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RESEARCH ARTICLE

Piccolo paralogs and orthologs display conserved patterns of alternative splicing within the C2A and C2B domains

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Abstract Piccolo is an organizational component of the presynaptic active zone, a specialized region of nerve terminals where synaptic vesicles fuse and release their neurotransmitter contents. Alternative splicing (AS) of the mouse Piccolo gene (*PCLO)* produces two primary splice isoforms: isoform-1 that includes two C2 domains (C2A and C2B) and isoform-2 with only C2A. Genome-wide association studies have identified variations located in or near the C2A domain of human Piccolo that predispose individuals to affective disorders and in rare cases leads to altered brain development. In zebrafish a genome duplication event led to the generation of *PCLO-a* and *PCLO-b*: gene paralogs that display strikingly similar genomic organization with other *PCLO* orthologs. Given this conservation in genomic structure, it is likely that AS patterns of zebrafish *PCLO* paralogs are similar to mammalian *PCLO*. We used a RT-PCR strategy to identify four zebrafish isoforms generated from zebrafish *PCLO*-a and *PCLO-b* that are equivalent to mouse Piccolo isoform-1 and isoform-2. Additionally, we identified an exon skipping event that leads to exclusion of a 27 nucleotide exon in both zebrafish Piccolo-a and Piccolo-b. Elimination of this exon in mammalian Piccolo alters the calcium binding property of the C2A domain. We also measured transcriptional levels of mouse and zebrafish Piccolo splice variants and demonstrate that despite similarities in AS, there are quantitative differences in gene expression. Our results indicate that AS of Piccolo is similar across diverse taxa and further support the use of zebrafish to study the role of Piccolo in neurodevelopment and synaptic signaling.

Keywords Synaptic · Alternative splicing · C2 domains · Zebrafish · Orthologs · Paralogs

Introduction

Rapid and efficient communication between neurons occurs at chemical synapses. A key structural component of synapses is the presynaptic active zone (AZ): a highly specialized structure composed of large molecular complexes that function to recruit and sequester synaptic vesicles to neurotransmitter release sites (Schoch and Gundelfinger 2006; Ackermann et al., 2015). Piccolo/Aczonin is a high molecular weight, multi-domain protein with restricted expression to the AZ (Cases-Langhoff et al. 1996; Wang et al. 1999; Fenster et al. 2000) and interacts with numerous synaptic proteins known to regulate vesicle recruitment and release including a number of proteins that regulate F-actin dynamics such as Profilin (Wang et al. 1999), GIT1 (Kim et al. 2003), Abp1 (Fenster et al. 2003), and Daam1 (Wagh et al. 2015). Piccolo also dynamically interacts with the synaptic-associated protein, Synapsin1a, to regulate synaptic vesicles exocytosis (Leal-Ortiz et al. 2008). Studies using shRNA in cultured hippocampal neurons show that the loss of Piccolo disrupts F-actin assembly in synapses leading to altered neurotransmitter release (Waites et al. 2011). In addition, acute knockdown

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of Piccolo and the closely related Bassoon protein from synapses results in disorganization and degradation of presynaptic terminals (Waites et al. 2013). Because Bassoon and Piccolo both interact with and negatively regulate the E3 ubiquitin ligase Siah, the synaptic degradation observed in synapses lacking these proteins is likely due to altered protein ubiquitination and turn over (Waites et al. 2013). Partial genetic deletion of Piccolo in mice results in alteration in the number of synaptic vesicles clustered within nerve terminals (Mukherjee et al. 2010) and Bassoon knockout mice show reduced synaptic transmission (Altrock et al. 2003) indicating that these AZ proteins play key roles in synaptic vesicle clustering and organization in presynaptic terminals.

In addition to numerous studies implicating Piccolo as an important regulator of synaptic vesicles dynamics, alterations in human *PCLO* appear to predispose individuals to major depressive disorder (MDD) and/or aberrant brain development. A number of genome-wide association studies have found an association between MDD and a non-synonymous single-nucleotide polymorphism (SNP) at rs2522833 (Ala-4814-Ser) located in the C2A domain of Piccolo (Sullivan et al. 2009; Hek et al. 2010; Aragam et al. 2011; Verbeek et al. 2012; Woudstra et al. 2012; Verbeek et al. 2013; Woudstra et al. 2013), suggesting that this variant is a causal risk factor for depression. Interestingly, in a transgenic mouse model where the C2A domain is overexpressed, mice display depression-like behavior. This suggests that changes in this calcium-sensing region of Piccolo negatively affect synaptic function (Furukawa-Hibi et al. 2010). In addition, an intronic SNP (rs13438494) located in the 3′ end of *PCLO* is significantly correlated with bipolar disorder (Choi et al. 2011). Further analysis revealed that rs13438494 negatively influences splicing efficiency through alteration of a splicing motif (Seo et al. 2013; Uno et al. 2015). While the physiological consequences of SNPs in human Piccolo are still under investigation, the presence of changes in intronic sequences that could affect post-transcriptional processing suggests that variations in *PCLO* may lead to aberrant splicing of Piccolo pre-mRNA transcripts (Uno et al. 2015). In a more recent study aimed towards identifying the genetic cause of a rare inherited disorder known as pontocerebellar hypoplasia type III, a homozygous nonsense variant in *PCLO* was identified in two affected individuals that introduces a STOP codon near the 3′ end of the gene (Ahmed et al. 2015) and hypothetically eliminates translation of the carboxy-terminal PDZ domain and both C2 domains. Taken together, these findings provide compelling evidence that alterations in the C2A domain adversely affect the function of Piccolo and that changes in both gene expression and splicing of Piccolo transcripts modify synaptic function and ultimately neuronal development.

Alternative splicing (AS) of genes is a fundamental process in eukaryotic organisms that ultimately leads to greater protein diversity. For protein components of the presynaptic nerve terminal, AS of precursor mRNAs can be extensive. For example, AS of the neurexin genes in mice leads to generation of over a thousand different transcripts (Ullrich et al. 1995; Treutlein et al. 2014). In addition to extensive AS, presynaptic genes also display a high degree of stabilizing selection relative to many other vertebrate genes (Hadley et al. 2006). AS patterns between orthologous presynaptic genes are likely similarly conserved, although such conservation has not been extensively studied. In mouse and rat brain, differential splicing of Piccolo results in formation of two primary splice variants: isoform 2 (I-2) and isoform 1 (I-1). Both isoforms are identical with the exception at the carboxy-terminus where Piccolo I-1 contains both the C2A and C2B domain and Piccolo I-2 contains only the single C2A domain (Wang et al. 1999; Fenster and Garner 2002). In addition to the primary I-1 and I-2 variants, minor splicing events in human, rat, and mouse Piccolo lead to additional alternatively spliced variants including transcripts lacking the 27-nucleotide (nt) exon 16 located in the highly conserved C2A coding region (Fenster and Garner 2002; Garcia et al. 2004). Inclusion of this 27 nt exon in the C2A domain of rat Piccolo and likely other Piccolo orthologs results in translation of an additional 9 amino acids that reduce the calcium and phospholipid binding affinity of this protein motif versus C2A domains that lack this short peptide region (Gerber et al. 2001; Garcia et al. 2004).

In zebrafish and other teleost fish species, a whole genome duplication (WGD) led to the generation of two paralogs of the Piccolo gene, *PCLO-a* and *PCLO-b* (Nonet 2012). Both zebrafish *PCLO* paralogs have strikingly similar genomic organization compared to mouse and human homologs with the exception of additional sequence coding for sixteen zinc fingers domains located in the 5′ end of zebrafish *PCLO-b* versus the two zinc fingers found in mammalian *PCLO* and zebrafish *PCLO-a* (Nonet 2012). Given the conserved genomic structure of *PCLO* between diverse vertebrate species, the goal of this study was to perform a comparative analysis of the AS patterns and mRNA expression levels of the splice isoforms generated from mouse *PCLO* and zebrafish *PCLO-a* and *PCLO-b* in adult brain. Through identification of multiple RNA transcripts from both species, we show that zebrafish *PCLO-a* and *PCLO-b* are both post-transcriptionally processed to yield two primary AS variants and a minor splice variant identical to splicing products generated from mouse *PCLO*. In addition, we quantified mRNA expression levels of Piccolo splice variants revealing measurable differences in gene expression between mouse and zebrafish transcripts.

Materials and methods

Animals

Zebrafish breeding, care, and maintenance were described previously (Posner et al. 2013) and were housed at Fort Lewis College (FLC) and Ashland University (AU). Mice were housed in the animal facilities at FLC and AU. Zebrafish were the AB wild-type line and purchased from the Zebrafish International Resource Center (Eugene, OR). Mice were from the C57BL/6 genetic background and purchased from The Jackson Laboratory (Bar Harbor, ME).

RT-PCR and real-time quantitative RT-PCR

Total RNA was isolated from zebrafish or mouse brain using RNAqueous®-4PCR Kit (Ambion) according to manufacturer' instructions. First-strand cDNA was generated from 100 ng of RNA using the cDNA Reverse Transcription Kit (Applied Biosystems) with random hexamers in 20 µl reactions at 25 °C for 15 min, 37 °C for 120 min and 85 °C for 5 min. For all PCR amplification of alternatively spliced transcripts, the reaction volume was 25 µl using 1 µl of cDNA, 12.5 µl of *Taq* 2X Master Mix (New England Biolabs) and 1 µl each of forward and reverse primers (10 µm final concentration) under the following cycling conditions: 95 °C for 3 min followed by 35 cycles of 95 °C for 3 min, 52–57 °C (depending on optimal primer annealing conditions) for 30 s, 72 °C for 1 min and 72 °C for 7 min. For real-time PCR, 2 µl of cDNA was amplified using a StepOne PCR System with PowerSYBR® Green PCR Master Mix (Applied Biosystems). Cycling parameters were 95 °C for 3 min, 95 °C for 15 s, 60 °C for 60 s. (40 cycles). Reactions (20 µl) were performed in triplicate. Standard curves for relative quantification were generated with 1–100 ng of total RNA. Primers were designed with DNA Workbench software (CLC BIO) (see Table 1 for primer sequences). Length of amplicons was variable due to limited choice of sequence and ranged from 83 to 277 bp (see Table 1). PCR products were verified by gel electrophoresis with single bands observed for each primer set and independently confirmed by DNA sequence analysis. Non-template controls (water) were included to control for contaminants. Normalization of target gene expression was based on mean-actin cycle thresholds (Ct) and was calculated according to established protocols (Fraga et al. 2012).

DNA sequence analysis

PCR amplicons with multiple splicing products were separated by DNA gel electrophoresis. Individual products were excised from the gel and purified via gel purification (Qiagen). PCR reactions with single bands were cleaned with ExoSAP-IT (Affymetrix) following manufacturer's instructions. Purified PCR products were sequenced off-site using a DNA sequencing service (Functional Bioscience, Madison, WI). Sequence files were downloaded into DNA Workbench (CLC BIO) for primer design, genomic structure analysis, and DNA alignment. For primer design and DNA sequence analysis and alignment, *PCLO* sequences for mouse, zebrafish, coelacanth, lizard, and frog were obtained from supplementary files (Nonet 2012). Predicted genomic *PCLO* DNA sequence files for human (gene ID: 27445), chicken (gene ID: 395319), and gar (gene ID: LOC102697692), were downloaded from the NCBI database (http://www.ncbi.nlm.nih.gov).

Results

Mouse Piccolo isoform-1 and isoform-2 are expressed at relatively equal levels in adult brain

Previous analyses of cDNA and EST clones of mouse and rat Piccolo indicated the presence of two primary splice isoforms, Piccolo I-1 and I-2 (Wang et al. 1999; Fenster and Garner 2002). I-1 and I-2 share an identical 5′ coding region, while the I-1 variant includes an additional five exons in the 3′ end of the gene. Generation of I-1 results from an alternative splice donor (ASD) site in exon 20 that leads to removal by splicing of an in-frame stop codon found in the shorter I-2 (Fig. 1a). Deduced amino acid sequence analysis indicated that the 5 additional exons included in I-1 (exons 21-25) are translated into a second predicted C2 domain (C2B) in addition to the single C2A domain found in I-2 (Wang et al. 1999; Fenster and Garner 2002). Although both I-1 and I-2 have been detected in adult rat and mouse brain, comparison and quantification of gene expression levels between these major splice variants has not been examined.

In order to measure and compare the expression levels of mouse Piccolo I-1 and I-2 transcripts, we devised a RT-PCR strategy to specifically amplify either the I-1 or I-2 transcript from purified mouse brain RNA. I-2 was amplified using a forward primer designed to anneal to sequence in exon 15, an exon common to both transcripts and not subject to AS and a reverse primer designed to anneal only to the unique coding region retained in exon 20 of I-2. I-1 was amplified using primer pairs designed to anneal to DNA sequence located in the I-1 specific C2B coding region (Table 1; Fig. 1b). Using this experimental strategy, we were able to detect PCR products representing exclusively I-1 or I-2 transcripts. Sequence analysis of amplicons confirmed the presence of the ASD site in I-2

Table 1 Oligonucleotide primer sequences used in RT-PCR and quantitative real-time qPCR analysis of mouse and zebrafish (ZF) alternatively spliced transcripts

Transcript isoform	Gene ID (NCBI)	Primer sequence	Amplicon size (bp)
Mouse I-1	26875	(For) CATTGCCACCCAAAAAAAGG	110 bp
Mouse I-1	26875	(Rev) AGTGGCCTGCAGGACTTAGG	
Mouse I-2	26875	(For) AGCCCTCTGTGATCAAAAGC	277 bp
Mouse I-2	26875	(Rev) TGCTGAGGAATTTATTTGCG	
Mouse (long)	26875	(For) CAGGACGAGGTGTTGAGTACA	83 bp
Mouse (long)	26875	(Rev) GGAAGTCGTTGGATGAGAATCTATC	
Mouse (short)	26875	(For) TCAAGTCATGGTTGTCCAGAATG	146 bp
Mouse (short)	26875	(Rev) TCCACTCGGGATTAAGAC	
ZF I-1a	100148002	(For) AATCTTCCCTGACCCTGCTA	221 bp
ZF I-1a	100148002	(Rev) GTCGTTTACTTGGTTGGAGA	
ZF 1-2a	100148002	(For) CATCAAGAAGAAGACCAGAG	174 bp
ZF 1-2a	100148002	(Rev) ACTTTATCCAGCCACACATA	
ZF I-1b	100149996	(For) GCTTTATGTGGTGAATGTGG	224 bp
ZF I-1b	100149996	(Rev) CTTTCTCATGTCCACCTTGT	
ZF I-2b	100149996	(For) CCCCTAAAACCTCAGTCATC	266 bp
ZF I-2b	100149996	(Rev) GTAAGGGTTACTTGGCTGG	
ZF-a (long)	100148002	(For) TTATGACAAACAGCTGGGA	138 bp
ZF-a (long)	100148002	(Rev) TGCACAACCATGACCTGA	
ZF-a (short)	100148002	(For) TTATGACAAACAGCTGGGA	133 bp
ZF-a (short)	100148002	(Rev) GTAGAGGTGCTGAAAACAAA	
ZF-b (long)	100149996	(For) CTTCAAATCAACTACGACAGG	143 bp
ZF-b (long)	100149996	(Rev) CTTGCGTTCTGGACAACC	
ZF-b (short)	100149996	(For) CTTCAAATCAACTACGACAGG	159 bp
ZF-b (short)	100149996	(Rev) CTGCTCCAGATGTATGTTT	

(including the in-frame STOP codon found only in I-2) and the C2B coding sequence found only in I-1 (data not shown).

We next used these same isoform-specific primer pairs to measure transcriptional levels of Piccolo I-1 versus I-2 using real time quantitative RT-PCR (qRT-PCR). Quantitative analysis of both transcripts in adult mouse brain indicated that the longer I-1 variant is expressed at approximately two-fold higher levels (1.8X) than the shorter I-2 (Fig. 1c).

Zebrafish PCLO-a and PCLO-b can be processed into Piccolo isoform-1 or isoform-2

Comparison of database *PCLO* DNA sequences across multiple and diverse vertebrate taxa indicated high levels of sequence similarity and conservation of genomic structure especially in the 3′ end of the gene (Fenster and Garner 2002; Nonet 2012). Because of the high degree of interspecies conservation, we hypothesized that AS patterns for *PCLO* are also conserved between evolutionarily diverse organisms. To provide evidence to support this hypothesis, we focused exclusively on the ASD region located in mouse *PCLO* exon 20 and the homologous ASD stretch in predicted database sequences for *PCLO* across a range of vertebrate species. Alignment of the mouse *PCLO* exon 20 ASD region including the distal intronic sequence that leads to the in-frame STOP codon with the predicted homologous ASD sequences for *PCLO* for human, frog, lizard, chicken, coelacanth, gar, and *PCLO-a* and *PCLO-b* of zebrafish indicated nearly identical splice junctional sequence in all examined species (Table 2; Fig. 2). Interestingly, even for zebrafish *PCLO-b*, where the 3′ end of the gene has less sequence similarity to *PCLO-a* and other orthologous *PCLO* genes, we detected the presence of a putative ASD site and in-frame stop codon.

Analysis and comparison of *PCLO* sequence from multiple taxa provides evidence that *PCLO* may be generated into two primary splice isoforms in other vertebrate species. To experimentally test this hypothesis, we designed a RT-PCR strategy (similar to that described above for murine *PCLO*) to detect putative Piccolo I-1 and I-2 transcripts in zebrafish. To identify the zebrafish Piccolo-a I-2 variant, a forward primer in the common exon 20

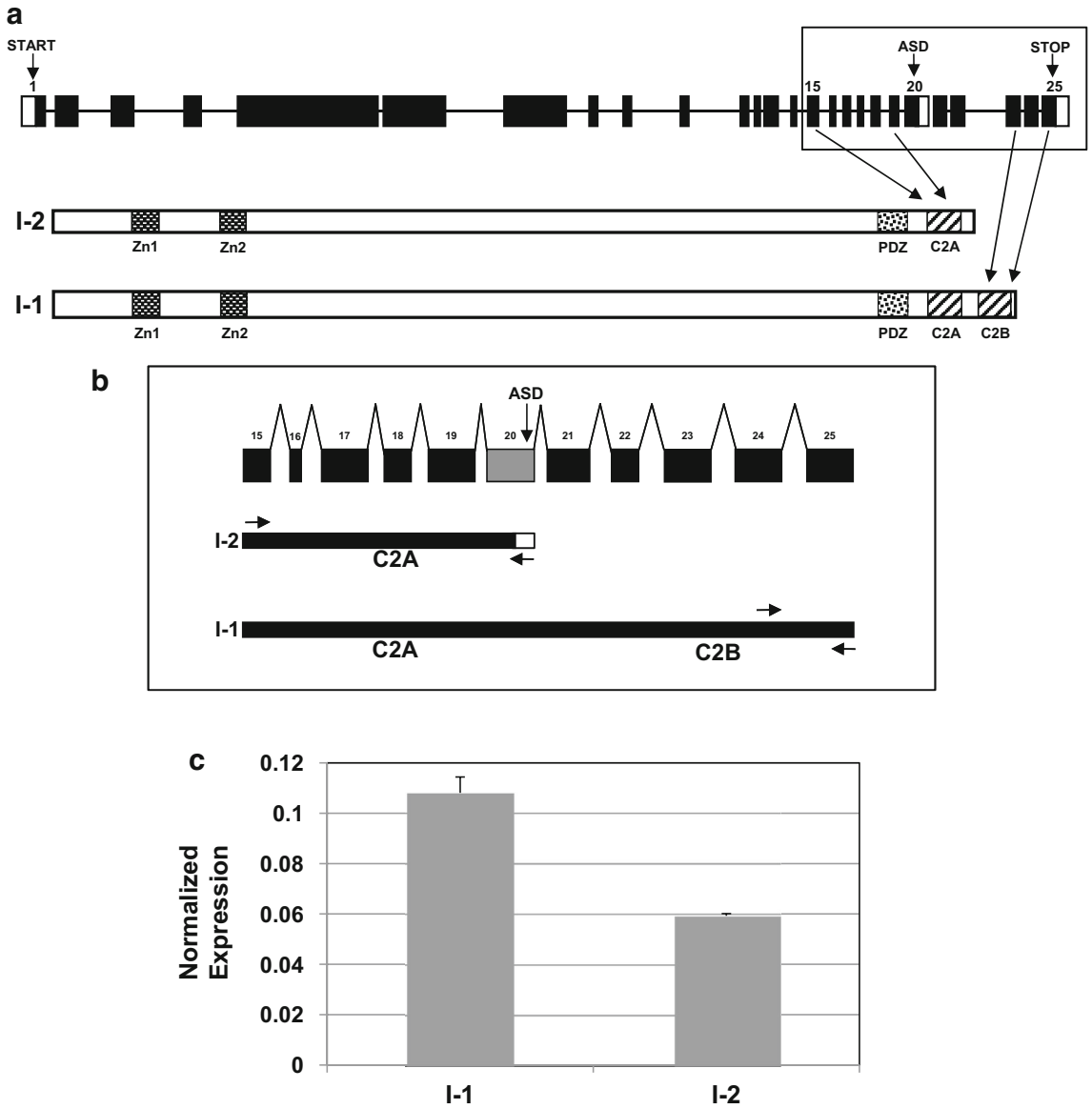

Fig. 1 Quantitative analysis of mRNA levels of mouse Piccolo isoform 1 (I-1) and isoform-2 (I-2). **a** Intron/exon schematic of mouse *PCLO*. Exon coding regions are represented by *black boxes* and the 5′ and 3′ untranslated regions by *open boxes*. *Diagonal lines* indicate corresponding exons that encode the C2A and C2B domain. An alternative splice donor site (ASD) located in exon 20 leads to an additional five exons incorporated into Piccolo I-1. **b** Exons located in the 3′ end of mouse *PCLO* encoding the alternatively spliced C2A and C2B domains (*top*). Isoform-specific primers were designed to amplify either Piccolo I-2 or I-1 (*bottom*). The *forward arrow* located in I-2 corresponds to the sense primer located in exon 15 and the *reverse arrow* indicates the anti-sense primer located in the unique sequence of exon 20 (*grey box*) retained only in Piccolo I-2 due to the skipped ASD site (*open box*). *Forward* and *reverse arrows* in I-1 indicate primers used to amplify unique sequence contained only in Piccolo I-1. **c** Relative expression of mouse Piccolo I-1 versus Piccolo I-2 analyzed using quantitative real-time RT-PCR (qPCR). The ΔΔCT method was used to calculate the qPCR fold change using mouse actin mRNA for normalization and all differences in expression are relative to actin control. Triplicate independent real-time PCR was performed for each transcript. *Error bars* represent ± SEM

and a reverse primer were designed to anneal only to the unique coding region found in exon 21 of I-2. For Piccolo-a I-1, primers were designed to amplify the C2B domain-coding region located only in the I-1 variant. A similar strategy was used for zebrafish Piccolo-b using a common forward primer for Exon 32 and an I-2 specific primer for exon 33 and primers designed for the C2B domain of zebrafish Piccolo-b to amplify the I-1 transcript (Table 1; Fig. 3a). Like the two Piccolo isoforms in mouse, we detected I-1 and I-2 variants for both zebrafish Piccolo-a and Piccolo-b (e.g. I-1a, I-2a, I-1b and I-2b). DNA sequence analysis of PCR products indicated that Piccolo

Table 2 Comparison of *PCLO* alternative splice donor (ASD) across evolutionarily diverse taxa

Species and Exon Number	Alternative Splice Donor Site to Generate Isoform 1 or 2
Human Isoform 2(Exon 20)	CAA CTC CGC ATT CAA CCA **AGT AAA AGA CGC AAA TAA** Q L R I Q P S K R R K *
Human Isoform 1(Exon 20)	CAA CTC CGC ATT CAA CCA **Agt aaa aga cgc aaa taa** Q L R I Q P
Mouse Isoform 2(Exon 20)	CAA CTC CGC ATT CAA CCA **AGT AAA AGA CGC AAA TAA** Q L R I Q P S K R R K *
Mouse Isoform 1(Exon 20)	CAA CTC CGC ATT CAA CCA **Agt aaa aga cgc aaa taa** Q L R I Q P
Chicken Isoform 2 (Exon X)	CAG CTT CGC CTT CAA CCA **AGT AAA AGA CGC AAA TAA** Q L R L Q P S K R R K *
Chicken Isoform 1 (Exon X)	CAG CTT CGC CTT CAA CCA **Agt aaa aga cgc aaa taa** Q L R L Q P
Lizard Isoform 2(Exon 18)	CAG CTC CGC CTT CAA CCA **AGT AAA AGA CAC AAA TAA** Q L R L Q P S K R H K *
Lizard Isoform 1(Exon 18)	CAG CTC CGC CTT CAA CCA **Agt aaa aga cac aaa taa** Q L R L Q P
Frog Isoform 2(Exon 19)	AGC CTT CGT CTT CAA CCA **AGT AAA AGA CGC AAA TAA** S L R L Q P S K R R K *
Frog Isoform 1(Exon 19)	AGC CTT CGT CTT CAA CCA **Agt aaa aga cgc aaa taa** S L R L Q P
Coelacanth Isoform 2(Exon 19)	CAG CTC CGC CTC CAA CCA **AGT AAA CGA CGC AAA TAA** Q L R L Q P S K R R K *
Coelacanth Isoform 1(Exon 19)	CAG CTC CGC CTC CAA CCA **Agt aaa cga cgc aaa taa** Q L R L Q P
Gar Isoform 2 (Exon X)	CAG CTC CGC CTT CAA CCA **AGT AAA CGG CGC AAA TAA** Q L R L Q P S K R R K *
Gar Isoform 1 (Exon X)	CAG CTC CGC CTT CAA CCA **Agt aaa cgg cgc aaa taa** Q L R L Q P
Zebrafish-a Isoform 2(Exon 21)	CAG CCC CGT CTC CAA CCA **AGT AAA CGA CGC AAA TAA** Q P R L Q P S K R R K *
Zebrafish-a Isoform 1(Exon 21)	CAG CCC CGT CTC CAA CCA **Agt aaa cga cgc aaa taa** Q P R L Q P
Zebrafish-b Isoform 2(Exon 33)	CAA TCC CGA CTC CAG CCA **AGT AAC CCT TAC TAC TTC ACA TGA** Q S R L Q P S N P Y Y F T *
Zebrafish-b Isoform 2(Exon 33)	CAA TCC CGA CTC CAG CCA **Agt aac cct tac tac ttc aca tga** Q S R L Q P

Sequence analysis of predicted database genomic sequence for *PCLO* from multiple species indicates the presence of a putative ASD (bold). An in-frame STOP codon (asterisk) generates the truncated I-2 (uppercase) or an alternative splicing event excludes the STOP codon in the mature RNA sequence that includes additional exons leading to the longer I-1 (lowercase)

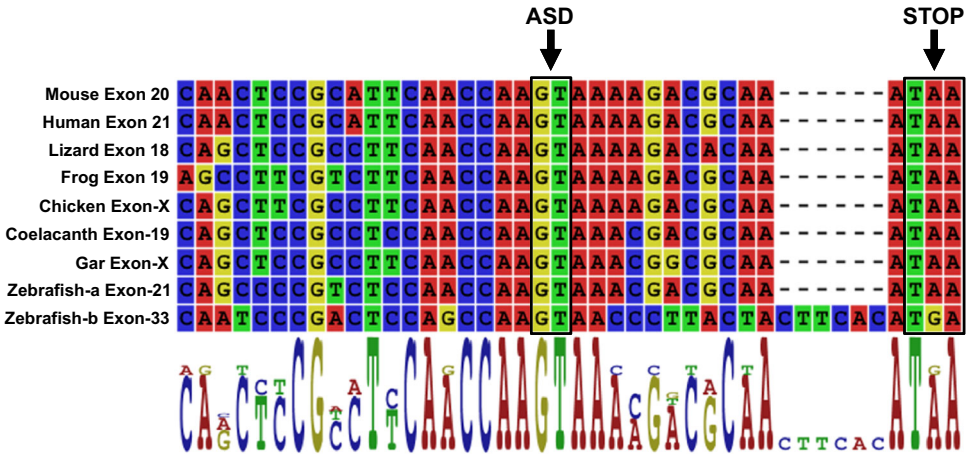

Fig. 2 DNA alignment of the *PCLO* alternative donor site (ASD) site from evolutionarily diverse vertebrate taxa. Shown are the putative 5′ ASD site and downstream sequence for selected vertebrate species including the predicted in-frame STOP codon that leads to the truncated I-1 transcript. Aligned sequences include predicted database genomic DNA sequences for *PCLO* from mammals (mouse-*Mus musculus* and human-*Homo sapiens*), reptile (lizard-*Anolis carolinensis*), amphibian (frog-*Xenopus tropicalis*), avian (chicken-*Gallus gallus*), and three evolutionarily distinct fish species (coelacanth-*Latimeria menadoensis*, gar- *Lepisosteus oculatus*, and zebrafish-*Danio rerio*). Corresponding exon numbers from each species containing the ASD are from published annotated sequence data. Chicken and gar genomes have not been carefully annotated and are thus indicated as exon X. Intron/exon junctions were predicted based on sequence homology with other *PCLO* orthologs

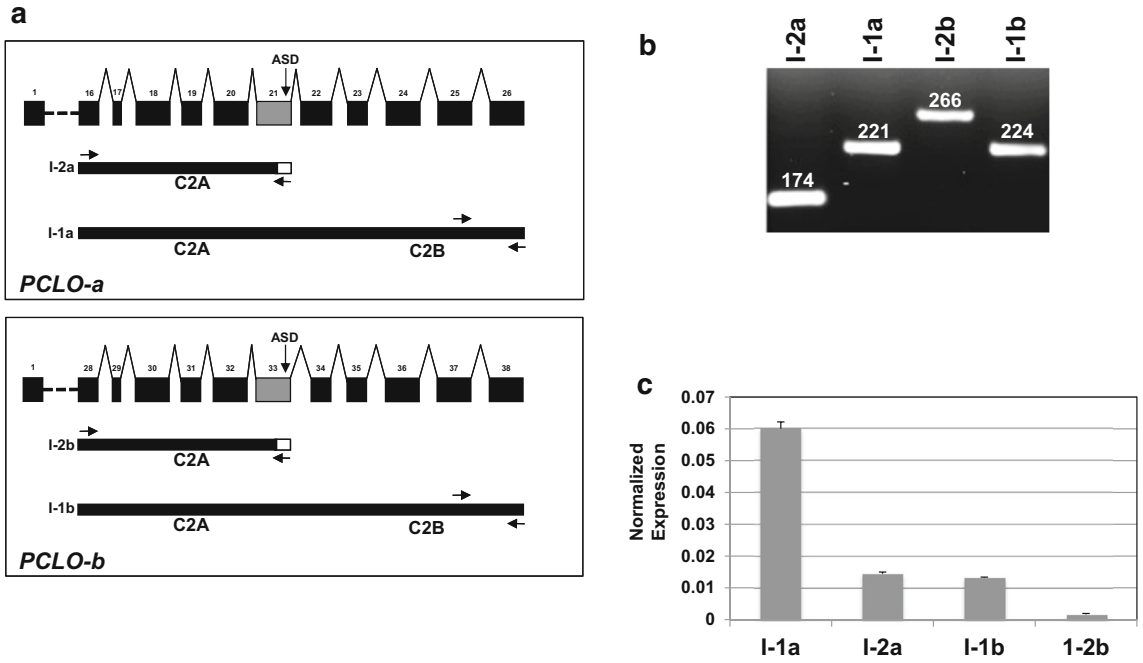

Fig. 3 Identification and gene expression analysis of isoform-1 and isoform-2 for zebrafish Piccolo-a and Piccolo-b. **a** 3′ genomic structure of zebrafish paralogs, *PCLO-a* (*top*) and *PCLO-b* (*bottom*). Both genes contain a putative ASD site (see Nonet, 2012 for complete genomic structure of *PCLO-a* and *PCLO-b*). Isoform-specific primers were designed to specifically amplify either zebrafish Piccolo-a I-1 (I-1a) or I-2 (1-2a) or Piccolo-b I-1 (I-1b) or I-2 (I-2b). For zebrafish Piccolo-a and Piccolo-b, *forward arrows* located in I-2 corresponds to the sense primer located in exon 16 (*PCLO-a*) and exon 28 (*PCLO-b*) and *reverse arrows* indicate the anti-sense primer located in the unique sequence of exon 21 (*PCLO-a*) or exon 33 (*PCLO-b*) retained only in truncated Piccolo I-2 transcripts (*open box*). *Forward and reverse arrows* in I-1 amplify unique sequence contained in zebrafish Piccolo I-1 for *PCLO-a* and *PCLO-b*. **b** PCR amplification of zebrafish Piccolo-a isoform 2 and isoform 1 (I-2a and I-1a) or Piccolo-b isoform 2 and 1 (I-1b and I-2b). All amplicons migrated at the predicted size and were confirmed by DNA sequence analysis. **c** The relative expression levels of the four zebrafish Piccolo isoforms (I-1a, I-2a, I-1b, and I-2b) was analyzed using qRT-PCR. The ΔΔCT method was used to calculate the qRT-PCR fold change using zebrafish beta-actin mRNA for normalization and all differences in expression are relative to actin control. Triplicate independent real-time PCR was performed for each transcript. *Error bars* represent ± SEM

zebrafish isoforms are spliced in a similar manner to mouse Piccolo (Fig. 3b). This indicates that the putative ASD sites observed in zebrafish paralogs *PCLO-a* (exon 21) and *PCLO-b* (exon 33) are recognized by the nuclear splicing machinery similarly to mouse *PCLO* to generate the four primary zebrafish Piccolo isoforms (Fig. 3b). Our results suggest that post-transcriptional processing of pre-mRNA Piccolo transcripts leading to the maturation of two primary isoforms is conserved across evolutionarily distinct vertebrate species.

Using the same primer pairs for our initial detection of I-1 and I-2 transcripts for both Piccolo-a and Piccolo-b, we conducted real time qRT-PCR to measure mRNA expression levels of all four primary isoforms (i.e. I-1a, I-2a, I-1b and I-2b) from adult zebrafish brain. In contrast to the relatively similar mRNA expression levels observed for mouse Piccolo I-1 and I-2 (Fig. 1c), distinct differences in expression levels were observed between the four zebrafish primary splice variants (Fig. 3c). The expression level of Piccolo-a isoform-1 (I-1a) was approximately 4–9 fold higher than the other three zebrafish Piccolo isoforms. Specifically, the expression of I-1a was detected at 4.2 fold higher than I-2a, 4.5 fold higher than I-1b, and 8.7 fold higher than I-2b.

An exon-skipping event in the Piccolo C2A coding region is conserved between mice and zebrafish

In addition to the AS events that generate the I-1 and I-2 variants, in both mouse and rat Piccolo several exon-skipping events have been detected in the 3′ end of the gene including one that leads to the exclusion of the 27 nt exon (exon 16) yielding a minor alternatively spliced variant of Piccolo (Fenster and Garner 2002). The exclusion of the 9 amino acids encoded by exon 16 in rat Piccolo dramatically alters the protein structure of the C2A domain thereby changing the calcium and phospholipid binding properties of this motif (Garcia et al. 2004). To explore the possibility that this functionally significant exon-skipping splicing event in *PCLO* is conserved across evolutionarily diverse species, we first aligned the putative homologous

27 nt exon *PCLO* DNA sequence from representative taxa and also included the upstream and downstream intron sequence in our analysis. DNA sequence alignment revealed near consensus between species within the exonic region but much less similarity in the intronic sequence proximal to the splice acceptor site and distal to the splice donor site (Fig. 4). This variability in flanking intron sequence between examined species suggests that this exon-skipping event may not be as highly conserved as the AS events that lead to generation of the I-1 and I-2 splice variants we detected in mouse and zebrafish Piccolo. To investigate whether this minor splice variant is also present in zebrafish, we used an RT-PCR strategy to amplify transcripts that include (long) or exclude (short) the 27 nt exon for both zebrafish Piccolo-a (exon 17) or Piccolo-b (exon 27) and for comparison, mouse Piccolo (Fig. 5). We first designed primer pairs that would anneal to exons flanking the skipped exon for all three Piccolo genes. For mouse Piccolo and for both zebrafish Piccolo-a and Piccolo-b, we observed two distinct PCR products separated by approximately 27 basepairs (bp). DNA sequence analysis of purified PCR products confirmed the inclusion (long) or exclusion (short) of the 27 nt exon in all amplified transcripts (Fig. 6a). Our analysis indicates that this minor splice variant previously identified in mammalian Piccolo is also present in zebrafish Piccolo-a and Piccolo-b and provides further evidence for conservation of AS patterns of *PCLO* across diverse vertebrate taxa.

A quantitative comparison of mRNA levels for transcripts containing or lacking this short but functionally significant exon may provide insights regarding the calcium sensing properties of Piccolo in mature synapses, as this may vary depending on which transcript is most highly expressed. For both mouse and zebrafish, we devised an RT-PCR strategy using primer pairs that would amplify either the long or the short transcripts (Fig. 5; Table 1). Using this strategy, we were able to detect transcripts for the two predicted mouse Piccolo minor splice variants (e.g. ms-short and ms-long) and the four predicted minor splice variants for zebrafish Piccolo-a and Piccolo-b (e.g. a-long, a-short, b-long, b-short)

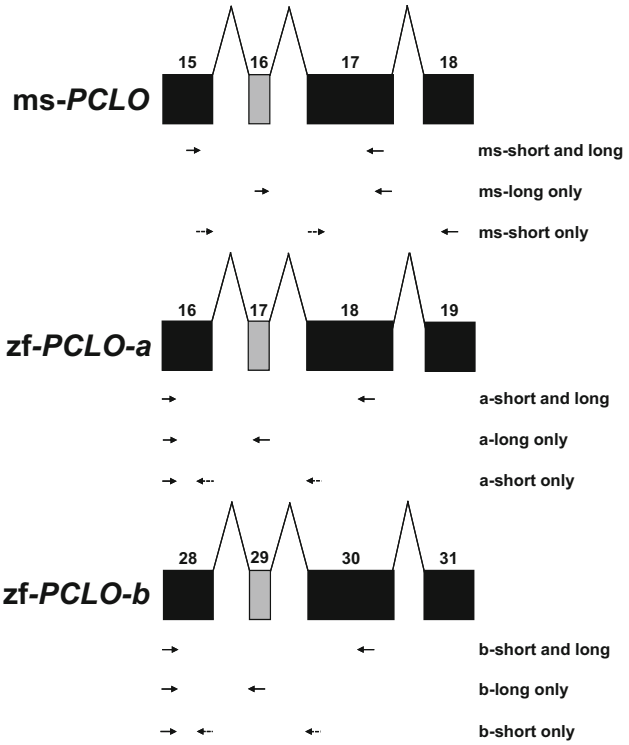

Fig. 5 RT-PCR strategy used to amplify transcripts for both mouse (ms) and zebrafish (zf) Piccolo including (*long*) or excluding (*short*) the 27 nt exon (*gray box*). Three sets of primers were designed per gene (e.g. ms *PCLO* and zf *PCLO-a*/*PCLO-b*) to amplify the following: both the long and short transcripts (*top*), only the long transcript (*middle*), or only the short transcript (*bottom*). For the short only transcript, primers were designed to anneal to exon sequence both distal and proximal to the skipped exon to preclude amplification of transcripts containing the 27 nt exon and amplifying only transcripts that lacked the skipped exon

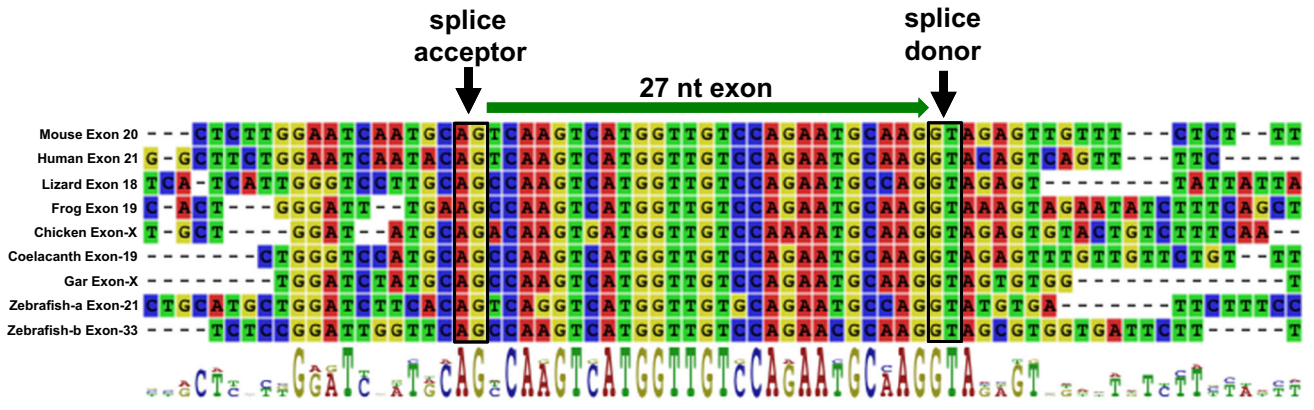

Fig. 4 A functionally significant exon-skipping event in Piccolo is likely conserved in evolutionarily diverse taxa. Alignment of predicted 27 nt exon (*green arrow*) and flanking intron DNA sequences from representative vertebrate taxa revealed a high degree of DNA sequence similarity in the 27 nt exon coding region but less similarity in the flanking intronic sequence

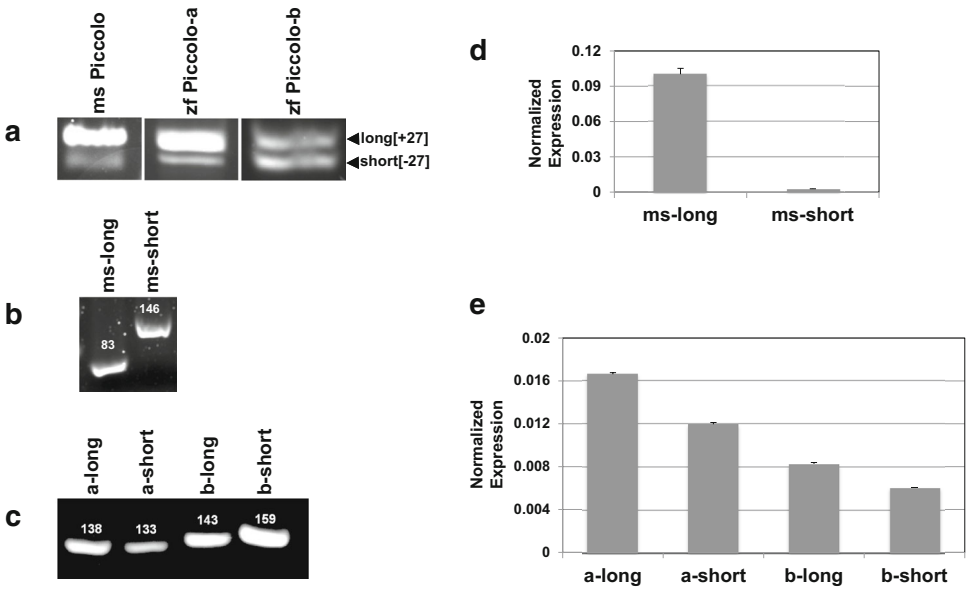

Fig. 6 Identification and gene expression analysis of mouse and zebrafish Piccolo-short and Piccolo-long transcripts. **a** Gel electrophoresis analysis of amplicons from RT-PCR using primers to amplify both the long (+27) and short (−27) transcripts. PCR products were gel purified and DNA sequence analysis confirmed insertion or deletion of the 27 nt exon for mouse Piccolo (exon 16), zf Piccolo-a (exon 17), and zf Piccolo-b (exon 29). **b** PCR primers were used to amplify mouse Piccolo transcripts including the 27 nt exon (i.e. ms-long) or excluding the 27 nt exon (i.e. ms-short). Both amplicons migrated at the predicted size and the presence or absence of the 27 nt exon was confirmed by DNA sequencing. **c** PCR primers were used to amplify zebrafish Piccolo-a and Piccolo-b transcripts including the 27 nt exon (a-long or b-long) or excluding the 27 nt exon (a-short or b-short). All four amplicons migrated at the predicted size and the presence or absence of the 27 nt exon was confirmed by DNA sequencing. **d** Relative expression of the two mouse transcripts was analyzed by real-time qRT-PCR. The ΔΔCT method was used to calculate the qPCR fold change using mouse actin mRNA for normalization. **e** Relative expression of the four identified transcripts was analyzed by qPCR. The ΔΔCT method was used to calculate the qPCR fold differences using zebrafish beta-actin mRNA for normalization. All differences in expression for Piccolo transcripts are relative to actin controls. Triplicate independent real-time PCR were performed for both mouse and zebrafish transcripts. *Error bars* represent ± SEM

(Fig. 6b, c). We used the same primer pairs for real-time qPCR to quantify gene expression levels of the long versus the short transcripts for both mouse and zebrafish alternatively spliced transcripts. Real-time qRT-PCR analysis of mouse brain mRNA indicate that the long transcript is expressed at much higher levels (approximately 36 fold higher) than the short transcript (Fig. 6d). For both zebrafish Piccolo paralogs, expression levels of the long transcripts were relatively equal to the short transcripts: 1.40 fold higher expression of a-long versus a-short and 1.35 fold higher expression of b-long versus b-short (Fig. 6e). Our results indicate that in adult mouse brain, the long piccolo transcript that codes for the extended C2A domain is the predominant transcript. In contrast, in adult zebrafish brain, long and short Piccolo-a and Piccolo-b transcripts are expressed at relatively equal levels.

Discussion

In the present study, we used a RT-PCR strategy to demonstrate conservation of AS patterns between mouse and zebrafish *PCLO*. Specifically, we detected the major I-1 and I-2 variants for both zebrafish paralogs of *PCLO* (*PCLO-a* and *PCLO-b*) that have been previously identified for mammalian *PCLO*. In addition, we show that a functionally significant minor splicing event that had been previously characterized for mammalian *PCLO* also occurs in the AS of zebrafish *PCLO*-a and *PCLO-b*. Finally, we used real-time qRT-PCR to measure the transcriptional levels of each of the identified major and minor splice variants for both mouse and zebrafish Piccolo and demonstrate that despite similarities in AS between organisms, there are distinct differences in the gene expression levels of Piccolo splice variants between these evolutionarily divergent vertebrate species.

The relationship between gene duplication and alternative splicing

In vertebrate organisms, the processes of AS and gene duplication (GD) play a major role in protein diversity and function. In the case of AS, generation of splice variants from the same gene can vary dramatically ranging from major splice events that lead to the inclusion of entirely new protein domains to minor splicing events that may

alter the property of only an individual protein motif in a multi-domain protein (Artamonova and Gelfand 2007). In the case of GD, the initial consequence is the generation of nearly identical paralogs but over evolutionary time the function of duplicated genes can diverge significantly resulting in functional diversification through the processes of subfunctionalization or neofunctionalization (Innan and Kondrashov 2010). Although a number of recent studies have carefully analyzed the relationship between AS and GD, the role these processes play in the generation of new distinct protein isoforms is still not well understand (Lambert et al. 2014; Abascal et al. 2015).

Teleost fish are ideal to study the interconnection between AS and GD due to the occurrence of a WGD between 320 and 370 million years ago and the incredible diversity of teleost species with over 27,000 estimated species (Volff 2005). AS can vary greatly among eukaryotic organisms. For example, it is predicted that over 95 % of multi-exonic human genes undergo differential splicing with less frequent AS detected in lower eukaryotic species (Pan et al. 2008). In teleost, AS can also be extensive ranging from a high level of estimated 43 % AS in fugu to a relatively lower level of 17 % AS in zebrafish (Lu et al. 2010). The completion of the zebrafish genome revealed that over 70 % of human genes have a clearly identifiable zebrafish orthologs (Howe et al. 2013). The identification and characterization of teleost gene paralogs that not only show conservation of AS events but also display shared AS patterns in orthologous genes across a wide range of vertebrate species including humans may provide valuable insight into the evolutionary relationship between AS and GD.

A comparison of conservation of AS events for paralogous and orthologous genes between teleost and mammals has only been examined for a handful of genes. Most studies have focused on the concept of subfunctionalization where the duplicated teleost genes fulfill the separate function of isoforms spliced from the single mammalian gene. For example, it was shown that for the Synapsin gene (*SYN*) duplicated in the teleost species fugu, the *SYN* paralogs (*SYN-a* and *SYN-b*) substitute for the two isoforms generated by AS of the single human gene (Yu et al. 2003). Another example is the single gene for microphthalmia-associated transcription factor (*MITF*) that in mammals and birds is expressed as at least four isoforms that are generated by use of alternative promoter sites and 5′ exons. In zebrafish this gene is duplicated (e.g. *MITF-a* and *MITF-b*) and each paralog serves the role of at least one of the single gene isoforms found in mammals (Altschmied et al. 2002). Other studies have identified genes associated with human disease in which the human gene exhibits AS, the zebrafish paralogs generated by GD are similar to human spliced isoforms. For example, the human septin-9 gene (*SEPT9*) which has been linked with pathogenesis in a number of human cancers can produce at least seven different mRNA transcripts through AS (McIlhatton et al. 2001). In zebrafish the gene is duplicated (e.g. *SEPT9-a* and *SEPT9-b)* and AS variants of the gene similar to the human orthologs, have been detected in zebrafish (Landsverk et al. 2010). The neural-expressed microtubule-associated protein tau gene (*MAPT*) can be generated into six isoforms in human brain. Changes in the expression of these isoforms are linked to dysregulation of microtubule assembly, a cellular condition implicated in Alzheimer's disease and other neurodegenerative disorders (Johnson and Jenkins 1999). Zebrafish have two paralogs of the tau gene (e.g. *MAPT-a* and *MAPT-b*) and can be generated into AS variants in a similar manner to *MAPT* isoforms observed in the brains of Alzheimer's patients (Moussavi Nik et al. 2014).

The rapid pace of whole genome sequencing and the advent of RNA sequencing for a number of vertebrate species has allowed for cross-species comparison of AS between distantly related species (Barbosa-Morais et al. 2012). For example, a recent study looking for examples of identical splicing patterns between orthologous genes in diverse vertebrate species identified nine genes that showed remarkable conservation of tissue-specific patterns of AS events between human, mouse, and zebrafish. Interestingly, some of these genes encode protein products associated with regulation of the neuronal microtubule network (Madgwick et al. 2015). Another study comparing human genes containing mutually exclusively spliced homologous exons with known orthologs in vertebrate fish species showed only a limited number of genes in which AS of homologous exons were entirely conserved between teleost and human orthologs (Abascal et al. 2015). These related AS events in orthologous genes between distantly related species likely have been maintained through natural selection due to their contribution to preserved processes important for vertebrate development.

In this study we explored the connection between AS and GD in the highly conserved vertebrate gene *PCLO*. Using the model genetic vertebrate organisms mouse (mammals) and zebrafish (teleost), we show that both major and minor AS events are conserved between zebrafish *PCLO* paralogs and mouse *PCLO* and speculate that equivalent AS events for *PCLO* likely occur in other vertebrate species including human. In addition, although AS events between mouse and zebrafish *PCLO* appear to be nearly identical, the gene expression patterns of both major and minor Piccolo transcripts between zebrafish and mouse display distinct differences in adult brain. For example, in mouse, Piccolo I-1 and I-2 are expressed at relatively similar levels suggesting that both isoforms are equally involved in cellular process restricted to mature synapses.

In contrast, zebrafish Piccolo-a I-1 is expressed at much higher levels in adult brain than the other three major Piccolo isoforms indicating that this isoform could be the more relevant player in adult zebrafish neurons.

Zebrafish as a model organism to study the neuronal function of Piccolo

Current advances in molecular and imaging techniques have allowed researchers to investigate synapse formation in zebrafish neurons (Leung et al. 2013). In mammalian synapses, one defined mechanism of delivering presynaptic components to developing synapses is via active zone precursor transport vesicles (PTVs) that contain a number of synaptic proteins including Piccolo and Bassoon (Zhai et al. 2001; Tao-Cheng 2007). A recent study examining the contribution of the synaptic protein Synapsin to newly forming synapses in developing zebrafish neurons, revealed that PTVs containing Piccolo and Bassoon are present in zebrafish neurons and that timing and delivery of these vesicular particles resembles that found in nascent mammalian neurons (Easley-Neal et al. 2013). *In situ* hybridization analysis indicates that RNA transcripts for both Bassoon and Piccolo paralogs are broadly expressed throughout the adult zebrafish brain much like mammalian Piccolo (Cases-Langhoff et al. 1996; tom Dieck et al. 1998; Nonet 2012). The presence of Piccolo and Bassoon in zebrafish PTVs and their similar distribution in adult zebrafish brain indicate that these presynaptic proteins may contribute to the processes of synapse formation and neurotransmitter release in zebrafish neurons in a manner comparable to their well-studied mammalian orthologs. What is not clear is the precise cellular distribution of Bassoon and Piccolo paralogs in zebrafish and whether certain paralogs have unique cellular functions that contribute to specific processes in synaptogenesis and later mature neurons. Zebrafish neurons represent an important experimental model to examine the unique contributions of Bassoon and Piccolo paralogs and their alternatively spliced products to synaptic function.

Conservation of minor AS events in PCLO orthologs and paralogs

The C2 domains of Piccolo are protein motifs that have been identified in a large number of proteins (Nalefski and Falke 1996; Rizo and Sudhof 1998). Much of what we know about the calcium-binding properties of C2 domains comes from studies on the synaptic vesicle protein, Synaptotagmin-1 (Fernandez-Chacon et al. 2001). The two C2 domains in Synaptotagmin-1 act as molecular sensors to mediate vesicle fusion and synchronous neurotransmitters in response to presynaptic calcium influx (Chapman 2008). The C2A domain of Piccolo is similar to the C2 domains of Synaptotagmin-1 and contains the required 5 charged amino acids that mediate calcium binding whereas the C2B domain lacks these requisite amino acids (Fenster et al. 2000). The C2A domain of Piccolo has an unusual calcium binding property that can be dramatically altered by the removal of the 9 amino-acid sequence encoded by an alternatively spliced 27 nt exon (Gerber et al. 2001; Garcia et al. 2004). The absence of the 9 amino-acid stretch in the short C2A domain allows for immediate high affinity calcium binding whereas the long C2A domain requires a conformational change in order to coordinate calcium binding (Garcia et al. 2004). Our identification of zebrafish splice variants for both Piccolo-a and Piccolo-b that either include (long) or exclude (short) this short amino acid region indicates that this exon-skipping event is highly conserved across distant vertebrate species and is preserved in paralogous genes. Furthermore, we measured gene expression levels of the long and short variants in both zebrafish and mouse brain showing that in zebrafish, the long variant of Piccolo-a is expressed relatively equal to the other three variants (e.g. Piccolo-a short, Piccolo-b short, and Piccolo-b long) (Fig. 6e) whereas in adult mouse brain, mRNA transcriptional levels of the long variant are much higher than the short one (Fig. 6d). Given that this exon-skipping event leads to a change in the calcium-sensing property of Piccolo, is conserved across diverse taxa, and occurs in both zebrafish Piccolo paralogs, differential expression of the long and short variants likely have important effects on synaptic function.

Conclusion

Recent studies suggest mutations in human Piccolo play a causal role in MDD (Sullivan et al. 2009; Hek et al. 2010; Aragam et al. 2011; Verbeek et al. 2012; Woudstra et al. 2012; Verbeek et al. 2013; Woudstra et al. 2013) and abnormal brain development (Ahmed et al. 2015). Here we show that AS patterns of *PCLO* between zebrafish and mammals are highly conserved especially within exons located with calcium-binding C2A domain coding region. Taken together with the conserved genomic structure and the similar distribution of Piccolo in mammalian and teleost brain, our study further supports the use of zebrafish as a relevant model for studying the role of Piccolo in neuronal development and synaptic signaling. Furthermore, our results provide an important example of conservation of AS of orthologous genes across diverse taxa.

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Compliance with ethical standards

Conflict of Interests The authors declare no conflict of interest.

Human and animal rights statement The respective Animal Use Care Committees at Ashland University and Fort Lewis College approved all animal procedures.

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