ankyrin-associated membrane proteins bind to the ankyrin membrane-binding domain. Exceptions are H^+/K^+ ATPase and Na/K ATPase which require additional binding sites on the ankyrin spectrin-binding domain (29, 39). Ankyrin polypeptides have been predicted to associate with a variety of diverse cytosolic proteins including tubulin (40-42), clathrin (43), obscurin (44, 45), the molecular co-chaperone Hdj1/Hsp40, and the Rho-GEF Tiam-1 (46). The physiological relevance of a number of these ankyrin interactions remain to be evaluated *in vivo*.

3. ANKYRIN-DYSFUNCTION AND ABNORMAL PHYSIOLOGY

The importance of ankyrins in vertebrate physiology has been illuminated by human disease and ankyrin-deficient organisms. The following section will detail specific experimental findings which have clearly defined key roles for ankyrins in the establishment and maintenance of specialized membrane domains for metazoan physiology.

3.1. Unc-44

Mutations to the *Caenorhabditis elegans unc-44* gene are associated with defective nematode axonal guidance (47-49). The normal nematode postdeirid axon extends from the lateral surface to the ventral nerve cord (47). In contrast, *unc-44* mutants display random outgrowth of the postdeirid axon which then contacts with the inappropriate binding partners (47). In 1995, Otsuka and colleagues cloned the *unc-44* gene and identified the cDNA as an orthologue of human ankyrin (50). Since this initial discovery, additional mutations in the *Caenorhabditis elegans* ankyrin-pathway have been shown to be linked with abnormal nematode physiology. Expression of dominant-negative forms of LAD-1 (for L1-like adhesion), the sole *Caenorhabditis elegans* L1CAM homologue (51), leads to improper germline and early embryo development as well as abnormal embryonic and gonadal morphogenesis. Together, these results implicate key roles for ankyrins and ankyrin-based pathways in normal metazoan physiology.

3.2. Ankyrin-R

Mutations in ankyrin-R lead to hemolytic anemia in humans and mice. The erythrocyte membrane is comprised of a spectrin-based lattice that links the actin-based cytoskeleton to membrane proteins including the anion exchanger via ankyrin-R. A spontaneous mutation in mice (*nb/nb*) leads to hemolytic anemia. Using a combination of genetics and biochemistry, Barker and colleagues showed that the *nb/nb* mutation produced severe hemolytic anemia due to truncations in ankyrin-R leading nearly to a complete loss of 210 kDa ankyrin-R expression

Table 1. Ankyrin-associated proteins

Protein	Ankyrin	Ankyrin-domain	Primary Reference(s)
Beta-spectrin	R, B, G	SBD	7
Anion Exchanger (AE1, AE2, AE3)	R	MBD	7, 19-21
Na/K ATPase	B, G	MBD,SBD	22-24
Na/Ca exchanger	R, B	MBD	25
NaCh	G	MBD, SBD	26, 27
InsP3 receptor	B	MBD	31, 32
Ryanodine receptor	B	MBD	30
CD44	R	MBD	33, 34
L1CAMs	R, B, G	MBD	35-38
Tubulin	R, B	MBD	40-42
Clathrin	R	MBD	43
H/K ATPase	G	MBD, SBD	29
Tiam-1	R, G	MBD	46
Hdj1/Hsp40	B	R-domain	71
Fas	G	Death	10
Sigma Receptors	B	?	76
Obscurin	R	R-domain	44, 45

(52). Interestingly, *nb/nb* mice also display degeneration of Purkinje cell neurons and cerebellar psychomotor defects (53). 210 kDa ankyrin-R is also lost in striated muscle in these animals. However a muscle phenotype in these animals has not been further explored.

Based in part on findings from the *nb/nb* mouse, Eber and colleagues identified defects in ankyrin-R expression as a primary cause of human hemolytic anemia (54). Loss or mutation of full-length ankyrin-R is associated with decreased spectrin and anion exchanger and instability of the erythrocyte membrane leading to spherocytosis and anemia. Currently, approximately 50% of Caucasian human hereditary spherocytosis cases are due to ankyrin-R mutations (55). The *nb/nb* mice predict potential cerebellar defects in humans with ankyrin-R dysfunction. However, to date there are no clear links between ankyrin-R deficiencies and human cerebellar phenotypes.

3.3. Ankyrin-G

Ankyrin-G polypeptides play critical roles in the development and maintenance of membrane domains in excitable cells including neurons and cardiomyocytes. A role for ankyrin-G in the maintenance of excitable membrane domains in the brain was predicted based on co-purification and co-localization of ankyrin-G with brain voltage-gated Na_v channels (56, 57) and co-localization of ankyrin-G with Na_v channel isoforms at the neuromuscular junction (58-60). Additionally, neurofascin, a member of the L1CAM family with ankyrin-binding activity, is co-localized with ankyrin-G at nodes of Ranvier in peripheral nerve and at Purkinje cell axon initial segments (56). The cerebellar-specific knock-out of ankyrin-G provided the key tool to study the role of ankyrin-G *in vivo* (61). Ankyrin-G mice display a number of phenotypes consistent with cerebellar dysfunction including decreased locomotion, abnormal gait, and significant tremor (61). At a molecular level, both Na_v channel isoforms and L1CAMs are not properly localized at critical membrane sites in the ankyrin-G mice leading to abnormalities in cerebellar Purkinje cell neuron action potentials (61, 62). Additionally, loss of ankyrin-G-based localization of the L1CAMs results in abnormal development of interneuron circuits (63). Specifically, in the absence of ankyrin-G, the normal gradient of 186 kDa neurofascin along the Purkinje axon initial segment-soma axis is lost (63). This loss results in abnormal directional basket axon growth and reduced GABAergic synapse formation (63). Therefore, ankyrin-G-dependent targeting in excitable cells in the brain is required for normal vertebrate nervous system function.

Ankyrin-G-dependent clustering of cardiac voltage-gated Na_v channel $Na_v1.5$ at excitable membrane domains in heart is required for normal human cardiac function. Recent findings implicate ankyrin-G in the targeting of $Na_v1.5$ to intercalated disc and T-tubule membranes in ventricular cardiomyocytes (64). As predicted from findings showing a role for ankyrin-G in Na_v isoform targeting to excitable membranes in brain, ankyrin-G co-immunoprecipitates with $Na_v1.5$ from detergent-soluble extracts from heart. Moreover, $Na_v1.5$ contains a nine amino acid sequence nearly identical to the ankyrin-binding motif identified in neuronal $Na_v1.2$ (65, 66). Ankyrin-G directly interacts with $Na_v1.5$ *in vitro* and this interaction is lost when the nine residue ankyrin-binding sequence of $Na_v1.5$ is removed (64). The potential importance of the ankyrin-G/$Na_v1.5$ interaction for vertebrate physiology was established by the identification of a mutation (E1053K) in the ankyrin-binding motif in a human patient with Brugada Syndrome (a cardiac arrhythmia associated with $Na_v1.5$ loss-of-function and sudden cardiac death) (64). This single mutation blocks $Na_v1.5$ association with ankyrin-G and $Na_v1.5$ E1053K (64). Strikingly, this single amino acid mutation blocks $Na_v1.5$ E1053K expression at the membrane surface of ventricular cardiomyocytes (64). Instead, loss of ankyrin-G binding results in $Na_v1.5$ E1053K accumulation in intracellular intermediates,

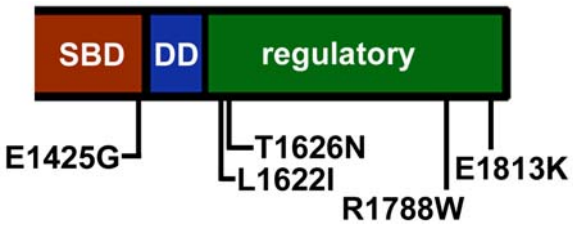

Figure 3. Spectrum of human mutations in *ANK2* spectrin-binding domain and C-terminal regulatory domain associated with ventricular arrhythmia and sudden cardiac death.

consistent with $Na_v1.5$ loss-of-function in the Brugada Syndrome patient (64). The cellular phenotype of E1053K is not due to mis-folded channel as expression of $Na_v1.5$ E1053K in cultured HEK293 cells results in normal channel expression at the plasma membrane. Interestingly, $Na_v1.5$ E1053K displays a lower threshold for voltage activation in HEK293 cells. Therefore, in addition to a role in $Na_v1.5$ cellular targeting, ankyrin-G may also gate $Na_v1.5$ at the cell surface. In summary, findings from brain and heart strongly support the requirement of an ankyrin-G-based cellular pathway for Na_v channel targeting to specialized membrane domains in excitable cells.

3.4 Ankyrin-B

Ankyrin-B is required for the establishment and maintenance of excitable domains in brain and striated muscle. A role for ankyrin-B in vertebrate physiology was first examined using mice homozygous for a null mutation in ankyrin-B (ankyrin-$B^{-/-}$ mice; lack 220 kDa and 440 kDa ankyrin-B (67)). Ankyrin-$B^{-/-}$ mice die at postnatal day 1-2. Loss of ankyrin-B in mice results in significant nervous system defects including hypoplasia of the corpus callosum and pyramidal tracts, dilation of lateral ventricles, and degeneration of long axon tracts (67). These phenotypes are associated with loss of L1CAMs throughout the brain, and strongly support a critical role for ankyrin-B in L1CAM organization in the brain as well as for normal vertebrate nervous system function.

Ankyrin-$B^{-/-}$ mice display a number of non-neurological defects. For example, ankyrin-B null mice display thymic atrophy which is associated with loss of $InsP_3$ receptor expression (15). Additionally, before death, ankyrin-$B^{-/-}$ mice display pronounced kyphosis and winged scapula (15), phenotypes present in humans with muscle disease. Moreover, skeletal muscle from ankyrin-$B^{-/-}$ mice displays occasional sarcomere disorganization, and ankyrin-$B^{-/-}$ mice display elevations in plasma creatine kinase levels indicating muscle damage (15). The molecular mechanism(s) underlying the thymus and musculoskeletal defects in ankyrin-$B^{-/-}$ mice are currently unresolved and will be a major goal of future research.

A major focus in ankyrin research for the past three years is the link between ankyrin-B dysfunction and cardiac arrhythmia. Similar to discoveries in the ankyrin-G field, breakthroughs for the role of ankyrin-B-based pathways in disease began in the mouse. Neonatal cardiomyocytes derived from both ankyrin-$B^{-/-}$ and mice heterozygous for a null mutation in ankyrin-B (ankyrin-$B^{+/-}$ mice) display abnormal calcium dynamics, spontaneous contraction rates, and abnormal expression and localization of ankyrin-associated membrane proteins including $InsP_3$ receptor, ryanodine receptor, Na/Ca exchanger, and Na/K ATPase (14, 68). Adult cardiomyocytes derived from ankyrin-$B^{+/-}$ heart display selective loss of ankyrin-B at T-tubule/sarcoplasmic reticulum membrane domains. This loss of ankyrin-B is paralleled by loss of Na/Ca exchanger, Na/K ATPase, and $InsP_3$ receptor from these same sites (68). Abnormal expression of these functionally-related ion channels and transporters leads to elevations in sarcoplasmic reticulum calcium load and sarcoplasmic reticulum Ca^{2+} transients in ankyrin-$B^{+/-}$ cardiomyocytes (68). Moreover, ankyrin-$B^{+/-}$ cells are susceptible to catecholaminergic-induced extrasystoles (68). Conscious ankyrin-$B^{+/-}$ mice display bradycardia, variable heart rate, and stress- and exercise-induced polymorphic ventricular arrhythmia and death (68).

A seminal finding in this field was the identification of a single ankyrin-B variant E1425G in a large French kindred with an atypical cardiac arrhythmia characterized by bradycardia, variable heart rate, and stress-induced sudden cardiac death (68, 69). When analyzed in a cardiomyocyte functional assay, this variant proved to be an ankyrin-B loss-of-function mutation (68). Therefore, cardiac ankyrin-dysfunction leads to cardiac arrhythmia and sudden cardiac death. These findings identified a new paradigm for cardiac arrhythmia based on mutations involved in the expression/localization of cardiac ion channels and transporters. The E1425G mutation is localized to the C-terminus of the spectrin-binding domain (Figure 3). A role for this region of ankyrin-B was unexpected and is an active focus of research. Since the initial discovery of the E1425G mutation, four additional loss-of-function mutations (L1622I, T1626N, R1788W, E1813K) have been associated with human polymorphic ventricular tachycardia (70). All of these mutations are found in the ankyrin-B C-terminal regulatory domain (Figure 3). One of these mutants (R1788W) blocks ankyrin-B interaction with the molecular co-chaperone Hdj1/Hsp40 (71). These mutations may affect ankyrin inter- or intra-molecular interactions, and will form the basis for future investigation.

4. CONCLUSIONS/PERSPECTIVE

Ankyrins play key roles in the development and maintenance of membrane domains in erythrocytes, kidney, brain, and heart. Animal models have proved a valuable resource to study the role of ankyrins in vertebrate physiology and have lead to major breakthroughs for understanding the pathophysiology of human diseases including hemolytic anemia and inherited cardiac arrhythmia. Ankyrin polypeptides are expressed in most cell types and have the potential to play critical roles in vertebrate physiology beyond the red cell, neuron, kidney, and cardiomyocyte. Therefore, a major unexplored area is evaluation of ankyrin-based pathways for physiology in other complex tissues. Recent findings demonstrate new membrane partners for ankyrins, and confirm the essential

roles for ankyrins in defining protein polarity in polarized epithelial cells. For example, Lopez et al. recently determined that direct interaction between ankyrin-G and the ammonium transporter (RhBG) is required for normal expression of the transporter on the basolateral membrane surface of Madin-Darby Canine Kidney (MDCK) cells (28). Additionally, findings in cultured human bronchial epithelial cells suggest that ankyrin-based pathways, in addition to targeting proteins to sites in polarized epithelial cells (24), are also responsible for the biogenesis of polarized membrane domains (72).

The molecular components underlying ankyrin-based pathways for protein targeting are largely unknown. We predict that ankyrin-based pathways for protein targeting have likely evolved for specialized metazoan-specific functions and therefore will operate separately from default pathways for protein biosynthesis and trafficking. Therefore, experimental context is crucial for uncovering ankyrin-based pathways. For example, the pathways for membrane targeting are not conserved between HEK293 cells and primary ventricular cardiomyocytes (64). In a larger context, these findings strongly indicate that ion channel and transporter trafficking must be studied in a physiological system to appropriately mimic the cellular environment (i.e. the presence of ankyrin-based pathways).

Ankyrins and ankyrin-related proteins (eg. protein 4.1, see review by Gascard and colleagues in this issue) are widely expressed, have complex gene expression, and are expressed at specific membrane domains. For example, a number of tissues (brain, heart, kidney) display multiple ankyrin gene products. Moreover, a single tissue may express multiple splice forms of the same ankyrin gene (kidney and brain express multiple ankyrin-B and -G polypeptides) and each polypeptide may display a unique distribution (5, 6, 57, 73-75). Two major unresolved questions in the ankyrin field include the potentially large scope of unidentified ankyrin-polypeptides as well as unlocking the code for ankyrin gene product specificity *in vivo*. Human genetics and structure/function studies suggest that the divergent C-terminal domain plays a major role in determining ankyrin-specific function *in vivo*. However, there are major unexplored regions in the spectrin-binding domain as well as critical splicing events, particularly the possibility of a diverse number of first exons leading to alternative ankyrin functions (61).

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