Exon Organization and Novel Alternative Splicing of Ank3 in Mouse Heart

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Abstract

Ankyrin-G is an adaptor protein that links membrane proteins to the underlying cytoskeletal network. Alternative splicing of the Ank3 gene gives rise to multiple ankyrin-G isoforms in numerous tissues. To date, only one ankyrin-G isoform has been characterized in heart and transcriptional regulation of the Ank3 gene is completely unknown. In this study, we describe the first comprehensive analysis of Ank3 expression in heart. Using a PCR-based screen of cardiac mRNA transcripts, we identify two new exons and 28 alternative splice variants of the Ank3 gene. We measure the relative expression of each splice variant using quantitative real-time PCR and exon-exon boundary spanning primers that specifically amplify individual Ank3 variants. Six variants are rarely expressed (<1%), while the remaining variants display similar expression patterns in three hearts. Of the five first exons in the Ank3 gene, exon 1d is only expressed in heart and skeletal muscle as it was not detected in brain, kidney, cerebellum, and lung. Immunoblot analysis reveals multiple ankyrin-G isoforms in heart, and two ankyrin-G subpopulations are detected in adult cardiomyocytes by immunofluorescence. One population co-localizes with the voltage-gated sodium channel NaV1.5 at the intercalated disc, while the other population expresses at the Z-line. Two of the rare splice variants excise a portion of the ZU5 motif, which encodes the minimal spectrin-binding domain, and these variants lack β-spectrin binding. Together, these data demonstrate that Ank3 is subject to complex splicing regulation resulting in a diverse population of ankyrin-G isoforms in heart.

Introduction

Normal excitation-contraction coupling in skeletal and cardiac myocytes requires that the relative arrangement of integral membrane proteins remains unperturbed throughout the contraction cycle. Some of these membrane proteins facilitate structural continuity between adjacent myocytes, while other membrane proteins mediate the ionic flux that underlies excitation-contraction coupling. Adaptor proteins like ankyrin are critical for the retention and scaffolding of integral membrane proteins to the underlying cytoskeleton. By scaffolding specific
membrane proteins and signaling molecules, ankyrins also contribute to the functional specialization of subcellular domains within myocytes.

Alternative splicing of an ankyrin gene produces different isoforms that display unique functions and subcellular distribution. In fact, alternative splicing of the *Ank3* gene results in numerous ankyrin-G isoforms that have been detected in various tissues including brain, skeletal muscle, lung, and kidney [1–6]. In heart, only one ankyrin-G isoform has been characterized, yet numerous membrane proteins have been shown to interact with ankyrin-G including connexin 43, β dystroglycan, and voltage-gated sodium channels [7–12]. These membrane proteins are expressed at distinct membrane domains in ventricular cardiomyocytes such as the intercalated disc, transverse(T)-tubule, and costamere [7, 8, 10–12]. Considering these findings, we hypothesize that the heart expresses more than one isoform of ankyrin-G.

This study is the first to report the comprehensive analysis of *Ank3* expression and alternative splicing in the heart. We demonstrate that the heart expresses multiple ankyrin-G isoforms and that ankyrin-G isoforms are detected at the intercalated discs and T-tubules of individually isolated cardiomyocytes. Using a PCR-based screen of cardiac mRNA, we identify two new exons in the *Ank3* gene and 28 novel splicing events in *Ank3* transcripts. We measure the relative ventricular expression of each splice junction by quantitative real-time PCR with transcript-specific primers. We demonstrate that expression of exon 1d, one of the five first *Ank3* exons, is restricted to heart and skeletal muscle. We evaluate some of the alternative splice isoforms for altered function and find that two rare isoforms of the ankyrin-G spectrin-binding domain lack spectrin binding. In summary, this study demonstrates that the *Ank3* gene is subject to complex splicing regulation resulting in numerous ankyrin-G isoforms in heart. We anticipate that these different isoforms underlie the diversity of ankyrin-G functions and subcellular distribution within cardiomyocytes.

**Materials and Methods**

**RNA isolation, reverse transcription, and PCR amplification of *Ank3* transcripts**

RNA was isolated from mouse tissues with GenEluteMammalian RNA kit (Sigma Aldrich). cDNA was synthesized using SuperScript III (Life Technologies). *Ank3* transcripts were amplified using nine overlapping primer sets using Phusion polymerase (Finnzymes) from mouse heart cDNA. PCR products were purified, ligated into pCR2.1-TOPO vector (Life Technologies), and sequenced.

**Quantitative RT-PCR analysis of alternative *Ank3* transcripts**

Exon-exon boundary spanning primers containing ~12 base pairs from each exon were designed to PCR-amplify specific *Ank3* splice junctions as previously described [13]. cDNA was synthesized from mRNA isolated from three age- and sex-matched mice. For each primer set, quantitative rt-PCR was performed in triplicate using SYBR Green Jumpstart Taq mix (Sigma Aldrich) and experiments were repeated three times. Analysis of the data was performed using a modified version of the Pfaffl method to incorporate primer efficiencies [14]. First, alternative *Ank3* splice junctions were grouped according to shared exons (e.g. E15/16 is grouped with E15/17). The fold difference was determined using the highest threshold cycle (*C*_T) value within that group as a reference. Percentage of abundance of each junction was calculated by comparing the *C*_T value of that junction to the sum of all *C*_T values for that group (e.g. the sum of E15/16 and E15/17 equal 100%). All errors were propagated through the fold difference and percentage calculations as standard deviations. The results were graphed with individual bars.
Molecular cloning of β-spectrins, ankyrin-G SBD and CTD variants

Spectrin repeats 13 to 17 of β1- and β2-spectrins were PCR-amplified from heart cDNA using primers for β1-spectrin (TCTATGTCATCTCCGATGAGATCCC, CTACAGTGACTCCAGGAACTAGAC) and β2-spectrin (AGAGTGCTGTCTCCATGTTG, CTAAGTATCCACTGCTGCTGGG). PCR products were sequenced to confirm the partial cDNAs of β1- and β2-spectrins. Products were subcloned into pGEX6p-1 for bacterial over-expression. Novel ankyrin-G SBD variants were generated by ligating Ank3 fragments PCR-amplified with primer sets 5 and 6 into pcDNA3.1. Ank3 fragments with novel splice junctions lacking exon 31 were PCR-amplified with primers (ATGACGGAGGAAATTATGACCAC, GGAGAGAGAACTTATCGTCCTT). Ank3 fragments PCR-amplified with primer set 6 used primers (GAACTTATCGCTCCCTGG, TCATGGTGATCGGCTTATG). Novel ankyrin-G CTD variants were PCR amplified with the primer set (ATGAGGATGGCGATAGTAGC, AACTTCTCCCTGCTTAGGCT) and subcloned into the lenti-viral vector pCDH1-MCS (Systems Biosciences).

In vitro binding assays

GST-fusion proteins of β1- and β2-spectrins were over-expressed and purified as described [13]. In vitro translated (IVT) products of AnkG-SBD variants (wt, ΔE31, ΔE28-31) were prepared using TnT T7-Coupled Reticulate Lysate System (Promega) and binding assays were performed as described [13].

Immunoblot analysis of ankyrin-G expression

Frozen mouse cerebellum and heart were pulverized with a mortar and pestle and resuspended in 4 volumes of RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 1 mM PMSF and 1X protease inhibitor cocktail (Sigma Aldrich)). Lysate was homogenized with a dounce homogenizer, incubated on ice for 30 minutes, and then centrifuged at 20,000 x g for 15 minutes at 4°C. Supernatants were collected and protein lysates were separated by SDS-PAGE and transferred to nitrocellulose membrane (GE Healthcare). Membranes were incubated with affinity-purified ankyrin-G IgG (0.5 μg/ml) or Na+/H+ exchange regulatory factor (NHERF) (Sigma Aldrich) overnight at 4°C. Immunoreactive polypeptides were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Isolation and imaging of individual adult mouse cardiomyocytes

Adult mouse cardiomyocytes were isolated as described previously [15]. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Welfare Committee at University of Texas Health Science Center at Houston (permit #13–083). 3 month-old mice were anesthetized with intraperitoneal injection of tribromoethanol-Avertin (Sigma T48402) at 0.2 ml/10g of 1.25% solution, euthanized by removing the heart, and all efforts were made to minimize suffering. Hearts from wild type mice were placed in ice-cold saline and the aorta was cannulated. Hearts were first perfused with warm perfusion buffer for a few minutes, followed by perfusion with digestion buffer containing collagenase (Worthington, Collagenase type II 305U/mg). Once digested, hearts were minced and
triturated, then centrifuged at 300 rpm x 5 minutes at 4°C. Supernatant was removed and cells were immediately fixed by adding ice-cold 100% ethanol in excess, and kept in -20°C until use.

Cells were washed in ice-cold phosphate-buffered saline (PBS, pH 7.4) 3x. Cells were then blocked with 5% normal goat serum and 0.075% TritonX-100 for 30 minutes at room temperature then incubated in primary antibodies overnight at 4°C. Primary antibodies used were: voltage-gated sodium channel NaV1.5 (1:500, [16], ankyrin-G (1:50, clone N106/20, UC Davis/NIH NeuroMab Facility), α-actinin (1:200, Sigma), and ankyrin-G (1:50, [17]). Secondary antibodies used were goat anti-rabbit conjugated to Alexa Fluor 488 and goat anti-mouse conjugated to Alexa Fluor 568 (1:500, LifeTechnologies). Hoechst 33258 (1:1000, LifeTechnologies) was used for nuclear staining after removal of the secondary antibody. ProLong Gold Antifade reagent (LifeTechnologies) was used for mounting coverslips. Images were obtained with a Nikon A1 confocal microscope (Nikon, Melville, NY) equipped with 40X oil, numerical aperture 1.4 objective.

Isolation and viral transduction of mouse neonatal cardiomyocytes
Primary cultures of cardiomyocytes were prepared from P1 wild-type neonatal mouse hearts as previously described [18]. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Welfare Committee at University of Texas Health Science Center at Houston (permit #13-083). Neonatal mice were euthanized by decapitation and all efforts were made to minimize suffering. Briefly, hearts were enzymatically and mechanically dispersed in Ham’s F10 with 0.05% trypsin for two 15-minute incubations at 37°C followed by a 50-minute incubation in collagenase type II (0.2mg/mL, Sigma). Pelleted and dispersed myocytes were pre-plated for 4 hours at 37°C in complete growth media (40% Ham’s F-10, 40% Dulbecco’s Modified Eagle’s Media with high glucose, and 20% fetal bovine serum). Non-adherent cardiomyocytes were pelleted, re-suspended in fresh complete growth media, and plated on MatTek plates (MatTek Corporation, Ashland, MA). 48 hours later, the media was replaced with defined growth media (1 μg/mL insulin, 5μg/mL transferrin, 1nM LiCl, 1nM NaSeO₄, 0.1nM thyroxine) to prevent overgrowth of fibroblasts. Following 3 days in culture, cardiomyocytes were transduced with lenti-viral constructs of GFP-tagged full-length or truncated AnkG-CTDs, and imaged 4 days later by confocal microscopy. Primary antibodies used were: GFP (1:500, SC-8334, Santa Cruz Antibodies), α-actinin (1:1000, Sigma), and ankyrin-G (1:500, [17]).

Statistical Analysis
Statistical analyses were performed using Prism (GraphPad Software, Inc; version 6.0). One-way ANOVA followed by Tukey’s multiple comparison test or unpaired Student’s t-test was used to analyze C_T values from qt-PCR experiments to determine whether the expression differences in splice junctions were statistically significant.

Results
The heart expresses a heterogeneous population of ankyrin-G isoforms
To determine if the heart expresses more than one ankyrin-G isoform, we performed immunoblot analysis of ankyrin-G protein expression in heart using a polyclonal antibody to ankyrin-G [10]. To confirm the specificity of the ankyrin-G antibody, we also performed immunoblot analysis of ankyrin-G expression in wild-type and ankyrin-G null cerebellums. In contrast to ankyrin-G null cerebellums, wild-type cerebellums express numerous ankyrin-G
immunoreactive polypeptides (Fig 1A). Likewise, we detected multiple ankyrin-G isoforms in heart lysate from adult mice.

We performed confocal microscopy to evaluate ankyrin-G subcellular localization with resident proteins of the intercalated disc and T-tubules in cardiomyocytes isolated from adult mice. Using a monoclonal antibody to ankyrin-G, we find a population of ankyrin-G co-localizes with the voltage-gated sodium channel NaV1.5 at the intercalated discs (Fig 1B). The commercially available monoclonal antibody was generated against the spectrin-binding, death, and C-terminal domains of human ankyrin-G. Additionally, using a polyclonal antibody to ankyrin-G, we demonstrate another population of ankyrin-G co-localizes with α-actinin, a cytoskeletal component of the Z-line that underlies 60% of the T-tubular network (Fig 1C)

The polyclonal antibody was generated against the death and C-terminal domains of human ankyrin-G [10]. Taken together, these data demonstrate that the heart expresses a heterogeneous population of ankyrin-G isoforms that localize to the intercalated disc and/or Z-lines of individual myocytes.

Identification of novel Ank3 exons and splice variants in cardiac mRNA

To examine Ank3 mRNA expression and identify novel alternative splice variants, we performed reverse-transcriptase PCR on ventricular mRNA using nine overlapping primer sets (Table 1) that spanned the length of the Ank3 gene (Fig 2A). The integrity of the mRNA was evaluated by ethidium bromide staining of a 1% agarose gel. The presence of sharp 28S and 18S rRNA bands at a ratio of 2:1 and the absence of low molecular weight smears demonstrate that the mRNA was intact and not degraded (S1 Fig). We identified a variety of novel alternative Ank3 mRNA transcripts including: 10 variants of the membrane-binding domain, 12 iterations of the spectrin-binding domain, and 6 permutations of the C-terminal regulatory domain (Fig 2B). The splicing patterns include exon-skipping (exon 16 in MBD), use of alternative 5’-donor site (exon 39 in CTD) or alternative 3’-acceptor site (exon 28 in SBD), and excision of internal exonic sequence (exon 44 in CTD). We also identified two new exons in the spectrin-binding domain (exons 27 and 30). Neither exon alters the open reading frame nor do they disrupt the minimal spectrin-binding domain. Table 2 includes an updated nomenclature for Ank3 exon organization in addition to the lengths of individual exons and the intervening introns. Transcripts encoding the muscle-specific ankyrin-G isoforms that lack the membrane-binding domain (e.g. G107) initiate at the alternative start site in exon 25 and include exons 46–49. We have limited the scope of this study to the alternative splicing of full-length Ank3 transcripts.

To determine the relative mRNA expression of particular Ank3 transcripts, we designed exon-exon boundary spanning primers that selectively PCR amplify transcripts based on their unique exon junctions. Specifically, one PCR primer spans the junction of two adjacent exons and the full-length primer is required to amplify a PCR product (Fig 2C). PCR conditions (primer lengths, annealing temperatures) are optimized for quantitative real-time PCR analysis such that the efficiency of each primer set falls within the range of 90–110%. Nucleotide sequences, annealing temperatures, and primer efficiencies for all 28 primer sets are listed in S1 Table. We have previously used this technique to demonstrate the expression of alternative ANK2 transcripts in mouse and human hearts [13].

Expression of five Ank3 alternative first exons in various tissues

The Ank3 gene has five first exons within ~409 kb of the second exon. To determine the relative expression of each first exon in heart, we performed quantitative real-time PCR analysis using exon-exon boundary spanning primers that selectively detect specific exon junctions.
**Fig 1. Immunoblot and immunofluorescent detection of ankyrin-G isoforms in heart.**

(A) Ankyrin-G immunoblot demonstrates expression of different isoforms in heart (arrows). Loss of ankyrin-G immunoreactive polypeptides in ankyrin-G null cerebellums demonstrates antibody specificity. NHERF1 immunoblot demonstrates similar loading of protein lysates. 

(B) Immunofluorescent co-localization of ankyrin-G (monoclonal antibody) and the voltage-gated sodium channel NaV1.5 at intercalated discs of an individually isolated adult cardiomyocyte (white arrows and highlighted in the inset).

(C) Immunofluorescent co-localization of ankyrin-G (polyclonal antibody) and α-actinin at Z-lines of individually isolated adult cardiomyocytes (white arrows and highlighted in the inset). Scale bar represents 10 microns.

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The expression of each first exon is represented relative to the expression of the other first exons. Therefore, the sum of expression of exons 1a, 1b, 1c, 1d, and 1e equals 100%. Rare alternative splice junctions (< 1%) are indicated by gray, dashed lines (Fig 3A) and not included in the summation, although the primary data is provided in S2 Table.

In heart, we compared the relative expression of the first exons in ventricular and atrial mRNA isolated from three mouse hearts. We found that Ank3 transcripts with first exons 1b, 1c, or 1d are more abundant than transcripts with first exons 1a or 1e (Fig 3C). In addition to heart, Ank3 gene products have been detected in numerous tissues including skin, skeletal muscle, lung, kidney, and brain [1–6, 20–22]. To determine if first exons display tissue-specific expression, we measured the relative expression of the first exons in mRNA transcripts isolated from kidney, brain, and skeletal muscle (whole tissue). In skeletal muscle, the relative expression of Ank3 transcripts with first exon 1b is ~60%, 1c is ~20%, and 1d is ~10% (Fig 3C). Ank3 transcripts with first exons 1a and 1e are expressed less than 5% in skeletal muscle. In kidney and brain, we found that Ank3 transcripts with first exons 1b and 1c are more abundant than transcripts with first exons 1a and 1e (Fig 3C). Interestingly, Ank3 transcripts with first exon 1d were virtually undetectable in kidney and brain. Exon 1d was also undetected in mRNAs isolated from cerebellum and lung (data not shown). To validate the similarity of mRNA quantity and quality isolated from the different mouse tissues, we demonstrated equivalent expression of the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) in mRNA samples from the ventricle, atria, skeletal muscle, brain, and kidney (Fig 3B).

### Cardiac expression of Ank3 splice variants encoding ankyrin-G MBD

Ank3 exons 1–21 encode the ankyrin-G MBD (Table 2). During the screen for alternative transcripts, we identified four alternative splice variants (± exon 16 and ± exon 18) of the MBD (Fig 4A). To measure the relative expression of these alternative splice variants, we performed quantitative real-time PCR using exon-exon boundary spanning primers on mRNAs isolated from mouse ventricular tissue. We found that splice variants including exon 16 or exon 18 are about four-fold more abundant than transcripts that lack these exons (Fig 4B).

### Cardiac expression of Ank3 splice variants encoding ankyrin-G SBD

During the screen for cardiac-specific Ank3 transcripts, we identified two new exons (27 and 30) in the SBD (Fig 5A and Table 2). We also identified 12 novel alternative spliced transcripts. We measured the relative expression of each splice junction by quantitative real-time PCR.

### Table 1. Ank3 primer sets.

| #  | 5’ primer | 3’ primer |
|----|-----------|-----------|
| 1  | ATGAGTGAAAGCCAAAGGAGAAG | GCAACGTGTAAGGGAGTGATGTC |
| 2  | CTGCTCGGAAAGCCACGAAAGG | GAGCCCCATCTTGAGGACGATAAC |
| 3  | GGTGACATCTATCCAGGCCGTAACC | GTTGAACATCGGCTTTCTACTCAG |
| 4  | GCCAAATCCGGAACGGCTTGAAGTC | CTTGAGATATATCCCATATCCAG |
| 5  | GAAGGTGTACCGGGAAGGAAATTATAG | CCGAAGGAGGATAAGTTCTTCTTC |
| 6  | CAGCGGACACGTAGATAATGTAAC | CTTGTACCATGGACAGCCCTTC |
| 7  | CTATACGCTCTTCCGAGGAGCGAAGC | CAAAGCTCTGCTCTCCCTTCT |
| 8  | GTGCTGTCTTCCAAACACGGTTCAG | CCACGTAGCTTACTATCCG |
| 9  | GATATCAGGATGCGATAGTAG | CATTCTCTTCTGCTTCCAC |

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Four splice junctions (E22/24, E26/32, E30/31, E29/32) are so rare (< 1%) that we excluded them from the determination of relative mRNA expression, although the primary data is provided S2 Table. We identified three Ank3 transcripts with unique 3'-splice junctions with exon 22: E22/23, E22/24, and E22/26. Expression of E22/24 is less than 1%, while E22/26 is ~1.5 fold more abundant than E22/23 (Fig 5B). We also identified three Ank3 transcripts with unique 5'-splice junctions with exon 26: E22/26, E23/26, and E24/26. While both E22/26 and E23/26 are equally abundant (~45% each), E24/26 is much less abundant (~5%). Lastly, we identified three Ank3 transcripts with unique 5'-splice junctions with exon 28: E26/28, E26/tr28 (tr:
Table 2. Ank3 exon organization.

| Exon # | Previous exon # | 5'-splice | 3'-splice | Length/CDS (bp) | 3' intron (bp) | Domain | Comments |
|--------|----------------|-----------|-----------|----------------|----------------|--------|----------|
| 1a     |                | AGCAG/gt  | 357/57    |                | 85099          | MBD    | Start    |
| 2a     | ag/GGTGA       | AAAAG/gt  | 39        |                | 49954          | MBD    |          |
| 1b     | 1              | AAAAG/gt  | 577/63    |                | 172527         | MBD    | Start    |
| 1c     | AAAAG/gt       | 861/114   | 79015     |                |                | MBD    | Start    |
| 1d     | AAGAGA/gt      | 358/36    | 1942      |                |                | MBD    | Start    |
| 1e     | AGAGAG/gt      | 343/24    | 20966     |                |                | MBD    | Start    |
| 2b     | 2              | AGCAG/gt  | 102       |                | 430            | MBD    | ANK Repeat 1 |
| 3      | 3              | AGATGG    | 99        |                | 199            | MBD    | ANK Repeat 2 |
| 4      | 4              | AAAGAG    | 99        |                | 6475           | MBD    | ANK Repeat 3 |
| 5      | 5              | AAATGG    | 99        |                | 6501           | MBD    | ANK Repeat 4 |
| 6      | 6              | AAGAGG    | 186       |                | 1924           | MBD    | ANK Repeat 5, 6 |
| 7      | 7              | AGTGGG    | 99        |                | 25689          | MBD    | ANK Repeat 7 |
| 8      | 8              | AGATGG    | 99        |                | 17180          | MBD    | ANK Repeat 8 |
| 9      | 9              | AGAAGG    | 99        |                | 3518           | MBD    | ANK Repeat 9 |
| 10     | 10             | AAATGG    | 198       |                | 3590           | MBD    | ANK Repeat 10, 11 |
| 11     | 11             | AAATGG    | 99        |                | 2941           | MBD    | ANK Repeat 12 |
| 12     | 12             | AGCTGGG   | 99        |                | 1964           | MBD    | ANK Repeat 13 |
| 13     | 13             | AGAGAG    | 99        |                | 2369           | MBD    | ANK Repeat 14 |
| 14     | 14             | AGATGG    | 99        |                | 4376           | MBD    | ANK Repeat 15, 16 |
| 15     | 15             | AAATGG    | 198       |                | 293            | MBD    | ANK Repeat 17 |
| 16     | 16             | AGAAGG    | 99        |                | 7084           | MBD    | ANK Repeat 18 |
| 17     | 17             | AAATGG    | 198       |                | 925            | MBD    | ANK Repeat 19, 20 |
| 18     | 18             | AGAGAG    | 99        |                | 4497           | MBD    | ANK Repeat 21 |
| 19     | 19             | AAATGG    | 99        |                | 89             | MBD    | ANK Repeat 22 |
| 20     | 20             | AAATGG    | 99        |                | 667            | MBD    | ANK Repeat 23 |
| 21     | 21             | AAATGG    | 99        |                | 5108           | MBD    | ANK Repeat 24 |
| 22     | 22             | AGATGG    | 99        |                | 177            | SBD    |          |
| 23     | 23             | AAATGG    | 63        |                | 15927          | SBD    |          |
| 24     | 24             | AGATGG    | 54        |                | 1628           | SBD    |          |
| 25     | 25             | AGCAG/gt  | 384/16    |                | 1503           | SBD    | 3' intron to ATG of E25/alt. start |
| 26     | 26             | AGCAG/gt  | 124       |                | 4575/4590      | SBD    |          |
| 27     | 27             | AGCAG/gt  | 12        |                | 2935           | SBD    | new exon |
| 28     | 28             | AGCAG/gt  | 103       |                | 18455          | SBD    |          |
| 28'    | 28             | AGCAG/gt  | 88        |                | 18455          | SBD    | 5'-truncated exon 28 |
| 29     | 29             | AGCAG/gt  | 107       |                | 2381           | SBD    |          |
| 30     | 30             | AGCAG-gt  | 33        |                | 2453           | SBD    | new exon |
| 31     | 31             | AGCAG-gt  | 225       |                | 2767           | SBD    | ZU5 motif |
| 32     | 32             | AGCAG-gt  | 155       |                | 14649          | SBD    | ZU5 motif |
| 33     | 33             | AGCAG-gt  | 212       |                | 2114           | SBD    |          |
| 34     | 34             | AGCAG-gt  | 208       |                | 1457           | SBD    |          |
| 35     | 35             | AGCAG-gt  | 97        |                | 661            | SBD    |          |
| 36     | 36             | AGCAG-gt  | 229       |                | 618            | SBD    |          |
| 37     | 37             | AGCAG-gt  | 126       |                | 828            | DD     |          |
| 38     | 38             | AGCAG-gt  | 123       |                | 1721           | DD     |          |
| 39     | 39             | AGCAG-gt  | 82        |                | 3891           | DD     |          |
| 39'    | 39             | AGCAG-gt  | 46        |                | 3927           | DD     | 3'-truncated exon 39 |
| 40     | 40             | AGCAG-gt  | 7694      |                | 589            | SBD    | brain-specific exon |

(Continued)
Interestingly, exon 27 is one of the newly identified exons and transcripts containing this exon (E27/28) are the most abundant (~55–60%), followed by transcripts with the splice junctions of E26/tr28 (~25%) and then E26/28 (~15%).

Two ankyrin-G SBD isoforms lack β-spectrin binding

In the spectrin-binding domain, the ZU5 motif represents the minimal β-spectrin binding domain and is encoded by exons 31 and 32 [23]. We identified two novel alternative Ank3 splice variants that lacked exon 31. While these transcripts in heart are rare (<1%) (S2 Table), we evaluated β-spectrin binding to GST-fusion proteins of these SBD isoforms (SBD ΔE31 and SBD ΔE28–31) (Fig 6A). The ankyrin-binding domain has been mapped to spectrin repeats 14 and 15 [24–26]; therefore, we generated GST-fusion proteins of spectrin repeats 13 through 17 for both β1- and β2-spectrins. Briefly, in vitro translated and 35S-labelled SBD protein fragments were incubated with GST, GST β1-spectrin, or GST β2-spectrin. Glutathione sepharose precipitated radiolabelled protein complexes that were resolved by SDS-PAGE and visualized by autoradiography. While β1- and β2-spectrins bind wild-type SBD, the SBD displays greater binding capacity for β1-spectrin than for β2-spectrin (Fig 6B). In contrast, neither SBD ΔE31 nor SBD ΔE28–31 bound to spectrin demonstrating that partial loss of the ZU5 motif completely disrupts SBD binding to β-spectrin. Coomassie Blue Stain demonstrated equal loading of GST fusion proteins β1- and β2-spectrin (Fig 6C).

Cardiac expression of Ank3 splice variants encoding ankyrin-G CTD

The focus of this study is to identify novel variants of full-length ankyrin-G. Ank3 exons 46, 47, 48, and 49 encode an obscurin-binding domain that is uniquely expressed in truncated ankyrin-G isoforms that were initially characterized in skeletal muscle (e.g. G107) [2–4]. We were unable to detect these exons in full-length Ank3 transcripts in heart; therefore, the characterization of alternative splicing of this domain was excluded from this study.
Fig 3. Relative mRNA expression of alternative Ank3 first exons. (A) Diagram of alternative splicing of 5 first exons of the Ank3 gene. Gray dashed lines represent rare alternative spliced junctions (<1%). (B) qPCR analysis of hypoxanthine-guanine phosphoribosyltransferase (HPRT) expression (measured as cycle threshold number) in ventricle, atria, skeletal muscle, brain, and kidney from three mice (labeled M1–M3). (C) Relative mRNA expression of alternative first exons was measured in heart (ventricle and atria), skeletal muscle, brain, and kidney by qPCR analysis. Bar graphs represent technical replicates of qPCR samples and error bars represent standard deviations. Statistical analysis was performed with one-way ANOVA with Tukey’s multiple comparison test (*** p-value ≤0.001, ns: not significant) to assess the significance of expression differences between different splice variants (i.e. 1a vs. 1b).

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We identified six novel Ank3 splice variants of the CTD (Fig 7A). Exon 40 was never detected in any cardiac transcripts and we identified two splice junctions that excise this brain-specific exon. The splice junction of E39/41 is predominant in heart and the use of an alternative 5'-donor site within exon 39 is exceedingly rare (<1%) (S2 Table). We identified three alternative splice variants of exon 44: E43/44, E43/tr44, and a medial excision of 267 base pairs from exon 44. Exon 44 is 679 bp in length and the 5'-truncation removes 588 bp (tr44). The splice variant E43/44 is more abundant than E43/tr44 (~55% versus ~40%), while the splice variant that lacks 267 base pairs in the middle of exon 44 is less common (~5%) (Fig 7B).

Exogenous ankyrin-G CTD isoforms display similar subcellular expression as endogenous ankyrin-G

Ankyrin-G is localized to the intercalated disc and Z-lines in adult ventricular cardiomyocytes (Fig 1B–1C). We have previously demonstrated that the C-terminal domain of ankyrin-B plays an important role in targeting this related adaptor protein to the sarcomeric M-line in cardiomyocytes [27]. To assess whether the C-terminal domain of ankyrin-G contains targeting motifs, we evaluated the subcellular localization of the two most abundant CTD variants in virally transduced neonatal cardiomyocytes. Lenti-viral expression in neonatal cardiomyocytes is preferable to adult myocytes because they survive in culture for 7–10 days compared to adult myocytes, which survive for ~24 hours. Lenti-viral constructs with GFP tags were made of the ankyrin-G CTD containing the full-length exon 44 (679 bps) or the 5’-truncated exon 44 (lacking 588 bps) (Fig 8A). Expression of lenti-viral constructs was confirmed by immunoblot analysis of the GFP tag (Fig 8B). We evaluated the co-localization of these CTD variants to α-actinin in neonatal cardiomyocytes. Both variants displayed diffuse cytosolic localization and lacked the defined patterning of sarcomeres. Moreover, both variants displayed similar subcellular distributions as endogenous ankyrin-G, which was detected using the polyclonal
ankyrin-G antibody (Fig 8C). One of the limitations of neonatal cardiomyocytes as a model system is that they lack fully developed subcellular domains, which may be required for the proper targeting of ankyrin-G.

### Discussion

This study is the first to describe a comprehensive analysis of Ank3 expression in heart. We identified two new exons and 28 novel alternative splicing events in the Ank3 gene. An update to the Ank3 exon organization and nomenclature is provided in Table 2. Using quantitative real-time PCR with exon-exon spanning primers, we demonstrate that alternative Ank3 splice variants are expressed at similar levels in three separate hearts. Moreover, we find that
expression of Ank3 transcripts initiated with exon 1d is restricted to the heart and skeletal muscle as these transcripts are undetectable in brain, kidney, cerebellum, and lung.

The majority of alternative splicing is situated within the coding region of the spectrin-binding domain, specifically within the exons immediately 5’ of the minimal spectrin-binding domain ZU5A, which is encoded by exons 31 and 32 [28, 29] (Figs 2 and 5). The newly identified exons 27 and 30 are also alternatively spliced, but neither exon alters the open-reading frame of ankyrin-G and exon 30 is rarely expressed in cardiac transcripts (Fig 5 and Table 2). Interestingly, exon 30 is included in three expressed sequence tags (CO430616, CF745086, CV555908) that were isolated from neural tissue. We identified two rare alternative splice variants that remove exon 31 (junctions of exons 26 to 32 and exons 29 to 32). In vitro translated products of these SBD isoforms lacked binding to both β1- and β2-spectrins (Fig 6). Moreover, wild-type ankyrin-G SBD bound more β1-spectrin than β2-spectrin. Ankyrin-G SBD binding to β-spectrin isoforms (β1, β2, β3, and β4) has yet to be studied extensively, much less the influence of alternative splicing on this process.

We demonstrated that the heart expresses five distinct ankyrin-G isoforms by immunoblot analysis. Imaging of individual cardiomyocytes reveals the expression of two ankyrin-G sub-populations. One population co-localizes with the voltage-gated sodium channel NaV1.5 at the intercalated disc (ICD), while the other population localizes to the Z-line. Interestingly, the ICD population is detected using the ankyrin-G monoclonal antibody, while the Z-line population is detected with the ankyrin-G polyclonal antibody. These differences may be the result of different antigens used for antibody production. The monoclonal antibody was generated
against an antigen encoded by exons 26, 28, 31–39, 41–45, and 50. In addition, splicing variations present in the antigen include 5’-truncation of exon 28, 3’-truncation of exon 39, and medial excision of 267 base pairs from exon 44. The antigen for the polyclonal antibody consisted of the death and C-terminal domains, which included the full-length exon 44.

The expression of numerous ankyrin-G isoforms in a specific tissue is quite common. Understanding the function of each isoform remains a challenge, but insights into an isoform’s function may be inferred from its subcellular localization. In kidney and muscle, the truncated AnkG119 isoform is located in the Golgi apparatus and is necessary for transport of sodium potassium ATPase (NKA) to the endoplasmic reticulum [1, 30]. Many alternative ankyrin-G isoforms that lack membrane-binding domains are expressed in skeletal muscle [2–4]. The obscurin-binding domains encoded by Ank3 exons 46–49 in the C-terminal domain regulate the sarcolemmal localization of these isoforms [2]. While a previous study demonstrated skeletal muscle expression of a full-length ankyrin-G isoform with exons 46–49 [3], we were unable to detect a similar isoform in cardiac muscle.

To evaluate whether Ank3 exon 44 encoded any targeting motifs, we examined the subcellular localization of the two most abundant CTD variants in neonatal cardiomyocytes. Neither isoform displayed sarcomeric localization, similar to expression of endogenous ankyrin-G (detected with the polyclonal antibody) (Fig 8C). While neonatal cardiomyocytes are viable to express proteins introduced by lenti-virus, they lack the elaborate T-tubular network found in
adult cardiomyocytes. Proper subcellular targeting of ankyrin-G most likely requires fully developed membrane domains in adult myocytes.

Given the diversity of functions and subcellular localizations ascribed to ankyrin-G, it is not surprising that the heart expresses multiple ankyrin-G isoforms. In ventricular cardiomyocytes,
ankyrin-G expression overlaps the expression of voltage-gated sodium channels (NaV) at the intercalated disc and T-tubules. Interestingly, the voltage-gated sodium channels display differential subcellular localization with NaV1.5 predominantly expressed at the intercalated disc, while the other sodium channels (NaV1.1, 1.3, and 1.6) are predominantly localized to the T-tubules [31–33]. In addition to interacting with NaV1.5 at the intercalated disc, ankyrin-G has also been shown to interact with plakoglobin and connexin 43 [11]. Whether the intercalated disc expresses a heterogeneous population of ankyrin-G isoforms is not known, but such a scenario could account for the diversity of ankyrin-G interactions. Finally, while it has been shown that ankyrin-G is required for the retention of dystrophin and β-dystroglycan at the costameres in skeletal muscle (and presumably cardiac muscle) [7, 8], the cDNA of this particular ankyrin-G isoform has yet to be characterized. Future research efforts will focus on identifying full-length Ank3 transcripts in heart and evaluating these isoforms for specific functions.

In closing, there are tremendous opportunities for discovery in examining the splicing regulation of ankyrin genes. Our data suggests that the Ank3 gene is subject to complex transcriptional regulation in the heart. We propose that alternative splicing of the Ank3 gene results in a diverse population of ankyrin-G isoforms with each isofrom aggregating a unique set of proteins that impart specific functionality to unique cardiomyocyte domains such as the intercalated disc, T-tubule, or costamere.

Supporting Information

- **S1 Fig.** EtBr-stained agarose gel of mRNA isolated from 3 mouse hearts. (TIF)
- **S1 Table.** Primer sequences for qPCR analysis of Ank3 splice junctions. (DOCX)
- **S2 Table.** Average Cq values of rare Ank3 transcripts in 3 mouse hearts. (DOCX)

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Author Contributions

Conceived and designed the experiments: GY SRC. Performed the experiments: GY HCW MM. Analyzed the data: GY SRC. Wrote the paper: GY HWC SRC.

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