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Alternative splicing: a key mechanism for ankyrin-B functional diversity?

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Ankyrin is a family of adaptor proteins encoded by three genes, *ANK1*, *ANK2* and *ANK3* (also known as ankyrin-R, ankyrin-B and ankyrin-G, respectively) [1]. The ankyrins serve to link specific membrane proteins to the cellular cytoskeleton and help membrane proteins remain stably positioned in the correct location within the cell. The ankyrins contain 24 “ankyrin repeats”, a 33 amino acid motif commonly found in diverse proteins that appears to enable protein-protein interactions. In heart cells ankyrin-B has been shown to be important in targeting of the Na⁺/Ca²⁺-exchanger (NCX), the Na⁺/K⁺-ATPase and the inositol-1,4,5-triphosphate receptor (IP₃R) [2,3], while ankyrin-G underlies the sarcolemmal positioning of the cardiac Na⁺ channel (Nav1.5) [4]. Defects in *ANK2* have been linked to the inherited long QT syndrome type 4 (LQTS4, part of the “ankyrin syndrome”) [3], highlighting the importance of the ankyrins in physiologic functioning and in disease.

In this issue of *Journal of Molecular and Cellular Cardiology*, Cunha *et al.* [5] are the first to describe the complex genomic organization of human *ANK2* gene, which encodes the ankyrin-B isoform. In this comprehensive study, the authors identify six new coding exons and demonstrate that the *ANK2* gene is subject to complex post-transcriptional regulation due to over thirty previously unknown alternative splicing events. In addition to post-translational modifications, alternative splicing is a key biological mechanism to generate a plethora of functionally diverse proteins from a relatively small number of human genes. Alternative splicing events are described for a large and growing number of genes, and contribute to proteome complexity by modulating the binding properties, intracellular localization, enzymatic activity, and stability of final protein products [6]. In addition to changes in promoter activity to alter actual protein levels, gene regulation by alternative splicing might be a cellular tool to adjust to different functional demands. On the other hand, aberrant splicing events are also known to play a causal role in human disease [7]. Here, Cunha and colleagues suggest complex alternative splicing of *ANK2* gene as a source for ankyrin-B functional diversity, opening a new era of studying ankyrin-B molecular function and its role in human disease.

The ankyrin-B protein structure consists of three functional domains: an amino terminal membrane binding domain, a spectrin-binding domain, and a regulatory domain (that includes both a “death subdomain” and a C-terminal region - see Fig. 1). These domains have defined roles in binding membrane-associated proteins (e.g., ion channels, transporters, phosphatases

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and cell adhesion molecules), in regulating intra- and intermolecular interactions, and in establishing a linkage of membrane proteins to the cytoskeleton [8,9]. The identification of alternative splicing throughout the *ANK2* gene suggests that these events may contribute to the functional variety of ankyrin-B isoforms and multi-protein domains in the heart.

Firstly, an alternative first exon (1') with an associated upstream non-coding exon (0) has been identified. This finding not only implies an alternative ankyrin-B protein structure, but also reveals a potential switch for the regulation of gene expression in the form of alternate promoter regions. The latter also provides a mechanism for yet to be identified isoform related restrictions in expression pattern and tissue specificity, as previously described for *ANK1* and *ANK3*, the genes coding for ankyrin-R and ankyrin-G, respectively [10,11].

In addition to the newly found alternative first exon, six additional alternative splicing events are identified in the membrane binding domain, namely skipping of individual exons 7, 17 and 21, and of combinations of exons 11, 12 and 13. The membrane binding domain codes for twenty-four ANK repeats of which few are known to be involved in protein-protein interactions [8]. To date, no ankyrin-B binding partners are known to interact with ANK repeats coded by exons 7 and 11-13. However, removal of exon 17 or 21, and thereby excluding ANK repeat 18 and 23 from the final protein, might alter the interaction with the previously identified binding partners NCX and IP₃R, respectively [12,13]. It is tantalizing to speculate that alternatively-spliced ankyrin-B proteins may direct these Ca²⁺ handling proteins to distinct subcellular domains in cardiomyocytes in order to optimize intracellular Ca²⁺ homeostasis.

Next, two new exons, exon 24 and 28, are identified in the ankyrin-B spectrin-binding domain. Both of these novel exons seem to be sensitive to exon skipping events. Noteworthy is that exon 28 is located closely to a Zu-5 motif, encoded by exons 29, 31 and 32, and important for β -spectrin binding [14]. Therefore, alternative splicing of exon 28 might affect the association with β -spectrin. However, using an *in vitro* binding assay the authors could not find a difference in the binding affinity of spectrin to ankyrin-B polypeptides resulting from alternative splicing of exons 24 and/or 28, although they raised the possibility that the novel ankyrin-B isoforms could selectively bind to specific β -spectrin isoforms. Alternatively, splicing variants in the ankyrin-B spectrin binding domain might combine with the alternative splicing in other domains to further affect the ankyrin-binding properties.

Additional splicing events in the spectrin-binding domain consist of partial deletion of exon 26, due to an alternative 3'-splice acceptor site, and skipping of exons 30, 38 and 40. However, no physiological effects are predicted for the 15 base pair deletion involving the partial exon 26 skip. As described above, however, the protein domain involved in β -spectrin binding is encoded by exons 29, 31 and 32 [14]. Therefore, inclusion of exon 30 would likely disrupt this domain and potentially result in an ankyrin-B isoform lacking β -spectrin binding capacity. Interestingly, mature transcripts harboring exon 30 have not yet been detected in heart tissue.

The C-terminal part of the spectrin binding domain was recently found to interact with B56 α , a subunit of the serine/threonine phosphatase protein phosphatase 2A (PP2A) [9,15]. This interaction might be affected by exclusion of exon 38 by alternative splicing.

With 3 newly identified exons, an alternative stop codon and 6 potential exon skipping events, some of them including multiple exons, the C-terminal domain of ankyrin-B is subject to complex post-transcriptional regulation, and this might have significant consequences in ankyrin-B functions in a physiological context. Considering the importance of the C-terminal domain of ankyrin-B in regulating the intra- and intermolecular interactions, the newly discovered variability in the structure of this domain will endow numerous ankyrin-B isoforms with a wide range of affinity for known partners, as well for as yet-to be discovered novel associate proteins. All this will occur as a consequence of two factors: a) the newly described

complex alternative exon splicing in this domain will yield mature polypeptides with a large variety in amino acid sequences, and b) the three newly discovered exons will yield additional peptide sequences which might contain additional protein-binding motifs, thereby increasing the number of potential ankyrin-B binding partners. For example, a sequence in exon 46 shows great similarity with a recently discovered domain in a small isoforms of ankyrin-R (small ankyrin 1) that binds the very large protein obscurin in striated muscle [16]. This finding suggests the possibility that certain ankyrin-B isoforms might also indirectly bind to proteins of the contractile apparatus, which would potentially expand the physiological role of ankyrin-B in cardiac cells. It would be interesting to determine whether those ankyrin-B isoforms regulate cytosolic Ca^{2+} levels or modulate cell contraction. An interesting possibility highlighted by Cunha *et al.* [5] in the current issue of the *Journal of Molecular and Cellular Cardiology*, is that by endowing the heart with multiple ankyrin-B isoforms it might be possible for each isoform to show specific affinity for a reduced number (or even one) of target proteins, rather than a single ankyrin-B with multiple binding capabilities.

Notably, the C-terminal domain harbors most of the loss-of-function mutations that underlie LQTS4 or the “ankyrin-B syndrome” characterized by a broad spectrum of cardiac alterations that include sinus node bradycardia, ventricular tachycardia, atrial fibrillation and sudden cardiac death in response to catecholaminergic stimuli [8]. At the cellular level, loss-of-function mutations in ankyrin-B dramatically alters $\text{Na}^+/\text{Ca}^{2+}$ -exchanger, IP_3R and Na^+/K^+ ATPase localization. As a result, Ca^{2+} homeostasis may be disrupted, leading to sarcoplasmic reticulum Ca^{2+} overload and spontaneous Ca^{2+} release that can lead to arrhythmogenic “early afterdepolarizations” or “delayed afterdepolarizations”. Clearly, the newly discovered exons in the C-terminal domain increase the possibility that additional loss-of-function mutations might add to the growing list of mutations already described in this domain.

With the discovery of multiple ankyrin-B isoforms in the human heart, it becomes important to perform new studies to determine whether these alternative ankyrin-B polypeptides have specific function with known or new target proteins, as well as to elucidate their subcellular localization. This work by Cunha *et al.* [5], nicely complements earlier findings showing that various isoforms of ankyrin-G and ankyrin-R occur in other tissues as a result of alternative splicing.

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Figure 1. *ANK2* genomic organization, alternative splicing events and potential physiological effects Schematic representation of revised *ANK2* exon organization as described by Cunha *et al.* [5]. Exons coding for ankyrin-B membrane-binding domain are depicted in blue, for spectrin-binding domain in green and for C-terminal regulatory domain in two colors (purple for the death sub-domain and yellow for the C-terminal region). Newly identified exons, including a non-coding (0) and an alternative first exon (1') are labeled in red. Exons in white have not been found in heart. An alternative 3'-splice acceptor site is located in exon 26. Inclusion of exon 50 results in a frameshift and the use of an alternative stop codon in exon 51. Alternative splicing events and possible effects on ankyrin-B function are displayed. See text for further details. NCX = Na⁺/Ca²⁺ exchanger, IP₃R = inositol-1,4,5-triphosphate receptor, PP2A = protein phosphatase 2A.