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A novel role for zebrafish *zic2a* during forebrain development

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Abstract

Patterns of transcription factor expression establish a blueprint for the vertebrate forebrain early in embryogenesis. In the future diencephalon, several genes with patterned expression have been identified, yet their specific functions and interactions between them are not well understood. We have uncovered a crucial role for one such gene, *zic2a*, during formation of the anterior diencephalon in zebrafish. We show that *zic2a* is required for transcription of the prethalamic markers *arx* and *dlx2a*. This function is required during early steps of prethalamic development, soon after its specification. *zic* genes are evolutionarily related to *gli*s, transcription factors that mediate hedgehog signaling. Intriguingly, the hedgehog signaling pathway also acts to promote development of the prethalamus. We asked if *zic2a* interacts with hedgehog signaling in the context of forebrain development in zebrafish. Our data show that hedgehog signaling and *zic2a* function at different times, and therefore act in parallel pathways during forebrain development. Taken together, our results identify Zic2a as a novel regulator of prethalamic development, and show that it functions independently of hedgehog signaling.

Keywords

Zic; arx; dlx2a; forebrain; prethalamus; preoptic area

Introduction

The vertebrate forebrain originates as a sheet of cells in the anterior neural plate during gastrulation. The forebrain gradually acquires its characteristic morphological complexity and cell type diversity, in part through progressive refinement of regional patterns. The genetic mechanisms underlying early forebrain patterning include intercellular communication via secreted growth factors and intracellular events, often involving activation of region-specific transcription factors (Wilson and Houart, 2004; Rhinn et al., 2006). The hedgehog (Hh) family of growth factors and the signaling cascade downstream of it are essential for early forebrain regionalization (Fuccillo et al., 2006; Ingham and Placzek, 2006; Bertrand and Dahmane, 2006). In humans, mutations that disrupt Hh signaling are a major cause of holoprosencephaly (HPE), a birth defect characterized by forebrain abnormalities (Dubourg et al., 2007; Monuki, 2007). Essential roles for Hh signaling in the developing forebrain have also been demonstrated in mouse (Hayhurst et al., 2007; Chiang et al., 1996; Rallu et al., 2002), chick (Kiecker and Lumsden, 2004) and zebrafish (Karlstrom et al., 1999; Tyurina et al., 2005; Scholpp et al., 2006), where Hh signaling promotes formation of the anterior diencephalon (AD).

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*ZIC*2, a zinc-finger transcription factor belonging to the *Zic* (zinc finger of the cerebellum) gene family (Benedyk et al., 1994; reviewed in Aruga, 2004; Merzdorf, 2007) is among the few genes outside of the Hh pathway also causally linked to HPE. HPE is observed in *Zic*2 knock-down mice (Nagai et al., 2000), demonstrating a critical role for Zic2 during mouse forebrain development. Mouse Zic2 is also required in more posterior brain subdivisions and in the spinal cord during neural tube closure (Nagai et al., 2000). Similarly, morpholino-mediated knock-down of zebrafish *zic2a* causes dorsal neural tube defects (Nyholm et al., 2007). Thus, Zic2 function in the forebrain is clearly important and conserved, yet the mechanism of this function remains largely unexplored.

All Zics share a highly conserved DNA binding domain composed of five zinc-finger motifs, as well as N-terminal and C-terminal domains of unknown functions (Aruga, 2004; Merzdorf, 2007). Within the zinc-finger domain, Zics share significant sequence similarity with Glis, components of the Hh pathway. This similarity suggests a common evolutionary origin of Zic- and Gli-encoding genes. DNA binding specificities of Zics and Glis have also been conserved since several Zics are able to bind Gli recognition sites *in vitro* (Mizugishi et al., 2001). Furthermore, biochemical studies have shown that Zic and Gli may modulate each other's function through direct protein-protein interactions (Koyabu et al., 2001). Altogether, this evidence suggests that Zics may modulate Hh signaling during vertebrate development, and that Zic2 in particular may do so in the context of the developing forebrain. However, a direct experimental test of such an interaction has not been reported.

The zebrafish genome contains two *Zic2* homologs, *zic2a* and *zic2b* (Toyama et al., 2004). We present evidence that *zic2a* plays an early role in the zebrafish forebrain in promoting formation of the prethalamus (PT), a division of the AD. Since Hh signaling plays a similar role in this tissue, we asked if *zic2a* and components of the Hh pathway genetically interact during PT formation. Our data show that Zic2a and Hh pathway functions are clearly separable in time, with Zic2a acting early in PT development, soon after its initial specification, and Hh signaling playing a later role in PT maturation. Thus, Zic2a acts independently of Hh signaling to promote early formation of the AD.

Methods and Materials

Zebrafish strains and embryo culture

Adult zebrafish were maintained according to established methods (Westerfield, 1995). Embryos were obtained from natural matings and staged according to (Kimmel et al., 1995). The following zebrafish strains were used: wild type AB, smu^{b641} (Varga et al., 2001), syu^{t4} (Odenthal et al., 2000), and *Tg(HuC:gfp)* (Park et al., 2000).

Mutant genotyping

syu^{t4} homozygous mutant embryos were positively identified either by PCR (forward: 5′-ACAGAAGGCCGTGAAGGAC-3′ and reverse: 5′-GCCACGTTCCCATTTGATAC-3′) after ISH or by lack of *shha* expression in a double ISH. smu^{b641} homozygous mutant embryos were identified by lack of *ptc1* expression in a double ISH.

In situ hybridization (ISH)

Antisense RNA probes were transcribed using the MAXIscript kit (Ambion) from the following plasmid templates:*arx* (Miura et al., 1997), *dbx1a* (Hjorth et al., 2002), *dlx2a* (Amores et al., 1998; (Akimenko et al., 1994)), *eomesa* (Costagli et al., 2002), *emx1* (Kawahara and Dawid, 2002), *fezf2* (Jeong et al., 2007), *foxg1* (Rohr et al., 2001), *gfp* (Koster and Fraser, 2001), *gli1*, *gli2a* (Karlstrom et al., 2003), *gli3* (Tyurina et al., 2005), *irx1b* (Lecaudey et al., 2005), *isl1* (Korzh et al., 1993), *itnp* (Unger and Glasgow, 2003) *lef1* (Dorsky et al., 1999),

lhx1a (Toyama and Dawid, 1997), *nkx2.2a* (Karlstrom et al., 2003), *otpb* (Eaton and Glasgow, 2007), *pax6a* (Krauss et al., 1991), *ptc1* (Vanderlaan et al., 2005), *rx3* (Jeong et al., 2007), *shha* (Etheridge et al., 2001), *sim1* (Serluca and Fishman, 2001) *six3b* (Seo et al., 1998), *titf1a*, *titf1b* (Rohr et al., 2001), and *zic2a* (Grinblat and Sive, 2001). ISH was carried out as previously described (Gillhouse et al., 2004). The PT domain, delimited by the expression of *foxg1* and *shha* on either side, was measured using the outline tool (AxioVision 3.0) on an Axioskop2 plus (Zeiss).

Proliferation analysis

BrdU incorporation in 10s and 17s embryos was carried out as previously described (Shepard et al., 2004). Embryos were fixed immediately after incorporation. After antibody staining and fluorescent detection, embryos were counterstained with SYTOX green and mounted in DABCO for confocal microscopy. The total cell number and the number of BrdU labelled cells in the approximate prethalamic area were counted manually in four sections per embryo. The prethalamic area was estimated from the *arx* expression pattern at 10s and the *dlx2a* expression pattern at 18s. Average total cell number at 10s or 17s were not significantly different between conMOs and zic2aMOs.

Immunohistochemistry and histology

Embryos were fixed in 4% paraformaldehyde in PBS and stained using the following antibodies: anti-human HuC/D (1:500, Molecular Probes, #A-21271), anti-activated caspase-3 (1:200, BD Pharmingen, #559565), anti-BrDU (1:100, Roche, #11170376001) Alexa488-conjugated goat anti-rabbit secondary (1:1000, Molecular Probes), and Alexa568-conjugated goat anti-mouse secondary (1:1000, Molecular Probes). Embryos were embedded in Eponate 12 medium (Ted Pella) and sections (4uM) were cut with a steel blade on an American Optical Company microtome. Nuclei were counterstained with Methyl Red. Confocal images taken with a 25X lens on an Axiovert 100M (Carl Zeiss MicroImaging, Inc.) with Lasersharp Confocal Package (model 1024, Bio-Rad) or with a 40X lens on an Olympus FV1000 with FV10-ASW software (Olympus).

Knockdown assays

Three antisense morpholino oligomers were used to knock down expression of Zic2a in this study: two translation-blocking MOs (zic2a AUG = CGATGAAGTTCAATCCCCGCTCACA, and zic2a PROX = CTCTTTCAAGCAGTCTATTCACGGC), and a splice-blocking MO (zic2aMO = CTCACCTGAGAAGGAAAACATCATA) (Nyholm et al., 2007). conMO = standard control MO (Genetools). MOs were diluted in 1X Danieau buffer (Nasevicius and Ekker, 2000) to 1–2ng/nl (zic2aMO), 4–6ng/nl (Zic2a AUG and PROX), or 3–4ng/nl (conMO).1nl was injected at 1–2 cell stage. Cyclopamine (Sigma #C4116 or Toronto Research Chemicals #C988400) was used at 10μM as previously described (Tyurina et al., 2005).

Results

Zic2a functions in the prethalamus during forebrain development

zic2a is expressed broadly in the anterior neural plate starting at mid-gastrulation (Grinblat and Sive, 2001; Toyama et al., 2004; Nyholm et al., 2007). By early somitogenesis (4s), *zic2a* transcription was restricted to several subdivisions of the forebrain primordium, including the prospective telencephalon, retina, and a domain fated to give rise to the PT (Staudt and Houart, 2007; arrow in Fig. 1A). *zic2a* expression in the PT primordium was transient, since it was not detected before the 4s stage (not shown) or after the 8s stage (Fig. 1B, C). Starting at 8s, *zic2a* was expressed in the thalamus, pretectum, and in part of the retina. Expression of *arx*, a

marker of the early PT (Staudt and Houart, 2007; Miura et al., 1997), overlapped the medial portion of the *zic2a* expression domain at 4s (Fig. 1D and data not shown). Subsequently *arx* was found in a domain adjacent to *zic2a* at 8s (Fig. 1E) and 12s (Fig. 1F).

The early and widespread expression of *zic2a* in the forebrain primordium suggested an early role for Zic2a. To test this hypothesis, Zic2a was knocked down using a splice-blocking antisense morpholino oligonucleotide specific for *zic2a* (zic2aMO), described previously (Nyholm et al., 2007). The overall telencephalic and diencephalic pattern formed correctly in zic2a morphants, as indicated by correct expression of telencephalic markers (*six3b* and *fezf2)*, eye field markers (*six3b* and *rx3*), as well as thalamic *(irx1b*) and hypothalamic (*fezf2*) markers (Supplementary Fig. 1). In contrast, expression of the PT marker *arx* was initiated correctly in zic2a morphants (4s, Fig. 1G), but was not maintained, becoming mildly reduced by 8s (Fig. 1H), and strongly reduced by 10s (Fig. 1I-L). *arx* expression was similarly reduced using non-overlapping translation-blocking MOs against *zic2a* (Supplementary Fig. 2). Interestingly, *fezf2* expression in the PT primordium was not affected in Zic2a-depleted embryos (Supplementary Fig. 1). These data suggest that initiation of *arx* transcription occurs independently of Zic2a, but its maintenance requires Zic2a function.

We next asked if Zic2a also functioned later in the developing forebrain, where it continues to be expressed. Forebrain pattern in zic2a morphants was assayed at the end of somitogenesis using a panel of markers (Supplemental Table 1). Several markers of the AD (PT and preoptic area), had reduced expression domains in zic2a morphants. These markers included *dlx2a* (Fig. 2A, B), *pax6a* (Fig. 2C, D), and *eomesa* (Supplementary Fig. 3). We confirmed the specificity of the AD morphant defect using non-overlapping translation-blocking MOs against *zic2a*, and found them to cause a similar *dlx2a* reduction (Supplementary Fig. 2). *dlx2a* reduction in the PT was evident by 17–18s (Supplementary Fig. 4), soon after it is first expressed there (Akimenko et al., 1994). The telencephalon and hypothalamus of Zic2a-depleted embryos showed a mild expansion of posterior markers *emx1* (Fig. 2E, F), *titf1b* (Fig. 2G, H), and *titf1a* (Supplementary Fig. 3). The thalamus, marked by *dbx1a*, was patterned normally (Fig. 2I, J).

We extended our analysis of the AD reduction observed in zic2a morphants by asking if it was smaller, or if it was correctly sized but mispatterned at the end of somitogenesis. The AD area was demarcated by telencephalic expression of *foxg1* anteriorly and expression of *shha*, a ZLI marker, posteriorly (Fig. 2K, L). The area between the *foxg1* and *shha* expression domains was significantly smaller in zic2a morphants compared to conMO injected embryos at the end of somitogenesis ($p=0.001$, Fig. 2M). Together, these data show that AD is significantly reduced in size, while the adjacent forebrain subdivisions develop correctly in Zic2a-depleted embryos (Fig. 2N).

We further examined Zic2a-depleted embryos for persistent patterning defects using markers of diencephalic neurons. *isl1* marks two primary neuron clusters in the diencephalon of prim-5 stage embryos: the dorsorostral cluster (DRC), formed in the preoptic area of the hypothalamus, and the ventrorostral cluster (VRC), located in the PT (Fig. 3A). In zic2a morphants, the DRC and VRC were strongly reduced or absent (Fig. 3B). In contrast, the telencephalic *isl1*-expressing cluster was only mildly affected. Expression domains of *sim1* and *otpb*, transcription factors that mark overlapping clusters of neurons in the preoptic area, were dramatically reduced in Zic2a-depleted embryos (Fig. 3D, F). *sim1* and *otpb* are required cell-autonomously for the formation of isotocin producing neurons marked by *itnp* (Eaton and Glasgow, 2006;Eaton and Glasgow, 2007). At 2 dpf, expression of *itnp* was lost in zic2a morphants (Fig. 3G, H). Diencephalic *lhx1a* expression in zic2a morphants remained normal (Supplementary Fig. 3), showing that not all neurogenesis in the diencephalon was affected.

Together, results of Zic2a knock-down experiments suggest that Zic2a is required between 4s and 12s to activate *arx* transcription in the newly formed PT primordium. Since *zic2a* transcripts were not detected in the PT primordium or the adjacent preoptic area after the 8s stage, it is likely that the growth and neuronal differentation defects observed at later stages were an indirect consequence of the early role Zic2a plays during somitigenesis.

Reduced proliferation, but not apoptosis or premature differentiation, contributes to the early anterior diencephalic defect in zic2a morphants

We have shown that the PT primordium is sized correctly by 12s, but becomes reduced by late somitogenesis stages. This reduction may be due to increased apoptosis, premature neuronal differentiation, or failure to proliferate sufficiently. To test apoptosis rates in zic2a morphants, we examined the distribution of apoptotic cell markers, acridine orange (Hill et al., 2003) and activated caspase-3 (Ryu et al., 2005). Neither method revealed an increase in the number of apoptotic cells in zic2a morphants at 10s and 14s (data not shown). We also used an antisense morpholino against p53 (p53MO, Robu et al., 2007) to block the apoptotic pathway in zic2a morphants. If Zic2a normally functions to prevent apoptosis, then in Zic2a depleted embryos apoptotic cell death should lead to the characteristic reduction in PT size, and in zic2a/p53 double morphants this defect should be alleviated (rescued). We did not observe rescue of the zic2aMO-induced defect in the presence of p53MO (Fig. 4). At the 15s stage, the *arx* expressing domain was reduced similarly in both zic2a morphants and in zic2a/p53 double morphants compared to p53MO injected controls (Fig. 4A–C). At the end of somitogenesis, *dlx2a* expression in zic2a morphants and in zic2a/p53 double morphantsalso showed equivalent reduction of the PT (Fig. 4D–F). Together, these data show that Zic2a promotes PT development independently of regulating apoptosis.

Another plausible explanation for the reduced size of the PT primordium in zic2a morphants is premature cell-cycle exit and differentiation of neuronal precursors. To test this hypothesis we used *Tg(HuC:gfp)*, a transgenic line that expresses Gfp in post-mitotic neuronal precursors (Park et al., 2000). *Tg(HuC:gfp)* embryos were injected with conMO or zic2aMO and examined for expression of Gfp by fluorescence at 10s and 14s (data not shown) and for expression of *gfp* by WISH at 8s and 12s (Fig. 4G, H). We found noevidence of increased *gfp* RNA or protein, suggesting that Zic2a does not regulate the timing of cell cycle exit and differentiation of neuronal precursors in the PT.

The remaining possible explanation for the smaller PT primordium in morphants is reduced proliferation. Wetested this possibility by examining BrdU incorporation, a method for marking cells in the S phase of the cell cycle. At 10s, the proportion of BrdU-labelled cells was somewhat reduced in zic2aMOs compared to conMOs, but this reduction was not statistically significant (Fig. 4I–K). However, by 17s the ratio of BrdU positive/total cells was significantly reduced in zic2aMOs (Fig. 4I, L–M, $p = .005$). These data show that Zic2a is required to promote the mitotic cell cycle, but that the PT patterning requirement (activation of *arx* expression) precedes the mitogenic requirement.

Zic2a cooperates with Hh signaling to promote anterior diencephalic formation

Hh signaling plays an important role in promoting AD development in zebrafish embryos. We asked if *zic2a* genetically interacts with the Hh pathway. We first examined expression of *dlx2a* in the forebrains of *sonic hedgehog* (*syu*^{t4}, Odenthal et al., 2000) and *smoothened* (*smu*^{b641}, Varga et al., 2001) mutant embryos. *dlx2a* expression was reduced in the PT of both mutants, while expression in the telencephalon remained relatively normal (Fig. 5C, E), as previously observed (Scholpp et al., 2006). To generate embryos depleted for both Zic2a and Hh signaling, zic2aMO was injected into progeny from a *syu*^{t4}/+ incross. While most embryos showed a *dlx2a* reduction similar to zic2a morphants (77% of 74 total), some of the zic2aMO/

syu^{t4} mutant clutch exhibited a greater loss of *dlx2a* expression in the PT (Fig. 5D, 23% of 74 total) than in either the homozygous mutant or zic2a morphant alone. In a separate experiment, we confirmed that these strongly affected embryos were genotypically *syu*^{t4}/*syu*^{t4} (9/9 embryos, see Materials and Methods for details). Similarly, when progeny from a *smu*^{b641}/+ incross were injected with zic2aMO, *dlx2a* expression was completely lost in 33% of the injected embryos (Fig. 5F, 60/195). Genotyping confirmed that all embryos exhibiting complete loss of *dlx2a* were *smu*^{b641}/*smu*^{b641} (17/17 embryos, see Materials and Methods). These data indicate that Zic2a acts in parallel with, but not epistatically to the Hh signaling pathway in the PT primordium.

Zic2a acts before Hh signaling to promote maturation of the prethalamic primordium

Since Zic2a function is required for *arx* transcription by 8s (Fig. 1), we next asked if Hh signaling is required at the same time in the PT primordium. Progeny from *smu*^{b641}/+ in crosses were analyzed for expression of *arx* at mid-somitogenesis. *smu*$^{b641/b641}$ embryos, identified by the absence of *ptc1* expression (Varga et al., 2001), showed normal *arx* expression at both 8s and 12s (Fig, 6, A–D). However, by 18s *dlx2a* expression in the PT was strongly reduced in *smu*^{b641}/*smu*^{b641} embryos (Fig. 6E, F). We next asked if Hh signaling and Zic2a may be playing synergistic roles in the early PT primordium. To test this hypothesis, we examined *arx* expression in embryos simultaneously depleted for Zic2a using zic2aMO, and for Hh signaling using exposure to an alkaloid inhibitor of Hh signaling, cyclopamine (Tyurina et al., 2005). *arx* expression in conMO-injected, vehicle treated embryos was indistinguishable from *arx* expression in conMO-injected, cyclopamine-treated embryos (Fig. 6G, I), confirming our conclusion that Hh signaling does not play arole in early PT patterning. zic2amorphants treated with vehicle showed the typical reduction of the *arx* expression domain that was indistinguishable from the defect seen in cyclopamine treated zic2a morphants (Fig. 6H, J). Together, these results argue that Hh signaling acts after 12s in the developing PT.

Zic2a and Hh signaling both promote development of the AD. Since Zic2a acts prior to Hh signaling, it is possible that Zic2a modulates the Hh pathway by controlling transcription of its genetic components. To address this possibility we asked if Zic2a regulates transcription of several members of the Hh signaling pathway. At mid-somitigenesis, *ptc1* (Fig. 7A, B) and *gli1* (Fig. 7C, D) were transcribed correctly in Zic2a-depleted embryos. The ZLI, the main Hh source in the diencephalon, was established normally (Fig. 7E, F). At prim-5 stage, Hh signaling was also unaffected in zic2a morphants as evidenced by correct expression of Hh targets *nkx2.2a*, *ptc1* and *gli1* (Cohen, 2003; Barth and Wilson, 1995; Fig. 7G–L). *gli2* and *gli3* were also expressed correctly(Fig. 7M–P). Conversely, *zic2a* expression was not affected in *smu*^{b641}/*smu*^{b641} mutants (Supp. Fig. 5). Together these results suggest that Zic2a and Hh signaling carry out similar, but independent functions in the PT primordium.

Discussion

Zic2 is essential for correct forebrain development in mammals, yet the mechanism of this function is not understood. We have characterized a novel role for *zic2a* during forebrain development of the zebrafish. We show that Zic2a is required for the correct formation of the AD (prethalamus and preoptic area), and identify an early requirement for Zic2a in the PT primordium that involves maintenance of *arx* transcription. We further demonstrate that, while Zic2a and Hh signaling function similarly in the AD, they act independently. This study is the first demonstration of a role for *zic2* in non-mammalian forebrain development.

Zic2a plays a patterning role in the forming prethalamus

While the mechanism of Zic2a function in the forming PT has yet to be fully elucidated, our current data argue in support of a primary patterning role for Zic2a, rather than a role in

modifying cell cycle progression. The relative timing of *zic2a* expression in the PT primordium and its role in regulating transcription of *arx* suggest that *arx* is a proximal transcriptional target of Zic2a. *arx*, a homeobox transcription factor, is required to activate transcription of *dlx* genes in the prethalamus of mammals (Seufert et al., 2005; Kitamura et al., 2002).

zic2a is expressed in the PT primordium for a brief period during early somitogenesis, but continues to be expressed in the adjacent thalamus throughout somitogenesis (Grinblat and Sive, 2001). Our data are consistent with the hypothesis that Zic2a is functioning in the PT shortly after its brief pulse of expression there. Alternatively, Zic2a may function in the thalamus, and the thalamus in turn may signal to the adjacent prethalamus to promote its growth. Correct formation of the thalamus and ZLI in zic2a morphants (Figs 2 and 7, respectively) argue against the latter explanation. Furthermore, recent studies show that thalamus and prethalamus are specified and maintained independently (Jeong et al., 2007; Scholpp et al., 2007).

High levels of *zic2a* transcript in the zebrafish telencephalon (Toyama et al., 2004; Grinblat and Sive, 2001), together with the prevalence of telencephalic defects in human HPE patients with mutations in ZIC2, predict a role for Zic2 in the telencephalon. Absence of significant telencephalic defects in zic2a morphants is likely due to functional redundancy with other Zic family members (Nyholm et al., 2007; Aruga et al., 2002; Ogura et al., 2001; Inoue et al., 2007). *zic2b*, *zic1*, *zic4*, and *zic5* are co-expressed with *zic2a* in the telencephalic primordium and may play partially redundant roles there (Nagai et al., 1997; Toyama et al., 2004; Grinblat and Sive, 2001).

Zic2a functions independently of Hh signaling to pattern the diencephalon

The hypothesis that Zic and Gli proteins interact *in vivo* is supported by several lines of evidence. *Zic* family members interact directly *in vitro* and bind to the same binding site sequence (Mizugishi et al., 2001). Moreover, co-overexpression of Zic1 and Gli1 in cultured mammalian cells results in Gli protein relocalizing from the cytoplasm into the nucleus (Koyabu et al., 2001). In our study, a careful temporal dissection allowed us to determine that a direct functional interaction between Zic2 and Gli proteins was not likely in the AD. However, Zic and Gli proteins may interact to pattern other developing tissues. Zic1 and Gli3 double-knockout mice show synergistic phenotypes in the vertebral arches, suggesting that these proteins may interact there (Aruga et al., 1999). The relative temporal requirements of these two factors have not been resolved. Likewise, Gli3 is involved in development of optic stalk and retina (Tyurina et al., 2005; Furimsky and Wallace, 2006). Analysis of a potential role for Zic2a in these tissues is in progress

Our finding that Zic2a and Hh pathway components do not cross-regulate at the level of transcription is in contrast to reports of aberrant *Zic2* expression in Hh-depleted mouse embryos (Hayhurst et al., 2007, Brown et al., 2003). Hayhurst et al. (2007) further show that Hh signaling activates forebrain Zic2 expression indirectly, likely through regulating FGF8. Cross-regulation between the Hh and Fgf signaling pathways is an important conserved mechanism of forebrain formation in vertebrates, including zebrafish (reviewed in Bertrand and Dahmane, 2006). In this study, we have begun to ask what role, if any, zebrafish *zic2a* plays in this interaction. In future studies, we will continue this examination by focusing on FGF signaling as a candidate regulator of *zic2a* transcription.

Zic2a and Wnt signaling in of anterior diencephalic development

We have previously shown that canonical Wnt signaling directly activates *zic2a* transcription in the midbrain and forebrain (Nyholm et al., 2007). Wnt signaling plays a major role in patterning the forebrain along the A-P axis (Houart et al., 2002), promoting posterior and

repressing anterior forebrain fates. Since *zic2a* is activated by Wnt signaling but functions to promote anterior diencephalic development, we speculate that Zic2a may be part of a feedback loop that limits the inhibitory action of Wnt signaling in the AD. A similar role was recently demonstrated for *fezf2*, a zinc-finger transcription factor that promotes PT formation by attenuating the posteriorizing effects of Wnt signaling (Jeong et al., 2007). We show that Zic2a does not regulate *fezf2* transcription, suggesting that *zic2a* likely acts downstream or in parallel with *fezf2* in the PT primordium.

The HPE connection—While the classical defining trait of HPE is the failure of cerebral hemispheres to separate (Sarnat and Flores-Sarnat, 2001), deletion of diencephalic structures is also frequently associated with HPE. The prethalamic and preoptic areas are diencephalic structures that are strongly reduced in zic2a morphants. In humans, the subthalamus (human equivalent of the PT) is important mainly for controlling skeletal muscle coordination (Colnat-Coulbois et al., 2005), and the preoptic area plays a major role in thermoregulation (Blatteis, 2007). While defects in the subthalamus or the preoptic area have not been described specifically in humans with HPE, spasticity and temperature dysregulation are symptoms commonly associated with HPE. Therefore, similar areas of the diencephalon may be impaired in zebrafish lacking Zic2a and in humans with reduced ZIC2 levels. Another disorder frequently associated with HPE is diabetes insipidus (Hahn et al., 2005, Dubourg et al., 2007) although this association has not been examined specifically in ZIC2-linked HPE cases. Diabetes insipidus can be caused by the loss of specific neuronal clusters which secrete oxytocin (Burbach et al., 2001). Zic2a-depleted embryos fail to express *itnp*, which encodes isotocin, the functional zebrafish analog of oxytocin. zic2a morphants also show reduced expression of *otpb* and *sim1*, transcription factors required for development of *itnp*-expressing neurons (Eaton and Glasgow, 2007). These data further suggest that Zic2a-depleted zebrafish may accurately recapitulate some aspects of human HPE, although they do not exhibit the full HPE phenotype, possibly due to functional redundancy with zic2b.

arx, a proximal target of zic2a according to this study, has not been associated with HPE. In humans, mutations in *ARX* are causally linked to XLAG (X-linked lissencephaly with abnormal genitalia). XLAG is characterized by many symptoms, including agenesis of the corpus callosum and poor temperature regulation (Kitamura et al., 2002). Interestingly both of these symptoms are also found in HPE patients. These findings taken together strongly suggest that we are uncovering aspects of forebrain development and Zic2a function that have been conserved during vertebrate evolution. Future zebrafish studies are likely to provide valuable insights into the genetic nature of human HPE, despite the overt differences in their forebrain morphogenesis (Wullimann and Rink, 2002).

A model for zic2a function in the developing diencephalon

Our findings show that zebrafish Zic2a acts to maintain the PT soon after it is specified, at least in part through transcriptional control of *arx*. *arx* in turn is likely to play an essential role in promoting growth and/or differentiation of the PT primoridum through regulation of other PT transcription factors such as *dlx2a* (Kitamura et al., 2002). These data place Zic2a in a key early position in the regulatory cascade of transcription factors that control development of the PT (Fig. 8). While Zic2a and Hh signaling carry out similar roles in the forming PT, they function consecutively rather than concomitantly, and therefore do not directly interact. Parallels between humans with HPE caused by Zic2 mutations and zic2a-depleted zebrafish suggest that we are uncovering conserved regulatory mechanisms that govern forebrain development in vertebrates.

Supplementary Material

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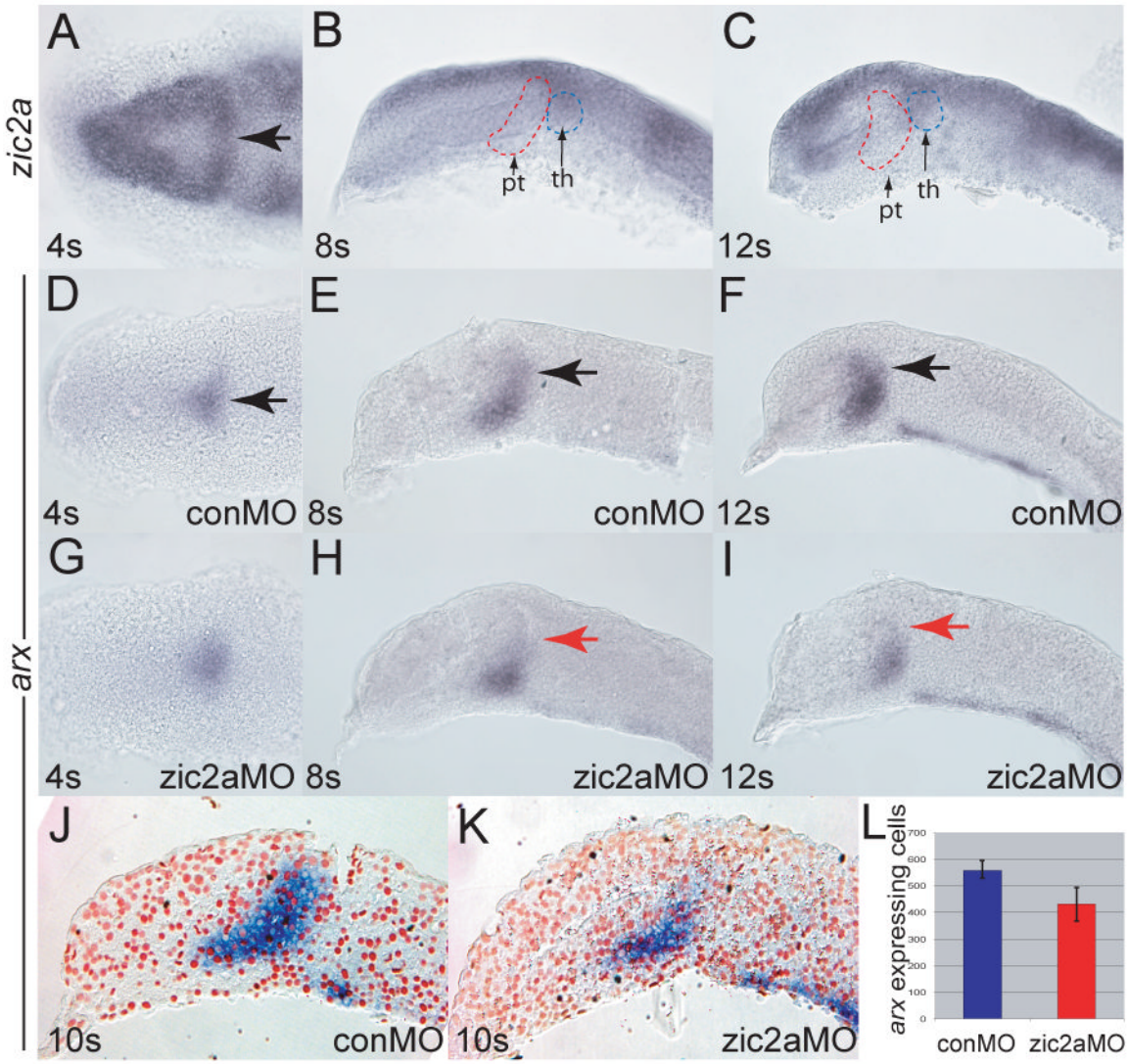

Fig. 1. Zic2a is required for maintenance, but not for initiation of *arx* expression in the PT primordium

Embryos were stained by ISH for expression of *zic*2a (A-C) or *arx* (D-I). (A) Uninjected embryos express *zic2a* transiently in the early PT at 4 somites. (B, C) *zic2a* is not expressed in the PT at 8 and 12 somites. Prethalamus (pt) and thalamus (th) are outlined for reference. (D-E) Normal *arx* expression in the PT primordium of control MO injected embryos at 4, 8 and 12 somites. (G-H) *arx* expression in *zic2a* morphants. (G) *arx* is expressed normally at 4s (44/50 embryos, 3 exp.). (H) *arx* is mildly reduced at 8 somites (8/23 embryos, 2 exp.). (I) *arx* is drastically reduced at 12 somites (28/33 embryos, 2 exp.). (J, K) Representative parasagittal sections of conMO-and zic2aMO-injected embryos stained for *arx* by ISH at 10s. (L) Graph of average number of *arx* expressing cells in conMO (n = 3) and zic2aMO-injected (n = 3) embryos (results significant at p = 0.05). All embryos are shown with anterior to the left. A, D and G are dorsal views, all others are lateral views. Arrows mark the PT primordium.

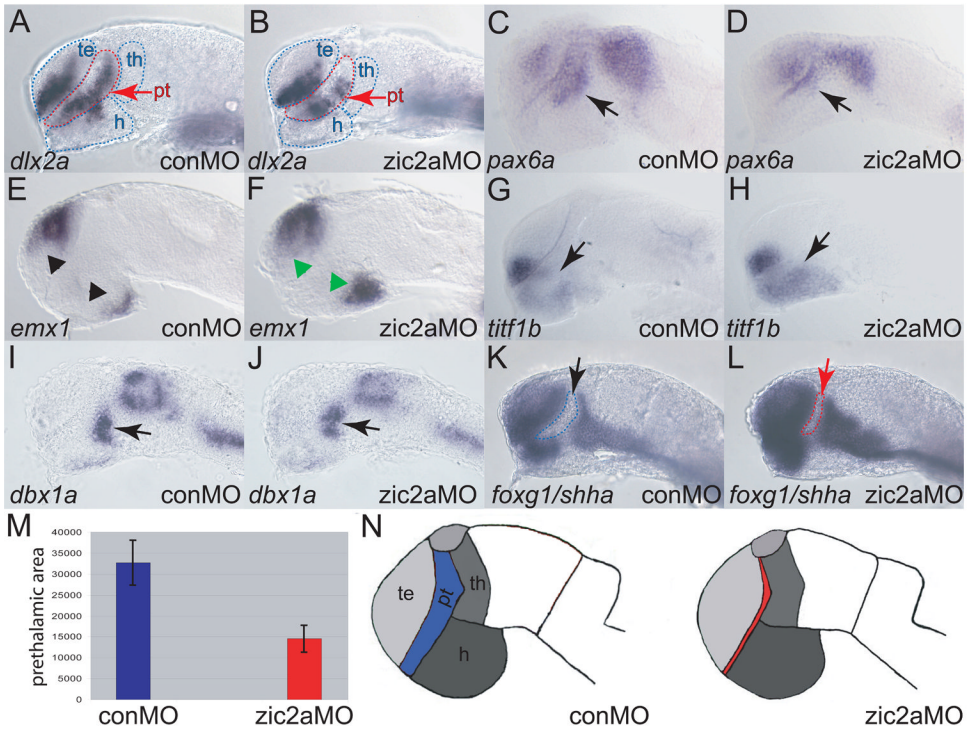

Fig. 2. Zic2a functions primarily in the PT during forebrain development

The effect of Zic2a depletion on overall pattern in the forebrain at Prim-5 was examined using ISH with several markers of forebrain subdivisions (see Supplemental Table 1 for numbers). B) *dlx2a* expression is reduced in the PT, but not affected in the telencephalon of zic2a morphants. (C, D) *pax6a* expression is reduced in the PT region of Zic2a depleted embryos (arrows), but not affected in the telencephalon. (E, F) Expression of *emx*1, a marker of posterior telencephalon and posterior hypothalamus is expanded in both domains. (G, H) *titf1b* expression in the hypothalamus is expanded anteriorly inzic2a morphants. (I, J) The thalamus, marked by *dbx1a*, is formed normally. (K, L) Expression of *foxg1* in the telencephalon and *shha* in the ZLI are normal in *zic2a* morphants. Note that the AD area, bordered by expression of *foxg1* anteriorly and *shha* posteriorly, is reduced in *zic2a* morphants. (M) The AD area was measured in pixels^2 using Axiovision software (Zeiss). The bar graphs represent average AD areas calculated from 12 conMO injected embryos and 18 zic2aMO injected embryos. Standard error bars shown, results significant at p = .001. (N) Summary of the effects of Zic2a depletion on forebrain regionalization. The strongest defect is observed in the AD, indicated in red. All embryos are at Prim-5 stage and are shown in lateral views with anterior to the left. Arrows mark the PT and arrowheads mark expanded domains in the telencephalon and hypothalamus. te = telencephalon, th = thalamus, pt = prethalamus, h = hypothalamus.

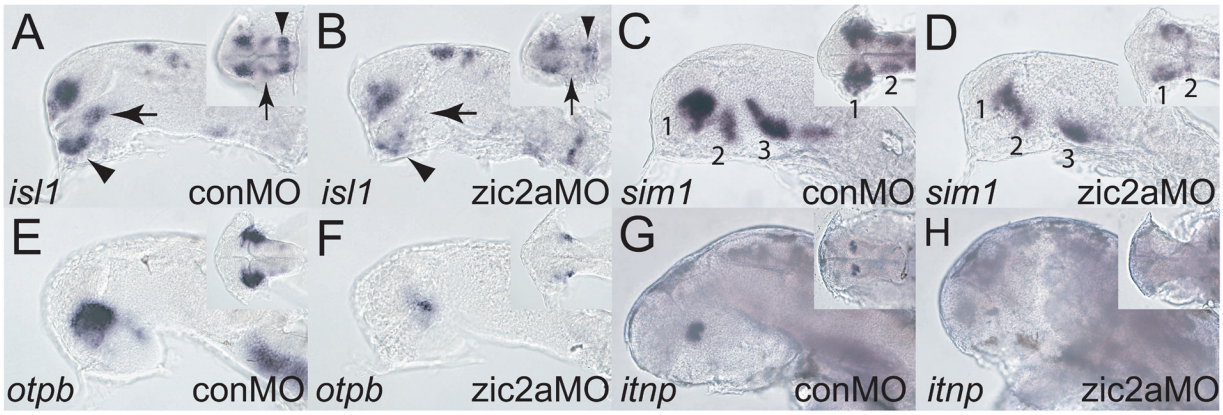

Fig. 3. Zic2a is required for diencephalic neurogenesis

The effect of Zic2a depletion on neurogenesis in the forebrain was examined using ISH with neuronal markers. (A, B) *isl1* expression in *zic2a* morphants at Prim-5 shows loss of the ventrorostral cluster (VRC, arrow), and a fusion of the dorsorostral cluster (DRC, arrowhead) (35/39 embryos, 2 exp.). (C, D) *sim1* expression in the preoptic area is strongly reduced (27/36 embryos, 2 exp.) at Prim-5. (E, F) *otpb* expression in the preoptic area is also strongly reduced (30/30 embryos, 2 exp.). (G, H) Expression of *itnp*, a marker of differentiated neurons at 2dpf, is lost in zic2aMO injected embryos (28/34 embryos, 2 exp.). Embryos are shown in lateral view with anterior to the left. Insets are ventral views of the same embryos, except in A and B, which are anterio-ventral views. Numbers in panels C and D mark different neural clusters.

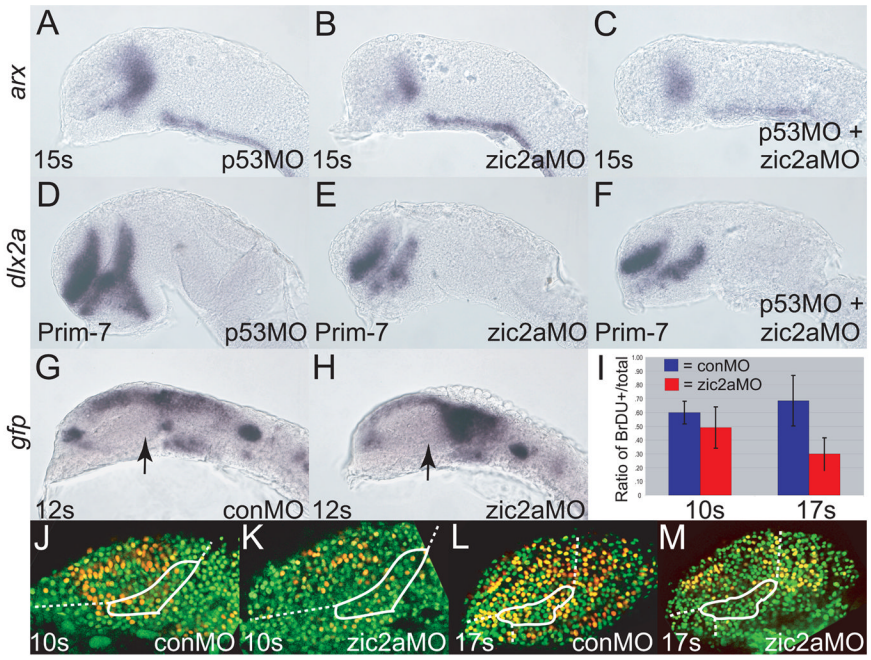

Fig. 4. Zic2a regulates proliferation, but not apoptosis or differentiation of PT precursors

Embryos were injected singly or co-injected with zic2aMO and p53MO and stained out for expression of *arx*, an early PT marker at 15 somites (A-C), *dlx2a*, a late PT marker, at Prim-7 (D-F), or *gfp* (G, H). (A) *p53* morphants show normal expression of *arx* (26/26 embryos, 2 exp.). (B) Embryos injected with zic2aMO alone show a strong reduction of *arx* expression at 15s (13/17 embryos, 2 exp.). (C) Co-injection of zic2aMO and p53MO leads to a similar reduction of *arx* expression (11/16 embryos, 2 exp.). (D) p53MO-injected embryos show no patterning defect at Prim-7 (29/30 embryos, 2 exp.). (E) zic2aMO-injected, or (F) zic2aMO and p53MO co-injected embryos show equivalent loss of *dlx2a* expression at Prim-7 (19/26 embryos, 2 exp. and 33/45 embryos, 2 exp. respectively). (G, H) Transgenic Tg(HuC:*gfp*) embryos express *gfp* in post-mitotic neurons. (G) conMO-injected transgenic embryos show no evidence of *gfp*-positive post-mitotic cells in the PT at 12s. (H) zic2aMO morphants do not contain prematurely differentiating cells in the PT at 12s (17/17 embryos, 3 exp.). (I) Ratios of BrdU positive cells/total cells in conMOs and zic2aMOs at 10s and 17s.10s analysis revealed no significant difference between conMO-injected (n = 5) and zic2aMO-injected (n = 4) embryos.17s analysis showed a significant difference (p = .005) between conMOs (n = 5) and zic2aMOs (n = 4). (J-M) Representative confocal sections of MO-injected embryos, showing BrdU-positive cells in yellow and BrdU-negative nuclei in green. White outlines the approximate prethalamic area determined by *arx* expression at the same stages. Embryos are shown in lateral view, anterior to the left. Arrows mark the PT.

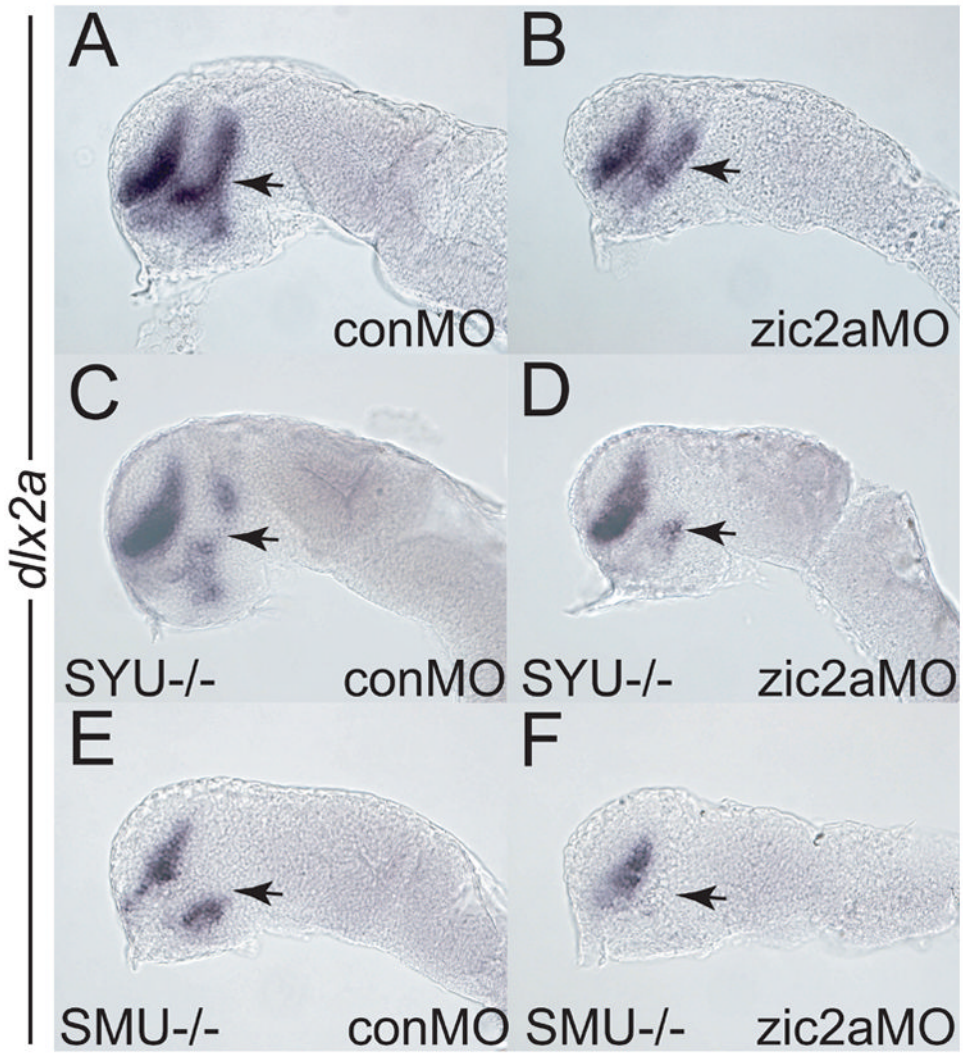

Fig. 5. Zic2a promotes PT formation in cooperation with components of the hedgehog signaling pathway

Embryos of different genetic backgrounds were injected with a conMO (A, C, E) or a zic2aMO (B, D, F) and stained for *dlx2a* expression by ISH at the prim-5 stage. (A, B) Wild type embryos depleted of Zic2a (B) show a typical reduction of PT relative to control morphants (A). (C) Homozygous *syu*^{t4} mutant embryos show reduction of PT *dlx2a* (9/35 embryos, 2 exp.). (D) *syu*^{14} mutants depleted of Zic2a exhibit an almost complete loss of *dlx2a* in the PT (17/74 embryos, 3 exp.). (E) homozygous *smo*^{b641} embryos show reduced *dlx2a* expression in the PT (25/83 embryos, 3 exp.). (F) *smo*^{b641} mutants depleted of Zic2a show complete loss of *dlx2a* in the PT (60/195 embryos, 4 exp.). Embryos are shown in lateral view, anterior to the left. Arrows point to the PT.

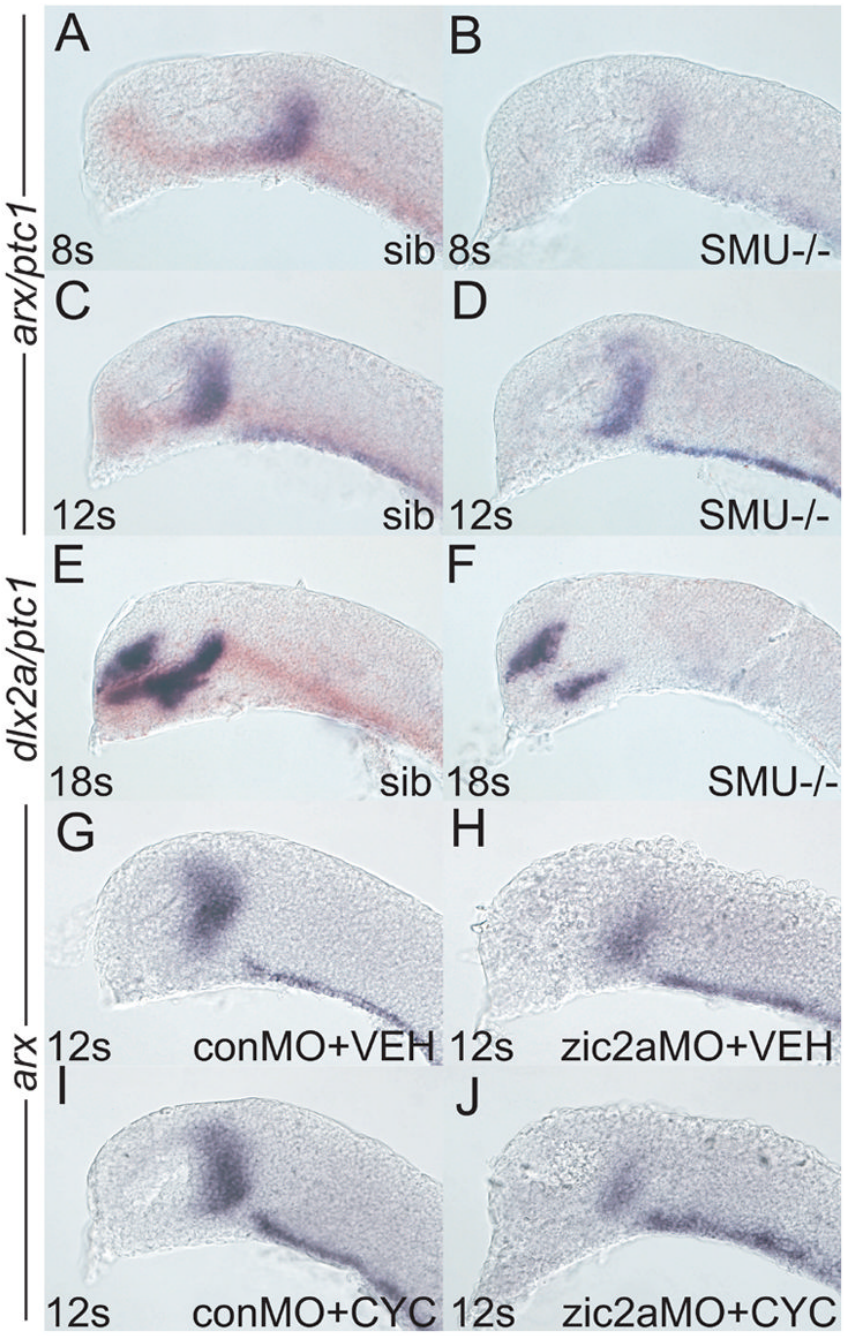

Fig. 6. Zic2a acts before hedgehog signaling to promote maturation of the PT primordium

Embryos of different genetic backgrounds were injected with conMO or zic2aMO and stained for *arx* expression by ISH, except in E, F, which were stained for *dlx2a*. (A-F) Embryos were derived from a $smo^{b641/+}$ incross. Wildtype and heterozygous siblings were identified by the presence of *ptc1* expression (orange), while mutant embryos lacked any *ptc1* expression. (A) Wildtype sibling embryos (84/117) have very similar *arx* expression as (B) mutant siblings (33/107 embryos) at 8s. (C) Wildtype embryos at 12s (70/95 embryos) are indistinguishable from (D) mutant siblings (25/95 embryos). (E) At 18s, *dlx2a* is strongly expressed in the PT of wildtype embryos (109/143 embryos). (F) Mutant embryos display a dramatic reduction of *dlx2a* expression by 18s (23/132 embryos). (G, H) Embryos were injected with either conMO

(G, 13/13 embryos) or zic2aMO (H, 7/7 embryos), treated with vehicle at 50–60% epiboly and fixed at 12s. (I, J) Embryos were injected with conMO (I, 12/12 embryos) or zic2aMO (J, 8/8 embryos), treated with 10μM cyclopamine at 50–60% epiboly and fixed at 12s. Embryos are shown in lateral view, anterior to the left.

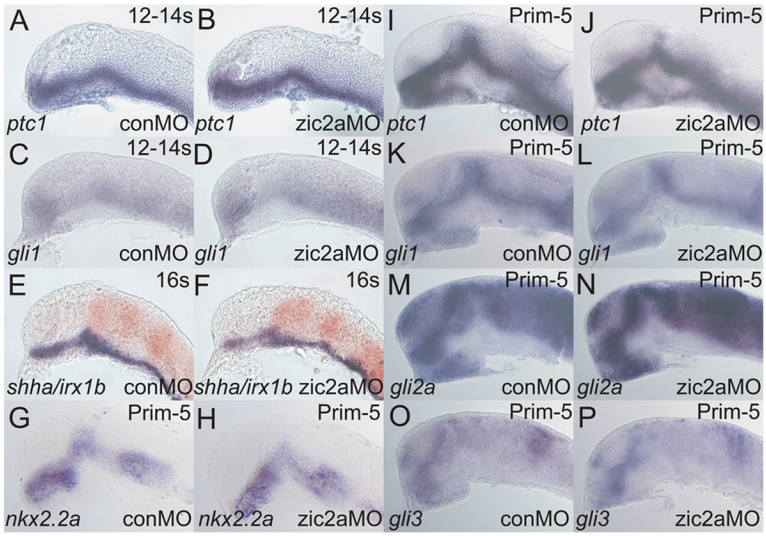

Fig. 7. Zic2a controls PT patterning independent of hedgehog signaling

Embryos were injected with conMO or zic2aMO, and examined by ISH for expression of the following markers, which were expressed correctly in zic2a moprhants. (A, B) *ptc1* at 12–14s (20/20 morphants, 2 exp.). (C, D) *gli1* at 12–14s (9/9 morphants, 2 exp.). (E, F)*shha* at 16s (38/38 morphants, 2 exp.). (G-P) Embryos are at Prim-5 (see Supplemental Table 1 for numbers). (G, H) *nkx2.2a*. (I, J) *ptc1*. (K, L) *gli1*. (M, N) *gli2a*. (O, P) *gli3*. Embryos are shown in lateral views, anterior to the left.

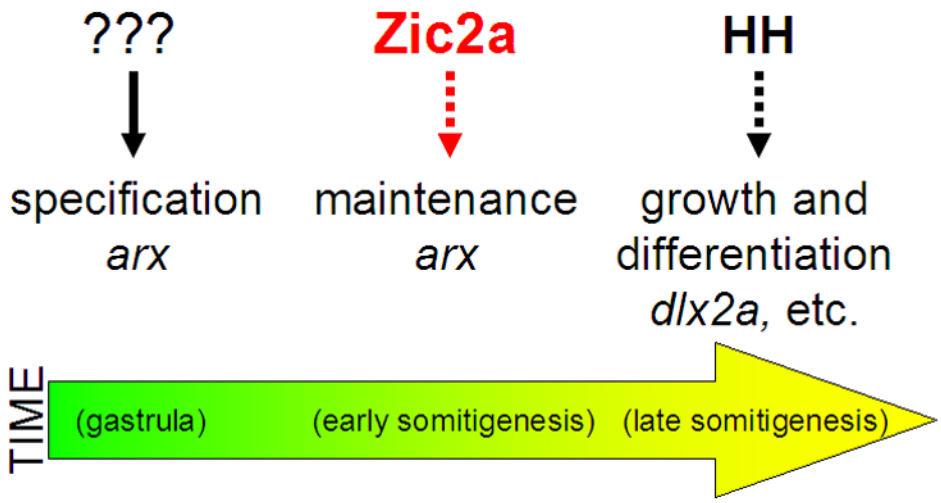

Fig. 8. A proposed model for Zic2a function in the developing prethalamus

zic2a is expressed transiently in the PT primordium during early somitogenesis and acts there to maintain transcription of *arx*. *arx*, and possibly other PT-specific transcription factors, are in turn required to promote growth of the PT primordium and correct neurogenesis in the PT and the adjacent preoptic area.