Chapter 9
Zebrafish Zic Genes Mediate Developmental Signaling

Cecilia Lanny Winata and Vladimir Korzh

Abstract The introduction of genomics into the field of developmental biology led to a vast expansion of knowledge about developmental genes and signaling mechanisms they are involved in. Unlike mammals, the zebrafish features seven Zic genes. This provides an interesting insight into Zic gene evolution. In addition, an unprecedented bioimaging capability of semitransparent zebrafish embryos turns to be a crucial factor in medium- to large-scale analysis of the activity of potential regulatory elements. The Zic family of zinc finger proteins plays an important, relatively well-established, role in the regulation of stem cells and neural development and, in particular, during neural fate commitment and determination. At the same time, some Zic genes are expressed in mesodermal lineages, and their deficiency causes a number of developmental defects in axis formation, establishing body symmetry and cardiac morphogenesis. In stem cells, Zic genes are required to maintain pluripotency by binding to the proximal promoters of pluripotency genes (Oct4, Nanog, Sox2, etc.). During embryogenesis, the dynamic nature of Zic transcriptional regulation is manifested by the interaction of these factors with distal enhancers and other regulatory elements associated with the control of gene transcription and, in particular, with the Nodal and Wnt signaling pathways that play a role in establishing basic organization of the vertebrate body. Zic transcription factors may regulate development through acting alone as well as in combination with other transcription factors. This is achieved due to Zic binding to sites adjacent to the binding sites of other transcription factors, including Gli. This probably leads to the formation of multi-transcription factor complexes associated with enhancers.

Keywords Zebrafish · Enhancer · Promoter · Transcription · Stem cells · Gastrulation · Left-right asymmetry · Neurogenesis · Developmental signaling

C. L. Winata (✉)
International Institute of Molecular and Cell Biology, Warsaw, Poland

Max-Planck Institute for Heart and Lung Research, Bad Nauheim, Germany
e-mail: cwinata@iimcb.gov.pl

V. Korzh (✉)
International Institute of Molecular and Cell Biology, Warsaw, Poland
e-mail: vkorzh@iimcb.gov.pl

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9.1 Introduction

The patterning of the embryo is achieved through a process involving the determination of embryonic body axes and defining which cell types develop in each embryonic coordinate. The Zic family proteins are known to be involved in such process (Nagai et al. 1997; Aruga 2004). They are characterized by the presence of a C2H2-type Zn finger DNA-binding domain, which is highly similar to that of the Gli family proteins (Aruga et al. 1994). The ability of Zic proteins to activate transcription has been demonstrated through reporter assays (Kuo et al. 1998; Salero et al. 2001; Sakurada et al. 2005), as well as identification of its binding sites near promoters of putative target genes (Lim et al. 2010). Their physical interactions with Gli proteins, as well as their ability to bind Gli consensus motif (Mizugishi et al. 2001), suggested an interaction with the Hh signaling pathway. However, the range of such interactions with signaling mechanisms seems to be much broader. Genome-wide profiling of Zic3-binding sites in zebrafish provided preliminary insight into a rather complex regulatory network, including components of Nodal, Hh, Wnt, and Notch signaling pathways (Winata et al. 2013).

9.2 The Zebrafish Zic Family of Transcription Factors

The Zic family proteins are known for their involvement in multiple aspects of embryonic patterning (Aruga 2004; Ware et al. 2006; Merzdorf 2007). Their study dates back more than 20 years ago, when the first gene in the family, murine *Zic1*, expressed abundantly in the granule cells of the cerebellum, was cloned (Aruga et al. 1994). Comparisons of DNA sequences and gene structures of the Zic genes revealed their homology to the *odd-paired* gene of *Drosophila* known to specify the anterior-posterior identity of the embryonic body segments (Benedyk et al. 1994). Additional vertebrate *Zic* genes were subsequently identified and characterized (Nakata et al. 2000; Ishiguro et al. 2004) making a total of five in frog, chicken, and mammals. Two additional *zic* genes are present in zebrafish. *zic2b* arose from the teleost-specific gene duplication (Toyama et al. 2004), and *zic6* (Parinov et al. 2004; Keller and Chitnis 2007) represents a molecular evidence of existence in the evolution of the third pair of Zic genes (Zic3-6) similar to that of the Zic1-4 and Zic2-5 pairs. To date, no evidence exists of the presence of Zic2b in tetrapods, latimeria, and sharks, which suggests that it never evolved outside of the teleost lineage; on the other hand Zic6 remains only in teleosts. Given the fact that this book contains several reviews on Zic genes in other species, we will limit our analysis to the zebrafish Zic genes.

In all vertebrates *Zic* genes are located on opposite DNA strands as head-to-head pairs. For instance, the *zic1-zic4* pair is located in this configuration on zebrafish chromosome 24, the *zic2-zic5* pair on zebrafish chromosome 3, and the *zic3* paired with *zic6* on zebrafish chromosome 14 (Parinov et al. 2004) (Fig. 9.1). Such close

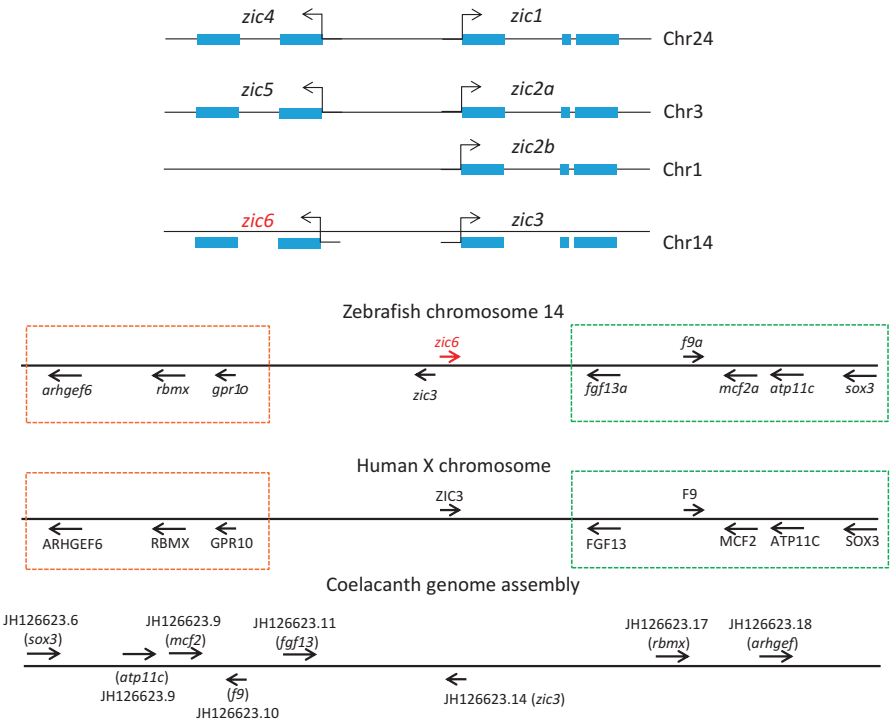

Fig. 9.1 The pairwise arrangement of *zic* genes in the zebrafish genome. The zebrafish genome contains seven *zic* genes, an additional two from that of higher vertebrates. These additions consist of *zic2b* located in chromosome 1 and *zic6* located in pair with *zic3* on chromosome 14. These two *zic* genes arose through chromosomal duplication in *Euteleostomi*, possibly excluding the *Coelacanthiformes*, and were subsequently lost in higher vertebrates. Nevertheless, the fragment of chromosome 14 containing *zic3* retains the syntenic relationship with that of the human X chromosome and the ancient *Coelacanth* (*Latimeria*) genome

proximity of pairs of Zic genes has been proposed to facilitate the sharing of regulatory regions. This idea is supported by the similarities in spatiotemporal expression patterns and somewhat overlapping functions between pairs of *zic* genes (Inoue et al. 2004; Ohtsuka et al. 2004; Merzdorf 2007; Nyholm et al. 2007). Members of the Zic family share many features of the characteristic expression pattern. In general, *zic* genes are expressed in the neural plate and later on in the dorsal neural tube. The role of *Zic* genes during neural development is conserved in all organisms that possess the nervous system (Lindgens et al. 2004), suggesting that these genes play an important role in the development and evolution of the nervous system. In particular, members of the Zic family positively regulate the proliferation of neural progenitors and negatively regulate the expression of proneural factors driving neural differentiation (Aruga 2004; Toyama et al. 2004; Nyholm et al. 2007). Based on the expression of a transgene in the ET33 transgenics that faithfully recapitulate the

expression of *zic3-zic6* pair, these genes may also play a role in the development of the brain meninges (Parinov et al. 2004; Kondrychyn et al. 2013; Chap. 12 of this book). Some of Zic genes are expressed in the mesoderm/mesendoderm, where they play an important role in the development of the body axis, etc.

Comparative analysis across different metazoan phyla revealed that *zic* genes probably evolved from an ancestral gene of the *gli/glis/nk*-like family that existed in the last common ancestor of the placozoans, cnidarians, and bilaterians. In these basal metazoans, *zic* genes are expressed in the endomesodermal tissues and highly neuralized developing tentacles, indicating that their function has likely been conserved since the early stages of metazoan evolution (Wada and Saiga 2002; Layden et al. 2010). Hence, a detailed study of regulation even of a single Zic gene could be rather informative in the context of regulation of all Zic genes.

9.3 Zic3 as a Transcription Factor

Application of next-generation sequencing (NGS) allows an unbiased genome-wide assessment of transcription factor (TF)-binding sites by ChIP-seq (Lim et al. 2010; Winata et al. 2013). This approach was applied to study the transcriptional activity of Zic3 in the developing zebrafish embryo at two different developmental stages. At 8 hpf Zic3 is expressed in the neural plate and mesoderm upon formation of germ layers (ectoderm, mesoderm, endoderm) and commencement of neural induction (Parinov et al. 2004). At 24 hpf Zic3 is expressed during neural differentiation in the dorsal neural tube (Winata et al. 2013). This analysis revealed that only a relatively small fraction of Zic3-binding events (8–9%) were associated with promoters. Most of these events were mapped to distant genomic locations. This is in line with the idea that Zic3, similar to other TFs, regulates gene activity through long-distance regulatory elements (Carroll et al. 2005; Wederell et al. 2008; Sanyal et al. 2012). Hence, the results of these studies led to the formulation of novel hypotheses regarding Zic3 function.

First, a difference in localization of Zic3-binding sites in stem cells and during embryogenesis possibly reflects changes in the role of this TF during different developmental periods. In stem cells that are in a relatively stable pluripotent state, Zic3 often acts as a general TF that binds to the core transcription machinery (Lim et al. 2010). This seems to be a common feature among TFs known to regulate ES cell pluripotency in mouse, such as Oct4, Stat3, and Klf4, all of which often bind sites within promoter regions (Chen et al. 2008). In contrast, during later development, when cells begin to differentiate, Zic3 binding to distal elements prevails. Such shift in site specificity of Zic3 suggests an acquisition of cellular functions specific for differentiating cells. A precise mechanism of this phenomenon remains unknown. At chromatin level it could be due to a decrease in the availability of binding sites in promoters or increase in the availability of distant binding sites. Both explanations suggest major epigenetic changes taking place during transition from a period of extensive cell proliferation to a period of cell fate determination and dif-

ferentiation. Epigenetic rearrangements in the form of changes in genome-wide histone methylation pattern on gene promoters have been well documented during the midblastula transition (Lindeman et al. 2010, 2011; Vastenhouw et al. 2010) and could thus support a model of TF-binding site accessibility. Equally important are changes at transcriptome level, which in principle could be both a cause and outcome of transcriptional regulation. A shift in Zic3 site specificity also correlates with a replacement of maternal transcripts by zygotic ones (Giraldez et al. 2006). Future studies could focus on investigating the relationship between these two events.

Second, a consensus-binding motif of Zic3 in zebrafish is highly similar to that found in mouse ES cells (Lim et al. 2010; Hong et al. unpublished). In sharp contrast, most of the surrounding regions appear to be poorly conserved in evolution. It is well documented that the evolution of divergent traits mostly involves modifications of regulatory elements rather than structural or functional changes in effector molecules, as the latter may impose dramatic changes in the gene regulatory network (GRN) controlling development (Carroll 2008; Wittkopp and Kalay 2012). In accordance with this idea, the binding sites of Zic3 diverge greatly, while their core structure and, possibly, their binding specificity remain largely conserved across metazoans (Layden et al. 2010; Winata et al. 2013).

Lastly, a large group of Zic3-binding sites fails to induce an expression of reporter. These sites could be nonfunctional or perform functions differently compared to regulatory elements that stimulate transcription. Analysis of such sites requires experimental output other than an increase in transient expression of reporter during embryogenesis in vivo used in this study. Possibly such sites could become functional at postembryonic stages or during adulthood. Zic3 may require interacting partners to induce transcription at these sites. This possibility is especially attractive since binding motifs of other TFs often are identified in proximity to Zic3 motifs of TF-binding sites, and enhancer studies have demonstrated that multiple TF-binding sites tend to co-localize with enhancers (Winata et al. 2013). Some of these enhancers form large regulatory regions up to 50 kb previously termed as “super enhancers” (Whyte et al. 2013). Co-binding of a particular TF with different partners has been shown to cause transcriptional outcome distinct from the one brought about by a single TF. The presence of other TF-binding sites nearby Zic3 peaks therefore suggests that Zic3 may act in multi-TF complexes. Among possible candidates for Zic3-binding partners are Gli proteins. These effectors of Hh signaling are structurally similar to Zic (Aruga et al. 1994). Gli-Zic physical interactions as well as Zic ability to bind Gli consensus motif (Mizugishi et al. 2001) suggested an interaction with the Hh signaling pathway. This is further supported by the fact that a deficiency of Zic2 has been linked to holoprosencephaly, the phenotype also connected to defects in Hh signaling (Brown et al. 1998; Sanek and Grinblat 2008). Finally, genome-wide analysis of Zic3-binding sites showed that almost half of all Zic3-binding sites contain both Zic3 and Gli motifs (Winata et al. 2013). This provided additional support for Zic-Gli interaction in the regulation of gene activity. Interestingly, the Hh signaling pathway is activated as a result of zygotic transcription, i.e., after a shift toward Zic3 regulation of enhancers. The

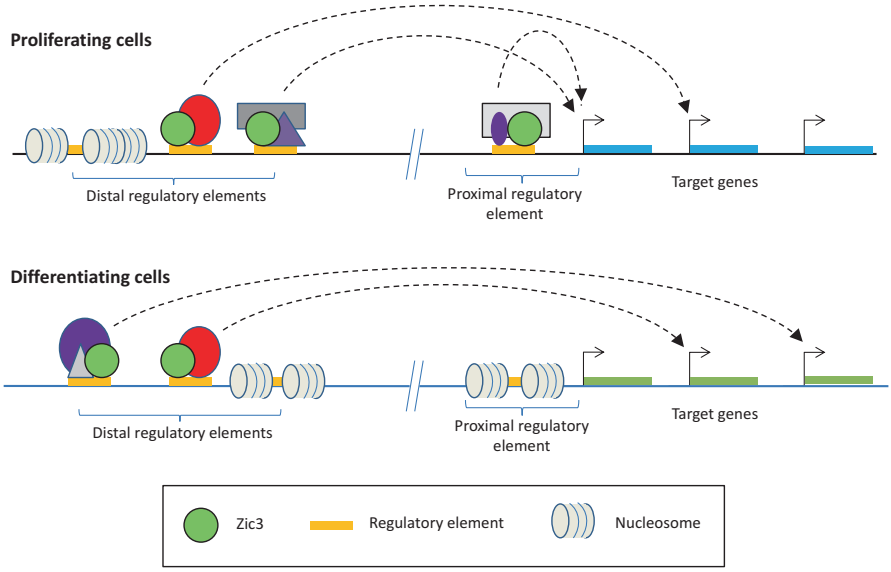

Fig. 9.2 Proposed model of Zic3 regulatory mechanism. In the developing embryo, Zic3 binds mainly to distal enhancer elements to regulate tissue-specific expression of target genes. The binding to different enhancer elements is regulated spatiotemporally through epigenetic mechanisms or recruitment by different transcription factors as binding partners

same could be true regarding other conserved binding sites detected in proximity of Zic3 motifs (Winata et al. 2013), which may become functional later on. At least for now, without detailed study of these potential interacting partners, it is difficult to determine the exact nature of their interaction with Zic3. Given the developmental shift from promoter-driven transcriptional regulation by Zic3 to enhancer-driven regulation and possible interaction with some other TFs, a mechanism involving Zic3-mediated transcriptional regulation in different spatiotemporal contexts could be illustrated as in Fig. 9.2.

9.4 Zic3 and Global Regulation of Development

The role of Zic3 in multiple, disparate aspects of development reflects its "mosaic pleiotropism" (Hadorn 1956; Carroll 2008). This property is exemplified by its involvement in the patterning of at least two different germ layers (ectoderm and mesoderm) and its role in activating different pathways at different developmental stages. The ability of TFs to perform multiple functions in different spatiotemporal contexts could be achieved through interactions with different partners which confer spatiotemporal specificity of its function (Carroll 2008). In the case of Zic3, the presence of this mechanism is supported by the co-localization of binding sites of

different TFs and Zic3, as well as evidence of possible physical interactions between Zic3 and Gli proteins. Within the wider context, comparative studies of metazoan evolution showed that the conserved Zic protein is repeatedly utilized in developmental processes. This is compatible with an idea of evolutionary "bricolage" (Wilkins 2007), which manifests itself as usage of an existing set of molecules during the evolution of new GRNs that eventually acquire novel developmental functions (Carroll 2008). The novel features generated from re-usage of a conserved TF often result in changes in the sequences of cis-regulatory elements (CREs) in the form of addition or deletion of a TF-binding site or modification of TF affinity or the strength of regulatory effects through changes in the number of binding sites (Carroll 2008). The case of Zic3 illustrates this principle – a majority of Zic3-binding sites are surrounded by poorly conserved regions, which may suggest distinct compositions of multiprotein complexes binding to the target CREs, resulting in evolutionary diversification. It is possible that an additional round of genome duplication in teleosts further contributed into relaxing selection pressure on CREs as it led to an even greater diversification of regulatory elements. This could be seen not only due to the genome-wide shift in a mode of Zic3 binding. It also correlates with a shift in Zic3 functionality, which is evident due to a difference in GO enrichment of associated genes during development. Importantly, the recognition motif of Zic3 involved in two developmental GRNs (8 and 24 hpf) remains the same, which highlights the importance of its pleiotropism.

In this context, it is worthwhile to mention *embryonic competence*, an actively acquired ability of developing cells to respond to an inductive signal. The developmental regulation of accessibility of regulatory regions, i.e., enhancers and promoters, in cells that respond to developmental signaling may be determined by the epigenetic state of chromatin, which reveals itself via embryonic competence. An analysis of developmental regulation of genetic activity by Zic3 revealed an important genome-wide switch from the regulation of the promoter-driven cellular functions during pluripotency state to the enhancer-driven regulation of functions associated with progressing development – cell migration, commitment and determination during gastrulation, as well as neural cell differentiation (Lim et al. 2010; Winata et al. 2013) (Fig. 9.3). Given the role of *Zic* genes in brain tumors (Aruga et al. 2010), it is easy to imagine that under pathological conditions such as dedifferentiation, a reversal from enhancer-driven regulation to promoter-driven general cellular activities such as cell proliferation may take place. When supported by experimental evidence, this emerging knowledge may help to formulate a novel paradigm of searching druggable targets.

9.5 Zic3 in Gastrulation and Left-Right (L-R) Patterning

Zic3 is distinguished from other Zic family members by its involvement in the L-R patterning (Aruga 2004). It was shown that Zic3 loss-of-function (LOF) causes laterality defects in *Xenopus* and zebrafish in support of the conserved role of Zic3 in

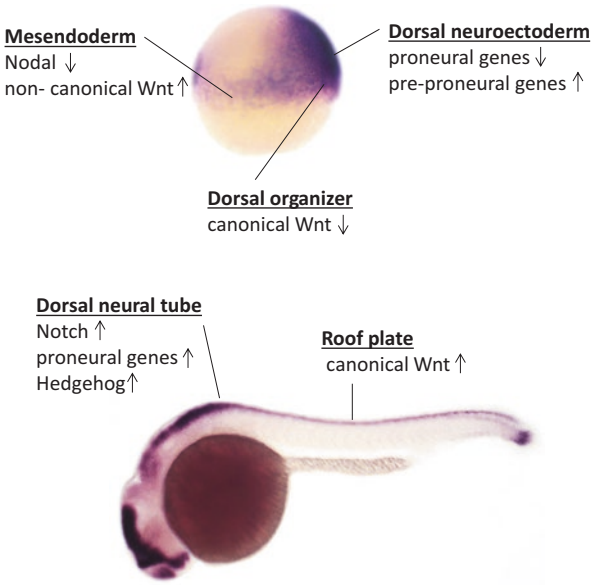

Fig. 9.3 Signaling pathways downstream of Zic3 in different spatiotemporal contexts in zebrafish development. Expression of *zic3* in 8 hpf and 24 hpf zebrafish embryo. Different signaling pathways within specific expression domains are subjected to regulation by Zic3. Spatiotemporal regulation of multiple developmental processes by Zic3 (right panel) is achieved by regulating distinct sets of target genes through binding different sets of regulatory elements at different developmental stages

regulating the L-R specification in vertebrates (Cast et al. 2012). Despite its role as a determinant of the L-R asymmetry, *Zic3* is not expressed unilaterally like some other determinants of this process (Nakata et al. 1997; Kitaguchi et al. 2000; Grinblat and Sive 2001). Moreover, organs, in which laterality is affected by Zic3 LOF, do not express *Zic3*. This raises a question as to how Zic3 confers the L-R patterning. Some studies suggested that an action of Zic3 in L-R asymmetry is an early developmental event, in which Zic3 regulates the formation of the dorsal organizer and therefore the midline structures (Fujimi et al. 2012), through its suppression of the canonical Wnt signaling (Winata et al. 2013). Defects of the midline structures are associated with aberrations in the L-R patterning (Danos and Yost 1996; Bisgrove et al. 2000). Furthermore, it was shown that Zic3 LOF defects in convergence extension (CE) correlate with subsequent defects in the L-R patterning (Cast et al. 2012). Therefore, Zic3 participates in establishing the L-R asymmetry by means of binary involvement as a regulator of the canonical Wnt signaling in the embryonic shield (organizer) and the noncanonical Wnt signaling during CE movements. ChIP-seq study in zebrafish suggested that this is indeed the case (Winata et al. 2013). Zic3 downstream targets include genes acting in the Nodal and canonical Wnt pathways that regulate early midline development. Zic3 also regulates genes directly implicated in the L-R patterning including members of the noncanonical Wnt (or planar cell polarity) signaling pathway, such as *dvl2*, *invs*, and *vangl2*, known to regulate ciliogenesis in zebrafish Kupffer's vesicle, which acts as an "organizer" (Morgan et al. 1998; Okada et al. 2005; Hashimoto et al. 2010; Wang et al. 2011). Of note is a differential tissue-specific activity of Zic3 in respect of canonical Wnt signaling, where, in the dorsal mesoderm, Zic3 inhibits Wnt

signaling and, in the neuroectoderm, it activates the same branch of this signaling pathway. This example serves as an illustration of the multiplicity of Zic3 developmental functions (Winata et al. 2013).

9.6 Zic Genes in Neural Development

Zic genes are some of the earliest TFs expressed in the neuroectoderm, where their expression precedes that of the proneural genes or starts at the same time as the expression of proneural genes (Nakata et al. 1997, 1998; Gamse and Sive 2001). In the zebrafish embryo, the earliest expression of Zic genes is represented by genes expressed during gastrula in the mesoderm – *zic2b* and *zic3* (Grinblat and Sive 2001; Toyama et al. 2004). Next, a domain of *zic3* expression appears in the posterior ectoderm. The expression of *zic1-zic4* is initiated at around mid-gastrula in the anterior neural plate (Elsen et al. 2008). By this time, *zic3* expression is higher in the posterior dorsal neuroectoderm in contrast to *zic1* and *zic2*, whose expression is higher anteriorly (Grinblat and Sive 2001). Later on, several *zic* genes are expressed in highly overlapping domains along the dorsal part of the neural tube and mesoderm – *zic1* is expressed throughout the brain, optic stalk, and dorsal somites (Rohr et al. 1999); *zic2* in the diencephalon, cerebellum, and optic stalk and weakly in the tail bud; *zic3* in the diencephalon, tectum, and optic stalk and strongly in the tail bud; and *zic5* and *zic6* at the beginning of neurulation (Toyama et al. 2004; Keller and Chitnis 2007; Kondrychyn et al. 2013). Hence, based upon expression patterns, the Zic genes of zebrafish seem to fall into at least three categories. The first one consists of genes that have only neural expression (*zic4*, *zic6*). The second is represented by their paired counterparts (*zic1*, *zic3*) expressed not only in neural tissues but also in the mesoderm and/or mesendoderm. In the third category, the Zic2a-Zic5 genes share expression in neural tissues, neural crest cells (NCC), and the apical ectodermal ridge (AER). Based on expression pattern, *zic2b* belongs to the same group of genes. Hence, it is possible to predict that the hypothetical counterpart of *zic2b* – *zic5b* – probably had similar expression, which could be one of the reasons for its elimination in evolution. The second prediction is based on a fact that since one of the genes with neural expression only (*zic6*) was lost in evolution, at some point another Zic gene with neural expression only (*zic4*) may follow the same fate.

Zic genes are negatively regulated by the BMP signaling and positively regulated by signaling emanating from the dorsal mesoderm. An inhibition of the ventralizing BMP activity induces expression of *zic1-3* in the dorsal neuroectoderm. This marks the earliest event in the determination of neural fate (Nakata et al. 1997; Grinblat and Sive 2001; Marchal et al. 2009). In the Bmp2b mutant *swirl*, *zic1* expression during late gastrulation expands significantly. In addition, expression in somites expands ventrally to encompass the whole somite. The opposite effect was observed upon Bmp gain-of-function (GOF) achieved by injection of *bmp2b* mRNA or upon decrease of dorsal mesoderm signaling in *chordino* mutant; both situations cause significant reduction of *zic1* expression (Rohr et al. 1999). Similarly, *zic3* expression

in the neural plate of dorsalized *swirl* (*bmp7*) and *snakehead* (*atp1a1a.1*) mutants expands into the ventral ectoderm. This supports the idea that BMP activity is necessary for restriction of *zic3* expression. Correspondingly, in the ventralized *chordino* mutant, *zic3* expression in the neuroectoderm was abolished (Grinblat and Sive 2001). The genome-wide analysis of Zic3-binding sites using ChIP-seq combined with gene expression profiling of Zic3 LOF demonstrated that Zic3 positively regulates genes essential to maintain neural progenitors during neuroectodermal specification (Winata et al. 2013). Some of these are direct targets of Zic3 (*dlx4b* and *msxe*), whereas others could be regulated indirectly (*msxc*, *irx1a*, *irx7*). Targets of Zic3 include also several Her genes implicated in the Notch signaling (*her4.2*, *her6*, *her9*). These genes are expressed in the neural plate, in which the marginal zone contains proliferating progenitors that contribute into dorsal neural tube and neural crest derivatives (Ekker et al. 1997; Thisse et al. 2001; Woda et al. 2003; Lecaudey et al. 2004; Thisse et al. 2004; Lecaudey et al. 2005). The identification of neural pre-pattern genes as downstream targets of Zic3, along with the repressive action of Zic3 on proneural genes (Winata et al. 2013), suggests that Zic3 acts to maintain a certain level of proliferation of neural progenitors. Thus, Zic3 establishes a particular number of neurons. It looks like Zic3 acts by maintaining an undifferentiated state of neural progenitors by positive regulation of repressors of the neural fate and, possibly, negatively regulating proneural genes. In support of this interpretation, Zic3 LOF caused an increase of neural differentiation markers, such as *neurog1* and *her9*. This indicates the repressive action of Zic3 on neural differentiation. Interestingly, binding sites of Zic3 were also found within 100 kb of *neurog1*, *neurod4*, and *ncam1a* promoters, which suggests that genes involved in neural differentiation could be direct targets of Zic3 also (Winata et al. 2013). Such mode of action is consistent with a model according to which Zic3 acts in parallel to Notch (Fig. 9.3).

9.6.1 Zic and Neural Crest

It has been demonstrated that several Zic proteins are expressed in and promote differentiation of the neural crest cells (NCC) (Teslaa et al. 2013). These cells originate from precursors located during gastrulation at the lateral edge of neural plate. As a result of neurulation, they converge at the dorsal neural tube together with precursors of the roof plate (see below). Subsequently, NCC undergo epithelial-to-mesenchymal transition (EMT), delaminate from the neural tube, and migrate out to differentiate into various cell types (Eisen and Weston 1993). Precursors of the NCC are specified through the combined action of Wnt, Fgf, and Bmp signaling. Wnt and Fgf signals along with Bmp antagonists are generated by the neural plate, while Bmp signals are generated by the adjacent epidermal ectoderm (Bang et al. 1999; Garcia-Castro et al. 2002). Taken together with the signaling activity of the dorsal mesoderm (i.e., Bmp antagonists), these signaling

pathways combined result in a dorso-ventral gradient of Bmp which increases ventrally and induces the expression of *zic* and *pax3/7* genes sufficient to induce NCC fate (Sato et al. 2005). In the zebrafish it was shown that *zic3* expression in the lateral neural plate depends upon Wnt signaling and an intermediate level of Bmp signaling. These signaling pathways seem to operate by regulating the two evolutionarily conserved enhancers located upstream and downstream of the *zic3-zic6* locus (Garnett et al. 2012). *Zic* genes are also involved in the subsequent migration and differentiation of the NCC. In zebrafish, *foxd3* and *pax3a* involved in NCC induction and migration (Bang et al. 1999; Stewart et al. 2006) were downregulated upon *zic3* knockdown. These genes are downstream targets of Zic3 (Winata et al. 2013), which acts as a positive transcriptional regulator. Although this result derives from analysis at 24 hpf, i.e., much later than when the NCC specification and migration from the dorsal neural tube take place (Raible et al. 1992; Kimmel et al. 1995), one needs to consider that *zic3* is constantly expressed in the NCC starting from gastrula. Its role in NCC migration can therefore be extrapolated based on this evidence.

9.6.2 Zic and Neurulation

Neurulation is a process in which the neural plate folds to form the neural tube. It involves apical constriction driven by the actin-based cytoskeletal microfilament network, which localizes at the subapical surface of the neuroepithelium to drive the folding movement (van Straaten et al. 2002; Sawyer et al. 2010; Korzh 2014). As a result of this movement, the neural folds are bent at a point known as the dorso-lateral hinge point (DLHP). This structure is crucial during neurulation as it determines the formation of the neural tube lumen and its shape and, eventually, the proper closure of the neural tube. It is also a landmark dividing the two lateral plates of the neural tube – the basal and alar plates. At the very beginning of neurulation, *zic2a* is expressed at the lateral edges of the neural plate giving rise to the future dorsal neural tube. As the neural plate folds to form the neural tube, *zic2a* and *zic5* expression continues to be dorsally restricted. Even prior to cavitation of the neural tube, the ventral border of the *zic2a* and *zic5* expression domain determines the position of the DLHP at the border of the dorsal alar plate and the ventral basal plate (Nyholm et al. 2007). The same two genes, Zic2a and Zic5, are required for the apical localization of F-actin and myosin II in the dorsal midbrain, particularly along the future luminal surface. The enrichment of myosin II, in turn, further ensures the structural integrity of the neuroepithelium. Interestingly, the canonical Wnt signaling is similarly required for the apical myosin II accumulation, further enhancing a link between Zic2a-Zic5 and Wnt signaling in the regulation of this process.

9.6.3 Zic and Roof Plate

Upon migration of the NCC out of a neural tube, the roof plate cells move dorsally to become the most dorsal cell lineage of the neural tube (Krispin et al. 2010a, b). Zic3 negatively regulates several proneural bHLH genes (*neurog1*, *neurod4*, and *her9*) (Winata et al. 2013). Probably this prevents differentiation of these cells as neurons and lets the roof plate cells to maintain their properties as signaling glia. In the zebrafish, Zic1, Zic3, and Zic4 control the expression of roof plate determinant *lmx1b* (Chizhikov and Millen 2004a, b; Elsen et al. 2008; Winata et al. 2013). In addition, Zic3-related Zic6 has been implicated in the regulation of cell adhesion in the dorsal neural tube during elongation of the roof plate. It is accepted that cell specification in the dorsal spinal cord depends mostly on Gli3-independent Wnt signaling. Hence, it comes as no surprise that several genes of the Wnt signaling pathway expressed in the dorsal neural tube are targets of Zic3 (Grinblat and Sive 2001; Fujimi et al. 2006; Winata et al. 2013). This in turn illustrates that at least Zic3 and, given Zic redundancy, other members of this family could act as dorsal developmental regulators alternative to Hh-independent Gli3. The developmental regulation mediated by Zic may play a role in a major morphogenetic rearrangement that prospective roof plate cells undergo between 24 hpf and 36 hpf. Being initially polarized along the medial-lateral axis, these cells rearrange polarity along the dorso-ventral axis, and a deficiency in the Zic genes affects this process (Kondrychyn et al. 2013). *lgl2* and *dlg2* are Zic3 targets expressed in the roof plate, where they regulate cell polarity at the level of cell adhesion (Bilder et al. 2003; Harris and Peifer 2004; Sonawane et al. 2005; Sabherwal et al. 2009; Sonawane et al. 2009; Winata et al. 2013). Hence, it is possible that Zic3 regulation of *lgl2* and *dlg2* plays an essential part in changes in cell adhesion necessary for reorientation of the prospective roof plate cells during stretching morphogenesis (Sonawane et al. 2009; Kondrychyn et al. 2013).

Subsequent stages of dorso-ventral patterning of the neural tube involve both Gli3-dependent and Gli3-independent mechanisms that mediate Wnt action in the intermediate and ventral neural tube. In the ventral neural tube, Wnts expressed in the floor plate contribute into the development of motor neurons (Ungar et al. 1995; Liu et al. 2000; Agalliu et al. 2009). The mechanisms by which the dorsal Wnts pattern the neural tube in a Gli3-dependent manner lack a few important details. It was proposed that Wnts acting in parallel with Bmps directly control the expression of homeodomain and basic helix-loop-helix (bHLH) TFs (Ulloa and Marti 2010). But in the absence of a mechanism for delivery of Wnts expressed dorsally into the ventral neural tube, this model remains incomplete. This is of particular importance since, unlike some other morphogens, the hydrophobic Wnts do not diffuse efficiently and act only at a short distance from Wnt-producing cells (Logan and Nusse 2004). In *Drosophila* the long-distance transport of the Wnt-related Wg is achieved by specialized cell extensions (cytonemes) (Roy et al. 2011) or transcytosis (Strigini and Cohen 2000; Marois et al. 2006; Gallet et al. 2008). Morphogens are known to be secreted by highly polarized cells such as the roof plate and floor plate. As a

matter of fact, the end feet of roof plate cells are tightly aligned with *glyt1*-positive stemlike cells adjacent to the primitive lumen/central canal prior to, during, and after stretching morphogenesis of the roof plate resulting in them spanning at least two-thirds of the diameter of the neural tube from cell bodies located in the dorsal neural tube. Such elongation of the roof plate may allow a long-distance transport of dorsal Wnts (Kondrychyn et al. 2013). Furthermore, it has been shown that the secreted Frizzled-related proteins enhance the diffusion of Wnt ligands to expand their signaling range (Mii and Taira 2009). Since Zic3 negatively regulates *sfrp1a* in the roof plate (Winata et al. 2013), this could be a mechanism to restrict a spread of Wnt signaling to a vicinity of a small apical footprint that the roof plate cells maintain at the dorsal aspect of the primitive lumen/central canal. Zic3 also negatively regulates the expression of ventral *wnt4b* [40], which could be a mechanism to restrict the effect of this ventral Wnt to the ventral-most aspect of the neural tube in close vicinity to the floor plate, where *wnt4b* is expressed (Liu et al. 2000). To summarize this part, it seems that the long-distance Wnt signaling could be regulated by the Zic3-based inbuilt transcriptionally regulated molecular system controlling Wnt signaling at multiple levels. It stimulates canonical Wnt signaling (by promoting *axin1*, *dvl2*, *lef1*) over noncanonical Wnt signaling (by blocking *vangl2* expression) as well as regulates expression of the Wnt receptors (Fzd8) and soluble Wnt-binding modulators (*sfrp1*) (Winata et al. 2013). In more general context, Zic expression is itself a subject of Wnt signaling regulation. Hence, Zic genes emerge as an important positive regulatory node enhancing Wnt signaling. Given a well-known role of Wnts as oncogenes and an activation of Zic expression in brain tumors (Chap. 16 of this book), the regulation of Zic genes and their targets in tumors should be explored further in search for anticancer therapy.

9.6.4 Zic and Midbrain

The regulation of Zic2a and Zic5 by the canonical Wnt signaling is well documented in the developing dorsal midbrain, which gives rise to the optic tectum. These two Zic genes are located in tandem with their transcription start sites separated by 4.6 kb of intergenic sequences. This suggests that they likely share regulatory elements. The expression of *zic5* does not initiate as early as that of *zic2a*. However, starting from late gastrula, the two genes are co-expressed at the future neural tube and retain very similar expression patterns in the dorsal brain. This sets another example, where a pair of Zic genes located in tandem could be transcriptionally co-regulated to some extent by the same *cis*- and *trans*-regulatory elements. Further analysis reveals that the *zic2a* and *zic5* locus contains transcriptional enhancers with the ability to drive expression in the midbrain. These enhancers are recognized by the canonical Wnt effectors, Tcf/Lef transcription factors, which activate the expression of *zic2a* and *zic5*. Although lacking any patterning defects, Zic2a and Zic5 knockdown resulted in a reduction of cell proliferation in the tectum

likely through the activation of *cyclinB1* transcription. Therefore, the canonical Wnt pathway activates *zic2a* and *zic5* expression during dorsal midbrain development to promote cell proliferation (Nyholm et al. 2007).

9.6.5 Zic and Hindbrain

In the hindbrain, a similar regulatory module operates downstream of Zic2a and Zic2b to regulate the patterning of the hindbrain motor neurons (Drummond et al. 2013). Here Zic2a and Zic2b act upstream of two enzymes acting in opposite manner. *aldh1a2* catalyzes the production of RA and activates the retinoic acid (RA) signaling (Niederreither et al. 2000). *cyp26a1* catalyzes the degradation of RA and blocks the RA signaling. Unlike *zic1*, two other Zic genes (*zic2a* and *zic2b*) are expressed early, starting from 5 hpf in the prospective anterior neural ectoderm. By 8 hpf, *zic1* expression became restricted to the anterior, while *zic2a* and *zic2b* exhibit a broader and more posterior expression pattern overlapping that of both *aldh1a2* and *cyp26a1*. Intriguingly, depletion of both Zic2 paralogs results in a reduction of the expression level and size of expression domains of both *aldh1a2* and *cyp26a1*. This reduction in expression occurs as early as at 7 hpf stage, i.e., during neuroectodermal specification. Nevertheless the overall effect is a reduction in RA signaling within the posterior hindbrain and spinal cord, which persists up to 26 hpf. Ultimately this results in the loss of vagal motor neurons. Interestingly, *zic3* is also expressed in a domain largely overlapping with that of *zic2a* and *zic2b*. However, in one set of Zic3 knockdown experiments, no obvious phenotype was noted, and authors did not pursue their analysis further (Drummond et al. 2013). This is seemingly at odds with our observation, according to which Zic3 morpholino caused prominent convergence extension and gastrulation defects (Winata et al. 2013). Moreover, Zic3-binding peaks were detected at 24 hpf upstream (−14 kb and −52 kb) of the *aldh1a2* locus, as well as upstream (−73 kb) of the locus encoding a *cyp26a1* paralog, *cyp26b1*. Hence, a combination of these two observations makes it likely that Zic3 may perform a role overlapping with those defined for Zic2a and Zic2b in hindbrain patterning. To resolve a difference in observations, this notion awaits more detailed analyses.

9.6.6 Zic and Disease

Dandy-Walker Syndrome (DWS) correlates with the reduction and displacement of the cerebellum and hydrocephalus of the fourth ventricle. Humans affected may have defects of motor development, hypotonia, and ataxia as well as mental retardation (http://omim.org/entry/220200; Chap. 13 of this book). This genetic defect was mapped to the Zic1-Zic4 pair of genes, and an analysis in animal models, including the zebrafish (Elsen et al. 2008), supported an idea that DWS could be due to

deficiency of this pair of genes. The expression patterns of *zic1* and *zic4* largely overlap, with *zic1* expression initiated at mid-gastrula and that of *zic4* initiated at late gastrula in the anterior neuroectoderm and at the lateral edges of the future neural plate. The expression of these two genes subsequently extends posteriorly to encompass the dorsal-most portion of the neural tube, including the roof plate at the level of the midbrain and hindbrain. Loss of Zic1-Zic4 function results in an improper opening of the hindbrain ventricle. As a result, the ventricle is fused from the level of rhombomere 2 toward the posterior. In addition, proliferation of progenitor cells of the dorsal hindbrain is reduced. The roof plate is disrupted or absent. This is particularly clear in the regions of fused ventricle. Zic1 and Zic4 positively regulate expression of genes known to play a role in the development of the roof plate – *lmx1b-1* and *lmx1b-2* and their downstream targets (*gdf6a*). Zic1-Zic4 deficiency also affects the dorsal aspect of *wnt1* expression at the rhombomere boundaries, but not its more dorsal expression, or that at the ventral aspect of rhombomere boundaries. Therefore, Zic1-Zic4 contribute in the development of some aspects of the roof plate and the dorsal aspect of the rhombomere boundaries. These processes seem to have some bearing on the canonical Wnt signaling. Importantly, disturbances of these processes are significant enough to cause defects in the development of the dorsal neural tube resulting in hydrocephalus.

In the hindbrain ventricular zone, Zic5 is involved in neurogenesis by promoting progenitor maintenance and proliferation and preventing commitment and cell-cycle exit resulting in the birth of a neuron. *zic5* expression is negatively controlled by miR-9, which promotes cell-cycle exit. This poises neural progenitors to be responsive to alternative signals, which promote commitment toward neural fate (Coolen et al. 2012). It remains unclear whether the expression of other zebrafish Zic genes is also controlled by miRNA, but given many similarities in Zic gene biology, this possibility may be not that far-fetched. This idea is supported by the fact that miR-564 negatively regulates the expression of Zic3 in human lung cancer (Yang et al. 2015). So it seems that Zic genes play various, albeit to some extent redundant, roles in neurulation and, in particular, the development of the dorsal neural tube.

Holoprosencephaly (HPE) is one of the prominent defects associated with the impairment of Zic function (Chap. 14 of this book). HPE is an outcome of the deficient axial mesoderm (notochord) and ventral neural midline tissues (floor plate) manifested in mammals by an incomplete separation of the bilateral hemispheres of the telencephalon and deficiency of ventral diencephalon including the eye fields (Fernandes and Hebert 2008). Mutations of Zic2 are known to cause HPE. This could be due to a role of Zic2 in directing events of early gastrulation prior to formation of the prechordal plate (Warr et al. 2008). In the zebrafish anterior neural tissue, Zic2a and Zic1 seem to have overlapping expression and function as their combined LOF results in the forebrain midline defect (Grinblat and Sive 2001). However, while the loss of Zic2a alone did not cause any midline defects, a single Zic1 knockdown strongly affects midline development and causes partial cyclopia. Unlike *zic2a*, the expression of *zic1* starts later during mid-gastrula, which excludes its

involvement in the formation of the prechordal plate. Zic1 LOF affects the optic stalk and diencephalon derivatives. The optic stalk cells convert into a more dorsal fate of pigmented cells, and optic vesicles ventralize as shown by the dorsally retracted darkly stained retinal pigmented epithelium (RPE). These phenotypes could be caused also by a decreased Nodal and Hedgehog (Hh) signaling, which similarly reduces the optic stalk markers, or by an increased RA signaling, which ventralizes the optic vesicle. It has been suggested that in regulating neural midline development, Zic1 acts upstream of these signaling pathways by controlling the Nodal signaling at the level of *cyc* expression, the Hedgehog signaling at the level of *shh* expression, and the RA signaling at the level of the RA-degrading enzyme *cyp26a1* expression (Abu-Abed et al. 2001). In support of these results, it was shown that Zic genes control Nodal signaling at multiple levels with Zic3 acting as the transcriptional inhibitor of Nodal co-receptor Oep (Winata et al. 2013).

Zic1 expression is under negative regulation of the Hedgehog (Hh) pathway. Shha GOF causes a reduction of *zic1* expression, resulting in the reduction of the anterior neuroectoderm expression domain and telencephalon later on and total absence of expression elsewhere (Rohr et al. 1999). The LOF experiments are more tedious due to inherent redundancy of genes encoding Hh ligands. As a result, changes in Zic expression in a single mutant of any of the Hh genes could be relatively mild. No wonder thus that *zic1* expression was unaffected in the *shha* mutant, which could be due to maintenance of an effect of Hh signaling by two other Hh-related genes (*shhb* and *ihhb*) expressed in dorsal mesodermal derivatives. Indeed, in mouse Shh mutants, where there is no such redundancy of Hh genes expressed in axial mesodermal derivatives, the midbrain expression of *zic1* decreased (Fogel et al. 2008). In this context, the analysis of zebrafish mutants affecting Hh receptors or mediators of Hh signaling could be more informative. Given that Zic3 acts as a negative regulator of Hh signaling at the level of transcription of *shha* and *shhb* (Winata et al. 2013), this may illustrate an autoregulatory feedback loop between Hh signaling and Zic proteins.

9.7 Conclusions

From the point of view of the evolution of Zic gene family in vertebrates, zebrafish having seven Zic genes is of particular interest. These genes could be segregated into categories that play roles in neural, mesodermal, and neural crest development with at least one gene in each pair combining roles in two germ layers. In many instances, these genes seem to perform redundant developmental roles. Analysis of Zic genes in zebrafish turned to be rather useful for rapid evaluation of functional and tissue-specific activity of regulatory elements driving the expression of these genes and identification of their transcriptional targets. Combining genomic studies (ChIP-seq) with bioimaging revealed developmental changes in the molecular regulatory mechanism involving the interaction of one of the Zic genes – Zic3 – with regulatory regions. This dynamic is reflected in the shift from a proximal

promoter-based regulation in proliferating cells to a distal enhancer-based regulation during the determination of cell fate. Such shift of Zic regulatory mode of action may involve epigenetic regulation at the chromatin level. In the near future, characterization of genetic and epigenetic factors behind the changes in spatiotemporal specificity of Zic transcriptional activity may provide more details about the molecular mechanism of the recruitment of different Zic proteins across developmental stages and cell lineages. These will contribute to the information necessary to understand the general mechanism of developmental regulation of pleiotropic transcription factors such as Zic.

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