A-kinase anchoring protein 4 has a conserved role in mammalian spermatogenesis

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Abstract

A-kinase anchor protein 4 (AKAP4) is an X-linked member of the AKAP family of scaffold proteins that anchor cAMP-dependent protein kinases and play an essential role in fibrous sheath assembly during spermatogenesis and flagellar function in spermatozoa. Marsupial spermatozoa differ in structural organization from those of eutherian mammals but data on the molecular control of their structure and function are limited. We therefore cloned and characterized the *AKAP4* gene in a marsupial, the tammar wallaby (*Macropus eugenii*). The gene structure, sequence, and predicted protein of *AKAP4* were highly conserved with that of eutherian orthologues and it mapped to the marsupial X-chromosome. There was no *AKAP4* expression detected in the developing young. In the adult, *AKAP4* expression was limited to the testis with a major transcript of 2.9 kb. *AKAP4* mRNA was expressed in the cytoplasm of round and elongated spermatids while its protein was found on the principal piece of the flagellum in the sperm tail. This is consistent with its expression in other mammals. Thus, *AKAP4* appears to have a conserved role in spermatogenesis for at least the last 166 million years of mammalian evolution.

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Introduction

The sperm tail controls sperm motility and effective sperm delivery, and also aids in penetration of the oocyte which is essential for fertilization (Miki *et al.* 2002). It is made up of the connecting, middle, principal, and end piece. The principal piece occupies approximately three quarters of the length of the flagellum and acts as the main structure driving sperm motility. The fibrous sheath is a unique cytoskeletal structure surrounding the axoneme and the outer dense fibers in the principal piece of the flagellum and is largely comprised of A-kinase anchor proteins (AKAPs) and a sperm-specific isozyme of glyceraldehyde 3-phosphate dehydrogenase (GAPDHS; Bunch *et al.* 1998, Mandal *et al.* 1999, Vijayaraghavan *et al.* 1999, Brown *et al.* 2003). During sperm maturation and capacitation many changes occur including modification of the mitochondria matrix and the mid-piece differentiation necessary for motility in marsupials (Temple-Smith & Bedford 1976). GAPDHS was identified as a conserved mammalian energy source for sperm motility in marsupials (Ricci & Breed 2005). All of these data suggest that marsupials employ the same molecular strategies as eutherians during spermatogenesis.

AKAP4, an X-linked member of the *AKAP* gene family (Turner *et al.* 1998) encodes the most abundant protein

of the sperm fibrous sheath in all mammals so far examined (Carrera *et al.* 1994, 1996, Fulcher *et al.* 1995, Turner *et al.* 1998, Colledge & Scott 1999, Eddy *et al.* 2003). *AKAP4* anchors cAMP-dependent protein kinase A (PKA) to the fibrous sheath of the spermatozoon where the kinase is likely to be required for the regulation of spermatozoal motility (Edwards & Scott 2000, Brown *et al.* 2003). Its protein is restricted to the spermatogenic cells from the round spermatid stage until spermatozoa mature. *Akap4* mRNA is expressed only in the post-meiotic phase of spermatogenesis (Miki *et al.* 2002). Since it is present only in germ cells and not in the somatic cells of the testis or in any other tissues (Fulcher *et al.* 1995), *AKAP4* was thought to be a scaffold protein involved in regulating flagellum function (Miki *et al.* 2002). *Akap4* deletion results in shorter sperm flagellum and an incomplete fibrous sheath formation causing infertility due to loss of motility (Miki *et al.* 2002). *AKAP4* interacts with a number of other proteins in the fibrous sheath. One of these is *AKAP3*, another important component of the fibrous sheath (Mandal *et al.* 1999, Vijayaraghavan *et al.* 1999, Brown *et al.* 2003). In *Akap4* null mice, the amount of *AKAP3* was reduced both in testis and sperm (Miki *et al.* 2002) suggesting a functional link in the recruitment of these two proteins.

GAPDHS is another major fibrous sheath protein that is essential for glycolysis providing an energy source for sperm motility. It is also distributed in the principal piece of the sperm flagellum (Bunch *et al.* 1998). While GAPDHS is present in *Akap4* null mice, it does not accumulate in the flagellum (Miki *et al.* 2002). Therefore, sperm motility is still disrupted in the absence of *Akap4* due to the interference with signal transduction and lack of association of glycolytic enzymes (Miki *et al.* 2002, Brown *et al.* 2003).

The fibrous sheath is a conserved structure that surrounds the outer dense fibers of the principal piece region of the sperm flagellum in both marsupial and eutherian mammals (Eddy 2007). Besides AKAP4, AKAP3 and GAPDHS, many other proteins are located on the flagellum. At least 17 major proteins have been identified in the fibrous sheath in rats (Kim *et al.* 1995b), 14 in humans (Jassim *et al.* 1992), and 10 in rabbits (Kim *et al.* 1995b, 1997). The amino acid composition of the purified fibrous sheath from human, rabbit, and rat spermatozoa are similar, indicating that mammalian sperm tail fibrous sheaths are composed of similar types of proteins (Kim *et al.* 1997). The outer dense fibers, another specific cytoskeletal component in mammals, encompasses the axoneme in the middle and principal pieces of the sperm tail, cooperating with the fibrous sheath to participate in signaling pathways to regulate sperm stability and motility. The genes that encode bovine and porcine outer dense fiber one proteins are highly conserved with those of human and rat (Kim *et al.* 1995a). In a marsupial (the brushtail possum *Trichosurus vulpecula*), there are seven major proteins in the outer dense fibers and 12 major proteins in the fibrous sheath (Ricci & Breed 2001). There is also strong cross-reactivity to the brushtail possum fibrous sheath proteins in the principal piece of the koala *Phascolarctos cinereus*, the dunnart *Sminthopsis macroura*, and the tammar wallaby *Macropus eugenii* as well as in that of the rat (Ricci & Breed 2001). Several other spermatozoon-specific proteins have been isolated from marsupial sperm. Two tammar sperm proteins, PSA-10 and WSA-1 (Harris & Rodger 1998, 2005) are present on the acrosome, midpiece, and principal piece of mature spermatozoa but as yet nothing is known of their function (Harris & Rodger 1998, 2005).

Although the 14 stages of spermiogenesis recognized in the tammar and sperm maturation follow a generally similar pattern to that of eutherians (Tyndale-Biscoe & Renfree 1987, Harris & Rodger 2005), the process in marsupials is complicated and dynamic and differs in some respects from that of eutherians (Lin *et al.* 1997). Sperm of most marsupials have a dorso-ventrally flattened nuclear surface (Temple-Smith 1994) and there is a more stable acrosome on the dorsal nuclear surface (Mate & Rodger 1991, Sistina *et al.* 1993). During epididymal transit, changes in acrosomal morphology occur, and the sperm head rotates on its axis from a T-shape relative to the tail to become aligned with it (Temple-Smith 1994, Lin

& Rodger 1999). Once in the female tract, the T-shape is resumed (Bedford & Breed 1994). While the marsupial sperm head has become more compact but there is no special stability of chromatin in the nucleus by disulphide bonding or in its head membranes (Temple-Smith & Bedford 1976, Cummins 1980), and although cAMP induces tyrosine phosphorylation of sperm proteins, it is not yet known whether capacitation occurs in marsupials (Sidhu *et al.* 2004). These differences in structural organization suggest that there may be differences in the genes controlling spermatogenesis and sperm function and motility. There has as yet been no study of the AKAP genes and proteins in marsupials. Given the importance of AKAP4 for sperm function in eutherian mammals, this study examined the conservation of AKAP4 sequences in marsupials and a monotreme, and examined its expression during spermatogenesis in a marsupial, the tammar wallaby *M. eugenii*.

Results

Cloning and characterization of the tammar AKAP4 gene

An ~2.5 kb partial AKAP4 cDNA sequence was obtained with cross-species primers from adult tammar testis cDNA. The full length cDNA was obtained with RACE. Sequence comparison with tammar partial genomic sequence (obtained from the trace archives at NCBI; (<http://www.ncbi.nlm.nih.gov/>)) confirmed the locations of all six exons, consistent with the genomic structure in human, mouse, and opossum. The full-length cDNA encodes a protein of 850 amino acids (Fig. 1) with exon 5 (the largest, about 2.2 kb) encoding the mature protein (Fig. 2A).

Amino acid alignment demonstrates that AKAP4 is highly conserved among the mammals. The entire wallaby AKAP4 protein shares 93.4% amino acid similarity with opossum (another marsupial), 80.0% with platypus (monotreme), 80.8% with human, 84.3% with mouse, 84.4% with rat, 86.7% with dog, 84.6% with cow, and 80.5% with horse. The cyclic AMP-dependent PKA plays a central role in sperm capacitation, motility and in the acrosome reaction. Two tethering domains of PKA, type I α regulatory subunit (RI α), and type II α regulatory subunit (RII α), are highly conserved in all AKAP4 proteins (Fig. 1). Protein tyrosine phosphorylation is one of the most important regulatory pathways to modulate events associated with sperm function, and conserved tyrosine sites have been found across all AKAP4 proteins (Fig. 1). Phylogenetic analysis with PHYLP 3.63 groups AKAP4 proteins into the same three main clusters using three different methods (maximum-likelihood, maximum parsimony, and neighbor-joining; only neighbor-joining tree shown). Tammar wallaby AKAP4 clusters with the opossum and platypus, forming a non-eutherian group, rodents form another cluster while the other eutherian mammals form a third group (Fig. 2B).

Figure 1 AKAP4 protein sequences alignment in known species. AKAP4 amino acid sequences were aligned with the wallaby predicted protein (ABL74506), opossum (XP_001363577), platypus (XP_001509793), human (NP_003877), dog (ENSCAFT00000025355), cow (NM_174235), horse (XP_001496170), mouse (Q60662), and rat (NM_024402). Regions shaded black indicate identical amino acids while those shaded in grey indicate similar amino acid residues. Protein encoded by the corresponding exons is shown for exons 2–6 based on the tammar AKAP4 gene structure. Two conserved tethering domains of PKA, RIIα/RIIα and RIIα, are labeled with yellow blocks. Conserved tyrosine residues are marked with Y.

AKAP4 was expressed in adult, but not developing testis

To determine the AKAP4 gene expression pattern in the tammar, RT-PCR was carried out in adult and developing testis with primers spanning the entire coding region. AKAP4 mRNA was detected strongly in adult testis and

weakly in the epididymis from the caudal region but there was no expression in any other adult tissue (Fig. 3A). No signal was detected in developing testes at any stage of pouch young or juvenile development (Fig. 3B). No AKAP4 expression was detected in

Figure 2 Tammar *AKAP4* gene structure and protein phylogenetic analyses. A) The tammar wallaby *AKAP4* gene consists of 6 exons. The black blocks represent the exons 1–6 of 129, 97, 53, 95, 2154, and 372 bp respectively. The sizes of intron 1 and 2 are 563 and 1841 bp respectively, but the size of introns 3 and 4 are unknown; intron 5 is 156 bp. B) *AKAP4* phylogenetic tree constructed with PHYLIP 3.63 forms three clusters: a marsupial and monotreme group (tammar, opossum, and platypus), rodent group (mouse and rat) and other eutherian mammals (human, cow, dog, and horse).

epididymal sperm (Fig. 3B). Northern blotting analysis was used to quantitate the relative mRNA expression profile of *AKAP4* in tammar wallaby. A primary transcript of 2.9 kb was present only in the testis, and not in the ovary, epididymis or any other somatic tissue examined (Fig. 3A). In addition, two smaller transcripts were detected, less than 1.5 kb in the testis, identical to that seen in mouse (Fulcher *et al.* 1995), the 2.9 kb hybridization band is consistent with predicted mRNA length and was taken to be the primary full-length transcript in tammar wallaby.

***AKAP4* mRNA was detected in round and elongated spermatids**

To gain insight into the conserved role of the *AKAP4* gene, we analyzed the gene expression pattern in adult testes by section *in situ* hybridization. Tammar *AKAP4* mRNA was restricted to the germ cells (Fig. 4A) specifically at the round spermatid (Fig. 4D) and elongated spermatid (Fig. 4G) stage and mRNA was not detected in spermatogonia, spermatocytes (Fig. 4) or the principal piece of sperm tail (Fig. 4J). This implies that

Figure 3 Tammar *AKAP4* RT-PCR expression and northern blotting. A) Expression of the *AKAP4* gene was assessed in a range of adult tissues. *AKAP4* was strongly detected in adult testis and weakly expressed in the epididymis with RT-PCR (~2.5 kb) (top) while only adult testis gave a predominant band of 2.9 kb with northern blotting (bottom). B) *AKAP4* gene expression was profiled in developing testes and purified mature spermatozoa. *AKAP4* was absent from developing testes from before birth until the juvenile state, while it was also absent from epididymal spermatozoa. 18S (100 bp) acted as a positive control.

AKAP4 expression begins post-meiotically. *AKAP4* mRNA was predominantly cytoplasmic with some nuclear staining (Fig. 4D and G). There was no evidence of *AKAP4* mRNA expression outside the tubules.

***AKAP4* protein was present in the sperm tail**

To further understand the role of *AKAP4*, we also investigated the protein distribution in adult testis by immunohistochemistry. Consistent with eutherian studies, very strong staining was observed on the mature spermatozoal tail and was undetectable in spermatogonia, primary spermatocytes, secondary spermatocytes, and round spermatids (Fig. 5A). However, there was some staining in the interstitial capillaries. Western blots were also conducted but yielded additional extra bands apart from the one of the expected size (82KB) (data not shown). Given this result, and the lack of *AKAP4* mRNA staining anywhere else but in the seminiferous tubules, we conclude that the staining in the capillaries was an artifact due to non-specific binding.

The *AKAP4* gene is on the marsupial X-chromosome

Gene mapping was performed to confirm that the location of *AKAP4* in the genome was on the X-chromosome as in all eutherian mammals so far examined. Tammar *AKAP4* was mapped with fluorescent *in situ* hybridization (FISH) to the distal end of the long arm of the X-chromosome (Fig. 6).

Figure 4 *In situ* hybridization of *AKAP4* in the adult tammar testis. *AKAP4* mRNA was detected largely in the cytoplasm of spermatids from the round to elongated stages up to the mature sperm stage. A, B, and C are low power ($\times 4$) images while D, E, F, G, H, I, J, K, and L are high power ($\times 40$). D, E, F show a seminiferous tubule containing round spermatids (RS) G, H, I show a seminiferous tubule containing elongated spermatids (ES) and J, K, L show a seminiferous tubule with spermatozoa (SP) in the last row. The left-hand column shows sections incubated with antisense probe; the middle column shows negative controls with incubated with sense probe and the right-hand column provides standard hematoxylin & eosin (H & E) stained sections. Scale bars=100 μ m.

Discussion

Despite the differences in structural organization of the marsupial and eutherian spermatozoon, it is clear that *AKAP4* encodes a functionally important protein in spermatogenesis. The *AKAP4* orthologues of the marsupial (tammar wallaby) and monotreme (platypus) were highly conserved with those of human and mouse and the expression patterns of tammar *AKAP4* mRNA and protein had similar profiles to those of eutherians. *AKAP4* was only detected in the adult testis of the tammar, and mRNA was observed only in round spermatids and elongated spermatids. The protein was also found in the sperm tail. This highly conserved expression pattern

suggests that tammar *AKAP4* is also the major structural protein of the fibrous sheath, tethering the regulatory subunits of PKA and other enzymes to organelles or cytoskeletal elements, and then initiating the signal-transduction pathways during sperm capacitation and motility (Fulcher *et al.* 1995, Miki *et al.* 2002).

AKAP4 encodes one of the most abundant proteins that form the sperm tail, so it is not surprising that it is highly conserved between tammar and human, mouse and rat. The full-length tammar *AKAP4* gene was 2.9 kb comprising six exons and encoding a predicted protein of 850 amino acids. This is consistent with the gene structure of *AKAP4* in all other mammals examined.

Figure 5 Immunolocalization of *AKAP4* protein in the adult tammar testis. *AKAP4* protein (brown staining) is mainly distributed in the principal piece of the sperm tail in tammar seminiferous tubules (A). There is also some staining in the interstitial tissue. The matching IgG negative control is shown on right panel (B). Scale bars=200 μ m.

Figure 6 Fluorescence *in situ* hybridization (FISH) of AKAP4. Hybridization signals (green) for wallaby AKAP4 are localized to the distal end of the long arm of the X-chromosome (ChX). Only one chromosome pair was labeled per metaphase plate as the cells were derived from a male tammar (the Y-chromosome is indicated: ChY).

With the exception of the non-coding exon 1, all exons are highly conserved, especially exon 5, the largest exon whose coding sequence forms the majority of the AKAP4 mature protein. The difference of N-terminus may reflect the species-specific protein or the different starting/splicing sites at 5'-end. Phylogenetic analysis grouped tammar AKAP4 most closely with that of another marsupial, the grey short-tailed opossum, and a distant relative from the South American marsupial fauna. In addition, we also aligned the major fibrous sheath protein from platypus, predicted to be AKAP4, and demonstrated that this gene is highly conserved in all mammal groups, and has been conserved for at least 166 million years. AKAP4 has not yet been isolated from any non-mammalian vertebrate groups. Mapping confirmed the localization of tammar AKAP4 on the marsupial X-chromosome as in eutherian mammals. This is consistent with the enrichment of genes involved in sex and reproduction on the mammalian X-chromosome (Ohno 1967, Wang *et al.* 2001). In the mouse, pro-AKAP4 (amino acids 1–840) is synthesized in the spermatids and then cleaved to form the mature AKAP4 protein (amino acids 180–840) before transported to the developing flagellum (Johnson *et al.* 1997, Nipper *et al.* 2006). In the tammar, the predicted pro-AKAP4 corresponds to amino acids 1–850 while the mature AKAP4 is encoded by amino acids 180–850 based on alignment with human and mouse. Tammar AKAP4 protein also has conserved functional regions including PKA binding sites and conserved tyrosine residues, suggesting conserved roles in both sperm motility and flagellum structure.

In humans and mice, AKAP4 is expressed exclusively in adult testis (Fulcher *et al.* 1995, Turner *et al.* 1998). Similarly, in the tammar, AKAP4 was only detected by RT-PCR in the adult testis and weakly in the epididymis. As expected, no expression was seen in the mature sperm. As sperm are stored in the epididymis, the weak band presumably reflects a small amount of residual mRNA that was shed into the seminiferous cord lumen and remains in the epididymis. Expression analysis in the developing testes from embryonic development through to adulthood demonstrated that the AKAP4 gene is only expressed in the mature (spermatogenic) testis and not in previous stages or in mature sperm. This is consistent with *Akap4* expression in the mouse (Fulcher *et al.* 1995). Northern blotting confirmed this result showing a major transcript of 2.9 kb only in adult testis. The transcript length is as predicted for the tammar as well as similar to that of mouse and human. *In situ* hybridization demonstrated that AKAP4 expression was restricted to the germ cells, and the expression was tightly regulated in the spermatogenic cycle. AKAP4 transcripts were observed in round spermatids and terminated at the late-stage spermatids (stage 14; Lin *et al.* 1997). Strong expression was restricted to round spermatids and elongated spermatids. Therefore, the *in situ* hybridization results suggest that AKAP4 mRNA is present during the whole period of sperm transformation and sperm tail formation. The distribution of AKAP4 protein predominantly on the sperm tail suggests that it is involved in its formation.

Tammar AKAP4 protein distribution on the mature spermatozoal tail, especially on principal piece, is similar to that seen in mouse (Brown *et al.* 2003), inferring that it functions as a scaffold protein during spermiogenesis in tammar wallaby and by analogy with the mouse, in making sperm motility possible.

This is the first study to examine the AKAP4 gene in a non-eutherian mammal. Its conserved expression pattern and sequence similarity between eutherians, marsupials, and monotremes suggests that AKAP4 has not recently evolved its role in spermatogenesis, but has retained that function at least since mammals diverged from their mammal-like reptile ancestors. As predicted, mRNA distribution and protein localization do not overlap in the adult tammar testis. We have shown that AKAP4 mRNA is present in the round and elongating spermatids, where it is translated and pro-AKAP4 is likely cleaved to form the mature protein. Mature AKAP4 is then transferred to the principal piece of the sperm tail where it likely acts as a scaffold, regulating flagellum function. This pattern of expression is consistent with that in mouse and human (Mohapatra *et al.* 1998). Together, these results show a high degree of conservation of AKAP4 for over 148 million years of divergent mammalian evolution and clearly demonstrate that it acts as a critical X-linked component of mammalian spermatogenesis.

Materials and Methods

Animals

Tammar wallabies *M. eugenii* from Kangaroo Island (South Australia) were maintained in open grassy yards in our breeding colony in Melbourne, Australia. Pouch young of various ages were removed from their mother's pouch for sampling. When the day of birth was uncertain, the age of pouch young was estimated using head length from published growth curves (Poole *et al.* 1991). All sampling techniques and collection of tissues conformed to Australian National Health and Medical Research Council guidelines (2004) and were approved by The University of Melbourne Animal Experimentation & Ethics Committees.

Tissues

Tissues (brain, pituitary, hypothalamus, olfactory bulb, thyroid, muscle, spleen, heart, lung, liver, kidney, adrenal, mammary gland, uterus, and ovary) were collected from three adult females, and from two adult males (epididymides and testes). All tissues were collected under RNase-free conditions. Developing testes from three fetuses sampled 1–2 days before birth (d25) and from pouch young aged d2 ($n=3$), d8 ($n=3$), d12 ($n=2$), d21 ($n=3$), d44 ($n=3$), d80 ($n=2$), d150 ($n=1$), d264 ($n=1$), d365 ($n=1$), juveniles (~14 months old ($n=3$)) (puberty/spermatogenesis in male tammar begins at around 19 months and males becomes fully mature at 25 months; Williamson *et al.* 1990) and mature adults ($n=2$) for molecular analysis were snap frozen in liquid nitrogen and stored at -80°C until use. Mature sperm were collected from adult epididymides, in 1 X PBS and snap-frozen. Tissue for *in situ* hybridization was fixed overnight in 4% paraformaldehyde, washed several times in 1 X PBS, and stored in 70% ethanol before paraffin embedding and sectioning at 8 μm .

Cloning of tammar AKAP4 and determining gene structure

AKAP4 was initially cloned from adult testis by RT-PCR using cross species primers (AF1 and AR1) designed to conserved regions of the gene. The resulting 2475 bp PCR product was then used to design tammar specific primers for 3' RACE and 5' RACE to fully characterize the AKAP4 transcript (the sequences of all primers are listed in Table 1).

Complementary DNA was reverse-transcribed from total RNA of adult testis of tammar wallaby, using the SMART cDNA library construction kit (Clontech). 5' RACE was performed using primer 5' PCR and primer AR1. Owing to an incomplete coding sequence at the 5' end, we designed a new primer AR2 as a nested primer according to the first sequence result and repeated 5' RACE. 3' RACE was performed using primer AF1 and CDS III nested PCR was performed using the AF2 and CDS III primers. PCR cycling conditions were: 35 cycles of 30 s, 95 $^{\circ}\text{C}$; 60 s, 55 $^{\circ}\text{C}$; 120 s, 72 $^{\circ}\text{C}$, in a 25 μl reaction with GoTaq Green Master Mix (Promega), 0.4 μM each.

To determine the tammar AKAP4 gene structure, comparative analysis was carried out using the gene structure of the human, mouse, rat, and South American grey short-tailed opossum *Monodelphis domestica* and the platypus

Table 1 Primers designed for analysis of AKAP4 expression by RT-PCR.

Primers	Sequence (5' → 3')	Function
AF1	TGTCTGATGATATTGACTGGTT	Cross species cloning & RT-PCR
AR1	TGGCAAACCTTCATGACCTC	Cross species cloning & RT-PCR
AF2	TTTACGGGAGATGAGGATG	3' RACE
AR2	AATAGGGTCCGAGGGTGCT	5' RACE
SMART IV	AAGCAGTGGTATCAACGCA-GAGTGGCCATTACGGCCGGG	5' RACE
CDS III	ATTCTAGAGGCCGAGCGCGCCGA-CATG-d(T) ₃₀ N ₋₁ N (N=A, G, C, or T; N ₋₁ =A, G, or C)	3' RACE
5' PCR primer	AAGCAGTGGTATCAACGCAGAGT	5' RACE
18S F	GATCCATTGGAGGGCAAGTCT	RT-PCR
18S R	CCAAGATCCAACACGAGCTTTT	RT-PCR
AF3	GTGGCCTCTGACATGATGGT	BAC screen
AR3	GCGTTTCAGCATAGCCTC	BAC screen

F denotes forward primers, R denotes reverse primers. All primers are shown in the 5'–3' direction; CDSIII, Smart IV and 5' PCR were synthesized by SIGMA (Genosys) according to the manual of the SMART cDNA library construction kit from Clontech.

Ornithorhynchus anatinus (<http://www.ensembl.org/>). Partial genomic sequence of *M. eugenii* was obtained from the trace archives at NCBI (<http://www.ncbi.nlm.nih.gov/>) to confirm the number of exons and the length of exons and introns.

Alignment and phylogenetic analysis

The AKAP4 protein sequences, human (NP_003877), mouse (Q60662), rat (NM_024402), cow (NM_174235), horse (XP_001496170), opossum (XP_001363577), and platypus (XP_001509793) were retrieved from GenBank (<http://www.ncbi.nlm.nih.gov/>). The sequence of dog (ENSCAFT00000025355) was retrieved from the Ensembl (<http://www.ensembl.org/>). The tammar AKAP4 (ABL74506) protein sequence has been submitted to GenBank. The above AKAP4 protein sequences were aligned with Clustal X 1.83 (<http://www.clustal.org/>), and edited by GeneDoc (<http://www.nrbsc.org/downloads/>). The phylogenetic tree was constructed with the PHYLIP 3.63 program (University of Washington) using standard settings, maximum-likelihood, maximum parsimony, and neighbor-joining analysis with 1000 replicates, and viewed with TREE-view 1.6.6.

RT-PCR expression analyses

In order to check the expression pattern of AKAP4 in developing testis and adult tissues as well as mature sperm, total RNA was isolated using RNAwiz (Ambion Inc., Austin, TX, USA) according to the manufacturer's instructions. The quality and quantity of total RNA was verified by two methods, gel electrophoresis, and optical density reading with a Nanodrop (ND-1000 Spectrophotometer, Wilmington, USA). 2 μg of total RNA was DNase-treated with DNase I (Ambion Inc.) for 30 min. 1 μg of total RNA was reverse-transcribed using SuperScript III kit (Invitrogen).

PCR was performed in a 25 µl reaction with GoTaq Green Master Mix with 0.4 µM of each primer (AF1 and AR1) and first-strand cDNA products. Amplification conditions were: 95 °C 30 s; 55 °C 60 s; 72 °C, 120 s for 35 cycles (AKAP4), or 25 cycles (18S). Samples were analyzed on a 1.2 and 2% agarose gel for AKAP4 and 18S respectively.

RNA isolation and northern blotting hybridization

Total RNA was isolated from snap-frozen adult tissues with RNAwiz (Ambion Inc). The concentration and quality of total RNA were examined same as above. Equivalent amounts of RNA (about 12 µg) were subjected to electrophoresis in 1% agarose gels containing formaldehyde (Sambrook *et al.* 1989). Northern blotting was performed according to standard methods, with hybridization at 42 °C in ULTRAhyb solution (Ambion Inc.) with [α -³²P]dCTP-labeled cDNA probe (a 2475 bp DNA fragment representing the conserved AKAP4 domain was generated by the PCR), and autoradiographed at -70 °C.

mRNA in situ hybridization

For *in situ* hybridization to adult testes sections, antisense, and sense RNA probes were prepared separately from a region including AKAP4 domain (~2.5 kb) of the tammar wallaby and labeled with digoxigenin-UTP, using SP6 or T7 RNA polymerase (Sp6 for production of sense probe, T7 for antisense probe). Tissues were fixed in 4% paraformaldehyde overnight at 4 °C, rinsed several times in 1 X PBS, embedded in paraffin, sectioned onto polysine slides (Menzel-Gläser, Braunschweig, Germany). After de-waxing, the sections were washed several times with 1 X PBS, glycine, Triton X-100, and triethanolamine buffer, and then were immediately hybridized (42 °C) and hybridization signals were detected by NBT/BCIP system according to the manufacturer's instructions (Roche GmbH, Mannheim, Germany). 0.1% nuclear Fast Red (Aldrich Chemical Corp., Milwaukee, WI, USA) was used for counterstaining.

Immunohistochemistry

Tissue sections (8 µm) were prepared as above and de-waxed. Antigen retrieval was performed in boiling 0.005 M Tris-EDTA (pH 9.0) for 20 min, and treated with 3% hydrogen peroxide in methanol for 15 min. The AKAP4 primary antibody (rabbit anti-mouse, gift from Dr Edward M Eddy) was applied to tammar adult testis tissue sections used at 1:150 dilutions at 4 °C overnight. Signal was amplified using the ABC/HRP kit (DAKO, New South Wales, Australia), visualized with DAB (DAKO), and counterstained with hematoxylin.

BAC library screening and chromosomal mapping

To determine the chromosomal localization of AKAP4 in the tammar, a *M. eugenii* BAC library was screened with the AKAP4 2475 bp probe, used for the Northern blots. Membranes were pre-hybridized for 2 h, the [α -³²P] dCTP-labeled

probe was hybridized at 65 °C overnight. Filters were washed with 2 X SSC/0.1%SDS, 1 X SSC/0.1%SDS, 0.1 X SSC/0.1%SDS 10 min each at 65 °C and autoradiographed at -70 °C for 2–5 days.

Chromosome preparations were made from peripheral blood according to standard methods with minor modifications (Schempp & Meer 1983). Chromosome FISH was performed as previously described with minor modifications (Wilcox *et al.* 1996). The BAC genomic DNA was labeled with dUTP-digoxigenin (DIG) by nick translation at 14 °C for 1 h and pre-blocked with tammar wallaby *Cot-1* DNA prior to hybridization. The probe was hybridized to tammar metaphase chromosome spreads at 37 °C overnight. Hybridization was detected using mouse anti-DIG-FITC antibody (Serva, Heidelberg, Germany). After hybridization, the chromosome preparations were stained with DAPI (4, 6-diamidino-2-phenylindole) to visualize the chromosomes.

Declaration of interest

There is no financial or other potential conflict of interest.

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