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SHORT COMMUNICATION

Insights into the evolutionary history of the vertebrate *zic3* locus from a teleost-specific *zic6* gene in the zebrafish, *Danio rerio*

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Abstract The *Zic* gene family of zinc-finger transcription factors includes five orthologues, *zic1–5*, that are common to the Euteleostian vertebrates (fish, frogs, birds, and mammals). The *Zic* genes have been implicated as regulators of a number of critical developmental processes, including neurulation, neuronal differentiation, neural crest specification, the establishment of left–right asymmetry, and regulation of cell proliferation. The different *Zic* genes encode proteins that are expressed in broadly overlapping spatial domains, have conserved DNA-binding domains that recognize a common motif, are capable of physical interactions, and can co-regulate one another's transcription. Thus, the transcriptional regulation of individual proteins and their effects on downstream targets must be assessed within the context of co-expression with other family members. We describe a novel gene, *zic6*, that is specific to the teleost fishes and lacks the lateral and rostral expression domains typical of the other *Zic* family members. We present evidence that *zic6* is an ancestral locus arising by chromosomal duplication early in the Euteleostomi that was subsequently lost in the terrestrial vertebrates.

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Introduction

The *Zic* gene family encodes a group of C_2H_2 zinc-finger transcription factors, which are important regulators of early vertebrate development. They are part of a larger *Gli/Zic/NKL* gene superfamily and, together with the *Gli* genes, are thought to provide positional information within the developing embryo (Brewster et al. 1998). The *Zic* genes are typically expressed in ectodermal tissues contributing to the nervous system and neural crest, as well as somitic mesoderm (Grinblat and Sive 2001; Toyama et al. 2004). There is strong experimental support for a combined role of the *Zic* genes in neurulation, neurogenesis, neural crest specification, and establishment of left–right asymmetry (reviewed by Aruga 2004). Deficits in *Zic* gene family members have been linked to developmental defects such as spina-bifida, holoprosencephaly, and X-linked heterotaxia (reviewed by Grinberg and Millen 2005).

Understanding the significance of *Zic* gene function during embryonic development is confounded by their broadly overlapping expression with the potential for competition for DNA-binding, sites as well as cross-regulatory and physical interactions among orthologues (Grinblat and Sive 2001; Koyabu et al. 2001; Mizugishi et al. 2001; Nakata et al. 2000; Toyama et al. 2004). It is therefore essential to define the combined expression of the *Zic* gene family members and understand their evolutionary relationships. Although there is significant conservation in the structure of the Zic protein DNA-binding domain, consisting of five zinc-fingers, there is also considerable divergence in other parts of the protein that may be

correlated with altered post-translational regulation, protein–protein interactions and repressor/activator activities (Aruga et al. 2006). The evolutionary diversification among family members may, however, be constrained by their physical arrangement as paired genes (bigenes) of divergent orientation in the genome that share a limited amount of "upstream" DNA. Four known vertebrate homologues occur as *zic1*/*zic4* and *zic2*/*zic5* bigenes, with the exception being *zic3*, which is a single-gene locus, located on the X-chromosome in mammals (Aruga et al. 2006).

We describe in this paper the structure, genomic context, and embryonic expression of zebrafish *zic6* and use those pieces of evidence to infer the evolutionary relationships of the *Zic* family members in the Euteleostomi. The *zic6* gene was found to be teleost-specific, occurring among a broad range of fishes, but absent from the genomes of frogs, birds, and mammals. Genomic analysis established that *zic6* is paired with *zic3*, in opposite orientation, as is the case with the *zic1*/*zic4* and *zic2a*/*zic5* gene pairs. Synteny of flanking genes confirmed that the *zic3* loci of fish and the other vertebrate taxa are true homologues, supporting the conclusion that *zic6* was the product of a chromosomal duplication before the divergence of fishes and tetrapods and was subsequently lost in the tetrapod lineage. The expression of *zic6* in the neural plate lacked the lateral and rostral domains typical of the other *Zic* gene orthologues, indicating it has evolved a highly derivative if not entirely new regulatory role during early embryonic development of fish.

Materials and methods

Fish maintenance

Zebrafish, *Danio rerio* (strain AB*), were maintained at 28.5°C on a 14:10 light/dark cycle. All fish were housed at a density of ≤15 individuals per 2-l tank on a recirculating water system and fed daily with dry flake/krill and live brine. Embryos were fixed overnight at 4°C in 4% paraformaldehyde/phosphate-buffered saline (PBS), manually dechorionated and stored in absolute methanol at −20°C. Procedures were approved by the NIH ACUC.

Sequence analysis

Predicted protein sequences were obtained for previously described loci by TBLASTN query of the GenBank database (Tables 1 and 2), and undescribed loci were predicted from the Ensembl genomic DNA assemblies (Table 3). Amino acid sequences were aligned with Multalin (http://bioinfo.genopole-toulouse.prd.fr/multalin/) using the Blossum62 matrix and formatted with Se-Al v2.0a11 (http://evolve.zoo.ox.ac.uk/). Genetic distance, identity, neighbor-joining, and maximum likelihood calculations were performed with Phylip3.66 (http://evolution.genetics.washington.edu/phylip/phylipweb.html). Synteny analysis utilized genome assemblies from NCBI Map Viewer and the Ensembl Genome Browser.

cDNA cloning and in situ hybridization

The zebrafish *zic6* coding region was amplified from total RNA of 24-h post-fertilization embryos by polymerase chain reaction (PCR) with the Qiagen One-Step enzyme mix, gel-purified with Qiagen Qiaquick columns and TA cloned into pCRII-TOPO vector (Invitrogen). Primers were designed from assembled genomic sequences (Fwd, 5′-cctcagccaagcttgcaacaaaac-3′; Rev, 5′-atgggaagcaactca cgactgtc-3′). The cloned complementary DNA (cDNA) was sequenced from plasmid by MWG (Gaithersburg, MD). DIG- and FITC-labeled riboprobes for *zic6* and *deltaA* (Haddon et al. 1998) were in vitro synthesized and used for in situ hybridization essentially as described by Thisse and Thisse (1998) and Liang et al. (2000), using 1% Roche blocking reagent in PBS+0.1% Tween-20 with Roche alkaline phosphatase-labeled antibodies and either Roche BM Purple or Fast Red substrates. In situ hybridization results were imaged with a ProgRes C14 camera mounted on a Leica MZ12 binocular microscope and post-processed with Adobe Photoshop CS.

Table 1 Comparisons of conservative domains among Zic protein family members, exclusive of the zinc-finger domains

Locus	ZOC domain	Percent identity	ZF–NC domain	Percent identity	C-Term domain	Percent identity
Zic1	FNSTRDFLFRNR		GAFFRYMRQ-PIKQEL		HS-TL-SSNFNEWYV	
Zic2a	FNSTRDFLFRSR	92	GAFFRYMRQQCIKQEL	87	HS-SL-SSNFSEWYV	87
Zic2b	FNSTREFLLRSR	83	RAFFRYMRQQCIKQEL	81	HN-SL-TSNFNEWYV	87
Zic3	FNSTRDFLFRNR	100	GAFFRYMRQ-PIKQEL	100	HSDGL-PPNFNEWYV	67
Zic4	LSAGRDFLIRRE	42	GAFFRYMRQ-PIKQEL	100	LSSSFQPGQLSEWDV	33
Zic5	YTTSRDFILRRE	33	GAFLRYMRQ-PIKQEL	94	SNLSPQVTNLNEWYV	40
Zic6	YTSHRLEPSPRG	17	DAFLRCSRQNP-KHEL	56	PNSPFQKSLVNGWYT	27

The numbers indicate the percent identity for each domain relative to Zic1.

Table 2 Accession numbers for amino acid sequences based on cDNA

Species	*zic1*	zic2	zic3	zic4	zic5	zic6
Brafl	CAB96573	n/a	n/a	n/a	n/a	n/a
Musmu	NP_033599	NP_033600	NP_033601	NP_033602	NP_075363	n/a
Xenla	BAA33406	BAA33407	BAA23874	BAF36750	BAA95699	n/a
Danre	NP_571008	NP_571633, NP_001001820	NP_001001950	EF526309	NP_991290	NP_001001837

Brafl, *Branchiostoma floridae*; *Musmu*, *Mus musculus*; *Xenla*, *Xenopus laevis*; *Danre*, *Danio rerio*.

Results and discussion

Zebrafish *zic6* is a novel member of the *Zic* gene family

A thorough analysis of the zebrafish expressed sequence tag (EST) and genomic DNA sequence databases predicted a novel member of the *Zic* gene family in addition to homologues of *zic1–5* from frog, chick, and mammals. The predicted 525 amino acid sequence of the coding region from cDNA matched that from the genomic sequences. This novel locus was also cloned independently by Parinov et al. (2004) from a Tol2 retrotransposon insertion screen for developmental enhancer traps and designated as *zic6*.

Table 3 Ensembl-predicted genes from fish genome projects used to determine amino acid sequences of homologues used in the maximum likelihood analysis

Species	Locus	Ensembl Gene ID[a]
Gasac	*zic1*	ENSGACG00000003678
	zic2	ENSGACG00000001901
	zic3	ENSGACG00000017212
	zic4	ENSGACG00000003685
	zic5	ENSGACG00000001900
	zic6	ENSGACG00000017211
Oryla	*zic1*	ENSORLG00000010050
	zic2	ENSORLG00000011117
	zic3	ENSORLG00000001654
	zic4	ENSORLG00000010055
	zic5	ENSORLG00000011124
	zic6	ENSORLG00000001641
Takru	*zic1*	SINFRUG00000141943
	zic2	SINFRUG00000149466
	zic3	SINFRUG00000161097
	zic4	SINFRUG00000141940
	zic5	SINFRUG00000156741
	zic6	SINFRUG00000136281
Tetni	*zic1*	GSTENG00023940001
	zic2	GSTENG00027912001
	zic3	GSTENG00031261001
	zic4	GSTENG00023942001
	zic5	GSTENG00027915001
	zic6	GSTENG00031259001

Gasac, *Gasterosteus aculeatus*; *Oryla*, *Oryzias latipes*; *Takiru*, *Takifugu rubripes*; *Tetni*, *Tetraodon nigroviridis*

[a] http://www.ensembl.org/

The Zic proteins have a characteristic five C_2H_2 zinc-finger domain that was highly conserved in the Zic6 protein as well (Fig. 1a). In addition, the Zic family proteins have two N-terminal conservative domains with suspected functional significance: (1) the ZOC domain, which is conserved between invertebrate Opa and vertebrate Zic1–3, and (2) the ZF–NC domain adjacent to the first zinc-finger (Aruga et al. 2006). There are also serine-rich and conserved N/SEWYV motifs in the C termini of the proteins, although their possible functions are not clear. The Zic6 protein had the highest, although moderate, overall identity with mouse Zic4 (Fig. 1b) and was similar to zebrafish Zic4 and Zic5 in having relaxed conservation of the ZOC and C-terminal domains relative to Zic1–3 (Table 1). Zic6 exhibited a striking lack of conservation in the ZF–NC domain, which has very high identity among the other *Zic* orthologues (Table 1). Cluster analysis of the ZF–NC/zinc-finger region from the mouse, frog (*Xenopus laevis*), and zebrafish *Zic* gene family members supported the status of *zic6* as a novel orthologue rather than a paralogue of another family member, which frequently is the case in zebrafish, as with *zic2a* and *zic2b* (Fig. 1c). This was significant because evidence of a sixth orthologue has not been reported from any other vertebrate, even in a recent comprehensive phylogenetic analysis of the gene family by Aruga et al. (2006).

The *zic6* gene is specific to teleosts

A survey of available genome databases yielded open reading frames (ORFs) for predicted proteins with 78–93% identity to the zebrafish *zic6* gene in other teleosts, including medaka (*Oryzias latipes*), stickleback (*Gasterosteus aculeatus*), fugu (*Takifugu rubripes*), and pufferfish (*Tetraodon nigroviridis*). In each of the fishes, the *zic6* gene structure consisted of two exons with conserved exon–intron boundaries. This organization is shared with *zic4* and *zic5* and contrasts with the conserved three-exon architecture of *zic1–3* (Aruga et al. 2006). No homologue of *zic6* was found in frogs (*X. laevis* and *X. tropicalis*), birds (*Gallus gallus*), or mammals (*Bos taurus*, *Canis familiaris*, *Homo sapiens*, and *Mus musculus*). Unfortunately, the limited data available for the Chondrichthyan fishes or agnathans precluded analyses of gene complements in vertebrates

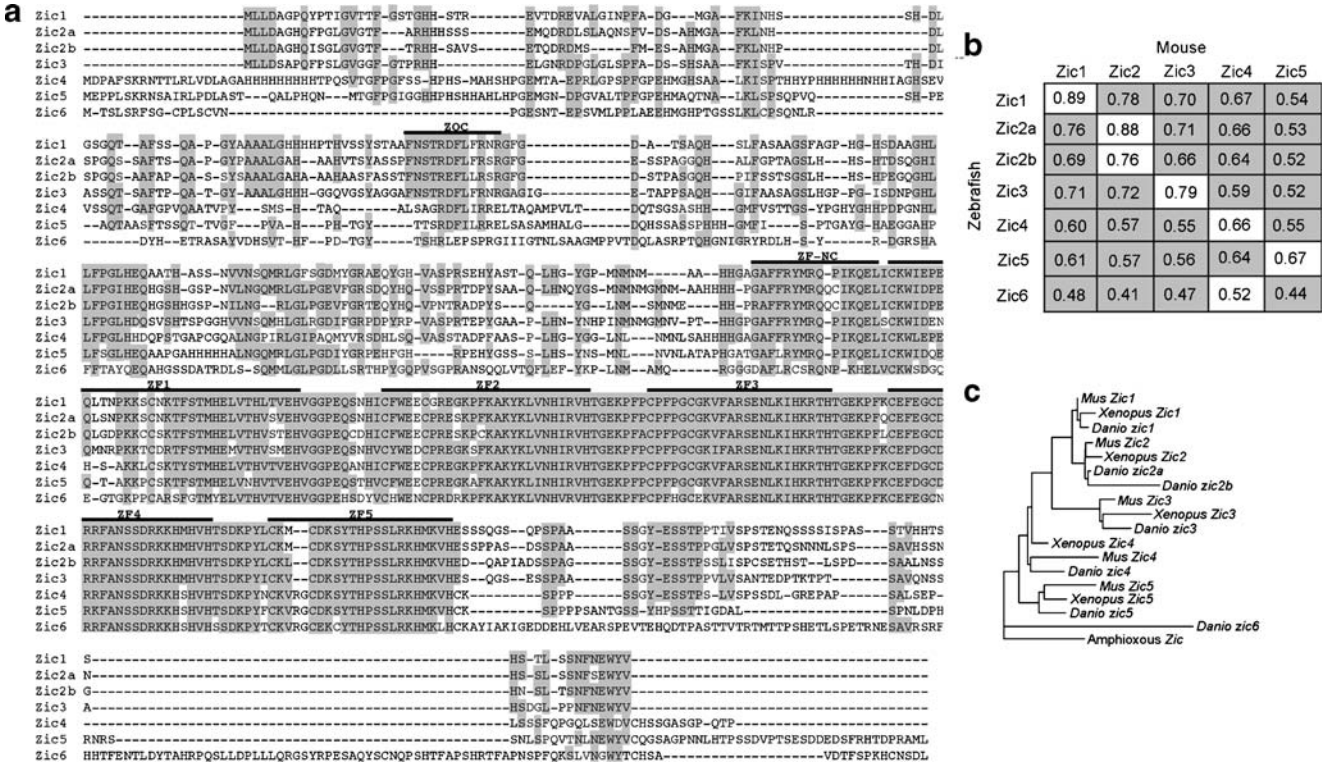

Fig. 1 Comparative analyses of predicted protein sequences from cDNA clones of the zebrafish *Zic* gene family members. **a** Amino acid alignments with conserved residues *shaded*. Previously defined conserved domains (Aruga et al. 2006) are indicated: *ZOC*, Zic-Opa conserved domain; *ZF–NC*, N-terminal to zinc-fingers conserved domain; *ZF1–ZF5*, five conserved zinc-finger domains in tandem. **b** Matrix of amino-acid identity among full-length Zic protein orthologues from zebrafish and mouse. The *white boxes* indicate cross-species pairs that share highest identity. **c** Phenogram for cluster analysis of zebrafish, *Xenopus*, and mouse Zic proteins constructed by neighbor-joining using pair-wise genetic distances for the ZF–NC and ZF1–ZF5 containing region. Amphioxus (*Branchiostoma floridae*) was used as an out-group to anchor the phenogram

basal to the Euteleostomi. However, only a single *Zic* gene homologue is known from the basal chordate, amphioxius (*Branchiostoma floridae*), indicating that the radiation of *zic1–6* occurred within the vertebrate lineage. The next closest living chordate group, the tunicates, experienced a separate radiation of the *Zic* genes (Aruga et al. 2006).

The teleost *zic3*/*zic6* locus is syntenic to the tetrapod *zic3* locus

Maximum likelihood analysis of the ZF–NC/zinc-finger region from teleost Zic protein homologues suggested that the *Zic* genes belonged to two symmetric clades, with common ancestors shared by *zic1–3* and *zic4–6* (Fig. 2a). This evolutionary pattern is consistent with the occurrence of vertebrate *Zic* orthologues as bigene pairs. The genomic architecture of vertebrate *Zic* genes is highly conserved, having loci consisting of paired orthologues in the case of *zic1*/*zic4* and *zic2*/*zic5*, with the exception being *zic3*. In all taxa, the bigene loci include one member from each clade, which we term the (+)-strand and (−)-strand orthologues, corresponding respectively to the three-exon (AB intron) and two-exon (A intron) groups of Aruga et al. (2006). An analysis of the genomic context of the *zic6* gene demonstrated that it is paired with *zic3* in divergent orientation, similar to the *zic1*/*zic4* and *zic2a*/*zic5* gene pairs; however, the *zic3*/*zic6* gene pair is only represented in teleosts (Fig. 2b). A comparison of the fish *zic3*/*zic6* bigene with the *zic3* locus in other vertebrates revealed synteny between flanking loci, although with an inversion in the orientation of *zic3* (Fig. 2b). The evolutionary position of *zic6* within the (−)-strand clade together with its genomic relationship to *zic3* and syntenic position among taxa suggest that it is an ancestral Euteleostian gene that was lost in the Tetrapoda. These results support the duplication–loss model proposed by Aruga et al. (2006) to explain the unitary *zic3* locus, rather than an equally likely single-gene duplication event, as appears to be the case for zebrafish *zic2b*. In the absence of data on basal vertebrates such as sharks, hagfish, and lampreys, it is not possible to confirm if the duplication generating the *zic3*/*zic6* bigene occurred in a common ancestor to the Vertebrata, as suggested by Aruga et al. (2006), or at a subsequent stage basal to either the Gnathostoma or Euteleostomi.

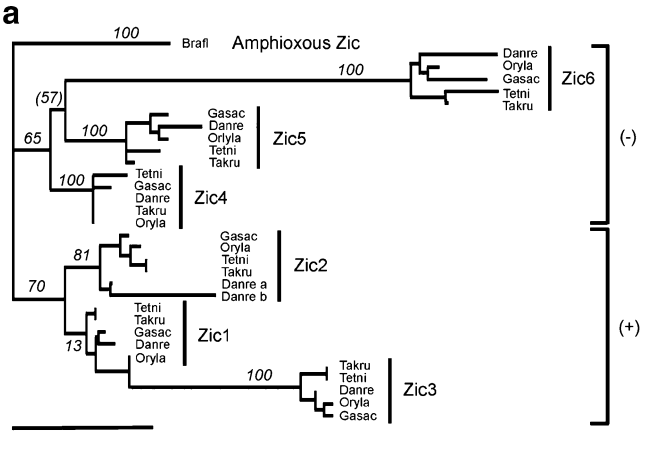

Fig. 2 Evolutionary and genomic analyses of the *zic6* locus. **a** Maximum-likelihood phylogeny of the teleost Zic protein orthologues using the ZF–NC and ZF1–ZF5 containing region, with Amphioxus as the outgroup. *Numbers* indicate reliability of critical branches among 100 bootstrap replicates (%); *parenthetical values* are associated with branch lengths not significantly different from zero ($p \geq 0.05$). *Brafl*, *Branchiostoma floridae* (Amphioxous); *Danre*, *Danio rerio* (zebrafish); *Gasac*, *Gasterosteus aculeatus* (stickleback); *Oryla*, *Oryzias latipes* (medaka); *Takifugu rubipes* (fugu); *Tetni*, *Tetraodon nigroviridis* (pufferfish). **b** Genomic analysis of synteny of genes flanking *zic3* and *zic6* in fish (same species as above), frogs (*Xenopus tropicalis*), birds (chick), and mammals (human, mouse)

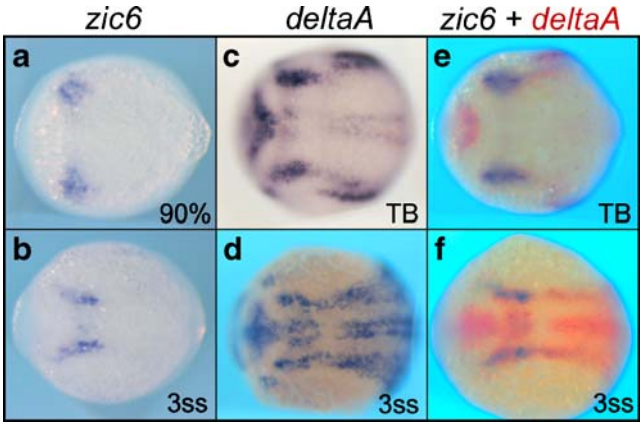

Fig. 3 Early embryonic expression of *zic6* in the zebrafish. Embryos are oriented with anterior to the right and dorsal up, with hindbrain (*hb*) and spinal cord (*sc*) divisions visible. **a**, **b** *zic6* mRNA appears during late gastrulation (**a**, 90% epiboly) in two spots in the anterior dorsal ectoderm that are maintained during early somitogenesis (**b**, three somites) as elongated patches in the anterior-medial neural plate. **c**, **d** The expression of the Notch ligand *deltaA* marks the diencephalic (*d*), trigeminal (*t*), and lateral (*l*), intermediate (*i*) and medial (*m*) spinal neurogenic domains during late gastrulation (**c**, tailbud) and early somitogenesis (**d**, three somites). **e**, **f** Two-color in situ hybridization with *deltaA* (*red*) demonstrates that the expression of *zic6* (*blue*) is specific to the intermediate neurogenic domain in the prospective hindbrain at tailbud and three-somite stages

The early expression of *zic6* is limited to the intermediate neurogenic domain

An analysis of zebrafish *zic6* messenger RNA distribution by in situ hybridization first detected weak expression at mid-gastrulation (70–80% epiboly) in the ectoderm that developed into a pair of strongly expressing patches by the end of gastrulation (Fig. 3a). The paired patches became elongated within the medial neural plate during early somitogenesis (Fig. 3b). Double in situ hybridization with the neurogenic domain marker *deltaA* (Fig. 3c,d) unequivocally demonstrated that the early expression of *zic6* was limited to the intermediate neurogenic domain of the prospective hindbrain (Fig. 3e,f).

The expression patterns of zebrafish *Zic* genes in general exhibit considerable conservation, especially between bigene pairs that may share common *cis*-regulatory elements. The expressions of the zebrafish *zic1*/*zic4* and *zic2a*/*zic5* bigenes are correlated during early development, such that the expression of *zic2a* and *zic5* is similar (Toyama et al. 2004), while that of *zic1* and *zic4* are spatially identical (Fig. 4a–d). Similarly, the unpaired paralogue *zic2b* has expanded expression but overlaps completely with *zic2a* (Fig. 4g). In comparison, the expression of *zic6* is more restricted than the other orthologues and is distinct from that of *zic3*, although the two bigene partners overlap where *zic6* is expressed.

A range of critical developmental processes occur during the open neural plate stage after gastrulation but before formation of the neural tube, including progenitor cell maintenance, neurogenesis, neural crest differentiation, and somitogenesis. In zebrafish, the common vertebrate *Zic* genes are all expressed to varying degrees in the lateral plate, forebrain/midbrain of the neural plate, and to a lesser extent in the hindbrain (Fig. 4a–e,g). The expression of zebrafish *Zic* family members in these domains is consistent with the patterns of their homologues in other vertebrates. The overlap between *zic1–5* in the neural plate border region underscores their critical function in the differentiation of neural crest (reviewed by Aruga 2004; Fujimi et al. 2006). In comparison, *zic6* is absent from the lateral and forebrain/midbrain regions and has very restricted hindbrain expression (Fig. 4f). The *zic3* gene is also expressed broadly in the hindbrain; therefore, the restricted expression of *zic6* could involve elements shared with *zic3* in the bigene promoter region. In general, however, the expression of *zic6* is highly derived and may reflect reduced- or neo-functionalization of the locus. After 1 day of develop-

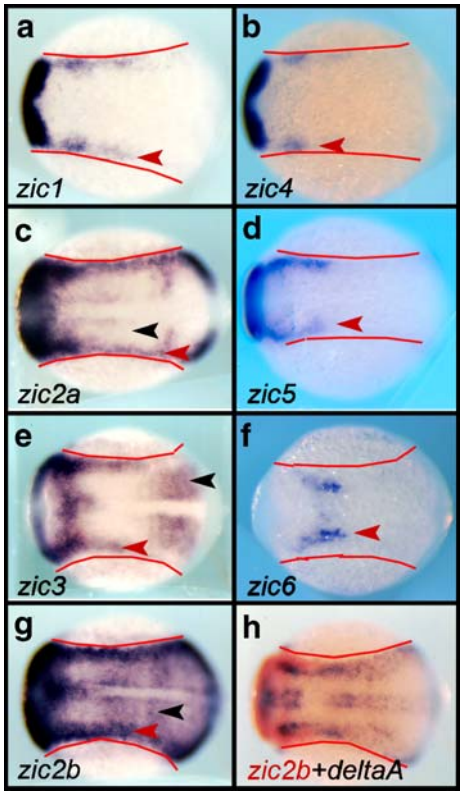

Fig. 4 Comparison of the expression of the zebrafish *Zic* gene orthologues in three-somite stage embryos. The *Zic* genes are organized as antipodal bigenes in the genome: **a**, **b** *zic1*/*zic4* on chr. 24; **c**, **d** *zic2a*/*zic5* on chr. 9; **e**, **f** *zic3*/*zic6* on chr. 14. **g** The exception is the paralogue *zic2b* on chr. 1, which is the result of a single-gene duplication within the zebrafish lineage. **h** The relative expression of the *Zic* genes in the neural plate was assessed by two-color in situ hybridization using *deltaA* as a reference transcript (shown: *zic2b* in *red* with *deltaA* in *purple*). The *red lines* indicate the lateral edges of the neural plate, the *red arrows* indicate ectodermal expression in the neural plate, and the *black arrows* indicate mesodermal expression

ment, however, *zic6* is expressed in the dorsal neural tube in a pattern similar to the other orthologues (Parinov et al. 2004).

Given the similarities among the various *Zic* gene family members, both among orthologues and across species, the highly restricted and very specific expression of *zic6* in the intermediate neurogenic domain of the zebrafish embryonic hindbrain is exceptional. This suggests that the expression of *zic6* may be a derived feature within the teleost fish lineage, possibly associated with a teleost-specific developmental feature. The fact that the other bigene pairs (*zic1*/*zic4* and *zic2a*/*zic5*) tend to exhibit similar expression patterns leads us to predict that the ancestral *zic3*/*zic6* bigene may also have shared regulatory elements. In that case, the loss of *zic6* in the tetrapods may not have been of great consequence if there was substantial redundancy of *zic3*. Alternatively, the novel expression pattern of *zic6* may have arisen in a common ancestor of the Euteleostomi and reflects a regulatory program that was subsequently lost in the Tetrapoda. However, the development of the intermediate neurogenic domain is very similar between zebrafish, which have *zic6*, and *Xenopus*, which lack *zic6*. This indicates that the function of *zic6* in that domain is not critical to its specification or development in vertebrates in general.

The synteny between the tetrapod *Zic3* locus and the teleost *zic3*/*zic6* locus together with the novel expression pattern of *zic6* in the zebrafish provides valuable insights into the evolutionary history of the *Zic* gene family (Fig. 5). The *zic3*/*zic6* bigene common to teleost genomes is one of three extant loci that resulted from repeated rounds of duplication early in the evolution of the vertebrates (Aruga et al. 2006; Meyer and Schartl 1999). The teleost *zic6* gene is a result of one such duplication that occurred in a common ancestor to the teleost fishes and the terrestrial tetrapods. A subsequent inversion/deletion event in the precursor to the mammalian X-chromosome resulted in a reversal of orientation of the *zic3* gene and a loss of the *zic6* gene. It is likely that the original function of *zic6* was redundant with other family members with overlapping ancestral expression and therefore not critical. This conclusion would also be consistent with extensive changes to the *cis*-regulatory program and amino sequence of *zic6* in comparison to other family members. However, the conserved zinc-finger DNA-binding domain provides evidence that *zic6* acts upon promoter elements common to the other *Zic* family members. Thus, *zic6* can be expected to contribute to a combinatorial code of *Zic* gene activity determining the transcriptional repression and/or activation of target genes during teleost development, which is not strictly analogous to that of the tetrapods. This is an

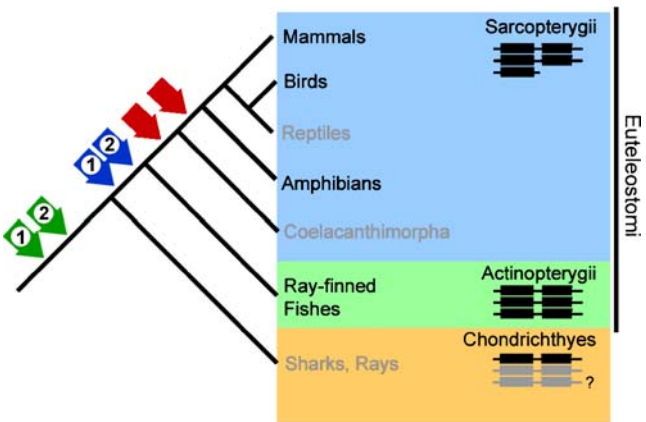

Fig. 5 The evolutionary sequence of chromosomal duplications leading to the expansion of the *Zic* gene family in the Euteleostomi. The chromosomal duplications involving the *Zic* bigenes could have occurred during whole genome duplications before the divergence of the Chondrichthyes (*green arrows*) or in a common ancestor of the Euteleostomi (*blue arrows*). The *red arrow* indicates possible points at which the loss of the *zic6* gene may have occurred relative to extant Sarcopterygian taxa. The taxa for which data on *Zic* gene complements were lacking are indicated in *gray* text

important caveat when using zebrafish as a model system for human functional genomics, but it also provides a window on the ancestral functions of the *Zic* genes that can yield insights not available with tetrapod model organisms.

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