FKBP8 is a negative regulator of mouse sonic hedgehog signaling in neural tissues

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Summary

Sonic hedgehog (SHH) is a secreted morphogen that regulates the patterning and growth of many tissues in the developing mouse embryo, including the central nervous system (CNS). We show that a member of the FK506-binding protein family, FKBP8, is an essential antagonist of SHH signaling in CNS development. Loss of FKBP8 causes ectopic and ligand-independent activation of the Shh pathway, leading to expansion of ventral cell fates in the posterior neural tube and suppression of eye development. Although it is expressed broadly, FKBP8 is required to antagonize SHH signaling primarily in neural tissues, suggesting that hedgehog signal transduction is subject to cell-type specific modulation during mammalian development.

Key words: FKBP8, FKBP38, Sonic hedgehog, Neural tube patterning, Signaling, Mouse

Introduction

During vertebrate development, distinct classes of neurons arise at stereotypical locations along the dorsoventral axis of the spinal neural tube. This pattern is largely generated through cell-cell signaling mechanisms involving locally secreted signaling molecules (Jessell, 2000). The diffusible ligand sonic hedgehog (SHH), which is produced by cells at the ventral midline of the spinal cord (notochord and floor plate), controls neural fate specification by signaling to cells and regulating their gene expression (Ingham and McMahon, 2001; Marti and Bovolenta, 2002). SHH is believed to function as a morphogen in the neural tube because it can specify different ventral fates as a function of its concentration (Ericson et al., 1997). SHH signaling regulates dorsoventral patterning of the neural tube by repressing genes that are associated with dorsal and lateral fates and by activating genes associated with ventral fates (reviewed by Briscoe and Ericson, 2001).

Consistent with the role of SHH defined in embryological experiments, null mutations in the murine *Shh* gene or in genes required to transduce the SHH signal abolish ventral fates in both the spinal cord and brain (Chiang et al., 1996; Wijgerde et al., 2002). The cells at the ventral pole of the neural tube instead acquire dorsal or lateral identities that are normally repressed by SHH signaling. Excessive SHH signaling results in an opposite phenotype: cells inappropriately adopt ventral identities in favor of dorsal identities (Echelard et al., 1993; Goodrich et al., 1997). Thus, increased or decreased levels of SHH signaling have pronounced effects on the patterning of the central nervous system.

Hedgehog signal transduction occurs through a series of poorly understood signaling events that modulate the activities of a family of transcriptional factors, the GLI proteins. In *Drosophila*, the transcription factor Cubitus interruptus (Ci, the ortholog of vertebrate GLI) mediates Hedgehog (Hh)-induced transcriptional activation (Methot and Basler, 2001). The hedgehog ligand binds to the multipass transmembrane protein Patched, preventing its normal inhibition of Smoothened (Chen and Struhl, 1996; Marigo et al., 1996). The Hh signal is transduced through Smoothened to a complex containing Ci (Robbins et al., 1997). Downstream of Smoothened, protein kinase A (Pka) phosphorylates Ci and facilitates its proteolytic processing into a truncated transcriptional repressor (Price and Kalderon, 1999). Hh signaling reverses this effect and promotes the accumulation of full-length Ci, which is a transcriptional activator of Hh-response genes (Ohlmeyer and Kalderon, 1998). Vertebrate orthologs of several *Drosophila* hedgehog signaling components have been identified and appear to be generally conserved in the vertebrate pathway (reviewed by Marti and Bovolenta, 2002).

The FK506-binding protein 8 (FKBP8, also known as FKBP38) (Pedersen et al., 1999; Shirane and Nakayama, 2003) is a member of the immunophilin family of proteins that bind immunosuppressant drugs such as cyclosporin A or FK506 (Snyder et al., 1998). FKBPs comprise a large family, the members of which share a conserved peptidyl-prolyl isomerase (PPI) domain. Proteins with PPI activity can alter the conformation of other proteins by allowing free rotation around prolyl peptide bonds. This activity may allow the immunophilins to function as accessory folding proteins or as

scaffold proteins that facilitate protein-protein interactions. FKBP12, a relatively well-studied member of the family, has several defined functions. Like other immunophilins, FKBP12 associates with and inhibits the Ca^{2+}-calmodulin activated protein phosphatase calcineurin upon binding FK506. In T-lymphocytes, FKBP12 activity prevents the dephosphorylation of NF-AT and prevents this transcription factor from entering the nucleus (Clipstone and Crabtree, 1992; Hemenway and Heitman, 1999). FKBP12 also binds to and regulates the activity of the inositol (1,4,5) triphosphate and ryanodine receptors, which regulate intracellular Ca^{2+} release (Cameron et al., 1995; Jayaraman et al., 1992). The presence of tetratricopeptide repeats (TPR) in addition to the PPIase domain in FKBP8 makes it more closely related to the larger members of the FKBP family such as FKBP52, which functions as a specialized co-chaperone (Young et al., 2003).

The in vivo function of FKBP8 is unclear, although recent data from cell culture experiments indicate that FKBP8 binds calcineurin in an FK506-independent manner and targets BCL2 and BCL2L to mitochondria, thus inhibiting apoptosis (Shirane and Nakayama, 2003). To define the in vivo role of FKBP8, we disrupted its function in mice by gene targeting. Our data show that FKBP8 has an essential role during development. Loss of FKBP8 leads to inappropriate activation of the SHH signaling pathway in the caudal neural tube, where ventral fates were dramatically expanded at the expense of dorsal fates. The direct role of FKBP8 in regulation of SHH signaling was confirmed genetically, because SHH-dependent neural fates are restored in *Shh*$^{-/-}$;*Fkbp8*$^{-/-}$ double mutants. Our data indicate that FKBP8 is primarily required for regulation of the hedgehog signaling pathway in neural tissues.

Materials and methods

Molecular cloning, antibodies, immunoblotting, and immunofluorescence

To obtain full-length sequences, we performed 5′ RACE (rapid amplification of cDNA ends) and DNA sequencing. The polyclonal anti-mouse FKBP8 antibody was generated in rabbit using the full-length recombinant protein as an antigen. Monoclonal antibodies α-SHH, α-FOXA2, α-NKX2.2, α-HB9, α-PAX6, α-PAX7 and α-MSX1/2 were obtained from the Developmental Studies Hybridoma Bank. An α-activated caspase 3 antibody (Promega) was used to monitor apoptosis. Rabbit α-PAX2 antibody was obtained from Zymed Laboratories. Immunofluorescence and immunoblotting were performed as described (Eggenschwiler and Anderson, 2000; Hong et al., 2001).

To determine the distribution of FKBP8, tissues were extracted in RIPA buffer/1 mM DTT/protease inhibitors. Total protein from adult tissues was quantified by the modified Lowry assay. In the immunoblot shown in Fig. 1B, 5 μg total protein was loaded per lane. To determine membrane association of FKBP8, rat retinal proteins were extracted under the following conditions: 20 mM Tris-HCl, pH 7.4, 0.15 N NaCl (labeled as 0.15 N NaCl in Fig. 1C); 1 N KCl (1 N KCl); 0.1 M $NaHCO_3$-Na_2CO_3 (pH 11.5); 20 mM Tris-HCl, pH 7.4, 0.15 N NaCl, 0.5% CHAPS (CHAPS); 6 M urea (6 M urea). All solutions also contained 1 mM DTT. Extracts were centrifuged at 200,000 ***g*** for 1 hour at 4°C. Both supernatants and pellets were analyzed by immunoblotting.

Gene targeting and mice

Genomic fragments, 3.4 kb and 2 kb long, were amplified by PCR from mouse (129/Sv) genomic DNA and inserted into the pGT-N29 (New England Biolabs) cloning vector (Fig. 2A). The resultant targeting construct, harboring a deletion of exons 4-6, was electroporated into mouse embryonic stem (ES) cells. Targeted clones were injected into C57BL/6 blastocysts to generate chimeras, which were crossed with C57BL/6 mice. Mouse genotyping was performed by PCR. Primer sequences are: KO9, GGCTTCCTGGGGCTT-AAGGAG; KO18, TGGTGAGCAGGTGTAGGGTGTGAC; KO21, GTCTGGCCATCCTACAGGCTGGC; KO26, GTCACCCTGAA-AGACGGCAGAGG; pnt3a, CGAGATCAGCAGCCTCTGTTCC-AC; and pnt6, CCGGAGAACCTGCGTGCAATC. The *Shh*-null mutation was previously described (Chiang et al., 1996). GenBank Accession Numbers are AY278608 and AY278607 for mouse and human FKBP8, respectively.

In situ hybridization

In situ hybridization was performed on fixed frozen sections and whole-mount embryos essentially as described (Belo et al., 1997; Schaeren-Wiemers and Gerfin-Moser, 1993). Riboprobes for in situ hybridization were synthesized from plasmids carrying mouse cDNA for *Wnt1*, *Gdf7*, *Pax3*, *Pax6*, *Dbx1*, *Isl2*, *Nkx2.2*, *Foxa2*, *Ptch1*, *Gli1*, *Pax1*, *Olig2*, *Ihh* (gifts from A. McMahon, T. Jessell, U. Deutsch, C. Walther, G. Fishell, C-C. Hui, E. Lai, M. Scott, and T. Caspary) and *Fkbp8*. The mouse *Shh* probe (a gift from A. McMahon) is from cDNA covering sequences roughly 150 bp upstream to 230 bp downstream of exon 2 (the exon deleted in the *Shh* mutant allele). The mouse *Dhh* and *Gli3* probes were generated from cloned PCR products (from positions 639-1164 and 1415-2327 in *Dhh* and *Gli3* cDNA sequences, respectively) and did not cross hybridize with related family members. *Fkbp8* sense and antisense probes were generated from a cDNA clone covering the entire coding region.

Results

FKBP8 is an integral membrane protein widely expressed in embryonic and adult tissues

The full-length mouse FKBP8 is 402 residues in length and consists of one PPIase domain, a TPR domain with three contiguous tetratricopeptide repeats (TPR) and a predicted membrane insertion site at the C terminus (Fig. 1A). Immunoblotting showed that FKBP8 is widely expressed in both adult and embryonic tissues (Fig. 1B). FKBP8 has a calculated molecular weight of 44 kDa, but migrated at an apparent molecular weight of 53 kDa, suggesting post-translational modification. Glycosylation is unlikely to account for the increase in molecular weight, as this remained unchanged upon digestion with endoglycosidases F and H, or O-glycosydase in combination with sialydase II (data not shown). We confirmed by biochemical extractions that FKBP8 is an integral membrane protein (Fig. 1C). FKBP8 associated with the membrane fraction and migrated at a higher molecular weight when translated in vitro in the presence of microsomal membranes, further indicating that it is post-translationally modified (Fig. 1D, left). In protease protection assays, intact membranous vesicles did not protect the translated protein from degradation (Fig. 1D, middle). These data suggest that FKBP8 adopts a topology in which the protein is exposed to the cytoplasm (Fig. 1D, right). Fractionation studies of cultured fibroblasts suggested that the protein is distributed to multiple membranous compartments including the ER and mitochondria (data not shown). By immunofluorescence, FKBP8 exhibited a mitochondrial localization pattern in COS7 fibroblasts (Fig. 1E, left). A similar mitochondrial localization

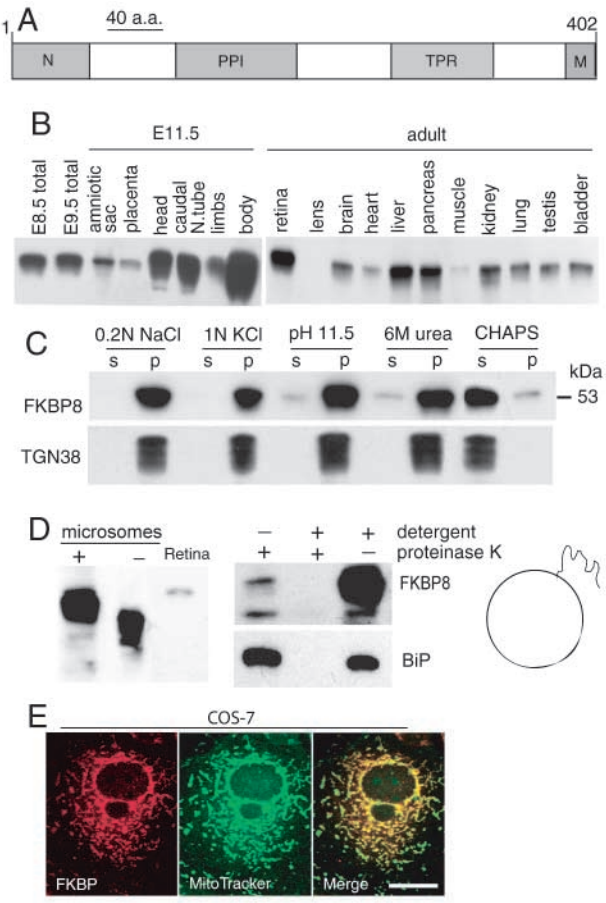

Fig. 1. FKBP8 is a widely expressed integral membrane protein. (A) Domain structure of murine FKBP8. Numbers on top indicate positions of amino acid residues. N is the N-terminal sequence missing in previous literature; PPI (residues 110-194), TPR (262-329) and the transmembrane domain (M; 381-400) are marked. (B) Immunoblots of mouse tissue homogenates. (C) FKBP8 is solubilized only with a detergent. TGN38, a known integral membrane protein shown here as a control. (D) Left, immunoblotting analyses of in vitro translated FKBP8 in the presence or absence of membranes. Middle, protease protection assay to determine membrane topography. FKBP8 was not protected by intact membranes. By comparison, the ER luminal protein BiP was fully protected. Right, a schematic diagram showing FKBP8 situated outside of the ER lumen. (E) FKBP8 is localized in the mitochondria of cultured fibroblasts (left), as shown by overlapping staining patterns of FKBP8 (red) and MitoTracker (green). Scale bar: 10 μm.

of FKBP8 in HeLa cells was previously described (Shirane and Nakayama, 2003).

FKBP8 is required for neural tube development

Both RNA and protein analyses indicated that disruption of the *Fkbp8* gene generated a null allele (Fig. 2A). We found that homozygous embryos died around embryonic day (E)13.5. The *Fkbp8*$^{-/-}$ mutant phenotype became morphologically apparent by E10.5 (Fig. 2B). At this stage, the mutant caudal neural tube appeared translucent and irregular. By E12.5, the caudal neural tube of the mutant embryo was dilated and appeared as a fluid-filled sac. In about 20% of the *Fkbp8*$^{-/-}$ mutant embryos, a clump of pigmented cells was seen in place of the eye (Fig. 2B), whereas the eyes appeared smaller in the remaining mutant embryos. The limb buds, branchial arches and somites of the mutants appeared normal. Histological examination revealed a dilated caudal neural tube and dorsally displaced, smaller dorsal root ganglia (Fig. 2B). By contrast, the rostral mutant neural tube appeared morphologically normal. The limbs developed normally in the mutant, and major organs such as the heart, lung and gut also appeared normal in histological sections (not shown).

Experiments using cultured cells (Shirane and Nakayama, 2003) indicate that FKBP8 blocks apoptosis by recruiting BCL2 and BCL2L to mitochondria. We considered the possibility that increased apoptosis could be the underlying primary defect for the phenotype we observed in the *Fkbp8*$^{-/-}$ mutant. We monitored apoptosis using an antibody against activated caspase 3 but found no difference between wild-type and mutant embryos in the affected lumbar neural tube at E10.5 (Fig. 2C). Similarly, no differences were observed between genotypes in the rostral neural tube or in non-neural tissues (data not shown). Thus, the phenotypic features observed in *Fkbp8*$^{-/-}$ mutants do not stem from excessive apoptosis.

To determine the basis for the tissue-restricted developmental defects of the mutant, we examined the tissue expression pattern of *Fkbp8* in E9.5 embryos by in situ hybridization and by immunostaining (Fig. 3). By whole-mount in situ hybridization (Fig. 3A), *Fkbp8* mRNA was found uniformly distributed throughout the E9.5 embryo. On sections, the in situ hybridization signals (Fig. 3B) showed no discernable variation along either the anteroposterior axis or the dorsoventral axis. Similar observations were made by immunostaining for the FKBP8 protein, either in whole-mount embryos (not shown) or on cross-sections through the neural tube (Fig. 3C). These data suggest that the rostral-caudal differences in the mutant neural tube morphology do not stem from regional differences in *Fkbp8* expression.

Loss of FKBP8 leads to expansion of ventral fates in the neural tube and disruption of optic vesicle patterning

Despite the normal frequency of cell death in the *Fkbp8* mutant, the caudal neural tube adopted a highly abnormal morphology. This suggested that some cellular process other than cell death was affected. Histologically, the cells throughout ventral mutant neural tube at E10.5 resembled floor-plate cells; they were arranged in a single-cell layer, showing columnar morphology with basally positioned nuclei. This phenotype suggested that the floor plate was greatly expanded and that cell identities along the dorsoventral axis of the neural tube may be altered.

To address this possibility in more detail, we examined the expression pattern of markers for dorsoventral neural cell fates in *Fkbp8*$^{-/-}$ mutants from E9.5 to E12.5 by in situ hybridization and immunohistochemistry (Fig. 4, data not shown). The expression domains of dorsoventral markers were clearly altered in mutant embryos in sections through the caudal neural tube just anterior to the hindlimbs. *Foxa2* expression normally marks the most ventral neural cell fate, the floor plate (Sasaki and Hogan, 1993). *Foxa2* expression was dramatically expanded in the *Fkbp8*$^{-/-}$ mutant, such that roughly the ventral half of the neural tube expressed this marker. *Pax6* and *Dbx1* are normally expressed at high levels

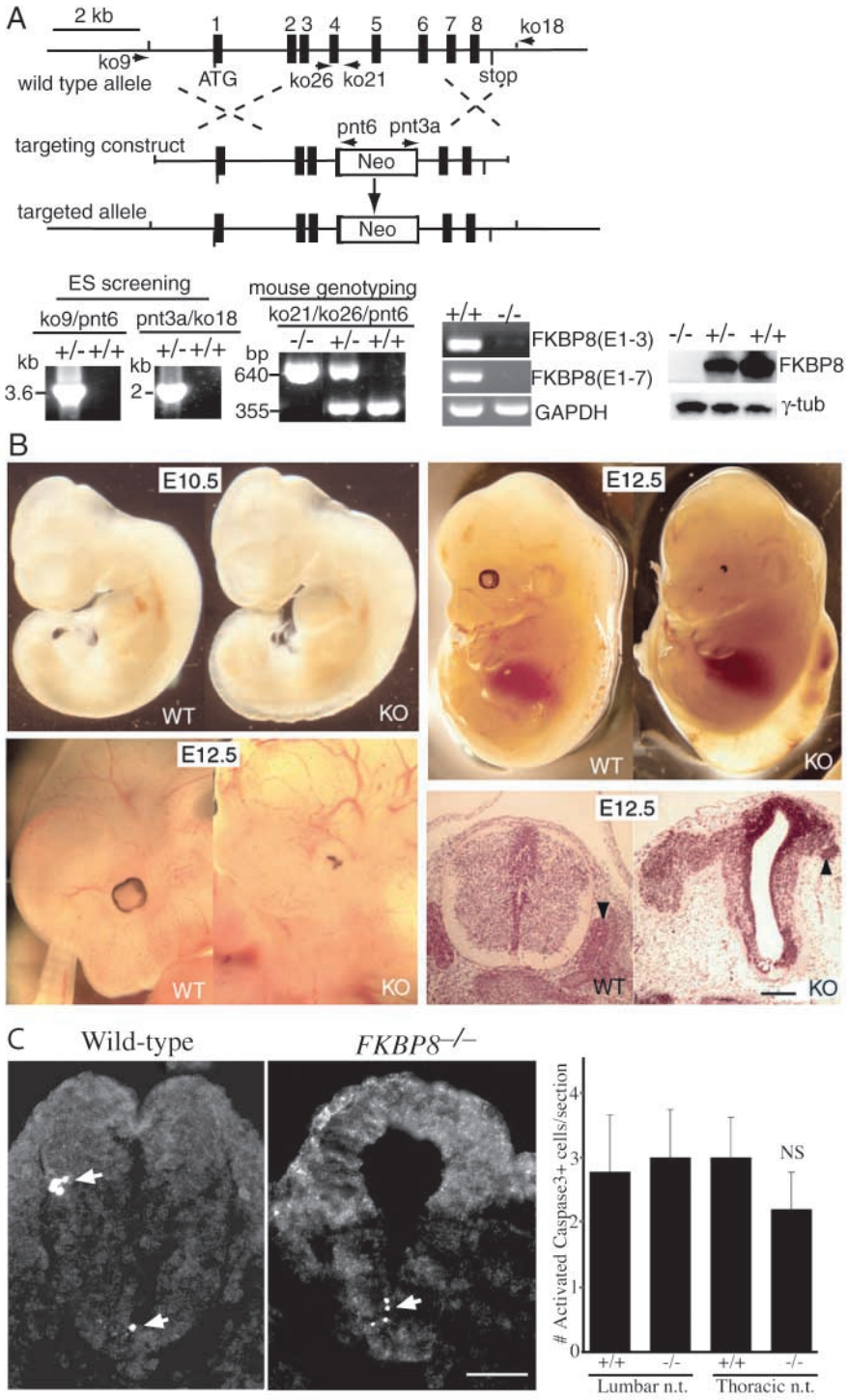

Fig. 2. Neural development defects in mice with targeted disruption of the *Fkbp8* gene. (A) (Top panel) targeting strategy. Black boxes, exons; arrowheads, PCR primers. (Bottom panels) Left two panels: PCR screening for targeted ES clones and mouse genotyping. Middle panel: RT-PCR analyses find the mutant transcripts reduced or absent. E1-3, primers spanning exons 1-3; E1-7, primers spanning exons 1-7. GAPDH amplification served as a control. Right panel: immunoblots showing complete ablation of FKBP8 protein. γ-tubulin is shown as a gel loading control. (B) Developmental defects in the CNS of *Fkbp8*$^{-/-}$ (knockout) embryos. The caudal neural tube defect (upper left and right) and failure of eye development (upper right and lower left) are apparent. The mutant neural tube is dilated (lower right). Dorsal root ganglia (arrowheads) are missing or disorganized. (C) Apoptosis monitored by immunofluorescence for activated caspase 3. Sections through the caudal neural tube of E10.5 wild type and *Fkbp8*$^{-/-}$ mutant embryos are shown (apoptotic cells are indicated by white arrows). Quantification (three embryos per genotype and four sections/embryo) showed no statistically significant difference between genotypes in the thoracic or lumbar neural tube (NS, not statistically significant). Scale bars: 100 μm in B; 50 μm in C.

in the lateral regions of the neural tube; *Dbx1* expression is strictly limited to a small population of lateral cells (including V0 interneuron progenitors), whereas *Pax6* expression is also observed at lower levels in ventrolateral and dorsal cells (Pierani et al., 2001; Shoji et al., 1996; Walther and Gruss, 1991). In the *Fkbp8*$^{-/-}$ mutant, both *Pax6* and *Dbx1* were expressed ectopically in dorsal regions instead of their normal lateral domains. Markers for the most dorsal fates, such as *Wnt1*, were not expressed in the mutant. The expression domains of other markers for specific dorsoventral fates (including *Shh*, *Nkx2.2*, *Isl2*, *Pax3*, *Pax7* and *Gdf7*, see below and data not shown) were all affected in a manner consistent with ventralization of neural cell fates. In the rostral neural tube, at the level of the forelimbs, all of the markers examined showed a normal expression pattern (data not shown). Thus, defective neural patterning in the *Fkbp8*$^{-/-}$ mutant is limited to the posterior of the prospective spinal cord.

Signaling through the bone morphogenetic protein (BMP) pathway appears to regulate the specification of dorsal neural fates (Lee et al., 1998; Liem et al., 1995), raising the possibility that FKBP8 acts in this pathway. However, the expression of MSX1 and MSX2, targets of the BMP signaling pathway (Alvarez Martinez et al., 2002; Furuta et al., 1997), was similar in the dorsal neural tube of *Fkbp8*$^{-/-}$ mutant and wild-type embryos (Fig. 4I,J). Thus, despite the absence of dorsal neural fates in the *Fkbp8*$^{-/-}$ mutant, BMP signaling appears normal in the mutant neural tissue.

By E9.5, *Shh* is normally expressed in the floor of the diencephalon located between the optic stalks (Ishibashi and McMahon, 2002). *Pax2* expression marks proximal fate (optic nerve) and *Pax6* expression marks distal fates (retina and lens) in the developing eye. Excess SHH signaling, caused by ectopic expression of *Shh* or blocking of the hedgehog pathway

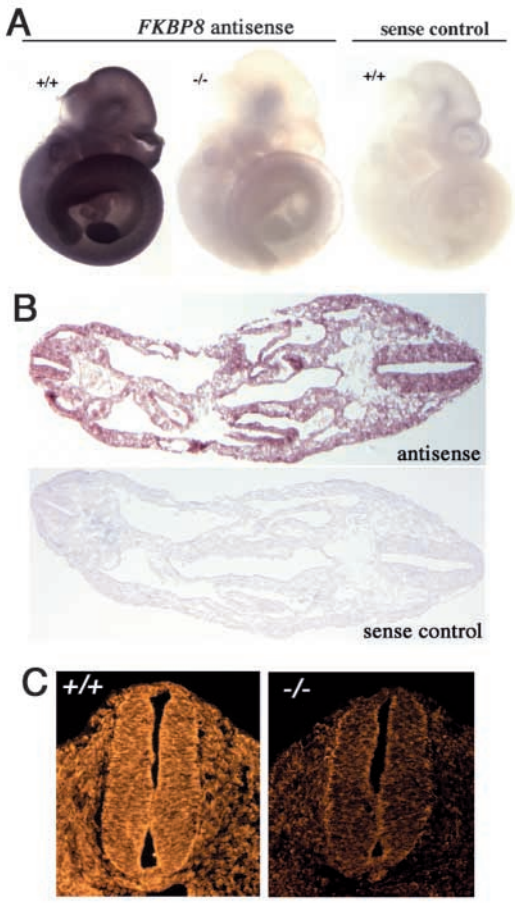

Fig. 3. Embryonic expression pattern of *Fkbp8*. (A) Whole-mount in situ hybridization in E10 embryos. *Fkbp8* appears uniformly expressed. Negative controls included the sense probe and *Fkbp8*$^{-/-}$ mutants hybridized with the antisense probe. The minimal signal in the mutant further indicated that the mutant *Fkbp8* transcript is destabilized. (B) In situ hybridization of sections. Thoracic neural tube is on the right, and lumbar neural tube is on the left. No differences in expression could be detected in the neural tube along the dorsoventral or anteroposterior axis. (C) Immunofluorescence of neural tube sections from E9.5 wild type (+/+) and *Fkbp8* mutant (–/–) embryos, stained with the FKBP8 antibody.

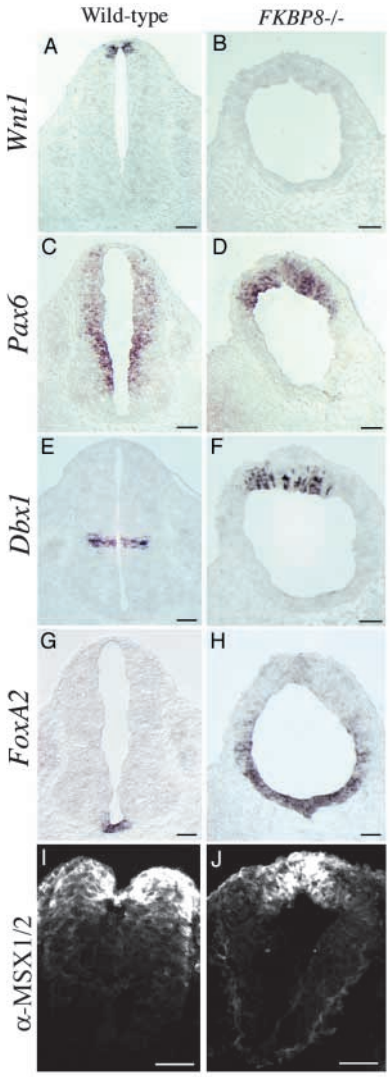

Fig. 4. The *Fkbp8*$^{-/-}$ neural tube is ventralized. Sections of E11.5 wild type and *Fkbp8*$^{-/-}$ neural tubes were analyzed by in situ hybridization. Sections at caudal levels of the neural tube just anterior to the hindlimbs are shown. Markers of dorsal fates, *Wnt1* (A,B) and *Gdf7* (data not shown) are not expressed in *Fkbp8*$^{-/-}$ mutants. In the mutant, dorsal cells adopt lateral fates marked by *Pax6* and *Dbx1* (D,F), and lateral cells acquire ventral fates marked by *Foxa2* (H) and *Nkx2.2* (data not shown). Expression of MSX1 and MSX2 in the E10.5 dorsal neural tube, monitored by immunofluorescence with an antibody that recognizes both proteins, is relatively unaffected in *Fkbp8*$^{-/-}$ mutants despite changes in neural tube morphology (I,J). Scale bars: 65 μm.

antagonist PKA, promotes *Pax2* expression at the expense of *Pax6* expression (Ekker et al., 1995; Macdonald et al., 1995; Perron et al., 2003). In E11.5 *Fkbp8*$^{-/-}$ mutant embryos, development of the retina and pigmented epithelium was severely reduced in the ventral half of the eye (Fig. 5A). A similar phenotype was observed when PKA function was blocked in *Xenopus* embryos (Perron et al., 2003). The eye defect in the *Fkbp8*$^{-/-}$ mutant embryos is reminiscent of the small eye phenotype caused by *Pax6* haploinsufficiency (Hill et al., 1991). In the E10.5 *Fkbp8* mutant, PAX2 was expressed ectopically in the dorsal optic vesicle (Fig. 5B). PAX6 expression was clearly reduced in the neural retina of the mutant at E11.5 (Fig. 5C). Similar defects have been observed in mutants for the hedgehog pathway antagonist *Rab23* (Gunther et al., 1994) (J.T.E., unpublished) and are consistent with inappropriate activation of the SHH pathway in the optic vesicle.

The SHH signaling pathway is constitutively active in the absence of FKBP8

The ventralization of the *Fkbp8*$^{-/-}$ mutant neural tube appeared to result from excessive SHH signaling. It is possible that FKBP8 normally acts as a negative regulator of SHH ligand production. Alternatively, FKBP8 may function as a negative regulator of the SHH pathway keeping it repressed in the absence of the SHH ligand. Patched 1 (PTCH1) and RAB23 have similar functions (Eggenschwiler et al., 2001; Goodrich et al., 1997). A third possibility is that FKBP8 acts positively

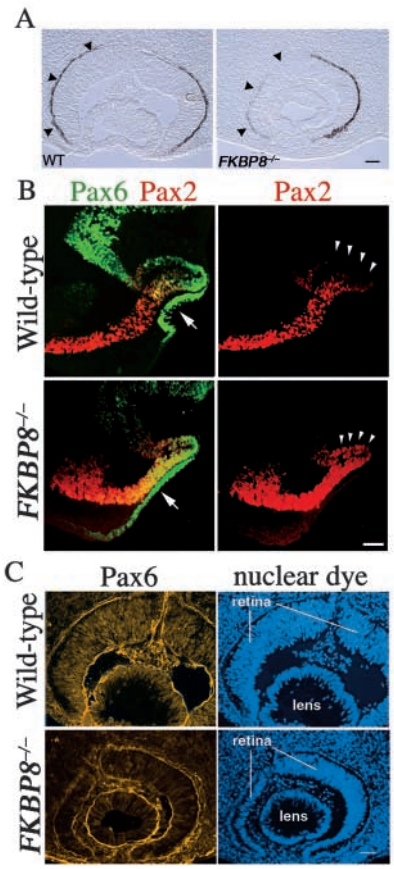

Fig. 5. Delayed eye development in *Fkbp8*$^{-/-}$ mutant embryos. (A) At E11.5, *Fkbp8*$^{-/-}$ mutant eyes are smaller, have a thinner retinoblast layer and exhibit a hypo-pigmented pigment epithelium in the ventral region. (B) PAX2 (red) and PAX6 (green) immunofluorescence staining of optic vesicles at E10.5. PAX2 is ectopically expressed in the dorsal regions of the optic cup (white arrowheads) in the mutant. PAX6 expression appears comparable with wild type in the dorsal retina and in the lens primordium (white arrows), although lens morphogenesis is clearly delayed. (C) At E11.5, PAX6 expression is apparently reduced in the mutant retinoblast layer and lens. Background staining in the lens capsule and skin is due to non-specific binding of the secondary antibody. Scale bar: 30 μm in A,C; 50 μm in B.

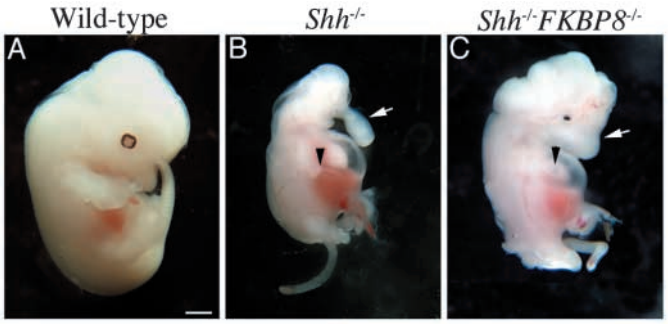

Fig. 6. Loss of *Fkbp8* partially restores CNS development in the *Shh*$^{-/-}$ mutant. Wild-type (A), *Shh*$^{-/-}$ (B) and *Shh*$^{-/-}$;*Fkbp8*$^{-/-}$ (C) littermates from an E12.5 litter are shown. Phenotype of the *Shh*$^{-/-}$ single mutant includes growth retardation, failure of limb outgrowth, severe holoprosencephaly (white arrow) and a single ventrally positioned eye. This phenotype is partially rescued in the *Shh*$^{-/-}$;*Fkbp8*$^{-/-}$ embryo (note rescue of cyclopia and holoprosencephaly, white arrow). Other features, such as limb outgrowth defects (arrowhead), are not rescued. Scale bar: 1 mm.

in a signaling pathway that dorsalizes the neural tube; when this is disrupted, SHH signaling is unopposed and ventralizes the neural tube.

To distinguish among these possibilities, we analyzed the phenotype of *Shh*$^{-/-}$;*Fkbp8*$^{-/-}$ double mutant. At E11.5, the *Shh*$^{-/-}$ mutant exhibited a variety of defects including severe growth retardation, cyclopia/holoprosencephaly, absence of ventral cell types in the neural tube, abnormal somites and deformed limbs (Fig. 6) (Chiang et al., 1996). In contrast to the *Shh*$^{-/-}$ mutant, the double mutant developed two distinct eyes separated by ventral neural tissue in the forebrain (Fig. 6). The double mutant caudal neural tube showed the dilated morphology characteristic of *Fkbp8*$^{-/-}$ single mutants. The somites and limbs were indistinguishable from that of the *Shh*$^{-/-}$ single mutant; the limbs of both *Shh*$^{-/-}$;*Fkbp8*$^{-/-}$ double mutants and *Shh* single mutants failed to grow out along the proximodistal axis and the somites of both mutants were small and abnormally shaped. These data suggest a partial rescue of SHH-dependent neural development in the *Shh*$^{-/-}$;*Fkbp8*$^{-/-}$ double mutant.

To examine this process on the cellular level, we examined markers of SHH-dependent cell fates in the caudal neural tube (Fig. 7). Motoneurons and their progenitors, marked by *Isl2*, HB9 and *Olig2* expression (Fig. 7, data not shown), are not specified in *Shh*$^{-/-}$ mutants, but were clearly present in the *Shh*$^{-/-}$;*Fkbp8*$^{-/-}$ double mutant. *Nkx2.2*, marking progenitors of V3 interneurons, and *Foxa2*, marking the floorplate, are dependent on SHH (Briscoe et al., 1999; Eggenschwiler et al., 2001; Sasaki and Hogan, 1993). Both these fates were specified in the *Shh*$^{-/-}$;*Fkbp8*$^{-/-}$ double mutant in ectopically expanded ventral domains. Markers of dorsal and lateral fates, such as *Pax3* and *Pax6*, were expressed across the ventral midline of the *Shh*$^{-/-}$ mutant and were shifted dorsally in the *Shh*$^{-/-}$;*Fkbp8*$^{-/-}$ double mutant. Patterning in the double mutant was generally similar to that in the *Fkbp8*$^{-/-}$ single mutant. A minor difference was observed for the lateral marker *Dbx1*, which at E11.5 was expressed in dorsal cells of the single mutant but in lateral cells of the double mutant. This might reflect a sensitivity of this marker to slightly different levels of SHH signaling in the two mutants (Pierani et al., 1999; Wijgerde et al., 2002). These data suggest that the loss of FKBP8 leads to excessive activation of the SHH pathway even in the absence of the SHH ligand.

We also examined the expression pattern of genes that regulate SHH signaling (Fig. 8). *Ptch1* and *Gli1* are direct targets of hedgehog signaling and are not expressed in the *Shh*$^{-/-}$ mutant neural tube (Litingtung and Chiang, 2000). *Ptch1* and *Gli1* were expanded dorsally in the *Fkbp8*$^{-/-}$ single and the double mutants. The expanded domain of *Ptch1* and *Gli1* expression in the *Shh*$^{-/-}$;*Fkbp8*$^{-/-}$ neural tube provides direct evidence for ligand-independent activation of the SHH pathway. Although SHH is required for *Gli1* expression, very high levels of SHH signaling repress this target (Motoyama et al., 2003). Consistent with this, the expression domain of *Gli1* was shifted dorsally in both the *Fkbp8*$^{-/-}$ single mutant and in the *Shh*$^{-/-}$;*Fkbp8*$^{-/-}$ double mutant.

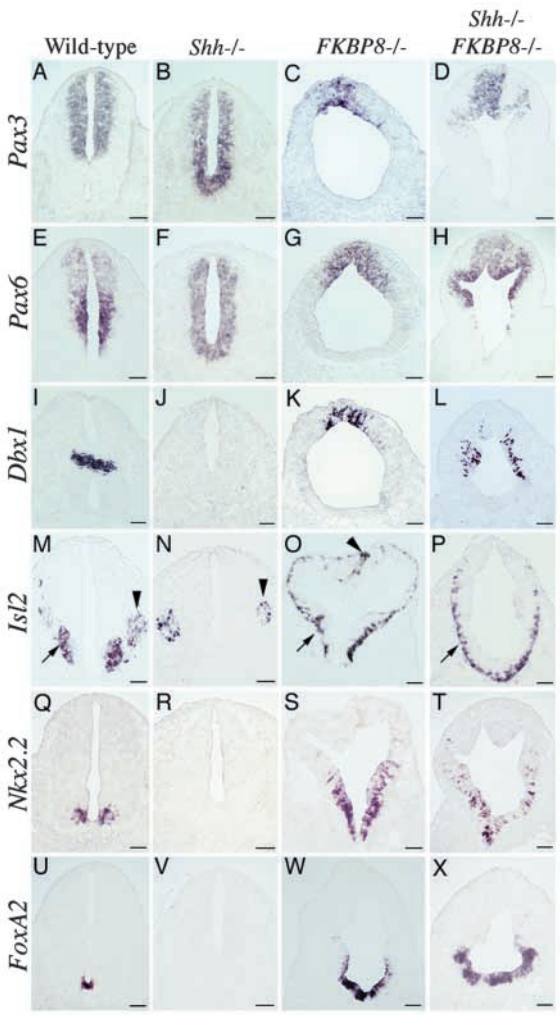

Fig. 7. SHH-independent dorsoventral neural patterning. E11.5 neural tube sections were assayed for expression of *Pax3* (A-D), *Pax6* (E-H), *Dbx1* (I-L), *Isl2* (M-P), *Nkx2.2* (Q-T) and *Foxa2* (U-X) in wild-type (A,E,I,M,Q,U), *Shh*$^{-/-}$ (B,F,J,N,R,V), *Fkbp8*$^{-/-}$ (C,G,K,O,S,W) and *Shh*$^{-/-}$;*Fkbp8*$^{-/-}$ (D,H,L,P,T,X) embryos. Dorsal and lateral cell fates (marked by *Pax3* and *Pax6*, respectively) are excluded from the ventral neural tube of wild-type and *Fkbp8*$^{-/-}$ embryos but were expressed across the ventral midline of *Shh*$^{-/-}$ mutants. Ventral repression of these cell fates was restored in the *Shh*$^{-/-}$;*Fkbp8*$^{-/-}$ double mutant. Note the specification in *Shh*$^{-/-}$;*Fkbp8*$^{-/-}$ mutants of lateral and ventral cell types such as *Dbx1*$^{+}$ V0 interneuron progenitors, *Isl2*$^{+}$ motoneurons (arrows in M,O,P), *Nkx2.2*$^{+}$ V3 interneuron progenitors, and *Foxa2*$^{+}$ floorplate. These cell types are absent in E11.5 *Shh*$^{-/-}$ mutants. In addition to motoneurons, dorsal root ganglia (DRG, arrowheads in M-O) also express *Isl2*. DRG, derived from the dorsally specified neural crest, are observed in wild-type and *Shh*$^{-/-}$ mutant embryos but are displaced dorsally or missing altogether in *Fkbp8*$^{-/-}$ and *Shh*$^{-/-}$;*Fkbp8*$^{-/-}$ mutants, consistent with disruption of dorsal fates in these embryos. Scale bars: 65 μm.

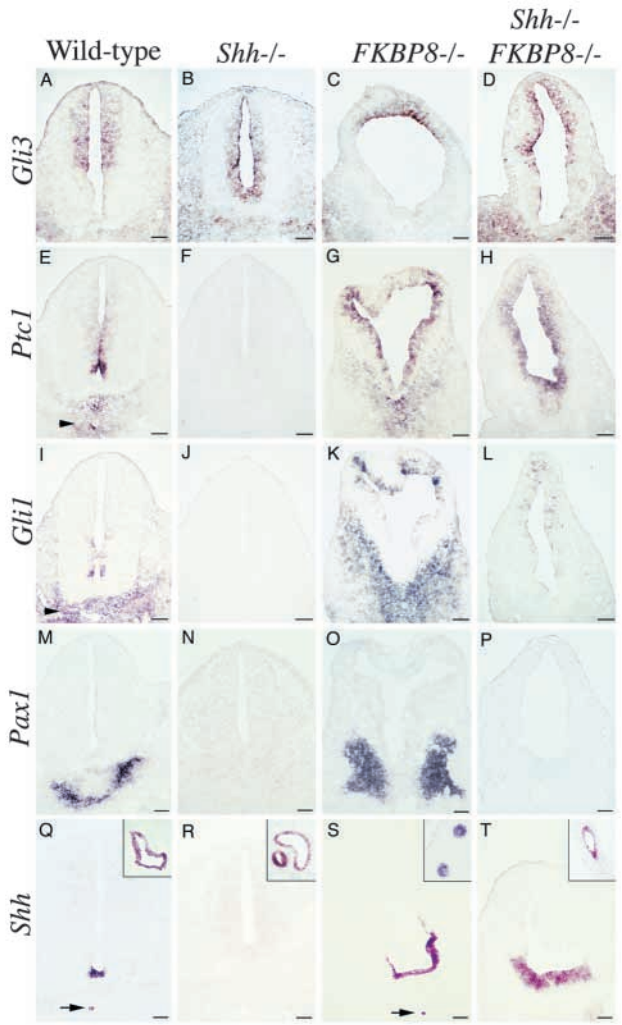

Fig. 8. Neural-specific effects of the *Fkbp8* mutation on hedgehog signaling. E11.5 neural tube sections stained for *Gli3* (A-D), *Ptch1* (E-H), *Gli1* (I-L), *Pax1* (M-P) and *Shh* (Q-T) expression. In the *Shh*$^{-/-}$ ventral neural tube, *Gli3* is ectopically expressed (B), whereas *Ptch1* and *Gli1* fail to be expressed (F,J). SHH is also required for expression of *Ptch1*, *Gli1* and *Pax1* in sclerotomal tissues (arrowheads in E,I). In *Fkbp8*$^{-/-}$ and *Shh*$^{-/-}$;*Fkbp8*$^{-/-}$ embryos, *Gli3* expression is repressed ventrally, whereas *Ptch1* and *Gli1* are expressed in the neural tube in dorsally expanded domains. Note that *Gli1* is not expressed in ventral neural tube regions of *Fkbp8*$^{-/-}$ and *Shh*$^{-/-}$;*Fkbp8*$^{-/-}$ embryos nor in the wild-type floor plate. Sclerotomal expression of *Ptch1*, *Gli1*, and *Pax1* is not rescued in *Shh*$^{-/-}$;*Fkbp8*$^{-/-}$ mutants (H,L,P). *Shh* is expressed in the gut of all four mutants (insets in Q-T) but not in the notochord or floorplate of *Shh*$^{-/-}$ mutants (R). The expanded floorplates of *Fkbp8*$^{-/-}$ and *Shh*$^{-/-}$;*Fkbp8*$^{-/-}$ mutants express *Shh*, but notochord expression (arrows in Q,S) is not seen in the *Shh*$^{-/-}$;*Fkbp8*$^{-/-}$ mutant. Scale bars: 65 μm.

Because GLI3 represses SHH target genes (Persson et al., 2002), SHH signaling is thought to activate targets in part by antagonizing the repressive activity of GLI3. SHH may also act by antagonizing GLI3 at the transcriptional level. Indeed, *Gli3* expression is normally restricted to lateral and dorsal neural tube and is found ectopically throughout the ventral neural tube of the *Shh*$^{-/-}$ mutant. *Gli3* expression was dorsally shifted in both the *Fkbp8*$^{-/-}$ single mutant and the *Shh*$^{-/-}$;*Fkbp8*$^{-/-}$ double mutant (Fig. 8). The dorsal shift of *Gli3* expression in the mutants may also contribute to the ligand-independent expression of SHH targets in the ventral neural tube of *Shh*$^{-/-}$;*Fkbp8*$^{-/-}$ double mutants.

FKBP8 appears dispensable for SHH signaling in non-neural tissues

The overall morphology of *Shh*$^{-/-}$ single and *Shh*$^{-/-}$;*Fkbp8*$^{-/-}$ double mutants suggested that non-neural tissues requiring SHH signaling, such as the limbs, branchial arches and somites, do not activate the SHH signaling pathway in the absence of FKBP8. In support of this, *Ptch1* and *Gli1* were expressed in the medial portion of wild-type somites but not in *Shh*$^{-/-}$ or *Shh*$^{-/-}$;*Fkbp8*$^{-/-}$ somites (Fig. 8). Maintenance of *Pax1* expression in the sclerotome depends on SHH signaling (Chiang et al., 1996). At E11.5, *Pax1* was not expressed in the *Shh*$^{-/-}$ mutants nor was it expressed in the somitic mesoderm of the double mutant, indicating that sclerotomal fate is not restored by the loss of FKBP8.

In addition to expressing *Shh*, the notochord requires SHH signaling for its own maintenance (Chiang et al., 1996). We confirmed the absence of the notochord in *Shh*$^{-/-}$ mutants using a 5′ RNA probe that could detect *Shh* transcript from the mutant allele; there was clear staining in the *Shh*$^{-/-}$ mutant gut (Fig. 8R, inset) but no staining in the position of the notochord (Fig. 8R). Although loss of FKBP8 clearly rescued neural tube *Shh* expression in the double mutant (Fig. 8T; in an expanded ventral domain), there was no morphological notochord and no *Shh* expression was observed in cells ventral to the neural tube. These data indicate that FKBP8 plays no detectable role in SHH signaling in the somites and the notochord.

The *Fkbp8* mutant phenotype is not caused by ectopic expression of Indian hedgehog or desert hedgehog

The other hedgehog family members, Indian hedgehog (*Ihh*) or desert hedgehog (*Dhh*), are not normally expressed in the neural tube or notochord although they are capable of inducing ventral neural fates (Pathi et al., 2001). To rule out ectopic activity of *Ihh* and *Dhh* as a cause for the ligand-independent activation of the SHH pathway, we examined their expression in the *Shh*$^{-/-}$;*Fkbp8*$^{-/-}$ double mutant. Although normal expression of *Ihh* and *Dhh* was observed in the gut and gonad, there was no ectopic expression in the neural tube or notochord by in situ hybridization (Fig. 9A). In addition, no hedgehog proteins were detected in the neural tube of the double mutant by immunofluorescence (Fig. 9B) using a monoclonal antibody (clone 5E1) that crossreacts with all three hedgehog ligands (Wang et al., 2000). Thus, activation of the SHH pathway in the double mutant is independent of all hedgehog family members.

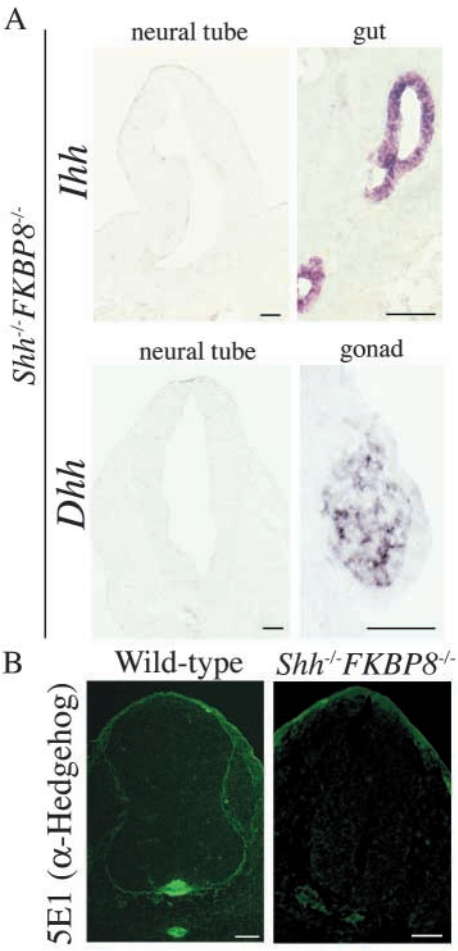

Fig. 9. Expression of hedgehog family members in *Shh*$^{-/-}$; *Fkbp8*$^{-/-}$ mutants. (A) Neither Indian hedgehog (*Ihh*) nor desert hedgehog (*Dhh*) expression is detected in the vicinity of the E11.5 *Shh*$^{-/-}$; *Fkbp8*$^{-/-}$ double mutant neural tube by in situ hybridization, although their endogenous expression (in the gut and gonad, respectively) is readily detected. (B) Hedgehog proteins are monitored using the monoclonal antibody 5E1. No staining is found within or around the E10.5 double mutant neural tube, although SHH protein is clearly detected in the notochord and floor plate of the wild-type embryo. Scale bars: 65 μm.

Discussion

This study demonstrates that FKBP8 is a negative regulator of the SHH pathway in the developing CNS. The conclusion is based in part on the ectopic expression of genes activated by SHH signaling in the *Fkbp8*$^{-/-}$ mutant neural tube. One of these genes (*Foxa2*) is known to be a direct transcriptional target of hedgehog signaling (Sasaki et al., 1997). In addition, genes that are normally repressed by SHH signaling are not expressed or are shifted dorsally in the *Fkbp8*$^{-/-}$ neural tube. The activation of SHH target genes in the *Fkbp8*$^{-/-}$ mutant is independent of SHH, as these targets are also expressed in the *Shh*$^{-/-}$;*Fkbp8*$^{-/-}$ double mutant. The SHH pathway appears maximally activated in the ventral neural tube of the double mutant because these ventral cells adopt the floor-plate identity, which requires the highest level of SHH signaling for its specification (Ericson et al., 1997; Roelink et al., 1995).

The TGFβ (BMP/GDF) and WNT signaling pathways are also important in the specification of dorsal cell fates in the spinal cord (Lee et al., 1998; Liem et al., 1995; Muroyama et al., 2002). Thus, it is formally possible that FKBP8 acts by promoting TGFβ or WNT signaling, which would account for the absence of dorsal neural fates in the *Fkbp8*$^{-/-}$ mutant. A role for FKBP8 in the BMP signaling pathway seems unlikely, given the normal expression of BMP signaling targets MSX1 and MSX2 in the *Fkbp8*$^{-/-}$ mutant. Moreover, the specification of ventral neural fates is not default; the lack of TGFβ or WNT signaling is not sufficient for the development of ventral fates in the absence of hedgehog signaling (Ericson et al., 1997; Liem et al., 2000; Megason and McMahon, 2002). Thus, the rescued development of ventral neural fates in the

Shh$^{-/-}$;*Fkbp8*$^{-/-}$ double mutant is most probably due to the loss of an essential antagonist in the hedgehog signaling pathway.

Hedgehog signaling antagonists

Several antagonists of the mouse hedgehog pathway have been characterized genetically, including PTCH1, RAB23, GLI3 and PKA. Mutations in any of these components lead to expansion of ventral cell types in the neural tube. *Ptch1*-null mutants show the strongest phenotype; cells at all positions in the *Ptch1*$^{-/-}$ neural tube acquire the floor plate fate, although cells also express markers of other ventral cell types, including V3 interneuron progenitors and motoneurons (Goodrich et al., 1997; Motoyama et al., 2003). No lateral or dorsal cell types (marked by *Pax6* and *Pax3* expression, respectively) are specified in the *Ptch1*$^{-/-}$ mutant. Mutants lacking *Fkbp8*, *Rab23*, or with partial loss-of-function of *Ptch1* or *Pka* (*Ptch1-plof* and *Pka-plof*) show a less severe phenotype in which the neural tube is partially ventralized (Eggenschwiler and Anderson, 2000; Huang et al., 2002; Milenkovic et al., 1999). The least severe phenotype is observed in the *Gli3*-null mutant, in which the expression domains of some lateral markers expand into slightly more dorsal regions (Persson et al., 2002).

There are interesting differences between the neural patterning phenotypes of *Fkbp8*$^{-/-}$, *Rab23*, *Pka-plof* and *Ptch1-plof* mutants. For example, the dorsal cell fates marked by expression of Pax3 and Pax7 are completely deleted in *Rab23*$^{-/-}$, *Pka-plof* and *Ptch1-plof* mutants but not in the *Fkbp8*$^{-/-}$ mutant. At the same time, ventral cell types, the floor plate and V3 progenitors are more strongly affected in the *Fkbp8*$^{-/-}$ mutant than in the other mutants of this class. Thus, Rab23 and PKA appear to be more important than FKBP8 in repressing hedgehog signaling in dorsal cells, and FKBP8 plays a more important role in ventral cells. These results suggest that the requirement for each of these factors in hedgehog signaling is not identical in all cells along the dorsoventral axis and may reflect differences in their mechanisms of action.

Although FKBP8 is required for neural tube patterning, this requirement varies along the anteroposterior axis. An overt dorsoventral patterning defect was seen only in the neural tube at lumbosacral levels, whereas patterning of the thoracic neural tube was normal. Further anteriorly, in the cephalic neural tube, a role for FKBP8 in SHH signaling is again apparent. Here, eye development was either delayed or disrupted in the *Fkbp8*$^{-/-}$ mutant, suggesting that excessive SHH signaling occurs in the ventral domain of the brain and optic vesicles. Consistent with this interpretation, the eye defects were accompanied by downregulation of PAX6 expression and ectopic PAX2 expression. In addition, ventral forebrain development in the *Shh*$^{-/-}$ mutant was rescued by the concomitant loss of FKBP8. The variable phenotype with respect to the anteroposterior neural axis does not appear to be related to regional differences in *Fkbp8* expression as the gene is expressed uniformly along the anteroposterior and dorsoventral axes of the neural tube. Similar anteroposterior differences have been observed in mutants lacking the hedgehog signaling antagonists RAB23 or PKA (Eggenschwiler and Anderson, 2000; Huang et al., 2002), and in mutants lacking both GLI3 and SHH (Litingtung and Chiang, 2000). In each of these cases, the embryos showed pronounced ventralization of caudal neural tube but minimal changes in the thoracic neural tube. Although the reason for this is unclear, it has been shown that the balance of activating and repressing functions of GLI3 vary along this axis (Litingtung and Chiang, 2000; Motoyama et al., 2003), suggesting that different effects of the *Rab23*, *Pka* and *Fkbp8* mutations on GLI3 and GLI2 activity might account for this phenomenon.

A tissue-specific role for FKBP8 in SHH signaling

We did not observe patterning defects in any of the non-neural tissues examined in the *Fkbp8* mutant. The limbs, somites or branchial arches all appeared normal. These are SHH-responsive tissues affected in other HH pathway mutants, such as *Shh*$^{-/-}$, *Smo*$^{-/-}$, *Ptch1*$^{-/-}$, *Rab23*$^{-/-}$ and *Gli3*$^{-/-}$ (Chiang et al., 1996; Eggenschwiler et al., 2001; Goodrich et al., 1997; Hui et al., 1993; Zhang et al., 2001). The *Shh* mutant phenotype was rescued only in the caudal neural tube and the brain of the *Shh*$^{-/-}$;*Fkbp8*$^{-/-}$ double mutant; there was no apparent rescue of the somite, limb or branchial arch defects. Indeed, *Ptch1*, *Gli1* and *Pax1* expression was not restored in the sclerotome of the double mutant somites, nor was *Shh* expression rescued in the double mutant notochord. These data suggest that the role of FKBP8 in the SHH signaling pathway is restricted to neural tissues.

There are several precedents for tissue-specific action of *Drosophila* HH pathway components. For example, in the *Drosophila* embryonic ectoderm, Fused kinase is required for HH responses in cells anterior to, but not posterior to, the *hh* expression domain, even though both types of cells respond to and require HH signaling (Therond et al., 1999). In the mouse, reduction of PKA activity affects neural tissues preferentially (Huang et al., 2002). Tissue-specific differences in the requirement for GLI transcription factors has also been observed. The effects of the *Gli3* mutation on limb development are much more dramatic than its effects on neural patterning, whereas the opposite is true for the *Gli2* mutation (Matise et al., 1998; Persson et al., 2002; te Welscher et al., 2002), indicating that different GLI transcription factors mediate responses to SHH signals depending on cell type. Thus, although some components of the hedgehog signal transduction mechanism, such as Ptch1 and Smo, are required in nearly all cell types (Goodrich et al., 1997; Zhang et al., 2001), signal transduction downstream of these components can vary significantly depending on context.

The hedgehog signaling pathway in mammals

FKBP8 joins a group of recently uncovered components of the mammalian hedghog signaling pathway including SIL, HIP, GAS1, megalin, RAB23, IFT172, polaris/TG737 and KIF3A (Chuang and McMahon, 1999; Eggenschwiler et al., 2001; Huangfu et al., 2003; Izraeli et al., 2001; Lee et al., 2001; McCarthy et al., 2002). Other components, such as PTCH1, dispatched, SMO, PKA, suppressor of fused, and the hedghog and GLI families play analogous roles in *Drosophila* and mammals (reviewed by Ingham and McMahon, 2001; Nybakken and Perrimon, 2002). However, in several cases novel components that are present in the mammalian genome have no clear orthologs in *Drosophila*, such as SIL, HIP and GAS1. *Drosophila* homologs of other components, such as polaris/*TG737* (*nompB*) and *Kif3a* (*Klp64D*), have been identified but these genes appear to have roles unrelated to hedgehog signaling in the fly (Kernan et al., 1994; Ray et al., 1999). FKBP8 shares sequence similarity with an

uncharacterized *Drosophila* gene product, CG5482 (http://flybase.bio.indiana.edu). The predicted CG5482 protein has the same overall domain structure, as well as a membrane insertion site at the extreme C terminus. There are no known mutations in the *CG5482* gene, nor are there mutations in other *Drosophila FKBP* genes that suggest their involvement in hedgehog signaling. Similarly, genes such as *Rab23* and *Ift172* have homologs in the *Drosophila* genome but the roles of these genes in *Drosophila* are not yet clear.

Taken together, these data suggest that during the evolution of mammals, significant differences in the mechanism of hedgehog signaling regulation have developed. These differences underscore the importance of complementing genetic studies of hedgehog signaling in *Drosophila* with similar studies in mice to gain insight into how the pathway functions in humans.

Biochemical functions of FKBP8

We do not understand how FKBP8 acts in the hedgehog pathway at the molecular level. We have observed that some endogenous FKBP8 protein is present in a complex with the RII subunit of protein kinase A in vivo (O.V.B. and T.L., unpublished), raising the possibility that FKBP8 has a role in PKA-dependent phosphorylation of the GLI proteins. This possibility is supported by the similarity in phenotype between mutants deficient in FKBP8 and PKA (Huang et al., 2002). Total PKA activity in *Fkbp8* mutant embryos, measured in vitro, is not diminished, suggesting that FKBP8 is not required for general PKA activity (O.V.B. and T.L., unpublished). Shirane and Nakayama (Shirane and Nakayama, 2003) found that FKBP8 associates with and inhibits the phosphatase calcineurin. This raises the possibility that activated calcineurin can promote hedgehog signaling. Regardless of the biochemical functions of FKBP8 in hedgehog signaling, it seems likely that its activities ultimately converge on the GLI transcription factors. Given that *Gli2*-null mutants have a considerably more severe neural patterning phenotype than *Gli3* or *Gli1* null mutants, we favor GLI2 as the principal target of FKBP8 function. This hypothesis is currently being tested though genetic epistasis.

FKBP8 is widely expressed in both adult and embryonic tissues. The function described in this study identifies the earliest requirement for FKBP8 during mouse development, leaving open the possibility that FKBP8 has other roles later in the embryo and in the adult. Failure to regulate hedgehog signaling properly in humans is associated with a variety of disorders ranging from birth defects such as holoprosencephaly to cancers of the brain and skin. Thus, identification of components of this signaling pathway and understanding how they function at the molecular level will have significant implications for the diagnosis and treatment of human diseases.

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