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A novel role for zebrafish *zic2a* during forebrain development

Nicholas A. Sanek [a,b], Yevgenya Grinblat [a,*]

[a] *Departments of Zoology and Anatomy, University of Wisconsin, Madison, WI 53706, USA*
[b] *Genetics Ph.D. Training Program, University of Wisconsin, Madison, USA*

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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 *glis*, 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., 2008; 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).

*ZIC*2, a zinc-finger transcription factor belonging to the *Zic* (*zi*nc finger of the *c*erebellum) 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

* Corresponding author.
E-mail address: ygrinblat@wisc.edu (Y. Grinblat).

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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 sig aling 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 (4 uM) were cut with a steel blade on an American Optical Company microtome. Nuclei were counterstained with Methyl Red. Confocal images taken with a 25× lens on an Axiovert 100 M (Carl Zeiss MicroImaging, Inc.) with Lasersharp Confocal Package (model 1024, Bio-Rad) or with a 40× 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 1× Danieau buffer (Nasevicius and Ekker, 2000) to 1–2 ng/nl (zic2aMO), 4–6 ng/nl (Zic2a AUG and PROX), or 3–4 ng/nl (conMO). 1 nl was injected at 1–2 cell stage. Cyclopamine (Sigma #C4116 or Toronto Research Chemicals #C988400) was used at 10 μM 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 (Figs. 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