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RESEARCH ARTICLE

Evolution of the Cdk-activator Speedy/RINGO in vertebrates

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Abstract Successful completion of the cell cycle relies on the precise activation and inactivation of cyclin-dependent kinases (Cdks) whose activity is mainly regulated by binding to cyclins. Recently, a new family of Cdk regulators termed Speedy/RINGO has been discovered, which can bind and activate Cdks but shares no apparent amino acid sequence homology with cyclins. All Speedy proteins share a conserved domain of approximately 140 amino acids called "Speedy Box", which is essential for Cdk binding. Speedy/RINGO proteins display an important role in oocyte maturation in *Xenopus*. Interestingly, a common feature of all Speedy genes is their predominant expression in testis suggesting that meiotic functions may be the most important physiological feature of *Speedy* genes. Speedy homologs have been reported in mammals and can be traced back to the most primitive clade of chordates (*Ciona intestinalis*). Here, we investigated the evolution of the *Speedy* genes and have identified a number of new Speedy/RINGO proteins. Through extensive analysis of numerous species, we discovered diverse evolutionary histories: the number of *Speedy* genes varies considerably among species, with evidence of substantial gains and losses. Despite the interspecies variation, Speedy is conserved among most species examined. Our results provide a complete picture of the *Speedy* gene family and its evolution.

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Introduction

Cyclin-dependent kinases (Cdks) are important regulators of the cell cycle in eukaryotic cells. The functions of Cdks are determined by their binding partners, cyclins, which activate the catalytic subunit and influence the substrate specificity both temporally and spatially. The members of Cdk and cyclin families expanded with increasing complexity of the animal kingdom. In budding yeast, *Saccharomyces cerevisiae*, a single Cdk (Cdk1/Cdc28p) and minimally two types of cyclins are sufficient to drive cell cycle progression. While mammals express several Cdks and cyclins, the functions of Cdks and cyclins became more specialized. A general concept of the somatic cell cycle is that cyclin D complexed with Cdk4/Cdk6 initiates G1/S transition, cyclin E/A complexed with Cdk2 sustain S-phase, and finally, cyclin B/Cdk1 drives mitosis. The transcriptional regulation and ubiquitin-mediated degradation of the cyclins are tightly controlled to ensure proper timing of cell cycle progression. The activity of Cdk/cyclin complexes is also modulated by

posttranslational modifications (inhibitory or activating phosphorylation, ubiquitylation, etc.) [1].

Speedy/RINGO (*R*apid *in*ducer of G_2/M progression in *o*ocytes) is a novel cell cycle regulator, which, despite the lack of sequence homology to any known cyclins, can catalytically activate Cdk1, Cdk2, and Cdk3 [2, 3]. Interestingly, the activation of Speedy/RINGO-Cdk complexes does not require activating phosphorylation by the Cdk-activating kinase (CAK), which is essential for all other known cyclins [4, 5]. *Xenopus* Speedy (Spy1) was originally identified in a screen for genes that confers resistance to UV irradiation in a *rad1*-deficient strain of *Schizosaccharomyces pombe* [6]. At the same time, an identical protein, RINGO, was identified in a screen for activators of the G2/M phase transition during *Xenopus* oocyte maturation [2]. The identification of Spy1/RINGO led to the discovery of new Speedy/RINGO members in mammals [7, 8]. It appears that, in mice and primates, several new Speedy/RINGO genes have evolved. Consistent with the function of Speedy/RINGO in *Xenopus* meiosis [9], expression in testis is a common feature of all *Speedy* genes. Comparative sequence analysis revealed a conserved region among the Speedy proteins. The conserved "Speedy domain" is responsible for Cdk binding. In cell culture-based studies, some of the Speedy proteins display potential of promoting the G1/S and G2/M transition and might also be involved in DNA damage control, while others appear to have negative effects on cell cycle progression [6, 10, 11]. In a recent study, it was reported that RINGO-activated Cdk1 and Cdk2 could inhibit Myt1, an inhibitory kinase of Cdk1/cyclin B, by phosphorylating it at several residues, which in turn triggers its inactivation and allows M-phase entry [12]. However, none of the *Speedy* genes has been knocked out in mice, which would provide important information about their physiological functions in mammals.

A preliminary sequence analysis of Speedy/RINGO proteins has been reported [7]. With the recent progress in genome sequencing of a variety of organisms, it is both interesting and necessary to revisit this topic to obtain a complete picture of the evolution of the Speedy/RINGO proteins. Here, we report the identification of several new *Speedy* genes from human and other species and we investigate the evolution of the *Speedy* genes relative to each other. Our results provide an overview of the evolution and possible physiological functions of the *Speedy* gene family.

Materials and methods

Identification of a *Speedy* gene in amphioxus

A TBLASTN search was performed against the amphioxus cDNA database (v.1.0), downloaded from the DOE Joint Genome Institute. The transcript with the best match was used to BLAT against the amphioxus genome assembly (Lancelet Mar. 2006 Assembly) on the UCSC genome browser.

Identification of *Speedy* genes in *Xenopus tropicalis*

We searched the *Xenopus tropicalis* assembly on the UCSC Genome Browser (*X. tropicalis* Aug. 2005 Assembly) using human and mouse *SpeedyB* genes. The top BLAT hit was found in the region that has been previously annotated as *Speedy* or *Speedyx* gene on scaffold_296. We designated this gene as *Xenopus SpeedyB* gene. We did not find any significant matches to *SpeedyB4* and *SpeedyB3* in the *Xenopus* genome assembly. Thus, we conclude that these two genes are missing in *Xenopus*.

Identification of *Speedy* genes in teleost fishes

Previously, a *SpeedyA* gene has been identified in the genome assembly of zebrafish. We used the sequence of zebrafish and elephant shark (*Callorhinchus milii*) *SpeedyA* genes to search for their orthologs in the genome assemblies of *Tetraodon* (*Tetraodon* Mar. 2007 Assembly), stickleback (Stickleback Feb. 2006 Assembly), and medaka (Medaka Oct. 2005 Assembly) on the UCSC Genome Browser. We could identify *SpeedyA* orthologs in the genomes of all three fishes. However, when we searched the zebrafish (Zebrafish Dec. 2008 Assembly) and the other three teleost genome assemblies for orthologs of elephant shark *SpeedyB* and mammalian *SpeedyB4* or *SpeedyB3* genes, there were no significant matches. Thus, we conclude that teleost fish genomes contain only the *SpeedyA* gene.

Cloning and expression analysis of elephant shark *Speedy* genes

A low-coverage (1.4×) sequence of the elephant shark genome has recently been generated [13]. We used protein sequences of *Speedy* genes from human, mouse, and zebrafish to search the elephant shark genome sequences (http://esharkgenome.imcb.a-star.edu.sg/) using TBLASTN and identified two scaffolds (AAVX01134848.1 and AAVX 01460574.1) that displayed similarity to Speedy sequences. These scaffolds contained partial coding sequences for *Speedy* genes. We designed appropriate primers for the coding sequences and generated full-length cDNA sequences for two elephant shark *Speedy* genes by doing 5′ and 3′RACE using cDNA from elephant shark testis.

The expression patterns of elephant shark *Speedy* genes were determined by qRT-PCR. Total RNA was extracted from various tissues of elephant shark by the TRIzol method. An amount of 1 µg of total RNA was

reverse-transcribed using the SMART rapid amplification of cDNA ends (RACE) cDNA Amplification kit (Clontech, Mountain View, CA, USA). qRT-PCR was carried out with 1 µl of the cDNA as a template with the following primer sets: *SpeedyA* (F: TAG CGA ATA CCA TGG AAG AAG ATG AAG and R: CTC CTC ACA GCA GCG CTT GCT); and *SpeedyB* (F: CTT TGG GCG CAG ATG AAT TTC C and R: TCC TTC TGC AGG TAA CTC CTG AC). *β*-actin was amplified as an internal control for normalization using primer set (F: GGT ATT GTC ACC AAC TGG GAC and R: AGA TGG GCA CAG TGT GGG TG). PCR was performed on an Applied Biosystems 7300 Real-Time PCR system (Life Technologies, Carlsbad, CA, USA) using KAPA SYBR FAST Universal qPCR kit (Genome Holdings), and relative quantification (RQ) was determined using the 7300 System SDS software v1.4 (Applied Biosystems). The PCR cycles comprised an initial denaturing step of 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. cDNA-starting material was normalized to *β-actin* expression and the mean threshold cycle (Ct) was used to determine relative levels of expression (RQ).

5′ and 3′RACE analysis were performed according to the manufacturer's instructions (Clontech). The sequences of gene-specific primers used in RACE are available on request. All PCR products were sequenced completely using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Identification of *Speedy* genes in the chicken genome

The human *SpeedyA* gene was searched against the chicken genome (NCBI Gallus_gallus-2.1 reference assembly). With the resulting alignment, part of the protein sequence from the high-scoring segment pairs was BLASTed against the chicken assembly on the UCSC Genome Browser (Chicken May. 2006). A 5.6-kb part of the region that contained the BLAT hit was retrieved for subsequent search (BLASTX) against the NCBI NR database. The protein sequence from the top hit, with the longest open-reading frame, was identified as the chicken *SpeedyA* gene. Searching the chicken genome with *SpeedyB1*, *SpeedyB3*, and *SpeedyB4* sequences from human or mouse did not identify any genes with significant similarity.

Identification of *Speedy*B genes in rat and mouse genomes

The rat genome assembly (Rat Nov. 2004 Assembly) was searched by BLAST using SpeedyB1 and SpeedyB2 sequences of mouse. This search identified two *SpeedyB* genes on rat chromosome12 located between the genes for Zfp316 and Rnf216, similar to the genomic context of mouse *SpeedyB1* on chromosome 5 of the mouse assembly (July 2007 NCBI37/mm9). The two genes are linked tail-to-tail similar to the mouse genes. We could predict the protein sequence only for the rat *SpeedyB1* gene, since the *SpeedyB2* gene contained frame-shifts, presumably due to sequencing errors. The rat *SpeedyB3* gene was identified using the mouse SpeedyB3 sequence as a query. The rat *SpeedyB3* is located on chromosome 3 flanked by *Bcl2-like11* gene upstream and *Anapc1* gene downstream. Searching of the rat and mouse genome assemblies using human *SpeedyB4* gene did not produce any significant matches. Therefore, we conclude that this gene is absent in rat and mouse. For cloning of mouse *SpeedyB3*, RT-PCR was carried out with 1 µl of the testis cDNA as a template with the following primers (F: CTC GAG CTA AGG GGT GCC CTG GCG [PKO968] and R: CAC GAC CCT GCT GAT GGTC [PKO969]). The PCR cycles comprised an initial denaturing step of 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. PCR products were cloned and sequenced completely using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). The expression patterns of mouse *Speedy* genes were determined by qRT-PCR. Total RNA was extracted using the Qiagen RNeasy mini kit according to the manufacturer's protocol. For each qPCR reaction, first-strand cDNA was synthesized from 0.2 µg total RNA using the SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. qRT-PCR was carried out with 1 µl of the cDNA as a template with the following primer sets: *SpeedyA* (F: CTG GCT AAT ACG GTT GAA G [PKO965] and R: CCA GAG TTG GTC CCT TAA C [PKO2696]); *SpeedyB1a* (F: ATG CCC AGC GCC CCA TGT TC [PKO2703] and R: GCG ATC TCG TGC CCA CAC CC [PKO2704]); *SpeedyB1b* (F: GAC CTG GCA GAG CTA TTT G [PKO991] and R: GAC CTT GTG GTG TTC AGG [PKO2695]); *SpeedyB3* (F: ATC TGC TGT CTA TGG TGG TG [PKO999] and R: CAT CCT CTT CCA TGT CAC AG [PKO998]); *β*-actin was amplified as an internal control for normalization using primer set PKR143 (F: ACG GCT CCG GCA TGT GCA AA, R: TTC CCA CCA TCA CAC CCT GG). PCR amplification was carried out using the Maxima SYBR Green qPCR Master Mix (Fermentas, K0252) and reactions were monitored continuously in a Rotor-Gene thermal cycler (Corbett Research) with the following program: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. All data were normalized to the expression levels of *β-actin* expression and the mean threshold cycle (Ct) was used to determine relative levels of expression (RQ).

Identification of *Speedy* genes in chimpanzee and human genomes

BLAST searches using human *SpeedyB1* gene resulted in several hits in the human genome. The query sequence indicated similarity to various SpeedyB2 or B2-like (SpeedyE or E-like) proteins. The human *SpeedyB1* and *SpeedyB4* genes were BLAST searched against the chimpanzee genome assembly on the UCSC Genome Browser (Chimp Mar. 2006 Assembly). The homolog of the chimpanzee *SpeedyB1* gene was identified on chromosome 17 while the homolog of *SpeedyB4* was identified on chromosome 11. The full-length protein sequences of chimpanzee genes were predicted based on similarity to their human orthologs. We searched the chimpanzee and human genome assemblies for the orthologs of mouse *SpeedyB3* gene and did not find any matches with significant similarity. Thus, we infer that *SpeedyB3* is missing in chimpanzee and human. The expression patterns of human *SpeedyB* genes were determined by RT-PCR. Total RNA was purchased from Stratagene. For each RT-PCR reaction, first-strand cDNA was synthesized from 0.2 μg total RNA using the SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. RT-PCR was carried out with 1 μl of the cDNA as a template with the following primer sets: *SpeedyB1* (F: GAT CCC AGC CCC CAG CCT CA [PKO2799] and R: GCC CAC ACA GCG TCT CCA CC [PKO2800]); *SpeedyB2* (F: GAT GAT GAG CCA GAG AAG GAG [PKO2471] and R: GGT ATG TGA GAG TGG GTC TTC C [PKO2472]); SpeedyB2-*like1* (F: GAC GAC GAG GAC TCC AAA C [PKO2467] and R: CTA GGA AAG GTG AGC GCG AT [PKO2468]); *SpeedyB2-like2* (F: GTG GTG GGA CAA ATC TGA GG [PKO2474] and R: GGT AGA GAG CCA GGA AGA AAT G [PKO2475]); *SpeedyB4* (F: CGC CAG TGC TGT GAG GAG GTC A [PKO2791] and R: GAA CAG TGG GGC GGC GAG AG [PKO2792]);. *β*-actin was amplified as an internal control using primer set (F: AGC GAG CAT CCC CCA AAG TT [PKO2481] and R: GGG CAC GAA GGC TCA TCA TT [PKO2482]).

Prediction of Speedy genes by HMM searches

In order to verify whether we have missed any Speedy genes in the above vertebrate genomes and superfamilies, a more detailed search was carried out using HMMER3.0 against a high quality curated family of Hidden Markov Model (HMM) database in Pfam (Pfam26.0). Whole genome ab initio *Ensembl* Genscan predictions from all the species were downloaded from the *Ensembl* FTP site. All Genscan-predicted proteins were searched for the presence of known HMM models using "hmmscan". The Genscan predictions with HMM hits to Speedy model (Spy1) were aligned with Speedy proteins previously identified, and Neighbor-joining trees were constructed to examine the identity of the predicted genes. However, this analysis did not identify any new *Speedy* genes in addition to those identified by us using BLAST searches.

All the Speedy proteins were searched against the conserved domains database (CDD) at NCBI using CD-search with default parameters. However, only the *Spy1* superfamily domain could be found in each of the sequences indicating that Speedy proteins do not contain any other known conserved domains.

Alignment of genomic regions of duplicated human and mouse *Speedy* genes

In order to identify the extent of conservation between closely linked duplicated human and mouse *Speedy* genes, we performed a global alignment of duplicated loci using MLAGAN and predicted conserved sequences with a criterion of >70 % sequence identity over a 100-bp window size. The conserved sequences were visualized using VISTA [40]. The genomic regions were retrieved from UCSC Genome Browser and included the promoter, untranslated, exonic and intronic sequences of the genes.

Phylogenetic analysis

Full-length protein sequences encoded by vertebrate *Speedy* genes together with the single Speedy protein in amphioxus were aligned using CLUSTALW. The Neighbor-joining tree was constructed using MEGA5 (http://www.megasoftware.net) with 5,000 bootstrap replications, assigning amphioxus *Speedy* as the out-group. The tree is presented as a phylogram in which the branch lengths are proportional to the substitution rates.

Results

The evolution of the *Speedy* genes

To understand the origin and evolutionary history of vertebrate *Speedy* genes and to gain an insight into their physiological functions, we have identified and analyzed Speedy genes from a number of different species including the most basal chordates. So far, *Speedy* genes in different organisms are known by different names (e.g., *Speedy* or *RINGO*) that carry suffixes (e.g., *SpeedyE5*, *SpeedyE7*, *SpeedyE8p* are present in human but have no orthologs in mouse or chicken), which do not actually reflect their orthologous relationships. This has been a major drawback in comparing *Speedy* genes and their functions in different vertebrates. To resolve such

confusions, we have classified the *Speedy* genes into two subgroups that were present in the common ancestor of jawed vertebrates (SpeedyA and SpeedyB) and renamed some of the genes based on their clustering pattern in the phylogenetic tree of Speedy proteins (Fig. 1).

A *Speedy* gene in amphioxus

Amphioxus represents the most basal group of chordates, the Cephalochordates, which are a sister group to the clade that includes Urochordates (e.g., sea squirt *Ciona*) and vertebrates. A partially predicted *Speedy* gene has been previously identified in the sea squirt, *Ciona intestinalis* (XP_002122378) [Table 1]. However, no Speedy homolog has been identified in amphioxus. Our extensive search for *Speedy* genes in the amphioxus genome assembly identified one single Speedy on the chromosome (chrUn: 251,260,657-251,269,524) flanked by the Synoviolin 1 isoform b (*SYVN1*) gene and the tRNA methyltransferase 61 homolog B (*Tmrt61b*) gene, upstream and downstream, respectively. We predicted the sequence of the complete *Speedy* gene in amphioxus based on similarity to the protein sequences and exon–intron organizations of *Speedy* genes in vertebrates. The amphioxus Speedy protein is 45 % identical to the Ciona Speedy. As it displays similarity to both elephant shark SpeedyA (49 %) and SpeedyB (47 %) proteins (described below) we have used it as a root for generating the neighbor-joining tree (Fig. 1).

The presence of a single *Speedy* gene in amphioxus indicates that duplication of *Speedy* genes occurred in the common ancestor of vertebrates after it diverged from the invertebrate lineage.

Speedy genes in elephant shark

The cartilaginous fishes (class chondrichthyes) comprising chimaeras, sharks, rays, and skates are the most basal group

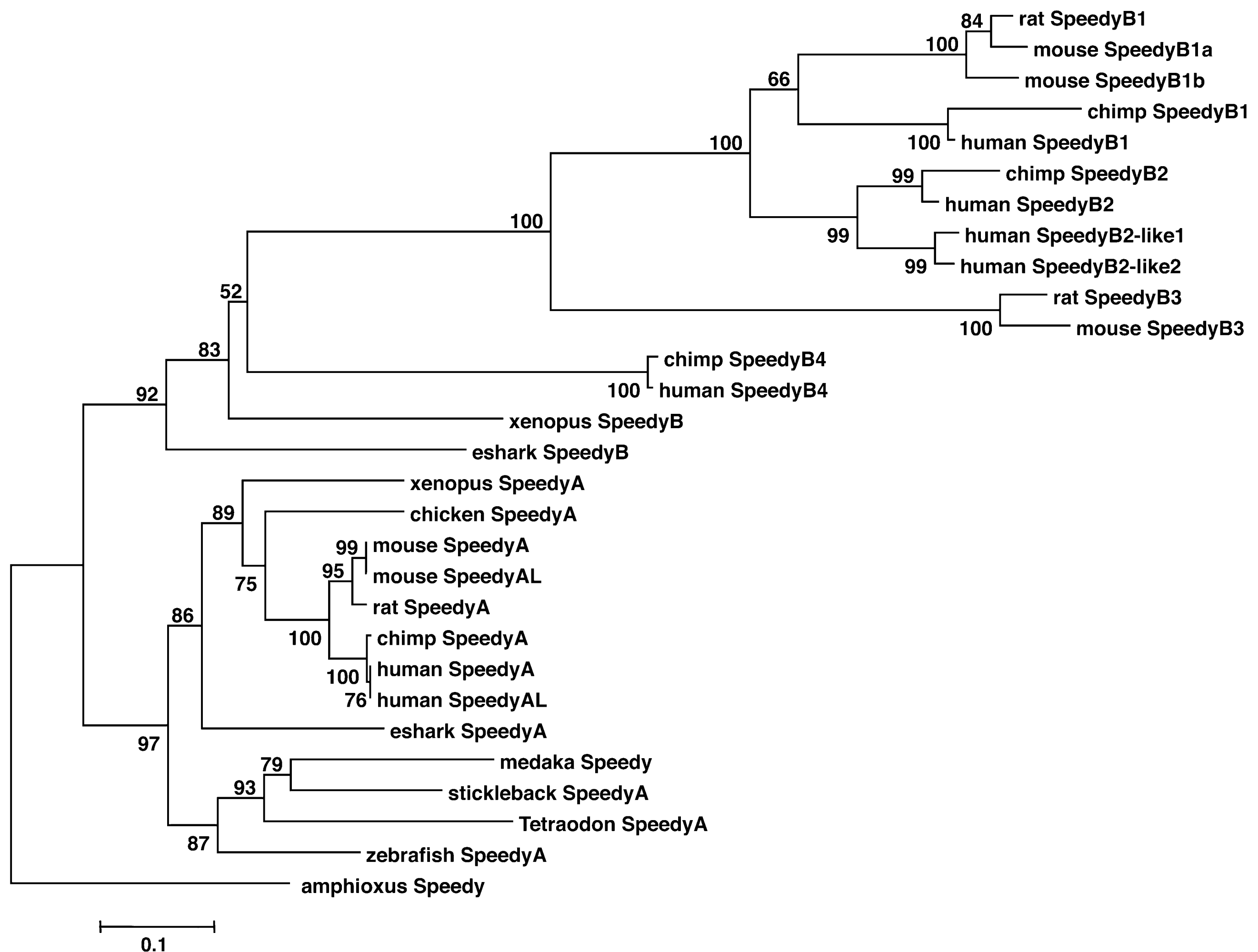


Fig. 1 Phylogenetic relationship of the Speedy protein family. Neighbor-joining phylogenetic tree displaying the evolutionary relationship of Speedy proteins based on their amino acid sequences. The amphioxus Speedy sequence was used as an out-group to root the tree. *Numbers* represent bootstrap values (given as percentages) for a particular node

Table 1 Speedy/RINGO proteins in different species

Rat_SpeedyA [NP_620210, 312aa, *Rattus norvegicus*]

MRHNQMCCETPPTVTVHVKSGSNRSHQTRKPVSLKRPILK
DSWQASEKNAHNNKPKRPRGPCLIIQRQEMTAFFKLFDDD
LIQDFLWMDCCCKIADKYLLAMTFVYFKRAKFTISEHTRI
NFFIALYLANTVEEDEEEAKYEIFPWALGKNWRKLFPNFL
KLRDQLWDRIDYRAIVSRRCCEEVMAIAPSHYIWQRERSV
HHSGAARNYNRDEVHLPRGPSATPVDCSLCGKKGRYVRL
GLSSSSSSSSDIVELTGKRSQELHNSLSMDMIGDPSQANTY
SQVANDHQSKKENETNFVKKTKSMGWFAESEE

Rat_SpeedyB1 [EDL89682.1 rCG42640, 268aa, *Rattus norvegicus*]

MGEGTPGVDSARVQEEGGIDQSPGFVERGIQVGRIVTAGQLSL
CSEEQSPQPGITRPSPGVVVDGEISGTAEPRVEARSQPPSS
SPKRKRDLSSDSEDDLAELLEPDPQPVWSVETLCGLRMRL
KRRRVSTVRPEHHKVFTRLLEDPVVKKFLNWDKMLRVSD
KYLLSMVIAYFSRAGLFSWQYRPIHFFLALYLANDMEEDN
QAPKQDIFYFLYGKSYAQRPMFHKLRFQFIRSMGWRIWVS
REECEEIQAYNPDLWVWARDRTNLT

Rat_SpeedyB3(D) [NP_001019483.1, 339aa, *Rattus norvegicus*]

MSTPVASETTQRLQKPKKGQKRKVPVKAMLAVTDRRSEMSP
VSPKVSCKQNDSGKQVPGDKVCLAQKAPQASSILASSDASA
GDVPEQRSKRKRGQRKRKLENIKTDPEACIVLASSDASAGD
VPVQRTKRKRVHKTKTLVDVKAATQENSMQASSTATPEA
APGTASELKFLRRGKRKSIWTVDRIEGTKLIMNKKRRPSYR
PEDLEAFYRLLEDPVVQNFLAADIFFRVTDKYLLSMVVEYF
GRVGLPGLYNRIHFFLALYIACDMEEDDPISKRSIFQFLLGR
DTWQDLYKDFLKLQRDFFQAMDYRAWVTPEQCVEIQNQN
PHHWVWSRVRQGTP

Mouse_SpeedyA [NP_001136103, 283aa, *Mus musculus*]

MRHNQMYCETPPTVTIHVKSGSNRSHQTRKPISLKRPILKDSW
EASENNAQNNKSKRPRGPCLIIQRQEMTAFFKLFDDDLIQD
FLWMDCCCKIADKYLLAMTFVYFKRAKFTINEHTRINFFIA
LYLANTVEEDEEEAKYEIFPWALGKNWRKLFPNFLKLRDQ
LWDRIDYRAIVSRRCCEEVMAIAPTHYIWQRERSVHHSGA
VRNYNRDEVHLPRGPSATPVDCSLCGKKGRYVRLGLSSSS
SSSSDTGELMEKGSQELHSAFSVDTAGDPPHTYSQGMA

Mouse_SpeedyAL [NP_083530, 310aa, *Mus musculus*]

MRHNQMYCETPPTVTIHVKSGSNRSHQTRKPISLKRPILKDSW
EASENNAQNNKSKRPRGPCLIIQRQEMTAFFKLFDDDLIQDF
LWMDCCCKIADKYLLAMTFVYFKRAKFTINEHTRINFFIAL
YLANTVEEDEEEAKYEIFPWALGKNWRKLFPNFLKLRDQL
WDRIDYRAIVSRRCCEEVMAIAPTHYIWQRERSVHHSGAVR
NYNRDEVHLPRGPSATPVDCSLCGKKGRYVRLGLSSSSSSS
SDTGELMEKDSQELHSAFSVDTAGDPPHTYSQVANDHQSN
KENETNFVKKNKSVEWFAESEE

Mouse_SpeedyB1a [NP_083324.3, 268aa, *Mus musculus*]

MGEGTPGVDSARVQEEGGRDQSLGFVEGRIQVGRIVTAGQL
SLCSEEQSPQPGITRPSPGVVVDGESSGLAEPRVEATPQPPSS
IQKRKRDESLDSEDDLAELFEPDPQPVWSVEMLCGLRMRL
KRRRVSTVRPEHHKVFTKLLEDPVVKKFLTWDKMLRVSD
KYLLSMVIAYFSRAGLFSWQYRPIHFFLALYLANDMEEDN
QAPKQDIFYFLYGKSYAQRPMFHKLRFQFIRSMGWKIWVS
REECEEIQAYNPDLWVWARDRTNLT

Mouse_SpeedyB1b [NP_808548.1, 236aa, *Mus musculus*]

MVMPWSSPLCTMPSKSAFFSQPRVEATPQPPSSIQKRKRDESS
DSEDDLAELFEPDPQPVWSVETPCGLRMTLQRQCVSTVRP
EHHKVFTKLLEDPVVKKFLTWDKMLRVSDKYLLSMVIAY
FSRAGLFSWQYRPIHFFLALYLANDMEEDNQAPKQDIFYFL
YGKSYAQRPMFHKLRFQFIRSMGWRIWVSQEECEEIQAYD
PELWVWTRDRTKLTQNPRVMDTRGHPPAIDPCA

Table 1 continued

Mouse_SpeedyB3(D) [JQ_023159.6, 341aa, *Mus musculus*]

MSTPVASDTTPRLQKPTKGQKKKVPVKATIGVSSDRRSEMSP
VSSKESCKQNDSGKQVPADSVCLAKKAPQVTSILASSDGS
AGDVPEQRSKRKKGQRKRKLDNIKMDPEVCVTLASSDAS
AGDVPVQRTKRKRAHKTRTLVDVKAAIQEGSVLASSTATP
EAAPGATSKLKFPRRGKKRSIWAVNRIEGMKLILNKKRRAS
YRPEDLEAFYRLLEDPVVQNFLAADIFFRVTDKYLLSMVV
EYFGRVGLPGHLYNRIYFFLALYIACDMEEDDPISKRSIFQ
FLLGRDTWQGLYKDFLKLQKDFILAMDYQAWVTPDQCA
EIQNQNPQHWVWSRVRQGTP

Chimp_SpeedyA [XP_515374, 313aa, *Pan troglodytes*]

MRHNQMCCETPPTVTVYVKSGSNRSHQPKKPITLKRPIFKDN
WQAFEKNTHNNNKSKRPKGPCLVIQRQDMTAFFKLFDDD
LIQDFLWMDCCCKIADKYLLAMTFVYFKRAKFTISEHTRIN
FFIALYLANTVEEDEEETKYEIFPWALGKNWRKLFPNFLKL
RDQLWDRIDYRAIVSRRCCEEVMAIAPTHYIWQRERSVHH
SGAVRNYNRDEVQLPRGPSATPVDCSLCGKKRRYVRLGLS
SSSSLSSHTAGVTEKHSQDSYNSLSMDIIGDPSQAYTGSEVV
NDHQSNKGKKTNFLKKDKSMEWFTGSEE

Chimp_SpeedyB1 [XP_511850.2, 252aa, *Pan troglodytes*]

MASGQARPPFEEESPQPSTTVRSPEVVVDDEVPGPSAAWIDP
SVQPQSLDLKRKSEWSDESEEELEEELELERAPEPEDTWV
VEMLCGLKMKLKRKRASSVLPEHHKAFNRLLGDPVVQKFL
AWDKDLRVSDKYLLAMVIAYFSRAGLFSWQYQRIHFFLAL
YVASDMEEDNQAPKQDIFSFLYGKNYSQRPLFHKLRYQLL
CSMRWRTWVSPEEMEENTGPRGDGNFQQEVYRDANARH
QEGREEPPVQI

Chimp_SpeedyB2 [XP_001149803, 265aa, *Pan troglodytes*]

MGQILGKIMMSHQPQPQEEQSPQRSTSGYPLQEVVDDEVSGP
SAPGVDPSPPRRSLGWKRKRECLDESDDEPEKELAPEPEET
WVAETLCGLKMKAKRRRVSLVLPEYYEAFNRLLEDPVIK
RFLAWDKDLRVSDKYLLAMVIAYFSRAGLPSWQYQRIHF
FVALYLANDMEEDDEAPKQNIIYFLYEETRSHIPLLHELWF
QLCRYMNPRARKNCSQIALFRKHRFHFFCYMHCRAWVSL
EELEEIQAYDPEHWVWVRDRAHLS

Chimp_SpeedyB4(C) [XP_508546.3, 293aa, *Pan troglodytes*]

MLWAIPELGSPCPISISYEMSDSQDSTTSPVVTTQVDLGGCSR
QGGGNGFLRFRQHQEVQAFLSLLEDSFVQEFLSKDPCFQI
SDKYLLAMVLVYFQRAHLKLSEYTHSSLFLALYLANDME
EDLEGPKCEIFPWALGKDWCLRVGKFLHQRDKLWARMG
FRAVVSRQCCEEVMAKEPFHWAWTRDRRPHHGGVQRVC
PQVPVRLPRGPGLSPPHCSPCGLPQHCSSHLLKPVSSKCPS
LTSECHRPPSQNYLSRVKNAWGGDFLIVLPAQMQLEPGSY
SLRIFPKPPARPGH

Human_SpeedyA [NP_001008779.1, 286aa, *Homo sapiens*]

MRHNQMCCETPPTVTVYVKSGSNRSHQPKKPITLKRPICKDN
WQAFEKNTHNNNKSKRPKGPCLVIQRQDMTAFFKLFDDDL
IQDFLWMDCCCKIADKYLLAMTFVYFKRAKFTISEHTRINFF
IALYLANTVEEDEEETKYEIFPWALGKNWRKLFPNFLKLRD
QLWDRIDYRAIVSRRCCEEVMAIAPTHYIWQRERSVHHSGA
VRNYNRDEVQLPRGPSATPVDCSLCGKKRRYVRLGLSSSSS
LSSHTAGVTEKHSQDSYNSLSMDIIGDPSQAYTGSEGMI

Human_SpeedyAL [NP_001136106, 313aa, *Homo sapiens*]

MRHNQMCCETPPTVTVYVKSGSNRSHQPKKPITLKRPICKDN
WQAFEKNTHNNNKSKRPKGPCLVIQRQDMTAFFKLFDDD
LIQDFLWMDCCCKIADKYLLAMTFVYFKRAKFTISEHTRIN
FFIALYLANTVEEDEEETKYEIFPWALGKNWRKLFPNFLKL
RDQLWDRIDYRAIVSRRCCEEVMAIAPTHYIWQRERSVHH
SGAVRNYNRDEVQLPRGPSATPVDCSLCGKKRRYVRLGLS
SSSSLSSHTAGVTEKHSQDSYNSLSMDIIGDPSQAYTGSE
VVNDHQSNKGKKTNFLKKDKSMEWFTGSEE

Table 1 continued

Human_SpeedyB1 [NP_001121548.1, 237aa, *Homo sapiens*]

MASGQARPPFEEESPQPSTTVRSPEVVVDDEVPGPSAPWIDPS
PQPQSLGLKRKSEWSDESEEELEEELELERAPEPEDTWVVE
TLCGLKMKLKRKRASSVLPEHHEAFNRLLGDPVVQKFLA
WDKDLRVSDKYLLAMVIAYFSRAGLFSWQYQRIHFFLALY
LASDMEEDNQAPKQDIFSFLYGKNYSQRPLFHKLRYQLLC
SMRWRTWVSPEEMEEIQAYDPEHWVWARDRTLIS

Human_SpeedyB2 [NP_001004351.3, 549aa, *Homo sapiens*]

MTSHQPQPQEEQSPQRSTSGYPLQEVVDDEVSGPSAPGVDPS
PPRRSLGCKRKRECLDESDDEPEKELAPEPEETWVAETLC
GLKMKAKRRRVSLVLPEYYEAFNRLLAPGVDPSPPRRSLG
CKRKRECLDESDDEPEKELAPEPEETWVAETLCGLKMKA
KRRRVSLVLPEYYEAFNRLLAPGVDPSPPRRSLGCKRKREC
LDESDDEPEKELAPEPEETWVAETLCGLKMKAKRRRVSLV
LPEYYEAFNRLLAPGVDPSPPRRSLGCKRKRECLDESDDEP
EKELAPEPEETWVAETLCGLKMKAKRRRVSLVLPEYYEAF
NRLLAPGVDPSPPRRSLGCKRKRECLDESDDEPEKELAPEP
EETWVAETLCGLKMKAKRRRVSLVLPEYYEAFNRLLEDP
VIKRFLAWDKDLRVSDKYLLAMVIAYFSRAGLPSWQYQRI
HFFLALYLANDMEEDDEAPKQKIFYFLYGKTHSHIPLRPKH
WFQLCRPMNPRARKNCSQIALFQKRRFQFFCSMRCRAWV
SPEELEEIQAYDPEHWVWARDRAHLS

Human_SpeedyB2-like1 [NP_001139682, 402aa, *Homo sapiens*]

MDRTETRFRKRGQITGKITTSRQPHPQNEQSPQRSTSGYPLQEV
VDDEMLGPSAPGVDPSPPCRSLGWKRKREWSDESEEEPEKE
LAPEPEETWVVEMLCGLKMKLKQQRVSSILPEHHKDFNSQL
APGVDPSPPHRSFCWKRKMEWWDESEESLEEEPRKVLAPEP
EEIWVAEMLCGLKMKLKRRRVSLVLPEHHEAFNRLLEDP
VIKRFLAWDKDLRVSDKYLLAMVIAYFSRAGFPSWQYQR
IHFFLALYLANDMEEDDEDSKQNIFHFLYRKNRSRIPLLRKR
WFQLGHSMNPRARKNRSRIPLLRKRRFQLYRSTNPRAR
KNRSRIPLLRKRRFQLYRSMNSRARKNRSQIVLFQKRRFH
FFCSMSCRAWVSPEELEEIQAYDPEHWVWARDRAHLS

Human_SpeedyB2-like2 [NP_778234.2, 336aa, *Homo sapiens*s]

MQKHYTVAWFLYSAPGVDPSPPCRSLGWKRKREWSDESEE
EPEKELAPEPEETWVVETLCGLKMKLKQQRVSPILLEHH
KDFNSQLAPGVDPSPPHRSFCWKRKMEWWDKSEESEEE
PRKVLAPEPEEIWVAEMLCGLKMKLKRRRVSLVLPEHHEA
FNRLLEDPVIKRFLAWDKDLRVSDKYLLAMVIAYFSRAGFP
SWQYQRLHFFLALYLANDMEEDDEDSKQNIFHFLYGKNR
SRIPLLRKRRFQLYRSMNPRARKNRSHIPLVRKRRFQLRR
CMNPRARKNRSQIVLFQKRRFHFFCSMSCRAWVSPEELEEIQ
AYDPEHWVWARDRARLS

Human_SpeedyB4(C) [NP_001008778.1, 293aa, *Homo sapiens*]

MLWAIPELGSPCPISISYEMSDSQDPTTSPVVTTQVELGGCSR
QGGGNGFLRFRQHQEVQAFLSLLEDSFVQEFLSKDPCFQI
SDKYLLAMVLVYFQRAHLKLSEYTHSSLFLALYLANDME
EDLEGPKCEIFPWALGKDWCLRVGKFLHQRDKLWARMG
FRAVVSRQCCEEVMAKEPFHWAWTRDRRPHHGGVQRVC
PQVPVRLPRGPGLSPPHCSPCGLPQHCSSHLLKPVSSKCPSLT
SECHRPPSQNYLSRVKNAWGGDFLIVLPPQMQLEPGTYSLRI
FPKPPARPGH

Xenopus_SpeedyA [NP_001081714.1, 300aa, *Xenopus laevis*]

MRHMQSVTRASSICGSGVKQVIGKGHPHARVVGARKAQIP
EREELSVKPKMVRNTHLNLQPQERQAFYRLLENEQIQEF
LSMDSCLRISDKYLIAMVLAYFKRAAGLYTSEYTTMNFF
VALYLANDMEEDEEDYKYEIFPWALGDSWRELFPQFLR
LRDDFWAKMNYRAVVSRRCCDEVMSKDPTHWAWLRD
RPMHHSGAMRGYLRNEDDFFPRGPGLTPASCTLCHKAG
VCDSGGVSHNNSSSPEQEIFHYTNREWSQELLMLPPELLL
DPECTHDLHILQEPLVGLEPDGTALEWHHL

Table 1 continued

Xenopus_SpeedyB [NP_001081976.1, 298aa, *Xenopus laevis*]

MRHMQSATRATLVCGSGVKQIIAKGHPNTRVFGARKA
KIPEREVLAAKPKITRITHLNLQPQERQAFYRLL
ENELIQEFLSMDSCLKISDKYLIAMVLAYFKRAGLYT
SEYTTMNFFVALYLANDMEEDEEDYKYEIFPWALG
DSWREFFPQFLRLRDDFWAKMNYRAVVSRRCCDEV
MAKDPTHWAWLRDRPIHHSGALRGYLRNEDDF
FPRGPGLTPASCALCHKASVCDSGGVS
HDNSSPEQEIFHYTNREWSQELLILPPELLL
DPESTYDIHIFQEPLVGLEPDGAALEWHHL

Zebrafish_SpeedyA [NP_001006091.1, 289aa, *Danio rerio*]

MIKLSLPWLETAPSGAAHSLQIRRGPRKTRPGSAGRNSADSSQQPR
TKTPGPTLLIQRQEMAAFFRLFDDDLIQDFLWMDCCCKLTDKY
LLAMTFVYFRRARFSIGEHSRINFFLALYLANTMEEDEEETKYEI
FPWALGKSWRKHFPRFLKQRDQLWARIEYRAAVSRRCCEEVM
AIVPSHFVWQRERAEHHSGAQRLQQNREEILVPRGPAASPEPCF
LCAKTSALPVPRPSSAGPRSSSAPLERKASHRASKTTKAQHTCK
YKPIAGSDVVSEMCHDHSMDWINEE

Eshark_SpeedyA [AEW46999.1, 302aa, *Callorhinchus milii*]

MRNNQLCCQTPPSITVHMKPGGSRLNQQKKVTKLK
QQPCIQKMRGKNTNVISKPKHARGPCLIIQRQEM
AAFFKLFDDDLIQDFLWMDCCCKVSDKYLLAMTFVY
FKRAGFQISDQTRMNFFVALYLANTMEEDEEEYKYEIFP
WALGKQWKKVYPDFLKQRDQLWARINYRAAVSKR
CCEEVMAIAPTHYIWQRGRPVHHSGAIRRYARND
VKLPRGPCDTPFHCSLCGQKEEFLGMYPS
SSSSCESQKDLCRNDLDEHLDGMEPDTCYSSA
EHQSFKNGGAKYCLAIKDRSMDWFIGNEE

Eshark_SpeedyB [AEW47000.1, 294aa, *Callorhinchus milii*]

MRHGQTHSHPHSSVTVQVKPGVTRLPGPDGEMLGWTPCPRE
GLAAEDSKPLDGFPAPDPSAAQRQELEAFLRLLDDNVIQD
FLWMDCCCRVADKYLLAMVLTYFKRASLLTSDYSRMNF
FLALYLANDMEEDEEQYKYEIFPWALGEDWRRHIPHFLS
LRVELWAQMNFRAAVSRRCCEEVISEIPSHYIWHRDRPV
HHGGAVRSYLQKETVPYPTGPNGTPPHCTLCHPAHSYLS
LNSHSSSSSPGSQTLLDVDWPQDLLILSPALLIDRSFEALQ
EVLGNGESDGQDYSCCL

Chicken_SpeedyA [XP_419361.2, 325aa, Gallus gallus]

MSVLTLVRSFVQPLLAAMRHSLVYHTPPAAHLKPSASRPQQQKK
LVRPKRHSSRNRRETSEKRAPRGTYGHPAASCLVVQRQEMTAF
FKLFDDDLIQDFLWMDCCCKIADKYLLAMTFVYFKRANFTVD
EHTRLNFFVALYLANTVEEDNEESKYEIFPWALGKNWRKLFPD
FLKLRDHLWSRIDYRAIVSRRCCEEVMAIAPTHYIWQRERSVY
HSGAIRNYNKDEAQLPRGPNNSPIPCYLCGKKGRFVRLGLSSS
SSSSTGSLEVTELCSSQDLKGTFAIEKMLVDSPASSAQDCQSL
SSKRRRDNTSNQDKSMDWFTSNEE

Medaka_SpeedyA [JN039025, 309aa, *Oryzias latipes*]

MKTPPSASLRVKRKNVRQIRRHLCQNSAFGPDAEGFSWKID
PSRDICYVTMLPAAVIQRQEMSSYFRLLDDKLIHDFLRMD
SCFKMTDKYLLAMTFVYFKRAHFTIAEYNRRNFFIALISQ
TPWRRMKSRASMKSFPGLWAKAGGNSFPASSSSETCCGL
ASSTEPLSAGAAVRVMAVVSSHSVWQRTRSDHHSGAQRQ
YSDPGSPFPRGPSASPPSCGLCNDGGRLHHRAVSLSVSRSS
PRQTLEKSHSFTTAASLEVTPPRVAAPQRKTVKSKSQAQP
SQRFCPEEMAREPFSYDSSYGIMDWMSEV

Table 1 continued

Stickleback_SpeedyA [AEW47002.1, 302aa, *Gasterosteus*]

MKLTRGRCQASPPVTVGVKPGSSHSLQTRRGLRPRRANRQD
AKSQAGPRREEMFRAQKTMPPTIVIQRQEMSSFFRLLDDD
VIHDFLWMDYCCKLTDKYLLAMTFVYFKRACFTIAQYTR
KNFFIALYLANTMEEDEEEGKYEIFPWALGKHWRKQFPRF
LKQRDMFWARVEYRAAVSRRCCEEVMDIVPSHLAWQRER
SEHHSGAQRQYGERDHTRIPRGPFASPVPCSLCKRGAASDQ
GPGSAASPSRGSSNTNHAFPQPFNFALSLEVTPPRAAACL
RRAAEAKYQTHPSVCCGKKVSQ

Tetraodon_SpeedyA [AEW47004.1, 311aa, *Tetraodon nigroviridis*]

MKHKSRRHHPPPYETVWVNPITG
SHSVPIRRELGLRRADNCQRVGKSRPGLRQADICCAKRSK
SVARTFAIDSQEMASFFNLFDDQLIRDFLLKDSCCKMTDK
YLLAMTFVYFKRARFAVAEYTRKNFFIALYLANTMEEDE
EEIKYEIFPWTLGKNWKKKFPVFLKQRDELWARMEYRAA
VSRHCCEEVMAIYPSHSLWQRERSEHHSGAQRQYGDHSLP
FPRGPSASPVFCALCNRSSVSDQGSSFSCSTSKENLIPVFVN
PFTTMDLIMLCTSLMLNYLVNSFDKHVQQRPLHGLVPDR
GRAPWPAVH

Lancelet_Speedy [AEW47001.1, 241aa, *Amphioxus*]

MEGQYEEWHKTAGPSPLVASKQETPILTRPLVPHVGKSLLSK
RSRTPERPPHPAPCPLAKRMKKPSLIVKTTEMDAFFRLIDDD
LIQDFLWMDRCCRIADKYLLAMVFAYFKRVGYSVQQYNR
MNFFVSLYLANDMEEDEDDMKYEIFPWALGVDWRSRYPR
FLRRRDHLWEAMHYRAAVSRKCCEEIMLIAPLHNIWQRLR
SDNHAGATRHYPKDEHDYEPRGPGYEPIYCALCQVEMIS

Ciona_Speedy [XP_002122378, 302aa, *Ciona intestinalis*]

MSKMNSNQVVTKYVLYLSNSQHKSKKRRLGKCQEAKHDIC
VPTKRSKESMEKLKNVTKPCLHITINEIDAFFSLFEDNTIQ
EFLALDSCFRISDKYLLAMVLTYFKRAHLHVSEYNVINFF
TALYLANDMAEDEEEFKYEIFPWALGEEWRDLYPGFLAQ
REKLWRKMKHRASVSRKCCEEAMEIQADHEIWSRERNEV
HGGAKRNHLKSKEEKEPFPRGPGRSPIPCSICVKINNSSGYY
SDDSIISDDMNILHVSTDSSPDEYFTHRNIGSKPRLKSKDIER
GDAEESSFDMEKLWETLS

of living jawed vertebrates (Gnathostomes). They diverged from bony vertebrates (ray-finned fishes, lobe-finned fishes, and tetrapods) about 420 million years ago. We selected elephant shark as a representative cartilaginous fish because it has been proposed as a model cartilaginous fish genome due to its compact genome size (900 Mb) and a low-coverage sequence (1.4×) of its genome has been generated [13]. We searched the low-coverage sequences of the elephant shark and identified two fragments of *Speedy* genes. By RACE, we were able to obtain full-length sequences for two *Speedy* genes, which were designated as *SpeedyA* and *SpeedyB* based on their high similarity to human and other vertebrate's *SpeedyA* and *SpeedyB* genes, respectively (Fig. 1). In elephant shark, *SpeedyA* is predominantly expressed in testis and at low levels in brain, whereas *SpeedyB* displays high levels of expression in ovary, spleen, and testis. Lower levels of expression of *SpeedyB* can be seen in other tissues too (Fig. 2).

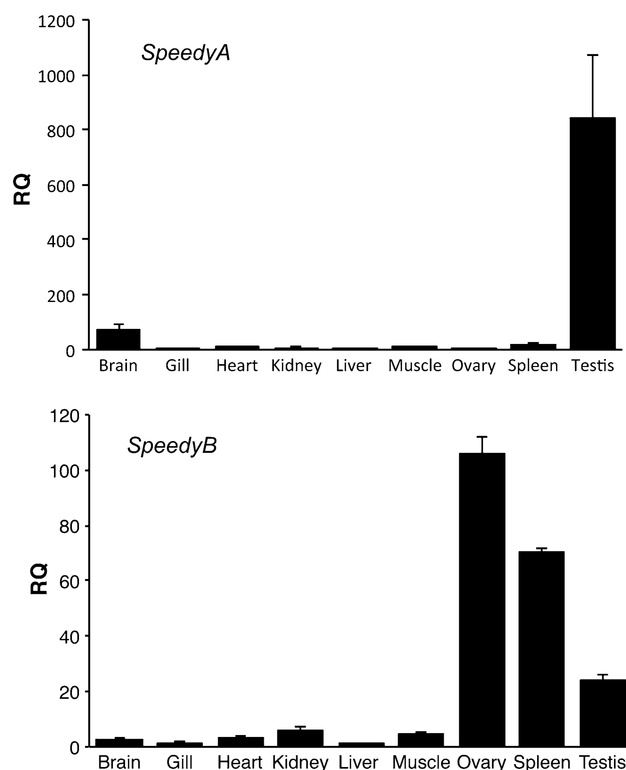


Fig. 2 Expression analysis of elephant shark (*Callorhinchus milii*) *SpeedyA* and *B*. qPCR analysis of elephant shark (eshark) *SpeedyA* and *SpeedyB*. qPCR was performed using total RNA from indicated tissues. *SpeedyA* (*upper panel*) and *SpeedyB* (*lower panel*) expression was analyzed using specific primers. Each *column* represents the average fold change compared to liver after normalization to β-actin

Speedy genes in bony vertebrates

Euteleostomi or bony vertebrates include more than 90 % of the living species of vertebrates. Expression of *Speedy* genes has already been reported in several members, which include mouse, rat, and human. However, during extensive database searches for *Speedy* genes in bony vertebrates, in addition to the previously known *Speedy* genes, we identified several new members of the *Speedy* gene family: *SpeedyB2*, *SpeedyB2-like1*, and *SpeedyB2-like2* in chimpanzee (data not shown) and humans (Figs. 1, 4b); and *SpeedyB3* in mouse (Figs. 1, 4a). The identities of these genes were confirmed by their similarity to *Speedy* genes in other vertebrates and their phylogenetic relationships (see below). For some genes, the identity was further confirmed by the conserved genomic context with their orthologs in other vertebrates. A summary of the Speedy protein sequence identified in various vertebrates is listed in Table 1.

Evolutionary history of *Speedy* genes in vertebrates

To gain insights into the evolutionary history of *Speedy* genes in vertebrates, we generated a neighbor-joining tree

for the full-length protein sequences of *Speedy* genes. The single Speedy gene in amphioxus was used as an out-group (Fig. 1). While most vertebrates contain two *Speedy* gene families, *SpeedyA* and *SpeedyB* (Fig. 1), all teleost fishes investigated encode only one *Speedy* gene (*SpeedyA*). Comprehensive analysis of the dataset revealed that whereas teleost fishes and the chicken lineage have lost *SpeedyB* during course of evolution, mammalian *SpeedyB* has undergone lineage-specific gene duplications giving rise to several *SpeedyB* genes. As we have uncovered more than one *SpeedyB* gene in mammals, we named them as *SpeedyB1/B2/B3/B4* based on the similarity to *SpeedyB* (Table 2). Whereas primates express one *SpeedyB1,* in rodents, lineage *SpeedyB1* has undergone additional tandem duplication—giving rise to two closely linked *SpeedyB1a* and *SpeedyB1b* genes (rat SpeedyB2/B1b was not included in the tree, as we could not predict its protein sequence). In addition to the B1 family, *SpeedyB* has undergone at least two rounds of independent duplications in the primate lineage—giving rise to *SpeedyB2* and *SpeedyB4* (RingoC) genes in human and chimpanzee. It has also duplicated once independently in the rodent lineage giving rise to *SpeedyB3* in mouse and rat. Thus, in contrast to a single *SpeedyB* gene in elephant shark and *Xenopus*, mammals contain four distinct families of *SpeedyB* genes (*SpeedyB1*, *SpeedyB2*, *SpeedyB3,* and *SpeedyB4*). The human and chimpanzee genes have undergone additional duplications resulting in several new *SpeedyB* (formerly *SpeedyE*) members. Since these *Speedy* genes display high sequence similarity to *SpeedyB2*, we named them as *SpeedyB2-like1* (SpeedyE2/E2L/E6/E5/WBSCR19-like protein) and *SpeedyB2-like2* (*SpeedyE1*/*Ringo1*). Interestingly, the *SpeedyB1*, *SpeedyB2,* and *SpeedyB3* genes in mammals have been evolving at a faster rate than other *Speedy* genes (Fig. 1). This suggests that their sequences have diverged considerably from other *Speedy* genes. It remains to be seen if this has resulted in altered functions of these fast evolving *Speedy* genes. We found that all the *SpeedyB2* and *B2-like* genes are located on human chromosome 7 (Suppl. Fig. 1A) and display high copy number variations (CNVs) that are, most likely, a result of tandem segmental duplications (Suppl. Fig. 1A–C). Expression analysis of human *SpeedyB2* and *B2-like* genes indicates their expression in several tissues with *SpeedyB2-like2* predominantly expressed in testis and heart (Fig. 4e).

Comparison of SpeedyA from different species

A comprehensive search for SpeedyA orthologs revealed that SpeedyA is conserved in all vertebrates from fish to human and displays similarity in the range of 46–80 % (Fig. 3a). Amphioxus, which is the most basal group of chordate, expresses only one Speedy protein similar to *Ciona*, and the amphioxus Speedy is 45 % identical to *Ciona* Speedy. Mouse SpeedyA protein is more than 75 % similar to human SpeedyA. In mammals, the SpeedyA protein exists as two isoforms, SpeedyA and SpeedyAL, which are products of alternative splicing. SpeedyAL contains additional 30 amino acids at the C-terminus (see Table 1). In general, the length of SpeedyA protein is restricted to a narrow range of 283–325 amino acids.

In *Xenopus laevis*, *SpeedyA* is expressed throughout oogenesis and during early embryogenesis [6]. The *Xenopus* SpeedyA protein is constantly turned over by $SCF^{\beta TrCP}$ and Siah-2, which regulate processing and degradation, respectively [14]. Human *SpeedyA* is expressed in variety of tissues and cell lines with higher levels of expression in brain and testis [7, 15]. Mouse *SpeedyA* also displays predominant expression in gonadal tissue [7] (Fig. 4a); however, lower levels of expression could be detected in other tissues (brain, kidney, lungs, spleen). Similar to human, elephant shark *SpeedyA* is expressed at high levels in testis; in addition, it displays low expression in the brain (Fig. 2). SpeedyA is a unstable nuclear protein whose expression is tightly controlled at transcriptional, translational, and posttranslational levels [16]. *SpeedyA* mRNA levels start to increase as cells enter mitosis and drops sharply at the end of G1. In vitro experiments with over-expressed protein indicated that SpeedyA protein accumulates in G1 phase, but low levels of SpeedyA are present in all phases of cell cycle. Interestingly, during mitosis, despite decreased expression, the human SpeedyA protein is present in a hyperphosphorylated form [16]. As cells exit G1 phase, the SpeedyA protein is targeted for ubiquitin-mediated degradation by two different ubiquitin ligases, SCF^{Skp2} ubiquitin ligase and Nedd4 [16, 17]. The N-terminal region of SpeedyA is essential for the degradation of the human SpeedyA protein by Nedd4 and is dependent on three phosphorylation sites: Threonine-15 (Thr-15), Serine-22 (Ser-22), and Threonine-33 (Thr-33). As these sites are conserved among human, mouse, and rat, we hypothesize that if these residues are essential for degradation of the human Speedy protein, they might be conserved in other species too. To investigate this, we analyzed SpeedyA protein sequences from various species and found that with exception of chicken, zebrafish, and amphioxus, Thr-15 is well conserved from human to medaka. Ser-22 and Thr-33 are present in mammals only (Fig. 3a, highlighted in yellow). Therefore, it is possible that Thr–15 phosphorylation plays a more important role in Speedy protein degradation than Ser-22 or Thr-33.

The *Xenopus* Speedy/RINGO protein was reported to be essential for oocyte maturation [2]. In accordance with that, a recent study has demonstrated that, also in porcine oocytes, SpeedyA over-expression accelerates meiotic maturation [18]. Comparable results were obtained in

mouse, where Speedy induces germinal vesicle breakdown in oocytes [9]. Recently, it has been reported that rat SpeedyA (LM23) is involved in spermatogenesis [19]. In addition, SpeedyA (LM23) knockdown in rat results in a complete meiotic arrest during spermatogenesis. All these results point in the direction that the meiotic functions of Speedy/RINGO may be conserved in all species. The predominant expression of *SpeedyA* in testis of elephant shark, mouse, and human supports this notion (Figs. 2, 4a).

Several reports indicated that, besides being a regulator of meiosis, the SpeedyA protein is also involved in the control of the somatic cell cycle. In vitro studies using osteocarcinoma cells (U2OS) revealed that overexpression of SpeedyA impairs cell proliferation in a Cdk2-dependent manner; moreover, silencing of SpeedyA prevents cell proliferation by inhibiting S-phase entry [15]. In addition, SpeedyA overexpression enhances mammalian cell survival in response to various genotoxic stresses. However, overexpression of dominant negative Cdk2 abolishes SpeedyA induced cell viability [20]. SpeedyA overexpression reduces apoptosis in response to UV irradiation and suppresses the activation of both S-phase and G2/M checkpoints. These effects are mediated by Cdk2, since mutant SpeedyA that cannot bind to Cdk2 fails to suppress the DNA damage response [21]. Taken together, these results suggest that interaction between SpeedyA and Cdks plays a prominent role in controlling cell cycle events.

Comparison of SpeedyB from different species

SpeedyB is another member of the *Speedy* gene family whose expression can be traced back to elephant shark. Whereas elephant shark and *Xenopus* express only one *SpeedyB* gene, higher vertebrates contain at least three *SpeedyB* genes with teleost fishes and chicken as an exception. During evolution of vertebrates, teleost fishes and the chicken lineages must have independently lost the *SpeedyB* gene. Homology searches for *SpeedyB* genes in human and chimpanzee revealed the presence of three *SpeedyB* genes, namely *SpeedyB1*, *B2*, and *B4*. Similar to other Speedy members, human SpeedyB protein can also bind and activate Cdk1, Cdk2, and Cdk5 [22].

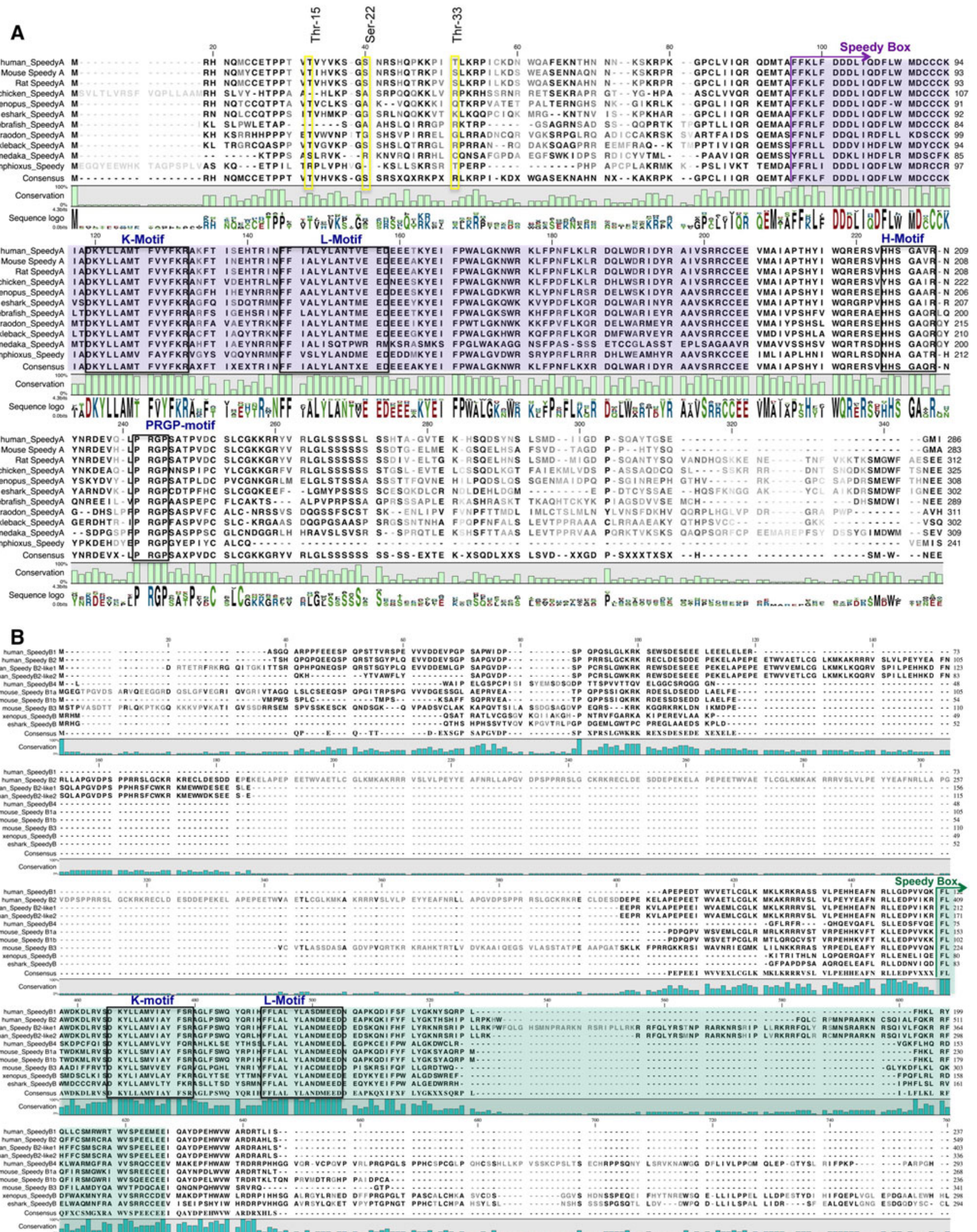
Human *SpeedyB1* is a novel *Speedy* gene we identified during homology searches. RT-PCR analysis of various tissues revealed that human *SpeedyB1* is predominantly expressed in testis (Fig. 4e).

Human *SpeedyB2* is the largest Speedy polypeptide, containing 549 amino acids. The *SpeedyB2* gene in primates has undergone additional gene duplication events giving rise to new members of the *Speedy* gene family: *SpeedyB2-like1* and *SpeedyB2-like2* (formerly known as Ringo1 or SPDYE1). While analyzing human SpeedyB2 and B2-like protein sequences, we noticed a stretch of 72 amino acids that has undergone several rounds of duplication, with five repeats of this stretch present in human *SpeedyB2*. These repeats are approximately 72 % conserved within the various human SpeedyB2 orthologs. However, *SpeedyB2-like1* and *2* contain only two copies of this repeat (Suppl. Fig. 4). These conserved repeats are entirely absent in human SpeedyB1 and SpeedyB4 and are also absent in all other known SpeedyB proteins. In the future, it would be interesting to investigate the physiological relevance of *SpeedyB2* gene duplication and these repeats. Expression analysis of human *SpeedyB2* genes by RT-PCR revealed that *SpeedyB2* and *B2-like* genes are ubiquitously expressed (Fig. 4e). Whilst most of the other Speedy proteins act as positive regulators of the cell cycle, SpeedyB2-like2 mostly functions as a negative cell cycle regulator. SpeedyB2-like2 overexpression inhibits the meiotic progression in *Xenopus* oocytes and results in an increased sub-G0 population in U2OS cells, which eventually leads to apoptosis [23].

Fig. 3 Sequence alignment of SpeedyA and SpeedyB proteins with conserved domains. SpeedyA is conserved across kingdoms, from fish to human (**a**). Sequence alignment of human, mouse, chicken, *Xenopus*, elephant shark, zebrafish, *Tetraodon*, stickleback, medaka, and amphioxus SpeedyA. Highlighted text in *purple* indicates the conserved Speedy box. Conserved motif-1, motif-2, H– and PRGP–motifs are indicated. The conserved threonine and serine phosphorylation sites are highlighted with individual *boxes*. Sequence alignment of SpeedyB from human, *Xenopus*, elephant shark, and mouse (**b**). Highlighted text in *green* indicates the conserved Speedy box

Human *SpeedyB4* is expressed in almost all tissues tested with colon as an exception (Fig. 4e, [7]). Similar to SpeedyA, SpeedyB4 is also involved in the regulation of somatic cell cycle, specifically in the regulation of S and G2 phase of the cell cycle [11]. Recently, it has been reported that SpeedyB4 might be important for proper execution of mitosis as depletion of SpeedyB4 resulted in precocious mitotic exit [24]. RNAi experiments revealed that in absence of *SpeedyB4*, cells escape from mitosis even in the presence of spindle damage. Genome searches to identify orthologs in other organisms revealed that *SpeedyB4* orthologs exist in cows and pigs. However, rat and mouse sequence searches did not yield any hits with significant similarity, suggesting that *SpeedyB4* is entirely missing from the rat and mouse lineages.

In addition to *SpeedyB1* and *B2*, rat and mouse express another member of the *Speedy* gene family; *SpeedyB3*, which is absent in human and chimpanzee. Comprehensive analysis of mouse and rat *SpeedyB1* and *B2* indicated that they are arranged in a tail-to-tail array on chromosome 5, which suggests that, recently, mouse and rat *SpeedyB1* have undergone tandem gene duplication giving rise to *SpeedyB2*. Since *SpeedyB1* and *B2* are products of same gene, i.e. *SpeedyB1,* we renamed them as *SpeedyB1a* (for



SpeedyB1) and *SpeedyB1b* (for *SpeedyB2*). Analysis of various mouse tissues by qRT-PCR revealed that *SpeedyB1a*, *B1b*, and *B3* are predominantly expressed in testis, though lower level of expression can also be seen in other tissues (Fig. 4a–d). Like other Speedy proteins, mouse *SpeedyB1a, B1b*, and *B3* can bind to Cdk2 and Cdk1

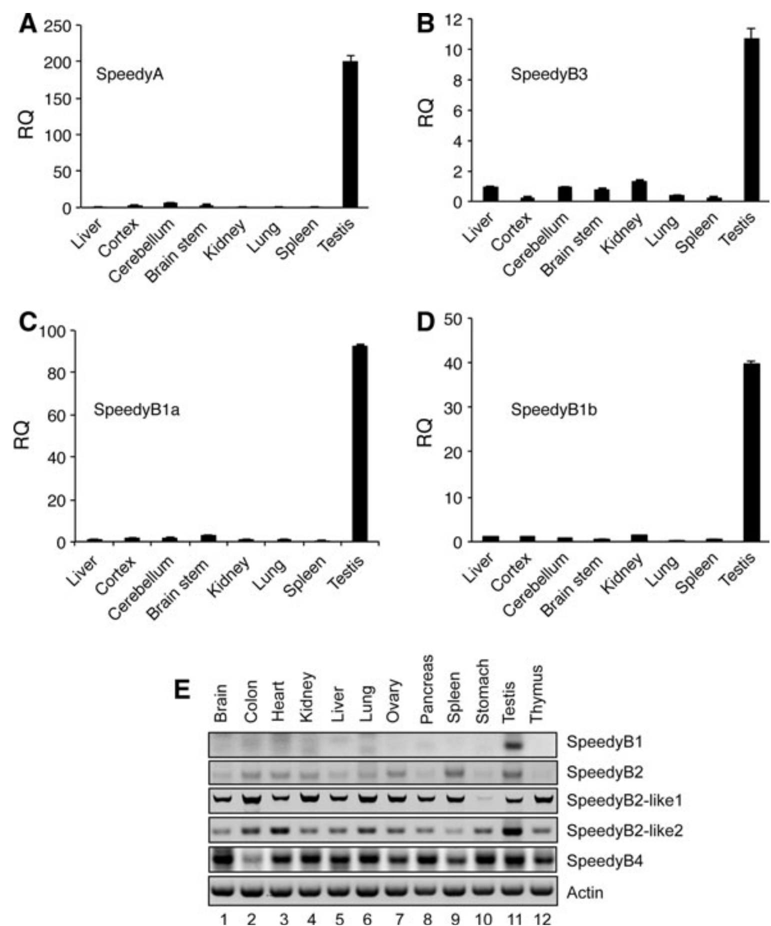


Fig. 4 Expression analysis of mouse and human *Speedy* genes. qPCR analysis of *SpeedyA* (**a**), *SpeedyB3* (**b**), *SpeedyB1a* (**c**), and *SpeedyB1b* (**d**) expression levels in the indicated mouse tissues. Each *bar* represents the average fold change compared to liver after normalization to β-actin. RT-PCR analysis of human *SpeedyB1*, *SpeedyB2*, *SpeedyB2-like1, SpeedyB2-like2* and *SpeedyB4* in the indicated human tissues. β-Actin was used as an internal control (**e**). Total RNA from human tissues was purchased from Stratagene

(Kaldis laboratory, unpublished data). Beyond this, little is known about the functions of *SpeedyB1a*, *B1b*, and *B3* in mice or rats. As evident from the sequence, all SpeedyB isoforms contain the conserved Speedy Box (see Fig. 3b, green box).

In future, it would be interesting to test if human *SpeedyB4* can compensate for the absence of *SpeedyB3* in rat or mouse. However, as there is very little sequence similarity between the two isoforms, it might well be possible that *SpeedyB4* and *SpeedyB3* are involved in entirely different functions.

Comparison of SpeedyA and SpeedyB

All Speedy proteins contain a highly conserved central region called the Speedy/Ringo box. As depicted in the alignment, Speedy proteins from various organisms display significant similarity in this conserved region (Fig. 3a,

Table 2 *Speedy* genes in vertebrates

Vertebrate	*SpeedyA*	*SpeedyB1*	*SpeedyB2*	*SpeedyB3(D)*	*SpeedyB4(C)*
Human	√	√	B2, B2-like1 & B2-like2	–	√
Chimpanzee	√	√	√	–	√
Mouse	√	*B1a & B1b*	–	√	–
Rat	√	*B1a & B1b*	–	√	–
Chicken	√	–	–	–	–
Xenopus	√	√	–	–	–
Zebrafish	√	–	–	–	–
Medaka	√	–	–	–	–
Stickleback	√	–	–	–	–
Tetraodon	√	–	–	–	–
Elephant shark	√	√	–	–	–

purple box). The major feature, which distinguishes SpeedyA from SpeedyB, is the positioning of the Speedy box. Whereas SpeedyA displays a centrally located Speedy box, the one in SpeedyB is located towards the C–terminal of the protein (Fig. 3a, b). A closer look within the Speedy box of SpeedyA and B identifies the presence of two conserved regions; the DKYLLxMxxxYFxR (K-motif) followed by the FFxALYLANxxEED (L-motif), which are almost 100 % conserved from human to fish (Fig. 3a, b). The extent of conservation of these boxes across different organisms suggest that within the Speedy box there are certain elements which might be essential for the function of Speedy proteins, and mutation within these boxes may impair its functions. However, detailed mutational analysis will be required to study the function of these motifs in Speedy proteins. Besides the presence of Speedy box, SpeedyA proteins share two unique sequence motifs: the HHSGAxR (H-motif) and the PRGPxxxPxxCxxC (PRGP-motif) at the C-terminal end of the Speedy box (Fig. 3a, b). Interestingly, mammalian SpeedyB proteins completely lack these unique signature motifs. It is unclear whether these unique sequences determine functional properties of the Speedy proteins. We speculate that the presence and positioning of these unique motifs might affect the expression and/or function of Speedy proteins thereby conferring different substrate specificity to SpeedyA/B. In future, it will be necessary to investigate the physiological relevance of these conserved motifs.

Discussion

The *Speedy* genes have evolved as a multigene family. Our analysis indicates that, whereas amphioxus expresses only one *Speedy* gene, mammals contain at least two distinct branches, *SpeedyA* and *SpeedyB*, of which the latter is further duplicated to give rise to several homologs (see below).

Among all *Speedy* genes, SpeedyA is conserved in all chordates. In higher vertebrates, SpeedyA is present as splice variants SpeedyA and SpeedyAL. The two variants differ by an extension at their C-terminus but we have yet to identify any obvious differences in expression or physiological function between SpeedyA and AL. Comprehensive analysis of the SpeedyB genes suggest that *SpeedyB* has undergone several lineage-specific gene duplications. However, we uncovered that, in teleost fishes or the chicken lineage, the *SpeedyB* gene is absent. It is unclear why *SpeedyB* has been lost in teleost fishes and chicken but retained in elephant shark (most basal jawed vertebrate) and *Xenopus*, and why mammals express duplicated forms. It would be interesting to examine the consequences of the loss of *SpeedyB* in fishes and chicken.

Gene duplication is considered as key factor in the evolution of new genes. This phenomenon is evident in a number of sequenced genomes ranging from bacteria to humans [25, 26]. Increases in gene copy numbers have been coupled to rises in organismal complexity and adaptive divergence at several points in the history of metazoans including during the chordate/vertebrate transition and during the teleost fish divergence [27]. Comprehensive analysis of *Speedy* gene family revealed that during course of evolution, the *Speedy* gene has undergone several rounds of gene duplication.

In mice, *SpeedyB1a* and *B1b* originated from tandem gene duplication and these genes are arranged in a tail-to-tail fashion. There are several genes, in human and mouse, which are arranged in tail-to-tail arrangement, e.g., insulin like growth factor binding proteins (IGFBP) [28]. The mechanism that leads to tail-to-tail arrangement after gene duplication is not yet understood. However, it is obvious that in addition to non-homologous breakage, it also requires an inversion step. Duplicate genes may display divergent functions. There are two major hypotheses that explain the functional retention of duplicated genes. The neofunctionalization hypothesis proposes that, after duplication, one daughter gene retains the ancestral function while the other acquires new functions [26]. The subfunctionalization hypothesis or duplication degeneration complementation hypothesis, asserts that the functions of the ancestral gene are partitioned between the duplicated genes, such that the duplicate genes complement each other by jointly performing the necessary subfunctions of the ancestral gene [29]. In the case of SpeedyB1a and B1b, we know that both can bind to Cdk2 and Cdk1, suggesting that, after duplication, this feature of the SpeedyB1 protein is conserved. However, we do not know whether SpeedyB1b has acquired any novel functions after duplication that differ from SpeedyB1a. To assess the extent of sequence conservation after the duplication, we aligned the entire sequence of mouse *SpeedyB1a* gene with corresponding sequence from mouse *SpeedyB1b* and human *SpeedyB1*. Interestingly, although the overall conservation is low, the 5′UTR (900 bp with 89 % identity) and two stretches of intronic sequences, one in intron 3 (874 bp with 95 % identity) and another in intron 5, display (~500 bp with ~88 % identity) high levels of conservation between the two mouse genes (Suppl. Fig. 2). The high conservation of these noncoding sequences suggests that they are under constraint, and hence they may encode some functional noncoding sequences such as noncoding RNA, cis-regulatory elements, suppressors, etc.

Similar to mouse, we found that the human *Speedy* gene has undergone several rounds of gene duplications giving rise to various *SpeedyE* (E1–E7) or *SpeedyE8P* (pseudogene) or E-like proteins (Suppl. Fig. 1A–B). Based on the

similarity and to avoid confusion, we renamed them as *SpeedyB2*, *SpeedyB2-like1*, and *B2-like2* (Table 3). Alignment of genomic sequences for these three genes indicated that their exonic and intronic sequences are highly conserved (94–100 % identity) over long stretches (Suppl. Fig. 3). This indicates that the duplication events that gave rise to the three genes occurred recently.

SpeedyB2-like1 displays copy number variation (CNVs) with multiple copies of the gene present in the chromosomal region 7q22. Careful analysis of the flanking chromosomal area revealed that the entire stretch has duplicated, giving rise to at least two copies of several genes: *POLRJ2, RASA4, UPK3BL, BC041025,* and *SpeedyB2-like1* (Suppl. Fig. 1C).

An interesting observation made during our genome searches was the chromosomal positioning of *SpeedyB2-like1* in the region 7q11.23 of chromosome 7. This region is widely associated with CNVs, that are the result of chromosomal deletions and duplications [30, 31], and contains large segmental duplications spanning centromeric, medial, and telomeric regions. The common deletion/duplications range in size from 1.5 to 1.8 Mb and encompasses approximately 28 genes resulting sometimes in diseases like heart defects in the case of FKBP6 microduplication [32] (Suppl. Fig. 1B). During crossing over, these duplicated segments cause unequal crossing-over or sometimes non-allelic homologous recombination, thereby resulting in deletions or paracentric inversions. Duplication in this region results in Williams–Beuren syndrome or 7q11.23 duplication syndrome, a neurodevelopmental disorder associated with distinctive behavioral characteristics [30, 33]. SpeedyB2-like1 (SpeedyE5/E2/E6) is flanked by some of these duplicated genes like FKBP6, WBSCR16, GTF2I, and GTF2IRD2. Similarly, *SpeedyB2-like2* (*SpeedyE1*), which is localized at chromosome 7p13, is also known as WBSCR19 and *SpeedyB2-like1* as WBSCR1-like protein3 (Table 3), due to its proximity to Williams–Beuren syndrome chromosome region 7q11.23 [34], potentially suggesting an involvement in these diseases.

Expression analysis of various *Speedy* genes in different species revealed that all *Speedy* genes are predominantly expressed in testis. However, a lower level of expression was also observed in brain and other tissues. The predominant expression in testis suggests that meiosis may be the most important function of *Speedy* genes. In future, it would be interesting to knockout *Speedy* genes in mouse and other organisms to investigate whether these genes confer essential roles in gametogenesis.

Until recently, Cdk2 was considered as a master regulator of G1/S-phase progression. However, two studies have broken this dogma by demonstrating that Cdk2 null mice are not only viable but also undergo normal postnatal development [35, 36]. Interestingly, Cdk2 plays an unexpected role in gametogenesis, since both Cdk2$^{-/-}$ males and females are sterile. A closer look at testis sections and chromosome spreads suggested that Cdk2 is required during the prophase I (pachytene stage) of male meiosis. It might also be involved in chromosome synapsis and have a telomeric function [35–37]. However, it is not clear which proteins assist Cdk2 in its meiotic functions. Deletion of cyclin A1, a putative interacting partner of Cdk2, also results in sterility with spermatocyte arresting in late diplotene [38, 39]. However, as Cdk2 deletion results in pachytene arrest, we speculate that there might be other proteins that may act as Cdk2 activators in meiosis. As Speedy/Ringo proteins have evolved as a new interacting partner of Cdk2 and have a well known function in

Table 3 Cross-reference of human and mouse *Speedy* genes

Gene	Accession number	Other names
Human Speedy		
SpeedyA (SPDYA)	NP_001008779.1	Speedy/RingoA1 [7]; Spy1 [15]
SpeedyAL (SPDYAL)	NP_001136106	Speedy/RingoA2 [7] Ringo3 [22]
SpeedyB1 (SPDYB1)	NP_001121548.1	SPDYE4
SpeedyB4 (SPDYB4)	NP_001008778.1	Speedy/RingoC [7]; Ringo2 [22]
SpeedyB2 (SPDYB2)	NP_001004351.3	SPDYE3
SpeedyB2-like1 (SPDYB2-L1)	JQ_023160	SPDYE2/SPDYE6/SPDYE5/*SPDYE2L*/WBSCR19-like protein3
SpeedyB2-like2 (SPDYB2-L2)	NP_778234	SPDYE1/Ringo1/WBSCR19 [22]
Mouse Speedy		
SpeedyA (*SpdyA*)	NP_001136103	Speedy/RingoA1 [7]
SpeedyAL (*SpdyAL*)	NP_083530	Speedy/RingoA2 [7]; Ringo3 [22]
SpeedyB1a (*SpdyB1a*)	NP_083324.3	Speedy/RingoB [7]; Ringo4 [22]
SpeedyB1b (*SpdyB1b*)	NP_808548.1	
SpeedyB3 (*SpdyB3*)	JQ_023159	

regulating meiosis in *Xenopus* and rat, we speculate that the meiotic function of Cdk2 is perhaps connected to its binding to Speedy proteins. To this end, we determined that Cdk2 from mouse testis could form complexes with SpeedyA, B1a, B1b, and SpeedyB3 (Kaldis laboratory, unpublished data). However, there may be additional Cdk2 partners awaiting discovery. Further studies are required to identify the substrates of Cdk2/Speedy complexes during meiosis and to understand how they are involved in the regulation of meiosis. A screen to identify new substrates of Cdk/Speedy in meiosis would increase our knowledge and understanding of Cdk2 functions and regulation.

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