Chapter 7
Amphibian *Zic* Genes

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Abstract Studies in *Xenopus laevis* have greatly contributed to understanding the roles that the Zic family of zinc finger transcription factors play as essential drivers of early development. Explant systems that are not readily available in other organisms give *Xenopus* embryos a unique place in these studies, facilitated by the recent sequencing of the *Xenopus laevis* genome. A number of upstream regulators of *zic* gene expression have been identified, such as inhibition of BMP signaling, as well as calcium, FGF, and canonical Wnt signaling. Screens using induced ectodermal explants have identified genes that are direct targets of Zic proteins during early neural development and neural crest specification. These direct targets include *Xfeb* (also called *glipr2*; hindbrain development), *aqp3b* (dorsal marginal zone in gastrula embryos and neural folds), *snail* family members (premigratory neural crest), genes that play roles in retinoic acid signaling, noncanonical Wnt signaling, and mesoderm development, in addition to a variety of genes some with and many without known roles during neural or neural crest development. Functional experiments in *Xenopus* embryos demonstrated the involvement of Zic family members in left-right determination, early neural patterning, formation of the midbrain-hindbrain boundary, and neural crest specification. The role of *zic* genes in cell proliferation vs. differentiation remains unclear, and the activities of Zic factors as inhibitors or activators of canonical Wnt signaling may be dependent on developmental context. Overall, *Xenopus* has contributed much to our understanding of how Zic transcriptional activities shape the development of the embryo and contribute to disease.

Keywords Zic genes · *Xenopus*

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7.1 Introduction

The Zic family of zinc finger proteins plays multiple roles during early development. In this chapter, we will examine how studies with *Xenopus laevis* embryos have contributed to our understanding of *zic* genes and their activities. Although complete gene knockout in early developmental stages of *Xenopus* is difficult, partly because maternal mRNAs can persist past MBT (Blum et al. 2015), gene expression levels can easily be altered in *Xenopus* embryos using morpholino oligonucleotides or injection of mRNAs. Further, *Xenopus* embryos readily lend themselves to physical manipulation. Therefore, studies in *Xenopus laevis* have contributed much to our understanding of the functional roles of *zic* genes during neural induction, early neural patterning, and formation of the neural crest. In addition, microarray screens have identified a number of direct targets of Zic proteins, prompting a number of new and ongoing studies. Due to years of study, a large body of knowledge has been amassed on *Xenopus* embryo development, gene regulation, and cell fate mapping, which helps put the roles of *zic* genes into context.

7.1.1 Experimental Approaches Unique to Xenopus

7.1.1.1 Ectodermal Explants (Animal Caps)

Ectodermal explants (animal caps) allow researchers to study gene expression in cells that are competent to respond to neural induction. At the same time, these explants allow the study of gene regulation free from the variety of inductive signals that characterize gastrulation and neural induction. For animal cap experiments, two-cell embryos are typically injected with mRNAs or other molecules into the animal hemisphere of both blastomeres. After maturing to late blastula (stage 9), ectodermal explants are harvested from the animal hemisphere of the embryos. The explants form characteristic balls, which can be aged to gastrula and neurula stages (using intact sibling embryos for staging), at which point they are processed in assays to determine gene expression (Sive et al. 2007). Ectodermal explants have been used extensively to identify gene regulatory relationships between *zic* and other genes, which can then be tested in whole embryos. In addition, ectodermal explants make *Xenopus* embryos uniquely suited to identify or confirm genes that are direct targets of transcription factors active during early development. A hormone-inducible transcription factor is constructed by fusing the glucocorticoid receptor domain (hGR) to the transcription factor, and mRNA for this inducible construct is injected into the embryos. The hGR domain forms a complex with endogenous HSP90, thus retaining the transcription factor in the cytoplasm (Kolm and Sive 1995; Mattioni et al. 1994). Treatment with the hormone dexamethasone allows the hGR-bound transcription factor to detach and enter the nucleus. In order to identify direct transcriptional targets, the hormone-inducible transcription factor is activated in the presence of protein synthesis inhibitors. More

detail is provided below in the description of two screens for direct targets of Zic1 (Cornish et al. 2009; Plouhinec et al. 2014).

7.1.1.2 Keller Explants

Keller open-faced explants are derived from the dorsal marginal zone of early gastrula embryos (Keller and Danilchik 1988). They comprise prospective mesoderm and ectoderm and allow powerful studies of the genes involved in regulating convergent extension movements (Keller et al. 1992). With regard to *zic* genes, this system is being used to study the role of *aqp3b*, a direct target of Zic1, in convergent extension (See and Merzdorf unpublished). Keller explants have also been used to study neural induction free from vertical signals, since the signals that pass from the mesoderm to the ectoderm portion of the explant are limited to planar signals. This system demonstrated that calcium transients are required for induction of *zic3* expression (Leclerc et al. 2003). With the identification of direct targets of Zic1 that play roles in noncanonical Wnt signaling (Cornish et al. 2009), Keller explants may help understand the roles that these genes play in convergent extension.

7.2 Zic Family Genes and Their Expression in Xenopus Embryos

7.2.1 Comparison of Zic *Genes in the Allotetraploid Genome of* Xenopus laevis

The genomes of both *Xenopus* species are nearly complete (Hellsten et al. 2010; Session et al. 2016). *Xenopus zic* genes show the same chromosomal arrangement as *Zic* genes in mouse and humans, with *zic* genes clustered on the same chromosome in a head-to-head orientation: *zic1* with *zic4*, *zic2* with *zic5*, and *zic3* on a different chromosome (Grinberg and Millen 2005; Aruga et al. 2006). In addition to the zinc finger (ZF) DNA-binding domain, the Zic-Opa (ZOC) and zinc finger-nucleocapsid (ZF-NC) domains (both N-terminal to the zinc fingers) are conserved between *Xenopus* and mammalian *zic* genes (ZOC is present only in *zic1-3*) (Houtmeyers et al. 2013).

Xenopus laevis and *Xenopus tropicalis* both have five *zic* genes, but due to the allotetraploid nature of *X. laevis*, its genome possesses two versions of each *zic* gene, one on either a longer or shorter chromosome. The *zic* genes are therefore named *zic.S* and *zic.L*. Table 7.1 shows the results of comparing nucleotide sequences of S and L *zic* gene-coding regions and the amino acid sequences of S and L Zic proteins. The S and L variants were also compared to the *X. tropicalis* versions of each Zic protein (Table 7.1) (Ricker et al. unpublished). The amino acid sequence identities indicate that the S and L versions of *X. laevis* Zic proteins are about

Table 7.1 Nucleotide and amino acid sequence identity between *Xenopus laevis* Zic.S and Zic.L versions and Zic proteins in *X. tropicalis*

Gene name	Sequence source	CDS nucleotide identity	Amino acid identity between S and L gene versions	Amino acid identity between S and L genes and *X. tropicalis* zics
zic1.S	NM_001090330.1	95%	98%:	98%
zic1.L	Sequence predicted from genome		7 aa substitutions (1 in ZF)	99%
zic2.S	NM_001085959.1	94%	96%:	96%
zic2.L	NM_001087724.1		11 aa substitutions (4 in ZF); 4 gaps in Zic2.S and 4 gaps in Zic2.L	95%
zic3.S	NM_001087619.1	96%	97%:	98%
zic3.L	Sequence predicted from genome		13 aa substitution (2 in ZF)	98%
zic4.S	Sequence predicted from genome	94%	92%:	93%
zic4.L	NM_001127780.1		33 aa substitutions (2 in ZF); 5 gaps in Zic4.S and 4 gaps in Zic4.L	93%
zic5.S	NM_001085657.1	95%	93%:	91%
zic5.L	Sequence predicted from genome		29 aa substitutions (4 in ZF); 3 gaps in Zic5.S and 4 gaps in Zic5.L	93%

The nucleotide sequences of the coding regions and the amino acid sequences of the *X. laevis zic* genes on the S and L chromosomes were compared using Blastn and Blastp, respectively, to determine their sequence identity. The number of amino acid differences is indicated and gaps comprise maximally three consecutive amino acids. The S and L versions in *X. laevis* are as different from each other as they are from the *X. tropicalis* versions of each *zic* gene

equally divergent from each other as they are from the Zic proteins in *X. tropicalis*. The differences include substitutions and small gaps spanning up to three consecutive amino acids. Outside of the coding region, in the 5′UTR and 3′UTR, the sequences are more divergent between the S and L versions. The untranslated regions of the *X. tropicalis zic* genes are significantly different from the UTRs of the *X. laevis zic* genes.

The presence of two versions for each *zic* gene in *X. laevis* allowed each gene to diverge and possibly even perform different functions. For example, the Zic1 direct target gene *aqp3b* is the L version of the X. *laevis aqp3* gene. It is expressed at gastrula and neurula stages, while *aqp3a*, the S version of the gene, is not expressed during early development (Cornish et al. 2009). In adult frogs, the tissues that express the two *aqp3* genes vary, although the composite of the expression patterns is similar to the overall expression pattern of the single *Aqp3* gene in mice (Cornish et al. 2009; King et al. 2004). Thus, the individual roles of the S and L copies of each *zic* gene may vary but, taken together, may perform similar functions as a single

copy in other species. Finally, while *X. tropicalis* is a useful model, to date it has not been used to study the roles of *zic* genes.

7.2.2 *Expression of* Zic *Genes in Xenopus Embryos*

The gene expression patterns of *zic* genes in *Xenopus* embryos overlap extensively (Fujimi et al. 2006, 2012), which is also the case for *zic* genes in other vertebrates, for example, mouse and chick embryos (Nagai et al. 1997; Furushima et al. 2000; Gaston-Massuet et al. 2005; McMahon and Merzdorf 2010). Despite the overlap, there are significant differences in the expression domains of *zic* genes.

7.2.2.1 Blastula Embryos

The *zic2* gene is the only maternally expressed *zic* gene in *Xenopus* embryos (Nakata et al. 1998). The expression of *zic1*, *zic3*, and *zic4* begins at stage 9, after midblastula transition, although the expression of *zic4* is initially very low (Fig. 7.1a). The *zic5* gene is not expressed in blastula embryos. In situ hybridization shows that in late blastula embryos (stage 9.5), *zic1*, *zic2*, and *zic3* are expressed in the dorsal marginal zone in both ectoderm and mesoderm (Fig. 7.1b). There does not appear to be significant expression of these *zic* genes in the roof of the blastocoel.

7.2.2.2 Gastrula Embryos

As gastrulation begins, the *zic1-3* genes are strongly expressed in the prospective neural ectoderm and moderately expressed in the mesoderm (stages 10.5 and 11; Fig. 7.1b). *zic4* expression is quite low, and *zic5* expression begins in late gastrula embryos (Figs 7.1a and 7.2). In late gastrula embryos (stage 11.5), *zic1*, *zic2*, *zic3*, and *zic5* are expressed to varying degrees in a broad region of the prospective neural ectoderm (Fig. 7.2), while *zic4* expression is extremely weak. These expression patterns are consistent with the significant roles that the *zic1-3* genes play during early stages of development and show that *zic* genes are among the earliest genes expressed in response to neural induction.

7.2.2.3 Neurula Embryos

During neurula stages, all five *zic* genes are expressed in the neural plate border. Only *zic2* and *zic3* are expressed within the neural plate. *zic3* is found in the midbrain-hindbrain region, and *zic2* is expressed at the midline of the neural plate (Fujimi et al. 2006) and in the progenitor cells located between the stripes of primary neurons (Brewster et al. 1998). At the neural plate border, *zic1-3* are strongly

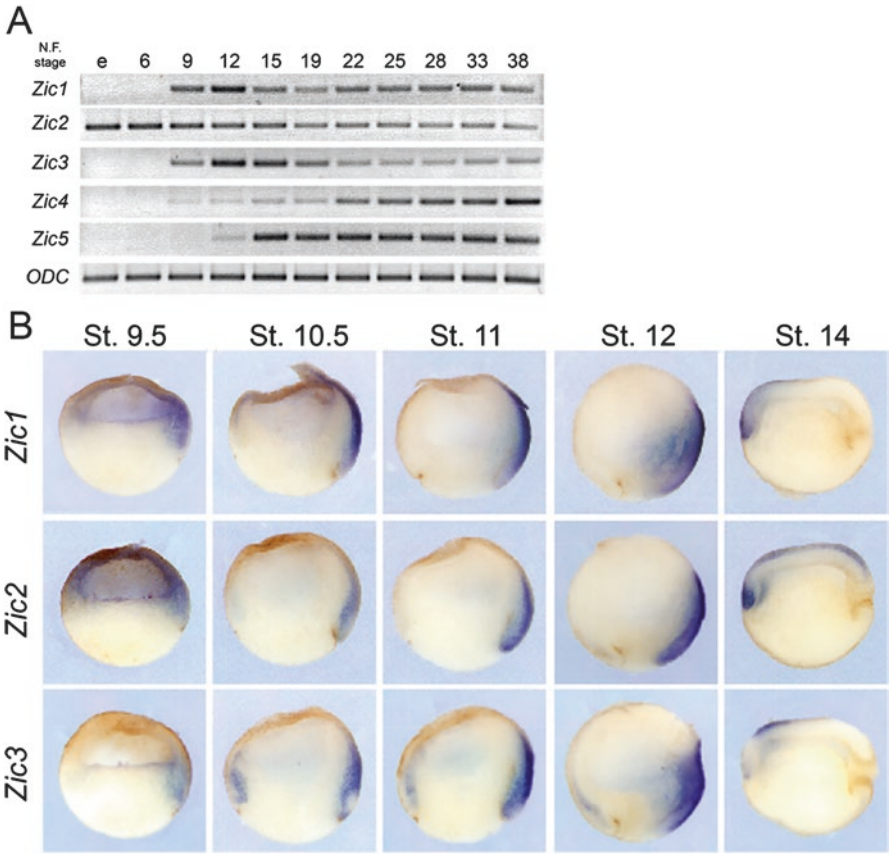

Fig. 7.1 Expression of *zic* genes in *Xenopus* embryos. (**a**) Expression of *zic1-5* determined by RT-PCR in unfertilized eggs (e) and *Xenopus* embryos at different developmental stages, including pre-MBT blastula (stage 6), post-MBT blastula (stage 9), late gastrula (stage 12), mid-neurula (stage 15), and tailbud stages (stage 19 and older). *zic2* is expressed both maternally and throughout early development. *zic1* and *zic3* are first detected at stage 9. The expression of *zic3* peaks in late gastrula/early neurula, while *zic1* expression remains strong. Weak expression of *zic4* is first detected at stage 9 and continues until tailbud (stage 22 and later stages), when it is more strongly expressed. Weak expression of *zic5* is detected by late gastrula (stage 12), and it is strongly expressed in neurula stages and beyond. (**b**) Expression of *zic1-3* by in situ hybridization in whole embryos. Dorsal is to the right. *zic2* mRNAs are more extensively present at stage 9.5, likely due to residual maternal mRNA. During late blastula (stage 9.5) and throughout gastrula (stages 10.5-12), the *zic1-3* genes are expressed in the dorsal ectoderm and in the involuting mesoderm. *zic3* is also expressed in the ventral and lateral involuting mesoderm (Fujimi et al. 2012; Kitaguchi et al. 2000). During neurula (stage 14) *zic1-3* are expressed in the neural plate and to some extent in the notochord (Reproduced from Fujimi et al. 2012 with permission of the publisher)

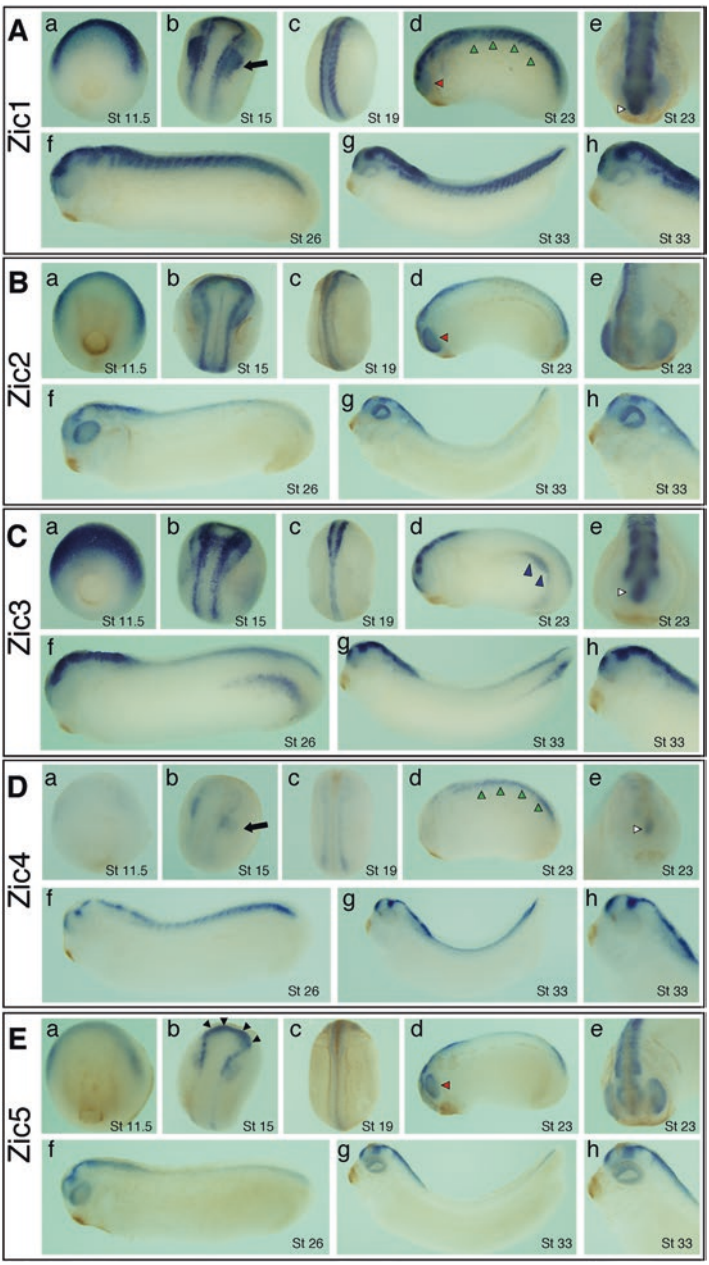

Fig. 7.2 Expression of *zic* genes in gastrula, neurula, and tailbud *Xenopus* embryos. The expression of the *zic1-5* genes was determined by in situ hybridization in whole embryos. During gastrulation (a: stage 11.5), all *zic* genes with the exception of *zic4* are expressed in the presumptive neural plate. During neurula (stages 15 and 19) and tailbud (stages 23 and later stages), all *zic* genes show expression in the dorsal neural tube. Other tissues also show *zic* gene expression, including the hyoid and branchial crest (black arrow), eye (red arrowheads), somites (green arrowheads), lateral mesoderm (blue arrowheads), and olfactory placode (white arrowheads) (Reproduced from Fujimi et al. 2006 with permission of the publisher)

expressed in wide regions, while *zic4* and *zic5* are more restricted to the regions of the neural folds (Fig. 7.2b). *zic1-3* and *zic5* are expressed strongly in anterior regions. After closure of the neural tube, all *zic* family members continue to be expressed in the dorsal neural tube (Fig. 7.2c). Thus, *zic* genes are expressed in areas required for neural patterning, neural crest specification, and neural tube closure.

7.2.2.4 Tailbud Stage Embryos

During tailbud and later stages, *zic1* is strongly and *zic4* is weakly expressed in the dorsal neural tube along the entire embryo (Fig. 7.2A, D d–g). *zic2*, *zic3*, and *zic5* are expressed more strongly in the anterior and posterior regions of the dorsal neural tube (Fig. 7.2B, C, E d–g). In tailbud stage embryos, *zic1*, *zic2*, and *zic5* show some expression in the region of the eye, both *zic1* and *zic4* are expressed in the somites (Nakata et al. 2000), and *zic3* is uniquely expressed in caudal lateral plate mesoderm (Fig. 7.2). Thus, *zic* gene expression patterns overlap extensively but also show unique aspects. Some of these correlate to known differences in *zic* gene function, although many of these differences in expression are not yet understood.

7.3 Upstream Regulators of *Zic* Gene Expression

Xenopus zic genes are expressed extensively during early development (Figs. 7.1 and 7.2), and a number of mechanisms are known to regulate *zic* gene expression.

7.3.1 Inhibition of BMP Signaling

The inhibition of bone morphogenetic protein (BMP) signaling is critical for neural induction (Sasai et al. 1996; Sasai and De Robertis 1997) and plays an early role in regulating *zic* gene expression. Signaling by BMP specifies ventral, non-neural fates and represses neural genes in *Xenopus* and other vertebrates. Thus, BMP signaling represses the expression of the *zic1*, *zic2*, and *zic3* genes (Gamse and Sive 2001; Nakata et al. 1997). Conversely, the inhibition of BMP signaling, often mediated by Noggin and Chordin, is essential for dorsal determination and specification of neural fate. Accordingly, misexpression of *noggin* (Mizuseki et al. 1998; Gamse and Sive 2001) or *FRL-1* (Yabe et al. 2003), which also represses BMP signaling, results in an increase in *zic* gene expression. Indeed, the promoter of *zic1* contains a 215 bp BMP inhibitory response module (BIRM) (−2.7 to −2.5 kb 5′ to the transcription start site). The BIRM is required for transcription of the *zic1* gene in the absence of BMP signaling in animal cap-based reporter assays (Tropepe et al. 2006). The BIRM contains consensus binding sites for several transcription factors, including

one Smad binding site and binding sites for the Ets, Oct, Lef/Tcf, and Sox transcription factors. Mutations in most of these putative transcription factor binding sites eliminate the ability of the BIRM to respond to Noggin, suggesting that multiple signals must cooperate to mediate *zic1* transcription in response to BMP inhibition. A dominant interfering Smad is able to induce expression of *zic1* in the absence of translation (Marchal et al. 2009), indicating direct regulation. However, mutation of the putative Smad binding site within the BIRM does not activate reporter gene expression (Tropepe et al. 2006). Thus, the inhibition of BMP signaling is required for *zic* gene expression, but which region of the *zic* gene directly responds to lack of BMP signaling, or exactly how the BIRM is responsive to suppression of BMP signaling remains to be answered.

7.3.2 Siamois and Twin

Organizer-specific transcription factors, such as Siamois and Twin, are responsible for the expression of BMP antagonists, including *noggin* and *chordin*. These BMP antagonists are secreted from the organizer (dorsal mesoderm) and block BMP in the neural ectoderm, which results in the upregulation of *zic* genes, as described above. Accordingly, *zic* genes are expressed in a wide domain in the neural ectoderm during gastrula stages (Fig. 7.2). Klein and Moody (2015) examined whether the expression of neural genes could be induced directly by organizer transcription factors, in addition to the indirect induction by BMP inhibitors. They found that in late blastula embryos, ectopic expression of the organizer genes *siamois* and *twin* induced ectopic *zic2* expression directly, in the absence of translation. Later in development, as gastrulation begins, *zic2* is present in the involuting dorsal mesoderm at moderate levels, with stronger expression in the neural ectoderm (Fujimi et al. 2012), while Siamois and Twin are limited to the dorsal mesoderm. This lack of overlap suggests that *zic2* expression in neural ectoderm is now regulated indirectly through induction of BMP inhibitors by Siamois and Twin. The significance for this bimodal regulation needs to be explored further. However, the direct induction of *zic2* by Siamois and Twin may serve to bias the dorsal region of the late blastula/early gastrula toward neural induction, and further studies demonstrated that maternal *zic2* is able to exert this bias as well (Gaur et al. 2016). This is supported by the finding that Zic1 is able to sensitize the future neural ectoderm for neural induction (Kuo et al. 1998).

7.3.3 Calcium Signaling

Calcium signaling helps mediate the activity of BMP inhibitors during neural induction. Noggin causes an increase in calcium transients in the prospective neural ectoderm, and experimentally increasing calcium demonstrated that it is a potent inducer of early neural genes and a repressor of epidermal genes (Moreau et al. 2008;

Leclerc et al. 2006). Thus, calcium signaling is required for neural ectoderm formation and is required for *zic3* gene expression. Blocking L-type calcium channels with specific antagonists in gastrula stage embryos and in Keller open-faced explants results in a reduction of *zic3* expression (Leclerc et al. 2000, 2003). The *xPRMT1b* gene, which codes for an arginine methyltransferase, is upregulated by Noggin in a calcium-dependent manner, and xPRMT1b can induce the expression of *zic3* (Batut et al. 2005). Thus, xPRMT1b appears to be a link between early calcium transients resulting from BMP inhibition and the expression of neural genes during neural induction, including *zic* genes.

7.3.4 FGF Signaling

FGF signaling is required for neural ectoderm formation in a variety of vertebrates (Patthey and Gunhaga 2014; Aruga and Mikoshiba 2011). In *Xenopus* embryos, FGF signaling in conjunction with Noggin activates *zic1* gene expression in ectodermal explants (Gamse and Sive 2001). In embryos with blocked BMP signaling, inhibition of FGF signaling only slightly reduced the induction of *zic1* expression but completely abolished the induction of *zic3* expression (Marchal et al. 2009). This suggests that FGF signaling increases the expression of *zic1* from the level established by BMP inhibition, while both FGF signaling and BMP inhibition are required for *zic3* gene expression. Further, *zic3*, but not *zic1*, is upregulated by FGF in the presence of cycloheximide, suggesting a direct mechanism for *zic3* expression. Conversely, *zic1*, but not *zic3*, expression is activated by Noggin in the presence of cycloheximide (Marchal et al. 2009). This suggests different regulatory mechanisms for the induction of these two *zic* genes, and it shows an important involvement for FGF signaling in their regulation.

7.3.5 Wnt Signaling

Wnt signaling contributes to early patterning of the neural ectoderm and promotes the expression of *zic* genes. During early anterior to posterior patterning in *Xenopus* embryos, *wnt* expression (in conjunction with Noggin) activates *zic1* expression in the posterior portion of the presumptive neural plate (Gamse and Sive 2001). Consistent with this finding, the BIRM regulatory element upstream of *zic1* contains a Lef/Tcf binding site. Mutation of this Lef/Tcf site eliminates the ability of the BIRM to respond to Noggin in reporter assays (Tropepe et al. 2006), supporting a requirement for Wnt signaling in *zic* gene regulation in posterior regions of *Xenopus* embryos.

7.3.6 *FoxD4 and Other Factors*

A regulator of *zic* gene expression in the early neural ectoderm is the forkhead transcription factor FoxD4 (also called FoxD5), which is expressed in tissue destined to become the neural ectoderm (Yan et al. 2009). During early *Xenopus* neural ectoderm formation, inhibiting *foxD4* expression causes a reduction of *zic2* expression but expands the expression domains of both *zic1* and *zic3*. Testing an activator construct of FoxD4 (FoxD4 fused to the VP16-activating domain) and a repressor construct of FoxD4 (FoxD4 fused to the EnR repressor domain) in whole embryos showed that FoxD4 acts as activator to induce *zic2* expression but as repressor to repress *zic1* and *zic3* expression (Yan et al. 2009). The acidic blob region in the N-terminal domain of FoxD proteins is required for induction activity, while interaction with a co-repressor at a site in the C-terminal domain is required for repressive activity (Pohl and Knochel 2005). Structure-function experiments indicate that the activating function of FoxD4 is a direct process, while its ability to inhibit genes requires intermediate factors. Further studies showed that the upregulation of *zic1* and *zic3* expression as a result of inhibiting *foxD4* expression can be rescued by *zic2* mRNA injections (Neilson et al. 2012). Thus, direct induction of *zic2* may contribute to the inhibition of *zic1* and *zic3* expression during the formation of the neural plate. Interestingly, this interaction is different in gastrula embryos, when FoxD4 has an activating effect on *zic1* and *zic3* expression (Yan et al. 2009). Thus, *zic* genes are regulated differently at different times during development, and individual *zic* genes are regulated by independent mechanisms.

Following the broad induction of *zic* gene expression by the inhibition of BMP signaling in conjunction with FGF and Wnt signaling, other factors help refine and limit the expression pattern of *zic* genes. A 5 kb region upstream from the transcription start site of *zic1* (a region containing the BIRM) encompasses additional binding elements that restrict *zic1* expression, since loss of this region caused an expansion of *zic1* expression (Tropepe et al. 2006). Candidate transcriptional repressors that limit the expression of *zic* genes are the Msx1 and Dlx1 transcription factors. Both are direct targets of intermediate levels of BMP signaling and are expressed in the epidermal-neural boundary region. Both repress *zic* gene expression in ectodermal explants, and Dlx1 was shown to repress *zic3* expression in *Xenopus* embryos (Tribulo et al. 2003; Feledy et al. 1999; Yamamoto et al. 2000; Monsoro-Burq et al. 2005). Thus, blocking BMP signaling creates a permissive environment for *zic* gene expression in the presumptive neural plate, while Dlx3 and Msx1 may prevent the expression of *zic* genes beyond the neural plate border region.

The TALE-family homeodomain proteins Pbx1 and Meis1 are important in early neural patterning, and their misexpression causes an increase in *zic3* expression (Maeda et al. 2001, 2002; Kelly et al. 2006). Further analysis showed that Pbx1 and Meis1 synergistically interact with a 3.1 kb region directly upstream of the *zic3* transcription start site (Kelly et al. 2006). In the anterior portion of the neural plate, the Six1, Six3, and Xrx1 transcription factors may promote expression of *zic* genes, since these transcription factors increase the transcription of *zic2* (Brugmann et al.

2004; Gestri et al. 2005; Andreazzoli et al. 2003). These transcription factors help refine *zic* gene expression patterns.

In summary, *zic* genes are expressed in the prospective neural ectoderm during gastrula stages and are among the first genes expressed in the early neural plate. Studies in *Xenopus* have greatly contributed to our understanding of the mechanisms that regulate *zic* gene expression. Inhibition of BMP, the resulting calcium transients, in conjunction with FGF and Wnt signaling are responsible for early zic gene expression. Nodal also counts among the upstream regulators of *zic* gene expression, which has mostly been explored in mouse (Houtmeyers et al. 2016). After initial induction of *zic* genes, their expression patterns are limited and refined by a number of other transcription factors and signaling mechanisms. Among these factors is expression of *shh* in the ventral neural tube, which represses *zic* gene transcription and therefore limits *zic* expression to the dorsal neural tube (Aruga et al. 2002). Overall, the mechanisms that are responsible for regulating *zic* genes individually at different times during development remain to be explored in greater detail.

7.4 Direct Transcriptional Targets of Zic Proteins

The DNA-binding domain of Zic transcription factors consists of five C_2H_2 zinc fingers. While the three-dimensional structure of this domain has not been determined for any of the Zic proteins, the significant similarity between the zinc fingers in Zic and Gli proteins allows the assumption that zinc fingers 2–5 interact with the major groove of the target gene, while zinc finger 1 engages in protein-protein interactions (Pavletich and Pabo 1993). Interestingly, Zic proteins have not been reported to act as homodimers (Brown et al. 2005).

7.4.1 Screens for Zic1 Direct Targets in Xenopus

Zic proteins are involved in the downstream regulation of a wide variety of genes. In *Xenopus*, two screens were conducted for direct target genes that are relevant during early neural development (Cornish et al. 2009) and during neural crest specification (Plouhinec et al. 2014). The unique ability to use ectodermal explants from *Xenopus* embryos makes the identification of direct targets more readily feasible than in other organisms. In these screens, an inducible *zic* construct (*zic1GR*) was used. Zic1GR is a fusion of Zic1 to the ligand-binding domain of the human glucocorticoid receptor, which renders Zic1GR inducible with dexamethasone (Kuo et al. 1998). In order to identify direct targets of Zic1, animal caps injected with *zic1GR* are aged to the desired stage and then first treated with cycloheximide to prevent protein synthesis, followed by treatment with dexamethasone to activate Zic1GR (Fig. 7.3). The animal caps are harvested and assayed for the transcription of new mRNAs, which are direct targets of Zic1. The Cornish et al. (2009) screen

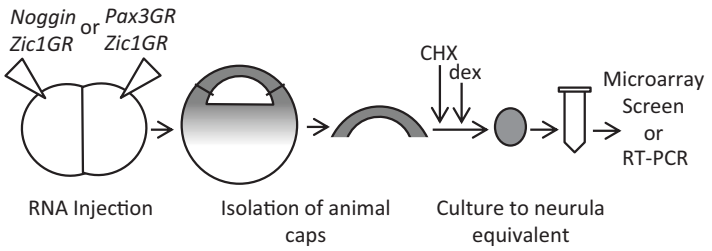

Fig. 7.3 Experimental design for microarray screens to identify direct transcriptional targets of Zic1. Embryos were injected at the two-cell stage into both cells with mRNAs for either *zic1GR/noggin* to induce early neural genes or *zic1GR/pax3GR* to induce neural crest genes. Control embryos were injected with mRNAs for *noggin* only or *pax3GR* only, respectively (Cornish et al. 2009; Plouhinec et al. 2014). Animal caps were dissected at stage 9. At the desired age, the isolated animal caps were treated first with cycloheximide (CHX) to prevent protein synthesis and later with dexamethasone (DEX) to induce the GR-conjugated transcription factors. The caps were then cultured to the correct stage and RNA isolated for microarray analysis and RT-PCR

aimed to identify early neural genes. Therefore, the animal caps were neuralized with a low dose of co-injected *noggin* mRNA. Plouhinec et al. (2014) set out to identify neural crest specifiers. Therefore, the animal caps were co-injected with hormone-inducible *zic1* and *pax3*. Both screens identified a number of genes, which are summarized in Table 7.2. Although both screens used a *zic1GR* construct to induce transcription of direct targets of Zic1, it is likely that the identified genes include direct targets of other Zic proteins, since the zinc finger domains of the Zic1-3 proteins are highly similar (Fujimi et al. 2006).

7.4.2 Direct Targets of Zic1 During Early Neurula Stages

A large number of genes were identified in the screen for direct targets of Zic1 during neural plate development (Cornish et al. 2009). The genes included in Table 7.2 are limited to direct targets that were confirmed by RT-PCR, and many were additionally shown to be regulated by Zic1 in whole embryos by in situ hybridization (Fig. 7.4). The screen was conducted at the equivalent of early neurula stages, and most of these genes are expressed in parts of the neural plate or in the neural plate border, overlapping with the expression patterns of Zic1 (Fig. 7.2).

7.4.2.1 Xfeb (Glipr2)

Among the direct target genes of Zic1, the putative metalloprotease Xfeb (Glipr2) was identified in both screens (Cornish et al. 2009; Plouhinec et al. 2014) and in an earlier spotted array (Li et al. 2006). It is expressed in the hindbrain and represses the expression of both the hindbrain gene *hoxB1* and the *otx2* gene, which is expressed

Table 7.2 Direct targets of Zic1 were identified in two screens

Accession number	Gene name	Confirmation by RT-qPCR	Second confirmation
NM_001095072.1	*Xfeb (glipr2)*[abc]	✓	*dnZic1*/in situ
NM_001094477.1	*aqp3b*[a]	✓	*dnZic1*/in situ
NM_001085780.1	*crabp2*[a]	✓	*dnZic1*/in situ
NM_001088044.1	*ptgds (cpl-1)*[a]	✓	*dnZic1*/in situ
NM_001088263.1	*ncoa3 (SRC-3)*[a]	✓	*ND*
NM_001088688.1	*prickle1*[a]	✓	*ND*
XM_018265023.1	*pkdcc2*[a]	✓	*dnZic1*/in situ
NM_001088196.1	*vegT*[a]	✓	*ND*
NM_001088341.1	*eomesodermin*[a]	✓	*ND*
NM_001085897.1	*myoD1*[a]	✓	*dnZic1*/in situ
NM_001085795.1	*hesx1 (Xanf2)*[a]	✓	*ND*
NM_001172199.1	*sall1*[a]	✓	*ND*
NM_001087226.1	*celf3*[a]	✓	*dnZic1*/in situ
XM_018244664.1	*Sp7 (osterix)*[a]	✓	*dnZic1*/in situ
NM_001088927.1	*lgals4*[a]	✓	*ND*
NM_001088044.1	*dgat2*[a]	✓	*dnZic1*/in situ
Xl.13309.1	*snail1*[b]	✓	*ZicMO1/RT-qPCR*
Xl.3818.1	*snail2*[b]	✓	*ZicMO1/RT-qPCR*
Xl.15393.1	*ets1*[b]	✓	*ZicMO1/RT-qPCR*
Xl.20029.1	*pdgfra*[b]	✓	*ZicMO1/RT-qPCR*
Xl.1946.1	*cyp26c1*[b]	✓	*ZicMO1/RT-qPCR*
Xl.5374.1	*dusp5*[b]	✓	*ZicMO1/RT-qPCR*
Xl.13925.1	*axin2*[b]	✓	*Unconfirmed by ZicMO1/ RT-qPCR*

Only direct targets that were verified by quantitative RT-qPCR are included. Several neural targets from the Cornish et al. (2009) screen ([a]) were additionally confirmed by injection of a dominant interfering *zic1* construct (*dnZic1*) into whole embryos and showed decreased target gene expression by in situ hybridization (see Fig. 7.4). Neural crest-specific targets from the Plouhinec et al. (2014) screen ([b]) were additionally confirmed by injection of *zic1MO* and *pax3MO*, which resulted in decreased expression of the target genes, as assayed by RT-qPCR of whole embryos. Li et al. (2006) found *Xfeb* (*glipr2*) in an earlier screen for direct targets of Zic1 ([c]). *Sp7* and *dgat2* were originally identified only by their unigene numbers Xl.8933 and Xl.25952. *pkdcc.2* corresponded to unigene number Xl.73297, which is updated here
ND not determined

anterior to the midbrain-hindbrain boundary (Li et al. 2006). This suggests that Xfeb contributes to patterning the neural plate and may be part of the regulatory mechanism that prevents expression of the *otx2* gene posterior to the midbrain-hindbrain boundary (Fig. 7.4). The identification of Xefb (Glipr2) in the Plouhinec et al. (2014) screen suggests that it also plays a role during neural crest specification. *Xfeb* and *gbx2* are both expressed in the hindbrain (Li et al. 2006; Rhinn and Brand 2001), and Gbx2 has neural crest specifier activity, which is dependent on the presence of Zic1

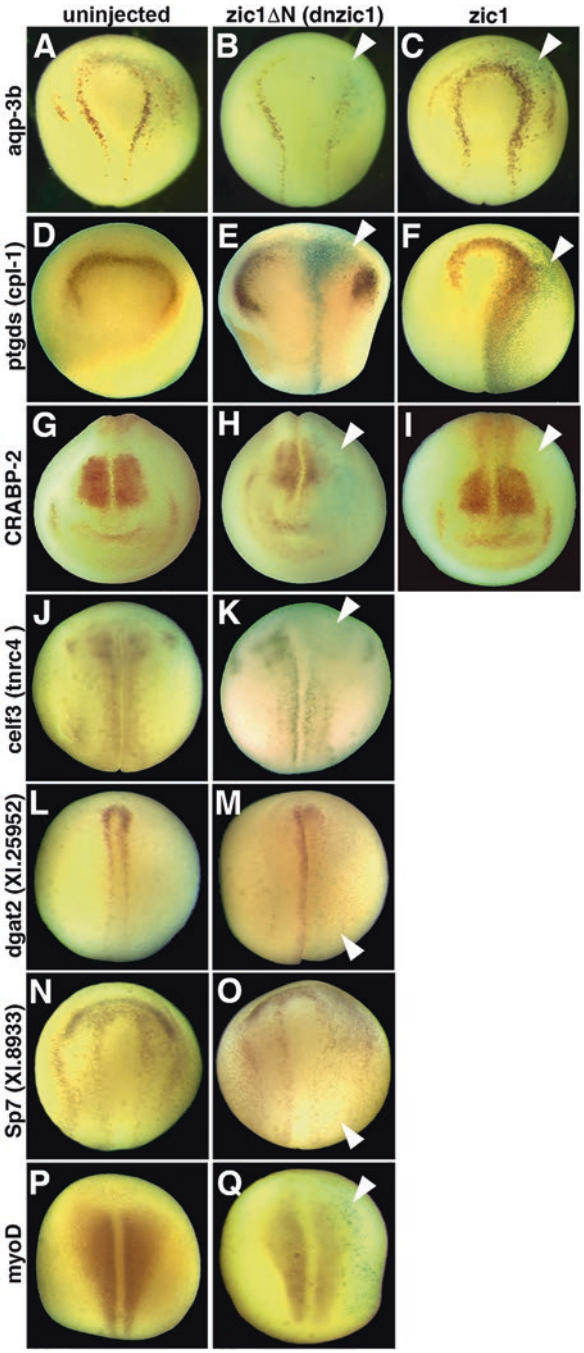

Fig. 7.4 Zic1 regulates the expression of direct target genes in neurula embryos (Cornish et al. 2009). Gene names are listed along the left with the original names or identifiers in parentheses. Shown are in situ hybridization expression patterns for neurula (stage 15–18) embryos that were uninjected (first column), injected with the dominant interfering construct *zic1ΔN* (*dnzic1*; second column), or injected with *zic1* mRNA (third column). Interfering with *zic1* activity reduced the expression levels of all direct target genes shown, indicating that Zic1 is required for their expression. Misexpressing *zic1* resulted in expansion of *aqp-3b*, *ptgds*, and *CRABP-2* expression. *Arrowheads* mark the injected sides (Reproduced from Cornish et al. 2009 with permission from publisher)

activity in ectodermal explants (Li et al. 2009). Thus, the induction of *Xfeb* by Zic1 may be required for the neural crest induction activity by Gbx2.

7.4.2.2 *aqp3b*

The *aqp3b* gene codes for an aquaporin, specifically an aquaglyceroporin. Aquaporins are channel proteins that allow passage of water and other small molecules (like glycerol) across cell membranes along their concentration gradients (Verkman 2005). In *Xenopus* neurula embryos, *aqp3b* is expressed in cells at the tips of the rising neural folds during neural tube closure (Fig. 7.4; Cornish et al. 2009). These cells, called "IS" cells (Schroeder 1970), separate the epidermal ectoderm and the neural ectoderm. During neurulation, the cells of the neural plate apically constrict, which allows the neural folds to rise and the neural tube to close (reviewed in Wallingford 2005). Compromising *aqp3b* expression in *Xenopus* embryos results in loss of apical constriction in neural plate cells and defective neural tube closure (Forecki and Merzdorf unpublished). Neural tube closure defects have been observed with mutations in human or mouse *zic2*, *zic3*, and *zic5* genes (Grinberg and Millen 2005). Thus, Aqp3b may be part of the mechanism that allows *zic* genes to control neural tube closure.

In gastrula embryos, aqp3b is expressed in the marginal zones and in the sensorial layer of the blastocoel roof (Forecki et al. 2018). Thus, *aqp3b* expression overlaps with *zic1-3* expression, which are expressed in the epithelial and sensorial layers of the dorsal marginal zone (Nakata et al. 1998; Fig. 7.1). Disrupting *aqp3b* expression in the dorsal marginal zone of whole embryos results in compromised border integrity between involuted mesendoderm and noninvoluted ectoderm and defective deposition of fibril fibronectin matrix at this boundary (Forecki et al. 2018). Further, inhibiting *aqp3b* expression in explants of the dorsal marginal zone region (Keller explants) interfered with their convergent extension, which was rescued with players in noncanonical Wnt signaling (See and Merzdorf unpublished). Although Zic proteins have not yet been examined for their roles in maintaining border integrities in gastrula embryos between involuted and noninvoluted cells, their expression patterns are consistent with this possibility. Further, involvement of Zic proteins in noncanonical Wnt signaling has not been demonstrated to date. However, identification in this screen of several genes that are involved in noncanonical Wnt signaling pathways suggests that Zic proteins may play such a role.

7.4.2.3 *pkdcc2* and Prickle Act in Noncanonical Wnt Signaling

The *pkdcc2* gene encodes a protein kinase, which regulates JNK-dependent Wnt/PCP signaling. It is important in both blastopore and neural tube closure (Vitorino et al. 2015). Prickle is a cytoplasmic protein that plays a key role in Wnt/PCP signaling as one of the six core components of Wnt/PCP signaling (reviewed in, e.g., Davey and Moens 2017). Accordingly, it is important for cell movements during

Xenopus gastrulation and neural tube closure (Takeuchi et al. 2003). Thus, these direct targets strongly suggest a new role for Zic proteins as regulators of noncanonical Wnt signaling.

7.4.2.4 *crabp2*, *ptgds*, *ncoa3*, and *cyp26cl* Are Genes Related to Retinoic Acid Signaling

The expression domains of the *crabp2* and *ptgds (*also called *cpl-1* or *lpgds)* genes overlap with the *zic1* expression domain (Fig. 7.4; Cornish et al. 2009). Both proteins function in regulating the cellular availability of retinoic acid during development. CRABP2 (cellular retinoic acid-binding protein 2) binds retinoic acid intracellularly and delivers it to the nucleus (Dong et al. 1999; Lepperdinger 2000). PTGDS acts dually as prostaglandin D2 synthase and as a lipocalin carrier for retinoic acid (Urade and Hayaishi 2000). Mutation analysis demonstrated that Zic1 acts only through the lipocalin function of PTGDS (Jaurena et al. 2015). The transcriptional coactivator Ncoa3 (also called SRC-3) activates the RAR/RXR nuclear receptor in response to retinoid binding in *Xenopus* (Kim et al. 1998). The direct target gene *cyp26cl* codes for a retinoic acid metabolizing enzyme, which is involved in anterior/posterior patterning of *Xenopus* embryos (Tanibe et al. 2008). Interestingly, in the pre-placodal ectoderm, Zic1 upregulates both the *cyp26cl* gene and the *raldh2* gene, which codes for a retinoic acid-synthesizing enzyme, although *raldh2* most likely is not a direct target of Zic1 (Jaurena et al. 2015). The authors hypothesize that retinoic acid synthesized by Raldh2 in *zic1*-expressing cells diffuses to and elicits signaling in surrounding cells, while the *zic1*-expressing cells themselves are not subject to signaling by the retinoic acid they produce due to the presence of Cyp26cl. Thus, a sharp boundary of retinoic acid-induced gene expression is created (Jaurena et al. 2015). Therefore, it appears that Zic1 regulates the expression of genes that control multiple aspects of retinoic acid signaling, which includes the synthesis and degradation of retinoic acid and aspects of its transport and availability.

7.4.2.5 VegT, Eomesodermin, and myoD Are Transcription Factors Important for Mesoderm Development

Eomesodermin acts very early in mesoderm development and regulates the expression of the t-box transcription factor VegT (Fukuda et al. 2010). VegT helps organize the paraxial mesoderm in *Xenopus* embryos (Fukuda et al. 2010). Experiments in chick embryos suggest that Zic1 may induce but not maintain *myoD* expression during somite development (Sun Rhodes and Merzdorf 2006). *zic* genes are known to play roles in mesoderm development, which have mostly been studied in other organisms.

7.4.2.6 Other direct targets of Zic1

Additional direct targets of Zic1 include *celf3, sall1, and hesx1,* which are associated with regulating gene expression in the developing nervous system. The *celf3* gene (also called *brunol1*) is broadly and strongly expressed in the neural plate border region. It codes for an RNA-binding protein with roles in regulating splicing events in the nucleus (Wu et al. 2010). The *sall1* transcription factor is expressed in the midbrain and in posterior regions of the neural plate (Hollemann et al. 1996). Sall1 is required for neural tube closure in mice (Böhm et al. 2008). The homeobox transcription factor Hesx1 is expressed in the anterior neural plate, where it promotes differentiation of the neural ectoderm and acts as a repressor of the *xbf-1*, *otx2*, and *pax6* genes (Ermakova et al. 1999).

Interestingly, no genes were identified in this screen, which are directly related to cell cycle control, and the gene most related to cell proliferation or cell differentiation is the *hesx1* gene, described above. Overall, the identified direct targets point to known and new activities for Zic transcription factors during early neural development.

7.4.3 Neural Crest-Specific Direct Targets of Zic1

The screen by Plouhinec et al. (2014) was a multi-step screen designed to limit identification of direct targets to only genes that act during neural crest specification. To this end, inducible *zic1GR* RNA was co-expressed with *pax3GR* RNA in animal caps (Fig. 7.3), and targets of Pax3GR alone were subtracted from the results. A variety of genes were identified, and those that were confirmed by an additional method are included in Table 7.2. Among these targets is the *Xfeb* gene (also called *glipr2*), which was identified in both screens and is discussed above.

The Plouhinec et al. (2014) screen identified the *snail1* and *snail2* (*slug*) genes as direct Zic1 targets, which are known to be expressed in the neural plate border region prior to neural crest migration. Snail1 has also been shown to induce s*nail2* and other neural crest markers, including *zic5* and *ets1* (Aybar et al. 2003). Further, there has been indication that Zic1 induces *snail2* acting as a repressor, indicating an indirect regulatory mechanism (a *zic1-EnR* construct activated *snail2* expression; Merzdorf unpublished). Thus, there may be more than one way in which Zic proteins can induce *snail2* expression.

Additional genes identified by Plouhinec et al. (2014) include the *ets1*, *dusp5*, and *pdgfra* genes. The gene for the Ets1 transcription factor is expressed in *Xenopus* premigratory neural crest cells destined to become cardiac tissues and has functions similar to Snail proteins (Nie and Bronner 2015). Dusp5 is a MAP kinase phosphatase and an important regulator of MAPK signaling (Caunt and Keyse 2013). MAPK signaling is essential for neural crest induction (Stuhlmiller and Garcia-Castro 2012a). Pdgfra is a receptor tyrosine kinase for PDGF. It is important for directed migration of cells in *Xenopus* gastrula embryos (Van Stry et al. 2005). During Wnt-induced cell proliferation of osteoblasts, Pdgfra is activated in a dishevelled-dependent manner (Caverzaiso et al. 2013).

The number of direct target genes for Zics identified in humans and mouse is relatively small. These targets include *ApoE*, *Math1*, *αCaM kinase II*, *dopamine receptor 1*, and *Pax3* (Salero et al. 2001; Yang et al. 2000; Ebert et al. 2003; Sakurada et al. 2005; Sanchez-Ferras et al. 2014). A ChIP-seq screen for direct targets of zebrafish Zic3 has yielded a large number of regulatory regions that drive a variety of genes involved in early development (Winata et al. 2013).

7.4.4 Interaction with Other Proteins

Zic proteins are transcription factors that bind DNA using C_2H_2 zinc finger domains, as stated earlier. There is some evidence that, like most transcription factors, their activity is regulated by interacting proteins. *Xenopus* Gli proteins, which are also C_2H_2 zinc finger transcription factors, interact with Zic proteins. Zic1, Zic2, and Zic3 and the Gli1, Gli2, and Gli3 proteins interact physically (through zinc fingers 3–5 of both Zic and Gli proteins) (Koyabu et al. 2001). In these Zic/Gli heterodimers, zinc fingers 3–5 would be occupied by binding to each other, thus preventing DNA binding by either protein. Therefore, in cases of co-expression, Zic and Gli proteins may regulate each other's activity as transcription factors. Indeed, in *Xenopus* embryos and in cell culture reporter assays, Zic and Gli proteins are able to reduce each others' activities as transcriptional activators (Brewster et al. 1998; Koyabu et al. 2001; Mizugishi et al. 2001). Zic2 has also been shown to interact with TCF1 and, via its zinc fingers, with TCF4, thereby interfering with Wnt/β-catenin signaling (Fujimi et al. 2012; Pourebrahim et al. 2011). In other organisms, there are not many proteins known to interact with Zic proteins. A yeast two-hybrid screen identified Imfa as a direct binding partner of Zic1, Zic2, and Zic3 in mouse (Mizugishi et al. 2004). In order to understand the activities of Zic factors, it will be important to learn more about proteins that modulate Zic activity by direct protein-protein interactions.

7.5 Biological Roles of Zic Transcription Factors

The *Xenopus* model lends itself to functional studies of genes. Loss and gain of function experiments combine to illustrate the activities of Zic transcription factors during embryonic development, particularly during gastrulation and early neural development.

7.5.1 Role of Maternally Expressed Zic2

Among the *Xenopus zic* genes, only *zic2* maternally expressed (Nakata et al. 1998). The role of maternally expressed *zic2* was studied using the host transfer method, where maternal *zic2* mRNA was depleted in oocytes that were then transferred back into *Xenopus* females for ovulation (Houston and Wylie 2005).

After fertilization and during development, this depletion resulted in exogastrulation, anterior truncations, thickened notochord, and axial abnormalities due to an overall increase in Nodal signaling (Houston and Wylie 2005). Similarly, double *zic2/zic3* morphants had a shortened body axis, smaller heads, and thicker, wider notochords (Fujimi et al. 2012). Thus, maternal expression of *zic2* is essential for early patterning of the embryo.

7.5.2 Zic *Genes During Gastrulation and Early Patterning of* Xenopus *Embryos*

Zygotic expression of the *zic1-4* genes begins shortly after midblastula transition, and all five *zic* genes are expressed during gastrulation, most strongly in the area of the presumptive neural plate (Fig. 7.1). As described above, the expression of *zic* genes appears to bias the ectoderm toward a neural fate in early embryos, since expression of *zic1* in animal cap ectoderm (from late blastula embryos) amplifies the neural inducing effects of Noggin (Kuo et al. 1998). In addition, maternal *zic2* and early zygotically expressed *zic2*, which is induced by the organizer transcription factors Siamois and Twin, also bias the presumptive ectoderm toward neural fate (Klein and Moody 2015; Gaur et al. 2016). The mechanism by which early *zic* gene expression is able to confer this predisposition for neural fate on the future neural ectoderm prior to gastrulation is currently not understood.

Zic3-null mice and *Xenopus zic3* morphants exhibit left-right (L-R) asymmetry defects (Purandare et al. 2002; Ware et al. 2006a; Cast et al. 2012). Of the *Xenopus zic* genes, *zic3* is most widely expressed in gastrula embryos (Fig. 7.2), and it is the only one among the *zic* genes that is involved in L-R asymmetry establishment. There are two prevailing models for the establishment of left-right (L-R) asymmetry in *Xenopus*. Evidence indicates asymmetry establishment either during early cleavage stages via ion flux or during gastrulation by cilia-driven flow (Blum et al. 2014). The result of breaking the symmetry by either mechanism is the asymmetric expression of the TGFβ-type growth factor *nodal* on the left side of the embryo. The *zic3* gene is a direct target of Nodal signaling, most likely via the activin response element found in the first intron of *zic3* (Weber and Sokol 2003). Zic3 then transmits this signal to downstream factors that determine left-sidedness (Kitaguchi et al. 2000). Zic3 has also been shown to regulate *nodal* expression in mice (Ware et al. 2006b). Thus, Zic3 may act upstream and downstream of the Nodal signaling that is required for L-R asymmetry formation.

7.5.3 *Zic Proteins and Wnt Signaling*

Zic proteins interact with canonical Wnt signaling, although the effects of these interactions appear to be dependent on *Xenopus* developmental stage. In late blastula embryos (stage 9.5), misexpression of *zic3* reduces the expression of the direct Wnt/β-catenin targets g*oosecoid* and *siamois*, which are genes expressed in the organizer, resulting in impaired notochord development. Zic3 is hypothesized to act as an early tuner of Wnt/β-catenin signaling in organizer mesoderm, where it is expressed at moderate levels (Fig. 7.1). It is likely that several Zic family members are able to affect Wnt/β-catenin signaling, since all five *zic* genes are able to reduce Wnt/β-catenin transcriptional activity in a luciferase reporter assay in *Xenopus* gastrula embryos and Zic3 was shown to physically interact with TCF1 (Fuijmi et al. 2012). Similarly, Zic2 binds directly to TCF4 and inhibits the ability of the β-catenin/TCF4 complex to activate transcription, thereby reducing the ability of β-catenin to induce Wnt targets in *Xenopus* animal caps (Pourebrahim et al. 2011). Further, the direct Zic1 targets Sp7 (also called Osterix) and Hesx1 (Cornish et al. 2009), both transcription factors, repress Wnt/β-catenin activity, and Hesx1 is expressed during late gastrula stage in the neural ectoderm (Andoniadou et al. 2011; Ermakova et al. 1999). These lines of evidence suggest that during gastrula stages and neural induction, Zic proteins inhibit canonical Wnt signaling.

Later in development, as Zic proteins contribute to patterning the neural plate, the effect of Zic1 on Wnt activity shifts. In neurula embryos, Zic1 acts as an activator of *wnt8b* expression, and it is able to activate *wnt1* and *wnt4* expression in neuralized animal caps. Further, Zic1 requires Wnt signaling to induce expression of the *engrailed-2* gene in ectodermal explants (Merzdorf and Sive 2006), indicating a role for Zic1 in promoting canonical Wnt signaling. Finally, the direct Zic1 targets *pkdcc2* and *prickle* (Cornish et al. 2009) suggest an unexplored role for Zic proteins in noncanonical Wnt signaling.

7.5.4 Zic *Genes During Patterning of the Neural Plate*

During *Xenopus* neurula stages, all five *zic* genes are expressed in overlapping yet distinct domains in the lateral neural plate and in the dorsal region of the closed neural tube (Fig. 7.2). Misexpression of each member of the *Xenopus zic* family expands the neural plate (*zic1*: Kuo et al. 1998; Mizuseki et al. 1998; Nakata et al. 1998), (*zic2*: Brewster et al. 1998; Nakata et al. 1998), (*zic3*: Nakata et al. 1997), (*zic4*: Fujimi et al. 2006), (*zic5*: Nakata et al. 2000). *zic* genes are expressed in relatively broad domains, as are other factors that pattern the neural plate. Combinations of these transcription factors, together with secreted factors, activate the expression of genes that are expressed in more limited domains. These include the *wnt* genes mentioned above (Merzdorf and Sive 2006): *wnt1*, which is expressed at the midbrain-hindbrain boundary, and *wnt4* and *wnt8b*, which are expressed at the forebrain/midbrain boundary and in the midbrain. Additional

genes induced by the expression of *zic1* include the dorsal neural marker *pax3*, the hindbrain markers *krox20*, *hoxD1* (Kuo et al. 1998), and *Xfeb (glipr2)* (Li et al. 2006). All *zic* genes induce the midbrain-hindbrain boundary marker *en-2* (Nakata et al. 1997, 1998, 2000; Kuo et al. 1998; Fujimi et al. 2006). *zic1-3* induce the forebrain and midbrain marker *otx2* and the cement gland markers *XAG-1* or *XCG*, while *zic4* and *zic5* are not able to induce these anterior genes (Kuo et al. 1998; Fujimi et al. 2006; Nakata et al. 2000). None of the *zic* genes are able to induce the posterior gene *hoxB9*. Most of these results were obtained in animal cap explants, although the regulation of the *pax3*, *en-2*, *wnt8b*, and *krox20* genes was confirmed in whole embryos (Kuo et al. 1998; Merzdorf and Sive 2006; Gutkovich et al. 2010). Thus, Zic proteins regulate genes in the neural plate regions that give rise to the brain but so far do not appear to be involved in regulation of genes important for spinal cord development.

The *zic1* gene is likely to play a role in the development of the midbrain-hindbrain boundary (MHB). Zic1 is required for expression of the MHB genes *en-2* and *wnt1*. Since Wnt signaling is required for activation of *en-2* expression by Zic1, Zic1 most likely induces *wnt1* transcription, which in turn induces expression of the *en*-2 gene (Merzdorf and Sive 2006). Zic1 may also help maintain the MHB through its direct target gene *Xfeb (glipr2)*. The *Xfeb* gene codes for a putative protease, which represses *otx2* expression (Li et al. 2006). *Xfeb* is expressed in the hindbrain up to the MHB. The transcription factors Otx2 and Gbx2 maintain the MHB by mutual repression (Rhinn and Brand 2001). Xfeb activity may help maintain a posterior limit to *otx2* expression during MHB formation. Thus, Zic1 may play a role in establishing and maintaining the midbrain-hindbrain boundary.

Zic family members appear to be essential for the formation of the hindbrain. Interfering with either *zic1* or *zic5* expression results in the loss of hindbrain cell fates (Gutkovich et al. 2010). Similar defects are observed when the transcription factor Xmeis is knocked down. In fact, defects in *zic1* and *zic5* morphants could be rescued with co-injection of *xmeis* RNA (Gutkovich et al. 2010). *hoxD1*, a gene that contributes to patterning the hindbrain, is a direct target of *Xmeis* and is known to be upregulated by *zic1* (Kuo et al. 1998). This indicates that *zic* genes work upstream of *xmeis* and *hoxD1* to promote formation of the hindbrain in *Xenopus* embryos. In addition, interfering with the expression of the Zic1 direct target gene *Xfeb (glipr2)* resulted in loss of *hoxD1* expression (Li et al. 2006). While it is not known if Xfeb may lie upstream of *xmeis1* or be part of a separate pathway, *zic* genes play an important upstream role during hindbrain development.

7.5.5 Zic Genes and the Neural Crest

zic genes act as neural crest specifiers, which has been shown in multiple organisms (reviewed in Merzdorf 2007; Houtmeyers et al. 2013). Neural crest cells are a migratory population of cells that originate from the neural plate border region. Multiple signaling pathways work together to specify the neural crest in two phases.

During phase one, BMP, Wnt, and FGF signaling induces the expression of transcription factors like *pax*, *msx*, and *zic* family members, which are neural border specifiers. During phase two, these neural border specifiers induce the expression of neural crest specifiers, including *snail1*, *snail2*, *ets1*, and *FoxD3* (Stuhlmiller and García-Castro 2012b). Accordingly, mutations in the mouse *Zic2 or Zic5* genes result in a reduction in neural crest cells and deformities in neural crest-derived structures (Inoue et al. 2004; Elms et al. 2003). In *Xenopus*, all *zic* family members are expressed in the neural plate border region (Fig. 7.2; Fujimi et al. 2006) and are important for the formation of neural crest cells. Misexpression of *zic1*, *zic2*, or *zic3* increases the extent of neural crest cell fate in whole embryos, and expression in animal cap explants results in the induction of neural crest markers (Nakata et al. 1997, 1998; Kuo et al. 1998). Similarly, misexpression of *zic4* in *Xenopus* embryos generates ectopic pigment cells, a neural crest-derived cell type (Fujimi et al. 2006). Misexpression of *zic5* in whole embryos causes strong induction of neural crest genes, but, unlike other *zic* family members, *zic5* is not as efficient at inducing neural genes (Nakata et al. 2000). Conversely, interfering with the expression of *zic* genes results in a reduction in the expression of neural crest genes (Hong and Saint-Jeannet 2007; Fujimi et al. 2006; Nakata et al. 2000; Gutkovich et al. 2010). Thus, while having slightly different roles, all members of the *zic* family contribute to induction of the neural crest.

The Zic1 and Pax3 transcription factors work jointly to induce neural crest cell fate in the developing embryo. The expression of *zic1* and *pax3* overlaps in the presumptive neural crest region (Sato et al. 2005; Hong and Saint-Jeannet 2007). Misexpression of either *zic1* or *pax3* alone increases neural crest marker expression only in the ectoderm bordering the neural crest field, while overexpression of both genes together induces ectopic neural crest formation in the ventral ectoderm (Sato et al. 2005) in a Wnt-dependent manner (Monsoro-Burq et al. 2005). When ectopically induced neural crest cells (by activating *zic1* and *pax3* in animal cap explants) are transplanted into embryos, they are able to migrate correctly and form differentiated cell types characteristic of neural crest cell fates. Interestingly, the cooperation between Zic1 and Pax3 is required for these fates, since transplanting cells in which *zic1* alone is activated results in the formation of neural tissue only (Milet et al. 2013). Thus, Zic1 and Pax3 can work together to induce a complete neural crest fate. While physical interaction between the Zic1 and Pax3 proteins was originally elusive (Sato et al. 2005), such an interaction was suggested by expressing these proteins in cultured cells (Himeda et al. 2013).

Since Zic1 and Pax3 together are able to induce a neural crest program in ectodermal explants, this synergy was employed to identify downstream neural crest genes (Plouhinec et al. 2014; Bae et al. 2014). The Plouhinec et al. (2014) screen focused on the identification of direct targets of Zic1/Pax3 and is described above. Bae et al. (2014) used a similar approach but did not limit their screen to direct targets. Both screens identified the *snail1 and snail2* (*slug*) genes. The latter screen identified a variety of additional neural crest genes that may or may not be direct targets. Overall, a variety of familiar and new genes were identified that are activated by Zic1 and Pax3 acting together. Among the neural crest specifiers, the

snail1, *snail2 (slug)*, and *ets1* genes were identified as direct target genes of the interaction between Zic1 and Pax3 (Plouhinec et al. 2014). Further, Zic1 is required for *snail1*, *snail2*, and *foxD3* expression (Plouhinec et al. 2014; Sasai et al. 2001; Gutkovich et al. 2010). FoxD3, which is not known as a direct target of Zic1 at this time, restricts cells to a neural crest fate and also aids in the migration of neural crest cells (Sasai et al. 2001). The Snail family and Ets1 are among the transcription factors that facilitate the delamination and migration of neural crest cells (Nie and Bronner 2015; Aybar et al. 2003). Both screens also identified the *pdgfra* gene, which codes for the alpha subunit of a platelet-derived growth factor (PDGF) receptor. PDGF receptor is important for migration of neural crest cells in mouse embryos (Soriano 1997), and in *Xenopus* it has been implicated in cell migration during gastrulation (Nagel et al. 2004; Van Stry et al. 2005). Thus, Zic1 is required for stabilizing neural crest fate and for the expression of genes that prepare neural crest cells for delamination and migration. While *zic1* is expressed in premigratory neural crest cells and is essential for the expression of genes required for the transition of neural crest cells to emigrate, studies in chick show that it ceases to be expressed as soon as neural crest cells become migratory (Sun Rhodes and Merzdorf 2006). Since *zic* genes can repress neural differentiation genes, their role may include keeping the premigratory neural crest population in an undifferentiated state until the time of cell migration. Overall, the two screens confirmed that the neural plate border specifier Zic1 acts to induce neural crest specifier genes, with some of these interactions identified as direct. This adds further detail to the role of the Zic transcription factors in the gene regulatory landscape that governs neural crest specification.

7.5.6 Zic *Genes and the Proliferation and Differentiation of Cells in the Nervous System*

Xenopus embryos undergo primary neurogenesis, during which six discrete stripes of N-tubulin-positive primary neurons differentiate in the early neural plate, while the remainder of the neural plate remains as undifferentiated progenitors. *zic2* is expressed in these undifferentiated progenitors between the stripes of primary neurons (Brewster et al. 1998). Misexpression of *zic2* in the regions of primary neuron differentiation resulted in a significant decrease in the number of N-tubulin-positive primary neurons, indicating a role for Zic2 in preventing the differentiation of primary neurons. Consistent with this finding, Zic2 has a repressive effect on transcription of the bHLH gene *neurogenin (ngnr-1)*, a gene that promotes neural differentiation (Brewster et al. 1998). Similarly, Sonic Hedgehog (Shh) signaling upregulates *zic2* expression, and overexpression of *Xenopus shh* during primary neurogenesis causes expanded expression of *zic2* and reduced N-tubulin-positive stripes (Franco et al. 1999). This indicates that *zic2* acts in maintaining progenitors

and preventing neurogenesis in certain areas of the neural plate, possibly under the regulation of Shh.

While Zic2 represses transcription of the neural differentiation factor *ngnr-1* (Brewster et al. 1998), Zic1 and Zic3 have inductive effects on the expression of the proneural genes *ngnr-1* and *neuroD* in animal cap explants (Nakata et al. 1997; Mizuseki et al. 1998). Further, *hesx1*, which promotes differentiation, is a direct target of Zic1 (Cornish et al. 2009). However, in mouse and chick embryos, Zic1 represses proneural gene expression (Ebert et al. 2003), misexpression of *Zic1* blocks neuronal differentiation, and mutations in *zic* genes cause a decrease in cell proliferation in the dorsal neural tube (Ebert et al. 2003; Aruga et al. 2002; Nyholm et al. 2007). In addition, *Zic1* promotes proliferation in the cerebellum and *Zic1* and *Zic3* in retinal precursors (Blank et al. 2011; Watabe et al. 2011). Consistent with these results from other organisms, interfering with *btg2* expression in *Xenopus* embryos (Btg2 reduces proliferation and promotes neuronal differentiation) results in increased *zic3* expression (Sugimoto et al. 2007), indicating that Btg2 downregulates *zic3* gene expression to allow neurogenesis to begin. Thus, it appears that Zic2 has a role in maintaining undifferentiated progenitors in the neural ectoderm, while the role of Zic1 and Zic3 in proliferation and differentiation is not completely clear and may be context dependent. Interestingly, the early neural transcription factor FoxD4 regulates these *zic* genes differently. It induces *zic2* transcription directly while indirectly repressing *zic1* and *zic3* expression (Neilson et al. 2012; Yan et al. 2009), which has been interpreted as FoxD4 keeping the neural ectoderm in a proliferative state by promoting *zic2* and repressing *zic1* and *zic3* expression. Since the expression domains of the *zic1*, *zic2*, and *zic3* genes overlap in the neural plate, it will be interesting and important to sort out the potentially opposite and context-dependent influences that these genes exert on neural differentiation.

7.6 *Xenopus* Studies Contribute to Our Understanding of Human Diseases

Xenopus embryos are increasingly employed as a model system in functional studies of human diseases (Kofent and Spagnoli 2016; Lienkamp 2016; Hardwick and Philpott 2015). With the near completion of the *Xenopus* genome and the advent of the TALEN and CRISPR-Cas9 systems of genome editing, such studies have become feasible (Tandon et al. 2016). With regard to diseases caused by mutations in human *ZIC* genes, *Xenopus* embryos were used to examine gene regulatory interactions in human craniosynostosis caused by mutations in the *ZIC1* gene. Craniosynostosis is the premature fusion of skull sutures that leads to abnormalities in brain development and brain function in human patients. Five independent families with a history of coronal craniosynostosis showed four different mutations in the third exon of ZIC1, C-terminal to the zinc finger region (Twigg et al. 2015). These mutations include one point mutation and three nonsense mutations that

result in truncations of the ZIC1 protein. The *ZIC1* and *engrailed* (*EN1*) gene expression domains overlap in the developing sutures (Twigg et al. 2015). Using the regulatory relationship between Zic1 and the *engrailed* (*en-2*) gene as a model, misexpression of wild-type *Xenopus zic1* or human *ZIC1* does not change the *engrailed* (*en-2*) expression domain at the midbrain-hindbrain boundary in *Xenopus* embryos (Merzdorf and Sive 2006; Twigg et al. 2015). In contrast, the human mutant *ZIC1* genes elicit increased and/or abnormal *en-2* expression in *Xenopus* embryos, indicating that the mechanism by which these C-terminal *ZIC1* mutations cause craniosynostosis may lie in dysregulation of the *EN1* gene in the developing sutures (Twigg et al. 2015). En1 has been shown to regulate osteogenic differentiation and induction of Osterix (Sp7) during the formation of mouse skull sutures (Deckelbaum et al. 2006). Interestingly, Osterix (Sp7) is a direct target of zic1 (Table 7.2). Thus, ZIC1 appears to participate in a gene regulatory network, which is disturbed by mutations in the C-terminal domain of ZIC1, resulting in abnormal bone development in the coronal sutures and craniosynostosis in human patients.

Xenopus embryos were used to study the mechanism by which a mutation in the first zinc finger of the human *ZIC3* gene causes TGA (transposition of the great arteries), which is a complex heart defect (Chhin et al. 2007). Zic3 plays a role in left-right axis formation and induction of the neural crest (Cast et al. 2012; Kitaguchi et al. 2000; Nakata et al. 1997, 1998), which are processes that may underlie the defects seen in the human patients. Injection of wild-type human *ZIC3* into *Xenopus* embryos induced misexpression of the left lateral plate mesoderm marker *pitx2* and the neural crest marker *snail2*. This induction activity was diminished when *Xenopus* embryos were injected with the mutant *ZIC3* gene (Chhin et al. 2007). Thus, it appears that the mutation in the first zinc finger (which does not bind to DNA but engages in protein-protein interactions) diminishes the overall activity of ZIC3 in both left-right axis formation and neural crest induction. Thus, *Xenopus* embryos have proven useful in studying the interactions of mutant forms of human *ZIC* genes with developmental mechanisms to identify a molecular basis for human disease.

7.7 Conclusion

Work with *Xenopus* embryos has greatly contributed to understanding the role of Zic transcription factors during development. While *zic* gene family members are important players in many developmental processes, much remains to be understood about the molecular mechanisms that govern *zic* gene expression and Zic activities. The screens for direct and indirect targets of Zic transcription factors have yielded a variety of genes that are supporting ongoing and new research and are giving rise to new insights. Important are the advent of new genetic tools, such as new methods for genome editing, and the sequencing of the *Xenopus laevis* genome. Thus, previous studies can now be combined with genomic studies that have long been the strengths of other model organisms to form a more complete understanding of how Zic proteins drive development. Zic gene expression overlaps and their

activities are partially redundant. Thus, it will be important to discover how individual *zic* genes are regulated and what distinguishes their functions. These studies will help with understanding the basis for human diseases. Indeed, *Xenopus* embryos have already been used to examine the molecular mechanisms underlying two human diseases caused by mutations in *ZIC* genes.

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