Tectonic, a novel regulator of the Hedgehog pathway required for both activation and inhibition

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We report the identification of a novel protein that participates in Hedgehog-mediated patterning of the neural tube. This protein, named Tectonic, is the founding member of a previously undescribed family of evolutionarily conserved secreted and transmembrane proteins. During neural tube development, mouse Tectonic is required for formation of the most ventral cell types and for full Hedgehog (Hh) pathway activation. Epistasis analyses reveal that Tectonic modulates Hh signal transduction downstream of Smoothened (Smo) and Rab23. Interestingly, characterization of *Tectonic Shh* and *Tectonic Smo* double mutants indicates that Tectonic plays an additional role in repressing Hh pathway activity.

Supplemental material is available at http://www.genesdev.org.

Received August 9, 2005; revised version accepted November 2, 2005.

Hh signals are secreted proteins essential for normal development and tissue homeostasis. Misregulation of Hh signaling in humans can lead to congenital defects and cancers (McMahon et al. 2003). The most extensively studied function of the Hedgehog (Hh) family member Sonic hedgehog (Shh) is its role in the developing neural tube. There, Shh acts as a morphogen to direct the production of particular neuronal subtypes at defined dorsoventral positions (Jacob and Briscoe 2003). Shh mediates its effects by binding to its receptor, Patched (Ptch). Ptch, in the absence of Shh, represses the downstream signaling pathway by inhibiting the activity of Smoothened (Smo), a seven-transmembrane protein. Binding of Shh to Ptch relieves the repression of Smo, triggering events that culminate in the activation of transcription factors of the Gli family. How Hh signals are transduced is incompletely understood.

Results and Discussion

Through a screen for genes encoding secreted and transmembrane proteins (Skarnes et al. 1995; Mitchell et al. 2001), we identified a novel gene which, because it is involved in a diverse range of developmental processes, we named *Tectonic* after the Greek word for builder. Conceptual translation of *Tectonic* indicates that it encodes a 63-kDa protein with no recognized domains other than an N-terminal signal peptide. Genomic database searches identify two other mammalian Tectonic family members, Tect2 and Tect3, which are 49% and 58% similar to Tectonic, respectively (Supplementary Fig. 1). The *Drosophila* genome contains a single *Tectonic* homolog. Thus, Tectonic is the founding member of an evolutionarily conserved family of proteins of undetermined function.

To assess whether Tectonic is secreted as predicted, we created a fusion between the putative Tectonic signal peptide and alkaline phosphatase. This fusion is robustly secreted by Cos7 cells, indicating that the signal peptide is functional (Supplementary Fig. 2). Interestingly, full-length Tectonic is not secreted by Cos7 cells, suggesting that its secretion may be regulated.

Insertion of the gene trap vector in *Tectonic* occurs in the first of 12 introns (Fig. 1A). The resultant mutant allele encodes a fusion between the first 57 amino acids of Tectonic and a membrane-spanning βgeo reporter (Mitchell et al. 2001). Given that no wild-type transcript is detectable in *Tectonic* mutants by RT–PCR and Northern blot analyses (Fig. 1B,C), and that transmembrane βgeo fusion proteins are retained in intracellular compartments (Skarnes et al. 1995), the *Tectonic* gene trap is likely to be a null allele.

During embryonic development, *Tectonic* is expressed in regions that participate in Hh signaling. *Tectonic* is first expressed during gastrulation stages in the ventral node (Fig. 1D,E). At embryonic day 9.5 (E9.5), *Tectonic* is expressed in the gut endoderm, limb buds, notochord, somites, neural tube and floorplate (Fig. 1F). Unlike regulators of Hh signaling such as *Ptch* and *Hhip* (Goodrich et al. 1996; Marigo and Tabin 1996; Chuang and McMahon 1999), *Tectonic* is not a transcriptional target of Hh signaling (Supplementary Fig. 3B,C).

Tectonic mutants die between E13.5 and E16.5 and display holoprosencephaly (Fig. 1G), a defect associated with reduced Hh signaling (Chiang et al. 1996). Shh mediates induction of the floorplate, a histologically distinct cell population at the ventral midline of the neural tube. Like *Shh* mutants and *Gli2* mutants (Chiang et al. 1996; Ding et al. 1998; Matise et al. 1998), *Tectonic* mutants fail to form floorplates and, instead, cells of neural morphology are present at the midline (Fig. 2A). Molecular analysis with the markers *Shh* and FoxA2 (Hnf3β) confirms that *Tectonic* is required for floorplate formation (Figs. 2B, 3B). However, the notochord forms normally in *Tectonic* mutants as judged by *Shh* and *Brachyury* expression (Fig. 2B; Supplementary Fig. 3D). Thus, axial defects in *Tectonic* mutants are confined to the floorplate.

In addition to the floorplate, high levels of Hh signaling are required for the induction of the adjoining V3 interneurons (Litingtung and Chiang 2000; Wijgerde et

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[*Keywords*: Shh; signal transduction; mouse development; neural patterning; open brain]

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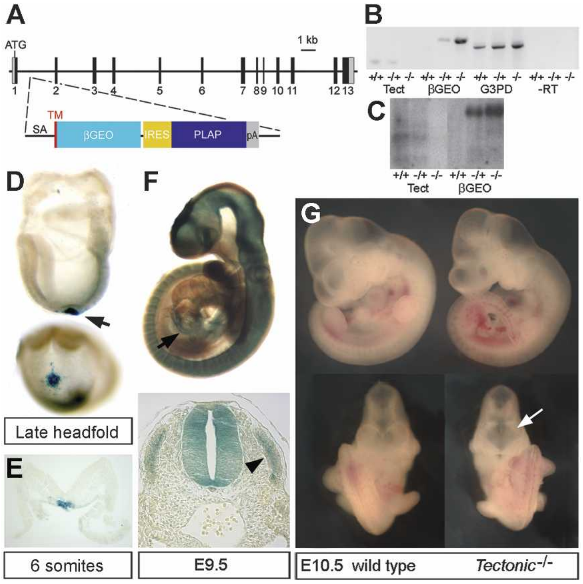

Figure 1. *Tectonic* is expressed in domains of Hh signaling, and is essential for embryonic development. (*A*) The mouse *Tectonic* gene is comprised of 13 exons on chromosome 5. The gene trap consists of a strong splice acceptor (SA) followed by an ORF encoding a transmembrane domain (TM) and βGEO. The gene trap also includes an IRES and PLAP coding sequence followed by a polyadenylation sequence (pA). (*B*) RT–PCR analysis of *Tectonic* gene expression in E11.5 wild-type, heterozygous, and homozygous mutant embryos. Primers are specific for the *Tectonic* coding sequence 3′ to the gene trap (Tect), the β*GEO* transcript, and *G3PD*. Included is a –RT control using *G3PD*-specific primers. (*C*) Northern blot analysis of *Tectonic* and β*GEO* expression in wild-type, heterozygous, and mutant embryos. (*D–F*) β-Galactosidase staining of *Tectonic* heterozygotes. (*D*) Lateral and distal views of late headfold stage embryos, demonstrating restricted *Tectonic* expression in the node (arrow). (*E*) *Tectonic* is expressed in the ventral epithelium of the node, as revealed in a transverse section through the node of a six-somite stage embryo. (*F*) At E9.5, *Tectonic* is expressed in the neural tube, gut epithelium (arrow), notochord, and somites (arrowhead), as seen both in whole-mount and transverse section. (*G*) E10.5 *Tectonic* mutants exhibit reduced telencephalon size and holoprosencephaly (arrow).

al. 2002). Analysis of neural tube patterning reveals that, like Shh, Tectonic is required for formation of the *Sim1*-expressing V3 interneurons (Fig. 2C). Nkx2.2, a marker of the progenitors of the V3 interneurons (Briscoe et al. 1999), is also lost in *Tectonic* mutants (Fig. 3C), suggesting that these defects are not due to defects in neuronal maturation, but in their specification. Moreover, the Tectonic-dependent defects in ventral neural development are not limited to the V3 interneurons. *Tectonic* mutants also display a variable reduction in the number of Islet1/2-positive motor neurons (Fig. 3D). However, Tectonic is not required for the expression of *Dbx1* or *Dbx2* (Fig. 2D; data not shown), indicating that Tectonic function is not essential for the development of more dorsal cell fates within the neural tube.

The loss of ventral neural markers in *Tectonic* mutants is accompanied by a ventral expansion of genes normally restricted to more dorsal domains. High levels of Hh signaling exclude expression of *Irx3* from the V3 and motor neuron progenitor (p3 and pMN) domains (Briscoe et al. 2000). In *Tectonic* mutants, *Irx3* expression expands to include all but a small number of ventral cells (Fig. 2E). Similarly, expression of Pax6, another factor repressed by high Hh signaling (Ericson et al. 1997), is dramatically expanded in *Tectonic* mutants (Fig. 3B). Taken together, these changes in marker expression indicate that Tectonic is essential for the induction of the ventral-most cell types of the neural tube. These patterning defects are qualitatively similar to those caused by mutations in *Shh* or *Gli2* (Chiang et al. 1996; Ding et al. 1998; Matise et al. 1998; Litingtung and Chiang 2000), suggesting that Tectonic participates in Hh signaling.

To test this hypothesis, we examined the expression of *Gli1* and *Ptch*, two general Hh transcriptional targets. Significantly, *Gli1* expression is reduced throughout *Tectonic* mutant embryos at E9.5 (Fig. 2F). In the developing neural tube of *Tectonic* mutants, expression of *Gli1* and *Ptch* is similarly dramatically reduced (Fig. 2G,H). Hh signaling in the neural tube is antagonized by Bmp activity (Barth et al. 1999; Kawakami et al. 2005). Expression of Msx1, a readout of Bmp pathway activity in the dorsal neural tube (Liu et al. 2004), is normal in

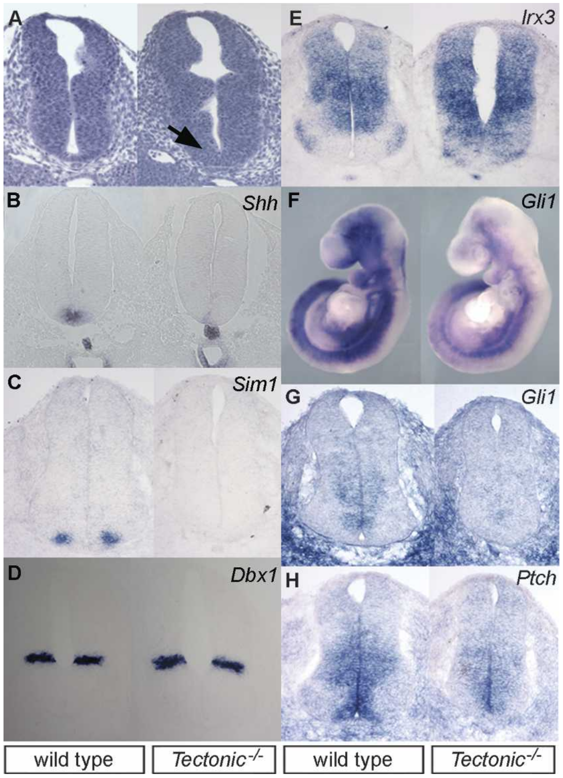

Figure 2. Tectonic is required for Hh-mediated patterning of the ventral neural tube. (*A*) Hematoxylin-and-eosin-stained transverse sections of E9.5 embryos. *Tectonic* mutants lack a histologically distinct floorplate (arrow). (*B–H*) In situ hybridization of E9.5 whole-mount embryos (*F*), or transverse sections of E9.5 (*B*) or E10.5 (*C–E,G,H*) embryos. (*B*) *Shh*, a marker of the floorplate, is not expressed in the *Tectonic* mutant neural tube. However, *Tectonic* mutants express *Shh* normally in the notochord and gut epithelium. (*C*) Similarly, *Sim1*, a marker of V3 interneurons, is not expressed in the *Tectonic* mutant neural tube. (*D*) Expression of *Dbx1*, a marker of V0 interneuron precursors, is expressed in *Tectonic* mutants. (*E*) Expression of *Irx3*, a gene normally expressed dorsal to the pMN domain, is expanded almost to the ventral midline of *Tectonic* mutants. (*F*) *Gli1*, a general transcriptional target of Hh signaling, is broadly diminished in *Tectonic* mutants. (*G*) Similarly, *Gli1* expression is reduced in the neural tubes of *Tectonic* mutants. (*H*) *Ptch*, another general Hh transcriptional target, is also down-regulated in the *Tectonic* mutant neural tube.

Tectonic mutants (Supplementary Fig. 4), suggesting that Tectonic does not influence Hh signaling indirectly by altering Bmp activity. Together, these results argue that Tectonic acts in neural patterning by positively regulating the Hh pathway.

Conceptually, Tectonic could contribute to Hh signaling by participating in the creation of the Hh protein gradient or in the interpretation of that gradient. To distinguish between these two possibilities, we carried out epistasis experiments with *Ptch* mutants. If Tectonic acts in Hh processing, release or distribution, *Ptch* should be epistatic to *Tectonic*. However, if Tectonic acts in Hh signal transduction, *Tectonic* should be epistatic to *Ptch*. Ptch-dependent defects in embryonic turning and dorsal neural tube closure are ameliorated in *Tectonic Ptch* double mutants (Fig. 3A). Embryos lacking Ptch function show marked expansion of the ventral domains of the neural tube (Fig. 3B–D; Goodrich et al. 1997). Examination of the dorsoventral patterning of the neural tube of *Tectonic Ptch* double mutants reveals a loss of ventral neural fates indistinguishable from those of *Tectonic* single mutants (Fig. 3B–D).

Like Ptch, Rab23 is a negative regulator of the Hh pathway (Eggenschwiler et al. 2001; Huangfu et al. 2003). Embryos homozygous for the *opb*^{2} mutation in *Rab23* display a ventralized neural tube (Fig. 3E,F; Eggenschwiler and Anderson 2000). As with *Ptch*, embryos mutant for both *Rab23* and *Tectonic* display neural tube patterning defects identical to those of *Tectonic* single mutants (Fig. 3E,F). Together, these results indicate that *Tectonic* is epistatic to both *Ptch* and *Rab23*. As Rab23 has been reported to act downstream of Smo (Huangfu et al. 2003), these data suggest that Tectonic modulates Hh transduction at a point downstream of Ptch, Smo, and Rab23.

To investigate whether the Tectonic-mediated effects on neural tube patterning reflect changes in Hh pathway activity, we assayed the expression of *Gli1* in *Tectonic Ptch* double mutants (Fig. 3G). Loss of Ptch function causes ectopic expression of high levels of *Gli1* in the dorsal neural tube. In contrast, *Tectonic Ptch* double mutants display uniform low levels of *Gli1* expression (Fig. 3G). These data confirm that *Tectonic* is essential for maximal activation of the Hh pathway. Furthermore, our results strongly suggest that Tectonic functions downstream of both Ptch and Rab23 in the Hh signal transduction pathway, and not in Hh production or release. Consistent with this conclusion, Shh protein is distributed in a dorsoventral gradient in *Tectonic* mutant neural tubes similar to that of wild-type neural tubes (Supplementary Fig. 5).

One of the most prominent defects displayed by *Shh* mutants is the severe reduction in forebrain development (Chiang et al. 1996). Strikingly, *Tectonic Shh* and

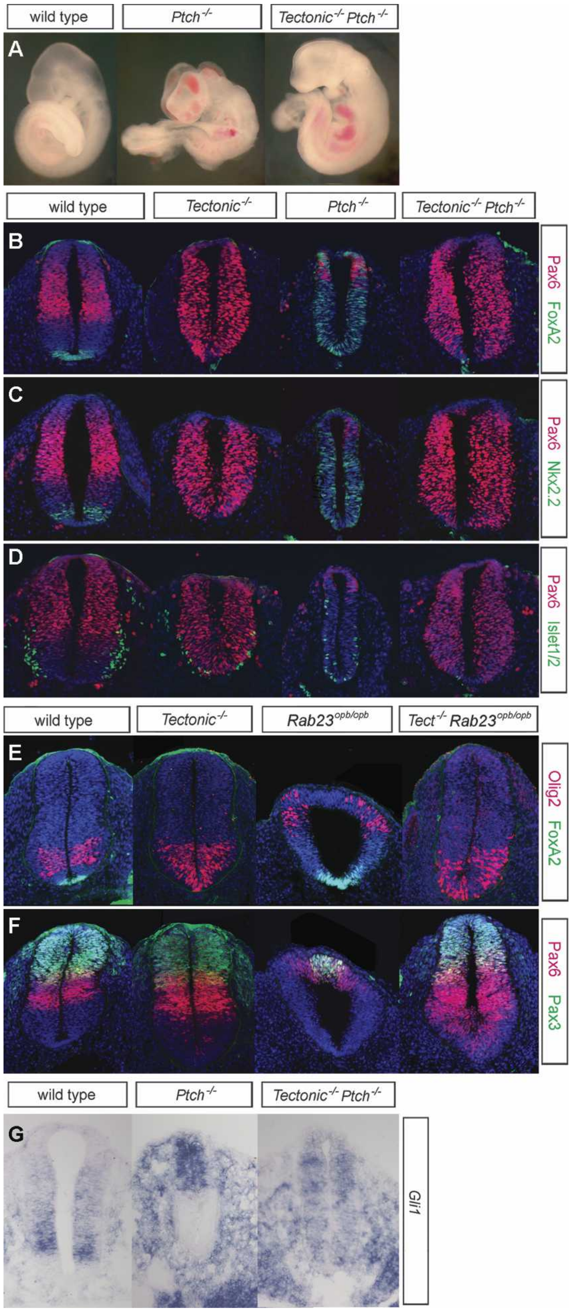

Figure 3. *Tectonic* is epistatic to *Ptch* and *Rab23*. (*A*) Lateral views of E9.5 littermates. *Ptch* mutants display a characteristic open neural tube and defective turning whereas normal turning is largely restored in *Tectonic Ptch* double mutants. (*B–D*) Transverse sections of E9.5 embryos stained for expression of Pax6 in red and, in green, FoxA2 (*B*), Nkx2.2 (*C*), or Islet1/2 (*D*). Nuclei are visualized with DAPI staining (blue). (*B*) *Tectonic* mutants lack floorplate expression of FoxA2 and show expanded Pax6 expression. Conversely, *Ptch* mutants display expanded FoxA2 expression and reduced Pax6 expression. *Tectonic Ptch* double mutants closely resemble *Tectonic* single mutants. (*C*) *Tectonic* mutants lack Nkx2.2 expression, a marker of the p3 domain, whereas *Ptch* mutants display expanded Nkx2.2 expression. *Tectonic Ptch* double mutants exhibit a loss of Nkx2.2 expression identical to that of *Tectonic* single mutants. (*D*) Motor neuron expression of Islet1/2 is reduced in most ($n = 4/5$) *Tectonic* mutants, expanded in *Ptch* mutants, and reduced in *Tectonic Ptch* double mutants. (*E*,*F*) Transverse sections of E10.5 embryos. (*E*) Similar to *Ptch* mutants, *Rab23* mutants exhibit an expansion of FoxA2 (green) and a dorsal shift in expression of Olig2, a marker of motor neuron precursors (red). In contrast, *Tectonic Rab23* double mutants resemble *Tectonic* mutants. (*F*) Expression of the dorsal markers Pax3 and Pax6 is shifted dorsally in *Rab23* mutants, but not in *Tectonic Rab23* double mutants. (*G*) *Gli1* in situ hybridization of transverse sections of E9.5 neural tubes. Whereas *Gli1* is normally expressed in a dorsoventral gradient, in *Ptch* mutants, *Gli1* is widely up-regulated and expressed ectopically in the dorsal neural tube. In *Tectonic Ptch* double mutants, *Gli1* is expressed at a uniform low level throughout the dorsoventral extent of the neural tube.

Tectonic Smo double mutants have considerably larger forebrains than do either *Shh* or *Smo* mutants (Fig. 4A; Supplementary Fig. 6). Although these results appear paradoxical given the reduced forebrains of *Tectonic* mutants, they suggest that there is a higher level of Hh activity in double mutants than in single mutants, implying that in addition to its role in pathway activation, Tectonic exerts a repressive effect on the pathway. To test whether this is the case, we examined neural tube expression of *Dbx1* and *Dbx2*, markers of the p0 and p1 precursors that are induced by low Hh levels (Wijgerde et al. 2002). If p0 and p1 formation in *Tectonic* mutants requires Shh activity, *Tectonic Shh* double mutants should show a reduction in *Dbx1* and *Dbx2* expression similar to that displayed by *Shh* mutants. However, our analysis reveals a dramatic increase in *Dbx1*- and *Dbx2*-expressing cells in *Tectonic Shh* double mutants as compared to *Shh* single mutants (Fig. 4B,C). These surprising results demonstrate that *Dbx1* and *Dbx2* expression in *Tectonic* mutants is independent of Shh, and suggest that Hh pathway activity is higher in *Tectonic Shh* double mutants than in *Shh* mutants.

To assess whether increased *Dbx1* and *Dbx2* expression reflects increased Hh pathway activation, we examined *Ptch* expression in *Tectonic Shh* double mutants. We found that the abrogation of *Ptch* expression exhibited by *Shh* mutants is indeed partially alleviated by loss of Tectonic function (Fig. 4D), indicating that levels of Hh pathway activation are in fact higher in *Tectonic Shh* double mutants than in *Shh* mutants. The genetic interaction between *Shh* and *Tectonic* is similar to that observed between *Shh* and *Gli3* (Litingtung and Chiang 2000) and suggest that Tectonic acts in a Shh-independent fashion to repress the Hh pathway. Taken with the forebrain data, these results reveal that Tectonic plays dual essential roles in both activating and inhibiting the Hh pathway in the anterior and posterior neural tube.

The loss of the activator function can be depicted as a rightward shift in the Hh pathway activity gradient of *Tectonic* mutants (Fig. 4E). Our additional finding that Tectonic inhibits Hh pathway activation in the absence of Shh can be represented graphically as a leftward shift in the Hh pathway activity gradient of *Tectonic Shh* double mutants relative to *Shh* mutants (Fig. 4E). This evidence that Tectonic functions in Hh signal transduction to fully activate the pathway in the presence of high Hh levels and to repress the pathway in the absence of Hh signals may reflect a combination of decreased function of both Gli activators and Gli repressors. In this regard, Tectonic joins a number of recently described regulators of Hh signal transduction including mouse IFT proteins (Huangfu et al. 2003; Liu et al. 2005) and

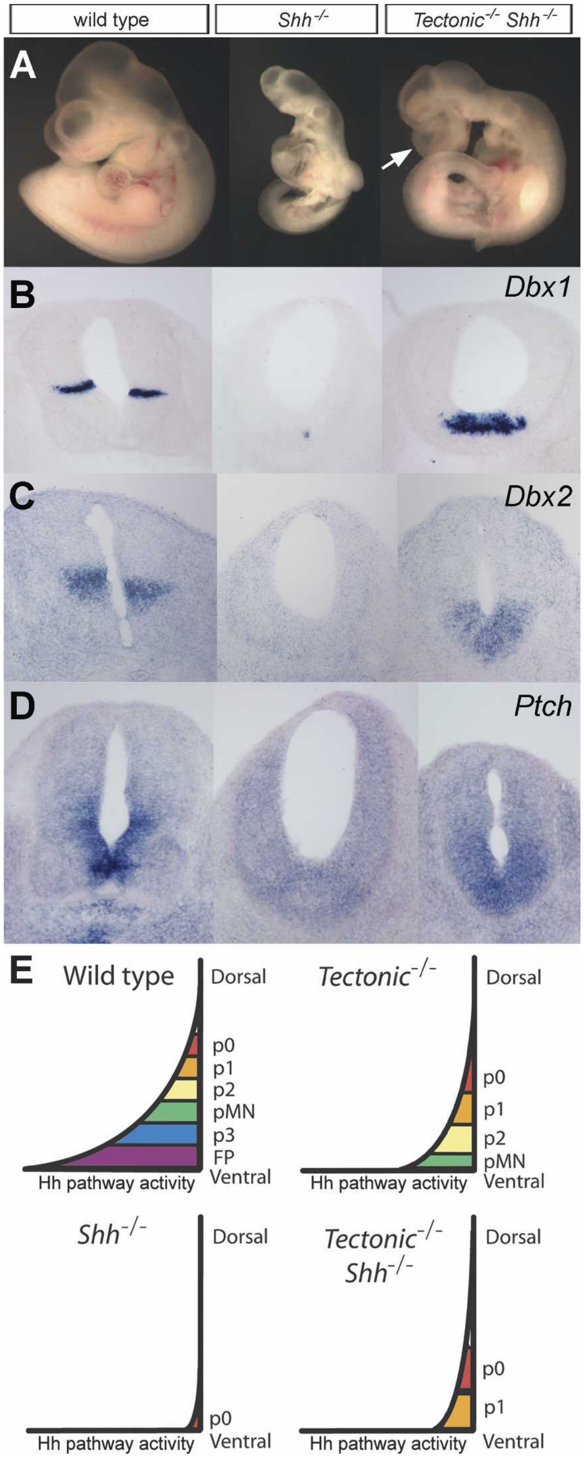

Figure 4. In addition to its role in mediating high levels of Hh signaling, Tectonic functions to repress low levels of Hh pathway activation. (*A*) Lateral view of E10.5 embryos. *Shh* mutants are one-third the size of littermates and show severely diminished forebrains. *Tectonic Shh* double mutants are larger than *Shh* single mutants and develop markedly larger telencephalons (arrow). (*B–D*) In situ hybridization of transverse sections of E10.5 embryos. (*B*) In *Shh* mutants, *Dbx1* expression, a marker of the p0 domain, is reduced to a very few cells at the ventral midline. *Tectonic Shh* double mutants exhibit increased *Dbx1* expression relative to *Shh* single mutants. (*C*) *Dbx2*, a marker of the p0 and p1 domains, is markedly reduced or not expressed in *Shh* mutants. In contrast, *Tectonic Shh* double mutants express *Dbx2* robustly at the ventral midline. (*D*) *Tectonic Shh* double mutants display higher levels of *Ptch* expression than do *Shh* single mutants. (*E*) Levels of Hh pathway activity within the developing ventral neural tube are translated into distinct fates, including floorplate (FP) and five neural precursor domains (p3–p0) at defined dorsoventral positions. In *Tectonic* mutants, neural fates that require the highest levels of Hh signaling are lost, represented as a rightward shift in the Hh pathway activity curve. Disruption of Shh function causes severe reduction of the p0 and p1 domains and loss of more ventral fates. In contrast, loss of both Shh and Tectonic function results in increased Hh pathway activity and restored p0 and p1 development, revealing an inhibitory role for Tectonic in the Hh pathway.

zebrafish Iguana (Sekimizu et al. 2004; Wolff et al. 2004). Additionally, a *Drosophila* protein complex that includes Cos2 is similarly required for full pathway activation (Robbins et al. 1997; Sisson et al. 1997; Wang and Holmgren 2000; Wang et al. 2000; Lefers et al. 2001) and inhibition (Methot and Basler 2000; Stegman et al. 2000; Wang et al. 2000; Lefers et al. 2001). Tectonic is the first extracytosolic factor shown to act in this dual capacity.

Although the molecular mechanism by which Tectonic functions is not clear, our double mutant analyses suggest that it modulates Hh signal transduction at a point fairly downstream in the pathway. As Rab23 acts in the same region of the pathway and is thought to control vesicle transport, it will be interesting to assess whether it regulates the trafficking of Tectonic.

Materials and methods

Mouse strains

The mouse embryonic stem cell line KST296 carrying an insertion of the pGT1pfs secretory trap vector in the *Tectonic* gene was isolated as described in Mitchell et al. (2001). *Tectonic* F1 heterozygotes were backcrossed to C57Bl/6 mice for six generations prior to intercrossing. Genotyping of *Tectonic* was performed using genomic PCR with a pair of wild-type-specific primers (5′-CGCCTCTTTAGCCCTCTGTT-3′ and 5′-AGAACCTCCACGAGAGCAGA-3′) and a mutant-allele-specific primer (5′-TCTAGGACAAGAGGGCGAGA-3′). *Ptch*, *Rab23*, *Shh*, and *Smo* embryos were genotyped as described (Chiang et al. 1996; Goodrich et al. 1997; Eggenschwiler et al. 2001; Zhang et al. 2001).

Secretion assays

Cos7 cells were transfected using Fugene6 (Roche) with APTag5 (GenHunter) or APTag5-TectSignal, a vector in which the SEAP signal sequence has been replaced with that of Tectonic. Alkaline phosphatase activity in the supernatant was chemiluminescently measured using the Phospha-Light Assay (Applied Biosystems) and a 20/20^{n} luminometer (Turner BioSystems).

RT–PCR and Northern blot analyses

RT–PCR was performed using exon-spanning primers complementary to *Tectonic* cDNA 3′ to the gene trap insertion (5′-AATCCGCTGTTCC TTCCAC-3′ and 5′-TGCGTCAGTGTGTGATTCAG-3′), to the β*GEO* transcript (5′-CTTGGGTGGAGAGGCTATTC-3′ and 5′-AGGTGAG ATGACAGGAGATC-3′), and to *G3PD* (5′-GTGTTCCTACCCCCAAT GTG-3′ and 5′-TGTGAGGGAGATGCTCAGTG-3′). Northern blots were hybridized to a *Tectonic* cDNA probe spanning exons 2–12 and a probe to β*geo*.

Immunohistochemistry and in situ hybridization

X-gal staining, in situ hybridization, and immunohistochemical staining were carried out using antibodies and protocols as previously described (Ericson et al. 1997; Briscoe et al. 1999, 2000; Takebayashi et al. 2000; Gritli-Linde et al. 2001) with the exception of rabbit α-Pax6 antibody (Covance Research Products), which was used at 1:300. The α-FoxA2, α-Nkx2.2, α-Islet1/2, α-Msx1/2, and α-Pax3 antibodies were obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa under contract NO1-HD-7-3263 from the NICHD.

Gene analysis and accession numbers

Sequences of Tectonic family members were aligned using ClustalW and Boxshade 3.21. Domain analysis was performed with SignalP 3.0 and HMMTOP 2.0. Mouse *Tectonic* cDNA sequence, GenBank accession number DQ278867; human *Tectonic* cDNA sequence, GenBank accession number DQ278868; mouse *Tect2* cDNA sequence, GenBank accession number DQ278869; human *Tect2* cDNA sequence, GenBank accession number DQ278870; mouse *Tect3* cDNA sequence, GenBank accession number DQ278871; human *Tect3* cDNA sequence, GenBank accession number DQ278872; Drosophila *dTectonic* cDNA sequence, GenBank accession number DQ278873.

Acknowledgments

We are grateful to Debbie Pangilinan for expert mouse husbandry, to Andrew Norman for technical assistance, and to Pao-Tien Chuang and Andrew McMahon for the anti-Shh antibody Ab80. We thank Kevin Mitchell, Chulho Kang, and Marc Tessier-Lavigne for help generating the KST296 ES cell line and the *Tectonic* mice. This work was assisted by valuable conversations with Gail Martin, Pao-Tien Chuang, Didier Stainier, and Tom Kornberg. This research was supported by funding from the Chicago Community Trust and grants from the NICHD and NIMH. J.R. is a UCSF Fellow generously supported by the Sandler Foundation and a March of Dimes Basil O'Connor Award.

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