Zebr­afish zic2a patterns the forebrain through modulation of Hedgehog-activated gene expression

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Holoprosencephaly (HPE) is the most common congenital malformation of the forebrain in human. Several genes with essential roles during forebrain development have been identified because they cause HPE when mutated. Among these are genes that encode the secreted growth factor Sonic hedgehog (Shh) and the transcription factors Six3 and Zic2. In the mouse, Six3 and Shh activate each other’s transcription, but a role for Zic2 in this interaction has not been tested. We demonstrate that in zebrafish, as in mouse, Hh signaling activates transcription of six3b in the developing forebrain. zic2a is also activated by Hh signaling, and represses six3b non-cell-autonomously, i.e. outside of its own expression domain, probably through limiting Hh signaling. Zic2a repression of six3b is essential for the correct formation of the prethalamus. The diencephalon-derived optic stalk (OS) and neural retina are also patterned in response to Hh signaling. We show that zebrafish Zic2a limits transcription of the Hh targets pax2a and fgf8a in the OS and retina. The effects of Zic2a depletion in the forebrain and in the OS and retina are rescued by blocking Hh signaling or by increasing levels of the Hh antagonist Hip, suggesting that in both tissues Zic2a acts to attenuate the effects of Hh signaling. These data uncover a novel, essential role for Zic2a as a modulator of Hh-activated gene expression in the developing forebrain and advance our understanding of a key gene regulatory network that, when disrupted, causes HPE.

KEY WORDS: Zic2, Six3, Hedgehog signaling, Pax2, Prethalamus, Optic stalk, Retina, Zebrafish

INTRODUCTION

The vertebrate forebrain is subdivided into the telencephalon, which gives rise to the cerebral cortex, and the diencephalon, which comprises the future thalamus, prethalamus (PT) and hypothalamus. The blueprint for these subdivisions is established early in development as patterned gene expression (Wilson and Houart, 2004; Rhinn et al., 2006). Although many of the genes play key roles in forebrain development, regulatory relationships between these genes are poorly understood. Hedgehog (Hh) proteins secreted from the ventral diencephalic midline play a central role in patterning the early forebrain primordium (Bertrand and Dahmane, 2006; Ingham and Placzek, 2006). Hh function results in transcriptional activation of several targets, many of which encode transcription factors (TFs). The homeobox TF gene six3 is transcriptionally activated by Hh signaling and directly activates shh transcription (Geng et al., 2008; Jeong et al., 2008). six3 function is essential for correct development of all forebrain derivatives, as mice and human with compromised six3 or shh function develop with a profound forebrain defect termed holoprosencephaly (HHE) (Krauss, 2007).

In addition to regulating genes important for development of the forebrain proper, Hh signaling regulates the transcription of genes involved in retinal development. The neural retina is initially part of the forebrain primordium, but separates from it early in development through evagination to form bilateral optic vesicles. A transient optic stalk (OS) connects each optic vesicle to the forebrain (Schmitt and Dowling, 1994). Owing to their common origin, the early OS and retinal domains are contiguous. Hh proteins secreted from the ventral-diencephalic-midline pattern the initially uniform OS/retinal field (Ekker et al., 1995; Varga et al., 2001; Schimmert et al., 2003; Lee, J. et al., 2008), partitioning it into three domains: the OS, the ventral retina and the dorsal retina (Lupo et al., 2005). The future OS lies closest to the Hh source and expresses several Hh-dependent homeobox TF genes, including pax2a (Macdonald et al., 1995; Takeuchi et al., 2003; Mui et al., 2005). The optic vesicles, distal to the Hh source, express pax6, a gene with key regulatory roles during retinal development (Gehring, 1996).

The zinc-finger TF Zic2 plays a key role during early forebrain development. Mutations in mammalian ZIC2 can cause HPE (Brown et al., 1998), and the zebrafish ortholog zic2a is required for correct formation of the ventral diencephalon (Sanek and Grinblat, 2008). In the developing retina, Zic2 and other Zic family members are required for retinal axon guidance in mouse and chick (Herrera et al., 2003; Zhang et al., 2004; Lee, R. et al., 2008). Although essential functions for Zic genes are well documented, the molecular basis for these functions during forebrain and eye development is unclear.

In this study, we further examine the function of zebrafish zic2a during forebrain development, in particular its regulatory relationship with Hh signaling and six3b. We show that zebrafish Zic2a negatively modulates Hh-dependent transcription of six3b in the forebrain. Zic2a repression of six3b is essential for the correct formation of the PT, a diencephalic derivative. Zic2a is also required in the OS and retinal primordium, where it inhibits Hh-dependent expression of pax2a. These data identify a novel role for Zic2a in the regulatory network, coordinated by Hh signaling, that patterns the vertebrate forebrain.

MATERIALS AND METHODS

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 (Kimmel et al., 1995). The following zebrafish strains were used: wild-type AB, smu6041 (Varga et al., 2001), syt4 (Odenthal et al., 2000), pax2a3C693 (Erickson et al., 2007), Tg(fou4f3:gap42-GFP)y256 (Xiao et al., 2005), and Tg–8.0cdhb:lynGFPzf106 (LeClandey et al., 2008).

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Accepted 1 September 2009
Mutant genotyping

 smo<sup>641</sup> homozygous mutant embryos were positively identified by characteristic morphological defects and a lack of ptc1 expression. pax2a<sup>2553</sup> embryos were identified by a lack of fgf8a expression in the mid-hindbrain boundary (MHB), and by morphological defects.

In situ hybridization (ISH)

Antisense RNA probes were transcribed using the MAXIscript Kit (Ambion, Foster City, CA, USA) from the following plasmid templates: arx (Miura et al., 1997), atoh7 (Masi et al., 2000), epha5a (Piker and Brand, 2005), fgf8a (Furthauer et al., 1997), fox1 (Rohr et al., 2001), pax2a (Hoyle et al., 2004), pax2b (Pfeffer et al., 1998), pair (Krauss et al., 1991), rzd3 (Jeong et al., 2007), sri3 (Seo et al., 1998), iil17rd (Tsang et al., 2002), spry4 (Furthauer et al., 2001), tbr1 (Take-uchi et al., 2003), vax2 (Gross and Dowling, 2005) and zic2a (Grinblat and Sive, 2001). Single-color ISH was carried out as previously described (Gillhouse et al., 2004). Double fluorescent analysis was carried out using digoxigenin (DIG)-labeled probes (Roche, Basel, Switzerland) detected with anti-DIG Fab fragments (Roche) and Fast Red substrate (Sigma, St Louis, MO, USA), combined with fluoresceinated-dextran (FLU-DEX) (Invitrogen, Carlsbad, CA, USA) detected with anti-substrate (Sigma, St Louis, MO, USA), and zic2aMO. Digoxigenin (DIG)-labeled probes were digoxigenin-labeled using digoxigenin (DIG)-labeled RNA polymerase (Roche, Basel, Switzerland) and DIG RNA labeling mix (Roche). Relative standard curves were produced using 1:4 serial dilutions of the template. Each template type was reverse-transcribed in duplicate using an iScript Select cDNA Synthesis Kit (BioRad) with oligo(dT) primers. Duplicate reactions were pooled prior to qPCR.

Real-time PCR methods

Primers were designed against sequences that spanned an intron near the 3′ end of each gene and produced an amplicon of ~200 bp. Primer specificity was checked by agarose gel electrophoresis and melting curve analysis. The 20 μl reactions comprised: 1× Power SYBR Green Master Mix (Applied Biosystems), 200 nM each of primer and 2 μl of 1:10 dilution of cDNA template. Components shared across reactions were mixed first and then aliquoted to ensure equivalent reaction conditions. Each template type was linear in log space (R² > 0.997 in each case; primer efficiency was nearly 100%).

Real-time PCR data analysis

Cycle threshold (Ct) values were filtered such that values greater than two standard deviations from the average Ct of each technical replicate were excluded from analysis. The remaining Ct values were analyzed using the ‘relative standard curve’ method as outlined in the ABI Prism 7700 User Bulletin #2. Relative standard curves were produced using 1:4 serial dilutions of cDNA as a template and the amplification for each primer set was linear in log space (R² = 0.997 in each case; primer efficiency was nearly 100%; data not shown). six3b primer sequences were 5′-ATCGAAGACA-GAGGGACAGG-3′ and 5′-ATACTGAGAGCTGGTCGT-3′; gadph primer sequences were 5′-ATCAAGAAGACTGCTAAGGCT-3′ and 5′-ATCTACTCTCTGGAGGCAATGT-3′.

Knockdown assays

Three antisense morpholin oligomers (MOs) were used to knock down expression of zic2a in this study: two translation-blocking MOs (zic2a AUG, CGATGAAATTCATATCCCCGCTACA; zic2a PROX, CTCCTCGAAGACTGCTTACCC) and a splicing-blocking MO (zic2aMO, CTCACCTGAGAAAGAACATCA) (Nyholm et al., 2007). Six3bMO (CTCTAAAGGAGACCTGAAACCATG) was purchased from Open Biosystems (Ando et al., 2005; McCollum et al., 2007). Control morphants were generated using the standard control MO (conMO; Genetools, Philomath, OR, USA). MOs were diluted in 1× Danieau buffer (Nasevicius and Ekker, 2000) to 1-2 ng/nl (zic2aMO), 8 ng/nl (six3bMO), 4-6 ng/nl (zic2a AUG and PROX), or 3-4 ng/nl (conMO), and 1 nl was injected per embryo at the 1- to 2-cell stage. Cyclospamine (Toronto Research Chemicals, North York, Ontario, Canada) was used at 100 μM as previously described (Tuyurina et al., 2005). SU5402 (Calbiochem, Darmstadt, Germany) treatments were carried out on embryos with chorions at embryonic day 3 (E3). SU5402 was dissolved in DMSO and used at 10 μM (Yamauchi et al., 2006).

Overexpression assays

Sense RNA transcripts were made with the mMessage mMachine Kit (Ambion) from the following templates: shha (Ungar and Moon, 1996) and hhip (Ochi et al., 2006). mRNA was purified using spin columns (Bio-Rad, Hercules, CA, USA) and diluted in RNAse-free water. Injections of 12.5 or 25 pg of shha mRNA and 3.8 pg of hhip mRNA were administered per embryo.

RESULTS

six3b transcription is activated by Hh signaling in the zebrafish forebrain

An important regulatory relationship between Hh signaling and six3 has been demonstrated in the mouse forebrain (Geng et al., 2008; Jeong et al., 2008). To test whether this relationship exists in zebrafish, we asked whether Hh signaling is required to activate six3b transcription in the zebrafish forebrain and retina. Embryos treated with cyclopinome to block Hh signaling showed reduced six3b expression in the forebrain (Fig. 1A,B), but not in the retina. Similarly, smo<sup>641</sup> mutants, deficient in Hh signaling (Varga et al., 2001), showed strongly reduced six3b expression in the forebrain (Fig. 1C,D), but continued to express six3b in the retina (Fig. 1C′,D′). These results demonstrate that Hh signaling is required for proper levels of six3b expression in the zebrafish telencephalon and diencephalon.

We next asked whether Hh signaling is sufficient to activate six3b transcription in the zebrafish forebrain. When six3b expression was assayed by ISH in embryos microinjected with low amounts of shha RNA, mildly expanded six3b expression was consistently observed in the telencephalon and diencephalon (Fig. 1G-H). Notably, this low level of shha RNA did not trigger ectopic expression of ptc1, a direct target of Hh signaling (Fig. 1E-F), suggesting that six3b is a more sensitive indicator of Hh signaling levels than ptc1. When shha-overexpressing embryos were assayed quantitatively by real-time PCR, six3b transcript levels were not found to increase significantly in proportion to increasing shha levels (see Fig. S1 in the supplementary material), suggesting that Hh signaling is not sufficient to activate six3b transcription. Together, these results indicate that, in zebrafish, Hh signaling is required for six3b transcription in the telencephalon and diencephalon and does not control six3b transcription in the retina.

Zic2a represses six3b transcription in the forebrain in an Hh-dependent manner

six3 and zic2 play crucial roles during forebrain development in mammals, yet a regulatory relationship between them has not been tested in this tissue. To determine whether six3b transcription is regulated by Zic2a in zebrafish, we employed antisense MO knockdown assays as previously described (Nyholm et al., 2007; Sanek and Grinblat, 2008). Zebrafish six3b is expressed in the anterior forebrain primordium from early somitogenesis (Seo et al., 1998). In Zic2a-depleted morphants, six3b was expressed normally...
at the 10-somite (10s) stage (Fig. 2A-B). However, at the 15s and prim-5 stages, zic2a morphants upregulated expression of six3b throughout the forebrain (Fig. 2C-F), suggesting that Zic2a functions to repress six3b starting at mid-somitogenesis. This upregulation was confirmed by qPCR analysis (Fig. 2G).

zic2a is expressed in the telencephalon and dorsal diencephalon (Sanek and Grinblat, 2008) (see Fig. S2 in the supplementary material), consistent with the possibility that Zic2a could act cell-autonomously, and perhaps directly, to repress six3b in these cells. However, in the diencephalon, zic2a is restricted to the distal OS and is not expressed in cells that ectopically activate six3b in morphants (see Fig. S2 in the supplementary material), suggesting that Zic2a functions indirectly in this area. As shha expression appears to be unaffected in Zic2a-depleted embryos (Sanek and Grinblat, 2008), we hypothesized that Zic2a might control transcription of a secreted factor that acts as an Hh agonist or antagonist to modulate the transcriptional activation of six3b. This hypothesis predicts that expression of six3b should be enhanced in zic2a morphants in an Hh signaling-dependent manner. six3b expression is greatly decreased, but not eliminated, in Hh signaling-defective smob641 embryos (Fig. 3A,C); the low level of six3b expression remaining in these mutants is likely to be due to residual Hh signaling through the maternally provided smo (Varga et al., 2001). Zic2a depletion in smo641 embryos did not result in increased six3b throughout the forebrain as is typical of zic2a morphants (Fig. 3A-D). An increase in six3b expression was observed relative to smo641 embryos, but this expression was weak and restricted anteriorly, consistent with an enhanced transcriptional response to residual Hh signaling in smo641 embryos. Similar results were obtained in zic2a morphants treated with cyclopamine to block Hh signaling (data not shown). These findings support the hypothesis that Zic2a functions to regulate six3b transcription in an Hh signaling-dependent manner.

If Zic2a functions to control the level or extent of Hh signaling, the addition of an exogenous Hh antagonist should also rescue the zic2a morphant defect. To test this prediction, we used RNA overexpression of Hhip, a membrane-bound and secreted Hh-binding protein that antagonizes Hh signaling in mice and zebrafish (Chuang and McMahon, 1999; Coulombe et al., 2004; Ochi et al., 2006). Although Zic2a depletion resulted in six3b expansion throughout the forebrain (Fig. 3E-F), and hhip mRNA injection did...
not affect six3b expression (Fig. 3G), embryos co-injected with hhip mRNA and zic2aMO showed significant rescue of six3b expression in the telencephalon and diencephalon (Fig. 3H). Together, these data suggest that Zic2a functions as a negative modulator of Hh-induced gene expression in the zebrafish forebrain.

**Zic2a function in the prethalamus requires six3b repression**

We have previously shown that Zic2a depletion causes defects in the ventral diencephalon-derived PT (Sanek and Grinblat, 2008). To test whether ectopic expression of six3b in zic2a morphants was responsible for this defect, we knocked down Zic2a and Six3b simultaneously using a six3-specific MO (Ando et al., 2005; McCollum et al., 2007), and assayed the resulting embryos for expression of dlx2a and arx, markers of the PT (arrowheads in Fig. 4). As previously described, dlx2a was reduced in the zic2a morphant PT (Fig. 4A,B). dlx2a was unaffected in six3b morphants (Fig. 4C). In zic2a; six3b double morphants, the dlx2a PT domain was similar to that in the controls in the amount of staining, although it was somewhat mispatterned, indicating that Six3b depletion partially rescues the PT defect in zic2a morphants (Fig. 4D,E). arx expression, reduced in zic2a morphants, was similarly rescued by Six3b knockdown (Fig. 4F-J). These data suggest that Zic2a promotes PT development through repression of six3b, and suggest that Six3 might play a role in the development of the diencephalon.

**Zic2a restricts the expression of optic stalk markers**

Because Zic2a modulates the transcriptional readout of Hh signaling in the developing forebrain, and another major role for Hh signaling is to pattern the adjacent OS and retinal precursors, we next asked whether Zic2a is involved in the development of the OS and retina. Zic2a-depleted embryos were examined at the prim-5 stage for expression of several regional markers. rx3, which marks the pre-optic area of the anterior diencephalon adjacent to the OS, was expressed normally (Fig. 5A,B). The expression of the proximal OS-restricted vax1 was also unaffected (Fig. 5C,D). By contrast, pax2a (Fig. 5E,F) and fgf8a (Fig. 5G,H), distal OS markers, were strongly expanded into the ventral retina, as were OS markers pax2b and il17rd (data not shown). The specificity of the pax2a expansion phenotype was confirmed in two ways: by using non-overlapping translation-blocking MOs against zic2a to produce the same defect.
The effect of Zic2a depletion was examined using ISH. (A-D) The following markers were expressed normally in control and zic2a morphants: rx3 in the pre-optic area of the hypothalamus (A, 36/36 control morphants, n=3; B, 42/44 zic2a morphants, n=3); vax1 in the proximal OS (C, 50/50 control morphants, n=4; D, 58/68 zic2a morphants, n=4). (E,F) pax2a is expressed normally in control morphants (E, 51/52 embryos, n=4), and is expanded into the retina in zic2a morphants (f, 123/131 embryos, n=6). (G,H) fgf8a is restricted to the OS in control morphants (G, 29/29 embryos, n=2), and expanded into the retina when Zic2a is depleted (H, 44/53 embryos, n=3). Green arrowheads in F,H point to abnormal expression in the ventral retina. A-H are lateral views, anterior to the left. A'-H' are ventral views of the same embryos, anterior to the left. All embryos are at prim-5.

(data not shown), and by knocking down Zic2a together with p53, to show that the pax2a defect in zic2a morphants was not due to increased cell death (see Fig. S3 in the supplementary material). The pax2a patterning defect first manifested in Zic2a-depleted embryos by 19s (see Fig. S4 in the supplementary material). By contrast, fgf8a was correctly patterned at 19s (see Fig. S4 in the supplementary material), and did not become expanded until 21s (data not shown).

The regulatory relationships between zic2a, fgf8a and pax2a were further analyzed using Zic2a MO assays in combination with pharmacological inhibition of fibroblast growth factor (FGF) signaling and pax2a mutants. This analysis demonstrated that zic2a acts upstream of pax2a and fgf8a (see Fig. S5 in the supplementary material), and that fgf8a promotes pax2a expression downstream of Zic2a function (see Fig. S6 in the supplementary material), which is consistent with previous findings (Nakayama et al., 2008).

We next asked whether early retinal development was disrupted in Zic2a-depleted embryos concomitant with expansion of OS markers into the retina. During normal development, the OS-retinal border is marked by expression of pax2a. In zic2a morphants, pax2a was expanded into the ventral retina and the OS-retinal border did not form properly (Fig. 6A,B), indicative of the incomplete retinal closure defect coloboma. pax6a expression in the morphant retina, assayed relative to nasally localized green fluorescent protein (GFP) in the Tg(−8.0;ldhb:lynGFP)z116 transgenic line (Lecaudy et al., 2008), was largely unaffected (Fig. 6C,D). The naso-temporal markers foxg1 and efna5a were also patterned correctly (see Fig. S7 in the supplementary material); however, the ventral retinal marker aldh1a3 was mildly expanded in zic2a morphants (Fig. 6E,F). Analysis of these retinal markers showed that retinal patterning is largely unaffected by Zic2a depletion, except in the ventral region. However, the morphological defect (coloboma) that was evident by prim-5 (Fig. 6A-F) persisted until later stages (Fig. 6G,H). Expression of atoh7 in the first differentiating neurons of the ventral retina was absent in zic2a morphants, although neurogenesis at later stages was not grossly affected (see Fig. S7 in the supplementary material). Together, these data show that Zic2a is dispensable for overall retinal patterning and neurogenesis, but functions to promote normal development of the ventral retina and to repress OS marker transcription in the ventral retina at stages prior to 19s.

To better understand the regulatory relationships between zic2a and the other OS markers, we mapped their expression relative to each other at two stages: at 16s, soon after these genes are first expressed in the presumptive OS-retina, and at 23s, near the end of somitogenesis. At 16s, pax2a was expressed in a broad domain that included the OS, the proximal optic vesicle and part of the distal optic vesicle (Fig. 7A). pax6a expression was limited to the distal optic vesicle (Fig. 7B), where it overlapped extensively with pax2a. fgf8a was expressed in the anterior forebrain but, at this stage, did not extend into the OS (Fig. 7C). zic2a was expressed strongly in the OS and weakly in the distal retina (Fig. 7D). vax1 was expressed broadly in the OS and in the presumptive retina at this stage (Fig. 7E). These expression patterns, summarized in Fig. 7F, suggest that the OS-retinal border is already established by 16s, with the expression of zic2a restricted on the OS side and pax6a on the retinal side; however, expression of pax2a and vax1 is not yet restricted at this border. By 23s, zic2a, pax2a and vax1 expression domains were largely restricted to the OS, where they overlapped almost completely (Fig. 7G,H; data not shown). Together, these data are consistent with a role for Zic2a in OS-retinal border formation during somitogenesis.

Hh activates zic2a transcription and Zic2a modulates Hh-activated gene transcription in the optic stalk and retinal primordium

Because Hh signaling is an essential activator of OS gene expression (Ekker et al., 1995; Macdonald et al., 1995; Varga et al., 2001; Stenkamp and Frey, 2003), we asked whether it was also required to activate zic2a expression in the OS. Hh signaling-deficient smoΔΔ mutants showed a marked reduction in OS zic2a expression (Fig. 8A,B,D,E). To determine whether Hh signaling was sufficient to activate zic2a expression, wild-type embryos were injected with shha mRNA. zic2a expression was...
dramatically expanded in the retina of embryos overexpressing shha, whereas zic2a expression outside of the OS and retina appeared reduced (Fig. 8C,F). These results show that Hh signaling is both necessary and sufficient to activate zic2a expression in the OS and retinal precursors.

Because Hh signaling activates OS gene expression and Zic2a represses it, we hypothesized that zic2a might negatively modulate the transcriptional outcome of Hh signaling in the OS. If this hypothesis were correct, OS marker expansion in response to Zic2a depletion should require intact Hh signaling. Zic2a-depleted embryos with functional Hh signaling (i.e. wild-type embryos and smo^{b644} heterozygotes) showed expanded pax2a relative to control morphants (Fig. 9A-B), and homozygous smo^{b644} mutants had little or no pax2a expression (Fig. 9C). Although smo^{b644} homozygotes depleted of Jic2a expressed some pax2a in the ventral retina, this expression was weaker, and the domain less expanded, than in zic2a morphants (Fig. 9D). Similar results were obtained for fgf8a expression in Zic2a-depleted smo^{b644} mutants and for pax2a expression in Zic2a-depleted shha^{d4} mutants (data not shown). Together, these results show that zic2a function during OS and retinal patterning is dependent on the presence of an intact Hh signaling pathway.

If Zic2a functions as a negative modulator of transcription downstream of Hh signaling, Zic2a depletion should sensitize the embryo to increased Hh signaling, i.e. exogenous Hh should activate pax2a expression more strongly in zic2a morphants than in controls. As shown in Fig. 9E-H, simultaneous introduction of zic2aMO and shha mRNA resulted in a more robust ectopic expansion of pax2a in the OS and retina than did shha mRNA injection alone. Similar results were obtained for OS markers fgf8a and spry4 (see Fig. S8 in the supplementary material). These results further support a role for Zic2a as a negative modulator of Hh-induced gene expression during OS and retinal patterning.

Zic2a function is required in the ventral retina before 19s, when pax2a expansion is first observed in zic2a morphants. However, zic2a is not transcribed in the ventral retina until late somitogenesis, after 23s (Fig. 7; see Fig. S2 in the supplementary material). These observations argue that in the ventral retina, as in the diencephalon, Zic2a acts to restrict expression of Hh target genes outside of its expression domain. Furthermore, this model predicts that an Hh antagonist should rescue patterning defects in Zic2a-depleted...
Zic2a limits Hh effects in the diencephalon

The central role of Hh signaling during forebrain development is well documented in non-mammalian vertebrates, particularly in the zebrafish (Karlstrom et al., 1999; Tuyrina et al., 2005). Similarly, mammalian Six3 and its two zebrafish orthologs, six3a and six3b, play essential roles in the developing forebrain and retina (Kobayashi et al., 1998; Lagutin et al., 2003; Inbal et al., 2007; Lavado et al., 2008). Although Six3 has been shown to act indirectly upstream of Zic2 transcription during early Xenopus development (Ge stri et al., 2005), the regulatory relationship between Hh signaling, Six3 and Zic2 has not been extensively explored. In this study, we show that the relationship between Hh and Six3 is at least partly conserved in zebrafish, as Hh is required for six3b expression. We further show that Zic2a is an essential modulator of Hh-induced expression of six3b in the zebrafish forebrain. Although we have not analyzed six3a expression in zic2a morphants, the six3MO used here has been reported to inhibit both six3a and six3b (Ando et al., 2005), raising the possibility that Zic2a regulates both six3 orthologs.

The forebrain of Zic2a-depleted embryos develops surprisingly normally given the extent of six3b expansion throughout the telencephalon and diencephalon, perhaps because this derepression embryos. We have shown that Hhip can rescue six3b expansion in the forebrain (Fig. 3), and have similarly tested the ability of Hhip to rescue pax2a expansion in the ventral retina. Although zic2a morphants showed the typical pax2a expansion (Fig. 10A-B) and embryos injected with zebrafish hhip mRNA developed normally (Fig. 10C), embryos co-injected with zic2aMO and hhip mRNA showed a nearly normal expression of pax2a (Fig. 10D), indicating a strong rescue of the OS-retinal defect in zic2a morphants by Hh antagonism. Taken together, these results argue that Zic2a controls OS and retinal patterning non-cell-autonomously and in an Hh-dependent manner.

**DISCUSSION**

Hh signaling, an essential regulator of vertebrate forebrain development (Bertrand and Dahmane, 2006; Fuccillo et al., 2006; Ingham and Placzek, 2006), functions, in part, through coordinating expression patterns of several target genes, among them six3 in the forebrain and pax2 in the OS and retina. Data presented here identify zebrafish zic2a as an Hh-regulated OS marker, and as an essential novel modulator of Hh-activated gene expression in the forebrain, OS and retina. We show that Zic2a restricts transcription of known Hh target genes: six3b in the telencephalon and diencephalon, and pax2a in the ventral retina. We propose that zic2a functions in the OS to regulate transcription of an Hh agonist or antagonist (Fig. 11). This novel regulatory mechanism is important for the formation of the PT and correct patterning of the ventral retina in zebrafish, and could possibly be conserved in mammals.

**Zic2a: a step towards understanding the molecular basis of HPE**

The majority of mutations identified in HPE patients are found in three genetic loci – SHH, ZIC2 and SIX3 (Cohen, 2006; Dubourg et al., 2007; Krauss, 2007) – suggesting that these genes may be part of a common regulatory network. In support of this idea, transcriptional crossregulation between Six3 and Shh was recently documented in the mouse forebrain and shown to be essential for correct formation of the ventral diencephalic midline (Geng et al., 2008; Jeong et al., 2008), but a role for Zic2 in this network has not been tested in the mouse model.

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**Fig. 8.** Hh is necessary and sufficient for zic2a expression in the OS and ventral retina. (A-F) zic2a is expressed in the OS at 16s (A). In smo−/− mutants, zic2a expression is reduced in the OS at this stage (B, 14/14 embryos, n=2), but not elsewhere. shha mRNA-injected WT embryos express zic2a ectopically in the OS and retina at 16s (C, 13/13 embryos, n=1). zic2a expression is still reduced in smo−/− mutants at 19s (D,E, 30/30 embryos, n=2), and expanded in shha-injected retina (F, 19/19 embryos, n=1). The same result was observed at prim-5 (39/42 embryos, n=2, data not shown). Arrowheads point to the OS. Embryos are in lateral view, anterior to the left.

**Fig. 9.** Zic2a patterns the OS and retina in an Hh-dependent manner. (A-D) Zic2a-depleted WT siblings show expanded pax2a (B, 99/99 embryos, n=4), whereas smo−/− mutants injected with conMO lose pax2a expression (C, 12/12 embryos, n=2). Zic2aMO-injected smo−/− mutants show a rescued pax2a phenotype (D, 30/30 embryos, n=4). (E-H) Co-injection of conMO and shha mRNA, or injection of zic2aMO alone, causes moderately expanded pax2a expression (F, 20/34 embryos, n=2; G, 30/30 embryos, n=2, respectively). Embryos injected with zic2aMO and shha mRNA show strong pax2a expansion (H, 23/33 embryos, n=2). Arrowheads mark the OS. Panels A-H are lateral view, anterior to the left. Panels A’-H’ are ventral views of the same embryos, anterior to the left. All embryos are at prim-5.
takes place relatively late in development, at mid-somitogenesis stages. Our data show that the forming PT is particularly sensitive to Six3b levels at mid-somitogenesis, consistent with the late role of mouse Six3 in the diencephalon (Lavado et al., 2008). These data suggest that Zic2 similarly modulates the regulatory relationship between Hh signaling and Six3 during mammalian embryogenesis, and offer a novel explanation of how ZIC2 mutations cause HPE.

Another Zic family member, zic1, was recently shown to play an essential, yet distinct, role during zebrafish forebrain development (Maurus and Harris, 2009). By contrast to Zic2a, which limits Hh signaling in the ventral forebrain, Zic1 promotes Hh expression and Zic1 depletion leads to loss of ventral forebrain tissue (cyclopia). This functional difference can be explained either by distinct DNA binding specificities of Zic1 and Zic2a or by differences in where and when they function during development. In support of the latter, the OS is likely to be the major site of Zic2a function in the forebrain, as zic2a is strongly expressed there beginning at mid-somitogenesis. In contrast, zic1 is not found in the OS until late-somitogenesis, when it is only weakly expressed there (Grinblat and Sive, 2001). The more severe phenotype caused by Zic1 depletion in zebrafish suggests that ZIC1 is more broadly required during mammalian development than ZIC2, and is consistent with the fact that ZIC1 mutations have not been identified in HPE patients.

**Is the retinal function of Zic2a conserved?**

Zebrafish embryos depleted of Zic2a develop a characteristic retinal defect. A similar defect, non-syndromic ocular colobomas (NSOC), is frequently associated with HPE; moreover, mutations in *SHH* have been correlated with this defect (Schimmenti et al., 2003). Human *ZIC2* mutations have not been associated with NSOC, and the underlying genetic causes of most ocular colobomas are not well understood (Schimmenti et al., 2003). Ocular defects, that may include coloboma, have been reported in homozygous *Zic2a*−/− mice, but not characterized (Elms et al., 2003; Herrera et al., 2003). The data presented here offer a potential explanation for these ocular defects and suggest ZIC2 as a candidate gene in human colobomas.

Coloboma in *zic2a* morphants may result from misregulation of either *pax2a* or *six3b*, as both genes have important functions in the developing OS and retina. Several lines of evidence argue that Zic2a function in the retina is not mediated through Six3b. First, OS and retinal mispatterning in *zic2a* morphants is not rescued by Six3 depletion (see Fig. S9 in the supplementary material). Second, retinal expression of *six3b* is not dependent on Hh signaling in mice (Geng et al., 2008) or zebrafish (Fig. 1). Third, *six3b* overexpression leads to a larger retina, and we do not observe an increase in retinal size in *zic2a* morphants. Although these data show that Zic2a is not a major regulator of retinal *six3b*, further analysis is required to determine whether Zic2a contributes to this regulation. Conversely, misregulation of *PAX2* has been causally linked to coloboma in human (Gregory-Evans et al., 2004) and chick (Sehgal et al., 2008), suggesting *pax2a* expansion in *zic2a* morphants as the likely cause of ventral retinal defects.

*zic2a* is expressed in the zebrafish retina, as is Zic2 in higher vertebrates (Nagai et al., 1997; Brown et al., 2003; Toyama et al., 2004). The relatively late onset of *zic2a* expression in the ventral retina allows us to distinguish its function in the OS precursors, as described in this manuscript, from its later functions. Retinal Zic2 controls guidance of ipsilaterally projecting retinal axons in mouse (Herrera et al., 2003; Garcia-Frigola et al., 2008; Lee, R. et al., 2008). This function of *zic2* is not conserved in zebrafish, where all retinal ganglion cell axons project contralaterally (Sakai and Halloran, 2006). Further studies to address retinal-specific functions of zebrafish *zic2a* will probably require conditional overexpression and genetic mutant analyses.

**How does zic2a function during forebrain patterning?**

Hh growth factors act as morphogens in the OS and retinal primordium. They are secreted from the diencephalic midline and diffuse laterally, instructing cells in proximal positions to develop as OS and cells located more distally to develop as ventral retina (Ekker et al., 1995; Macdonald et al., 1995; Take-uchi et al., 2003; Lupo et al., 2005). Hh-induced *pax2a*, *vax1* and *vax2* promote OS development, at least in part through repression of the retinal marker *pax6* (Macdonald et al., 1995; Schwarz et al., 2000; Take-uchi et al.,
Zic2a limits Hh effects in the diencephalon

2003; Mui et al., 2005). We have now shown that zic2a is also activated by Hh signaling in the OS, but unlike the other targets, does not promote OS or inhibit retinal fates. Instead, Zic2a acts to modulate the Hh-regulated pattern of gene expression. Zic2a function provides the means for Hh signaling to negatively modulate, and thus refine, its downstream signaling activity. Although negative-feedback regulation such as this has been described for several signaling pathways, including Hh signaling (Chuang and McMahon, 1999; Ochi et al., 2006; Lee, J. et al., 2008) and Fgf signaling (Furthauer et al., 2001), this negative regulation is not typically mediated via induction of a transcription factor.

As a transcription factor, Zic2a probably acts cell-autonomously to directly activate or repress transcription of its target genes. However, our data clearly indicate that Zic2a downregulates the transcription of several genes outside of its own expression domain. A plausible explanation is that Zic2a directly regulates transcription of a secreted factor, which then acts on neighboring cells to modulate Hh signaling. Although zic2a is expressed in several domains in the forebrain, the OS is the most likely site for this function because of its proximity to the tissues affected in mutants. Likely candidate targets of Zic2a include genes that facilitate or hinder the transport of Hh ligands, e.g. the Hh antagonist hhip (Chuang and McMahon, 1999; Coulombe et al., 2004; Ochi et al., 2006), and extracellular proteins such as Glypican or Megalin (Low-density lipoprotein-related protein 2 – Zebrafish Information Network), which can aid or hinder transcytosis and affect long-range signaling (Willow et al., 1996; McCarthy et al., 2002; Beckett et al., 2008). The zebrafish megalin gene is not expressed in the OS (McCarthy et al., 2002) and therefore may be repressed by Zic2a. The known zebrafish hhip gene is not expressed in the OS and is not a candidate target for Zic2a (data not shown), but at least two other hhip genes are likely to exist (Katoh and Katoh, 2006; Ochi et al., 2006). A concerted effort to identify direct transcriptional targets of Zic2a is in progress and is likely to uncover novel Hh modulators that act downstream of zic2a in OS and retinal patterning. This novel Hh negative-feedback control might also be used in other developmental contexts where Hh signaling is involved, such as the limb bud.

Acknowledgements
We thank Steve Wilson, Jeff Gross, Mary Halloran, Mary Ellen Lane, Ann Ungar, Monte Westerfield and the Zebrafish International Resource Center (ZIRC) for providing plasmids and zebrafish lines; and Jessica Pierson and other members of the Grinblat laboratory for insightful discussions throughout the course of this work and for constructive comments during preparation of this manuscript. This work was funded by an NIH RO1 grant to Y.G. Deposited in PMC for release after 12 months.

Author contributions
N.A.S. carried out the bulk of data collection and analysis; A.B.T. performed real-time PCR and analyzed the data; M.K.N. assisted with morphant analysis and real-time PCR experiments; and Y.G. participated in data analysis. All four authors contributed to manuscript preparation.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/2/3791/DC1

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