The Cell Surface Membrane Proteins Cdo and Boc Are Components and Targets of the Hedgehog Signaling Pathway and Feedback Network in Mice

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Summary

Cdo and Boc encode cell surface Ig/fibronectin superfamily members linked to muscle differentiation. Data here indicate they are also targets and signaling components of the Sonic hedgehog (Shh) pathway. Although Cdo and Boc are generally negatively regulated by Hedgehog (HH) signaling, in the neural tube Cdo is expressed within the Shh-dependent floor plate while Boc expression lies within the dorsal limit of Shh signaling. Loss of Cdo results in a Shh dosage-dependent reduction of the floor plate. In contrast, ectopic expression of Boc or Cdo results in a Shh-dependent, cell autonomous promotion of ventral cell fates and a non-cell-autonomous ventral expansion of dorsal cell identities consistent with Shh sequestration. Cdo and Boc bind Shh through a high-affinity interaction with a specific fibronectin repeat that is essential for activity. We propose a model where Cdo and Boc enhance Shh signaling within its target field.

Introduction

Hedgehog (HH) signals regulate the specification of complex patterns within embryonic fields as diverse as imaginal discs in Drosophila larvae and the neural tube and limb of vertebrate embryos (McMahon et al., 2003). In the neural tube, the induction of all ventral cell identities requires direct Sonic hedgehog (Shh) signaling; the actual cell fate choice is determined by the concentration of Shh ligand (reviewed in Briscoe and Ericson, 2001; Jessell, 2000). Shh is initially released from the midline notochord underlining the ventral neural plate/tube and later from the floor plate. The floor plate, a population of ventral midline support cells within the neural tube, is itself a target of Shh signaling that requires the highest levels of ligand for its induction (Ericson et al., 1997). Shh from these sources forms a gradient that extends over the ventral half of the neural tube (Gritli-Linde et al., 2001).

In these patterning processes, feedback mechanisms acting at the level of ligand binding play a critical role in determining both the number and full range of ventral cell types (reviewed in Ingham and McMahon, 2001). Patched-1 (Ptch1) encodes the vertebrate HH receptor while Hedgehog-interacting protein-1 (Hhip1) encodes an unrelated membrane-associated protein that similarly binds all mammalian HH ligands. Ptch1 and Hhip1 are upregulated in response to HH signaling; their feedback functions serve to modify the range of signaling and regional response of target cells (Chuang and McMahon, 1999; Jeong and McMahon, 2005). A third HH binding factor, Growth arrest-specific-1 (Gas1), is thought to inhibit Shh signaling; Gas1 is itself repressed in response to HH signaling (Lee et al., 2001). Here we present evidence that Cdo and Boc, which encode cell surface bound members of the Ig/fibronectin domain superfamily, are novel feedback components that act in a different manner, to enhance Shh signaling within subregions of Shh’s neural target field.

Results and Discussion

Cdo and Boc Are Targets of Shh Signaling that Cell Autonomously Enhance Shh Signaling

To attempt to identify novel, general feedback components, we compared transcriptional profiles (data not shown) from early, somite-stage mouse embryos, where HH signaling is either normal (wild-type embryos), absent (Smoothened [Smo] mutant embryos [Zhang et al., 2001]), or enhanced (Ptch1 mutant embryos [Goodrich et al., 1997]), with profiles generated from microdissected tissues from later stage embryos where Shh signaling is lost (head and limb fractions from E10.5 Shh mutant embryos [St-Jacques et al., 1998]). Among those genes encoding cell surface or secreted proteins downregulated in response to Shh (enhanced expression in Smo and Shh mutants and repressed in Ptch1 mutants), we identified Gas1, as expected, and two genes that encode related members of an Ig/fibronectin repeat-containing superfamily of cell surface, membrane-spanning proteins, Cdo (sometimes Cdon [Kang et al., 1997]) and Boc (Kang et al., 2002).

Cdo and Boc represent a subfamily within the Ig superfamily, consisting of an ectodomain comprised of four (Boc) or five (Cdo) Ig repeats, followed by three fibronectin type III (FNIII) repeats and a long, divergent intracellular domain (Kang et al., 1997, 2002). Interestingly, Cdo mutant mice exhibit a microform holoprosencephaly, wherein midline facial structures are absent, a phenotype reminiscent of a partial loss of Shh signaling (Cole and Krauss, 2003; Cooper et al., 1998; Tian et al., 2005). A further link to the HH pathway comes from an siRNA screen in Drosophila that lists a Boc/Cdo relative, CG9211, as a putative effector of HH signaling (Lum et al., 2003).

Cdo and Boc expression were examined in the developing mouse and chick embryo. In both, Cdo and Boc expression are excluded from most HH-signaling domains, consistent with negative regulation by HH signaling (Figure S1 [see the Supplemental Data available with this article online], data not shown, and Mulleri et al. [2000, 2002]). In the neural tube and somites,
both genes are expressed dorsally, whereas in the limb, mesenchymal expression is restricted to the anterior two-thirds. On removal of HH signaling in Smo and Shh mutants, Cdo and Boc expression is enhanced, expanding ventrally in the somites and neural tube and to the posterior margin of the limb (Figure 1). In contrast, normal expression is lost, or markedly downregulated, both when HH signaling is derepressed in Ptc1 mutants (Figure 1A) or ectopically activated following Smo expression in the somites and neural tube and to the posterior mesenchyme of Shh mutant forelimb buds at E10.5, but are broadly repressed on activation of HH signaling following ectopic expression of SmoM2 throughout the limb mesenchyme. Anterior at top, lateral views.

(B) Boc and Cdo expression expand into the posterior mesenchyme of Shh mutant forelimb buds at E10.5 but are broadly repressed on activation of HH signaling following ectopic expression of SmoM2 throughout the limb mesenchyme. Anterior at top, dorsal views.

(C) Cdo and Boc expression in the E10.5 neural tube at the forelimb level. Dorsal Cdo and Boc expression is upregulated and their expression domains expand ventrally in the Shh mutant neural tube. Cdo also shows expression in the floor plate (arrow) and notochord (arrowhead); the former is lost in Shh mutants (see also Figure S1). Dorsal at top.

To address this possibility, we determined whether Cdo and Shh genetically interact. Cdo−/− mutants have a mild holoprosencephalic phenotype; midline structures are lost, and left and right nasal processes, while separate structures, are positioned closer to the midline (Figure 2A) (Cole and Krauss, 2003). Although Shh−/− embryos exhibit an extreme holoprosencephalic phenotype, Shh+/− embryos are comparable to wild-type (Chiang et al., 1996). When Shh gene dosage is lowered in a Cdo mutant background (Shh+/−; Cdo−/−), the Cdo phenotype is dramatically enhanced; the nasal processes fuse into a single, proboscis-like structure, a hallmark of Shh deficiency (Figure 2A) (Chiang et al., 1996; Muli et al., 2000). The observed genetic interaction suggests that Cdo may normally promote Shh signaling.

Given Cdo expression in the floor plate, a structure induced by high levels of Shh signaling (McMahon et al., 2003), we characterized ventral patterning in the neural tube of these mutants. During normal floor plate development there is a transitory period wherein ventro-medial progenitors are Nkx2.2+ and Foxa2+. At later stages, Foxa2 is restricted to the definitive floor plate and Nkx2.2 to ventro-lateral vp3 progenitors of the V3 class of spinal interneurons (for reviews, see Briscoe and Ericson, 2003; Jessell, 2000). At this stage, Shh is activated in the floor plate; activation requires the activity of Foxa2, which binds directly to Shh cis-regulatory transcriptional control regions (Jeong and Epstein, 2003). At E10.5, Foxa2+ and Nkx2.2+ cell populations are largely independent cell populations in the neural tube of both wild-type and Shh−/− embryos (Figure 2B and data not shown). However, in Cdo mutants, few midline cells are Foxa2+
only; most remain both Foxa2+ and Nkx2.2+ (Figure 2B). The total number of Foxa2+ cells is also reduced (Figure 2C). Coupled with this reduction in Foxa2+ cells there is a corresponding decrease in the Shh-producing floor plate (Figure 2B). In Cdo−/−; Shh+/− mutants, this phenotype is enhanced; in some embryos a few remaining Shh+, Foxa2+ cells are present at the midline and all such cells are also Nkx2.2+ (Figures 2B and 2C); in others, Foxa2 is entirely absent and vp3 Nkx2.2+ progenitors are also reduced (Figure 2C and data not shown). However, vpMN, Olig2+ motor neuron progenitors that are positioned more dorsally are unaffected (Figure 2B). The reduction in vp3, Nkx2.2+ progenitors most likely reflects reduced levels of normal floor plate-derived Shh, because in Gli2 mutants, FP specification is lost and a reduction in vp3 progenitors is also observed (Ding et al.,...
In summary, Cdo is essential for normal floor plate specification and interacts with the Shh signaling pathway in this process.

To further address interactions between Boc, Cdo, and the Shh pathway, we ectopically expressed Boc and Cdo in the neural tube of the developing chick embryo. Shh patterns the presumptive spinal cord by modulating the expression of transcriptional regulators that determine specific neural cell fates (Briscoe and Ericson, 2001; Jessell, 2000). For example, class I genes, such as Pax6 and Pax7, are repressed by Shh signaling, while class II genes, which include Nkx2.2 and Olig2, are activated (Briscoe et al., 2000). Ectopic expression of cDNA constructs encoding either full-length (fl) Cdo or Boc, or truncated forms (t) of both factors that lack the intracellular domain, results in common phenotypes: cell autonomous repression of Pax6 and dorsal expansion of Nkx2.2 (fl) progenitors, Olig2 (fl) motor neuron progenitors (pMN), and Foxa2 (fl) floor plate (Figures 3A–3C, Figure S3, and data not shown). Importantly, where different ectopic cell identities are observed in the same section, progenitors show a normal, relative distribution. For example, ectopic Olig2 (fl) pMN progenitors always lie dorsal to ectopic Nkx2.2 (fl) progenitors (Figure 3B and data not shown).

Pax7 broadly marks dorsal cell identities, the ventralmost of which lie at the normal limit of Shh signaling and overlap the dorsal limits of detectable Shh protein (Grüttlin-Lin et al., 2001; Wijgerde et al., 2002; C. Chamberlain and A.P.M., unpublished data). Ectopic expression of Cdo and upregulation of Boc in this region (Boc is weakly expressed normally; see Figure S2), but not in more dorsal positions, leads to a cell autonomous repression of Pax7 (Figure 3A, far left panel, and Figure 3D). In contrast, when Boc or Cdo are extensively expressed within the Shh target field just ventral to the normal Pax7 domain, a cell nonautonomous expansion of Pax7 (fl) cells is observed into the normal Shh target field (Figure 3A, far left panel, and Figure 3E).

These results lead to several conclusions. First, expression of Boc and Cdo promotes the adoption of more ventral neural identities than is appropriate for cells at a given D-V position. However, the relative position of ectopic cell identities to one another is normal, suggesting that polarity cues are still observed, as expected if Cdo- and Boc-mediated inductions are Shh dependent. Further, while elevated levels of Cdo and Boc can repress Pax7 (fl) fates, repression is only observed close to the D-V boundary, where low levels of Shh are both present and active based on direct analysis of Shh protein distribution and the expression of Pch1 and Gli1, transcriptional targets of the pathway. In all these instances we only observe a cell autonomous action of Cdo and Boc, consistent with their directly modulating Shh signaling input to the ectopically expressing cells. Second, the cell nonautonomous appearance of more dorsal cell fates above a strong area of Boc or Cdo expression is suggestive of phenotypes observed when Shh is sequestered by ectopic expression of Hhip1 (Stamatakis et al., 2005). This may indicate that either Boc or Cdo...
themselves bind Shh, or their activity promotes Shh retention indirectly. Third, both Cdo and Boc appear to have similar properties, as each generates a similar phenotype. Although Cdo and Boc can associate with each other (Kang et al., 2002), this association does not appear to be necessary to promote ventralization. Fourth, in their molecular action, the intracellular domain is dispensable for ventralizing activity, whereas the transmembrane domain is not (data not shown). Together, these data are consistent with a model in which the ectodomains of Cdo and Boc bind to and sequester Shh ligand, thereby enhancing Shh signaling cell autonomously where ligand is available but also potentially limiting Shh movement to more dorsal positions in the normal target field.

To address whether the action of Cdo and Boc are indeed specific for HH signaling, we performed coelectroporation studies with HH pathway-specific components that are known to act at the level of ligand binding and ligand-dependent feedback regulation of membrane signaling. Ptch1loop2 encodes a modified form of the HH-receptor Ptch1 in which removal of one of two extracellular loops prevents ligand binding (Briscoe et al., 2001). As ligand binding to Ptch1 is required to block Ptch1-mediated inhibition of Smo activity, and Smo activity is required for the specification of all HH-dependent cell fates, expression of Ptch1loop2 specifically inhibits ligand-dependent signaling at the level of Ptch1-Smo. As expected if the ectopic induction of ventral cell identities by Cdo and Boc is dependent on Shh ligand-based signaling, coexpression of Ptch1loop2 with Boc results in a cell autonomous inhibition of Boc-mediated ventralization (Figure 4A, lower panel). Importantly, where cells express only Boc, ectopic ventral cell identities are observed (Figure 4A, upper panel). Expression of Ptch1loop2 is associated with ventral cells ectopically activating the dorsal marker Pax7; inhibition of Pax7 is the lowest Shh threshold response reported to date: less than 500 pM of Shh is sufficient in in vitro assays to abolish Pax7 expression, whereas greater than 4 nM is required for floor plate induction (Ericson et al., 1997). As expected, ectopic ventral expression of Ptch1loop2 results in ectopic Pax7+ cells (Figure 4B, upper panel). However, coexpression of Boc suppresses this phenotype (Figure 4B, lower panel). These results suggest that, where ligand is available, Boc enhancement of Shh signaling is sufficient to overcome the inhibitory effects of Ptch1loop2, providing sufficient, minimal level signaling to enable Pax7 repression, but insufficient for ectopic induction of Nkx2.2+ vp3 progenitors. In support of this model, electroporation with a constitutive repressor form of Gil3 (Gil3R) that is insensitive to Shh signaling results in a cell autonomous ventral expansion of Pax7+ cells that cannot be inhibited by coelectroporation with Boc (Figure S4). Hhip1 encodes a second, membrane-associated feedback antagonist that binds ligand directly (Chuang and McMahon, 1999). Hence, ectopic expression of Hhip1 acts to downregulate HH signaling cell autonomously in HH responding cells. We also coexpressed Boc with Hhip1; however, ectopic expression of Hhip1 alone leads to a severe growth defect and an apparent loss of viability in ventral progenitors, precluding further study (data not shown).

Figure 4. Coexpression of Boc with Ptch1loop2 Abrogates the Effects of Boc-Mediated Enhancement of Ventral Cell Fate Specification
Plasmid constructs encoding cytoplasmically truncated Boc(t) (GFP coexpression) or Ptch1loop2 (DsRed coexpression) were electroporated into the chick neural tube, and the expression of (A) vp3 (Nkx2.2+) and (B) dorsal (Pax7+) progenitors was assayed by immunohistochemistry as indicated. Control DNAs consist of base vectors producing only GFP or DsRed.
expressing Hhip1, Boc, and Cdo bind N-Shh::AP, binding is Shh-dependent (i.e., AP alone does not bind), and both Boc and Cdo bind N-Shh::AP as effectively as Hhip1 (Chuang and McMahon, 1999). We next examined whether binding represents a direct association of Shh with either Boc or Cdo. When N-Shh::AP and epitope-tagged, secreted forms of Boc or Cdo (BocΔTMCD and CdoΔTMCD) are cotransfected into Cos7 cells and supernatants are assayed, we detect N-Shh::AP/BocΔTMCD and N-Shh::AP/CdoΔTMCD complexes, indicating that Shh binds to both Boc and Cdo ectodomains (Figures 5C and 5D). The use of secreted forms reduces the possibility that unknown, cell surface bound factors promote binding or contribute directly to the complex.

Figure 5. Cdo and Boc Bind Shh
(A) Binding of N-Shh::AP to Cdo- and Boc-expressing cells. Scale bar, 50 μm.
(B) Quantitation of N-Shh::AP binding to Cdo and Boc. Error bars represent the mean ± SD of four identical treatment groups. Significant differences between binding of N-Shh::AP and AP conditioned medium (CM) are indicated by * (two-tailed student t test, p < 0.01).
(C and D) (C) Immunoprecipitation of Boc and (D) immunoprecipitation of Cdo extracellular domains (BocΔTMCD and CdoΔTMCD, respectively) with Shh. Cos7 cells were transfected with BocΔTMCD or CdoΔTMCD alone (lane 1), or cotransfected with N-Shh::AP (lane 2) or AP (lane 3). Complexes were immunoprecipitated from supernatants with anti-AP beads. Epitope-labeled BocΔTMCD was detected following Western blot analysis of immunoprecipitates with anti-Myc antibody, and epitope-tagged CdoΔTMCD with anti-HA antibody. N-Shh::AP was detected with anti-Shh antibody or anti-AP; the latter was also used to detect AP.
(E) Dissociation constant (Kd) measurements. Saturation binding curves and Scatchard analysis (insets) of N-Shh::AP binding to Hhip1 (left), Boc (middle), and Cdo (right). Each point on the graphs represents the average of three identical treatment groups.
Figure 6. Mapping of Shh Binding Domains in Cdo and Boc

(A) Cos7 cells transfected with N-Shh::AP alone (lane 1) or cotransfected with various Cdo ectodomain Fc fusion constructs (lanes 2-6). Complexes were immunoprecipitated from conditioned medium with Protein A agarose. Top panel: Detection of N-Shh::AP with anti-Shh. Bottom panel: Identification of Fc fusion proteins with anti-human IgG. Asterisks highlight the various Fc fusion proteins as confirmed by comparison with the migration of molecular weight markers (left).

(B) Quantitation of relative Shh binding to each Cdo-Fc fusion proteins. Binding is expressed as a ratio of Shh band intensity/Cdo-Fc band intensity.

(C) Schematic of Cdo mutant constructs. Full-length Cdo (top) is contrasted with constructs expressing only the FNIII(3) domain of the Cdo [CdoFNIII(3)TMCD, middle] or constructs that express the entire extracellular domain except for the FNIII(3) domain [CdoΔFNIII(3), bottom]. Identical constructs were also generated for Boc.

(D) Binding of NShh::AP to Cdo and Boc constructs containing the third FNIII repeat [CdoFNIII(3)TMCD and BocFNIII(3)TMCD, respectively] or lacking the third FNIII repeat [CdoΔFNIII(3) and BocΔFNIII(3), respectively]. Scale bar, 50 μm.

(E) Quantitation of N-Shh::AP binding to full-length and truncated Cdo and Boc constructs. Error bars represent the mean ± SD of four identical treatment groups.
The FNIII(3) Domains in Both Cdo and Boc Are Necessary for Enhancement of Shh Signaling in the Developing Chick Neural Tube

(A) Expression of Nkx2.2+ (red) or Pax7+ (blue) neural cell progenitors in chick neural tubes electroporated with full-length Cdo (top left panels), CdoΔFNIII(1) (top right panels), CdoΔFNIII(2) (bottom left panels), or CdoΔFNIII(3) (bottom right panels). Green cells indicate GFP expression in electroporated cells.

(B) Expression of neural cell progenitor markers in chick neural tubes following electroporation with full-length Boc (left panels) or BocΔFNIII(3) (right panels).

To determine the affinity of the Shh-Boc and Shh-Cdo interactions, saturation binding experiments were performed using COS7 cells transfected with Cdo, Cdo, or Hhip1 for comparison (Figure 5E). Calculation of the dissociation constants (Kd) for NShh::AP binding to Cdo and Cdo yielded similarly high affinities (approximately 3 and 4 nM, respectively), while a Kd of 1 nM for Hhip1 is in close agreement with previously published data (Chuang and McMahon, 1999).

Having established that Boc and Cdo interact with Shh with high affinity, we performed domain-mapping analysis to define the region of Boc and Cdo that binds to Shh (Figure 6). Immunoprecipitation experiments using Fc-fusion constructs that contain either the Ig-like domains or FNIII domains of Cdo indicate that the fibronectin repeat-containing region plays the major role in Shh binding (Figure 6A). Furthermore, analysis of constructs expressing each FNIII domain singly suggests that most binding can be ascribed to the FNIII(3) domain, the most highly conserved of these repeats (Figures 6A and 6B). To further confirm the importance of this domain in Cdo and Boc, NShh::AP binding assays were performed using constructs whose extracellular domains consist of only the third FNIII repeat (CdoF-NIII(3)TMCD and BocF-NIII(3)TMCD) or the entire extracellular domain except for the third FNIII repeat (CdoΔF-NIII(3) and BocΔF-NIII(3), Figures 6C–6E). In these assays, the FNIII(3) domain of Cdo and Boc is both necessary and sufficient to specifically mediate NShh::AP binding to Cos7 cells, indicating that this region plays a critical role in these interactions.

Figure 7. The FNIII(3) Domains in Both Cdo and Boc Are Necessary for Enhancement of Shh Signaling in the Developing Chick Neural Tube

Cdo and Boc Binding to Shh Is Necessary, but Not Sufficient, to Ectopically Activate Shh Signaling

To test whether the FNIII(3) domains of Cdo and Boc are necessary to augment Shh signaling, chick electroporation experiments were performed with a series of Cdo and Boc constructs (Figure 7 and Figure S5). Expression of full-length Cdo or Boc results in ectopic activation of Nkx2.2 (Figure 7A, top left panels, and Figure 7B, left panels, respectively), as does expression of Cdo constructs lacking either FNIII(1) (Figure 7A, top right panels) or FNIII(2) (Figure 7A, bottom left panels). In contrast, Cdo or Boc lacking FNIII(3) fails to ectopically activate Nkx2.2, despite strong ventral expression of these variants (Figure 7A, bottom right panel, and Figure 7B, right panels, respectively). Despite the clear requirement for FNIII(3) in Shh binding and activity, the FNIII(3) domain alone of either Boc or Cdo is not sufficient to reproduce Cdo- or Boc-dependent phenotypes within the neural tube (Figure S5). Thus, Cdo and Boc most likely promote signaling by binding Shh in conjunction with interactions requiring other regions of their extracellular domains. Understanding these interactions may provide some insight into why some Shh binding proteins, such as Boc and Cdo, function as positive regulators of Shh signaling, while others, such as Hip1, function as negative regulators. These studies, together with those in the accompanying paper (Zhang et al., 2006), identify Boc and Cdo as novel components of the vertebrate Hedgehog signaling pathway. A report that a related gene is required for normal HH signaling in Drosophila tissue culture cells suggests this function is conserved (Lum et al., 2003). Considering all the genetic, biochemical, and expression analyses, we propose a model wherein Cdo and Boc enhance HH signaling at two critical positions within a postulated HH activity gradient: (1) where the highest signaling levels are required in FP specification and (2) at the fringes of a HH target field, close to the D-V intersect in the neural tube and possibly also at the anterior limit of signaling in the limb bud. At these latter positions, where ligand levels are expected to be low, this mechanism may increase the robustness of signaling. Additionally, the negative regulation of Boc and Cdo expression by HH signaling would restrict Cdo/Boc-mediated enhancement of HH signaling to the relevant region, establishing this feedback system an appropriate domain for their action. While the current model is clearly speculative, the future analysis of Boc mutants and Cdo; Boc compound mutants, along with further biochemical and cellular analyses, should provide important tests of these ideas.

Our data provide two critical mechanistic insights; first, Cdo and Boc both bind Shh via their FNIII(3) domains, and second, Cdo and Boc binding to Shh is necessary for enhancement of Shh signaling. We suggest...
that Shh/Cdo or Shh/Boc complexes either facilitate presentation of active ligand to Ptc1, or that binding counteracts feedback-mediated sequestration of ligand and ligand turnover, increasing effective levels of signaling in a responding cell. Thus, Cdo and Boc appear to represent a new class of factors in the increasingly complex Shh feedback network; expression of each is broadly negatively regulated by HH signaling, but their activity stimulates HH signaling. Importantly, the accompanying work of Zhang et al. (2006) identifies Cdo as a modulator of Shh signaling in holoprosencephaly, implicating these genes as potential interacting factors in Shh-related human pathologies.

Experimental Procedures

Transcriptional Profiling

The transcriptional profiling will be described in detail elsewhere (TT and APM, in preparation). Briefly, RNA was prepared from 6-8 and 10-13 somite stage wild-type, Ptc1<sup>+/−</sup> (Goodrich et al., 1997) and Smo<sup>−/−</sup> (Zhang et al., 2001) embryos, and from head and limb buds isolated from E10.5 wild-type and Shh<sup>−/−</sup> (St-Jacques et al., 1998) embryos. RNAs were used in standard procedures to generate probes for analysis of transcript expression on Affymetrix U74Av2 and M430 A and B microarrays. Data were statistically analyzed using Resolver software (Rosetta).

Mice

Mouse experiments were carried out largely on a 129 background as in the original Cdo report; hence the “weak” midline defects in the Cdo<sup>−/−</sup> embryos in this study. The Shh mutant allele on a 129/Sv; C57BL/6J; CBA/J hybrid background was crossed with Cdo<sup>−/−</sup> stock (129/Sv; C57BL6) and the phenotypes of littermates examined in this mixed background (Cdo<sup>−/−</sup>, n = 14; Cdo<sup>−/−</sup>; Shh<sup>−/−</sup>, n = 13). The strongest midline defects in Cdo<sup>−/−</sup> littermates were always observed in those carrying the Shh null allele.

Generation of Cdo and Boc Constructs

All constructs were generated using standard molecular biology procedures (Maniatis et al., 1982). Briefly, cytoplasmic truncations of Boc and Cdo [Boc(t) and Cdo(t)] deleted aa 79-1115 of human Boc and aa 968-1251 of mouse Cdo. Soluble versions of Boc and Cdo that lack both the transmembrane and cytoplasmic domains (Boc(TMCD) and Cdo(TMCD)) were truncated at aa 855 and aa 958 of Boc and Cdo, respectively. Constructs encoding the third FNIII repeat, transmembrane, and cytoplasmic domains of Boc(FNIII3(TMCD) and Cdo(FNIII3(TMCD)) were fused to their respective signal peptides at aa 712 of Boc and aa 830 of Cdo. Constructs encoding Boc and Cdo that lack the third FNIII repeat [Boc(FNIII3) and Cdo(FNIII3)], respectively deleted aa 710-809 of Boc and aa 832-919 of Cdo. All constructs were cloned into pCIG.

Cdo-Fc fusion proteins were generated by PCR amplification of each of the indicated regions as follows: CdoG1(1−5−)Fc (aa 1−575), CdoFNIII1(1−3−)Fc (aa 534−599), CdoFNIII1(1−3−)Fc (aa 534−711), CdoFNIII2(1−3−)Fc (aa 862−814), and CdoFNII3(1−3−)Fc (aa 802−858). The FNIII constructs were then fused in-frame to native mouse Cdo start codon and signal sequence (aa 1−41), followed by the cloning of all constructs into the Iagt vector (Bergemann et al., 1995).

AP Binding Assays

These experiments were performed essentially as described previously (Flanagan et al., 2000). Briefly, Cos-7 cells were transfected with either AP or N-Shh:AP alone, or cotransfected with full-length mouse Hhip1 (Chuang and McMahon, 1999), mouse Cdo (Kang et al., 1997), human Boc (Kang et al., 2002), or mouse Fz3. Bound AP protein was visualized with BMW purple AP substrate (Roche) for cell surface staining, or with AP yellow liquid substrate (Sigma) to quantify AP binding in cell extracts. Scatchard analysis were performed using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA). K<sub>s</sub> measurements were determined by nonlinear regression analysis of the saturation binding data.

Immunoprecipitation Analysis

Cos-7 cells were transfected using Lipofectamine 2000 (Invitrogen), and conditioned medium was collected 48 hr after transfection. Immunoprecipitation of AP and N-Shh:AP from conditioned medium was performed by incubation with anti-AP agarose beads (Sigma) overnight at 4°C on a rotator. Beads were washed three times with buffer (50 mM Tris [pH 7.6], 500 mM NaCl, 1% Triton-X-100, resuspended in Laemmli sample buffer, heated at 95°C for 5 min, and analyzed by SDS-PAGE and Western blot analysis. AP and N-Shh:AP were detected with rabbit anti-AP antibody (Biomedia). Myc epitope-tagged Boc(TMCD) was detected with mouse anti-myc antibody (clone 9E10; Developmental Studies Hybridoma Bank). HA-tagged Cdo(TMCD) was identified with mouse anti-HA antibody (Covance). Rabbit anti-Shh antibody has been described previously (Bumcrot et al., 1995).

In Situ Hybridization and Immunofluorescence

Whole-mount digoxigenin in situ hybridization was performed as described on wild-type and mutant embryos (Wilkinson, 1992). Section in situ hybridization was carried out on 30 µm sections with digoxigenin probes at forelimb-levels. Immunofluorescence analysis was performed on 10 µm frozen sections; image collection was carried out on a Zeiss LSM510 confocal microscope. The following antibodies were used: rabbit anti-Olig2 (1:5000), mouse anti-Foxa2 (1:5), rabbit anti-Nkx2.2 (1:4000), mouse anti-Pax7 (1:20), rabbit anti-Olig2 (1:5000), mouse anti-Shh (1:25, Developmental Studies Hybridoma Bank), Alexa 568 or 633 goat anti-rabbit or goat anti-mouse (1:300, Molecular Probes) and rabbit anti-DsRed (1:400, BD Bioscience).

Chick Electroporation

Boc or Cdo and their derivatives and GI3R (a gift of S. Vokes) were cloned into pCIG vector (Megasen and McMahon, 2002) to enable coexpression of Boc and Cdo with GFP to visualize electroporated cells. Ptch<sup>flshphot</sup> (Briscoe et al., 2001) was cloned into pCIR. In this vector, the GFP-encoding cDNA of pCIG was replaced by one encoding Red fluorescent protein (DsRed-Express, Invitrogen). For electroporation, Qiang purified, supercoiled plasmid DNA was injected into the neural tube of Hamburger-Hamilton (HH) stage 11–12 chicken embryos (Hamburger and Hamilton, 1992). Boc and Cdo were injected at concentrations of 1.0 or 0.7 µg/ml in PBS, respectively, with 50 ng/ml Fast Green. In coelectroporation studies, both DNAs were at a concentration of 1.5 µg/ml. Electrodos were made from 0.5 mm diameter platinum wire (Aldrich) and were 5 mm long and spaced 5 mm apart. Electrodes were placed flanking the neural tube, covered with a drop of PBS, and pulsed five times at 25 V for 90 ms with a BTX Electroporator (Genetronics). Embryos were recovered after 48 hr at HH stage 21–22 and fixed for immunohistochemistry. Each analysis was repeated a minimum of 20 times.

Supplemental Data

Supplemental Data including five figures are available at http://www.developmentalcell.com/cgi/content/full/10/5/647/DCl/.

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References


Supplemental Data

The Cell-Surface Membrane Proteins Cdo and Boc Are Components and Targets of the Hedgehog Signaling Pathway and Feedback Network in Mice

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Figure S1. In Situ Hybridization Analysis of Cdo and Boc Expression in the Developing Mammalian Neural Tube

Cdo is expressed weakly, and transiently, in both the floor plate (arrow) and notochord (arrowhead). Expression in the floor plate region at the forelimb level is first observed at E9.0. Both floor plate and notochordal expression are lost by E11.5.
Figure S2. Comparison of Boc Expression in the E10.5 Neural Tube with Pax7

**A**, In situ hybridization analysis of Boc, Cdo, Ptch1, and Gli1 expression in the E10.5 mouse neural tube at the forelimb level. Black arrowheads indicate the dorsal limit of Ptch1 expression, while brackets indicate the regions of overlap between Ptch1 expression and Boc and Cdo expression, respectively. **B**, In situ hybridization (Boc) and immunofluorescence analysis (Pax7 and Olig2) indicates that Boc expression extends ventral to the Pax7 domain, overlapping the distal limits of Shh signaling. White arrowheads indicate the ventral limit of the Pax7 expression domain. Brackets mark the
region ventral to the Pax7 expression domain in which *Boc* expression is detected. **C,** Wholemount in situ hybridization analysis of *Boc, Cdo, Ptch1,* and *Gli1* expression in the E10.5 mouse forelimb bud. Dotted lines indicate the anterior limit of *Ptch1* expression; brackets denote overlap between *Ptch1* expression, and *Boc* and *Cdo* expression, respectively. Note that the *Boc* panel is duplicated from Figure 1.

Figure S3. *Boc* and *Cdo* Induce Ectopic Expression of Nkx2.2+ and Olig2+ Cells in a Cell-Autonomous Manner

**A,** Detailed inspection of the far left panels from Figure 3A, including a separate panel for Boc expression (GFP, green) demonstrates that all ectopic Nkx2.2+ cell progenitors are also GFP+. **B,** Detailed inspection of the second panel from left in Figure 3B, demonstrates that all ectopic Olig2+ cell progenitors also express Cdo.
Figure S4. Gli3R Induces Cell-Autonomous Expression of Pax7 Independent of Boc Expression

Electroporation of chick neural tubes with a Gli3 repressor construct (Gli3R, left panels), co-electroporation of Gli3R with full-length Boc (middle panels) or electroporation with full-length Boc alone (right panels). Electroporated cells are indicated by GFP expression (green). Immunofluorescent analysis of antibody staining denotes Pax7⁺ cell progenitors (red). White arrowheads demarcate areas of Gli3R-mediated cell-autonomous Pax7 expansion. White arrows indicate cell-non-autonomous expansion of Pax7 expression in Boc-electroporated neural tubes.
Figure S5. Ectopic Expression of the FNIII(3) Domain of Either Cdo or Boc Is Not Sufficient to Promote Shh-Dependent Cell Fate Specification

Specification of vp3 (Nkx2.2+) and dorsal (Pax7+) neural progenitors is unaltered by ectopic expression of either CdoFNIII(3)TMCD or BocFNIII(3)TMCD as visualized by the production of GFP following electroporation of plasmid constructs in the chick neural tube.