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LETTERS

# Boc is a receptor for sonic hedgehog in the guidance of commissural axons

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**In the spinal cord, sonic hedgehog (Shh) is secreted by the floor plate to control the generation of distinct classes of ventral neurons along the dorsoventral axis[1]. Genetic and *in vitro* studies have shown that Shh also later acts as a midline-derived chemoattractant for commissural axons[2]. However, the receptor(s) responsible for Shh attraction remain unknown. Here we show that two Robo-related proteins, Boc and Cdon, bind specifically to Shh and are therefore candidate receptors for the action of Shh as an axon guidance ligand. Boc is expressed by commissural neurons, and targeted disruption of *Boc* in mouse results in the misguidance of commissural axons towards the floor plate. RNA-interference-mediated knockdown of Boc impairs the ability of rat commissural axons to turn towards an ectopic source of Shh *in vitro*. Taken together, these data suggest that Boc is essential as a receptor for Shh in commissural axon guidance.**

Growing axons respond to attractive and repulsive guidance cues to navigate to their targets[3–5]. During spinal cord development, commissural neurons in the dorsal neural tube send axons towards then across the floor plate at the ventral midline, forming axon commissures (Supplementary Fig. 1). Commissural axons project towards the midline in part because they are attracted by netrin-1, a long-range chemoattractant secreted by the floor plate[6–8]. *In vivo* and *in vitro* analyses of mice and explants mutant for *Netrin-1* indicated that the floor plate secretes an additional diffusible attractant for commissural axons[7]. We previously showed that Shh mimics the floor plate's additional chemoattractant activity and acts through Smo to attract commissural axons to the ventral midline[2]. However, Smo does not bind Shh[9], and the binding receptor(s) acting with Smo to mediate the effect of Shh in axon guidance remain unknown.

Cdon (cell-adhesion-molecule-related/downregulated by oncogenes) and Boc (biregional Cdon-binding protein) are type I transmembrane orphan receptors consisting of four or five immunoglobulin and two or three fibronectin type III (FNIII) repeats in the extracellular domain, and an intracellular domain with no identifiable motifs (Supplementary Fig. 1a); they have been implicated in enhancing muscle differentiation[10,11]. This domain architecture is closely related to that of axon guidance receptors of the Robo and DCC (deleted in colorectal cancer) families[3]. Both Cdon and Boc share a high degree of homology in their extracellular domains and are expressed during early stages of development of the central nervous system[12,13]. In zebrafish forebrain, the morpholino-mediated knockdown of Boc resulted in axon guidance defects[14], although how Boc participates in axon guidance was not explained. Mice with homozygous mutations in Cdon display a microform of holoprosencephaly (HPE)[15], a developmental defect of the forebrain and midface caused by a failure to delineate the midline[16,17]. In both humans and mice, many forms of HPE are caused by disruptions in the Shh signalling pathway, indicating that Cdon and Boc might regulate Shh signalling, a possibility supported by the finding that a Cdon/Boc homologue in *Drosophila*, CG9211/iHog, is required for hedgehog (Hh) signalling[18] and by recent reports demonstrating that Cdon, Boc and iHog bind and signal through the Hh pathway[19–21]. Taken together, these observations indicated that Cdon and/or Boc might function in Shh-mediated axon guidance in vertebrates.

To explore their involvement in commissural axon guidance, we first examined the expression of Cdon and Boc in the mouse spinal cord during development when axons project to the floor plate. We examined RNA expression by *in situ* hybridization and reporter-gene expression in *Boc* and *Cdon* mutant mice generated by targeting these loci with a genetrap cassette encoding a β-galactosidase–neomycin fusion (β-geo) and human placental alkaline phosphatase (PLAP) reporter genes with the use of the 'targeted trapping' method[22] (Supplementary Figs 1 and 2). *In situ* hybridization on spinal cord sections from embryonic day (E) 11.5 showed that *Boc* and *Cdon* are expressed in domains consistent with their expression in the progenitors of commissural neurons (Fig. 1a, b, and Supplementary Fig. 2a). During this period, *Cdon* is expressed exclusively in the dorsal ventricular zone (VZ), a domain composed of mitotically active progenitors (Supplementary Figs 1c and 2a, b). In comparison with *Cdon* expression, the *Boc* expression domain extends more ventrally and laterally, and encompasses the region where postmitotic commissural interneurons initiate their differentiation, as demonstrated by overlap of the sites of expression of *Boc* and *Robo3*/*Rig-1*, a gene expressed by commissural neurons and known to be essential in commissural axon guidance[23] (Fig. 1a, and Supplementary Fig. 2a). In the case of *Boc* (but not *Cdon*), the PLAP reporter also labelled axons projecting to the floor plate, which seemed to be commissural axons as assessed by co-expression of the surface glycoprotein TAG1 (Fig. 1b, and Supplementary Fig. 2e). This result supports the idea that *Boc* mRNA is expressed in differentiating commissural neurons. However, *Boc* expression seems transient, as demonstrated by its narrow overlap with the domain of *Robo3*/*Rig1* expression (Fig. 1a). To test more directly whether Boc protein is expressed by commissural axons, we generated antibodies against Boc. Although these antibodies did not work on tissue sections, they revealed Boc immunoreactivity on the cell bodies, axons and growth cones of dissociated commissural neurons (but not most hippocampal neurons, which do not express *Boc*) in culture (Fig. 1c, and data not shown). Taken together, these data suggest that *Boc* is expressed by differentiating commissural neurons and Boc protein is expressed by commissural axons during their growth to the midline.

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To assess the functions of these receptors in commissural axon guidance, sections of spinal cord from *Boc* and *Cdon* homozygous mutant E11.5 embryos were stained with antibodies against TAG1. Although commissural axon projections seemed normal in $Cdon^{+/-}$ and $Cdon^{-/-}$ animals, abnormal projections of $TAG1^{+}$ commissural axons, which also expressed PLAP, were observed in *Boc* mutants (Fig. 2, and Supplementary Fig. 2f). The axons were highly dispersed and invaded the ventral spinal cord with ectopic projections extending over the motor columns (Fig. 2a, d). This phenotype is similar to that observed in mice after conditional removal of the Shh signalling mediator *Smo* in commissural neurons, using a Wnt-1 promoter to drive Cre expression[2]. At E11.5, $Boc^{-/-}$ mice seemed morphologically normal; neural tube patterning, including the specification of dorsal cell types, also seemed normal in *Boc* mutants, as assessed by the expression of multiple neural tube markers (Supplementary Fig. 3). These data indicate that Boc might act in the same pathway as Smo to guide commissural axons in response to Shh.

To test whether Boc functions as a receptor for Shh in commissural axon guidance, we expressed a Boc–green fluorescent protein (GFP) fusion protein, Cdon–GFP fusion protein and a variety of control proteins in COS cells, assaying them for binding to Shh. Only cells expressing Hhip, Cdon or Boc, but not surrounding cells or cells transfected with controls, showed specific binding to Shh (Fig. 3a). Quantitative assessment of the affinity of these interactions with the use of Shh–AP, a fusion protein of Shh with alkaline phosphatase, revealed a dissociation constant ($K_d$) similar to that between netrin-1 and its receptor DCC[24] (Fig. 3b, c). The interaction between Shh and Boc or Cdon is direct, because purified N-Shh bound to the affinity-purified ectodomains of Boc and Cdon with the same affinity as to COS cells expressing Boc or Cdon (Boc-ecto–Fc; Fig. 3c, d). Deletion analysis suggested that the Shh-binding activity of Boc was primarily attributable to the third FNIII domain (FNIIIc; Supplementary Fig. 4), and neither Boc nor Cdon bound to Slit in either assay (data not shown). Together, these results show that Boc and Cdon bind specifically and directly to Shh, which is consistent with recent reports[19–21].

Although defects in *Boc* mutant mice are consistent with Boc's being required for Shh-mediated axon guidance, they are also potentially consistent with an involvement in mediating responses to netrin-1. We therefore examined the ability of *Boc*-deficient neurons to respond to netrin-1 *in vitro*[8]. Netrin-1 stimulated similar levels of axon outgrowth in *Boc* heterozygous and mutant explants (Supplementary Fig. 5), indicating that *Boc* is not required for the growth-promoting activity of netrin-1.

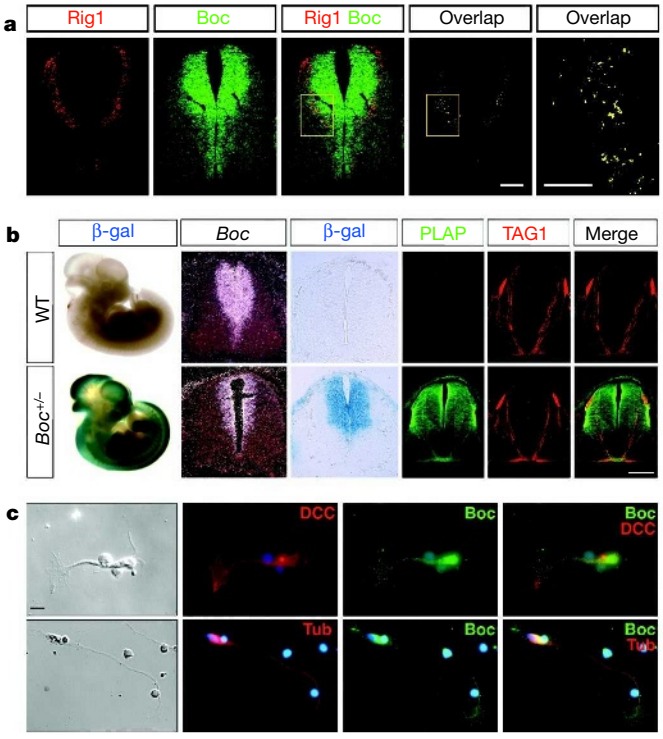


**Figure 1 | Boc is expressed by young commissural neurons undergoing axon guidance decisions in the spinal cord.** **a**, Fluorescent antisense probes to *Robo3/Rig1* (red) and *Boc* (green) on E11.5 spinal cord detect expression overlap, indicating that *Boc* might be expressed by commissural neurons. **b**, Expression of *Boc*, β-gal, PLAP and TAG1 in spinal cord of E11.5 wild-type (WT) and $Boc^{+/-}$ embryos. *Boc*-promoter driven PLAP (green) is expressed by dorsal progenitors and by $TAG1^{+}$ commissural neuron axons (red) in $Boc^{+/-}$ mice. **c**, Boc (green) was detected on the cell body, axon and growth cone of $DCC^{+}$ commissural neurons (red) in dissociated dorsal spinal cord cultures. Acetylated tubulin (Tub; red) labels cell bodies and axons. Scale bars: 100 μm (**a**, left panels), 50 μm (**a**, right panel), 200 μm (**b**) and 20 μm (**c**).

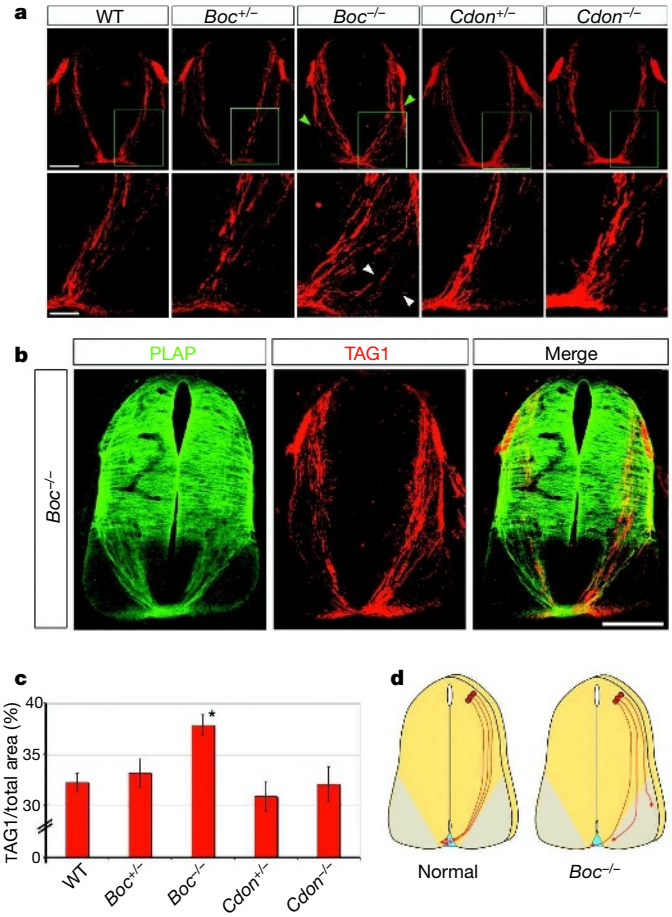


**Figure 2 | Aberrant axon targeting in $Boc^{-/-}$ but not $Cdon^{-/-}$ spinal cord.** **a**, Aberrantly projecting $TAG1^{+}$ commissural axon bundles (green arrowheads) and laterally displaced axons invading motor columns (white arrowheads) in E11.5 $Boc^{-/-}$ spinal cord; axon projections in $Boc^{+/-}$, $Cdon^{+/-}$ and $Cdon^{-/-}$ animals seem normal. **b**, *Boc*-promoter driven PLAP is expressed in misguided $TAG1^{+}$ axons in $Boc^{-/-}$ embryos (magnification in Supplementary Fig. 2f). **c**, Axon projection deviation was determined by using methods detailed in Supplementary Methods[2]. Number of embryos analysed: wild type (WT), 10 sections (3 embryos); $Boc^{+/-}$, 21 (7); $Boc^{-/-}$, 22 (8); $Cdon^{+/-}$, 13 (4); $Cdon^{-/-}$, 10 (3). Asterisk, $P < 0.0001$ (ANOVA); error bars show standard deviations. **d**, Left: normal commissural axons (red) project from dorsal spinal cord towards the ventral floor plate (blue). Right: in $Boc^{-/-}$ mice, axons deviate laterally and invade developing motor columns but seem ultimately to reach the floor plate. Scale bars: 200 μm (**a**, top), 80 μm (**a**, bottom) and 200 μm (**b**).

We next used an *in vitro* commissural axon turning assay to test the function of Boc in Shh-mediated axon turning[2] (Fig. 4a). After 40 h of co-culture with a source of chemoattractant, commissural axons (which normally project ventrally towards the floor plate) deviate in their trajectories and extend towards the chemoattractant. So far we have successfully used this assay with explants from rat, but not mouse, spinal cord (data not shown); thus, we were unable to examine axon turning directly in *Boc* mutant explants. We therefore designed small interfering RNAs (siRNAs) directed against rat and mouse, but not human, *Boc* (siBoc) to knock down its expression in rat spinal cord neurons. These siRNAs effectively decreased Boc protein levels in COS cells transfected with a mouse (but not a human) *Boc* expression vector and in endogenous rat spinal cord tissue (Supplementary Fig. 6). siBoc was co-electroporated with a GFP expression plasmid into the dorsal region of rat spinal cord explants, which were then co-cultured with COS cells expressing Shh or netrin-1. Axons of commissural neurons electroporated with a control siRNA were attracted towards both Shh and netrin-1 (Fig. 4a, b), as observed in control explants[2]. Commissural axons from explants treated with siRNA against *Boc* showed normal growth and responsiveness to netrin-1 (Fig. 4d), but their ability to turn towards a source of Shh was markedly diminished (Fig. 4b, c). The human Boc expression plasmid, which is impervious to the effects of siBoc in COS cells, successfully rescued the effect of siBoc treatment (Fig. 4c, and Supplementary Fig. 6). Collectively, these results indicate that Boc is required for commissural axons to respond to the chemoattractive effect of Shh.

Our findings show that Boc is expressed in regions of spinal cord harbouring developing commissural neurons, and genetic loss of *Boc* results in commissural axon misguidance without seeming to disrupt spinal cord patterning or the netrin-mediated outgrowth of axons from spinal cord explants. In contrast, loss of *Cdon* does not affect commissural neuron axon guidance, a finding consistent with the more restricted expression of *Cdon* within the dorsal spinal cord to progenitor cells, rather than postmitotic commissural neurons. In addition, our evidence that Boc is expressed by commissural neurons in dissociated culture, that *Boc* promoter-driven PLAP is expressed in the misguided commissural axons, and that Boc is cell-autonomously required for Shh-mediated axon turning are all consistent with the model that Boc is a receptor for Shh during axon guidance.

An earlier study showed defects in axon guidance in zebrafish treated with morpholinos directed against Boc, but the results were interpreted to reflect a role for Boc as a repulsive ligand[14]. Our results argue that in the mammalian spinal cord, Boc functions as a receptor for Shh-mediated attraction; whether it can also function in repulsion in other contexts in mammals remains to be explored. Our

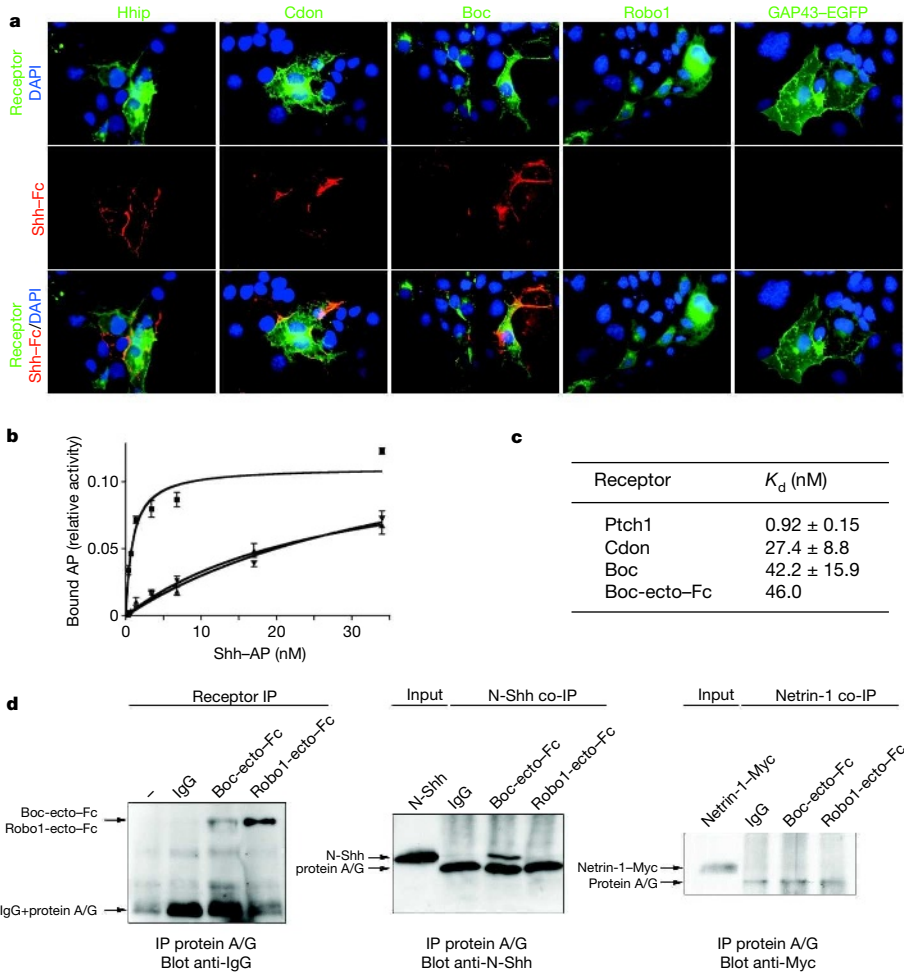


**Figure 3 | Boc and Cdon bind directly to Shh. a**, COS cells expressing Hhip, Boc and Cdon, but not Robo1 or membrane-localized GFP (GAP43–EGFP), bind Fc-epitope-tagged Shh (Shh–Fc; red). **b**, **c**, Quantification of Shh binding affinity to Boc and Cdon. Scatchard analysis was performed on Ptch1-expressing (squares in **b**), Boc-expressing (downward triangles) and Cdon-expressing (upward triangles) cells incubated with a Shh alkaline phosphatase (Shh-AP) fusion protein to determine dissociation constants ($K_d$; $n = 4$) (**c**). Errors are s.e.m. **d**, Boc interacts directly with Shh. The affinity-purified ectodomain of Boc epitope-tagged with Fc (Boc-ecto–Fc; left) co-immunoprecipitated with purified N-Shh, whereas the ectodomain of Robo1 did not (Robo1-ecto–Fc; middle). Purified netrin-1 did not interact with Boc-ecto–Fc or Robo1-ecto–Fc (right). IP, immunoprecipitation.

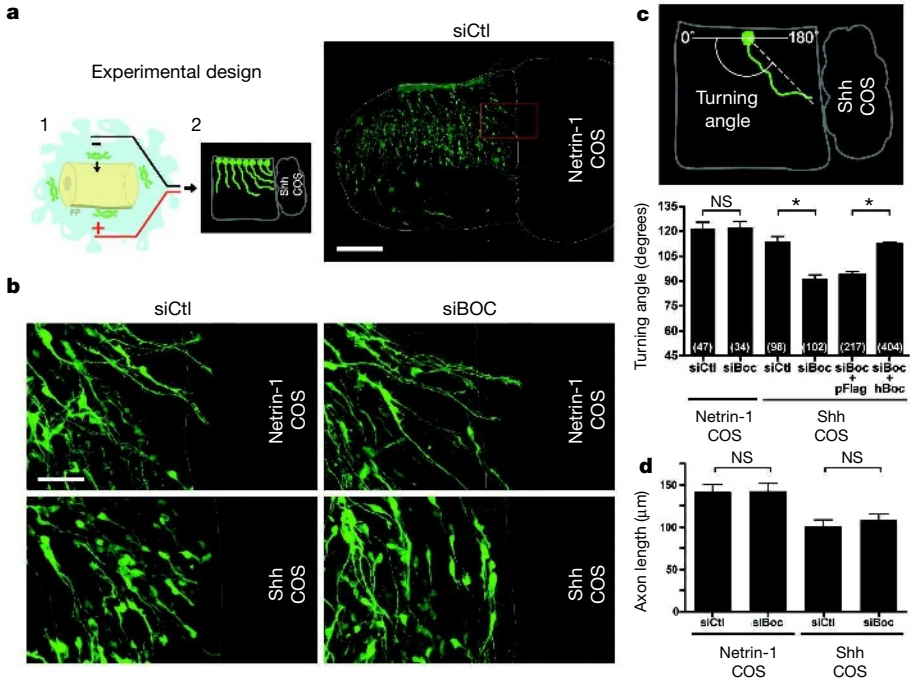


**Figure 4 | Boc is required for commissural axons to respond to the chemoattractive effect of Shh.** **a**, Left: siRNAs against *Boc* (siBoc) or an unrelated sequence (siCtl) were co-electroporated with GFP into the dorsal region of spinal cord explants (1) and co-cultured with COS cells expressing Shh or netrin-1 (2). Right: axons of commissural neurons co-electroporated with GFP (green) and siCtl are attracted towards netrin-1. Scale bar, 200 μm. **b**, High magnification. Explants electroporated with siBoc showed normal responsiveness to netrin-1 but exhibit markedly diminished ability to turn towards Shh. Scale bar, 50 μm. **c**, Quantification and rescue of siBoc-mediated inhibition of Shh attraction. Top: method of quantification of axon turning angle. Bottom: siBoc decreases axonal attraction by Shh; co-electroporation of human Boc (hBoc, which is impervious to the effects of siBoc), but not control plasmid (pFlag), rescues Shh-mediated attraction. NS, not significant; asterisk, $P < 0.001$. The numbers in parentheses show the number of axons quantified (three to ten explants each; error bars show s.e.m.). **d**, siBoc does not affect axon length. A significant difference in axon length is observed between netrin-1 and Shh ($P < 0.01$), but not between siCtl and siBoc. For siCtl/netrin 21 axons were quantified, for siBoc/netrin 22, for siCtl/Shh 20, and for siBoc/netrin 26.

previous findings established that Shh is a chemoattractant for commissural axons and that its rapid effects are mediated by Smo, as these are inhibited by cyclopamine[2]. Smo itself does not bind to Shh, and our findings suggest a model in which Boc is expressed by young commissural neurons, directly binds Shh, and enables the axons of these neurons to respond to Shh, presumably by interacting with Smo either directly or indirectly. However, commissural neurons also express Ptch (data not shown), raising the possibility that Boc might act in collaboration with other Shh receptors during axon guidance. Although the role of Shh in cell fate specification clearly involves the regulation of transcription in the cell nucleus, axon guidance events at the growth cone are largely independent of transcriptional events[5]. These observations raise the possibility that other, yet to be identified, mechanisms for Shh signalling are deployed downstream of Boc and Smo during growth cone guidance.

## METHODS

See Supplementary Information for detailed methods.

***In situ* hybridization.** $^{35}$S and fluorescent *in situ* hybridization was performed with previously described methods[23,25]. Template for RNA probes to the cytoplasmic and 3′ untranslated regions of murine Boc and Cdon were generated from complementary DNA isolated from embryonic stem (ES) cells or cDNA obtained from the I.M.A.G.E consortium with the use of standard polymerase chain reaction (PCR) methods.

**Generation and analysis of mutant mice.** ES cells with mutations in the *Boc* or *Cdon* loci were generated by targeting a gene trap vector 5′ to the exon encoding the transmembrane domain, as described previously[22]. Chimaeric mice were generated from the targeted ES cells, and offspring transmitting the mutant allele were crossed three or more generations into CD1 background.

**Binding assays.** The binding of Shh to a variety of receptor proteins was examined with published methods[26–28]. Full-length Boc and Cdon cDNAs used in the binding experiments were obtained and modified from the I.M.A.G.E. consortium. Ptch1 was obtained from C. C. Hui, Shh–AP from A. McMahon, and Shh–Fc from Genentech. Robo1, Robo1-ecto–Fc and gap–EGFP were described previously[18,29].

**Analysis of axonal projections.** Axonal projections were detected by immunohistochemistry and enzymatic reactions as described previously[30]. Quantification of circumferential misguidance in commissural axons was performed by deriving the ratio of the area of spinal cord spanned by the TAG1-labelled axons to the total area of the spinal cord[2]. Analysis of variance and a two-tailed Student's *t*-test were used to perform statistical analysis.

**Turning assays.** Rat E11 spinal cord explants were dissected as described previously[2], then placed in L15 medium containing a β-actin–GFP plasmid and siRNA. The explants were positioned between the electrodes to target the dorsal side preferentially, then electroporated. Explants were subsequently embedded within a collagen matrix, cultured, and analysed as described previously[2].

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**Supplementary Information** is linked to the online version of the paper at www.nature.com/nature.

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