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 Hhip, 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 (HPE) (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; Schimmenti 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; Take-uchi 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, *smo^b641* (Varga et al., 2001), *syu^t4* (Odenthal et al., 2000), *pax2a^b593* (Erickson et al., 2007), *Tg(pou4f3:gap43-GFP)^s356t* (Xiao et al., 2005), and *Tg(–8.0cldnb:lynGFP)zf106* (Lecaudey et al., 2008).

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Mutant genotyping

smo^b641 homozygous mutant embryos were positively identified by characteristic morphological defects and a lack of *ptc1* expression. *pax2a^b593* 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* (Masai et al., 2000), *efna5a* (Picker and Brand, 2005), *fgf8a* (Furthauer et al., 1997), *foxg1* (Rohr et al., 2001), *pax2a* (Hoyle et al., 2004), *pax2b* (Pfeffer et al., 1998), *pax6a* (Krauss et al., 1991), *rx3* (Jeong et al., 2007), *six3b* (Seo et al., 1998), *il17rd* (Tsang et al., 2002), *spry4* (Furthauer et al., 2001), *vax1* (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 fluorisceinated-dextran (FLU-DEX) (Invitrogen, Carlsbad, CA, USA) detected with anti-FLU Fab fragments (Roche) and enzyme-labeled fluorescence (ELF)-97 substrate (Invitrogen). Differential interference contrast (DIC) and epifluorescent imaging was carried out on a Zeiss Axioskop2 Plus microscope with AxioVision 3.0 software (Zeiss, Oberkochen, Germany). Confocal imaging was carried out on an Olympus FV1000 microscope with FV10-ASW software (Olympus, Tokyo, Japan).

Quantitative PCR (qPCR) analysis

Preparation of cDNA

Total RNA was extracted from embryos using Trizol (Invitrogen). The RNA was then cleared of genomic DNA with a Turbo DNA-free Kit (Ambion), and 250 ng 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 of each primer and 2 μl of a 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 analyzed in triplicate during each 'technical replicate'. No RT (reverse transcriptase) and no template-control reactions were performed for each template type. Reactions were run on an Applied Biosystems ABI 7500 as follows: denaturation at 94°C for 4 minutes; amplification at 94°C for 30 seconds, 58°C for 1 minute and 72°C for 1 minute (40×). Two technical replicates were performed for each template type.

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 ($R^2 > 0.997$ in each case; primer efficiency was nearly 2; data not shown). *six3b* primer sequences were 5′-ATCGAAGACA-GAGGGACAGG-3′ and 5′-ATACTGGAGACGCTGGTCGT-3′; *gapdh* primer sequences were 5′-ATCAAGAAAGTCGTCAAGGCTG-3′ and 5′-ATCTACTCCTTGGAGGCCATGT-3′.

Knockdown assays

Three antisense morpholino oligomers (MOs) were used to knock down expression of *zic2a* in this study: two translation-blocking MOs (zic2a AUG, CGATGAAGTTCAATCCCCGCTCACA; zic2a PROX, CTCTTTCAAGCAGTCTATTCACGGC) and a splice-blocking MO (zic2aMO, CTCACCTGAGAAGGAAAACATCATA) (Nyholm et al.,

2007). *Six3b*MO (CTCTAAAGGAGACCTGAAAACCATG) 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 (*zic2a*MO), 8 ng/nl (*six3*MO), 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. Cyclopamine (Toronto Research Chemicals, North York, Ontario, Canada) was used at 100 μM as previously described (Tyurina 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 cyclopamine to block Hh signaling showed reduced *six3b* expression in the forebrain (Fig. 1A,B), but not in the retina. Similarly, *smo^b641* 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 *shh*-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
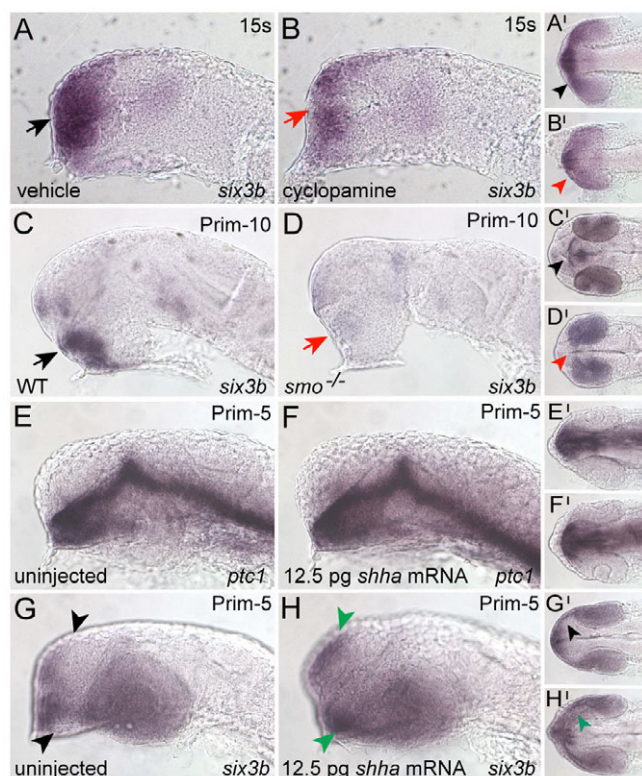
Fig. 1. Hh signaling activates *six3b* expression in the developing zebrafish forebrain. (**A**,**B**) At 15s, *six3b* is expressed normally in the anterior forebrain of vehicle-treated embryos (black arrowheads in A, 15/15 embryos) and dramatically reduced in cyclopamine-treated embryos (red arrowheads in B, 14/14 embryos treated from the 2- to 4-cell stage onwards). (**C**,**D**) At prim-10, ventral forebrain *six3b* expression is strong in wild-type (WT) embryos (black arrowheads in C, 67/92 embryos) and absent or greatly reduced in *smo⁻ᐟ⁻* mutants (red arrowheads in D, 25/92 embryos). (**E-H**) Embryos injected with a low amount of *shha* mRNA (12.5 pg) show expanded *six3b* expression [green arrowheads in H, 10/23 embryos, 2 experiments (*n*=2)], but no change in *ptc1* (F, 31/31 embryos, *n*=2). Embryos shown in panels A-H are lateral views. Panels A'-H' are ventral views of the same embryos, anterior to the left.

Fig. 2. Zic2a restricts *six3b* expression throughout the forebrain beginning at mid-somitogenesis. (**A**,**B**) *six3b* is similarly expressed in conMO- and *zic2a*MO-injected embryos at 10s-12s (A, 41/41 embryos, *n*=2; B, 57/57 embryos, *n*=2). (**C**,**D**) At 12s-15s, *six3b* expression is normal in control morphants (black arrowheads in C, 38/38 embryos, *n*=3), and expanded throughout the forebrain in *zic2a* morphants (green arrowheads in D, 58/58 embryos, *n*=3). (**E**,**F**) *six3b* expansion in *zic2a*MO-injected embryos persists at prim-5 (black arrowheads in E, 42/42 control morphants, *n*=3; green arrowheads in F, 40/41 embryos, *n*=3). (**G**) Real-time PCR showed that *six3b* levels, normalized to *gapdh*, were increased (*P*=0.0001) in ZicMO-injected embryos relative to control morphants (based on three biological replicates, with two technical replicates per biological replicate). A-F are lateral views; eyes have been removed from embryos in C-F. A'-B' are ventral views of the same embryos. C'-F' are dorsal views of the same embryos, anterior to the left.

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.
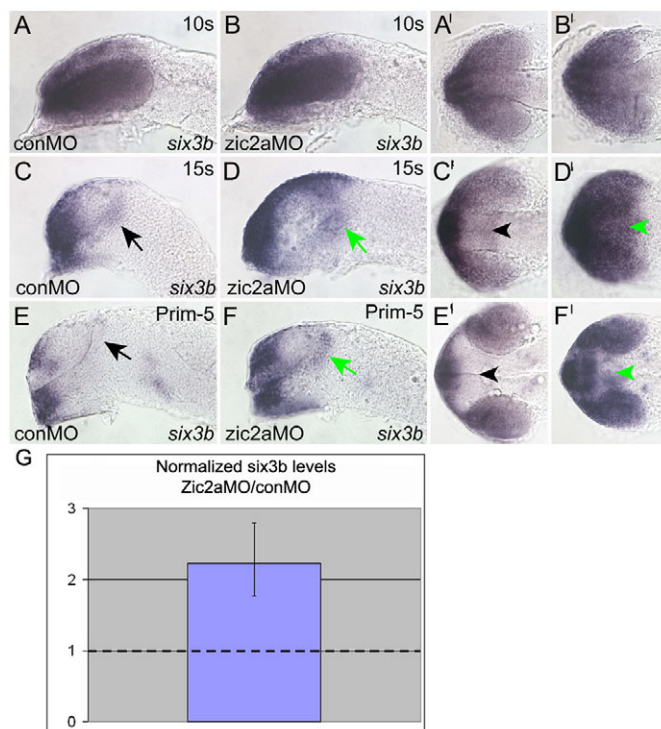
3A,C); the low level of *six3b* expression remaining in these mutants is likely to be due to residual Hh signaling through the maternally provided *smo* (Varga et al., 2001). Zic2a depletion in *smoᵇ⁶⁴¹* embryos did not result in increased *six3b* throughout the forebrain as is typical of *zic2a* morphants (Fig. 3A-D). An increase in *six3b* expression was observed relative to *smoᵇ⁶⁴¹* embryos, but this expression was weak and restricted anteriorly, consistent with an enhanced transcriptional response to residual Hh signaling in *smoᵇ⁶⁴¹* 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
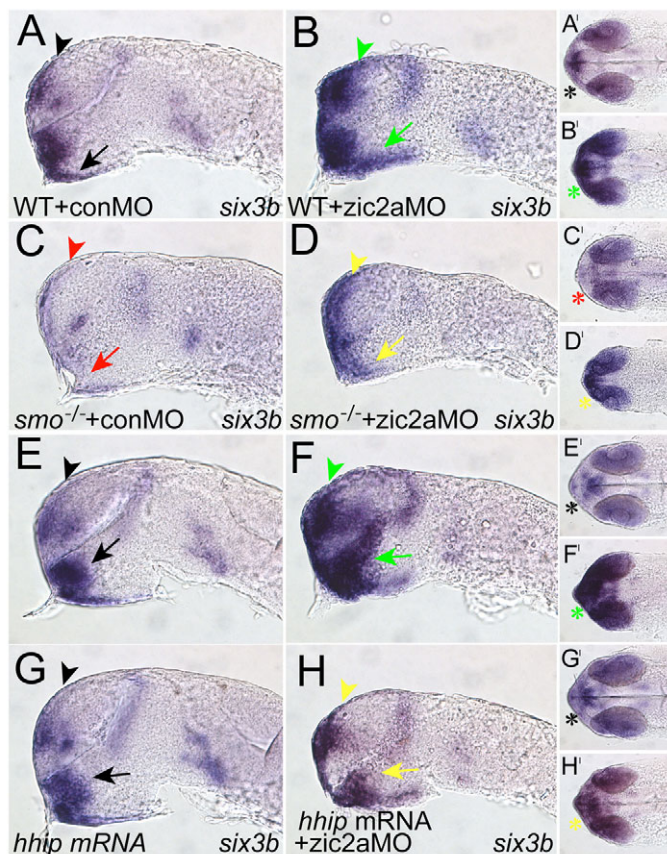
Fig. 3. Reduced Hh signaling in *zic2a* morphants prevents *six3b* expansion. (**A-D**) Zic2a depletion in *smo*$^{-/-}$ mutant embryos. Zic2a-depleted WT siblings show expanded *six3b* in the telencephalon and diencephalon (green arrowheads in B, 120/151 embryos, *n*=2), whereas *smo*$^{-/-}$ mutants injected with conMO lose *six3b* expression (C, 25/25 *smo*$^{-/-}$ embryos, *n*=2). *zic2a*MO-injected *smo*$^{-/-}$ mutants show upregulated, but anteriorly restricted, *six3b* expression (yellow arrowheads in D, 14/17 *smo*$^{-/-}$ embryos, *n*=2). (**E-H**) Zic2a depletion in the presence of exogenous Hhip. Embryos injected with *zic2a*MO alone show expanded *six3b* (green arrowheads in F, 45/45 embryos, *n*=2), whereas embryos injected with *hhip* mRNA express *six3b* normally (black arrowheads in G, 43/43 embryos, *n*=2). Embryos co-injected with *zic2a*MO and *hhip* mRNA show rescued *six3b* expression (yellow arrowheads in H, 17/40 embryos, *n*=2). A-H are lateral views, anterior to the left. A'-H' are ventral views of the same embryos, anterior to the left. Arrowheads point to the telencephalon and arrows to the diencephalon. Asterisks in A'-H' illustrate *six3b* expression in the optic stalks. All embryos are shown at prim-5.

not affect *six3b* expression (Fig. 3G), embryos co-injected with *hhip* mRNA and *zic2a*MO 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;
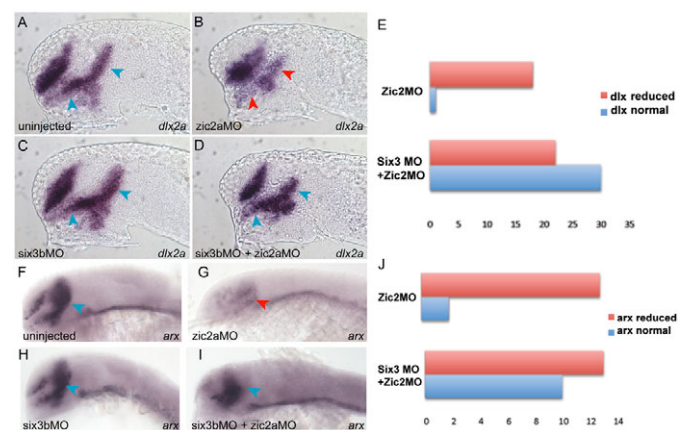

Fig. 4. Six3b depletion rescues prethalamic patterning defects in Zic2a-depleted embryos. (**A**) Uninjected embryos show normal *dlx2a* expression in the diencephalon (29/29 embryos, *n*=2). (**B**) *zic2a* morphants exhibit a strong reduction of diencephlic *dlx2a* (18/19 embryos, *n*=2). (**C**) *six3b* morphants show normal *dlx2a* patterning (23/23 embryos, *n*=2). (**D**) Embryos co-injected with *six3b*MO and *zic2a*MO exhibit partially rescued prethalamic *dlx2a* expression (30/52 embryos, *n*=2). (**E**) A graphic summary of the *dlx2a* rescue experiments. (**F**) Normal *arx* expression is observed in the PT of uninjected control embryos. (**G**) *arx* expression is strongly reduced in *zic2a* morphants (13/15 embryos, *n*=2). (**H**) *six3b* morphants show normal *arx* expression. (**I**) Embryos co-injected with *six3b*MO and *zic2a*MO show partial rescue of *arx* (10/23 embryos, *n*=2). (**J**) A graphic summary of the *arx* domain rescue experiments. Red, strongly reduced *dlx2a* or *arx* in PT; blue, normal or nearly normal expression of *dlx2a* or *arx* in PT. Arrowheads point to the diencephalic domains of *dlx2a* or *arx* expression. Lateral views with anterior to the left shown at prim-5 (A-D) or 20s (F-I).

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
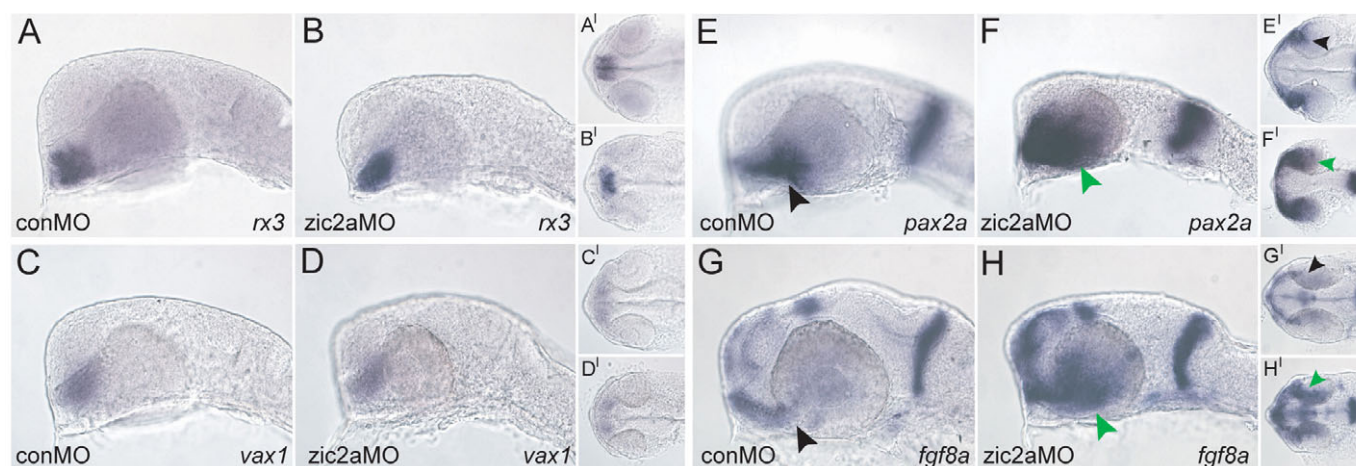
Fig. 5. Zic2a limits distal OS marker expression in the ventral retina. The effect of Zic2a depletion was examined using ISH. (**A-D**) The following markers were expressed normally in control and *zic2a* morphants: *rx3* in the pre-optic area of the hypothalamus (A, 36/36 control morphants, *n*=3; B, 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, 20/20 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, *pax*2a 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(–8.0cldnb:lynGFP)zf106* transgenic line (Lecaudey 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 *vax*1 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 *smo[b641]* 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
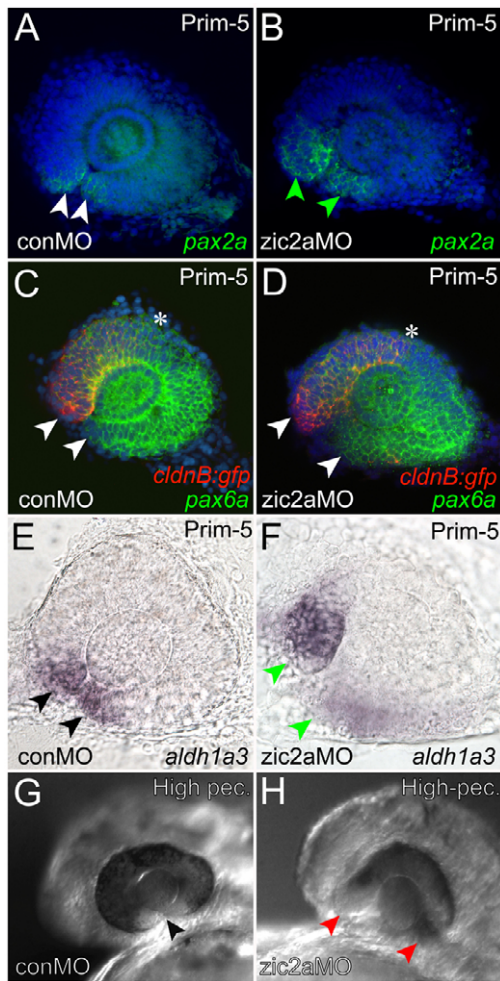
Fig. 6. Ventral retinal defects in *zic2a* morphants. (**A**,**B**) *pax2a* is normally expressed at the OS-retinal border of control morphants (A) and is expanded into the ventral retina of *zic2a* morphants (B). (**C**,**D**) *pax6a* expression is seen throughout the retina of *Tg(–8.0cldnb:lynGFP)zf106* embryos injected with conMO (C) or *zic2a*MO (D). Arrowheads in C,D point to the anterior limit of *pax6a* expression, and asterisks mark the posterior limit of *cldnb:gfp* expression in the nasal retina. (**E**,**F**) *aldh1a3* expression is normal in conMO-injected embryos (arrowheads in E, 44/44 embryos, *n*=2). The *zic2a* morphant retina fails to close and *aldh1a3* expression is expanded (arrowheads in F, 35/38 embryos, *n*=2). The choroid fissure is closed in uninjected embryos (arrowhead in **G**), but open in *zic2a* morphants (red arrowheads in **H**, 21/28 embryos, *n*=2). A-F show dissected retinae at prim-5, anterior to the left. A-D are confocal *z*-stacks. Embryos in G,H are at the high-pec stage.

Fig. 7. Patterned gene expression in OS and retinal precursors. (**A-F**) Wild-type embryos at 16s stained using wholemount in situ hybridization (WISH, green) for expression of *pax2a* (A), *pax6a* (B), *fgf8a* (C), *zic2a* (D) and *vax1* (E). Nuclei were counterstained using DAPI (red). The schematic (F) illustrates the overlap between *zic2a* and *pax2a* expression domains in the presumptive OS, and the overlap between *pax2a* and *pax6a* domains in the presumptive retina. (**G-I**) Expression of *zic2a* (G), *pax2a* (H) and *pax6a* (I) detected at 23s. Expression patterns, imaged using confocal microscopy, are shown as *z*-stacks in ventral view, anterior up.

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^{b641}* heterozygotes) showed expanded *pax2a*
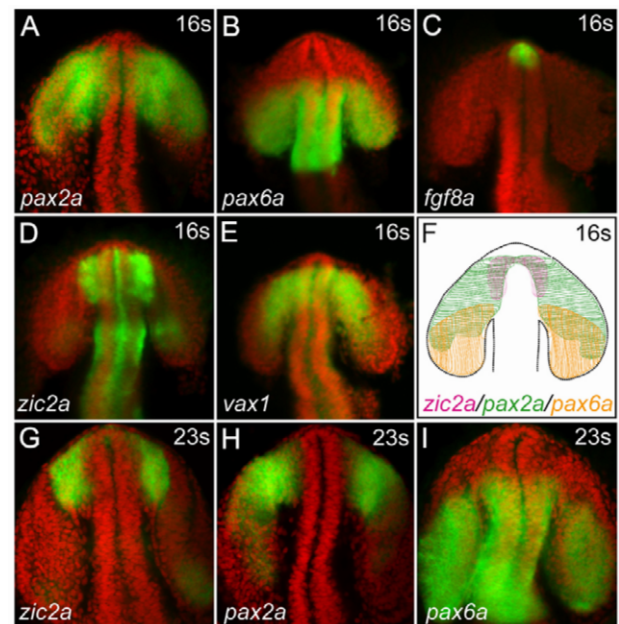
relative to control morphants (Fig. 9A-B), and homozygous *smo^{b641}* mutants had little or no *pax2a* expression (Fig. 9C). Although *smo^{b641}* homozygotes depleted of Zic2a expressed some *pax2a* in the ventral retina, this expression was weaker, and the domain less expanded, than in *zic2a* morphants (Fig. 9D). Similar results were obtained for *fgf8a* expression in Zic2a-depleted *smo^{b641}* mutants and for *pax2a* expression in Zic2a-depleted *shha^{t4}* 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 *zic2a*MO 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
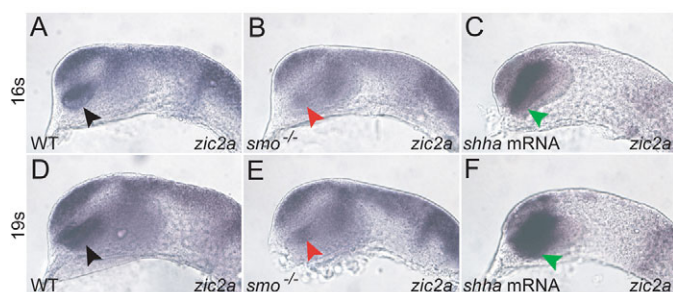
Fig. 8. Hh is necessary and sufficient for *zic2a* expression in the OS and ventral retina. (A-F) *zic2a* is expressed in the OS at 16s (A). In *smo*$^{-/-}$ mutants, *zic2a* expression is reduced in the OS at this stage (B, 14/14 embryos, *n*=2), but not elsewhere. *shha* mRNA-injected WT embryos express *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.

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 *zic2a*MO 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.
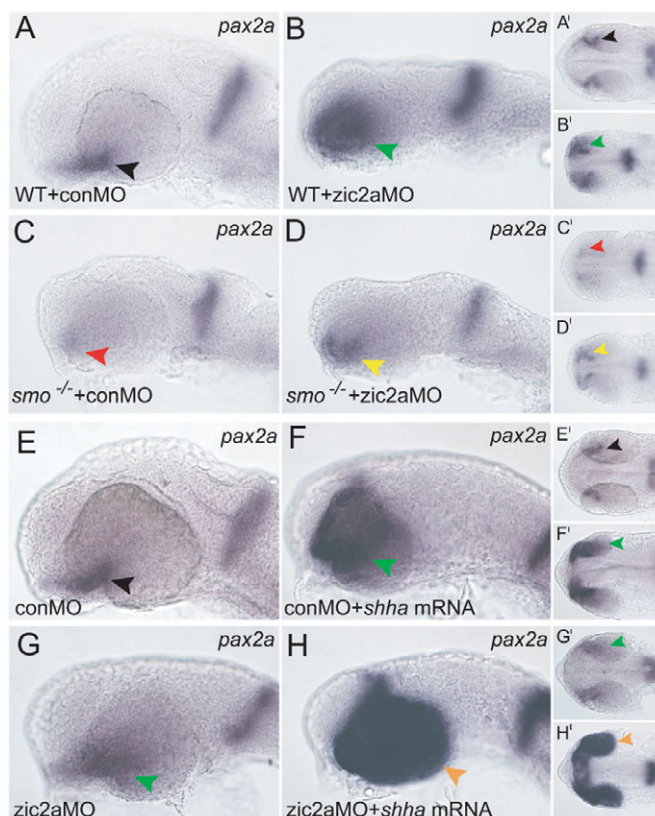

Fig. 9. Zic2a patterns the OS and retina in an Hh-dependent manner. (A-D) Zic2a-depleted WT siblings show expanded *pax2a* (B, 99/99 embryos, *n*=4), whereas *smo*$^{-/-}$ mutants injected with conMO lose *pax2a* expression (C, 12/12 embryos, *n*=2). *zic2a*MO-injected *smo*$^{-/-}$ mutants show a rescued *pax2a* phenotype (D, 30/30 embryos, *n*=4). **(E-H)** Co-injection of conMO and *shha* mRNA, or injection of *zic2a*MO alone, causes moderately expanded *pax2a* expression (F, 20/34 embryos, *n*=2; G, 30/30 embryos, *n*=2, respectively). Embryos injected with *zic2a*MO 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.

The central role of Hh signaling during forebrain development is well documented in non-mammalian vertebrates, particularly in the zebrafish (Karlstrom et al., 1999; Tyurina 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 (Gestri 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 *six3*MO 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
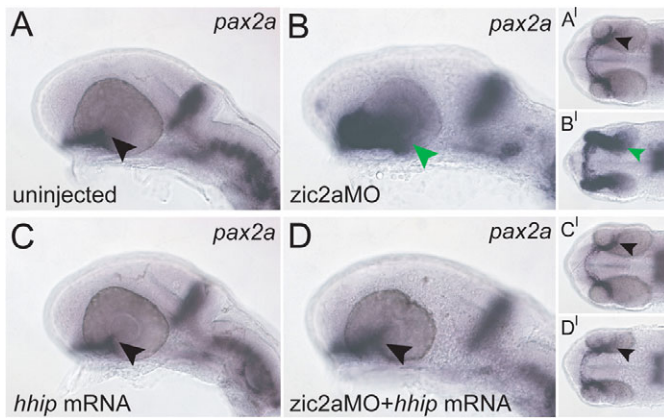
Fig. 10. Exogenous Hh antagonist rescues OS and retinal patterning in Zic2a-depleted embryos. (**A**) Wild-type *pax2a* expression. (**B-D**) Embryos injected with *zic2a*MO show expanded *pax2a* (B, 43/43 embryos, *n*=3), whereas embryos injected with *hhip* mRNA show normal *pax2a* expression (C, 8/8 embryos, *n*=1). Embryos co-injected with *zic2a*MO and *hhip* mRNA show normal *pax2a* expression (D, 30/41 embryos, *n*=3). A-D are lateral views, anterior to the left. A'-D' are ventral views of the same embryos, anterior to the left. Arrowheads mark the posterior limit of *pax2a* expression in the retina. 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

Fig. 11. A model of Zic2a function in the zebrafish forebrain primoridum. *zic2a* is required to restrict expression of *six3b* in the forebrain and *pax2a* in the ventral retina. We propose that, in both tissues, *zic2a* acts non-cell-autonomously through negative modulation of Hh signaling. Hh ligands are produced at the diencephalic midline and trigger a signaling cascade in neighboring cells that activates transcription of several targets, including *zic2a* in the OS. Zic2a, in turn, may control transcription of a secreted Hh agonist or antagonist in the OS. These data suggest that Zic2a and Hh signaling are engaged in a novel negative-feedback loop in the developing forebrain.

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 *zic*2a 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.,
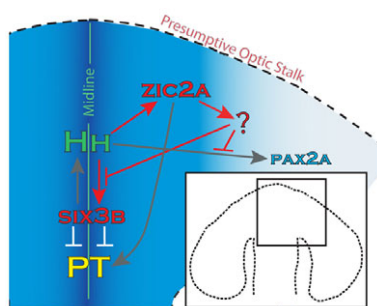
2003; Mui et al., 2005). We have now shown that *zic2a* is also activated by Hh signaling in the OS, but unlike the other targets, does not promote OS or inhibit retinal fates. Instead, Zic2a acts to modulate the Hh-regulated pattern of gene expression. Zic2a function provides the means for Hh signaling 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 morphants. 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 aide or hinder transcytosis and affect long-range signaling (Willnow 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.

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/136/22/3791/DC1

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