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# Molecular evolution of a-kinase anchoring protein (AKAP)-7: implications in comparative PKA compartmentalization

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## Abstract

**Background:** A-Kinase Anchoring Proteins (AKAPs) are molecular scaffolding proteins mediating the assembly of multi-protein complexes containing cAMP-dependent protein kinase A (PKA), directing the kinase in discrete subcellular locations. Splice variants from the AKAP7 gene (AKAP15/18) are vital components of neuronal and cardiac phosphatase complexes, ion channels, cardiac Ca<sup>2+</sup> handling and renal water transport.

**Results:** Shown in evolutionary analyses, the formation of the AKAP7-RI/RII binding domain (required for AKAP/PKA-R interaction) corresponds to vertebrate-specific gene duplication events in the PKA-RI/RII subunits. Species analyses of AKAP7 splice variants shows the ancestral AKAP7 splice variant is AKAP7 $\alpha$ , while the ancestral long form AKAP7 splice variant is AKAP7 $\gamma$ . Multi-species AKAP7 gene alignments, show the recent formation of AKAP7 $\delta$  occurs with the loss of native AKAP7 $\gamma$  in rats and basal primates. AKAP7 gene alignments and two dimensional Western analyses indicate that AKAP7 $\gamma$  is produced from an internal translation-start site that is present in the AKAP7 $\delta$  cDNA of mice and humans but absent in rats. Immunofluorescence analysis of AKAP7 protein localization in both rat and mouse heart suggests AKAP7 $\gamma$  replaces AKAP7 $\delta$  at the cardiac sarcoplasmic reticulum in species other than rat. DNA sequencing identified Human AKAP7 $\delta$  insertion-deletions (indels) that promote the production of AKAP7 $\gamma$  instead of AKAP7 $\delta$ .

**Conclusions:** This AKAP7 molecular evolution study shows that these vital scaffolding proteins developed in ancestral vertebrates and that independent mutations in the AKAP7 genes of rodents and early primates has resulted in the recent formation of AKAP7 $\delta$ , a splice variant of likely lesser importance in humans than currently described.

**Keywords:** A-Kinase anchoring protein, Protein evolution, PKA regulatory subunit, PKA compartmentalization, AKAP15/18

## Background

cAMP-dependent protein kinase A (PKA) is a broad-specificity serine/threonine protein kinase that is ubiquitously distributed among tissue types, and identified in organisms from fungi to humans. Inside cells, PKA exists as a tetrameric protein composed of two catalytic subunits and a regulatory subunit dimer. The PKA regulatory subunit (PKA-R) functions to maintain kinase inactivity in the absence of cAMP and form protein-protein interactions that compartmentalize the kinase.

The selective sequestration or compartmentalization of intracellular PKA has been supported by several studies showing that PKA is concentrated in discrete intracellular locales by A-kinase anchoring proteins, or AKAPs [1-4]. AKAPs bind to the regulatory (i.e., PKA-RI, PKA-RII or both) subunit dimer of the tetrameric PKA holoenzyme at an antiparallel four-helix domain termed the dimerization and docking (D/D) domain [5-7]. The AKAP-PKA-R interaction functions to sequester PKA in the vicinity of its phosphorylation substrates [2,8,9] and this selective concentration of PKA activity in part, explains how multiple diverse extracellular stimuli can result in highly specific and discrete PKA-mediated phosphorylation events [10,11].

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Originally named according to their apparent molecular weight, AKAPs are now designated by gene number (i.e., AKAP15/18 splice variants are referred to as AKAP7 splice variants). Currently there are 14 annotated AKAPs (AKAP1 to AKAP14). The PKA-regulatory subunit binding domain of AKAPs is the most recognizable element among the otherwise structurally diverse protein family. AKAPs are classified according to binding specificity for the PKA regulatory subunits; RI-, RII- or both RI and RII PKA regulatory domains (dual specific) [12]. The primary structure (i.e., amino acid sequence) of the PKARI/RII-binding domain is highly variable among AKAPs, though the secondary structure, which forms an amphipathic helix, contains several conserved hydrophobic residues that determine PKA-R-subunit specificity and binding affinity [13-15]. AKAPs also contain unique localization signal(s) which are responsible for AKAP targeting to sub-cellular compartments, and in many cases, AKAPs also contain enzyme-binding sites that enable the formation of multi-protein signaling complexes [16,17]. Various AKAP targeting domains have been shown to interact with G-protein coupled receptors (GPCRs; [18]), adenylyl cyclases [19], Exchange protein activated by cAMP (EPAC; [20]), and phosphodiesterases (PDEs) [21,22]. Hence, AKAPs function as signal processing hubs specifically linking upstream activation signals to downstream effector proteins.

AKAPs have been shown to regulate neuronal ion channels [23,24], memory and cognition [25-27], reproduction function [22,28], as well as renal [29] and cardiac function [11,30-35]. One AKAP that has been identified in many tissues and a variety of cell signaling pathways is AKAP7 (also known as AKAP15/18). Splice variants of the AKAP7 gene (AKAP7 $\alpha$ ,  $\beta$ ,  $\delta$ , and  $\gamma$ ) are dual-specificity AKAPs that have been extensively studied in central nervous system (CNS), striated muscle ion channel regulation [2,36,37], cardiac calcium cycling [38], and renal aquaporin transport [29].

AKAP7 $\alpha$ , the first AKAP7 splice variant identified, is a membrane bound protein containing a lipid modification domain in the amino terminus [39]. AKAP7 $\alpha$  binds to L-type Ca<sup>2+</sup> channels where it regulates the PKA-dependent phosphorylation of the ion channel [40]. AKAP7 $\alpha$  has also been shown to interact with sodium channels in the brain [2], and is required for feedback inhibition of the epithelial Na<sup>+</sup> channel (ENAC) [41]. AKAP7 $\alpha$ -mediated PKA anchoring to ion channels (Ca<sup>2+</sup> and Na<sup>+</sup>) provides a regulatory mechanism for channel function, whereby anchoring of PKA regulates the phosphorylation state of the ion channel and has a profound effect on channel "open probability" and ion movement [1,40,42,43]. Another extensively studied AKAP7 splice variant is AKAP7 $\delta$ , which was originally identified in rat kidney [29]. In rat renal principle cells, the AKAP7 $\delta$  splice variant functions to anchor PKA to intracellular vesicles containing the aquaporin water

channel and is required for aquaporin fusion to the plasma membrane [29]. Also in rats, AKAP7 $\delta$  has been identified in the cardiac sarcoplasmic-endoplasmic reticulum ATPase (SERCA) where it regulates PKA phosphorylation of a SERCA negative regulatory protein, phospholamban (PLN) and controls Ca<sup>2+</sup> re-uptake into the sarcoplasmic reticulum (SR) [38]. AKAP7 $\delta$  has become a prime example for the pharmacological potential of PKA-AKAP disruptor molecules, particularly in the treatment of human heart failure [44,45]. Much less is known about the AKAP7 $\beta$  and  $\gamma$  splice variants. AKAP7 $\beta$  differs from AKAP7 $\alpha$  by the inclusion of a third exon which imparts a unique membrane localization that differs from that found for AKAP7 $\alpha$  [46]. AKAP7 $\gamma$  has been cloned from human pancreas [46] and mouse oocytes [47], though the functional significance of this splice variant remains relatively unknown.

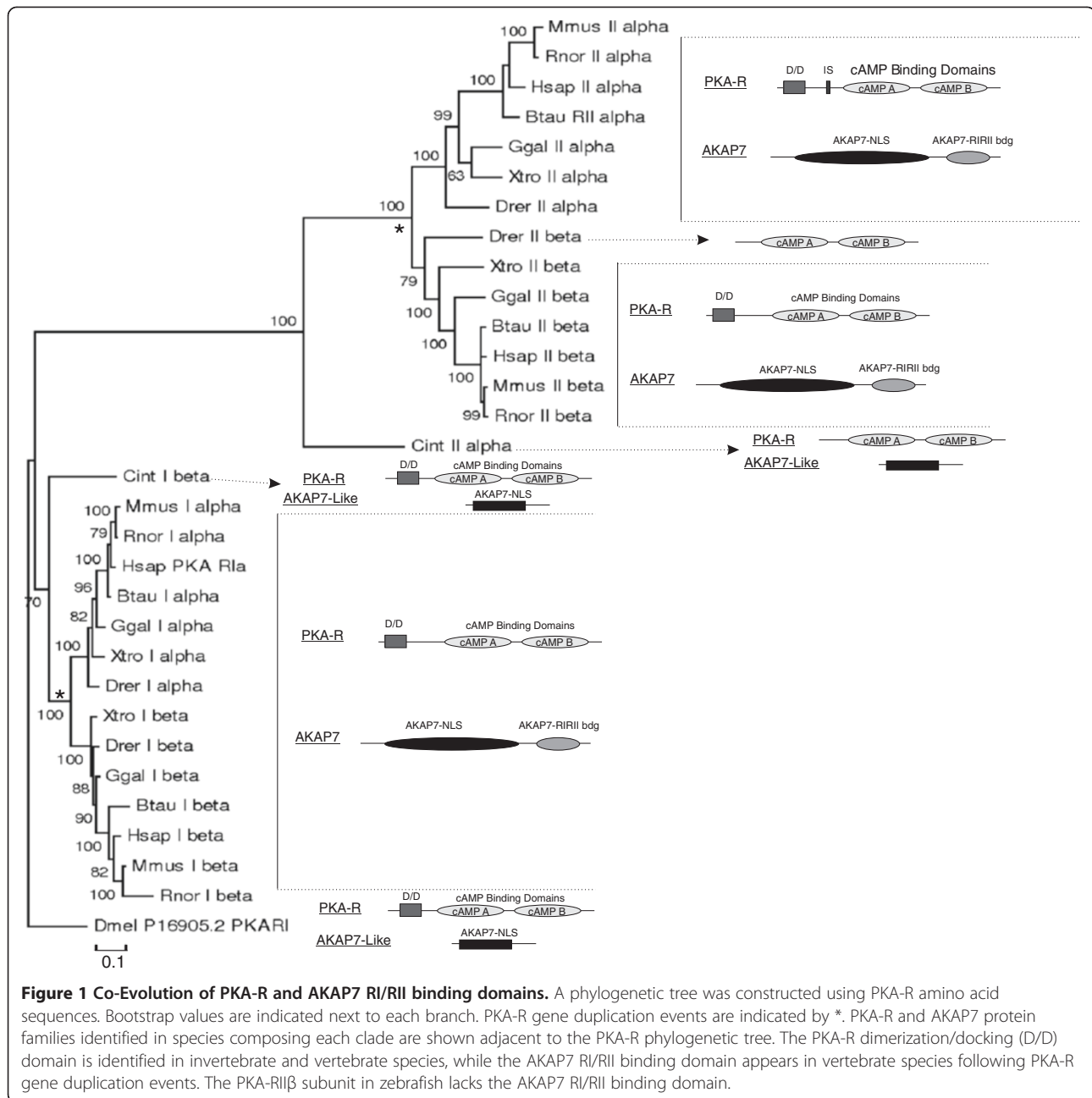
Though appreciation for the importance of AKAPs in cell signaling has grown exponentially in recent years, an analysis of the evolution and development of an AKAP remains absent. This study addresses several key questions in the molecular evolution of AKAP7 proteins including; 1) evolution/genesis of the AKAP7 RI/RII binding domain in relation to the PKA-R D/D domains 2) evolution of the AKAP7 gene structure 3) species analyses of AKAP7 splice variants, 4) sequence determination of previously un-reported mouse and human AKAP7 $\delta$  and 5) evolutionary conservation of key protein regions in short AKAP7 splice variants and rapid change in AKAP7 long form splice variants.

## Results

### Evolution of AKAP7 and PKA-R interaction motifs

The AKAP7 protein domain required for PKA-R binding (AKAP7 RI/RII binding domain; pfam 10470) is detected in transcripts of species from zebrafish to humans (Figure 1). The 3' terminal exon encoding the AKAP7 RI/RII binding domain first appears in the AKAP7 gene of lamprey and is conserved throughout vertebrates, placing AKAP7 binding domain genesis at the base of the vertebrate lineage (Figures 2 and 3). Transcripts containing (encoding) the AKAP7RI/RII binding domain were not identified in *Drosophila* and *Ciona*, though transcripts containing high sequence identity to the AKAP-7 NLS were identified (termed AKAP7-like; Figure 1). AKAP7-like genes were also identified in *Strongylocentrotus purpuratus* (Additional file 1: Figure S1), *Chlorella variabilis*, and *Daphnia pulex* (not shown). The resultant putative protein products of these AKAP7-like genes lack the AKAP7RI/RII binding domain and likely the PKA binding functionality of derived AKAPs.

The PKA-R subunit protein domain necessary for AKAP interaction (the Dimerization/Docking, D/D domain; pfam 02197) is conserved in the PKA-R subunits of insects, urochordates, chordates, and vertebrates (with

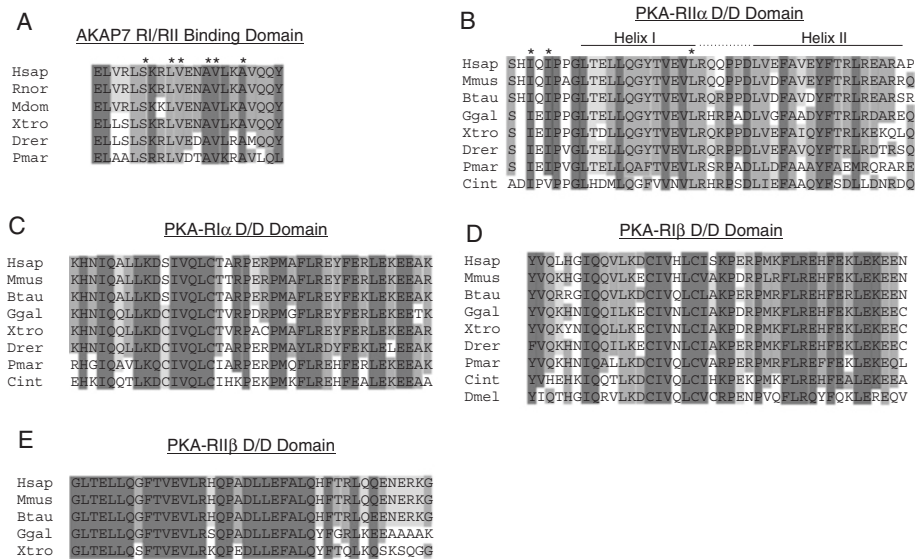


the exception of Zebrafish PKA-RII $\beta$ ; Figures 1 and 2). Specifically, the residues necessary for PKA-R/AKAP7 interaction [5,7,13,15] are conserved in all species examined (Figure 2). Appearance of the AKAP7 RI/RII binding domain corresponds with the vertebrate specific PKA-R gene duplication (Figure 2).

#### AKAP7 Splice variant evolution

AKAP7 gene structure, exon numbers and orientation were determined for multiple species (Figure 3, Additional file 1: Figure S1). AKAP7 $\alpha$  coding sequence in the AKAP7 gene and EST transcript evidence first

appears in basal teleosts (*Danio rerio*), and is conserved from teleosts to humans (Figure 3, Additional file 1: Figure S1). AKAP7 $\beta$  ESTs are only identified in mammals (pig; *Sus scrofa*; Additional file 1: Figure S1), though the AKAP7 $\beta$ -specific gene coding region is detectable from marsupials to mammals (Figure 3). AKAP7 $\delta$  is the most recent AKAP7 splice variant, with EST evidence supporting transcript presence in rats through humans. However exon data suggests this splice variant is absent from AKAP7 genes in species below laurasiatherian mammals (Additional file 1: Figure S1, Figure 4). AKAP7 $\delta$  is formed by the incorporation of a 19-nucleotide exon



**Figure 2 Amino Acid Alignments of AKAP7 RI/RII binding domain and PKA-R D/D domains.** Protein regions from the AKAP7 RI/RII binding domain (A), PKA-RIIα dimerization/Docking domain (D/D; B), PKA-RIIα D/D (C) PKA-RIIβ D/D, (D), and PKA-RIIβ (E) were aligned using Clustal X. (A) Residues in the AKAP7 RI/RII binding domain that have been previously identified as components of an amphipathic  $\alpha$ -helix necessary for PKA-R subunit interaction are indicated by (\*) and residues that are conserved are shaded. In the AKAP7 RI/RII binding domain,  $\alpha$ -helix forming residues are conserved in vertebrates from lamprey to humans. Residues in the PKA-RIIα (B), PKA-RIIα (C), PKA-RIIβ (D), and PKA-RIIβ (E) D/D domains forming the docking surface for the AKAP helix which are highly conserved are indicated by shading. Residues in PKA-RIIα that are essential for AKAP binding [48,49] are indicated by (\*). The spacer region between helices in PKA-RIIα is indicated by a dotted line.

located between 7- and 10Kb upstream of the putative AKAP7 $\gamma$  5' exon from cow to human (Figure 4). The appearance (expression) of the AKAP7 $\delta$  splice variant coincides with the loss of expression for AKAP7 $\gamma$  in both rat and marmoset (*Calithrix jacchus*) EST databases, as well as the integration of a smaller AKAP7 $\gamma$  5' exon in the mouse transcript which results in a truncated AKAP7 $\gamma$  amino terminus unique to mouse (Figure 4).

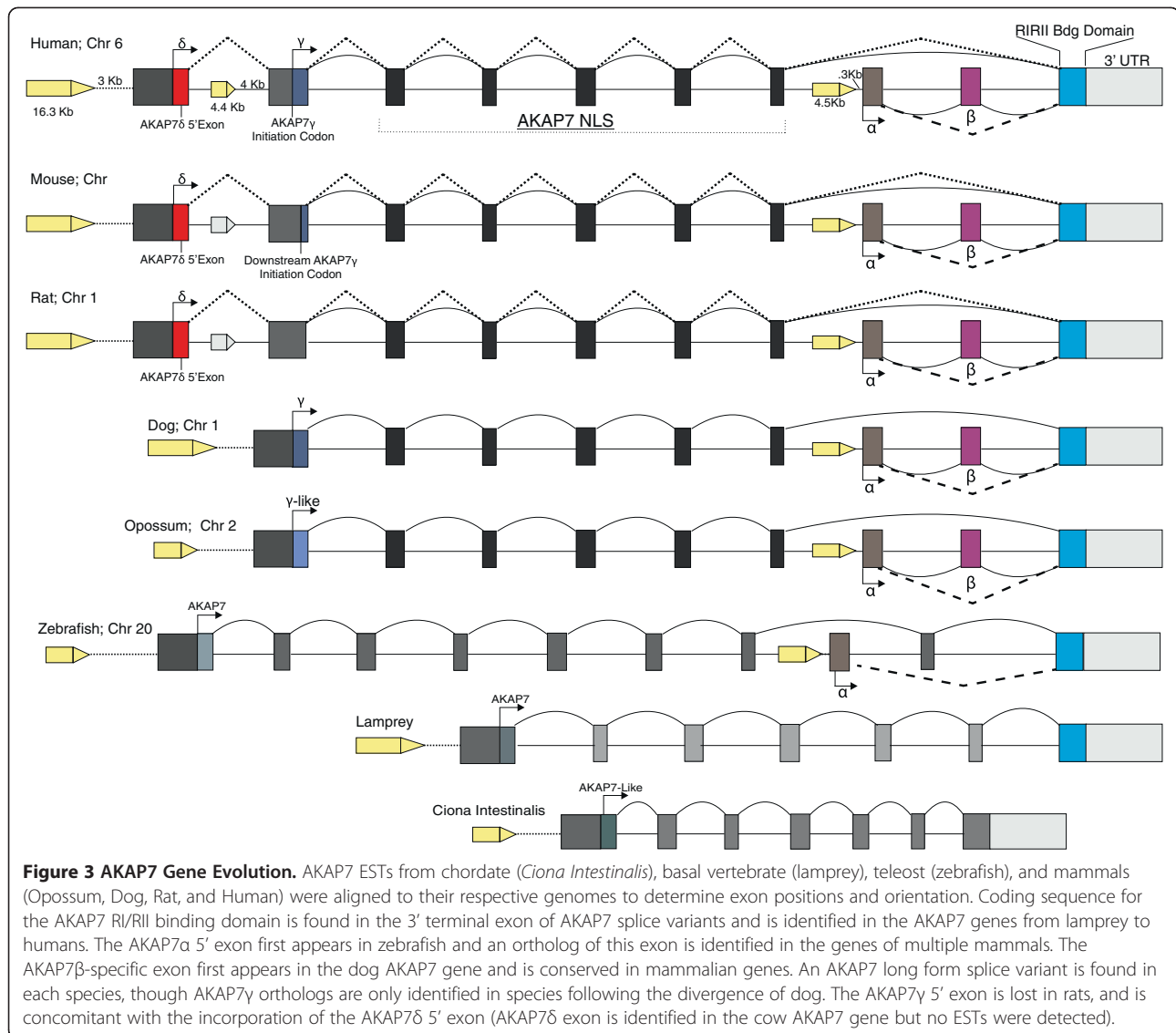
The sequencing of human and mouse AKAP7 $\delta$  and alignment with rat AKAP7 $\delta$  validates the presence of the multiple nucleotide mutations resulting in the truncation of AKAP7 $\gamma$  in mice and loss of AKAP7 $\gamma$  in rats (Additional file 2: Figure S2). Mouse AKAP7 $\delta$  has a 79% nucleotide sequence identity compared to human AKAP7 $\delta$  (74 % amino acid identity), and rat AKAP7 $\delta$  has 78% nucleotide sequence identity to human AKAP7 $\delta$ . Both rat and mouse AKAP7 $\delta$  splice variants contain an "RGD" amino acid sequence that is absent in human AKAP7 $\delta$  (Additional file 2: Figure S2).

#### Replacement of AKAP7 $\gamma$ with AKAP7 $\delta$ indicates similar function

Multiple sequence nucleotide alignments identified mutations in the AKAP7 genes of rat, mouse, and marmoset which result in the loss of (in rat and marmoset) or truncation of (in mouse) the AKAP7 $\gamma$  splice variant and incorporation of the AKAP7 $\delta$  splice variant

(Figure 4). In the rat AKAP7 gene, the putative AKAP7 $\gamma$  initiation codon contains two nucleotide mutations where "ATG" in the Kozak sequence is mutated to "GGG", the result of a transition at position 1 of the codon (A  $\rightarrow$  G), and a transversion at position 2 of the codon (T  $\rightarrow$  G) yielding the missense mutation Met  $\rightarrow$  Gly. In the mouse AKAP7 gene, a cognate mutation was also identified in the putative AKAP7 $\gamma$  initiation codon as a single transition at nucleotide position 1 (A  $\rightarrow$  G) replaces the AKAP7 $\gamma$  start codon with "GTG", resulting in the missense mutation Met  $\rightarrow$  Val. Also identified in the mouse AKAP7 gene, a second mutation downstream of the AKAP7 $\gamma$  initiation codon results in the formation of an internal start site where an "ATG" is formed as the result of a nucleotide transversion (C  $\rightarrow$  A), yielding the amino acid Leu  $\rightarrow$  Met mutation which yields a truncated AKAP7 $\gamma$  splice variant in mouse (Figure 4). The AKAP7 $\gamma$  mutations in the rodent lineage seem to be specific to rats and mice, as another rodent (*Oryctolagus cuniculus*; rabbit) lacks these mutations, and also altogether lacks the AKAP7 $\delta$  5' exon (Figures 3 and 4). The loss of AKAP7 $\gamma$  is also evident in marmoset, as "ATG" is replaced by "ATA", the result of a transition at position 3 of the initiation codon (G  $\rightarrow$  A) replacing Met with Ile.

Two-dimensional western analysis of recombinant rat and mouse AKAP7 long-form splice variants confirms the loss of AKAP7 $\gamma$  in rats, and shows that both

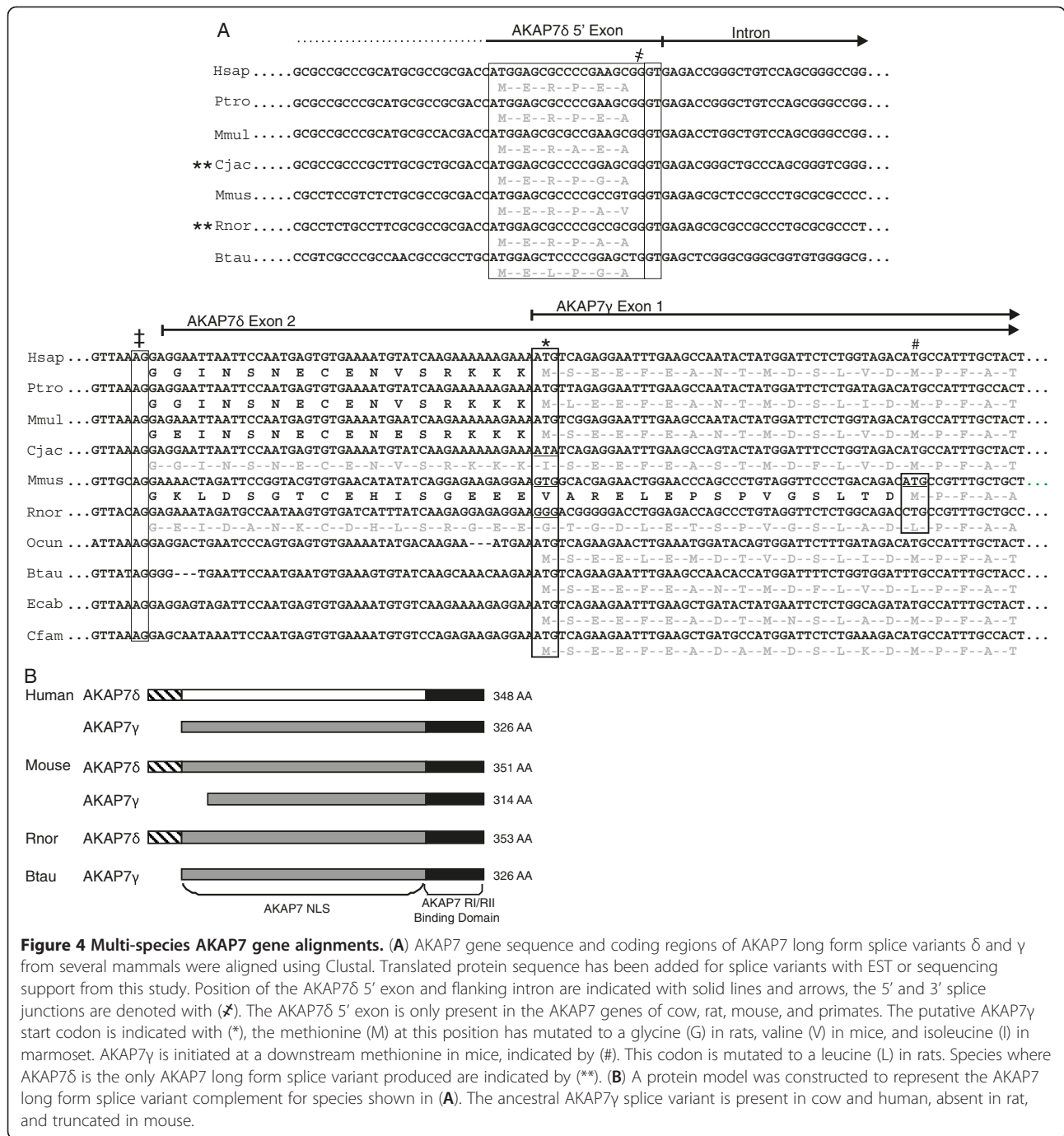


AKAP7δ and AKAP7γ are produced from AKAP7δ cDNA from mice (Figure 5). It is also apparent that mouse AKAP7γ is the major product from AKAP7δ cDNA (Figure 5A), which is likely the result of an AKAP7γ internal translational start site that is present in mice, but absent in rats (Figure 4). AKAP7 immunoprecipitations (IPs) identified AKAP7δ and a variant of AKAP7δ (see below) in rat heart, while AKAP7δ, AKAP7γ, and unknown variants were seen from mouse heart IPs (Figure 5). Comparison of rat and mouse AKAP7 cardiac localization by immunofluorescence (Figure 6) shows identical localization compared between rat (AKAP7δ only) and mouse (predominantly AKAP7γ but also AKAP7δ). The pattern of AKAP7 cardiac immunostaining in rat and mouse is consistent with localization to the sarcoplasmic reticulum, as previously reported for AKAP7δ in rat [38].

### Insertion-deletions in human and Rat AKAP7δ yield novel splice variants

Sequencing of human AKAP7δ identified splice variants containing nucleotide sequence insertions and deletions (indels). Human AKAP7δ variant-1, contains a seventy five nucleotide insertion between the first and second exons of AKAP7δ (located 5' to the AKAP7γ start codon; Figure 7), and an exon deletion (AKAP7δ exon 6; Figure 7). AKAP7δ variant-2 contains two insertions; a sixty nine-nucleotide insertion between AKAP7δ exons 1 and 2, and a sixty nine nucleotide insertion between AKAP7δ exons 7 and 8 (Figure 7). The upstream insertion sequence in AKAP7δ variant-1 has 100 % sequence identity to a region in the human AKAP7 gene (located on chromosome 6) between the AKAP7δ 5' exon and AKAP7δ exon 2. The upstream insertion sequence in variant-2 has 96% sequence identity to the human

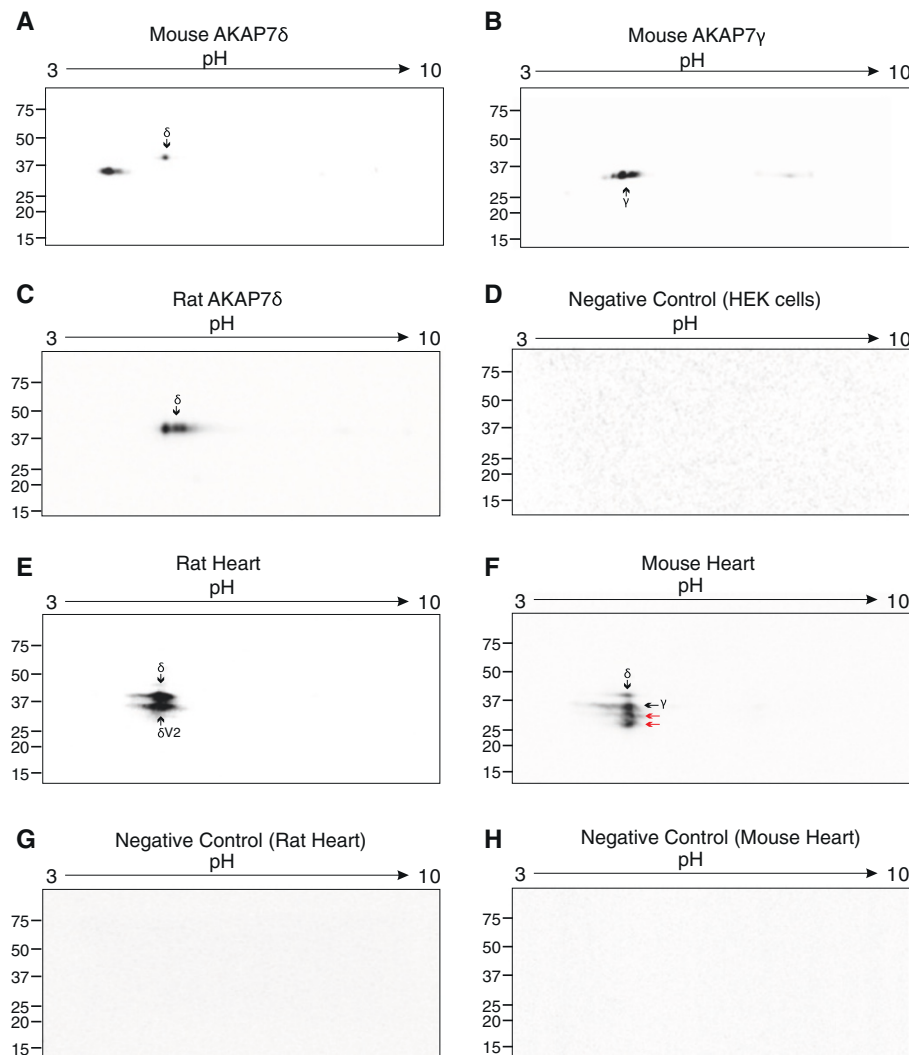




**Figure 4 Multi-species AKAP7 gene alignments.** (A) AKAP7 gene sequence and coding regions of AKAP7 long form splice variants  $\delta$  and  $\gamma$  from several mammals were aligned using Clustal. Translated protein sequence has been added for splice variants with EST or sequencing support from this study. Position of the AKAP7 $\delta$  5' exon and flanking intron are indicated with solid lines and arrows, the 5' and 3' splice junctions are denoted with (X). The AKAP7 $\delta$  5' exon is only present in the AKAP7 genes of cow, rat, mouse, and primates. The putative AKAP7 $\gamma$  start codon is indicated with (\*), the methionine (M) at this position has mutated to a glycine (G) in rats, valine (V) in mice, and isoleucine (I) in marmoset. AKAP7 $\gamma$  is initiated at a downstream methionine in mice, indicated by (#). This codon is mutated to a leucine (L) in rats. Species where AKAP7 $\delta$  is the only AKAP7 long form splice variant produced are indicated by (\*\*). (B) A protein model was constructed to represent the AKAP7 long form splice variant complement for species shown in (A). The ancestral AKAP7 $\gamma$  splice variant is present in cow and human, absent in rat, and truncated in mouse.

AKAP7 gene. The downstream insertion sequence in AKAP7 $\delta$  variant-2 has 100% sequence identity to the AKAP7 $\beta$ -specific sequence. PCR amplification of the human AKAP7 $\delta$  indel regions show the 5' insertion is predominant relative to the native form, while the deletion-variant is less prominent than the native transcript form (Figure 7C). The AKAP7 $\delta$  splice variant containing the AKAP7 $\beta$  exon is a minor product but is identified in most tissues (Figure 7C).

Open reading frame (ORF) analysis of Human AKAP7 $\delta$  transcript variants-1 and 2 (Figure 8) show that the AKAP7 $\delta$  5' insertions shifts the reading frame downstream to begin initiation at the AKAP7 $\gamma$  coding region (Figure 8A), while the downstream exon deletion seen in AKAP7 $\delta$  variant-1 results in a truncated protein that contains the AKAP7 nuclear localization signal (NLS), but lacks the AKAP7 RI/RII binding domain (Figure 8B). The open reading frame for AKAP7 $\delta$  variant-2 begins at



**Figure 5 2D Western Analysis of AKAP7 $\delta$  and AKAP7 $\gamma$ .** Recombinant mouse AKAP7 $\delta$ , AKAP7 $\gamma$ , and rat AKAP7 $\delta$  were analyzed by two-dimensional western blotting. V5-tagged Mouse AKAP7 $\delta$  (A), AKAP7 $\gamma$  (B) or rat AKAP7 $\delta$  (C) were detected from transfected HEK293 cell lysates using a V5-HRP antibody. (D) Mock-transfected HEK293 cell lysates were probed with V5-HRP as a negative control. (E) Rat heart lysate used in AKAP7 immunoprecipitations (using AKAP7 antibody) was probed with protein A-HRP. Two products are identified, rat AKAP7 $\delta$ , and a variant of rat AKAP7 $\delta$  identified in this study (rat AKAP7 $\delta$  variant 1, see Supplemental Information Figure 3). (F) Mouse heart lysate subjected to AKAP7 immunoprecipitation followed by detection with protein A-HRP. Mouse AKAP7 $\delta$  and AKAP7 $\gamma$  are identified in addition to two unknown splice variants (indicated by red arrows). For negative control immunoprecipitations rat heart lysate (G) or mouse heart lysate (H) was immunoprecipitated with rabbit IgG (pre-immune serum) followed by detection by protein A-HRP. The pH range and molecular weight are indicated at the top and left (respectively) of each blot.

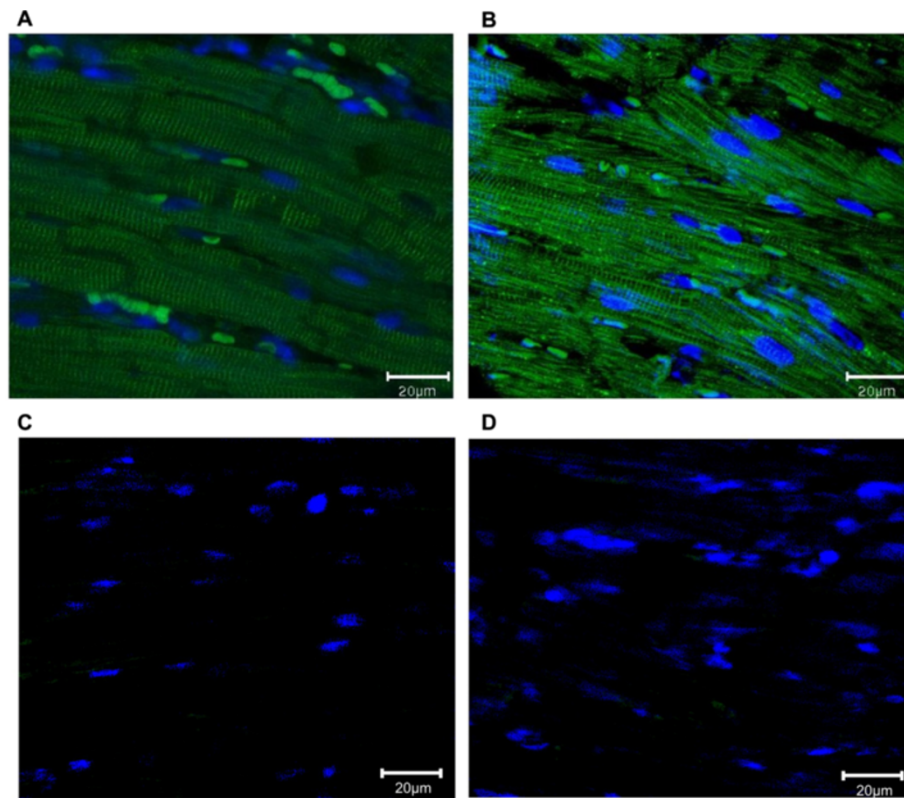
the AKAP7 $\gamma$  initiation codon and produces a protein containing the AKAP7 NLS, AKAP7 RI/RII binding domain and the AKAP7 $\beta$  protein region (Figure 8C).

Two exon-deletion transcript variants were also identified in rat AKAP7 $\delta$  (Additional file 3: Figure S3). AKAP7 $\delta$  variant-1 contains deletions at exons 3–6, while variant-2 contains a deletion of exon 6. Of these variants, only rat AKAP7 $\delta$  variant-2 contains the AKAP7 RI/RII binding domain. No indels were detected from sequencing mouse AKAP7 $\delta$ , however a short (3-exon) AKAP7 $\gamma$  splice variant was detected in mouse ESTs

(Acc# BC099487.1), and is marginally expressed in mouse spleen (Additional file 4: Figure S4).

#### Conservation in AKAP7 short splice variants

AKAP7 short splice variants (AKAP7 $\alpha$  and  $\beta$ ) have pronounced sequence conservation across the vertebrate phyla. Nucleotide sequence alignments of the AKAP7  $\alpha$ / $\beta$  5' exon show that this exon is highly conserved from zebrafish to humans (Additional file 5: Figure S5), as the nucleotide sequence homology for this exon ranges from 66 to 98 % identity. Amino acid alignment of the



**Figure 6 AKAP7 Localization in rat and mouse heart.** Merged immunofluorescence images of (A) adult rat and (B) mouse hearts were taken using a confocal microscope. Immunostaining was performed using a rabbit anti-AKAP7 antibody, the 2<sup>o</sup> Ab is Alexa fluor 488 anti-rabbit. Nuclei are stained with Dapi (blue). AKAP7 immunostaining is prominent in the sarcoplasmic reticulum in both rat (expressing AKAP7 $\delta$  only) and mouse (predominantly AKAP7 $\gamma$  but also AKAP7 $\delta$ ). In the negative controls for rat (C) and mouse (D) normal rabbit serum (AKAP7 pre-immune serum) was used in place of the AKAP7 primary antibody. Images were taken at 60X magnification.

AKAP7 $\alpha/\beta$  amino terminus (Additional file 3: Figure S3) shows this protein region has a sequence homology of 70 to 100% from zebrafish to humans.

AKAP7 $\beta$  is distinguished from AKAP7 $\alpha$  by the inclusion of a 69-nucleotide exon between the AKAP7  $\alpha/\beta$  5' exon and the AKAP7 3' terminal exon. This AKAP7 $\beta$ -specific exon is found in the genomes of several mammals (Figure 3, Additional file 1: Figure S1), and is highly conserved (Additional file 5: Figure S5). Amino acid alignment of the AKAP7 $\beta$ -specific protein region shows sequence homology of 65 to 95% from pigs to human (Additional file 5: Figure S5).

#### Tissue Expression of AKAP7 splice variants in rodents and humans

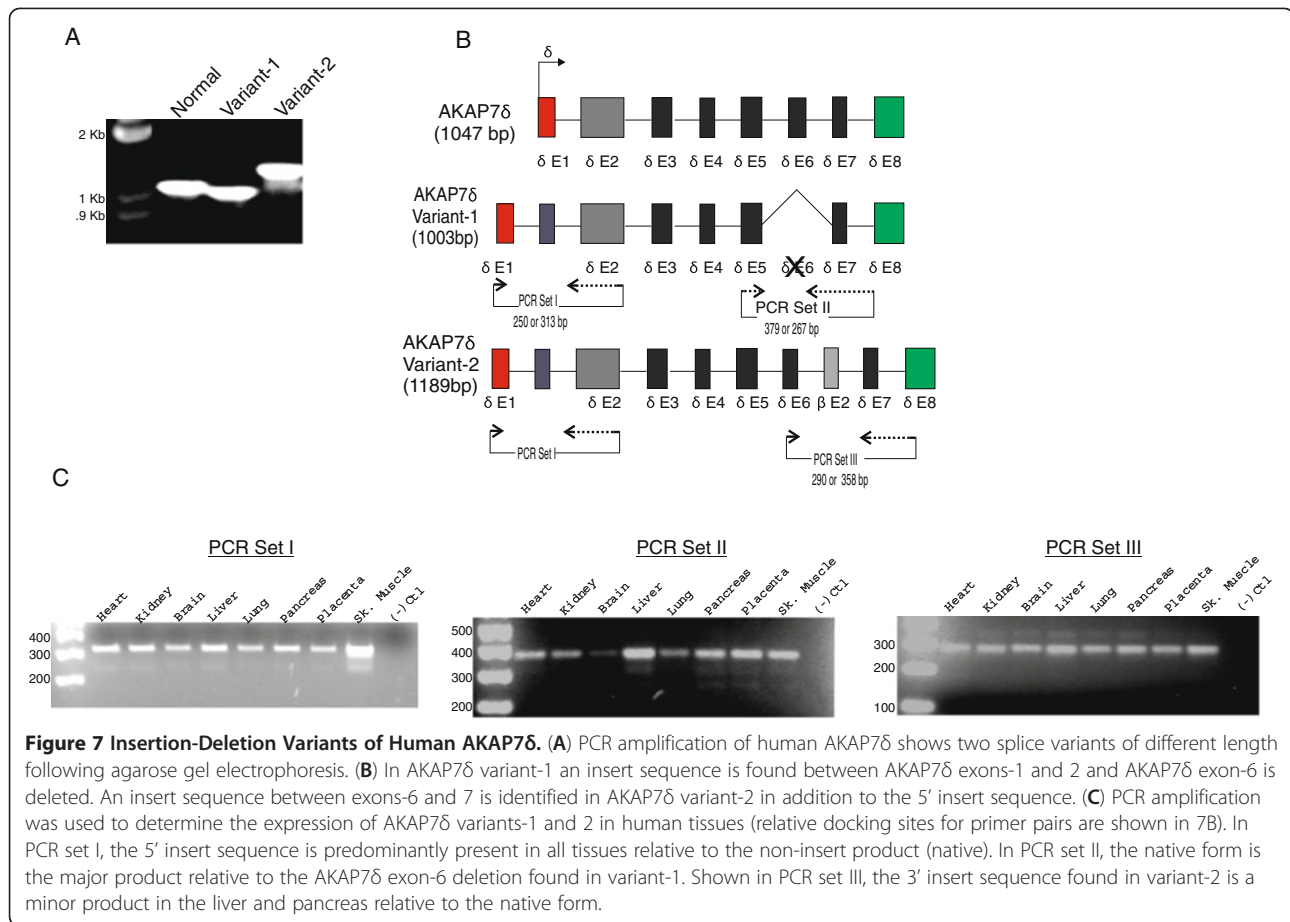
AKAP7 $\delta$  is ubiquitously expressed in human, mouse and rat tissues (Figure 9). AKAP7 $\alpha$  expression is expressed in several rodent and human tissues, with conserved expression seen between rat, mouse and human heart, kidney, brain, liver and lung (Figure 9). AKAP7 $\beta$  mRNA expression is also detected in multiple tissues in rodent and human, though conserved expression between

rodents and humans is only seen in the kidney, with the predominant AKAP7 $\beta$  expression site is the pancreas in humans (Figure 9).

#### Discussion

In this study we have provided the first evolutionary analysis of an AKAP. The significance of PKA signaling has been shown throughout eukaryotic organisms. Here, we show the AKAP7 RI/RII binding domain appears in basal vertebrates, corresponding to a PKA-R subunit gene duplication event. In the molecular evolution of the AKAP7 gene, AKAP7 short splice variants appeared early in the vertebrate lineage and have remained highly conserved. In the AKAP7 long form splice variants, multiple nucleotide mutations and insertions-deletions have resulted in the concurrent loss of AKAP7 $\gamma$  and formation of AKAP7 $\delta$  in rats. Specifically in rats, the replacement of AKAP7 $\gamma$  with AKAP7 $\delta$ , as shown in this study, has likely lead to an over-statement of the significance of AKAP7 $\delta$  in species other than rat [29,38,44,50,51]. Evolutionary pressures in the form of nucleotide insertions and mutations in the Human AKAP7 $\delta$  coding sequence





have likely driven preference for AKAP7 $\gamma$  production in humans. Hence, it is likely that AKAP7 $\delta$  is of minimal significance in human AKAP7-mediated PKA signaling.

#### Integration of the AKAP7-RI/RII binding domain and PKA-R Dimerization/Docking domain in vertebrate evolution

While AKAP7-like proteins were identified in invertebrate and chordate species, the appearance of the RI/RII binding domain (and formation of AKAP7 proteins) occurs in basal vertebrates (lamprey). The formation of AKAP7 proteins from AKAP7-like proteins corresponds with PKA-R gene duplication events where the PKA-RI gene duplicates to form PKA-RI $\alpha$  and PKA-RI $\beta$ , and PKA-RII forms PKA-RII $\alpha$  and PKA-RII $\beta$ . Evolution of the PKA-R subunits has been previously described [52]. Here we provide greater resolution of the timing of PKA-R gene duplications, and show that the amino acid sequences of PKA-R D/D domains are highly conserved, even prior to the gene duplication events. The relative fixation of PKA-R D/D sequences prior to and following the appearance of the AKAP7 RI/RII binding domain suggests that AKAP7 evolved subsequent to the increased presence of PKA (the result of PKA-R gene

duplications). This is supported by the relatively rapid development of the AKAP7 RI/RII binding domain which is absent in chordates (ciona), but present in vertebrates (lamprey).

#### Origin of AKAP7 $\delta$ , the need for an AKAP7 long-form splice variant

As seen in our AKAP7 gene and splice variant phylogenetic analyses, AKAP7 $\gamma$  is the ancestral AKAP7 long form splice variant in mammals. Several nucleotide mutations identified in the murine lineage (as well as a basal primate) resulted in the loss of AKAP7 $\gamma$  through mis-sense mutations in the translational start codon. The most dramatic of these mutations occurs in rats, where the AKAP7 $\gamma$  initiation codon contains a successive transition and rare transversion that results in glycine replacing methionine. The results of these mutations have been confirmed in NCBI database searches, as rat AKAP7 $\gamma$  has been removed from the database (acc. #XM\_001053676.1 has been removed), mouse AKAP7 $\gamma$  is truncated (acc. #NM\_018747.4) and marmoset EST search results show AKAP7 $\gamma$  is not present whereas AKAP7 $\delta$  is expressed (accession number XM\_002746958.1). The combination of the AKAP7 $\gamma$  initiation codon loss combined with the

**A**

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:ATG GAG CGC CCC GAA GCG GGA GGA: AAG GAA AGC CTG GAG GTC AAG TAA CTT GTT GAT TTC TAC GCA GCT GTC AAC ATTT AC TAA CGT GAG GAA TTAATT CCA ATG AGT GTG AAA ATGT AT CAA
: M...E...R...P...E...AG...G...: K E S L E V K * LV D FY AA V N IY * R E E LI PM S V K MY Q
      |
GAA AAA AGA AA ATG TCA GAG GAA TTT GAA GCC AAT ACT ATG GAT TCT CTG GTA GAC ATG CCA TTT GCT ACT GTA GAT ATT CAG GAT GAC TGT GGA ATC ACT GAT GAA CCT CAA ATA AAT TTG
E K R M S E E F EA NT M D S LV D M PF AT V D I Q D D C G IT D E PQ I N L

AAG AGA AGT CAA GAA AAT GAA TGG GTC AAG AGT GAT CAA GTA AAG AAG AGG AAA AAA AAG AGA AAA GAT TAT CAA CCC AAC TAT TTC CTG TCC ATT CCA ATC ACC AAC AAA GAG ATT ATA AAA
K R S Q E N E W V K S D Q V V K K R K K K R K DY Q PN YF LS I PI TN K E I I K

GGA ATT AAG ATC CTG CAG AAT GCA ATA ATA CAA CAA GAT GAG CGA CTG GCC AAA GCA ATG GTC AGT GAT GGT TCC TTT CAT ATT ACC CTG CTG GTG ATG CAA TTA TTA AAT GAA GAT GAA GTA
G I K I LQ NA I I Q Q D E R L AKAM V S D G S F H IT LL V M Q LL N E D E V

AAC ATT GGT ATT GAT GCT CTT TTG GAA TTG AAA CCA TTC ATA GAA GAA CTC CTC CAG GGA AAA CAT TTG ACT TTG CCC TTT CAA GGG ATT GGT ACT TTT GGA AAT CAG GTT GGA TTT GTG AAG
N I G I DA LLE LK PF I E EL LQ G K H LT LP F Q G I GT F G N Q V G F V K

CTG GCA GAA GGA GAT CAT GTA AAC TCA CTT TTG GAG ATA GCA GAG ACT GCA AAT AGG ACA TTT CAA GAA AAA GGC ATC CTG GTA GGA GAG AGC AGA AAT TTT AAA CCT CAT TTG ACC TTC ATG
LA E G D H V N S LL E IA ET AN RT F Q E K G I LV G E S R N F KP H LT F M

AAG TTG TCA AAA TCA CCG TGG CTC CGT AAG AAT GGA GTG AAA AAA ATA GAT CCT GAT TTA TAT GAA AAG TTT ATC AGT CAC AGA TTT GGA GAA GAA ATA TTA TAT CGC ATA GAT CTT TGC TCC
K L S K S PW LR K N G V K K I D PD LY E K F I S H R F G E E I LY R I D LC S

ATG CTG AAG AAA CAA AGT AAT GGT TAT TAT CAC TGT GAA TCT TCC ATT GTG ATT GGT GAA AAG AAC GGA GGG GAG CCC GAT GAC GCT GAA CTA GTA AGG CTC AGT AAG AGG CTG GTG
M LK K K Q S N GY YH CE S S I V I G E K N G G E PD DA E LV R LS K R LV

GAG AAC GCG GTG CTC AAG GCT GTC CAG CAG TAT CTG GAG GAA ACA CAG AAT AAA AAC AAG CCG GGG GAG GGG AGC TCT GTG AAA ACC GAA GCA GCT GAT CAG AAT GGC AAT GAC AAT GAG
E NA EA AD Q N G N D N E

AAC AAC AGG AAA TGA
N N R K *
    
```

**B**

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:ATG GAG CGC CCC GAA GCG GGA GGA: AAG GAAA GC CTG GAG GTC AAG TAA CTT GTT GAT TTC TAC GCA GCT GTC AAC ATTT AC TAAC GT GAG GAAT TAATT CCA ATGA GT GTG AAA ATGT AT CAA
: M...E...R...P...E...AG...G...: K E S L E V K * LV D FY AA V N IY * R E E LI PM S V K MY Q
      |
GAA AAA AGA AA ATG TCA GAG GAA TTT GAA GCC AAT ACT ATG GAT TCT CTG GTA GAC GAT GAA CCT CAA ATA AAT TTG AAG AGA AGT CAA GAA AAT GAA TGG GTC AAG AGT GAT CAA GTA AAG
E K R M S E E F EA NT M D S LV D D E I N LK R S Q E N E W V K S D Q V V K K R K K K R K DY Q PN YF LS I PI TN K E I I K G I K I LQ NA I I Q Q D E R L

AAG AGG AAA AAA AAG AGA AAA GAT TAT CAA CCC AAC TAT TTC CTG TCC ATT CCA ATC ACC AAC AAA GAG ATT ATA AAA GGA ATT AAG ATC CTG CAG AAT GCA ATA ATA CAA CAA GAT GAG CGA
K R K K K K R K DY Q PN YF LS I PI TN K E I I K G I K I LQ NA I I Q Q D E R L

CTG GCC AAA GCA ATG GTC AGT GAT GGT TCC TTT CAT ATT ACC CTG CTG GTG ATG CAA TTA TTA AAT GAA GAT GAA GTA AAC ATT GGT ATT GAT GCT CTT TTG GAA TTG AAA CCA TTC ATA GAA
LA KA M V S D G S F H IT LL V M Q LL N E D E V N I G I DA LL E LK PF I E

GAA CTC CTC CAG GGA AAA CAT TTG ACT TTG CCC TTT CAA GGG ATT GGT ACT TTT GAA AAT CAG GTT GGA TTT GTG AAG CTG GCA GAA GGA GAT CAT GTA AAC TCA CTT TTG GAG ATA GCA
E L LQ G K H LT LP F Q G I GT F G N Q V G F V K LA E G D H V N S LL E IA

GGG AGT GAA AAA AAT AGA TCC TGA
G S E K N R S *
    
```

**C**

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:ATG GAG CGC CCC GAA GCG GGA GGA: AAG AAG GAA AGC CTG GAG GTC AAG TAA CTT CAA GTT GAT TTC TAC GCA GCT GTC AAC ATTT AC TAA CGT GAG GAA TTAATT CCA ATG AGT GTG AAA ATGT AT
: M...E...R...P...E...AG...G...: G K E S L E V K * LQ V D FY AA V N IY * R E E LI PM S V K MY Q
      |
CAA GAA AAA AGA AA ATG TCA GAG GAA TTT GAA GCC AAT ACT ATG GAT TCT CTG GTA GAC ATG CCA TTT GCT ACT GTA GAT ATT CAG GAT GAC TGT GGA ATC ACT GAT GAA CCT CAA ATA AAT TTG
Q E K R M S E E F EA NT M D S LV D M PF AT V D I Q D D C G IT D E PQ I N L

AAG AGA AGT CAA GAA AAT GAA TGG GTC AAG AGT GAT CAA GTA AAG AAG AGG AAA AAA AAG AGA AAA GAT TAT CAA CCC AAC TAT TTC CTG TCC ATT CCA ATC ACC AAC AAA GAG ATT ATA AAA
K R S Q E N E W V K S D Q V V K K R K K K R K DY Q PN YF LS I PI TN K E I I K

GGA ATT AAG ATC CTG CAG AAT GCA ATA ATA CAA CAA GAT GAG CGA CTG GCC AAA GCA ATG GTC AGT GAT GGT TCC TTT CAT ATT ACC CTG CTG GTG ATG CAA TTA TTA AAT GAA GAT GAA GTA
G I K I LQ NA I I Q Q D E R L AKAM V S D G S F H IT LL V M Q LL N E D E V

AAC ATT GGT ATT GAT GCT CTT TTG GAA TTG AAA CCA TTC ATA GAA GAA CTC CTC CAG GGA AAA CAT TTG ACT TTG CCC TTT CAA GGG ATT GGT ACT TTT GGA AAT CAG GTT GGA TTT GTG AAG
N I G I DA LL E LK PF I E E LL Q G K H LT LP F Q G I GT F G N Q V G F V K

CTG GCA GAA GGA GAT CAT GTA AAC TCA CTT TTG GAG ATA GCA GAG ACT GCA AAT AGG ACA TTT CAA GAA AAA GGC ATC CTG GTA GGA GAG AGC AGA AGT TTT AAA CCT CAT TTG ACC TTC ATG
LA E G D H V N S LL E IA ET AN RT F Q E K G I LV G E S R S F K PH LT F M

AAG TTG TCA AAA TCA CCG TGG CTC CGT AAG AAT GGA GTG AAA AAA ATA GAT CCT GAT TTA TAT GAA AAG TTT ATC AGT CAC AGA TTT GGA GAA GAA ATA TTA TAT CGC ATA GAT CTT TGC TCC
K L S K S PW LR K N G V K K I D PD LY E K F I S H R F G E E I LY R I D LC S

ATG CTG AAG AAA CAA AGT AAT GGT TAT TAT CAC TGT GAA TCT TCC ATT GTG ATT GGT GAG TTG GAA AGC TCG TCC TCT GCA GTC CTA CAG AGA TAC AGC AAG GAT ATA CCC AGT TGG TCA
M LK K K Q S N GY YH C E S S I V I G E L E S S S SA V LQ RY S K D I PS W S

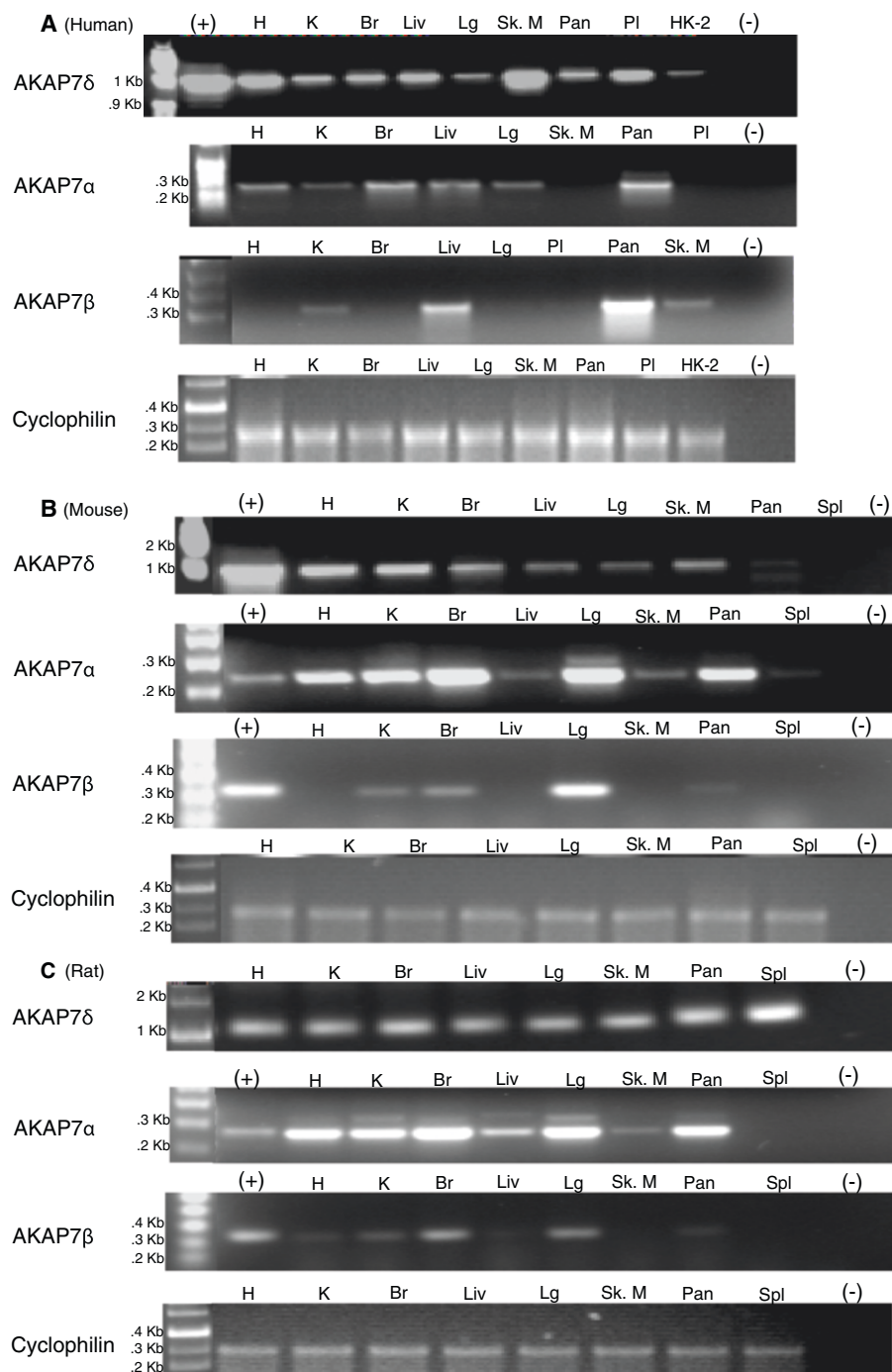
AGT GGT GAA AAG AAC GGA GGG GAG CCC GAT GAC GCT GAA CTA GTA AGG CTC AGT AAG AGG CTG GTG GAG AAC GCG GTG CTC AAG GCT CAG CAG TAT CTG GAG GAA ACA CAG AAT
S G E K N G G E PD DA E LV R LS K R LV E NA V L KAV Q QY LE ET Q N

AAA AAC AAG CCG GGG GAG GGG AGC TCT GTG AAA ACC GAA GCA GCT GAT CAG AAT GGC AAT GAC AAT GAG AAC AAC AGG AAA TGA
K N K P G E G S S V KT EA AD Q N G N D N E N N R K *
    
```

**Figure 8 Open Reading Frame Analysis of Human AKAP7δ insertion-deletion variants.** Human AKAP7δ splice variants were assessed for open reading frames using the NCBI ORF finder. (A-C) Coding and protein sequence for the AKAP7δ amino terminus is indicated by a dotted box, reading frame initiation is indicated by an arrow, and the AKAP7 NLS has a dotted underline. The AKAP7 RI/RII binding domain (if present) is identified in a solid box and termination codons are indicated by an (\*). (A) The 5' insertion identified in the majority of human tissues contains two termination codons and results in a reading frame shift yielding the AKAP7γ splice variant. (B) The 5' insertion combined with deletion of exon-6 results in a truncated protein with the AKAP7γ amino terminus. (C) The 5' insertion combined with the insertion of the AKAP7β exon yields a protein with the AKAP7γ amino terminus, the AKAP7 NLS, the protein region previously identified only in AKAP7β, and the AKAP7 RI/RII binding domain.

concurrent absence of a downstream initiation codon utilized in mice (and present in the AKAP7 genes of several other species), likely yielded the evolutionary pressure for the incorporation of the AKAP7δ 5' exon in rats.

In vitro analyses of mouse AKAP7γ, AKAP7δ and rat AKAP7δ show that both γ and δ splice variants are produced from the mouse AKAP7δ transcript, suggesting that AKAPγ is an internal translational start site product of AKAP7δ production. This is consistent with our



**Figure 9 Tissue Expression of AKAP7 Splice Variants.** PCR amplification and agarose gel electrophoresis of AKAP7 $\delta$ ,  $\alpha$  and  $\beta$  splice variants from (A) human, (B) mouse and (C) rat tissue cDNA. AKAP7 $\alpha$  tissue expression is conserved among human, mouse, and rat heart, brain, liver and lung. AKAP7 $\beta$  tissue expression is conserved in the kidney, and AKAP7 $\delta$  is ubiquitously expressed in humans, mice and rats.

AKAP7 gene analysis and provides a different model for AKAP7 long form splice variants than have been previously reported [29,53]. Our results also suggest that AKAP7 $\gamma$  is the major product of AKAP7 $\delta$  transcription and translation. In rats, only a single protein product was identified from recombinant AKAP7 $\delta$  expression

which is consistent with gene and EST alignments showing the loss of AKAP7 $\gamma$  in rats. Findings from previous studies that have addressed AKAP7 $\gamma$  synthesis and function in rats [53,54] should be solely attributed to AKAP7 $\delta$  as the AKAP7 $\gamma$  splice variant is absent in rats. The significant role of AKAP7 $\delta$  in the renal aquaporin

shuttle [29], and regulation of cardiac  $\text{Ca}^{2+}$  cycling [38] has led to a concerted effort to develop AKAP7 $\delta$  peptidomimetics for use in treating human heart failure [44,50]. Results from this study show that AKAP7 $\delta$  is the sole AKAP7 long form splice variant found in rats, and the significance of this splice variant is very likely over-stated. Combined with the identification of nucleotide insertions in the 5' end of human AKAP7 $\delta$  that would exclusively promote the production of AKAP7 $\gamma$  in all tissues, the evidence presented here indicates that the functional significance of AKAP7 $\delta$  is minimal in species other than rats. In mice, where AKAP7 $\gamma$  appears to be the dominant product of AKAP7 $\delta$  transcription and translation, cardiac AKAP7 immunolocalization is consistent with the SR indicating AKAP7 $\gamma$  largely replaces AKAP7 $\delta$  at the SR in species other than rat and supports the possibility that AKAP7 $\delta$  and AKAP7 $\gamma$  are functionally redundant in mice. Further studies are required to determine the functional consequences and distinction between AKAP7 $\delta$  and AKAP7 $\gamma$  in mice and human skeletal muscle.

#### **Evolutionary pressure for replacing AKAP7 $\delta$ with AKAP7 $\gamma$**

AKAP7 $\delta$  has been identified as a vital component in cardiac  $\text{Ca}^{2+}$  handling at the sarcoplasmic reticulum in rats [38] and has been proposed as a pharmacological target in treating heart failure [44,50]. Our evolutionary analysis of the AKAP7 gene has shown that the origin of AKAP7 $\delta$  was brought about through a series of nucleotide mutations in the AKAP7 $\gamma$  coding sequence in rats, hence likely leading to exaggeration of the functional significance of AKAP7 $\delta$ . This is supported by *in vitro* analysis of mouse AKAP7 $\delta$ , where AKAP7 $\gamma$  (through a likely internal translation initiation site) is the major product. AKAP7 immunoprecipitations from rat and mouse heart tissue also show AKAP7 $\delta$  and an AKAP7 $\delta$  variant in rat while in mouse heart AKAP7 $\delta$  is a minor product and AKAP7 $\gamma$  is prominent. Furthermore, sequencing of human AKAP7 $\delta$  (this study) showed the dominant presence (in multiple tissues) of a nucleotide sequence insertion immediately downstream of the AKAP7 $\delta$  5' codon, resulting in a reading-frame shift that promotes the production of AKAP7 $\gamma$ . Therefore AKAP $\gamma$  is the predominant, functional AKAP7 long form splice variant in humans and pharmacological efforts in designing AKAP7 $\delta$  disruptor peptides should instead be directed at AKAP $\gamma$ .

#### **AKAP7 short form splice variant evolution**

Found only in the vertebrate lineage, AKAP7 $\alpha$  is the ancestral AKAP7 splice variant and has conserved tissue expression in rodents and human heart, kidney, brain, liver and lung. AKAP7 $\alpha$  has been extensively studied in the heart and brain, where this protein regulates PKA

localization to (and phosphorylation of) ion channels such as the L-type  $\text{Ca}^{2+}$  channel [1,40,55,56] and epithelial  $\text{Na}^{+}$  channels [2,36,37,41]. Deletion of a specific region in the cardiac voltage-sensitive  $\text{Ca}^{2+}$  channel abolishes AKAP7 binding leading to cardiac hypertrophy and premature death in mice, and demonstrates the importance of AKAP7 $\alpha$  mediated PKA signaling in adrenergic stimulation [57]. As seen in the expression results in this study, it is also likely that AKAP7 $\alpha$  has a conserved role in ion channel regulation in kidney, liver and lung. AKAP7 $\alpha$  amino-terminal amino acids that have been previously identified [1] in membrane targeting through myristoylation (glycine at position 2; Figure 9) or palmitoylation (cysteins at positions 5 and 6; Figure 9) are absolutely conserved from zebrafish to humans. Furthermore, hydrophobic residues (leucine-4, phenylalanines-7 and 9, arginine-11) in the AKAP7 $\alpha$  amino terminus which are likely to be critical for membrane localization are also conserved between zebrafish and humans. Given the high degree of sequence conservation in the AKAP7 $\alpha$  amino terminus, it is likely this protein functions in membrane ion channel regulation throughout vertebrate species following the divergence of lamprey.

Development of the AKAP7 $\beta$  exon occurred in mammals (opossum), and while AKAP7 $\beta$  and  $\alpha$  splice variants share the same amino terminus, the AKAP7 $\beta$ -specific exon confers a cellular localization distinct from the AKAP7 $\alpha$  splice variant [46]. The functional significance of the AKAP7 $\beta$  splice variant in mammals remains unknown, though the sequence conservation in this protein is certainly suggestive of its importance. AKAP7 $\beta$  is primarily conserved in the kidney of rodents and human, and it seems likely that this splice variant has a conserved function in renal ion channel regulation. Importantly, in this study we have identified a novel AKAP7 long-form splice variant containing the  $\beta$ -exon. Further studies are required to address the unique membrane-localizing function of this AKAP7 protein region.

#### **Conclusions**

Our results show that the formation of AKAP7 proteins occurred in basal vertebrate species, likely in response to the increased presence and distribution of intracellular PKA following PKA-R gene duplication events. Short form splice variants from AKAP7 genes have remained highly conserved across species, while long forms have undergone rapid evolutionary change. Independent mutations in the AKAP7 genes of rats, mice and marmoset have resulted in loss or truncation of AKAP7 $\gamma$  and a likely compensatory incorporation of AKAP7 $\delta$ . These results suggest that studies of AKAP7 $\delta$  are not necessarily comparable among rats, mice and humans.



The specific targeting of human AKAP7 $\delta$  in pharmacotherapeutics should be undertaken with caution, as this splice variant is largely absent in humans.

## Methods

### Sequence data acquisition

Human and rodent AKAP7 splice variant sequences were obtained from NCBI and ENSEMBL (release 61) databases by searching for the gene "AKAP7" and AKAP7 transcripts. Annotated AKAP7 proteins were analyzed for conserved AKAP7 protein families (Pfam) using both the NCBI Conserved Domain Database (CDD) and Sanger Institute Protein Family Database (<http://pfam.sanger.ac.uk/>) [58]. Conserved AKAP7 Pfams were identified as the AKAP7 Nuclear Localization Signal (AKAP7 NLS; Pfam 10469) and the AKAP7 RI/RII binding domain (pfam 10470). Human, rat and mouse AKAP7 Nucleotide and protein sequences obtained from NCBI (<http://www.ncbi.nlm.nih.gov>) and ENSEMBL were used in BLASTP, TBLASTN and EST search algorithms to identify AKAP7 transcripts, proteins and genes in multiple species (listed below). Search results were verified to contain both AKAP7 Pfams (AKAP7 NLS and AKAP7 RI/RII). Sequences obtained from these searches that contained only the AKAP7 NLS Pfam were classified as "AKAP7-like proteins. Multiple species spanning the vertebrate and invertebrate lineages were used for this study, specifically: primates-*Homo sapiens* (*H. sap*), *Pan troglodytes* (*P. tro*), *Macaque mulatta* (*M. mul*) *Callithrix jacchus* (*C. jac*), rodents-*Mus musculus* (*M. mus*), *Rattus norvegicus* (*Rnor*), *Oryctolagus cuniculus* (*O. cun*); laurasiatheria- *Canis familiaris* (*C. fam*), *Equus caballus* (*E. cab*), *Bos Taurus* (*B. tau*), *Sus scrofa* (*S. scr*), metatheria- *Monodelphis domestica* (*M. dom*), reptiles-*Anolis carolinensis* (*A. car*), amphibians- *Xenopus tropicalis* (*X. tro*), teleosts- *Danio rerio* (*D. rer*), *Takifugu rubripes* (*T. rub*), *Oryzias latipes* (*O. lat*), *Gasterosteus aculeatus* (*G. acu*); vertebrates- *Petromyzon marinus* (*Pmar*); chordates- *Ciona intestinalis* (*C. int*).

### Development of PKA-R and AKAP7 interaction

To determine the relationship between AKAP7 RI/RII binding domain and PKA-R gene evolution, we constructed a PKA-R phylogenetic tree from the PKA-R cAMP binding domains, and identified AKAP7 RI/RII binding domain presence/absence for each species used in the PKA-R phylogenetic tree. PKA-R translated amino acid sequences were aligned using the Clustal function of Mega v5 [59]. PKA-R subunit protein relationships were determined using the algorithm of Mr. Bayes 3.1.2. Mr. Bayes analysis was run under a fixed rate Dayhoff model. The tree is a representative of 4 independent runs, of four million generations each. The tree was

rooted with *Drosophila melanogaster*. Pictorial representation of AKAP7 RI/RII presence/absence is shown next to the PKA-R clades that contain this motif.

### EST to Gene alignments

To determine AKAP7 splice variant exon positions and gene structures, the AKAP7 genes of human, rat, dog, opossum, zebrafish, lamprey, and ciona were analyzed using GENESCAN [60] and Promoter 2.0 Prediction Server [61] FPROM (Softberry, Inc., Mt Kisco, NY) programs. AKAP7 splice variant exon positions and splice junctions were verified by using NCBI two-sequence BLAST (bl2seq) and manually aligning cDNA sequences to the genomic sequences using Mega v5 [59].

### Animals

All experiments involving animal subjects were carried out with the approval of the University of Illinois-Chicago (UIC) and Jesse Brown VA Medical Center (JBVAMC) Institutional Animal Care and Use Committees (IACUC). Adult male rats and mice were used for cardiac and renal tissue preparations in DNA sequencing, western blotting and immunofluorescence applications.

### AKAP7 Splice variant detection and sequencing

Human multiple tissue cDNA (MTC panel I, CloneTech, CA) was used in PCR amplification and sequencing applications. Rat and mouse total RNA were prepared using Trizol reagent (Invitrogen). Five micrograms of total RNA was used in first-strand cDNA synthesis using SuperScript III Reverse Transcriptase (Invitrogen) primed with random hexamers. PCR was performed using KOD Hot Start DNA polymerase (EMD Biosciences). Primers used for the amplification of human, mouse and rat AKAP7 $\alpha$ ,  $\beta$ , and  $\delta$  splice variants are listed in Table 1. PCR products were extracted from agarose gels and cloned into the pGEM-T vector system (Promega) and sequenced using a capillary sequencer (Applied Biosystems).

### Transfections

Mouse AKAP7 $\delta$ , AKAP7 $\gamma$  and rat AKAP7 $\delta$  transcripts were PCR amplified from heart or kidney cDNA templates and cloned into a pcDNA3.1/V5-His expression vector (Invitrogen) to produce V5-tagged proteins. For transient transfection purposes,  $2 \times 10^5$  HEK293 cells (American Tissue Culture Collection) were grown on 6-well plates for 24 h in Dulbecco's Modified Eagle Medium, 10 % Fetal Bovine Serum (FBS) (BioWhittaker), 100 U/mL penicillin and 50  $\mu$ g/mL streptomycin. Cell transfections were performed by using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. Cells were transfected with



**Table 1 Primers used in PCR amplifications and Sequencing**

Name	Sequence 5' to 3'
Human AKAP7δ For	ATGGAGCGCCCCGAAGCGGGAGGAATTAAT
Human pan AKAP7 Rev	TCATTTCTGTTGTTCTCATTGTCCATTCTGATCA
Human AKAP7α For	ATGGGCCAGCTTTGCTGCTTTCTTTCTCAAG
Human AKAP7β For	GAGTTGGAAAGCTCGTCTCTGCAGTCTAC
Human AKAP7δ-indel set 1 For	ATGGAGCGCCCCGAAGCGG
Human AKAP7δ-indel set 1 Rev	TTTCTCTTTACTTGATCACTCTTGACCCATTTCATT
Human AKAP7δ-indel set 2 For	TTCATAGAAGAACTCCTCCAGGGAAAACATTTGACTTTG
Human AKAP7δ-indel set 2 Rev	GAAGATTCACAGTGATAATAACCATTACTTTGTTTT
Human AKAP7δ-indel set 3 For	CCTGGTAGGAGAGAGCAGAAGTTTTAAACCTCATTGAC
Human AKAP7δ-indel set 3 Rev	TCCACCAGCCTTACTGAGCCTTACTAGTTC
Mouse AKAP7δ For	CACCATGGAGCGCCCCGCGGT
Mouse pan AKAP7 Rev	CTCCGGTTGTTATCACTGCCATCACCATTCCGAT
Mouse AKAP7γ For	CACCATGCCGTTTGCTGCTGTAGATATTCAAGATGA
Mouse AKAP7α For	ATGGGCCAGCTTTGCTGCTTCCCTTTTCG
Mouse AKAP7β For	GAGTTGGAAATCCATCTTCTGCAGACCTACAGAG
Mouse AKAP7γ-short Rev	TTAGTGCCAGGGTGGAGCTTTGGAGCAGT
Rat AKAP7δ For	CACCATGGAGCGCCCCGCGGGGA
Rat pan AKAP7 Rev	CTCCGGTTGTTATCACTGCCATCGCCATTCC
Rat AKAP7α For	ATGGGCCAGCTTTGCTGCTTCCCTC
Rat AKAP7β For	CAGTTGGAAAGTCCATCTTCTGCAATCCTACAGAGAT

mouse AKAP7δ, mouse AKAP7γ, or rat AKAP7δ. Transfected and mock-transfected cells were harvested 48 hours after transfection and washed with PBS followed by sonication in deionized urea-thiourea-chaps buffer (UTC; 8 M urea, 2 M Thiourea, 2 % chaps) with protease and phosphatases inhibitors (Pierce). Supernatants were then used in 2D western applications.

#### Immunoprecipitations

Immediately following removal, rat and mouse hearts were fast-frozen in liquid nitrogen and dounce homogenized at 4°C in 50 mmol/L of Tris-HCl (pH 7.4), 150 mmol/L of NaCl, 1 mmol/L of EDTA, 0.25 % sodium deoxycholate, 1 % triton X-100 and 1X HALT protease and phosphatases inhibitor cocktails (Pierce). The homogenate was centrifuged at 10,000X RCF for 15 minutes at 4°C, the supernatant was collected and protein concentrations were determined using the Dc Protein Assay (BioRad). Five hundred micrograms of the heart lysates were incubated in 500 µl lysis buffer containing 1 µg of rabbit polyclonal AKAP7 antibody with slow rotation on a rocker overnight at 4°C. Negative control immunoprecipitations were carried out using rabbit IgG (pre-immune serum) instead of the AKAP7 antibody. Lysate-immunoglobulin samples were then incubated with 50 µl of protein A/G agarose beads (EMD) for 2 hours under constant rotation. The samples

were then centrifuged, supernatants were removed and agarose bead pellets were successively washed in PBS containing 1.00 %, 0.5 %, and 0.05 % Tween-20. The beads were resuspended in deionized UTC buffer with protease and phosphatases inhibitors, mixed and incubated at room temperature for 10 minutes. Following centrifugation, the supernatants were used in 2D Western applications.

#### Two-dimensional western analysis

Samples from transfections and heart lysate immunoprecipitations were centrifuged for 10 minutes at 10,000 RCF and the protein content of the supernatants was estimated using the RC/DC protein assay (BioRad). Fifty micrograms of total protein was isoelectrically focused (IEF) using 7 cm isolated pH gradient (IPG, pH 3–10 non-linear gradient) strips on an IEF cell (BioRad). Second-dimensional separations were performed on 4–15 % gradient tris-glycine SDS-PAGE gels. Proteins were transferred to PVDF membranes for western analysis. To detect recombinant AKAP7 proteins from transfections, membranes were probed using an anti-V5 HRP-conjugated antibody (Invitrogen) at a 1:5000 dilution. Proteins from AKAP7 immunoprecipitations were detected with a protein-A HRP conjugate. In negative control immunoprecipitations, rabbit IgG was used in

place of the AKAP7 antibody. Western blot images were taken on a Kodak image station 4000R pro.

### Immunofluorescence microscopy

Affinity-purified rabbit polyclonal pan-AKAP7 antisera was produced by Genscript corp. (Piscataway, NJ) for use in immunoprecipitations and immunofluorescence applications in rat and mouse hearts. The epitope used for AKAP7 antisera production was "LVRLSKRLVE-NAVC" which is common to rodent and human AKAP7 splice variants. Following removal, rat and mouse hearts were fixed overnight with 10% formalin in 0.1 M phosphate buffer (pH 7.2). Tissues were dehydrated in graded ethanol solutions and embedded in paraffin. Nonspecific staining was blocked by incubation with 1.5 % normal goat serum in PBS for 1 hr at room temperature in a humidified atmosphere. The sections were incubated with the anti-AKAP7 antibody or normal rabbit serum (for negative control) overnight at 4 C. After three washes of 10 min each in PBS-T, the sections were incubated with Alexa fluor 488-conjugated anti-rabbit secondary antibody (Invitrogen) for 2 hours at room temperature and washed three times. Nuclei were counterstained with Dapi.

### Additional files

**Additional file 1: Figure S1. AKAP7 Splice Variant Evolution.** EST classification and EST to gene matching shows the differential incorporation of AKAP7 splice variants throughout evolution. A cladogram was constructed using the NCBI taxonomy common tree tool and matches known species phylogenetic relationships. The accession numbers from NCBI or Ensemble databases and corresponding exon-intron structures in each species (determined by EST to gene matching) for the AKAP7 splice variants is shown in each column. A splice variant is indicated as "not present" if the exon(s) for that splice variant are absent from the AKAP7 gene. A splice variant is indicated as "not detected" if the necessary exons are identified in the AKAP7 gene but no EST was identified. Splice variants with a non-orthologous AKAP7 $\gamma$  exon-1 are listed as AKAP7 $\gamma$ -like.

**Additional file 2: Figure S2. Nucleotide and Amino acid alignments of human, mouse and rat AKAP7 $\delta$ .** (A) Human and mouse AKAP7 $\delta$  splice variants were sequenced and aligned to the previously reported rat AKAP7 $\delta$  nucleotide sequence. Predicted mutations in the AKAP7 $\gamma$  initiation codon are confirmed, and indicated by an (\*). There is 78-89 % sequence identity between rat, mouse, and human AKAP7 $\delta$  nucleotide sequence. The least similar region is found between nucleotides 180 and 200, (B) translated amino acid sequences were aligned using ClustalX2. Rat and mouse AKAP7 $\delta$  contain an "RGD" domain between amino acids 70 and 75, this domain is absent from the human sequence.

**Additional file 3: Figure S3. Splice variants of Rat AKAP7 $\delta$ .** (A) PCR amplification of AKAP7 $\delta$  from rat heart cDNA yielded two smaller splice variants in addition to the native form. (B) Rat AKAP7 $\delta$  variant-1 has an exon-7 deletion, whereas AKAP7 $\delta$  variant-2 consists of exons 1, 2 and 8, while exons 3-7 are deleted. Rat AKAP7 $\delta$  variant-1 contains the AKAP7 NLS (dotted underline) but not the AKAP7 RI/RII binding domain. Rat AKAP7 $\delta$  variant-2 lacks the AKAP7 NLS but contains the AKAP7 RI/RII binding domain (box).

**Additional file 4: Figure S4. Mouse AKAP7 $\gamma$  splice variant.** A short splice variant consisting of AKAP7 $\gamma$  exons 1 and 2, and a unique 3' exon was identified in mouse ESTs. (A) This truncated splice variant contains

the AKAP7 NLS (dotted box), but lacks the AKAP7 RI/RII binding domain. A model was constructed (B) to show the relative positions of constituent exons. (C) The mouse AKAP7 $\gamma$  short variant is not widely expressed, and is detected only in the spleen.

**Additional file 5: Figure S5. Nucleotide and amino acid alignments of AKAP7 short form splice variants.** (A) The amino terminus of AKAP7 $\alpha$  and AKAP7 $\beta$  was aligned using clustalX2. Amino acid sequences are highly conserved from zebrafish to humans. Residues that have been previously identified as lipid-modified [1] are indicated by (\$) and hydrophobic residues are indicated with down-arrows (↓). (B) The AKAP7 $\beta$ -specific region is identified in species from pig to humans, with several highly conserved residues which are indicated with (\*).

### Abbreviations

AKAP: A-Kinase Anchoring Protein; PKA-R: PKA regulatory subunit; PKA-R D: PKA regulatory subunit dimerization/docking domain; SERCA: Sarcoplasmic-Endoplasmic Reticulum Calcium ATPase; PLN: Phospholamban; ORF: Open Reading Frame.

### Competing interests

The authors declare that they have no competing interests

### Authors' contributions

KRJ conceived the study, designed the experiments, obtained data, interpreted data, and drafted the manuscript. JNJ constructed the PKA-R phylogenetic tree and edited the manuscript. GKC provided technical advice and edited the manuscript. RSD edited the manuscript and supported study. All authors have read and approved the manuscript for publication.

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