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Original Article

The *zic1* gene is an activator of Wnt signaling

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ABSTRACT The *zic1* gene plays an important role in early patterning of the *Xenopus* neurectoderm. While Zic1 does not act as a neural inducer, it synergizes with the neural inducing factor Noggin to activate expression of posterior neural genes, including the midbrain/hindbrain boundary marker *engrailed-2*. Since the *Drosophila* homologue of *zic1, odd-paired (opa),* regulates expression of the *wingless* and *engrailed* genes and since Wnt proteins posteriorize neural tissue in *Xenopus,* we asked whether *Xenopus* Zic1 acted through the Wnt pathway. Using Wnt signaling inhibitors, we demonstrate that an active Wnt pathway is required for activation of *en-2* expression by *zic1*. Consistent with this result, Zic1 induces expression of several *wnt* genes, including *wnt1, wnt4* and *wnt8b*. *wnt1* gene expression activates expression of *engrailed* in various organisms, including *Xenopus,* as demonstrated here. Together, our data suggest that *zic1* is an upstream regulator of several *wnt* genes and that the regulatory relationships between *opa, wingless* and *engrailed* seen in *Drosophila* are also present in vertebrates.

KEY WORDS: *zic, wingless, wnt, Xenopus, engrailed, neural*

Introduction

Wnt signaling is involved in many developmental processes and despite the considerable understanding of this pathway, the upstream events that regulate *wnt* gene expression are not well understood (Wodarz and Nusse, 1998, Logan and Nusse, 2004, Wang and Wynshaw-Boris, 2004, Ciani and Salinas, 2005). In *Drosophila*, the *odd-paired (opa*) gene is required for activation of *wingless* gene expression (Benedyk *et al.*, 1994).

Vertebrate *opa* homologues are members of the *zic* gene family of zinc finger transcription factors (Kuo *et al.*, 1998, Mizuseki *et al.*, 1998, Nakata *et al.*, 1998). *zic* genes have been implicated in patterning the dorsal neural tube, in neural crest development and in cerebellar development (Aruga, 2004). Do *zic* genes regulate Wnt signaling in vertebrates? The *zic1* gene (also called *opl* and *zicr-1*) (Kuo *et al.*, 1998, Mizuseki *et al.*, 1998, Nakata *et al.*, 1998) may be involved in regulating *wnt* expression. Throughout early development in *Xenopus*, *zic1* and several *wnt* genes show extensive overlap in their expression patterns in the presumptive neurectoderm, in the dorsal neural tube, at the forebrain/midbrain boundary and at the midbrain/hindbrain boundary (McGrew *et al.*, 1992, Wolda *et al.*, 1993, Cui *et al.*, 1995, Chang and Hemmati-Brivanlou, 1998, Kuo *et al.*, 1998), suggesting there may be a regulatory connection between *zic1* and *wnt* genes. Further, *wnt* genes are known to posteriorize neural tissue in animal cap (undifferentiated ectoderm) assays (McGrew *et al.*, 1995, McGrew *et al.*, 1997, Chang and Hemmati-Brivanlou, 1998, Domingos *et al.*, 2001). This activity is shared by *zic1* (Kuo *et al.*, 1998). In particular, when the BMP inhibitor Noggin is expressed in animal caps from *Xenopus*, these animal caps express panneural genes and a subset of anterior neural genes (Lamb *et al.*, 1993), while co-expression of Wnt proteins activates more posteriorly expressed genes (McGrew *et al.*, 1995). Zic1 alone does not induce neural gene expression. Neither does Zic1ΔC, a C-terminally truncated form of Zic1 that shows enhanced transcriptional activation activity compared to full length Zic1 (*zic1ΔC* = *oplΔC*; Kuo *et al.*, 1998). However, like Wnt proteins, both Zic1ΔC and full length Zic1 synergize with Noggin to induce expression of posterior neural genes that are not activated by Noggin alone. One such gene is the midbrain/hindbrain boundary marker *en-2* (Kuo *et al.*, 1998), which is a target of Wnt signaling (Danielian and McMahon, 1996, McGrew *et al.*, 1999).

Since both *zic1* and *wnt* genes are able to posteriorize neural tissue and because *zic1* is a homologue of the *Drosophila odd-paired* (*opa*) gene that is involved in the regulation of *wingless (wg)* and *engrailed (en)* expression (Benedyk *et al.*, 1994), we asked whether *zic1* acts through the Wnt pathway to induce *en-2*. Our

Abbreviations used in this paper: C-terminus, carboxy-terminus; opa, *odd-paired* gene; PCR, polymerase chain reaction; RT, reverse transcriptase.

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data suggest that regulatory relationships between the *opa*, *wg* and *en* genes in *Drosophila* are conserved among the vertebrate homologues of these genes.

Results

Zic1 induces expression of en-2 *via the Wnt pathway*

zic1 and *en-2* expression domains overlap in early neurula (stage 14) embryos (Kuo *et al.,* 1998). We began this study by showing a similar overlap in mid/late neurula (stage 17) embryos between the *zic1* expression domain (Fig. 1A) and the expression domain of the *en-2* gene (Fig. 1B).

In order to investigate the regulatory relationships between *zic1*, *en-2* and *wnt* genes, we utilized animal cap assays. *In vitro* synthesized *zic1ΔC* and *noggin* RNAs were injected into 2-cell embryos, animal caps were isolated at late blastula (stage 9) and maintained in culture until control embryos reached early tailbud (stage 22), when they were harvested for RT-PCR analysis. Control experiments showed that animal caps injected with either *β-globin* or with *zic1ΔC* alone did not show expression of either the pan-neural gene *N-CAM* or the anterior neural marker *otx2* (Fig. 2; lanes 1 and 2). On the other hand, animal caps neuralized with Noggin showed induction of both *N-CAM* and *otx2* expression (lane 3). In contrast, *en-2* expression was induced when *zic1ΔC* and *noggin* were co-expressed in animal caps (lane 4). These data are consistent with previous studies showing that inhibition of BMPs is required for the induction of *en-2* by *zic1* in animal cap explants (Kuo *et al.,* 1998).

We next asked whether an active Wnt signaling pathway is required for the induction of *en-2* by *zic1*. We examined the necessity for Wnt signaling by using two different inhibitors of the canonical Wnt pathway. The first was a dominant interfering *wnt8* construct (*dnWnt*) (Hoppler *et al.*, 1996). Co-expression of *dnWnt* RNA in *zic1ΔC*-injected neuralized animal caps blocked induction of *en-2* expression (Fig. 2, lane 5). The second inhibitor was *GSK3* (He *et al.*, 1995), which promotes degradation of β-catenin, thus blocking the Wnt pathway. Co-expression of *GSK3* RNA and *zic1ΔC* in neuralized animal caps also inhibited induction of *en-2* expression (lane 6). The same experiments were conducted with full length *zic1*, where activation of *en-2* expression was also abolished by co-expression of full length *zic1*plus *noggin* with either of the two Wnt pathway inhibitors (not shown). Thus, induction of *en-2* expression by Zic1 requires an active Wnt pathway.

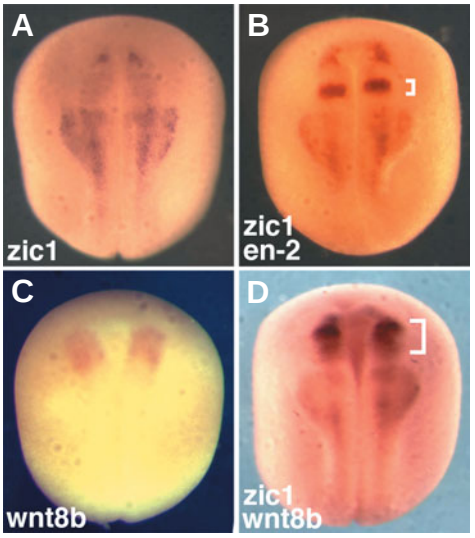


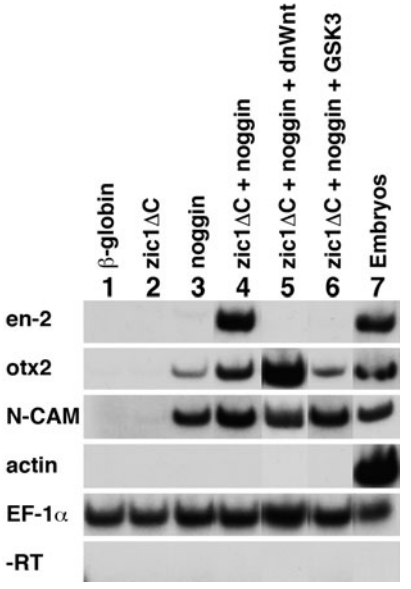
Fig. 1. The *zic1* expression domain overlaps with those of both *en-2* and *wnt8b*. In situ *hybridization of stage 17 neurula embryos.* **(A)** zic1 *expression domain.* **(B)** *Double* in situ *hybridization with* zic1 *and* en-2 *probes shows overlap between the two expression domains. The bracket indicates* en-2 *expression.* **(C)** wnt8b *expression domain in the midbrain.* **(D)** *Double* in situ *hybridization with* zic1 *and* wnt8b *probes indicates that their expression domains overlap. The bracket indicates* wnt8b *expression.*

Zic1 activates expression of a subset of wnt *genes*

One interpretation of these results is that *zic1* might regulate the expression of *wnt* genes. To examine this possibility, we expressed *zic1ΔC* or full length *zic1* with or without *noggin* and assayed *wnt* gene expression in animal caps (Fig. 3). Animal caps were examined by RT-PCR analysis for expression of the *wnt1*, *wnt3a*, *wnt4*, *wnt5a*, *wnt5c*, *wnt7b*, *wnt8*, *wnt8b* and *wnt11* genes. Animal caps taken from embryos injected with RNAs for either *β-globin*, *noggin*, *zic1ΔC,* or full length *zic1* (Fig. 3A, lanes 1-3 and 5) showed low or undetectable expression levels of *wnt1*, *wnt4* and *wnt8b*. In contrast, caps removed from embryos co-injected with *zic1ΔC* plus *noggin* showed strong induction of *wnt1, wnt4* and *wnt8b* expression (lane 4) relative to control caps. Expression of full length *zic1* in combination with *noggin* activated lower levels of *wnt1* and *wnt8b* expression than did *zic1ΔC* and did not induce significant expression of *wnt4* (lane 6). In the absence of *noggin*, *zic1ΔC*, but not full length *zic1*, activated appreciable levels of *wnt1* expression (lanes 3 and 5).

In the same assay, expression of *wnt8, wnt3a* and *wnt7b* was strong or detectable in animal caps that were not expressing Noggin (Fig. 3B, C; lanes 1, 3 and 5) and weak or absent in caps expressing Noggin (lanes 2, 4 and 6). Although Zic1ΔC activated expression of *wnt8* (lane 3), the relevance of this is unclear. When ectopically expressed in dorsal mesoderm during gastrulation, *wnt8* causes a loss of anterior structures (Christian and Moon, 1993), although the expression of *en-2* is unchanged (Fredieu *et al.*, 1997). Thus, the induction of *wnt8* by *zic1ΔC* is probably not relevant to the induction of *en-2*. What is the relevance of high *wnt7b* and *wnt3a* expression in control or *β-globin*-expressing animal caps (Fig. 3C, lane 1) and lack of expression in neuralized caps (lanes 2, 4, 6)? Since *wnt7b* is expressed not only in the neural plate but also in the epidermis (Chang and Hemmati-Brivanlou, 1998), Noggin may inhibit expression of the epidermal component of *wnt7b* expression in our assays. However, because *wnt3a* is expressed in the dorsal neural tube in an extensively overlapping domain with *zic1* (McGrew *et al.*, 1997, Kuo *et al.*, 1998) and *wnt3a* is known to induce *en-2* expression in neuralized animal caps (McGrew *et al.*, 1995), we had not expected downregulation of *wnt3a* by Noggin.

We also tested induction of *wnt5a, wnt5c and wnt11* expression in this assay, however none of these genes showed activation of expression by the *zic1ΔC/zic1+noggin* RNA com-



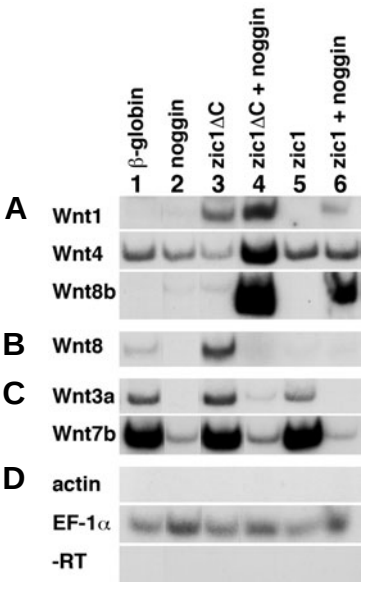


Fig. 2 (Left). Zic1 acts through the Wnt pathway to activate en-2. *Both cells of 2-cell stage embryos were injected with the* in vitro *synthesized RNAs listed along the top. A C-terminal truncation of the* zic1 *coding sequence was used (zic1ΔC). Animal caps were isolated at stage 9 and cultured until sibling embryos reached stage 22. Total RNA was isolated and subjected to RT-PCR analysis with the primers shown on the left.* **(Lanes 1,2)**, β-globin *or* zic1ΔC *injected animal caps did not show expression of neural markers.* **(Lane 3)** Noggin *mRNA induced the anterior neural marker* otx2 *and the general neural marker* N-CAM. **(Lane 4)** *Co-injected* zic1ΔC *plus* noggin *induced expression of the midbrain/hindbrain boundary marker* en-2. *Induction of* en-2 *expression is inhibited by* dnWnt **(lane 5)** *or* GSK3 **(lane 6)**, *which are inhibitors of the Wnt pathway. This demonstrates that Zic1 requires an active Wnt pathway to induce* en-2 *expression.* N-CAM *and* otx2 *were expressed in all samples that received noggin (lanes 3-6).* **(Lane 7)** *Whole embryos at stage 22 served as positive control. Muscle* actin *controlled for mesodermal contamination,* EF-1α *served as loading control and -RT samples controlled for DNA contamination. After culture to the equivalent of stage 22, animal caps expressing* β-globin *and* zic1 *constructs were always completely round.* Noggin*-expressing animal caps were occasionally elongated, but the amount of* noggin *used was low enough that most explants were round. Co-injection of* dnWnt *or* GSK3 *RNA did not influence the shape of the animal caps beyond the effects of* noggin. *Injections were as follows: 200 pg* β-globin, *200 pg* zic1ΔC, *5 pg* noggin, *150 pg* dnWnt8, *80 pg* GSK3.

Fig. 3 (Right). Zic1 induces *wnt* expression. *Embryos were injected into both blastomeres at the 2-cell stage with the indicated RNAs.* zic1ΔC *was tested in addition to full length* zic1. *Animal caps were isolated at stage 9 and cultured until sibling embryos reached stage 22. Total RNA was isolated and subjected to RT-PCR analysis with the primers shown on the left.* **(Lane 1)** β-globin *injected animal caps.* **(Lane 2)** Noggin*-injected.* **(Lane 3)** zic1ΔC*-injected.* **(Lane 4)** zic1ΔC *plus* noggin*-injected.* **(Lane 5)** *Full length* zic1*-injected.* **(Lane 6)** *Full length* zic1 *plus* noggin*-injected.* **(A)** wnt1, wnt4 *and* wnt8b *expression was induced by* zic1ΔC *plus* noggin *(lane 4).* zic1ΔC *alone induced* wnt1 *expression (lane 3), while full length* zic1 *plus* noggin *did not induce* wnt4 *expression (lane 6).* **(B)** zic1ΔC *induced* wnt8 *expression (lane 3).* **(C)** wnt3a *and* wnt7b *were expressed in* β-globin, zic1ΔC *and* zic1*-injected animal caps (lanes 1, 3 and 5), but not in samples that had been co-injected with noggin (lanes 2, 4 and 6).* **(D)** *Muscle actin controlled for mesodermal contamination,* EF-1α *served as loading control and -RT samples controlled for DNA contamination. Injections were as follows: 200 pg* β-globin, *200 pg* zic1ΔC, *200 pg* zic1, *5 pg* noggin.

binations (data not shown). In sum, these assays showed selective activation of *wnt* gene expression by combinations of activated or full length Zic1 plus Noggin.

Expansion of the wnt8b *expression domain by Zic1 in a whole embryo assay*

The strong induction of *wnt8b* expression in animal caps co-expressing *zic1ΔC/zic1+noggin* RNAs prompted us to investigate the ability of *zic1* to induce *wnt8b* expression in whole embryos. First, we established that *wnt8b* is expressed in the midbrain during neurula stages (Fig. 1C) and overlaps with the *zic1* expression domain (Fig. 1D). Subsequently, *zic1ΔC* or full length *zic1* RNAs were co-injected with *lacZ* tracer RNA into albino embryos. *In situ* hybridization showed that the region of the embryo expressing *wnt8b* was expanded by expression of *zic1ΔC* (Fig. 4A) and to a lesser degree by expression of full length *zic1* (Fig. 4B). Since the *in situ* assay is not quantitative and the responding tissue different in the animal cap and whole embryo assays, it is not clear whether the observed expansion is equivalent in the whole embryo and animal cap assays.

Importantly, the region in which *wnt8b* expression was expanded was contiguous with the endogenous *wnt8b* expression domain. Thus, whole embryos must contain factors that regulate where *wnt8b* expression can be modulated and *zic1* expression cannot be solely responsible for this modulation. Nonetheless, this result indicates that the animal cap assay accurately indicated the responsiveness of the embryo to *zic1*.

Regulatory relationships between wnt1 *and* en-2 *are conserved in* Xenopus

The *wnt1* and *en-2* expression domains overlap at the *Xenopus* midbrain/hindbrain boundary (Li *et al.*, 2006). Although regulatory connections between the *wnt1* gene and *en-2* have been shown in various organisms (McMahon *et al.*, 1992, Sugiyama *et*

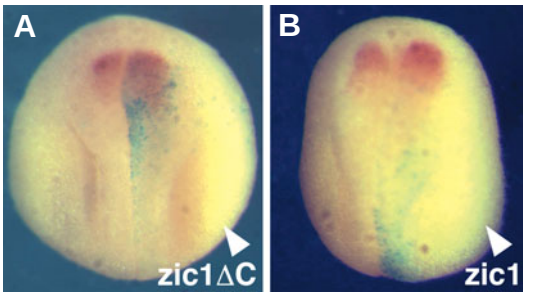


Fig. 4. Zic1 induces ectopic *wnt8b* expression. *One cell of 2-cell albino embryos was injected with* **(A)** *100 pg* zic1ΔC *RNA or with* **(B)** *100 pg full length* zic1 *RNA together with 25 pg* lacZ *RNA as tracer.* In situ *hybridization with* wnt8b *probe at neurula stage 18 showed that* zic1ΔC *and full length* zic1 *upregulate* wnt8b *expression on the injected (arrowheads) side.*

al., 1998), this has not been as fully explored in *Xenopus*. We therefore tested Wnt1 for its ability to induce *en-2* expression and found that Wnt1 induces robust *en-2* expression in neuralized animal caps (Fig. 5, lane 3). At the same time, we confirmed that Wnt3a induces *en-2* expression in neuralized animal caps (lane 4) (McGrew *et al.*, 1995) and showed that Wnt8 induces *en-2* expression (lane 5). In contrast, Wnt8b did not induce *en-2* expression (lane 6) and Chang and Hemmati-Brivanlou (1997) showed previously that Wnt7b does not induce *en-2* in neuralized animal caps. This confirms that a regulatory pathway exists between the *wnt* and *en-2* genes in *Xenopus* (McGrew *et al.*, 1995, McGrew *et al.*, 1999) and demonstrates that only a subset of Wnt proteins can activate *en-2* expression.

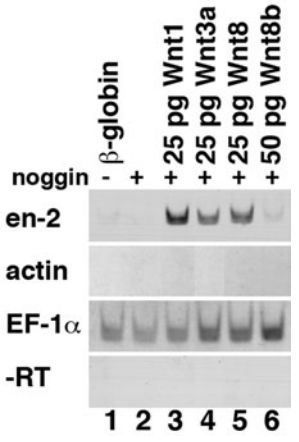


Fig. 5. Wnt1, Wnt3a and Wnt8 induce *en-2* expression. *Embryos were injected at the 2-cell stage into both cells with the sense RNAs listed along the top. Animal caps were isolated at stage 9 and cultured to the equivalent of stage 22. RT-PCR analysis was performed with the primers shown on the left.* **(Lanes 1,2)** β-globin *or* noggin *injected animal caps did not express* en-2. **(Lanes 3-5)** *25 pg* wnt1*, 25 pg* wnt3a *and 25 pg* wnt8 *RNA induced* en-2 *expression.* **(Lane 6)** *50 pg* wnt8b *failed to induce* en-2 *expression. All* wnt *RNAs were co-injected with 3 pg* noggin *RNA. The shape of the animal caps was round in all cases since very low levels of* noggin *and* wnt *RNAs were used. Muscle* actin *controlled for mesodermal contamination,* EF-1α *served as loading control and -RT samples control for DNA contamination.*

Having established in the explant assays that *zic1* forms a regulatory pathway with the *wnt1* and *en-2* genes, we asked whether Zic1 is able to induce ectopic *wnt1* and *en-2* expression in whole embryos. Misexpression of Zic1ΔC in whole embryos resulted in an increase in the intensity of *en-2* staining, as assayed by *in situ* hybridization, that was particularly evident in late neurula (stage 19) and older embryos (Fig. 6A). This increase in *en-2* expression was limited to the normal *en-2* expression domain, suggesting that other factors restrict the *en-2* expression domain. Expression of a dominant interfering form of *zic1* (*dnzic1*) strongly reduced expression of *en-2* (Fig. 6B) and *wnt1* (Fig. 6C) in whole embryos, suggesting that Zic1 is necessary but not sufficient for the activation of *wnt1* and *en-2* expression.

Discussion

We show that Zic1 requires an active Wnt pathway to induce expression of the *en-2* gene in neuralized *Xenopus* ectodermal explants. This relationship is conserved in *Drosophila*, where *opa* is required for expression of the *wg* and *en* genes (Benedyk *et al.*, 1994).

The ability of zic1 to induce neural genes in animal cap explants is dependent on the inhibition of BMP signaling to give the explants neural character (Kuo *et al.,* 1998). In whole embryos, BMP antagonists secreted from the organizer, such as Noggin and Chordin, sequester BMP proteins and therefore allow dorsal determination and formation of neural tissues (Sasai and De Robertis, 1997, Munoz-Sanjuan and Brivanlou, 2002). Indeed, *zic1*, which is among the first molecular indicators of neural fate determination, is expressed in direct response to interruption of BMP signaling (Tropepe *et al.,* 2006).

In animal caps, Zic1 acts through the Wnt pathway to activate *en-2* expression and we found that the expression of three *wnt* genes (*wnt1*, *wnt4* and *wnt8b*) is activated by Zic1ΔC in neuralized ectodermal explants. This suggests that Zic1 interfaces with *wnt* gene function by activating *wnt* gene expression. The expression domains of these three *wnt* genes overlap with that of *zic1* during early embryonic stages. During gastrula stages, *zic1* expression overlaps with *wnt4* expression in the dorsal ectoderm (McGrew *et al.*, 1992). During neurula stages, *zic1* expression becomes restricted to the lateral edges of the neural plate and subsequently to the dorsal neural tube (Kuo *et al.*, 1998), where its expression overlaps with that of *wnt1* and *wnt4* (McGrew *et al.*, 1992, Wolda *et al.*, 1993). Further, *zic1* expression extends to the forebrain/midbrain boundary, where the *zic1* and *wnt8b* expression domains overlap in the midbrain during neurula stages (this study) and at the forebrain/midbrain boundary during tailbud stages (Cui *et al.*, 1995). At the midbrain/hindbrain boundary, *zic1* expression overlaps with expression of *wnt1* and *en-2* (Brivanlou and Harland, 1989, Wolda *et al.*, 1993, Chang and Hemmati-Brivanlou, 1998, Kuo *et al.*, 1998, Li *et al.*, 2006).

Three consensus binding sites for LEF/TCF are present in the *Xenopus en-2* promoter (McGrew *et al.*, 1999), suggesting that the *en-2* gene is a direct target of canonical Wnt signaling. In the animal cap system, *wnt1, wnt3a* and *wnt8*, but not *wnt8b* or *wnt7b*, activated *en-2* expression in neuralized animal caps (this study, McGrew *et al.*, 1995, Chang and Hemmati-Brivanlou, 1998). Based on these considerations and on spatial expression patterns, *wnt1* is the most likely candidate through which *zic1* acts to

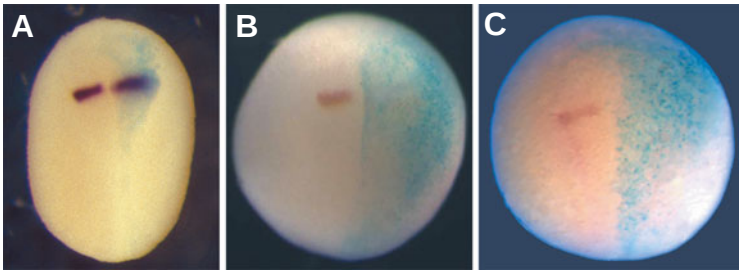


Fig. 6. Zic1 is required for *en-2* induction. (A) *One cell of 2-cell albino embryos was injected with 100 pg* zic1ΔC *RNA, resulting in an increase of* en-2 *expression levels within the* en-2 *expression domain. Embryos injected with 100 pg of a dominant interfering* zic1 *construct* (dnzic1) *showed very significant decrease* **(B)** *in* en-2 *expression and* **(C)** *in* wnt1 *expression. All embryos were co-injected with 25 pg* lacZ *RNA as tracer and the injected sides are shown on the right.*

activate expression of *en-2* in *Xenopus*. This would be consistent with findings in mice, chick, *Xenopus* (this study) and *Drosophila* that connect *wnt1* activity with activation of *engrailed* expression (DiNardo *et al.*, 1988, Martinez Arias *et al.*, 1988, Bally-Cuif *et al.*, 1992, McMahon *et al.*, 1992, Danielian and McMahon, 1996, Sugiyama *et al.*, 1998).

Our data using dominant interfering constructs suggest that Zic1 is necessary for activation of *wnt1* and *en-2* expression. However, although Zic1 causes an increase in *en-2* expression levels within its normal expression domain, ectopic Zic1 expression is not sufficient to induce ectopic *en-2* expression. Thus, other factors must be required for induction of *en-2* expression and helps explain why *zic1*, which is broadly expressed, can activate the expression of genes that are expressed in very restricted domains. We note that expression of other *zic* genes overlaps temporally and spatially with that of *zic1* (Nakata *et al.,* 1997, Nakata *et al.,* 1998, Nakata *et al.,* 2000). Our data do not distinguish whether other genes also act or synergize with Zic1 to activate *wnt* and *en-2* expression.

Genetic studies reveal a similar situation in *Drosophila*. Although loss of *opa* function gives rise to pair-rule defects in body pattern, *opa* is different from all other pair-rule genes in that it is expressed in a broad, unsegmented domain rather than in a segmented fashion. *opa* is required for the proper level and timing of *wg* and *en* expression but not for correct positioning of the expression domains of *wg* and *en* (Ingham *et al.*, 1988, Benedyk *et al.*, 1994). Similarly, *zic1* is expressed in a much broader domain than *wnt1* or *en-2* (Kuo *et al.*, 1998). Perhaps, analogous to *opa* activity in *Drosophila*, *zic1* may act to regulate the timing (and possibly the maintenance and/or level) of *wnt1* and *en-2* expression but not the position of their respective expression domains.

In *Drosophila*, the genes *ftz*, *prd* and/or *eve* may positively regulate the position of *en* expression (Howard and Ingham, 1986, DiNardo and O'Farrell, 1987) and the *runt* gene may do so negatively in areas where *ftz* is not present (Kania *et al.*, 1990, Swantek and Gergen, 2004). Expression of *en-2* in vertebrates may also require further regulatory influences. For example, two *pax* binding sites are required for the expression of a mouse *En-2* transgene (Song *et al.*, 1996) and *pax2* expression begins before *engrailed* expression at the midbrain/hindbrain boundary in *Xenopus* (Heller and Brandli, 1997).

As reflected in our model (Fig. 7), the data indicate that Zic1 induces *en-2* via activation of Wnt1 signaling. Alternatively, there is evidence that Wnt signaling may induce *en-2* by an indirect mechanism that is dependent upon FGF signaling (Domingos *et al.*, 2001). Expression of an FGF receptor in neuralized ectoderm results in the upregulation of *en-2* and *wnt1* expression (Umbhauer *et al.*, 2000). Thus, Zic1 may induce *en-2* by activating *wnt1*, which activates an FGF family member, which in turn activates *en-2* expression. Consistently, Zic1 appears to be able to induce *fgf8* (Li and Merzdorf, unpublished results), whose expression domain overlaps with the *zic1*, *wnt1* and *en-2* expression domains in *Xenopus* (Wolda *et al.*, 1993, Kuo *et al.*, 1998, Glavic *et al.*, 2002). McGrew *et al.* (1997) find that Fgf induces *en-2* in the presence of Wnt pathway inhibitors and Lee *et al*. (1997) show that *wnt1* regulates *en-2* expression via the Fgf pathway in mouse. Thus, there may be two pathways of *en-2* induction by *wnt1*, one direct via *LEF/TCF* sites in the *en-2* promoter and one indirect via FGF signaling. It will be important to determine whether the initiation and the maintenance of *en-2* expression rely on different parts of the pathway, as may be the case in mouse and in *Drosophila*.

In conclusion, we have shown that regulatory connections described for *Drosophila opa* are conserved in *Xenopus*. As in *Drosophila*, Zic1 may be responsible for the level and timing of *en-2* expression, rather than for its positioning. Further, the mechanisms underlying Zic1 activities may include activation of the expression of several *wnt* genes.

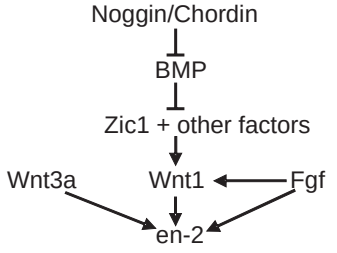


Fig. 7. Model of *en-2* induction by Zic1. *zic1 expression is activated after inhibition of BMP signaling by Noggin or Chordin. Zic1 and possibly other Zic proteins synergize with other factors to activate expression of* wnt1 *and other* wnt *genes. Wnt proteins activate expression of* en-2*. Wnt3a may act via a Zic1-independent pathway. An alternate pathway, by which* en-2 *expression is activated, may involve Fgf8 signaling. The activation events indicated by arrows need not necessarily be direct.*

Materials and Methods

Growth, microinjection, dissection and culture of embryos and explants

Xenopus laevis eggs were collected, fertilized and cultured as in (Sive *et al.,* 1989). Embryos were staged according to (Nieuwkoop and Faber, 1967). Microinjection techniques were as described (Kolm and Sive, 1995). For animal cap explant assays, both cells of 2-cell embryos were injected with a total of 100-200 pg *β-globin* RNA, 200 pg *zic1* or *zicΔC* RNA (= *oplΔC*) (Kuo *et al.,* 1998); and 3-5 pg *noggin* RNA in various combinations. Further, 150 pg *dnWnt8*, 80 pg *GSK3*, 25 pg *wnt1*, 25 pg *wnt3a*, 25 pg *wnt8* and 50 pg *wnt8b* RNAs were injected in combination with other RNAs as detailed in the figure legends. For animal caps, late

blastula (stage 9) animal hemisphere ectoderm was isolated and incubated in 0.5x MBS until sibling embryos reached stage 22.

In vitro transcription

For production of *in vitro* transcribed RNAs, the entire coding sequence of *zic1* was cloned into pCS2+ by PCR (several C-terminal amino acids were absent in the original *zic1* construct used in Kuo *et al.,* 1998). Capped sense RNAs for microinjection were synthesized for *zic1* and *zic1ΔC* RNA in pCS2+ by SP6 transcription of a NarI/NarI fragment. The dominant interfering *zic1 (dnzic1)* construct was made by PCR amplification of the zinc finger domain and C-terminus of the *zic1* coding region. This PCR product was cloned into the Nco1 and Xba1 sites of the pCS2+ATG plasmid. The pCS2+ATG was made by inserting a Kozak sequence between the BamH1 and EcoR1 sites of the pCS2+ vector. The coding sequence of *wnt1* was cloned into pCS2+ by PCR. *wnt1* sense RNA was synthesized by SP6 transcription from the NotI-digested pCS2+*wnt1* plasmid. Other sense RNAs were synthesized as published: *noggin* (Smith and Harland, 1992), *β-globin* (Krieg and Melton, 1984), *wnt3a* (Wolda *et al.,* 1993), *wnt8* (Christian *et al.,* 1991), *wnt8b* (Cui *et al.,* 1995), *dnWnt8* (Hoppler *et al.,* 1996), *GSK3* (He *et al.,* 1995) and *lacZ* (Turner and Weintraub, 1994).

Antisense probes for *in situ* hybridization were transcribed as previously described: *zic1* (Kuo *et al.,* 1998), *en-2* (Hemmati-Brivanlou and Harland, 1989), *wnt1* and *wnt3a* (Wolda *et al.,* 1993). Since the antisense probe synthesized from the *wnt8b* construct, kindly provided by Jan Christian, does not hybridize to neurula embryos, we synthesized *wnt8b* antisense RNA probe using T7 polymerase on a PCR product as template. The primers for the PCR product were:
forward: 5'-GACCTTCTTATCCCGTCTCCA-3' and
reverse: 5'-CTAATACGACTCACTATAGGCTAAACCACAGTCACCACAAA-3', where the underlined bases represent the T7 RNA polymerase promoter sequence.

In situ hybridization

Whole mount *in situ* hybridization was performed with albino embryos as described in (Harland, 1991). One cell of 2-cell embryos was injected with 100 pg *zic1ΔC* RNA, 100 pg *zic1* RNA, or 100 pg *dnzic1* RNA together with 25 pg *lacZ* RNA as tracer. β-galactosidase staining was performed as in (Kolm and Sive, 1995). The alkaline phosphate substrate NBT/BCIP (Sigma) was used and for double *in situ* hybridizations, BCIP in combination with NBT/BCIP was used, although the color differences were lost during the second staining.

RT-PCR

RNA from pools of 14-25 animal cap explants or from 2 embryos was analyzed by RT-PCR as described (Kuo *et al.,* 1998). The following primers were used: *N-CAM* and *en-2* primers (Hemmati-Brivanlou and Melton, 1994), *EF-1α* primers (Gammill and Sive, 1997), *otx2* primers (Pannese *et al.,* 1995), *actin* primers (Stutz and Spohr, 1986),
wnt1 primers (forward: 5'-ATCGGGACTGTATTGCCAAG-3'
reverse: 5'-ACCATTTGCCGCTGTTATTC-3')
wnt3a primers (forward: 5'-CTGGGGAAGGCTGGAAGTG-3'
reverse: 5'-TTGGGGGAGCTCTCATAGTAAATC-3')
wnt4 primers (forward: 5'-GAGTCGCCTTTTCCCAGTCAT-3'
reverse: 5'-GTAGCCCCATCAAATTTCTCCTTA-3')
wnt5a primers (forward: 5'-CCCCGGGACTGGCTATGG-3'
reverse: 5'-CGGGCTGGGGTCGATGTAAAC- 3')
wnt5c primers (forward: 5'-CCGCGGAGAGGAGCAACAT-3'
reverse: 5'-CACCCCGGCGGAACTGATAGC-3')
wnt7b primers (forward: 5'-ATGAAACTGGAGTGCAAATGTC-3'
reverse: 5'-GCTGTCCTCCTCACAGTAGTTG-3')
wnt8 primers (forward: 5'-AGATGACGGCATTCCAGA-3'
reverse: 5'-TCTCCCGATATCTCAGGA-3')
wnt8b primers (forward: 5'-CCGGACGAAAGGCAGTAAAGAG-3'
reverse: 5'-TAACGGCTAAACCACAGTCACCAC-3')
wnt11 primers (forward: 5'-CATTGCGCGTGCCTGTGC-3'
reverse: 5'-GAGTGCCGGTCTGTCTGTGGATG-3').

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