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REVIEW

The ZIC gene family encodes multi-functional proteins essential for patterning and morphogenesis

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Abstract The zinc finger of the cerebellum gene (*ZIC)* discovered in *Drosophila melanogaster* (*odd-paired*) has five homologs in *Xenopus*, chicken, mice, and humans, and seven in zebrafish. This pattern of gene copy expansion is accompanied by a divergence in gene and protein structure, suggesting that Zic family members share some, but not all, functions. *ZIC* genes are implicated in neuroectodermal development and neural crest cell induction. All share conserved regions encoding zinc finger domains, however their heterogeneity and specification remain unexplained. In this review, the evolution, structure, and expression patterns of the *ZIC* homologs are described; specific functions attributable to individual family members are supported. A review of data from functional studies in *Xenopus* and murine models suggest that *ZIC* genes encode multifunctional proteins operating in a context-specific manner to drive critical events during embryogenesis. The identification of *ZIC* mutations in congenital syndromes highlights the relevance of these genes in human development.

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Structural features of the ZIC family proteins

The Zic genes are orthologs of the *Drosophila melanogaster* pair rule gene *odd-paired* (*opa*). The first vertebrate member of this family was identified during a screen for cDNAs enriched in the murine cerebellum [1]. Since that time, the isolation and sequencing of ZIC genes from a wide variety of organisms [2–6] has enabled extensive comparative analysis of ZIC protein sequences, which has revealed a variety of structural elements (Fig. 1). The ZIC proteins are defined by the presence of a zinc finger domain that consists of five Cys2His2-type zinc fingers and which is most closely related to the zinc finger domain of the GLI, GLIS, and NKL families. The Zic zinc finger domain is distinguished by an atypical first zinc finger. Generally, one to five amino acid residues separate the two cysteines of a C2H2 zinc finger. In Zic proteins, however, this number is both increased and highly variable, ranging from 6 to 38 in species so far examined [6, 7]. Several functions have been ascribed to the zinc finger domain. Firstly, the zinc finger domain is able to bind DNA [8, 9]. Secondly, the zinc finger domain participates in protein/protein interactions [10, 11]. Thirdly, none of the ZIC proteins contain a canonical nuclear localization signal but the ZIC3 zinc finger domain (fingers 2–5) has been shown to be essential for this function and presumably harbors an interspersed nuclear localization signal [12, 13]. As recently reviewed [14], interactions between ZIC and other proteins have also been shown to alter the sub-cellular location of the ZIC proteins and it is possible that the nucleocytoplasmic shuttling of the ZIC proteins is controlled by one or more interacting proteins.

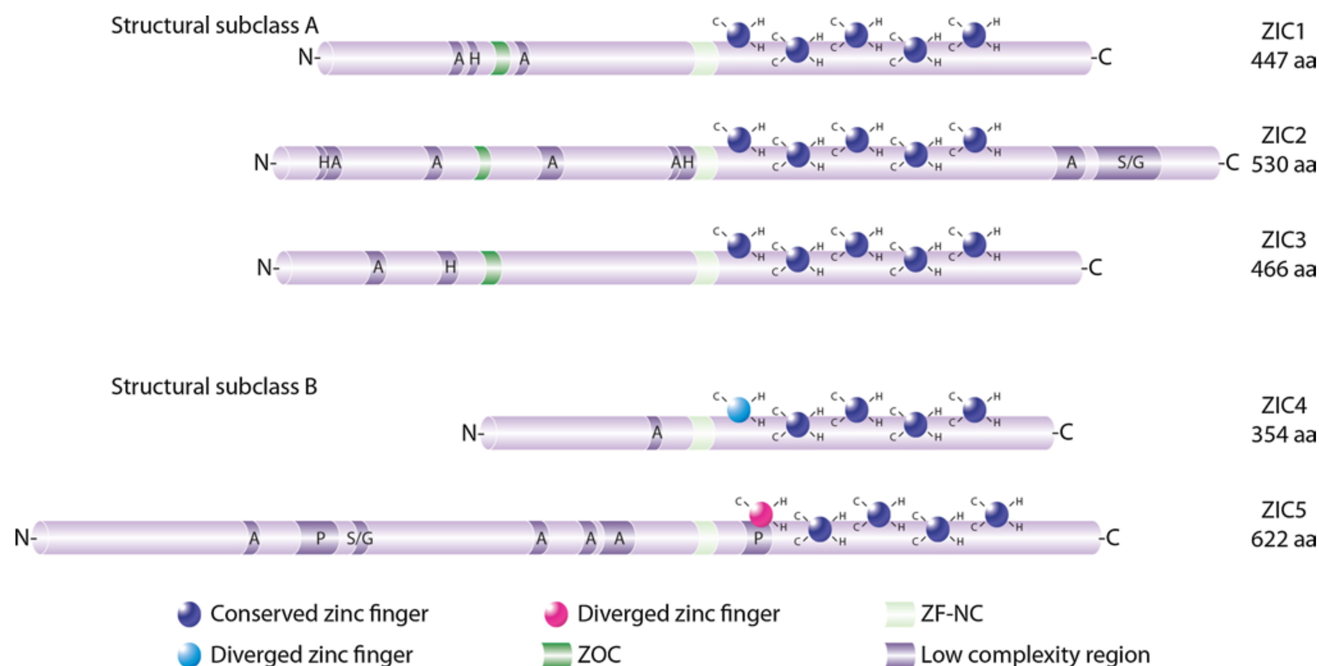

Fig. 1 Structural features of the Zic proteins. Each of the five human Zic proteins is depicted. All contain a zinc finger domain that consists of five tandem C2H2-type zinc fingers. This domain is highly conserved with only the first zinc fingers of ZIC4 and ZIC5 showing some divergence. All five proteins also contain a short (14–21 amino acid) highly conserved domain immediately upstream of the zinc fingers, called the ZF-NC domain. Three of the proteins (ZIC1, ZIC2, and ZIC3) share another small domain (9–10 amino acid) of homology towards the N terminal of the protein called the ZOC motif. All proteins also contain low complexity regions with the major amino acid found at each low complexity region shown by the associated letter (*A* alanine, *H* histidine, *P* proline, *S/G* serine/glycine). Expansion of the alanine repeat region located C-terminal of the ZIC2 zinc finger domain or at the N-terminus of the ZIC3 protein is associated with human pathology. On the basis of the presence or absence of the ZOC motif and the degree of conservation within the first zinc finger domain the Zic proteins can be divided into two distinct structural subclasses

Domain mapping experiments have not conclusively identified the fingers responsible for the DNA and protein binding activities. A missense mutation that alters the second cysteine of the fourth zinc finger of murine Zic2 ablates DNA binding and causes a loss-of-function phenotype, indicating that this finger may be required for DNA binding. The mutant protein is able to interfere with the trans-activation ability of wild-type ZIC2 in cell-based assays, implying that the inactivation of the 4th zinc finger does not interfere with a critical protein/protein interaction [15, 16]. Additionally, structural analysis of the ZIC3 protein indicates that the first two zinc finger domains may not be canonical (DNA binding) C2H2-type zinc fingers. Instead, these fingers may together form a single structural unit called the tandem CWCH2 motif, the hallmark of which is a tryptophan residue located between the two canonical cysteines of each zinc finger [17]. This motif occurs in the first and second zinc finger of the Zic proteins in a wide range of metazoan species [6]. The strong conservation of the motif suggests biological significance as does the fact that a missense mutation of the tryptophan in the first zinc finger of *ZIC3* is associated with congenital heart malformation. This mutation reduces protein stability and perturbs the nuclear localization of the protein [18]. The function of the CWCH2 motif is not known but it may be imagined that it could modulate the DNA binding capacity of the remaining canonical zinc fingers (for example, it may influence the position of the transcription factor with respect to the DNA), it could serve as a cap or insulator structure to isolate the DNA binding fingers from the N-terminal portion of the protein, or it could participate in protein–protein interactions.

Two further domains have been identified on the basis of sequence homology. A small, highly conserved domain (14–21 aa in size) is located immediately N-terminal of the zinc finger domain in each of the vertebrate ZIC proteins. This region has been termed the zinc finger N-terminally conserved domain (ZF-NC) but its function is not known [6]. Additionally, a small (9–10 aa in size) N-terminally located domain is conserved amongst a subset of the vertebrate ZIC proteins. The *Drosophila odd paired* gene also contains this domain and it was therefore named the ZIC/Odd paired conserved motif (ZOC motif) [2, 7]. This domain has been found to be required for protein–protein interactions, for example, each of the mammalian ZIC proteins that contain this domain (ZIC1, ZIC2, and ZIC3) have been shown to bind MDFI (I-mfa), an inhibitor of the basic helix–loop–helix type of transcription factors via the ZOC motif [19]. Other structural features of

the ZIC family include low-complexity regions located outside the zinc finger domain (poly-alanine, -histidine, -proline, and -serine/glycine tracts). Interestingly, expansion mutations of the C-terminal alanine tract of ZIC2 and the N-terminal alanine tract of ZIC3 are associated with human pathology. Alanine expansion mutations of ZIC2 are associated with holoprosencephaly (HPE) patients [20] and one ZIC3 expansion mutation is found in a Heterotaxy proband [21]. Experiments in vitro reveal that the ZIC2 mutations lead to a marked decrease in DNA binding activity of ZIC2 protein while the zinc finger domain remains intact [16].

As shown in Fig. 1, we propose that based on the presence or absence of the ZOC motif and on the spacing between the cysteine residues of the first zinc finger domain, the ZIC proteins can be subdivided into two distinct subclasses. Structural subclass A consists of ZIC1, ZIC2, and ZIC3 and is characterized by the presence of the ZOC box and conserved nature of zinc finger 1. Structural subclass B consists of ZIC4 and ZIC5, both of which lack the ZOC box and have a diverged first zinc finger.

Molecular function of the ZIC proteins

The ZIC proteins are thought to regulate the transcriptional activity of target genes. As recently reviewed [14], there is now good evidence that the ZIC proteins can influence gene expression, not only as classical transcription factors through direct DNA binding via their zinc finger domains but also as co-factors by binding to other transcription factors. Recently, we and others established that the ZIC proteins can repress canonical WNT signaling target genes by binding directly to the members of the TCF (transcription factor) protein family through its zinc finger domain. This ZIC-mediated inhibition of WNT signaling occurs without the ZIC proteins contacting DNA [10, 22]. These recent studies augment previous observations that suggest that the ZIC proteins can influence the transcriptional outcome of Hedgehog signaling without contacting DNA by binding to the GLI proteins [23].

Genomic arrangement and evolution of the vertebrate ZIC genes

The sequencing, assembly, and annotation of multiple genomes over the past decade has revealed that the *Zic* genes have a relatively unusual arrangement within the genomes of all vertebrates examined. Figure 2 shows the arrangement of the *Zic* genes (as shown in the Ensembl database) in the teleost lineage (as represented by zebrafish) as well as in amphibia (represented by *Xenopus*) and in mammals (represented by human and mouse). It can be seen that in each case at least some of the genes exist as tandem copies, divergently transcribed from an intervening bidirectional promoter. There are few examples of these types of paralog gene clusters being retained over large evolutionary distances but notably three other well-characterized examples (the *Hox*, *Dlx*, and *Fox* genes) all encode transcription factors critical for embryonic patterning and morphogenesis. In the case of the Zic genes, each of the retained pairs consists of one member from each structural subclass. Based on phylogenetic analysis of Zic gene sequences and intron/exon position, Aruga et al. [6] have proposed a hypothetical model of Zic gene evolution, shown in Fig. 3. In this model, the ancestral single-copy Zic gene contained one intron and a full set of conserved structural domains. Following tandem duplication of this gene, some variation occurred such that one gene copy acquired an additional intron and the other gene copy underwent sequence divergence with the breakdown of the ZOC motif and increased divergence of zinc finger 1. Subsequently, the genome duplication events proposed to have taken place early in the vertebrate lineage, presumably led to the generation of eight Zic genes arranged in four bigene clusters with each cluster containing a Zic gene from structural subclass A and B. Gene loss would largely account for the Zic gene copy number now seen in each particular vertebrate. As can be seen in Fig. 2, the zebrafish has seven Zic genes while other characterized vertebrates have five. This discrepancy is accounted for by two factors (1) the retention in the teleost lineage of the *Zic3* bigene pair (designated *zic6*) and (2) independent duplication of the *Zic2* gene leading to two copies of this gene, one paired with *Zic5* as in other vertebrates (and designated *zic2a* in zebrafish) and the other an unlinked copy (designated *zic2b*) [24]. The zebrafish *zic6* is distinguished by the lack of the ZF-NC domain [24], the only of the vertebrate genes depicted in Fig. 2 to lack this domain. In addition to the differences in the teleost lineage, Fig. 2 demonstrates that in both mammalian genomes examined here the ZIC4 gene has become less compact with the addition of introns between the coding and non-coding exons and a novel intron that divides the sequence corresponding to the first coding exon in each of the other depicted genes. Examination of other mammalian genomes suggests that this pattern is not unique to mice and man but may be more widespread in the mammalian lineage.

The different features of the Zic gene arrangement in the zebrafish genome provide additional opportunities for functional divergence and there is some evidence that the function of individual Zic genes is different in zebrafish and mammals. This is exemplified by the finding that in zebrafish *zic1* loss-of-function leads to the distinctive phenotype of cyclopia (the formation of a single, ventrally displaced eye) [25], whereas in mouse and man this phenotype is associated with loss of ZIC2 function [16, 26]. It has long been recognized that gene family expansion accompanied by the unusual arrangement of the Zic genes generates particular difficulties when studying their function. The genes are likely to exhibit broad overlap in gene expression and

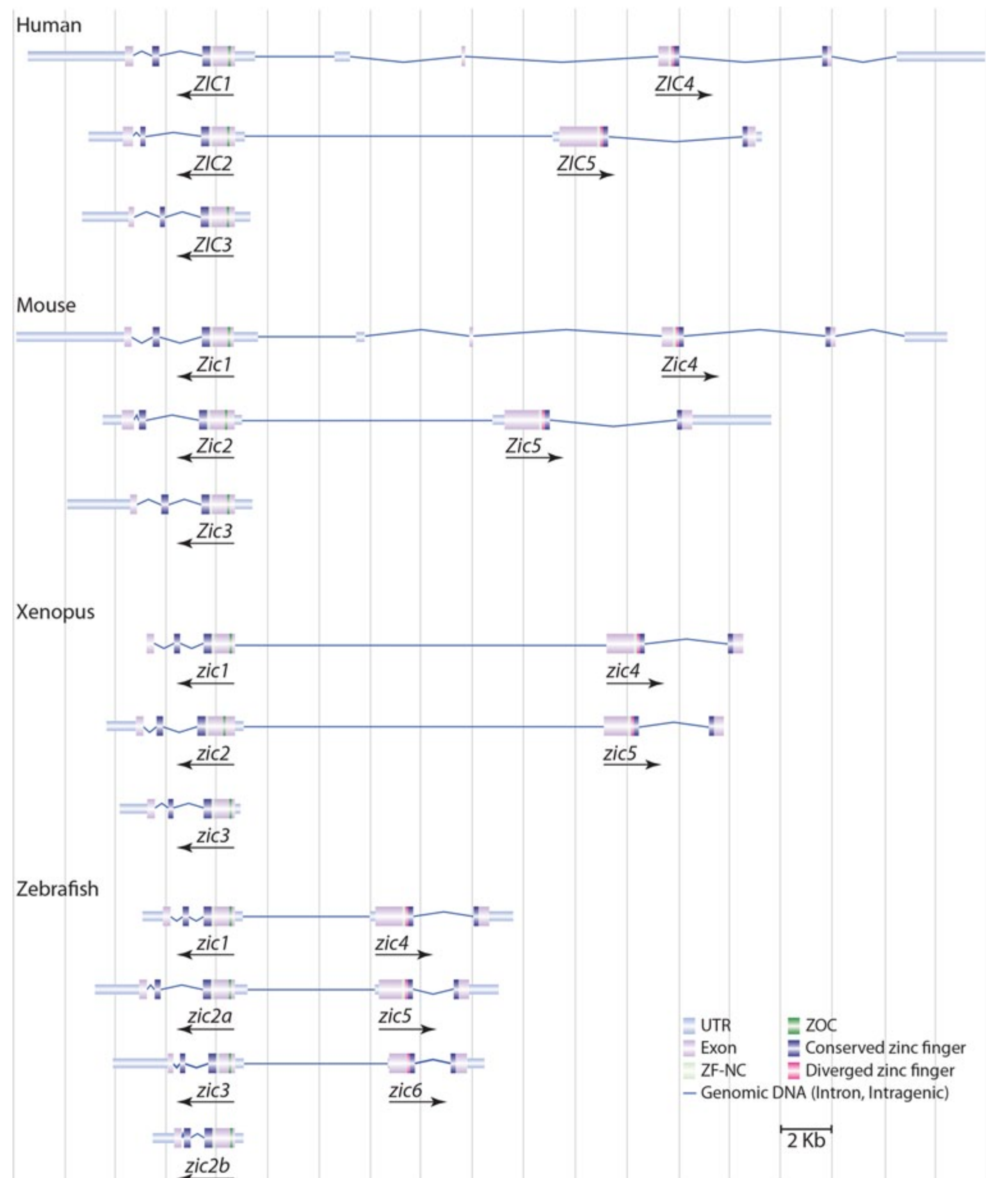

Fig. 2 The genomic arrangement of vertebrate Zic genes. The genomic arrangement of the Zic genes (as shown on the Ensembl genome browser: Human Assembly GRCh37. p8, Feb 2009, Mouse Assembly GRCm38, Jan 2012, *Xenopus tropicalis* Assembly JGI 4.2, Nov 2009. Zebrafish Assembly Zv9, Apr 2010) is depicted. The structural subclass A Zic genes are shown on the *left* and the structural subclass 2 genes are shown on the *right*. The human, mouse and *Xenopus* genomes contain five Zic genes arranged as two tandem pairs (*ZIC1/4* and *ZIC2/5*) with an unpaired *ZIC3* gene. The Zebrafish genome has seven Zic genes, three tandem pairs (*zic1/4*, *zic2a/zic5*, and *zic3/6*) and an additional gene *zic2b* most likely derived by independent duplication of *zic2a*

are likely to recognize the same DNA-binding motifs; they may also share protein partners and could participate in cross regulation of family members at both the gene expression and protein interaction levels.

Human and model organism genetic studies have repeatedly demonstrated that the Zic genes are required for embryonic development (as reviewed in [27–29]) and in some cases we have now been able to identify the embryonic stage and tissue in which Zic gene function is required in order to prevent the respective associated congenital defect. More study is required, however, to determine the precise molecular mechanisms of Zic gene function and regulation. Additionally, it is likely that both germ-line and somatic mutation of the Zic genes constitute as-yet-unrecognized risk factors for other diseases and disorders. As a basis for furthering our understanding of how the Zic genes contribute to human embryonic development and adult homeostasis we now review data from gene expression studies and from experiments that remove or increase Zic gene expression. We focus on experiments conducted in the two model organisms (mouse and *Xenopus laevis*) for which current genome annotations indicate a largely conserved gene arrangement with the human genome and for which considerable knowledge has now been amassed regarding Zic gene expression and the consequence of altered Zic gene dosage.

Spatial–temporal expression of the vertebrate *ZIC* genes

Murine Zic gene expression

The tandem gene arrangement of the vertebrate ZIC genes suggests the paired members may share regulatory regions

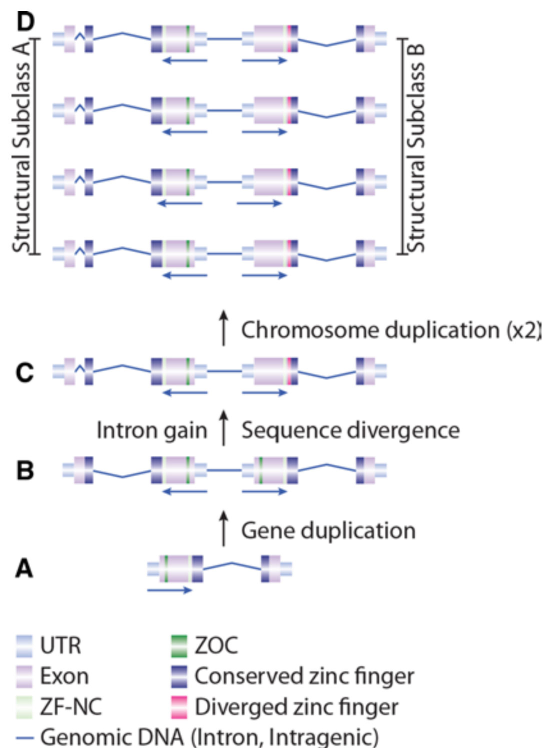

Fig. 3 The vertebrate Zic gene family expansion. A hypothetical model of Zic gene expansion early in the vertebrate lineage is depicted (adapted from ARUGA, 2006). The ancestral zic gene, which contained a single intron and each of the conserved protein domains (ZOC motif, ZF-NC, and zinc finger domain), first underwent tandem duplication to generate two divergently transcribed genes. Prior to chromosome duplication, the founding member of the structural subclass A genes gained an intron and the founding B class member lost the ZOC motif and changed the amino acid sequence of the first zinc finger. Two genome duplications resulted in eight Zic genes with subsequent gene loss, duplication, and intron gain giving rise to the genomic arrangements depicted in Fig. 2

and may be co-expressed. The finding that individual murine Zic genes when mutated give distinct phenotypes suggests however that Zic expression patterns may have at least partially diverged during evolution. Analysis of murine Zic mRNA expression patterns shows that the family members can be divided into two broad groups and supports the notion that some regulatory regions have been conserved while others have diverged. The expression of *Zic2*, *Zic3*, and *Zic5* initiates prior to, and is maintained throughout, gastrulation. During the later events of patterning, morphogenesis and early organogenesis *Zic2*, *Zic3*, and *Zic5* expression continues in a tissue-specific manner and the expression of *Zic*1 and *Zic4* is initiated. All five Zic genes are therefore co-expressed in some cells during embryonic development, providing the opportunity for heterotypic protein interactions and/or functional redundancy amongst the family members.

Zic2 appears to be the earliest of the murine Zic genes to be expressed, and expression occurs within the inner cell mass of the blastocyst at 3.5 days post-coitum (dpc) [30]. The expression of *Zic3* and *Zic5* has also been detected in embryonic stem cells (ES) cells in which ZIC3 proteins have been immunoprecipitated [9], and *Zic*5 transcripts detected [31]. By analogy, it seems likely that *Zic2* and *Zic*3 are expressed in the inner cell mass at the blastocyst stage. Consistent with this possibility *Zic*2, *Zic3*, and *Zic5* transcripts have been detected in the embryonic and extraembryonic ectoderm (or epiblast) of the immediate post-implantation stage embryo (the tissue derived from the inner cell mass of the blastocyst) [32, 33]. The expression of *Zic2, Zic3*, and *Zic5* is maintained in the ectoderm during gastrulation and transcripts are also found in some of the newly formed mesoderm that ingresses through the primitive streak [32, 33]. Notably, none of the *Zic* genes are expressed in the mesoderm that moves into the extraembryonic region. Despite the broad similarities in expression of these three genes during gastrulation, there are some differences. For example, *Zic3* is uniquely expressed in the anterior of the early gastrula in a region that corresponds to the prechordal plate and anterior definitive endoderm. At mid-gastrulation, *Zic2* is transiently and uniquely expressed in the node (Fig. 4), whereas at early somite stages *Zic3* is the only *Zic* family member expressed at the node [32]. By the early headfold stage of development the expression of all three *Zic* genes in extraembryonic ectoderm has ceased and expression in the embryonic ectoderm is restricted to neuroectoderm. As neurulation progresses, *Zic* expression in neuroectoderm becomes limited to the most dorsal region; the future site of neural crest cell and dorsal neuron production. Additionally, *Zic* expression is maintained in both the pre-somitic (*Zic2*, *Zic3*, and *Zic5*) and somitic (*Zic*1 5) populations of the lateral mesoderm.

Zic1 was initially reported to be expressed in a similar but more restricted pattern compared with *Zic2* and *Zic3* during murine gastrulation [34]. These in situ hybridization studies were not reproduced by Elms et al. [32], and an absence of *Zic*1 mRNA was confirmed by RT-PCR analysis of gastrulation stage embryos. It appears that during murine development both *Zic1* and *Zic4* are first expressed at the somite stage of development in the neuroectoderm and somitic mesoderm. By 9.5 dpc, transcripts from all five murine *Zic* genes are found in the dorsal spinal cord, the dorsal cranial neural tube, and in the somites. Subtle differences in the expression domains of the *Zic* genes persist, for example *Zic*4 is never expressed within the roof plate of the neural tube and has a more restricted expression domain in the dorsal sclerotome of the somites [32–35]. One day later, the expression of *Zic2, Zic3*, and *Zic5* is initiated in newly developing eye and in the limb buds. Several *Zic* family members are expressed at later stages of eye development in

the population of progenitor cells of the developing retina. Nine different types of cells originate from these progenitor cells and microarray analysis found that *Zic1*, *Zic2*, and *Zic3* are expressed in SSEA-1-positive cells at 14 dpc. This expression of *Zic1* and *Zic2* decreased and disappears over time, while in contrast, *Zic3* expression remains until adulthood [34, 36–39].

At later stages of development, neural *Zic* expression patterns become spatially limited. The progenitor cells of the thalamus, preoptic area, the septum, cortical hem, and the retina all share a specific pattern of *Zic* gene expression and have been investigated in *Zic* gene functional analyses over the past few years. The populations of progenitor cells in the brain, such as the granular layer of the cerebellum express all *Zic* family members, even in adult stages [34, 36]. The expression signal becomes generally more restricted at embryonic day 12.5 when it becomes limited to the dorsal neural tube and the dorsal midline in the brain. Remarkably, expression of *Zic4* at these stages can be mainly found in the septum.

From 12 dpc onwards, *Zic1-3* are all expressed in medial neural tissues including the cortical hem, the septum and the ventricular and sub-ventricular zone. In the marginal zones around the brain, the expression of *Zic*3 is less compared with the other two members; all are expressed in the nucleus of the meningeal fibroblasts. These regions of the brain are thought to be the source of Cajal–Retzius cells and continue to express the *Zic* family members until adulthood [34, 36].

Xenopus zic gene expression

The *Xenopus zic* gene family is characterized by gene-specific but also partially overlapping expression patterns over time and is mostly limited to progenitor tissue of the future central nervous system (CNS). Prior to fertilization, *zic2* transcripts have been be detected in the oocyte. Zygotic expression of all *zic* family members (except *zic4*) can be found at the onset of gastrulation and in prospective neural tissues. Very weak *zic4* expression was detected at the prospective neural fold region of the stage 11.5 mid-gastrula [40]. Expression of all *zic* family members continued at the neural plate border during early neurulation, neural crest formation, and at the dorsal central nervous system at later neurula stages. The spatio-temporal and partially overlapping expression patterns of this family make this model organism complex but simultaneously attractive to study.

In a functional screen using a maternal cDNA library from *Xenopus laevis*, Houston and Wylie [41] discovered high expression levels of maternal *zic2*. This supported the previous findings of Nakata et al. [42] who had screened for the primary expression patterns of all *zic* family members. They also detected *zic5* transcripts in the same cDNA extract, although this was not confirmed by Houston and Wylie.

All *zic* family members (except for *zic4*) are expressed at mid-blastula stages. During gastrulation, *zic* expression becomes limited to the prospective neural plate. Unlike for murine *Zic* expression patterns at this stage, most of the *Xenopus zic* genes are expressed in overlapping patterns, so only subtle spatial–temporal differences in expression can be observed. In general, *zic*1 and *zic*3 expression is more widespread compared with *zic*2 and *zic5*. The uncoupled *zic3* is uniquely expressed in the more caudal lateral plate mesoderm. *Zic2* and *zic3* are the only members that are expressed along the complete AP axis while *zic1* and *zic4* expression is found in the future hyoid and branchial arches. The selective expression of *zic5* is found mostly at the anterior neuroectodermal ridge [40, 42–44].

Nevertheless, all *zic* genes are reportedly expressed at the neural fold where they play an essential role during neural patterning [42–44]. To date, *Xenopus laevis* is the only vertebrate model where the expression of all *zic* family members at the neural plate border and at the dorsal side of the neural tube after closure is linked to neural crest cell production. Alterations in *zic* expression result in clear phenotypic differences in terms of the production and migration of neural crest cells. At later neurula stages, expression of all *zic* members continues in the dorsal CNS while their expression patterns elsewhere become further restricted. In the somites, expression patterns are mostly restricted to *zic1* and *zic4* transcripts although *zic2* is expressed to a lesser extent and is more ventrally located. During the early tailbud stages *zic1-3* are all dorsally expressed in the forebrain, midbrain, and hindbrain and are found at later stages in the telencephalon and di-/mesensephalon border, in a largely overlapping pattern. *Zic5* transcripts are only detected in the midbrain and hindbrain. Clear differences are observed at the eye vesicle where *zic2* and *zic5* expression are mostly observed, further highlighting the coupled expression of these two head-to-head placed genes. The expression of *zic2* and *zic5* is limited here to the ciliary marginal zone of the retina. In contrast, *zic2* expression is observed during somitogenesis and accompanied by *zic1* and *zic4* but not by *zic5* expression. As organogenesis progresses, these expression patterns are more restricted and remain present until later stages [3, 40].

Functional analysis of the Zic genes

Murine Zic genes

Mutational analysis of the murine Zic genes has played an important role revealing the function of these proteins in mammalian biology and there is now an extensive catalogue

Table 1 Murine *Zic* loss-of-function phenotypes

Zic phenotypes	*Zic* genotypes	Authors (reference)
Primitive streak dysgenesis	$Zic3^{-/-}$, $Zic3^{-/Y}$	Ware et al. [48]
Failure of anterior notochord production	$Zic2^{Ku/Ku}$	Warr et al. [26]
Left–right axis defect	$Zic3^{Bn/+}$, $Zic3^{Bn/Bn}$, $Zic3^{Bn/Y}$, $Zic3^{-/-}$, $Zic3^{-/Y}$	Carrel et al. [55], Purandare et al. [45]
Patterning and morphogenesis		
Neural tube defect: exencephaly	$Zic2^{Ku/Ku}$, $Zic2^{Kd/Kd}$ $Zic3^{Bn/Bn}$, $Zic3^{Bn/Y}$, $Zic3^{-/-}$, $Zic3^{-/Y}$ $Zic5^{-/-}$ (a), $Zic5^{-/-}$ (b)	Elms et al. [15], Nagai et al. [61], Klootwijk et al. [44], Purandare et al. [45], Inoue et al. [31], Furushima et al. [57]
Neural tube defect: spina bifida (and curled tail)	$Zic2^{Ku/+}$, $Zic2^{Kd/Kd}$ $Zic3^{Bn/Bn}$, $Zic3^{Bn/Y}$	Elms et al. [15], Nagai et al. [61], Garber et al. [74], Johnson et al. [96], Klootwijk et al. [56]
Classical holoprosencephaly	$Zic2^{Ku/Ku}$	Warr et al. [26]
Roof plate abnormality/middle interhemispheric variant Holoprosencephaly	$Zic2^{Kd/Kd}$ $Zic5^{-/-}$ (b)	Nagai et al. [61], Furushima et al. [57]
Hindbrain patterning	$Zic2^{Ku/Ku}$	Elms et al. [15]
Dorsal spinal cord hypoplasia	$Zic1^{-/-}$	Aruga et al. [98]
Cranial neural crest deficit/ craniofacial skeletal defect	$Zic2^{Ku/Ku}$ $Zic5^{-/-}$ (a), $Zic5^{-/-}$ (b)	Elms et al. [15], Inoue et al. [31], Furushima et al. [57]
Trunk neural crest deficit	$Zic2^{Ku/Ku}$	Elms et al. [15]
Omphalocele	$Zic3^{Bn/Bn}$, $Zic3^{Bn/Y}$	Klootwijk et al. [44]
Cleft lip	$Zic3^{Bn/Bn}$, $Zic3^{Bn/Y}$	Klootwijk et al. [44]
Skeletal defect: tail (reduced number of and/or dysmorphic caudal vertebrae)	$Zic2^{Kd/Kd}$ $Zic3^{Bn/+}$, $Zic3^{Bn/Bn}$, $Zic3^{Bn/Y}$, $Zic3^{+/-}$, $Zic3^{-/-}$, $Zic3^{-/Y}$ $Zic5^{-/-}$ (a)	Nagai et al. [61] Gruneberg [63], Purandare et al. [45], Inoue et al. [31]
Organogenesis		
Forebrain: dysgenesis of medial structures	$Zic3^{-/+}$, $Zic3^{-/-}$, $Zic3^{-/Y}$ $Zic5^{-/-}$ (b) $Zic1^{-/-}$, $Zic3^{Bn/Bn}$, $Zic1^{-/-}$; $Zic3^{Bn/Y}$	Purandare et al. [45], Furushima et al. [57] Inoue et al. [64]
Forebrain: enlarged ventricles	$Zic5^{-/-}$ (b)	Furushima et al. [57]
Cerebellum: reduced size	$Zic1^{-/-}$ $Zic3^{Bn/Bn}$, $Zic3^{Bn/Y}$ $Zic1^{+/-}$; $Zic4^{+/-}$, $Zic1^{-/-}$; $Zic4^{-/-}$	Aruga et al. [29, 65] Blank et al. [66], Grinberg et al. [67]
Cerebellum: folia patterning	$Zic1^{-/-}$ $Zic3^{Bn/Bn}$, $Zic3^{Bn/Y}$ $Zic1^{+/-}$; $Zic4^{+/-}$, $Zic1^{-/-}$; $Zic4^{-/-}$, $Zic1^{+-}$; $Zic2^{Kd/+}$	Aruga et al. [29, 65], Grinberg et al. [67], Blank et al. [66], Aruga et al. [98]
Microcephaly	$Zic2^{Kd/Kd}$ $Zic5^{-/-}$ (b)	Furushima et al. [57], Nagai et al. [61]
Skeletal defect: sternum/ribs	$Zic1^{-/-}$ $Zic5^{-/-}$ (a)	Inoue et al. [31], Aruga et al. [72]
Skeletal defect: vertebral arches	$Zic1^{-/-}$ $Zic2^{Kd/Kd}$ $Zic3^{Bn/+}$, $Zic3^{Bn/Bn}$, $Zic3^{Bn/Y}$, $Zic3^{+/-}$, $Zic3^{-/-}$, $Zic3^{-/Y}$ $Zic5^{-/-}$ (a) $Zic2^{Kd/+}$; $Zic3^{Bn/Y}$	Aruga et al. [72], Nagai et al. [61], Gruneberg et al. [63], Purandare et al. [45], Inoue et al. [31], Inoue et al. [64]
Skeletal defect: vertebral bodies	$Zic2^{Kd/+}$; $Zic3^{Bn/Y}$	Inoue et al. [64]
Skeletal defect: limb	$Zic2^{Kd/Kd}$ $Zic3^{Bn/Bn}$, $Zic3^{Bn/Y}$	Nagai et al. [61], Garber [74]
Eye: microphthalmia/anophthalmia	$Zic2^{Kd/Kd}$ $Zic3^{Bn/Bn}$, $Zic3^{Bn/Y}$ $Zic5^{-/-}$ (b)	Herrara et al. [69], Klootwijk et al. [44], Furushima et al. [57]
Eye: optic chiasm dysmorphology	$Zic2^{Kd/Kd}$	Herrara et al. [69]

Table 1 continued

Zic phenotypes	*Zic* genotypes	Authors (reference)
Eye: aberrant ipsilateral projection of retinal ganglion cells	$Zic2^{Kd/+}$, $Zic2^{Kd/Kd}$	Herrara et al. [69]
Post birth		
Viability: embryonic lethality	$Zic2^{Ku/Ku}$ $Zic3^{Bn/Bn}$, $Zic3^{Bn/Y}$, $Zic3^{-/-}$, $Zic3^{-/Y}$	Elms et al. [15], Garber et al. [74], Purandare et al. [45]
Viability: peri-natal lethality	$Zic2^{Kd/Kd}$ $Zic5^{-/-}$ (b)	Inoue et al. [31], Nagai et al. [61]
Viability: juvenile lethality	$Zic1^{-/-}$ $Zic2^{Kd/Kd}$ $Zic5^{-/-}$ (a) $Zic1^{+-}$; $Zic2^{Kd/+}$, $Zic1^{+/-}$; $Zic4^{+/-}$, $Zic1^{-/-}$; $Zic4^{-/-}$	Aruga et al. [65], Nagai et al. [61], Inuoe et al. [31], Aruga et al. [98], Grinberg et al. [67], Blank et al. [66]
Small at birth	$Zic2^{Kd/Kd}$ $Zic3^{Bn/Bn}$, $Zic3^{Bn/Y}$ $Zic5^{-/-}$ (a) $Zic1^{+/-}$; $Zic4^{+/-}$, $Zic1^{-/-}$; $Zic4^{-/-}$	Nagai et al. [61], Garber et al. [74] Inoue et al. [31], Grinberg et al. [67], Blank et al. [66]
Behavioral: pre-pulse inhibition	$Zic2^{Kd/+}$	Ogura et al. [68]
Behavioral: motor control	$Zic1^{+/-}$, $Zic1^{-/-}$ $Zic3^{Bn/Bn}$, $Zic3^{Bn/Y}$ $Zic1^{+/-}$; $Zic4^{+/-}$, $Zic1^{-/-}$, $Zic4^{-/-}$	Ogura et al. [68], Aruga et al. [29], Grinberg et al. [67], Blank et al. [66]
Behavioral: abnormal gait and posture	$Zic5^{-/-}$ (a) $Zic1^{+-}$; $Zic2^{Kd/+}$	Inoue et al. [31], Aruga et al. [98]
Hydrocephalus	$Zic5^{-/-}$ (a) $Zic1^{+-}$; $Zic2^{Kd/+}$	Inoue et al. [31], Aruga et al. [98]

(a) $Zic5^{Tm1Jaru}$ strain; (b) $Zic5^{Tm1Sia}$ strain

of processes, tissues, and organs known to be affected by loss of normal activity of one or more Zic proteins. The majority of available mouse alleles carry severe loss-of-function mutations and are primarily useful for studying the earliest function of each of the Zic proteins. The analysis of these alleles shows that Zic gene expression is required for the development of the embryonic axes, for many processes during the development of the CNS and for the development of the visual and musculo-skeletal systems. Importantly, where tested, the murine gene mutations give rise to phenotypes associated with mutation of the orthologous human *ZIC* gene, suggesting that the mouse mutants serve as good models for investigating the etiology of *ZIC*-associated human disorders (summarized in Table 1).

Zic function at gastrulation

The first developmental process known to require Zic function is gastrulation. In mouse embryos, the onset of gastrulation is marked by the formation of the primitive streak at the future posterior of the embryo. Cells delaminate from the embryonic ectoderm, ingress through the primitive streak, and differentiate into mesoderm and endoderm. The ectoderm at the future anterior of the embryo does not ingress through the primitive streak but differentiates in situ into the neural and non-neural ectoderm of the conceptus. Embryos that lack *Zic3*, either due to a spontaneous (bent tail, *Bn*) or targeted ($Zic3^{Tm1Bca}$) deletion of the locus, exhibit a range of phenotypes [44, 45]. Some embryos have defects that are obvious by the onset of gastrulation and which imply a role for Zic3 in the formation of the primitive streak and in the differentiation of the mesoderm and endoderm [46]. This is consistent with the broad expression of *Zic3* in the ectoderm of the early gastrula and in the primitive streak [32, 47]. Other *Zic3* null embryos do not exhibit a defect until late in gastrulation at a stage when the left–right (L–R) embryonic axis is being established. The L–R axis is crucial to normal development of vertebrate embryos since despite their external symmetric appearance, all species contain characteristic internal asymmetries. The term heterotaxy is used to describe a particular disturbance of L–R axis formation and mutations in both murine *Zic3* and human *ZIC3* are associated with the condition [13, 45, 47]. Heterotaxy results in congenital abnormalities of a wide range of midline structures and frequently includes heart defects. At mid-gestation, approximately 50 % of *Zic3* null embryos have overt heart abnormalities that are consistent with incorrect establishment of the L–R axis [48]. The correct establishment of

the L–R axis requires NODAL signaling during the early somite-stages at a specialized part of the primitive streak called the node. *Zic3* is expressed at the node of the early somite embryo [32] and the penetrance of $Zic3^{-/-}$ heart phenotypes is increased in embryos that are also haploinsufficient for *Nodal* [48]. It is likely that ZIC3 plays a direct role in the establishment of the L–R axis, but it is difficult to exclude the possibility that the observed L–R axis defects are secondary to earlier abnormalities in the establishment of the primitive streak and node.

Zic2 function is also required during gastrulation to ensure that the cells of the anterior notochord are produced. These cells should emerge from the node of the mid-gastrula, but an insufficient number of anterior notochord cells are found in embryos homozygous for a missense mutation (kumba: *Ku*) that prevents DNA binding of ZIC2 protein [15, 16, 26]. The incorrect formation of the anterior notochord results in an arrest of prechordal plate development, most likely because the anterior notochord normally generates a prechordal plate maintenance signal [26, 49]. The prechordal plate in turn is the site of expression of Sonic Hedgehog (SHH) a secreted molecule that is crucial for patterning the overlying ventral forebrain. If a functioning SHH signal is not produced by the prechordal plate, or not received by the forebrain neuroectoderm, then the developing brain does not separate into the left and right hemispheres, leading to HPE. In the most severe cases of HPE, the formation of the eye field is also affected and can produce two eyes that are abnormally close together (hypotelorism), two eyes that are fused at the midline (synophthalmia), or a single, ventrally displaced eye (cyclopia). Embryos homozygous for the severe loss-of-function *Ku* allele exhibit the full range of ventrally induced HPE phenotypes mimicking the range of ventral forebrain HPE phenotypes (or classical HPE) associated with heterozygous loss-of-function mutation of human *ZIC2* [26, 50, 51]. *Zic2* is not expressed within the ventral forebrain and it should be noted that despite the fact that the classical forms of HPE are considered defects of forebrain development, the finding of *Zic2*-associated HPE in *Ku/Ku* embryos reveals a role for ZIC2 during gastrulation rather than during forebrain development per se. *Zic2* is the only *Zic* gene expressed in the node of the mid gastrula and it is possible that in *Zic2* mutants this is the only tissue at this stage of development in which ZIC2 function is not at least partially rescued by the expression of *Zic3* and/or *Zic5*.

Zic *function at patterning and morphogenesis*

Once the germ layers are formed and the three embryonic axes are established, the embryo enters a phase of patterning the newly formed germ layers with respect to the axes and of morphogenesis so that by 9.5 dpc an embryo with the rudiments of almost every organ and system is produced. *Zic* genes are required to ensure that several aspects of patterning and morphogenesis occur correctly. One of the earliest morphogenetic events during this period is primary neurulation; the process that converts a flat sheet of neuroepithelium into the neural tube. Neural tube closure first requires that the neuroepithelium bends and that the two lateral margins of the neuroepithelium are brought into opposition before fusing at the dorsal midline of the embryo. In the mouse neural tube, closure begins at 8.5 dpc (when the embryo has ~ six somites) and initiates at three different points along the length of the embryo. A failure of closure at the different points results in discrete classes of neural tube defects. For example, a failure of cranial neurulation results in exencephaly, while a delay of trunk closure results in spina bifida. In mouse embryos, a failure to complete the closure of the trunk neural tube at the posterior neuropore before the process of secondary neurulation (which forms the neural tube of the tail region) begins to produce a curled tail [52]. Evidence that *Zic* gene expression is required for primary neurulation comes from the analysis of phenotypes associated with *Zic* mutated alleles. This includes an incompletely penetrant curled tail and spina bifida in animals heterozygous for the *Zic2 Ku* allele ($Zic2^{Ku/+}$), frequent exencephaly, and delayed posterior neuropore closure in all homozygous *Ku* embryos $Zic2^{Ku/Ku}$ embryos [15], incompletely penetrant exencephaly and delayed posterior neuropore closure in embryos homozygous or hemizygous for either of the *Zic3* null alleles ($Zic3^{Bn}$ or $Zic3^{Tm1Bca}$) [45, 53–56] and incompletely penetrant exencephaly in both targeted null alleles of *Zic5* ($Zic5^{Tm1Jaru}$ and $Zic5^{Tm1Sia}$) [31, 57]. Molecular investigation of the cause of delayed posterior neuropore closure in $Zic2^{Ku/Ku}$ embryos suggests that elevated bone morphogenetic protein (BMP) signaling (caused by decreased expression of BMP antagonists) may prevent the formation of the dorsal lateral hinge points required for neural tube closure at rostral levels of the trunk [58].

A process concomitant with, but independent of, neurulation is the formation of the neural crest. The neural crest is a transitory population of cells that arises at the boundary of the neural and non-neural ectoderm [59, 60]. In the mouse, the emigration of the cranial neural crest is complete before cranial neural tube closure (~18 somites) and trunk crest begins to emerge from the dorsal neural tube at early somite stages and before neural tube closure begins. Although all murine *Zic* genes are expressed in the dorsal neural tube, only *Zic2*, *Zic3*, and *Zic5* are expressed in the neuroectoderm at early somite stages when the neural crest is first being formed. There is good evidence that *Zic* gene expression is required for neural crest generation in the mouse embryo. Loss of *Zic2* function results in depletion of neural crest cells at all axial levels [15] and embryos that lack *Zic5* produce less cranial neural crest than normal embryos [31]. In the case of loss of *Zic2* function, the defect

has been shown to occur at an early stage of neural crest development, which is likely to reflect the inability of the ZIC2-depleted neuroectoderm to respond to neural crest induction [15].

Additional roles for *Zic2* expression in the development of the CNS have been found. Firstly, $Zic2^{Ku/Ku}$ embryos exhibit a defect in the establishment of the transient segmented structures (rhombomeres) that form within the hindbrain [15]. In addition, embryos homozygous for the hypomorphic $Zic2^{Kd}$ allele (which was generated via an incorrect targeting event leading to reduced expression of wild-type *Zic2* gene product) exhibit defects of the dorsal forebrain [61]. During neural patterning, the specialized roof plate structure forms at the dorsal midline of the neural tube. This region participates in patterning the dorsal–ventral axis of the neuroectoderm and its ablation in mouse embryos leads to a mild, dorsal form of HPE called the middle interhemispheric (MIH) variant [62]. $Zic2^{Kd/Kd}$ embryos lack a morphologically recognizable roof plate and exhibit a phenotype that may correspond to human MIH HPE. In humans, only heterozygous *ZIC2* mutations are found, resulting in partial loss-of-function. These mutations are associated with multiple central nervous system defects ranging from classical (alobar) to the milder MIH HPE. The only human *ZIC2* variant associated with MIH HPE behaves as a hypomorphic allele in cell-based transcription assays [16] and it seems likely that partial loss of ZIC2 function causes MIH HPE in both mouse and man. Conversely, the $Zic2^{Kd/Kd}$ mutants do not present with classical HPE and do not have neural crest or hind brain patterning defects, indicating that a small amount of functional ZIC2 protein is sufficient to prevent these phenotypes. The molecular basis of the hindbrain and dorsal forebrain *Zic2*-associated phenotypes has not been investigated and no other *Zic* gene mutations have been reported to give a hindbrain patterning phenotype, indicating that the requirement in this process may be *Zic2* specific.

Throughout this period of embryogenesis, the mesoderm generated during gastrulation also becomes patterned. The lateral mesoderm which arises from the posterior primitive streak becomes segmented into the somites that give rise to the musculoskeletal system. Heterozygous loss-of-function of either *Zic2* or *Zic3* genes is sufficient to produce tail kinks caused by dysmorphic vertebrae [15, 63] and animals that have a partial loss of *Zic2* function ($Zic2^{Kd/Kd}$) or which lack *Zic3* function ($Zic3^{Bn/Y}$) have a decreased number of tail vertebrate, which is exacerbated in $Zic2^{Kd/+}$; $Zic3^{Bn/Y}$ animals. These phenotypes indicate a deficit of the precursor cells from which the somites arise and defective somitogenesis. Analysis of somitogenesis in *Zic* mutant embryos suggests a role in the establishment of somite compartments [64]. These defects are consistent with the expression of *Zic2* and *Zic3* in the primitive streak and the emerging lateral mesoderm [32] and in the pre-somitic mesoderm [35]. Defects in the axial skeleton have also been described from loss of *Zic5* function but this has not been well studied [64].

Zic *function during organogenesis*

Once the embryonic pattern is established, individual tissues begin to differentiate during organogenesis. Mutational analysis has revealed further functions for the *Zic* genes at these stages in the developing brain, visual system, and in the axial and appendicular skeleton. It is at this stage that the expression of the additional *Zic1/4* gene pair begins and roles for these genes have been established in organogenesis.

Either primary or redundant roles for each of the five *Zic* genes have been described within the developing CNS. There is good evidence that *Zic1* and *Zic4* interact at the genetic level to regulate the development of the hindbrain-derived cerebellum, which plays an important role in motor control. Loss of *Zic1* function alone ($Zic1^{Tm1Jaru}$) produces morphological abnormalities of the cerebellum (hypoplasia and abnormal foliation), and corresponding behavioral abnormalities (a failure to develop the righting reflex and ataxia) [65, 66]. Animals that lack *Zic4* function alone ($Zic4^{tm2Kjmi}$) do not display overt cerebellar morphology or behavioral abnormalities, but *Zic4* ablation on the *Zic1* null background exacerbates the phenotype [66]. Similarly, animals heterozygous null for either *Zic1* or *Zic4* alone have no discernible phenotype, whereas animals heterozygous for a targeted mutation that removes both *Zic1* and *Zic4* function ($Zic1/Zic4^{tm1Kjmi}$) have cerebellar abnormalities that mimic the defect of human hindbrain development known as Dandy-Walker malformation (DWM) and a subset of DWM patients are hemizygous for both *ZIC1* and *ZIC4* [67]. The reduction in cerebellar size results from decreased proliferation of the granule cell progenitor population, a process that requires SHH signaling. *Shh* is expressed normally in the developing cerebellum of the mutant embryos, suggesting that ZIC1 and ZIC4 proteins are directly or indirectly required to receive or transduce the SHH signal. The molecular basis of the abnormal foliation pattern is not known, but SHH signaling is not required for this process [66]. As with *Zic2* function in forebrain patterning, this work has revealed sequential, temporally separate and molecularly disparate requirements for *Zic1* and *Zic4* gene function during cerebellar development. *Zic2* has also been shown to play a secondary role in cerebellar function. $Zic2^{Kd/Kd}$ animals have no cerebellar phenotype, yet one copy of this allele in combination with heterozygous loss of *Zic1* produces a phenotype similar to the *Zic1* null allele. It appears that haploinsufficiency for *Zic1* sensitizes the development of the cerebellum such that minor perturbations in other *Zic* genes can produce a phenotype similar to that of the *Zic1* null. This suggests some overlap in function, but that *Zic1* also has some function that cannot be compensated for by

Zic4 or *Zic2* expression. Animals that are null for *Zic3* also exhibit regional hypoplasia of the cerebellum and mild foliation defects demonstrating a non-redundant role of *Zic3* in cerebellum development.

Zic5 function has not been implicated in cerebellar development, but *Zic5* null mice exhibit subtle forebrain defects including poor differentiation of dorso-medial telencephalic structures, a phenotype that may be analogous to the roof plate defect seen in $Zic2^{Kd/Kd}$ embryos [57]. Additionally, several regions of the forebrain are underdeveloped, which corresponds to enlarged forebrain-derived ventricles [57], a feature that may be related to the occasional hydrocephalus documented in homozygous null animals [64]. Hydrocephalus also occurs in a proportion of animals with decreased dosage of *Zic1* and *Zic2* expression ($Zic1^{+/-}$; $Zic2^{Kd/+}$) [68]. In contrast to *Zic5*, a role for *Zic1*, *Zic2*, and *Zic3* function in forebrain development is revealed only when compound mutants are generated [36], suggesting a high level of functional redundancy between the *Zic* genes during forebrain development. As with the cerebellar phenotypes, where investigated, the forebrain phenotypes are associated with decreased proliferation of neuronal precursor cells, increased exit from the cell cycle, and an increased number of cells expressing markers of differentiated neurons at early stages of neurogenesis. Paradoxically, the early increase in the neuron number culminates in an eventual depletion of neurons and a reduced size of the affected brain region. Apparently *Zic* genes are required to promote self-renewal of the neuronal precursors to ensure a sufficient number of differentiated neurons. This conclusion is supported by that fact that mis- or over-expression of either *Zic1* or *Zic3* in the developing CNS inhibits neuronal differentiation. The molecular basis of this function is not known and whether ZIC proteins trans-activate cellular proliferation pathways, repress gene expression required for differentiation, or both remains to be determined. It is hypothesized that the *Zic* genes act via the Notch pathway to exert this influence, but to date this has not been genetically tested.

Zic gene mutation also leads to a variety of largely uncharacterized eye phenotypes. For example, *Zic5* null embryos are reported to have occasional microphthalmia or anophthalmia [44] as do $Zic2^{Kd/Kd}$ embryos [69]. One well-characterized role in the visual system for *Zic* genes is the requirement for *Zic2* expression to generate ipsilateral projections from retinal ganglion cells. The decision of retinal axons to either cross the midline at the optic chiasm (to project contralaterally) or to avoid the midline (and project ipsilaterally) is essential for binocular vision (high numbers of ipsilateral projections correlate with highly developed binocular vision). Partial loss of *Zic2* function is sufficient to reduce ($Zic2^{Kd/+}$) or eliminate ($Zic2^{Kd/Kd}$) the ipsilateral projection of retinal ganglion cells, [69], whereas expression of *Zic2* in neurons that normally project contralaterally is sufficient to redirect them to an ipsilateral location [70]. Ectopic expression and axon redirection is associated with the upregulation of the EphB1 receptor [70, 71], but some axon redirection occurs when *Zic2* is ectopically expressed in *EphB1* null embryos, suggesting EPHB1-dependent and -independent mechanisms are involved in ZIC2-driven axon guidance [70, 71].

As alluded to above, the Zic genes play a variety of roles in the development of the somite-derived structures. Each somite initially undergoes a mesenchymal-to-epithelial transition and the epithelium becomes partitioned into the presumptive sclerotome (ventromedial), myotome (dorsomedial), and dermatome (lateral) regions. The sclerotome cells then revert to being mesenchymal, break away from the somite, migrate around the neural tube, and form the major components of the axial skeleton (vertebrae, ribs, and sternum). All three of the ZOC-containing Zic genes are required for this process. Mutation of *Zic1*, *Zic2*, or *Zic3* alone is sufficient to cause defects in the dorsal part of the vertebrae (the vertebral arches) and in each case analysis precludes an early effect on somite formation implicating a requirement for Zic gene function during the later process of sclerotome differentiation [64, 72]. Animals that lack *Zic3* and retain only a small amount of *Zic2* function ($Zic2^{Kd/+}$; $Zic3^{Bn/Y}$) have defects of the vertebral arches as well as the ventral vertebral bodies, suggesting functional redundancy in the development of ventral vertebral structures [64]. Each of these Zic genes is also expressed in the dorsomedial myotome, which will give rise to much of the body musculature. Zic2 has been proposed to contribute to expression control of the muscle differentiation gene Myf5 in the mouse myotome [73]. Mutation of Zic genes has also been reported to give rise to defects of the appendicular skeleton, in particular the distal limb structures. When the *Bn* allele of *Zic3* was first isolated, Garber reported some $Zic3^{Bn/Y}$ animals had malformed forelimbs [74], and recently the targeted null allele of *Zic3*, while not itself producing limb defects, has been shown to rescue the heterozygous *Gli3* loss-of-function phenotype of the extra toes mutant. Gli3 is a transcriptional mediator of the Shh signaling pathway, which acts as an activator or repressor depending upon specific post-translational modifications. It appears that Zic3 can influence the balance of Gli3 activator and repressor forms and therefore promote Shh expression and signaling in the limb bud [75]. Depletion of Zic2 function is associated with defects of the limb skeleton but the molecular basis of this remains unknown [61].

Xenopus zic genes

Most of the functional studies on the *ZIC* gene family have been carried out in *Xenopus laevis* on the orthologous *zic* genes and zic proteins. The complete removal

of gene function is difficult in *Xenopus*; therefore a combination of gene knockdown and overexpression studies has been used to quickly identify phenotypic defects and transcriptional alterations. These experiments have both identified novel roles for the *zic* gene family and confirmed functions found in other model organisms. Since many of these experimental approaches differed between independent laboratories, the interpretation and comparison of the data has proven difficult, therefore the resultant phenotypes are mostly discussed separately in this review (Table 2).

Since *zic2* is maternally expressed in *Xenopus laevis*, it is difficult to use morpholino-based approaches to identify its function in vivo. Therefore, the host transfer method was used to eliminate maternal *zic2* expression by injecting anti-sense oligonucleotides into unfertilized oocytes that were planted back into a mother animal. This method led to phenotypes including exogastrulation, anterior truncations, and axial defects [10, 41]. Although initiation of gastrulation occurred normally, a delay in blastopore closure was evident, in 30 % of the exogastrulae. The other surviving embryos exhibited deep involution of the marginal zone, reduced head size, wrinkled epidermis, and a stunted dorsal axis. Despite the fact that these abnormalities can be due to secondary effects, clear forebrain defects were evident. In addition, neural tube formation and somitogenesis were clearly disrupted. The notochord seemed to be thickened and in some cases duplicated. Analysis of nodal target gene expression revealed a general increase in nodal signaling in these embryos. Injection of the nodal antagonist *cerberus* short in part rescued the phenotype, indicating indeed that Nodal signaling was disturbed but was not the only signaling pathway affected. This was further confirmed by our group and others when zic2 and other zic proteins were able to repress canonical Wnt signaling [10, 22]. This finding was partially based on injection of *zic3* mRNA that was found to alter the expression of organizer markers including *goosecoid* and *siamois*, which are direct Wnt target genes, and impaired notochord development. Due to the possible non-specific nature of this overexpression phenotype, a loss-of-function approach was also used. The injection of *zic3* morpholinos alone did not generate obvious abnormalities but co-injection with *zic2* morpholinos revealed a clear phenotype. In accordance with the maternal *zic2* knockdown phenotype, the notochord was thickened and widened, A-P axis was shortened and the size of head structures were reduced [22]. Although maternal zic2 proteins cannot be directly targeted, targeting of zygotic expression by morpholino technology revealed clear phenotypes attributable to altered zygotic *zic2* and *zic3* expression. This again demonstrated the functional redundancy in this gene family. Other early functions associated with *zic3* expression in *Xenopus* include regulation of left–right (L–R) patterning and convergent extension (C–E) morphogenesis. Similarly, and as previously mentioned, in humans, *ZIC3* mutations result in X-linked heterotaxy, and *Zic3* null mice exhibit

Table 2 *Xenopus zic* gene gain- and loss-of-function phenotypes

Zic phenotypes	Genotypes	References
Gain-of-function		
Organizer dysgenesis	*zic3*	Fujimi et al. [94]
Impaired notochord formation	*zic3*	Fujimi et al. [94]
Induced neuroectoderm formation	*zic*1, *zic*2, *zic*3	Nakata et al. [3, 42, 43], Kuo et al. [78], Misuzeki et al. [76]
Neurogenesis inhibition	*zic*2	Brewster et al. [79]
Induced neural crest cells	*zic*1, *zic*2, *zic*3, *zic4*, and *zic*5	Nakata et al. [3, 42, 43], Fujimi et al. [40]
Larger head structure	*zic*1, *zic*2, and *zic*3	Nakata et al. [3, 43]
Smaller eyes	*zic*1, *zic*2, and *zic*3	Nakata et al. [3, 43]
Induced retina	*zic*1, *zic*2, and *zic*3	Nakata et al. [3, 43]
Loss-of-function		
Exo-gastrulation, anterior truncations, and axial defects	Maternal *zic*2	Houston et al. [41]
Delay in gastrulation, left–right patterning, convergent extension morphogenesis, midline abnormalities, abnormal heart and gut looping	*zic*3	Cast et al. [47]
Thickened notochord, A–P axis shortening, and reduced head structures	*zic2* + *zic3*	Fujimi et al. [94]

Summarized are phenotypes occurring in *Xenopus* embryos injected with *zic*-specific morpholino or (dominant negative) mRNA

defects in gastrulation, neural tube closure, and axial patterning [45, 48]. *Xenopus zic3* morphants revealed significant impairment of C–E and in L–R patterning. The A–P axis was shortened, gastrulation was delayed, and abnormal heart and gut looping [47]. Collectively, these data support an evolutionarily conserved role of *Zic3* gene function during early vertebrate embryonic development.

At the prospective neural plate region, *zic3* was found to be ectopically expressed upon BMP4 signaling blockade. As for other neural inducers, it was postulated that repression of *zic3* expression was relieved when neural inducing signals altered BMP4 signaling [3]. Similarly, in addition to *zic3, zic1* and *zic2* were shown to be downstream targets of BMP4 signaling. The expression of both genes was upregulated in the neuroectoderm and both were potent inducers of neuroectodermal differentiation, though the potency of induction was less with *zic1* compared with *zic2* expression [43, 76–78].

Furthermore, *zic2* was shown to be expressed in stripes that alternate with those in which primary neurons differentiate in the neuroectoderm, and overlap the domains of floor plate and neural crest progenitors (stage 14). *Zic2* overexpression leads to induced neural crest cell differentiation and inhibition of neurogenesis, elevating *zic2* to the rank of vertebrate pre-pattern gene. The same expression pattern was found for *zic5* but this was not further investigated [42, 79]. More recent studies indicate, in addition to BMP signaling, Ca^{2+} accumulation can also regulate *zic* expression in *Xenopus* and that BMP signaling blockade together with fibroblast growth factor (FGF) activation triggers *zic* gene activation to induce neuroectoderm formation [80].

While it has only been demonstrated for *zic1* and *zic3*, it appears likely that *zic2* expression is also under control of BMP and FGF signaling. However, mice harboring mutations in both *Bmpr1a* and *Bmpr1b* genes, which results in a clear MIH HPE phenotype, maintain *Zic2* expression [81], suggesting that BMP is not required for *Zic2* expression in this context. Bearing in mind that this signaling cascade is essential for neuroectodermal differentiation, it is however very probable that specific members of the *ZIC* gene family act through these pathways during normal neural development in these animal models [82–85]. The genetic regulation of *Xenopus zic* genes is also discussed in more detail in the light of vertebrate neuroectodermal differentiation [80].

Both knockdown and overexpression of *zic3* resulted in clear neuroectodermal defects and lead to disturbed neurulation events. Although it is difficult to determine if the later defects are direct effects of altering *zic3* expression, clear phenotypes arose after ectopic expression. Induced pigmented cells were accompanied by a general expansion of the head structures without mesodermal induction. This does not account for effects in the eyes, which were smaller compared with the wild-type embryos while markers of neural retina were upregulated. Essentially the same characteristics were found when *zic1* and *zic2* were overexpressed, namely a thickening of the neural tube and ectopically expressed pigmented cells. Marker-based analysis revealed that the epidermal cell fate was shifted to neural and neural crest cell fate without changing the expression of mesodermal markers [43].

Primary neurons and hindbrain cells were lost using a morpholino against *zic1* expression and by injection of a dominant negative zic5 protein. Also, the induction of neural crest cells was disrupted. Similar defects were shown when XMeis3, a transcription factor that can induce hindbrain cell fates in a non-cell-autonomous manner, was knocked down, while the expression of all *zic* genes was maintained in this setting. This would suggest a role downstream of *zic* gene expression for XMeis3 function. This hypothesis was confirmed when the phenotype of the *zic1* morphant was rescued following co-injection of Xmeis3 [86, 87]. Since Xmeis3 is shown to activate the hindbrain induction center that regulates early A–P cell fate in the developing CNS, its potential loss of activity can directly explain the phenotype in the *zic1* and *zic5* loss-of-function models.

Overexpression of *zic5* in *Xenopus* led to a very strong neural crest cell-induced phenotype. Although *zic5* is expressed only from the onset of neural crest cell induction, it induces ectopic melanin-forming cells very efficiently. Marker gene analysis shows that overexpression of *zic5* also leads to the epidermal fate switched to neural and neural crest cell fate. A notable difference in the effects from overexpression of the different *zic* genes are to the anterior neural makers, which are unaffected in *zic5* gain-of-function assays while they were disrupted in *zic1-3* overexpression models. This would indicate that there are not only structural and expression-based differences, but that *zic5* function appears at least in part to have been modified during evolution compared with the *zic 1-3* genes [42].

Overexpression of *zic4* results in an overlapping phenotype compared with the other *zic* gene family members. As for all *zic* genes, melanocyte formation was increased leading to an observed increase in neural crest cell formation. A general induction of neural tissue differentiation was evident, although no anterior neural markers (similar to *zic5* overexpression) were altered. Hypoplastic eyes were observed with *zic4* overexpression, which did not occur when other *zic* genes were overexpressed. Since *zic4* expression is not observed in the *Xenopus* normal eye, this could be interpreted as an indirect effect. In general, the *zic4* overexpression phenotype largely mimics that observed for *zic5* overexpression leading again to the separation of the family into two groups that can be traced back to the chromosomal duplication where the *zic4* and *zic5* genes seem to have lost intrinsic characteristics that remain in the *zic1*, *zic2*, and *zic3* genes [40].

Discussion

Protein structure and synexpression subfamilies

Current investigations regarding Zic gene structure, expression, and function lead to an emerging picture in which gene duplication followed by loss of conserved protein domains and some alteration in expression control mechanisms has resulted in overlapping and unique functions of the orthologous and paralogous Zic family members. The most parsimonious chain of events would be that early in the vertebrate lineage, following an initial tandem gene duplication event, one copy of the tandem gene pair underwent structural changes that include loss of the ZOC motif and alteration in the amino acid composition of the first zinc finger. The subsequent genome duplications of the early vertebrate lineage therefore established two subclasses of Zic proteins. Here we define structural subclass A proteins as those that possess the ZOC motif and have retained sequence conservation across the first zinc finger (ZF1). Structural subclass B proteins are those that lack the ZOC motif and have a diverged amino acid composition of the residues immediately following the tryptophan residue of zinc finger 1. The ZOC motif is implicated in protein/protein interactions and it may therefore be anticipated that the structural subclass A Zic proteins (Zic1, 2, and 3) have some protein partners (and functions) in common but not shared by the structural subclass B proteins (Zic4 and 5). In contrast, the DNA binding ability of the Zic proteins is usually attributed to the more conserved zinc fingers (ZF3-5), suggesting that all five Zic proteins likely bind the same DNA sequences perhaps with varying affinity. Where investigated, this seems to be the case [11, 88].

This structural subclasses division persists amongst orthologous genes but there are some structural features of the Zic proteins that have undergone more rapid divergence such that differences can now be found between orthologs. This is exemplified by the location and composition of the low-complexity regions found outside of the ZOC, ZF-NC, and zinc finger domains, which can vary remarkably amongst orthologous genes (Fig. 5). Expansion mutations of the c-terminal alanine tract in *ZIC2* have been associated with HPE in humans, a congenital defect known to be caused by *ZIC2* loss-of-function, suggesting functional significance of this domain. An alanine repeat is found at a similar region in murine *Zic2* but not in *Xenopus zic2*. The precise function of low-complexity regions in the Zic proteins and in general is not well characterized, but there is evidence that they are associated with protein-binding activity and that their position within a sequence may determine their binding properties and biological function [89]. Differences between the sequences of orthologs should be taken into consideration when interpreting data from apparently analogous experiments in different organisms.

The finding that the genomic arrangement of the Zic genes, primarily as bigenes, has been conserved over large evolutionary differences suggests it may have functional significance. In particular it is possible that the bigenes share regulatory regions. A corollary of this hypothesis is that the expression pattern of bigene members would be more similar to one another than to the other paralogs. It remains difficult to determine whether this is the case for practical and technical reasons. First, there are few biological systems where the expression of all five genes has been investigated in detail and, second the hybridization efficiency of the probes used to detect different *Zic* mRNA species via in situ hybridization varies. In particular, both *Zic4* and *Zic5* transcripts seem difficult to detect (i.e., they lead to low signal; see for example *Zic5* signal in Fig. 4) using this technique. In order to complete surveys of Zic gene expression, it may be necessary to develop better probes or protocols for the in situ detection of *Zic4* and *Zic5* transcripts or to augment mRNA localization studies with additional techniques such as RT-PCR or to develop Zic isoform specific antibodies suitable for tissue hybridization studies.

Despite these caveats, the currently available data can be interrogated to assess the evidence for bigene co-expression. In *Xenopus* it appears that *zic2* and *zic5* are often

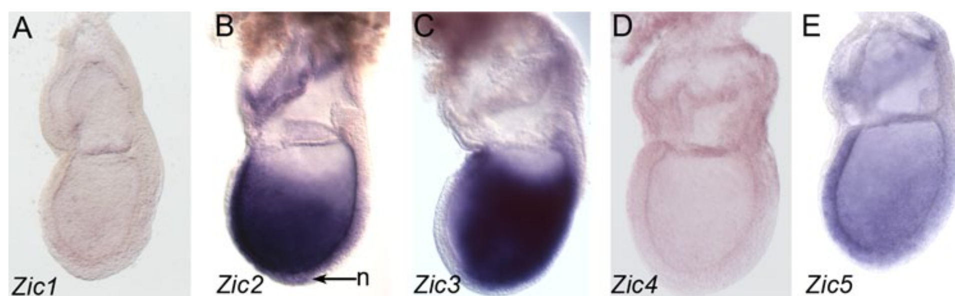

Fig. 4 Co-expression of the murine Zic genes during mouse gastrulation. Lateral view of 7.5 dpc mouse embryos following whole-mount in situ hybridization to *Zic1*- *5* (anterior is to the *left*). *Zic1* and *Zic4* are not expressed whereas *Zic2*, *Zic3*, and *Zic5* are all expressed in the ectoderm and mesoderm of the embryonic region and *Zic2* is uniquely expressed in the node of the embryo. *n* node

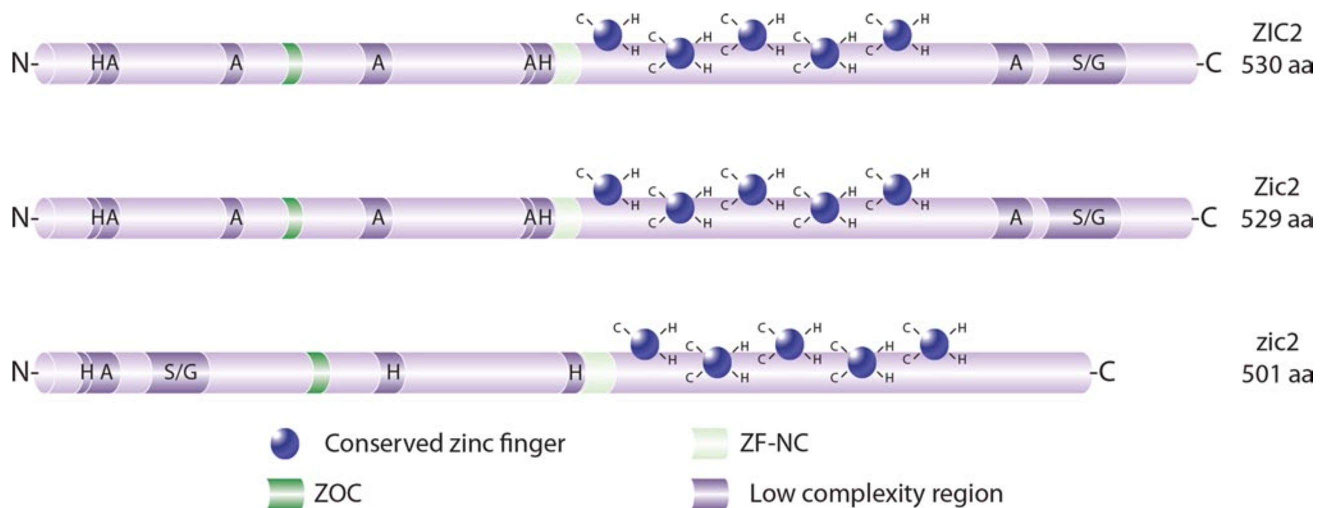

Fig. 5 The continued evolution of low-complexity regions in the vertebrate Zic genes. The ZIC2 protein from three species is depicted. The human (ZIC2) and mouse (Zic2) proteins are near identical with respect to the location, size, and amino acid involved in the depicted low-complexity regions. The *Xenopus* protein (zic2) differs in each of these respects; note in particular the absence of the C-terminal alanine and serine/glycine tracts. The major amino acid found at each low-complexity region is shown by the associated letter (*A* alanine, *H* histidine, *S/G* serine/glycine)

co-expressed (in the oocyte, the prospective neural tissue of the gastrula and subsequently at the neural plate border and in the developing retina), but uncoupled expression can also be found. For example, in the neurula, *zic2* expression is found along the axis, whereas *zic5* expression is restricted to the anterior neural ridge and *zic2*, but not *zic5*, is detected in the somites. Similarly, there are many sites at which both *zic1* and *zic4* are expressed (the branchial arches and the somites) but also sites at which *zic1* alone is detected (notably *zic4* expression initiates later than all other Zic genes being first detected in the prospective neuroectoderm of the mid-gastrula). The uncoupled *zic3* gene has elements in common with and unique from the bigene pair expression patterns. It seems possible that in *Xenopus* the bigenes do share regulatory elements but further studies are needed, for example to confirm whether some sites of *zic4* and *zic5* expression are currently undocumented.

In contrast to the situation in *Xenopus*, it seems clear from the murine expression studies that in the mouse the Zic genes can be divided into two synexpression groups with the *Zic1/4* bigene forming a distinct group from that of the *Zic2/5* bigene and the *Zic3* gene. Notably, this division mirrors the genomic arrangement rather than the structural subclass division of the Zic genes (Fig. 6). In general, during mouse development, the regions where *Zic1* and *4* are expressed are sites where all five Zic family members are transcribed. In contrast, there are several sites where only *Zic2*, *3*, and *5* are expressed (such as during gastrulation, in the presomitic mesoderm and developing limb bud). The pattern of expression therefore seems different from that in *Xenopus* where, for example, at least one member of the *zic1/4* bigene is expressed during gastrulation. This suggests that some Zic gene regulatory mechanisms have been significantly altered between *Xenopus* and the mouse, perhaps by the loss or gain of entire enhancers, rather than by the alteration of transcription binding sites within a conserved enhancer [25]. This raises the question of where the ZIC genes are expressed during human embryonic development. The strong correlation between loss-of-function phenotypes in mouse and man and the conserved genomic arrangement suggests that the expression patterns in human likely resemble those of the mouse.

Non-redundant and redundant function

In some cases, the inactivation of individual Zic genes can produce unique phenotypes (i.e., phenotypes not seen when other family members are alone mutated). Human and mouse genetics demonstrates that phenotypes in this category include *ZIC2*-associated classical HPE and *ZIC3*-associated heterotaxy indicating that the function of *ZIC2* and *ZIC3* in these processes is not fully redundant. This lack of redundancy may be caused a lack of co-expression. In this case, the unique phenotypes found upon individual Zic gene inactivation would reveal sites where only one Zic gene is expressed with the function of the missing member being fully compensated for (i.e., redundant) in areas of Zic gene co-expression. This idea is exemplified by the association between *ZIC2* mutation and classical HPE, which has been examined by analysis of the *Ku* mouse mutant. In this case, the first overt molecular defect found in $Zic2^{Ku/Ku}$ embryos is at the mid-gastrula node, a structure required

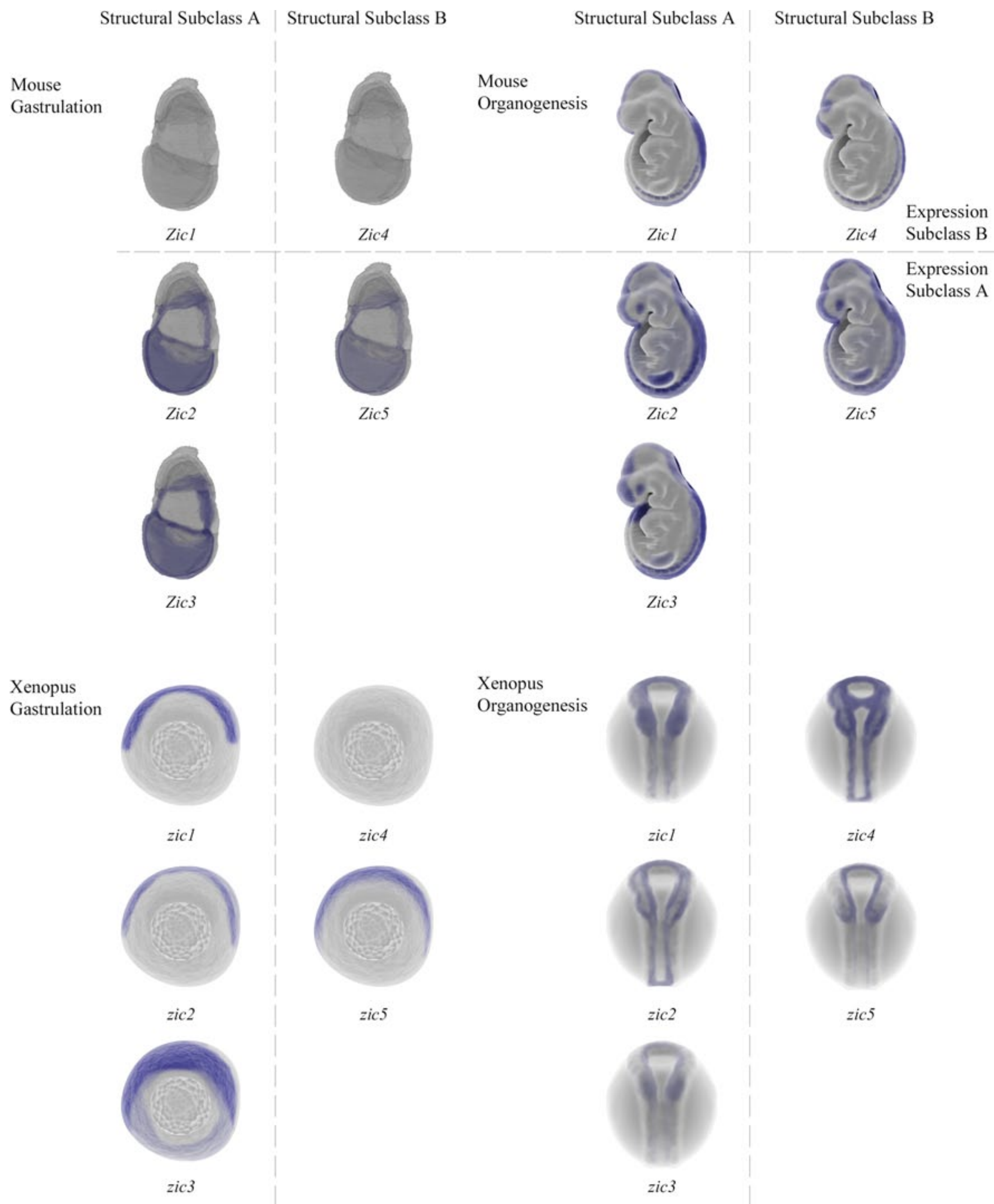

Fig. 6 The murine Zic gene expression subclasses reflect their genomic arrangement. The analysis of the mRNA expression patterns of the murine and *Xenopus* Zic genes reviewed here are depicted on images of mouse and *Xenopus* embryos at the stages shown. The data suggest that a new expression subclass has arisen during vertebrate evolution. All Zic genes are expressed in the ectoderm and mesoderm and tissues arising from these germ layers. In the mouse, two clear groups of gene expression patterns are seen; the Expression subclass A genes are expressed prior to and throughout gastrulation, during neurulation, and in organogenesis. The expression of the subclass B genes does not initiate until after neurulation and is absent from the optic cup, the forelimb bud and pre-somitic mesoderm at early organogenesis. In *Xenopus*, it seems most likely that there is only one synexpression group with the expression of all five Zic genes initiating prior to or during gastrulation and continuing in some developing central nervous system and mesoderm structures

for the development of the prechordal plate, the function of which is essential to prevent HPE [26]. This localized defect belies the widespread expression of *Zic2* transcripts at pre- and mid-gastrula stages, which a priori may be expected to cause widespread molecular defects. *Zic2* is, however, the only Zic gene expressed at the node with both *Zic5* and/or *Zic3* sharing other sites of *Zic2* expression at these developmental stages ([32, 33] and Fig. 4). It remains to be tested whether ablation of *Zic3* or *Zic5* on the *Ku* mutant background would reveal the additional defects expected if functional redundancy, coupled with a site of *Zic2*-specific expression, has produced an unusually mild defect in the *Zic2* mutant embryos.

In contrast to the HPE phenotype, the Zic gene murine mutational analysis suggests there are sites of full or partial Zic gene redundancy. Full functional redundancy is demonstrated when single mutants have no phenotype but the double mutant does, whereas partial redundancy is said to occur when one or both of the single mutants exhibits a phenotype that is enhanced in the double mutant. Care must be taken when performing and interpreting double mutant analyses since differences in genetic background and the use of hypomorphic alleles are both factors that can confound these experiments. Despite these caveats, there seems good evidence that the Zic genes do in some cases exhibit functional redundancy with one of the best characterized examples being the partial redundancy between *Zic1* and *Zic4* during cerebellum development [66, 67]. Here, null mutation of *Zic1* is associated with small size and abnormal foliation of the cerebellum. Although animals null for *Zic4* exhibit no detectable cerebellar phenotype the size and foliation defects are enhanced (relative to the *Zic1* null) in the double mutant. Moreover, *trans*-heterozygous loss of both *ZIC1* and *ZIC4* leads to cerebellar size and foliation defects in both man and mouse. This type of non-allelic non-complementation can occur when the two genes involved are both redundant and dose-dependent [90]. This combined haploinsufficiency implies that the amalgamated gene products fall below a threshold level necessary to achieve the wild-type phenotype and is consistent with a situation in which the expression or activity of the paralogs depends upon each other's expression. Here the null mutation in one or both of the genes causes decreased activity of the other leading to apparent haploinsufficiency.

The function of redundant family members can also be investigated by gain-of-function approaches, a strategy well suited to *Xenopus* embryos. All *Xenopus zic* family members are expressed at the neural plate border that forms the dorsal part of the neural tube and is the site of differentiation of the neural crest. The overexpression of each *zic* gene leads to the expansion of neural crest marker gene expression but not to any changes in expression of dorsal mesodermal markers. This highlights the ability of zic gene expression to induce anterior neural tissue and neural crest formation. In contrast with other *zic* genes, *zic4* and *zic5* were unable to induce the expression of forebrain and cement gland markers. It is interesting that this apparent functional difference mirrors the structural subclass divisions and suggests for example that the ability of *zic1-3* to induce anterior markers may be dependent upon the ZOC motif.

The Zic proteins are multi-functional

Several features of Zic gene genetics suggest that they are multi-functional proteins. Firstly, some Zic genes are required for multiple steps of the development of a particular organ as exemplified by the role of *Zic2* in dorsal–ventral forebrain patterning, by *Zic1* and *4* in cerebellar development and by *Zic2* and *3* in skeletal development. Although iterative function is a feature of many developmental proteins, the frequency of Zic gene re-use appears remarkable and is consistent with a multi-functional protein. Secondly, many transcription factors act in response to one particular signaling pathway, for example the Gli transcription factors are mediators of hedgehog signaling and the Lef/Tcf proteins are mediators of Wnt signals. In these cases, mutation of the transcription factors produces similar phenotypes to (or phenocopies) loss-of-function mutations in other pathway members such as receptors and ligands. One factor that has obscured the role of the Zic proteins is that they fail to phenocopy mutations in a particular signaling pathway and instead generally exhibit phenotypes reminiscent of a variety of signaling pathways. Additionally, Zic function is required for a myriad of biological processes and different signaling pathways are dominant in the respective biological processes. Zic gene genetics therefore suggest that the family members interact with many of the key embryonic signaling pathways and in doing so may serve to integrate and balance multiple signaling pathways. This idea is supported by biochemical experiments that demonstrate protein/protein interactions between Zic proteins and key transcriptional mediators of both the Shh and Wnt signaling pathways. These interactions appear to alter the way the pathway specific member can act, for example Zic proteins convert the Tcf transcription factors to repressors and Zic proteins can alter Gli3 repressor function [10, 23, 91].

As reviewed by Ali et al. [14] the molecular basis of Zic protein pleiotropy is beginning to be understood. The Zic genes have previously been shown to display phenotypes that result from the loss of DNA binding activity [16, 26], thus establishing that they can act as classical transcription factors. Consistent with this, the same consensus Zic DNA binding motif has now been identified by both a ChIP/Chip experiment and the UNIPROBE experiment [92, 93]. Biochemical experiments have shown that Zic proteins can also act as transcription co-factors to influence transcription of

target genes without contacting DNA. In particular, they can physically interact with TCF transcription factors to inhibit Wnt signaling [10, 94] and they can also regulate Gli transcriptional activity independent of DNA binding [95]. In addition to the established ability of Zic proteins to act as transcription factors and co-factors, the expansion of the Zic gene family provides the opportunity for homo or hetero dimerization and/or dominant negative interactions between the Zic proteins and these possibilities are yet to be fully explored.

Promoting pluripotency: a unifying Zic function?

Despite our inability to link the Zic proteins with one particular signaling pathway or even one class of protein function, can a consistent biological function be found for the Zic proteins? The reason behind many of the described Zic-associated phenotypes has not been fully investigated. One function that has been ascribed to the Zic genes multiple times is that of promoting pluripotency. Zic mutant end-phenotypes are often associated with small size (of the affected organ or of the entire animal). Where investigated, the reduced size has been attributed to the premature differentiation of a progenitor population. This has the effect of ultimately decreasing the size of the organ since the progenitor differentiation occurs at the cost of increasing the size of the progenitor pool via self-renewal. Investigations in murine ES cells have also ascribed the task of promoting pluripotency to Zic3 [96, 97] and it may be that functional redundancy has obscured this role during early embryogenesis in murine Zic mutants. Although a proportion of Zic-associated phenotypes can be explained by a failure of self-renewal, not all can be; for example, in the case of the *Zic2*-associated neural crest cell defect it is the number of differentiated cells that is depleted, not the progenitor pool. Clearly, the Zic family members are involved in differentiation as well as the control of pluripotency. Further work is needed to determine whether many of the Zic-associated phenotypes are caused by altered fate, renewal, or other cellular properties.

Conclusions

We conclude that the Zic genes encode proteins that truly are partially redundant, multi-functional molecules that operate in a context-specific manner to drive a large number of critical events during embryogenesis. Their ancestral role in the generation of neuroectoderm and mesoderm-derived tissues seems to have been largely conserved but specific details have been altered through evolution. Our comparison here has revealed differences in protein structure, genomic arrangement, and gene expression patterns between *Xenopus* and mouse. These differences may explain variation in the outcome of apparently equivalent loss-of-function and gain-of-function experiments between these two organisms. They can also be used to inform ongoing investigations regarding the molecular mechanism of Zic function.

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The templates in Fig. 6 were rendered from the EMAGE gene expression database (http://www.emouseatlas.org/emage/); EMA: 17 (TS11) and EMA:36 (TS15) [99].

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