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Exon organization and novel alternative splicing of the human *ANK2* gene: Implications for cardiac function and human cardiac disease

Shane R. Cunha¹, Solena Le Scouarnec^{3,4,5}, Jean-Jacques Schott^{3,4,5,6}, and Peter J Mohler^{1,2}

¹ Department of Internal Medicine, Division of Cardiovascular Medicine; University of Iowa Carver College of Medicine, Iowa City, IA 52242

² Department of Molecular Physiology and Biophysics; University of Iowa Carver College of Medicine, Iowa City, IA 52242

³ INSERM, UMR915, l'institut du thorax, Nantes, F-44000 France

⁴ CNRS, ERL3147, Nantes, F-44000 France

⁵ Université de Nantes, Nantes, F-44000 France

⁶ CHU Nantes, l'institut du thorax, Service de cardiologie, Nantes, F-44000 France

Abstract

Recent findings illustrate a critical role for ankyrin-B function in normal cardiovascular physiology. Specifically, decreased expression of ankyrin-B in mice or human mutations in the ankyrin-B gene (*ANK2*) results in potentially fatal cardiac arrhythmias. Despite the clear role of ankyrin-B in heart, the mechanisms underlying transcriptional regulation of *ANK2* are unknown. In fact, to date there is no description of *ANK2* genomic organization. The aims of this study were to provide a comprehensive description of the *ANK2* gene and to evaluate the relative expression of alternative splicing events associated with *ANK2* transcription in heart. Using reverse-transcriptase PCR on mRNA isolated from human hearts, we identify seven new exons associated with the *ANK2* gene including an alternative first exon located ~145 kb upstream of the previously-identified first exon. In addition, we identify over thirty alternative splicing events associated with *ANK2* mRNA transcripts. Using real-time PCR and exon boundary-spanning primers to selectively amplify these splice variants, we demonstrate that these variants are expressed at varying levels in human heart. Finally, ankyrin-B immunoblot analysis demonstrates the expression of a heterogeneous population of ankyrin-B polypeptides in heart. *ANK2* consists of 53 exons that span ~560 kb on human chromosome 4. Additionally, our data demonstrates that *ANK2* is subject to complex transcriptional regulation that likely results in differential ankyrin-B polypeptide function.

Address correspondence to: Shane R. Cunha, Ph.D., 285 Newton Road, CBRB, Room 2283, Iowa City, IA 52242, shane-cunha@uiowa.edu.

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Keywords

ankyrin-B; *ANK2*; cardiomyocytes; alternative splicing

1. Introduction

Ankyrins are adapter proteins that link integral membrane proteins to the actin/spectrin-based cytoskeleton in excitable and non-excitable cells [1]. In humans, three ankyrin genes *ANK1*, *ANK2*, and *ANK3* encode polypeptides termed ankyrin-R, ankyrin-B, and ankyrin-G, respectively. Ankyrins –B and –G are expressed in most tissues, while ankyrin-R displays a restricted expression pattern [1].

A canonical ankyrin is comprised of three functional domains that interact with a variety of proteins including integral membrane proteins, cytoskeletal elements, and signaling molecules. The ankyrin ‘membrane-binding domain’ (MBD, comprised of 24 consecutive *ANK* repeats, see Figure 1A, yellow) interacts with a host of integral membrane proteins including ion channels, transporters, and cell adhesion molecules. Some of these membrane proteins include voltage-gated Na⁺ and K⁺ channels, Na/K ATPase, anion exchanger, Na/Ca exchanger, InsP₃ receptor, as well as L1 family cell adhesion molecules [1, 2]. The 62 kD ankyrin ‘spectrin-binding domain’ (SBD, Figure 1A, red) displays binding activity for β-spectrin isoforms [3] and B56α, the regulatory subunit of protein phosphatase 2A [4]. Finally, the ‘death’ (DD) and ‘C-terminal’ domains (CTD) constitute the ‘regulatory domain’ (Figure 1A, green and blue) that mediates both ankyrin inter- and intra-molecular interactions [5–8].

Ankyrins are critical for normal vertebrate physiology. Ankyrin-R dysfunction results in loss of erythrocyte membrane integrity, spherocytosis, and hemolytic anemia in humans and mice [9–12]. Disruption of ankyrin-G in the cerebellum causes abnormal targeting of voltage-gated Na⁺ and K⁺ channels in Purkinje and granule cell neurons, irregular action potentials, and ataxia [13–16]. Mice lacking ankyrin-B manifest significant central nervous system defects including degeneration of long axon tracts and die at post-natal day 1 [17]. Recently, we demonstrated that ankyrin-B plays a critical role in normal cardiovascular physiology. Specifically, ankyrin-B^{+/-} mice display bradycardia, heart rate variability, polymorphic arrhythmia, syncope, and sudden cardiac death [18]. In humans, *ANK2* (encodes ankyrin-B) loss-of-function variants are linked to dominantly-inherited arrhythmia termed “ankyrin-B syndrome” [18–20]. Phenotypes associated with this syndrome include bradycardia, atrial fibrillation, and ventricular arrhythmia. To date, nine human ankyrin-B loss-of-function variants in six different *ANK2* exons have been identified that result in arrhythmia, or susceptibility to drug-induced arrhythmia [18–20].

Despite the critical role for ankyrin-B in normal cardiac physiology, virtually nothing is known regarding the regulation of *ANK2* transcription and translation in heart. Furthermore, there has yet to be a detailed analysis of *ANK2* genomic organization. This report describes the first analysis of *ANK2* genomic organization and the complex alternative splicing events associated with the assembly of a diverse set of cardiac *ANK2* mRNA transcripts. We report the unanticipated identification of seven novel human *ANK2* exons including a previously unidentified alternative first exon nearly 145 kb upstream of the previous first exon. Using human left ventricular tissue, we characterize a host of cardiac *ANK2* mRNAs that result in the addition or subtraction of key functional domains throughout the ankyrin-B polypeptide. Together, our new data demonstrates that *ANK2* is subject to complex transcriptional regulation that likely result in differential ankyrin-B function.

2. Methods

2.1 Human tissue, RNA isolation, and reverse transcription

RNA was isolated from left ventricular muscle tissue from six healthy donor hearts that were not suitable for transplantation (subclinical atherosclerosis, age, no matching recipients) through the Iowa Donors Network and through the National Disease Research Interchange. The investigation conforms with the principles outlined in the Declaration of Helsinki [21]. Age and sex were the only identifying information acquired from the tissue providers, and the Iowa Human Subjects Committee deemed that informed consent from each patient was not required. None of the patients died from cardiac-related causes. RNA was isolated from the apex of flash frozen left ventricular samples (fat, coronary arteries excluded). RNA (with a 260/280 ratio of 1.9 to 2.1) was reverse-transcribed into cDNA using SuperScript III reverse transcriptase (20 µl). 1 µl of this reaction was used in subsequent PCR or real-time reactions. Human tissue for RNA experiments in Figures 2–5 was isolated at the University of Iowa (six hearts). Additional assays to search for novel *ANK2* exons in humans were conducted in two non-diseased human hearts obtained from the University of Szeged (Szeged, Hungary) that were technically unusable for transplantation based on logistic considerations. Before cardiac explantation, organ donor patients did not receive medication except dobutamine, furosemide, and plasma expanders. The investigations conformed to the principles outlined in the Helsinki Declaration of the World Medical Association. All experimental protocols were approved by the Ethical Review Board of the Medical Center of the University of Szeged (no. 51–57/1997 OEJ). Total RNA from each cardiac sample was isolated and DNase treated with the RNeasy Fibrous Tissue Mini Kit (QIAGEN) following the manufacturer's instructions. The quality of total RNA was assessed with PAGE (2100 Bioanalyzer; Agilent Technologies). Absence of genomic DNA contamination was verified by PCR.

2.2 PCR amplification and isolation of alternative *ANK2* cardiac transcripts

PCR products were amplified from reverse-transcribed ventricular mRNA and human heart cDNA library using Advantage 2 polymerase (Clontech). Eleven primer sets were designed to subdivide ankyrin-B cDNA into overlapping sections of 600 to 800 bp in length.

The primers sets are as follows: 1: (GACAGTGGAGAGAAGTTCAACGGC, GGTGTCATTCTCCAAGAGGATGGC), 2: (CCTTTATACATGGCTGCCCAAGAG, CTTACACATTCCACGTGGTCTCC), 3: (CACTTCACTGTGCTGCACGAAGTG, CCATACTTGGCTGCTACATGCAGG), 4: (CAGTCCTATTGGAAGCAGGAGCAG, GACGTTGATGATGTGCGTGTGACC), 5: (GTGGCCTGTCACTATGGAAATGTG, CATTGTGTCTGCATCCTCGCATAG), 6: (CACTGAGAACTTAGACAACGTGGC, GCCTAGGATCTTCTTAACCAGCTC), 7: (CAGTGGTGTCTCGTATCAAACAGG, GATAGTCGTCCGCAAGGTTCCTGA), 8: (TCAGGAACCTTGCGGACGACTATC, GTCCAGCTGAAGCCAAGGTGATCA), 9: (TGATCACCTTGCTTCAGCTGGAC, GGAGAGCTGGGTACTATCATAGCC), 10: (CCTGCCTGAAGAGTCATCTCTGG, GCCCTCTTCTGTGTGATGGCTTTACTC), 11: (ATGTTGCAAAAGTCTGACAGCAAT, GGTGTCATTCTCCAAGAGGATGGC). PCR products were separated and extracted from 2.5% agarose gel, then ligated into pCR2.1-TOPO vector (Invitrogen) for sequence analysis.

2.3 Quantitative real time (qt)-PCR analysis of alternative *ANK2* transcripts

Based on the alternative *ANK2* transcripts amplified from reverse-transcribed ventricular mRNA and human heart cDNA library, PCR primers that spanned exon boundaries were designed to selectively amplify particular *ANK2* transcripts. For example, to amplify the *ANK2* transcript that lacks exon 7, we used a 5'-primer internal to exon 6 and a 3'-primer (24 bp) that spanned the junction of exons 6 (12 bp) and 8 (12 bp). To test the specificity of exon-spanning primers, we repeated the PCR amplification using a 3'-primer to either half of the

exon-spanning primer (e.g. first 12 bp corresponding to exon 6 or last 12 bp corresponding to exon 8). A detailed list of the 35 primer sets used for real-time PCR analysis is provided in Supplemental Table 1. We used a modified ΔC_t method that accounts for primer efficiencies to calculate the mRNA expression of *ANK2* splice variants relative to the expression of the exon 22/23 splice variant [22]. Specifically, to measure the fold difference, we calculated the primer efficiency (of the particular splice variant) to the power of the average of the C_t values for the particular splice variants minus the average of the C_t values of exon 22/23 transcripts. We expressed this number as a percentile in which expression of exon 22/23 was normalized to 100%. Experiments were replicated twice and each condition was performed in triplicate. Error bars represent standard deviation with a sample set of 3.

2.4 Immunoblot and co-immunoprecipitation assays

Immunoblots and co-immunoprecipitation assays were performed as described using ankyrin-B and NHERF1 (Sigma) Igs [23]. Briefly, hearts were extracted from post-natal day one ankyrin-B^{+/+} and ankyrin-B^{-/-} mice and lysed in four volumes of 50 mM Tris pH 7.4, 10 mM NaCl, 0.32 M sucrose, 5 mM EDTA, 2.5 mM EGTA, 1 mM PMSF, and 1X protease inhibitor cocktail (Sigma). The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Protein levels were measured using the Bicinchoninic Acid protein assay kit (Pierce) and equal amounts of protein lysate were separated by SDS-PAGE on 3–8% Tris-Acetate or 10% Bis-Tris gels (Invitrogen). Lysates were transferred to a nitrocellulose membrane (Whatman) which was incubated with affinity-purified ankyrin-B Ig (2 μ g/ml) or Na⁺/H⁺ exchange regulatory factor 1 (NHERF1) (Sigma) overnight at 4°C. Ankyrin-B and NHERF1 immunoreactive polypeptides were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

2.5 In vitro binding assays

In vitro translated (IVT) products of alternative ankyrin-B SBDs were prepared using the TnT T7-Coupled Reticulate Lysate System (Promega) and binding experiments were performed as described [24]. Briefly, IVT products were incubated with GST-fusion proteins at 4°C in 500 μ l binding buffer (50 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 0.1% Triton X-100) for 3 hours (for β_1 -spectrin). Binding reactions were washed five times in wash buffer (binding buffer supplemented with 1% Triton X-100 and 1 M NaCl). Binding reactions were pelleted, resuspended in SDS-sample buffer, separated by SDS-PAGE, and visualized with a phosphorimage machine (courtesy of Dr. Michael Welsh, University of Iowa).

3. Results

Identification of novel *ANK2* exons

Ankyrin-B is critical for targeting and stabilization of ion channels and transporters at transverse-tubule (T-tubule) and sarcoplasmic reticulum (SR) membranes in ventricular cardiomyocytes [18,23,25,26]. Furthermore, variants in the human *ANK2* gene may lead to fatal cardiac arrhythmias [18–20]. Surprisingly, we know very little regarding the regulatory mechanisms of transcription and translation of *ANK2* in heart. To identify cardiac-specific *ANK2* transcripts, we performed reverse-transcriptase PCR on mRNA isolated from left ventricular tissue from human hearts. PCR products were also amplified from a human heart cDNA library. We used ten different over-lapping primer sets that spanned the entire ankyrin-B cDNA (Figure 1B). We unexpectedly identified seven novel *ANK2* exons (numbered 0, 1', 24, 28, 46, 49, and 50) (Figure 1C and Table 1). The presence of these seven exons was confirmed in human genomic sequence (Table 1) and homologous exons were identified in mouse *Ank2*. Table 1 displays the most current account of human *ANK2* genomic organization

including the new exon nomenclature, 5'- and 3'-splice sites, exon length, proceeding intron length, and the corresponding functional domain.

A novel alternative first exon (E1') was characterized in addition to a non-coding exon (E0) that is located ~86.2 kb upstream of E1'. These exons were initially identified in a BLAST search of translated expressed sequence tags (DA809405). The new alternative first exon lies ~269.9 kb upstream of exon 2 and ~145 kb upstream of the previously-reported first exon (Figure 1E and Table 1) [18]. The non-coding exon (E0) is only found in transcripts containing E1' and is not associated with the previously-reported first exon. With the identification of these two new exons, the region encompassing the *ANK2* gene locus on human chromosome 4 dramatically increased in size from ~330 kb to ~560 kb (Figure 1D–E).

Regarding the other newly-identified exons, exon 24 is 54 bp in length and is located at the junction of the membrane-binding and spectrin-binding functional domains (Figure 1C). Exon 28 is located in the spectrin-binding domain and, given its close proximity to the Zu-5 motif, may modulate β -spectrin binding [3]. Novel exons 46, 49, and 50 are located in the ankyrin-B C-terminal domain and may contribute to the regulatory function of this domain (Figure 1C). Interestingly, exon 50 is the only exon that shifts the open reading frame (+2) such that an alternative stop codon is utilized in exon 51. This shift extends the open reading frame for an additional 61 amino acids that terminates with the sequence “Gln-Glu-Ala-Lys”. In the absence of exon 50, the open reading frame terminates in exon 51 after four amino acid residues (Asp-Asn-Asn-Glu). Finally, we identified an alternative 3'-splice acceptor site in exon 26 that removes the first 15 bp, but has no effect on the open reading frame.

Characterization of novel *ANK2* splice variants

While screening for cardiac-specific *ANK2* transcripts, we identified a surprising number of novel alternative splice variants. Alternative *ANK2* splice variants were identified in regions encoding the domains MBD, SBD, and CTD. To measure the relative mRNA expression of individual *ANK2* transcripts, we performed real-time PCR analysis of mRNA isolated from human heart using exon boundary-spanning primers [27]. Exon boundary-spanning primers allow for the selective PCR-amplification of individual alternative transcripts. For example, *ANK2* transcripts lacking exon 7 were selectively amplified using a 3'-primer that spanned the splice junction of exons 6 and 8 (Figure 2A, column 5). Likewise, transcripts containing exon 7 were selectively amplified using a boundary-spanning primer consisting of the junction of exons 6 and 7 (Figure 2A, column 4). To validate that specific transcripts were only amplified when using the full-length exon boundary-spanning primer, we repeated PCR using each individual half of the exon-spanning primer (Figure 2A, columns 1–3). These findings demonstrate that a full-length exon boundary-spanning primer is necessary for the amplification of a PCR product. Moreover, transcripts containing exon 7 are expressed markedly less than transcripts lacking this exon. These findings are corroborated by results from real-time PCR analysis (Figure 3D). Another representative example of selective PCR-amplification using full-length exon boundary-spanning primers is presented in Figure 2B that demonstrates the amplification of *ANK2* transcripts $\pm$ exon 24. This technique was used to confirm the selective amplification of individual *ANK2* transcripts for all novel alternative splice variants identified. For all transcripts, full-length exon boundary-spanning primers are necessary for the amplification of PCR products.

To ensure the accurate analysis of mRNA expression levels within a 1000-fold concentration gradient, we optimized the annealing temperatures for each primer set such that the efficiency fell within the range of 90 to 110% (see Supplemental Table 1 for annealing temperatures and primer set efficiencies). In calculating the relative mRNA expression levels, we used a modified Δ Ct technique to incorporate primer efficiency (see *Methods*). Values were expressed as a percentage of the expression of the *ANK2* transcript containing exons 22 and 23, which was

normalized to 100%. Based on the previous observation that disrupting exon 23 effectively eliminates all ankyrin-B isoforms [17, 28], we propose that all *ANK2* transcripts contain exon 22.

Relative mRNA expression levels of alternative *ANK2* splice variants were evaluated in ventricular tissue from four different human hearts. Experiments were replicated twice with each condition performed in triplicate. For each alternative transcript, results were displayed for individual hearts to observe any variability in expression between hearts. Finally, we performed real-time PCR analysis of the reference gene hypoxanthine-guanine phosphoribosyl transferase (HPRT) to assess relative cDNA amounts between hearts.

Novel alternative first exon in *ANK2* transcript

The novel alternative first exon E1' is 60 nucleotides long, but the coding sequence begins with the terminal 21 nucleotides (Table 1). This exon is ~269.9 kb upstream of exon 2, while the previously-identified first exon (E1) is ~124.6 kb upstream of exon 2 (Figure 3A). Real-time PCR analysis using exon boundary-spanning primers demonstrated that *ANK2* transcripts containing alternative first exons E1 or E1' were expressed at approximately the same level (Figure 3B). It is noteworthy that expression of these transcripts was significantly less (3.5–7.5% of E22/23 expression that is normalized to 100%) than all other *ANK2* transcripts. Considering the tremendous expanse of intronic sequence separating the first and second exons (~269.9 kb and ~124.6 kb for E1'/E2 and E1/E2, respectively), it is possible that these transcripts may in fact be significantly less abundant.

Novel *ANK2* splice variants encoding ankyrin-B membrane-binding domain

For the MBD, we identified novel *ANK2* transcripts that removed individual exons such as exons 7, 17, and 21 (Figure 3C). We also identified complex splice variants consisting of various combinations of exons 11, 12, and 13 corresponding to *ANK* repeats 10 and 11, 12, and 13, respectively (Figure 3C). Based on our previous studies, we predict that splicing events that remove exon 17 (encoding *ANK* repeat 18) or exon 21 (encoding *ANK* repeat 23) will disrupt the binding sites for Na/Ca exchanger (*ANK* repeats 16–18) [25] or InsP₃R (*ANK* repeats 22–24) [26].

In terms of relative mRNA expression, the general trend for each transcript is consistent across the four human hearts. In all hearts, the expression of –E7 transcripts (lacking exon 7) is much more abundant (>50 fold) than expression of +E7 transcripts (Figure 3D). While exon 7 is not abundantly expressed in cardiac transcripts, it was found in transcripts isolated during the screen of human ventricular mRNA; therefore, it was not designated as non-cardiac like exons 30 and 40. Expression of –E12/13 transcripts is the most abundant followed by –E12 transcripts and +E12/13 transcripts that are expressed at similar levels (Figure 3D). Transcripts lacking exons 11, 12 and 13 are the least abundant. In general, +E17 transcripts are expressed more abundantly than –E17 transcripts (~2 fold), although there is greater variability in –E17 transcript expression between the four hearts (Figure 3D). Both +E21 and –E21 transcripts are expressed at similar levels in all four hearts (Figure 3D). Together, these findings challenge the notion that the heart solely expresses a single, multivalent ankyrin-B polypeptide (e.g. 220 kD ankyrin-B), which contains distinct binding sites for the Na/Ca exchanger, Na/K ATPase, and InsP₃R [18, 23, 25, 26]. In fact, these findings suggest that alternative splicing of *ANK2* may produce distinct polypeptides that interact exclusively with the Na/Ca exchanger or InsP₃R.

Novel *ANK2* splice variants encoding ankyrin-B spectrin-binding domain

When screening *ANK2* for alternative splice variants, we identified multiple transcripts of the spectrin-binding domain that included or excluded exons 24, 28, or 38 (Figure 4A). In addition,

we also characterized an alternative 3'-splice acceptor site within exon 26 that removes the first 15 bp. Exons 30 and 40 were never found in any *ANK2* cardiac transcript. Furthermore, we were unable to detect exons 30 or 40 in ventricular mRNA by reverse transcriptase PCR using internal primers to exons 30 or 40; therefore, these exons are not likely expressed in cardiac *ANK2* transcripts. Interestingly, the inclusion of exon 30 would disrupt the Zu-5 motif, which is encoded by exons 29, 31, and 32 and is important for β -spectrin binding activity [3]. Exon 40 encodes the large 220 kD insert that is found in the brain-specific 440 kD ankyrin-B isoform [29].

We performed real-time PCR analysis to determine the relative mRNA expression levels of these alternative splice variants. While there was variability between hearts in the expression of transcripts $\pm$ exon 24, transcripts lacking this exon were more abundant than +E24 transcripts (Figure 4B). For exon 26, transcripts with the first 3'-splice acceptor site are more prevalent (~ 3 fold) than transcripts with the second 3'-splice acceptor site (Figure 4B). In addition, +E28 transcripts are more abundant (~ 2 – 5 fold) than –E28 transcripts (Figure 4B). Finally, transcripts containing exon 38 (+E38) are far more prevalent (~ 25 fold) than –E38 transcripts (Figure 4B).

Novel *ANK2* splice variants encoding ankyrin-B C-terminal domain

ANK2 CTD is subject to complex alternative splicing events (Figure 5A). In fact, eleven distinct *ANK2* transcripts were identified during the screen. We observed splice variants that simply include or exclude individual exons such as exon 46 or exon 48. +E46 transcripts are more common (~ 7.5 – 9 fold) than –E46 transcripts (Figure 5B), while +E48 transcripts are more abundant (~ 5 – 6 fold) than –E48 transcripts (Figure 5B). The majority of alternative transcripts are the result of many exons (45, 47, 48, 49, and 50) forming independent splice junctions with the final exon (exon 51; Figure 5A). All of these transcripts display similar expression levels with the exception of the transcript lacking exons 48, 49, and 50, which is the least abundant (Figure 5B). As mentioned above, inclusion of exon 50 alters the open reading frame (+2) such that an alternative stop codon is introduced in exon 51. It is noteworthy that the most extensive alternative splicing occurs within the C-terminal domain and that this domain regulates the intra- and inter-molecular interactions of ankyrin-B.

The heart expresses a heterogeneous population of ankyrin-B polypeptides

To determine whether the complex mRNA splicing events detailed in Figures 3–5 actually result in a diverse set of ankyrin-B polypeptides, we performed immunoblot analysis on protein lysates from ankyrin-B^{+/+} and ankyrin-B^{–/–} neonatal hearts. Mouse hearts were lysed on ice and in the presence of protease inhibitors to prohibit protein degradation. Equal loading of protein lysate was validated by Coomassie Blue stain (Figure 6B) and immunoblot analysis of the reference protein Na⁺/H⁺ exchanger regulatory factor 1 (NHERF1) (Figure 6C). To maximize the resolution of high molecular weight products, protein lysates were separated on a 3–8% Tris-Acetate gel, while low molecular weight products were resolved on a 10% Bis-Tris gel (Figure 6A, upper and lower panels). These findings demonstrate that ankyrin-B^{+/+} hearts express a heterogeneous population of ankyrin-B polypeptides. Specifically, canonical 220 kD and 160 kD ankyrin-B isoforms are only detected in the ankyrin-B^{+/+} hearts (Figure 6A, upper panel). Furthermore, numerous ankyrin-B immunoreactive polypeptides ranging in size from 45 to 105 kD are solely detected in ankyrin-B^{+/+} hearts. Interestingly, we also observed a 100 kD band in protein lysates from the ankyrin-B null hearts. To evaluate the expression of alternative ankyrin-B polypeptides in human heart, we performed immunoblot analysis on protein lysate isolated from human left ventricular tissue. Consistent with our findings from the mouse heart, the human heart expresses multiple isoforms of ankyrin-B, including the predominant 220 kD isoform (Figure 6D). Small variations between the alternative ankyrin-B isoforms identified in mouse heart versus human heart likely represent

minor differences in tissue source (whole mouse heart versus pure human left ventricular wall muscle tissue) and chemistry of the SDS-PAGE resolving gels (resolution of low molecular weight ankyrin-B polypeptides is improved on 10% Bis-Tris gels). These findings support the hypothesis that *ANK2* is subject to complex mRNA splicing events that translate into a heterogeneous population of ankyrin-B polypeptides.

4. Discussion

This is the first study to detail the large genomic organization and complex transcriptional regulation of human *ANK2*. Molecular analysis of human heart tissue revealed seven previously unidentified *ANK2* exons. To date, *ANK2* now includes 53 exons that span over 560 kb, with an alternative first exon nearly 270 kb upstream of exon 2. Our results show a complex splicing profile for *ANK2* in human cardiac muscle. In fact, we observed thirty new splicing events in key functional domains of ankyrin-B. Based on known sites for ankyrin-B interacting partners, our results suggest that ankyrin-B polypeptides are dynamically regulated at the transcriptional level for specific functions (e.g. NCX targeting to T-tubule membrane vs. InsP₃ receptor targeting to SR membrane). Together, our findings detail the complex transcriptional regulation of *ANK2* and identify new exons to be screened for disease-variants in the human population.

Our study characterized multiple splicing events in the *ANK2* gene that would alter ankyrin-B topology. The newly identified exon 1' lies ~145 kb upstream of the previously identified first exon. Alternative first exons have also been identified for *ANK1* [30] and *ANK3* [31]. Obvious future goals are to characterize the full-length *ANK2* transcripts associated with these alternative first exons and to address the possibility of differential gene regulation mediated by distinct promoter regions. In MBD, there are three predominant sites of alternative splicing that would alter *ANK* repeat organization. Interestingly, splicing at two of these sites disrupts known binding sites for Na/Ca exchanger and InsP₃R [25,26]. The third site of complex splicing consists of exons 10–13 encoding *ANK* repeats 9–13. To date, this region has not been associated with an ankyrin-B interacting partner. In the related polypeptide ankyrin-R, a binding site for the cell adhesion molecule neurofascin has been broadly localized to *ANK* repeats 7–18 [32]. Future experiments to characterize binding partners and binding sites will focus on *ANK* repeats 9–13.

We identified two novel exons (24 and 28) located in the coding region for the spectrin-binding domain. An alternative 3'-splice acceptor site was also characterized in exon 26 that removes the first 15 bp. Considering the different possible splicing events characterized in the spectrin-binding domain (Figure 4), we were intrigued by the prospect that alternative splicing could differentially regulate association of ankyrin-B with β -spectrin. For example, deletion of 70 amino acids preceding the minimal spectrin-binding domain in ankyrin-R reduced β -spectrin binding affinity by 20-fold [33]. In ankyrin-B, the minimal spectrin-binding domain is encoded by exons 29, 31, and 32 [3]. Using an *in vitro* binding assay, we were unable to detect differences in the interactions of β_1 -spectrin with four different alternative isoforms of ankyrin-B SBD (Figure 7). While these results suggest that alternative splicing of the SBD does not influence ankyrin-B/ β_1 -spectrin interactions, another intriguing possibility is that alternative SBD isoforms may differentially associate with specific β -spectrin isoforms in heart. Furthermore, exon 30 was not identified in any cardiac *ANK2* transcripts. Inclusion of this exon would disrupt the minimal spectrin-binding domain. Future experiments will address whether exon 30 modifies ankyrin/ β -spectrin interactions in a tissue/cell-specific manner.

We report the association of three new exons (46, 49, and 50) with human cardiac *ANK2* transcripts encoding the C-terminal domain, the site of nearly all human *ANK2* gene variants associated with cardiac arrhythmias [18–20]. The C-terminal domain plays an important role in regulating the intra- and inter-molecular interactions of ankyrins. For example, the C-

terminal and membrane-binding domains of ankyrin-B have been shown to interact by yeast 2-hybrid analysis [5]. This interaction is necessary for the proper subcellular targeting of InsP₃ receptor in cardiomyocytes [5]. Furthermore, the localization of the InsP₃ receptor at the SR membrane specifically requires the C-terminal domain of ankyrin-B and cannot be recapitulated with the C-terminal domain of ankyrin-G [8].

Studies addressing the association of ankyrin-R with the anion exchanger and β -spectrin suggest that intra-molecular interactions between the functional domains negatively regulate inter-molecular interactions. Specifically, an alternative ankyrin-R isoform lacking 161 residues in the C-terminal domain exhibits increased affinity for the anion exchanger and β -spectrin [6,7]. Together, these findings suggest that the C-terminal domain regulates the association of ankyrin with integral membrane and cytoskeletal proteins. The addition of exons 46, 49, and 50 likely add further complexity to the regulatory functions of the ankyrin-B C-terminal domain. For example, exon 46 contains a site homologous to an obscurin binding domain found in small Ank1.5 [34,35] leading to the intriguing possibility that ankyrin-B interacts with the large sarcomeric protein obscurin.

On a clinical note, the three new exons in the C-terminal domain will provide additional sequences to analyze for genetic variants associated with human cardiac arrhythmias. The nine loss-of-function variants previously associated with arrhythmia are illustrated in the context of our current understanding of *ANK2* genomic organization (Figure 8). Furthermore, we propose a revised nomenclature for these variants. Specifically, we suggest that the variants be referred to using the nucleotide change and location starting from exon 1 (e.g. E1425G becomes A4463G). This new nomenclature will hopefully mitigate any potential confusion arising from the existence of multiple cardiac ankyrin-B polypeptides.

In the ankyrin family, there is precedence for tissue expressing multiple alternative splice variants of ankyrin polypeptides. For example, multiple isoforms of ankyrin-R have been characterized in erythrocytes, brain, kidney, and muscle [30,36–38]. Multiple isoforms of ankyrin-G have also been identified in epithelial tissue, muscle, and neural tissue [31,39–42]. For ankyrin-B, multiple isoforms have been identified in brain and thymus [28,29,43,44]. Many of the alternative isoforms of ankyrin polypeptides display differential subcellular localization and most likely are associated with distinct functions. For example, two alternative isoforms of ankyrin-G express a unique serine-rich domain that facilitates the subcellular localization of these isoforms to the axon initial segments [45].

In metazoans, ankyrin genes are uniquely designed to facilitate the modular assembly of polypeptides reflecting a diversity of combinatorial possibilities. For example, one or two *ANK* repeats are encoded by individual exons. In *ANK2*, alternative splicing of these exons (as we observed for exons 17 and 21, Figure 3C) would disrupt the binding sites for Na/Ca exchanger or InsP₃R. Instead of the heart expressing a single canonical 220 kD ankyrin-B with numerous functions, our data suggests that the heart expresses multiple ankyrin-B polypeptides specifically tailored for unique functions and distinct subcellular targeting. Moreover, these alternative splicing events could regulate the ability of ankyrin to interact with cytoskeletal elements (e.g. β -spectrin). Prior studies addressing ankyrin-B function/dysfunction in heart have generally relied on the ‘canonical’ 220 kD ankyrin-B polypeptide [3, 4, 8, 18–20, 23, 25, 26, 46]. These experiments were likely successful as this polypeptide is the primary ankyrin-B isoform in heart and as past assays have primarily focused on well-characterized 220 kD ankyrin-B protein partners including InsP₃ receptor, β -spectrin, and Na/Ca exchanger [3, 25, 26]. Based on our new findings, it will be important in future studies to characterize specific functions associated with distinct ankyrin-B polypeptides in the cardiomyocyte. In summary, the modular exon organization and diverse combinatorial assembly of *ANK2* would allow for the multifaceted ankyrin-B gene products to function in highly specialized domains

in cardiomyocytes such as costameres, SR/T-tubule junctions, A-bands, and intercalated discs [18, 31, 42, 47–50].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig.1. Organization of ANK2 and ankyrin-B, and location of ANK2 exons on human chromosome 4

(A), Ankyrin-B domains include a membrane-binding domain (MBD, yellow), spectrin-binding domain (SBD, red), death domain (DD, green), and C-terminal domain (CTD, blue). Diagram denotes ankyrin-B-binding sites for NCX, InsP₃R, β -spectrin, and Hdj-1. B56 α binds to ankyrin-B SBD residues 305–486 [4]. (B), *Previous ANK2 exon organization*. Exons in white have not been identified in cardiac ANK2 transcripts. Exon 38/40 (previous/current) encodes the 220 kD insert found in brain-specific 440 kD ankyrin-B [29]. Numbered lines represent ten overlapping regions PCR-amplified to identify alternative ANK2 transcripts. (C), *Revised ANK2 organization*. Newly identified exons encoding ankyrin-B are labeled in black. Preceding exon 26, black bar represents an alternative 3'-splice acceptor site. In exon 51, 'b' represents an alternative stop site. Exons are not drawn to scale. (D), *Previous location of ANK2 exons*. The 46 exons of ANK2 span ~330 kb on human chromosome 4 (114,190,334–114,522,076 on NC_000004.10, represents NCBI Build 36) with substantial intronic sequence (~124.6 kb) separating exons 1 and 2. (E), *Current location of ANK2 exons*. With the identification of non-coding exon (E0) and an alternative first exon (E1'), ANK2 now spans ~560 kb and includes 53 exons 113,958,714–114,522,245 on NC_000004.10). New exons are indicated in red. Note extensive intronic sequence that separates E1' from E1 (~145.3 kb) and E0 from E1' (~86.2 kb).

Fig.2. PCR amplification of alternative splice variants

PCR products for specific alternative splice variants are only amplified when exon boundary-spanning primers are used. (A), Transcripts containing the exon junctions of 6/7 and 6/8 are only amplified using the full-length boundary-spanning primers (column 4,5). In contrast, no products are detected using half the boundary-spanning primer (columns 1–3). Asterisk underlies a faint PCR product detected for boundary-spanning primer E6/7-3'. Findings are in agreement with the real-time PCR results presented in Fig.3D. (B), Transcripts containing the exon junctions of 23/24 and of 23/25 are only amplified using the full-length boundary-spanning primers (columns 4–5). In contrast, no PCR products are detected using half the boundary-spanning primer (columns 1–3).

Fig.3. Relative mRNA expression of alternative ANK2 transcripts encoding ankyrin-B MBD
 (A), *Alternative splicing of ANK2 first exons*. Exon size represents relative exon length. Note lengthy intronic sequences separating the first exons from the second exon (~269.9 kb, ~124.6 kb). (B), *Relative mRNA expression of splice variants with alternative first exons*. Bars represent real-time PCR analysis in triplicate of mRNA isolated from four human hearts. (C), *Alternative splicing of ANK2 exons encoding ankyrin-B MBD*. Exon size represents relative exon length. Note complex splicing events associated with exons 10–14. (D), *Relative mRNA expression of ANK2 splice variants encoding ankyrin-B MBD*. Bars represent real-time PCR analysis in triplicate of mRNA isolated from four human hearts. Relative mRNA expression is presented for splice variants $\pm$ exon 7; with various combinations of exons 11, 12, and 13;

±exon 17; and ±exon 21. Relative expression of reference gene hypoxanthine-guanine phosphoribosyl transferase (HPRT) is also presented. For all data, cycle threshold (Ct) values were weighted according to primer efficiency (Supplemental Table 1) and normalized to Ct values of E22/E23 that was set to 100%. Error bars represent standard deviation ($n = 3$).

Fig.4. Relative mRNA expression of alternative ANK2 transcripts encoding ankyrin-B SBD (A), *Alternative splicing of ANK2 exons encoding ankyrin-B SBD*. Exon size represents relative exon length. Exon 30 (white) is considered non-cardiac. (B), *Relative mRNA expression of ANK2 splice variants encoding ankyrin-B SBD*. Bars represent real-time PCR analysis in triplicate of mRNA isolated from four human hearts. Ct values were weighted according to primer efficiency (Supplemental Table 1) and normalized to Ct values of E22/E23 that was set to 100%. Relative mRNA expression of transcripts $\pm$ exon 24, using alternative 3'-splice acceptor sites (a or b) in exon 26, $\pm$ exon 28, and $\pm$ exon 38. Relative expression of HPRT is also presented. Error bars represent standard deviation ($n = 3$).

Fig.5. Relative mRNA expression of alternative ANK2 transcripts encoding ankyrin-B CTD
 (A), *Alternative splicing of ANK2 exons encoding ankyrin-B CTD*. Exon size represents relative exon length. Exon 40 is not included in this diagram because it encodes the 220 kD insert found in brain-specific AnkB 440 kD. Note complex splicing events associated with exons 45–51.
 (B), *Relative mRNA expression of ANK2 splice variants encoding ankyrin-B CTD*. Bars represent real-time PCR analysis in triplicate of mRNA isolated from four human hearts. Ct values were weighted according to primer efficiency (Supplemental Table 1) and normalized to Ct values of E22/E23 that was set to 100%. Relative mRNA expression is presented for splice variants $\pm$ exon 46 and $\pm$ exon 48. Relative mRNA expression is also displayed for

alternative transcripts that form splice junctions with exon 51 including E45/E51, E47/E51, E48/E51, E49/E51, and E50/E51. Error bars represent standard deviation ($n = 3$).

Fig. 6. Heterogeneous population of ankyrin-B polypeptides in heart

(A) Ankyrin-B immunoblot of protein lysates from ankyrin-B^{+/+} and ankyrin-B^{-/-} hearts. Upper panel, lysates were separated on a 3–8% Tris-Acetate gel, while the lower panel depicts lysates separated on a 10% Bis-Tris gel. Equal loading was demonstrated by Coomassie Blue (B) and NHERF1 immunoblot (C). Note multiple ankyrin-B polypeptide products in wild-type hearts. Asterisks denote 220 kD and 160 kD ankyrin-B isoforms. (D) Ankyrin-B immunoblot of protein lysate from left ventricular tissue of human heart. Arrows indicate multiple ankyrin-B isoforms. Asterisk denotes the predominant 220 kD ankyrin-B isoform.

Fig. 7. β_1 -spectrin binding to alternative ankyrin-B SBD isoforms

(A–D), Diagram represents the alternative ankyrin-B SBD splice variant evaluated for binding to β_1 -spectrin. Representative binding between ^{35}S -labeled ankyrin-B SBD and GST-fusion β_1 -spectrin is presented in the phosphorimage. Conditions were repeated in triplicate and quantified results are presented in the graph. Error bars represent standard deviation ($n = 3$).

Fig. 8. Revised nomenclature for ankyrin-B loss-of-function variants

Nine loss-of-function (LOF) variants have been previously characterized in ankyrin-B encompassing the CTD (blue), DD (green), and a neighboring portion of the SBD (red) [18–20]. The locations of these variants are indicated on the corresponding exons for previous and updated *ANK2*. An updated nomenclature for the LOF variants includes the nucleotide change and its location in full-length *ANK2* that includes all exons starting with E1.

Table 1

ANK2 Exon Organization

Exon #	Previous exon #	5' splice	3' splice	Length (bp)	3' intron (bp)	Domain	Comments
0			TCCCA/gt		86190		non-coding
1'		ag/GTAAG	AAAAA/gt	60/21*	269901	MBD	alt. start
1	1		AGAAG/gt	84*	124603	MBD	start
2	2	ag/TCTGA	ATCAG/gt	102	21850	MBD	ANK repeat 1
3	3	ag/AATGG	CTAAG/gt	99	2544	MBD	ANK repeat 2
4	4	ag/AAGGG	CTCAG/gt	99	33051	MBD	ANK repeat 3
5	5	ag/AATGG	CAGAG/gt	99	4727	MBD	ANK repeat 4
6	6	ag/GATGG	CCAAG/gt	186	426	MBD	ANK repeats 5 & 6
7	7	ag/ATGAT	CTGAG/gt	24	2862	MBD	
8	8	ag/AGTGG	CCAGG/gt	99	1527	MBD	ANK repeat 7
9	9	ag/AATGG	CTAGG/gt	99	7554	MBD	ANK repeat 8
10	10	ag/GATGG	CTAAG/gt	99	5872	MBD	ANK repeat 9
11	11	ag/AATGG	CCCTG/gt	198	2117	MBD	ANK repeats 10 & 11
12	12	ag/AATGG	CAGAG/gt	99	164	MBD	ANK repeat 12
13	13	ag/TCTGG	ACATT/gt	99	6485	MBD	ANK repeat 13
14	14	ag/CGTGG	CCAGG/gt	99	9456	MBD	ANK repeat 14
15	15	ag/GAGGA	CCAAG/gt	198	3187	MBD	ANK repeats 15 & 16
16	16	ag/AAGGG	GGAAAG/gt	99	524	MBD	ANK repeat 17
17	17	ag/AACGG	CCAAG/gt	99	4116	MBD	ANK repeat 18
18	18	ag/AATGG	CTAAG/gt	198	4732	MBD	ANK repeats 19 & 20
19	19	ag/AGTGG	CAAAG/gt	99	684	MBD	ANK repeat 21
20	20	ag/CTTGG	CCAAG/gt	99	3929	MBD	ANK repeat 22
21	21	ag/AACGG	CTGGG/gt	99	925	MBD	ANK repeat 23
22	22	ag/AATGG	CCACA/gt	99	9228	MBD	ANK repeat 24
23	23	ag/ACTAT	AGAGG/gt	73	1384	MBD	
24	24	ag/CTCTT	TTCAG/gt	54	6977	MBD	
25	24	ag/GTGTAT	GACAG/gt	145	6307	SBD	
26a	25	ag/CCCTC	ACCAG/gt	103	707	SBD	
26b	25	ag/TTCCG	ACCAG/gt	88	707	SBD	alt. 3' splice site
27	26	ag/GTGTG	TCAAG/gt	104	5138	SBD	
28	27	ag/CCGCG	AGCAG/gt	36	6451	SBD	
29	27	ag/TTTCC	CTTGG/gt	225	1501	SBD	Zu-5 motif
30	28	ag/TAAAC	CTTGG/gt	99	983	SBD	
31	29	ag/GCCTG	TGAAAG/gt	155	2637	SBD	Zu-5 motif
32	30	ag/TACTG	TGCAG/gt	212	519	SBD	Zu-5 motif
33	31	ag/GCTCA	AACAG/gt	205	2444	SBD	
34	32	ag/GTGGG	GCCAG/gt	97	2365	SBD	
35	33	ag/GTCTC	TGGAG/gt	229	1100	SBD	
36	34	ag/GTGTG	TCAAG/gt	126	2757	SBD	
37	35	ag/GTACG	CAAAAG/gt	123	2253	SBD	
38	36	ag/GAATC	AAGAG/gt	33	1919	SBD	
39	37	ag/ATCGA	AAAAA/gt	22	2795	SBD	
40	38	ag/ATGAT	TGAAG/gt	6255	1523		220 kD insert
41	39	ag/ATCCA	GACAG/gt	75	2440	DD	
42	40	ag/AATTA	TACAG/gt	132	1569	DD	
43	41	ag/ATACC	TGAAG/gt	144	2383	DD	
44	42	ag/GGTTC	TATTG/gt	286	1662	CTD	
45	43	ag/TTTGA	AAAAA/gt	292	2727	CTD	
46	43	ag/GAGCT	TTCAG/gt	93	464	CTD	
47	44	ag/GGAGA	AGAAG/gt	84	111	CTD	α -helix

Exon #	Previous exon #	5' splice	3' splice	Length (bp)	3' intron (bp)	Domain	Comments
48	45	ag/GTTAC	CAGAG/gt	165	1314	CTD	α -helix
49		ag/GTCAC	AAGAG/gt	183	3143	CTD	
50		ag/GC'TTT	AAAAAG/gt	92	3275	CTD	
51a	46	ag/GACAA		15**		CTD	stop
51b		ag/GACAA		184**		CTD	alt. stop

Fields represent: new and previous exon numbering, 5'- and 3'-splice sites, exon length (bp), preceding intron length (bp), corresponding functional domain, and additional comments. For exon 1' and 1, the length is measured from the beginning of the open reading frame and is denoted with an asterisk. Exon 26a/b represents alternative 3'-splice acceptor sites that allow for the addition or subtraction of 15 bp. The Zu-5 motif constitutes the minimal binding domain for β -spectrin [3]. Exon 40 encodes the 220 kD insert that is found in brain-specific 440 kD ankyrin-B [29]. Exon 51a/b represents alternative stop sites depending on the open reading frame. The terminal amino acid sequence for 51a is "DNNIE", while the terminal sequence for 51b is "QEAK". Length of exons 51a/b corresponds to the distances to the in-frame stop codons and is marked with double asterisks.