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Overlapping roles and collective requirement for the co-receptors Gas1, Cdo and Boc in Shh pathway function

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Summary

Secreted Hedgehog (Hh) ligands signal through the canonical receptor Patched (Ptch1). However, recent studies implicate three additional Hh-binding, cell surface proteins, Gas1, Cdo and Boc, as putative co-receptors for Hh ligands. A central question is to what degree these co-receptors function similarly and their collective requirement in Hh signal transduction. Here we provide evidence that Gas1, Cdo, and Boc, play overlapping and essential roles during Hh-mediated ventral neural patterning of the mammalian neural tube. Specifically, we demonstrate two important roles for these molecules: an early role in cell fate specification of multiple neural progenitors, and a later role in motor neuron progenitor maintenance. Most strikingly, genetic loss-of-function experiments indicate an obligatory requirement for Gas1, Cdo and Boc in Hh pathway activity in multiple tissues.

Keywords

Mouse; Hedgehog; Neural Tube; Development; Gas1; Cdo; Boc; motor neuron

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Introduction

Hh signaling is essential for the patterning of both invertebrate and vertebrate embryos (McMahon et al., 2003). In mammals, Sonic hedgehog (Shh) signaling regulates numerous developmental processes, including craniofacial development, digit specification in the limb, and patterning of the ventral neural tube (McMahon et al., 2003). Biochemical and genetic studies performed over a decade ago identified the twelve-pass transmembrane protein Patched 1 (Ptch1) as the canonical Hh receptor (Marigo et al., 1996; Stone et al., 1996). Several additional Hh ligand-binding proteins have been identified that modulate Hh pathway activity at the cell surface. These include the Hh antagonist Hh interacting protein (Hhip1; Chuang and McMahon, 1999), and three additional Hh-binding proteins, Growth arrest-specific 1 (Gas1), CAM-related/down-regulated by oncogenes (Cdo) and Brother of Cdo (Boc; Allen et al., 2007; Martinelli and Fan, 2007a; Tenzen et al., 2006; Yao et al., 2006; Zhang et al., 2006). Cdo and Boc are structurally related and conserved from *Drosophila* to mouse, whereas Gas1 is a distinct, vertebrate-specific Hh pathway component. Despite strong evidence that Gas, Cdo and Boc promote Hh signaling, the general requirement for their action remains to be determined.

Cdo and Boc are cell surface integral membrane proteins that have an extracellular domain comprised of a series of immunglobulin and fibronectin-like repeats; structural analyses have identified the fibronectin repeats as critical for Shh binding (McLellan et al., 2006; McLellan et al., 2008; Tenzen et al., 2006; Yao et al., 2006). Initial studies in *Drosophila* identified a role for *ihog*, a *Drosophila* homolog of Cdo and Boc in Hh signaling (Lum et al., 2003), while more recent studies indicate that ihog, together with boi (a second Cdo and Boc homolog) are essential for transducing the Hh signal in the developing wing imaginal disc (Camp et al., 2010; Zheng et al., 2010). In mice, *Cdo* mutant embryos display microform holoprosencephaly, a defect commonly associated with mutations in the Shh pathway (Cole and Krauss, 2003), while *Boc* mutants are defective in Shh-dependent commissural axon guidance (Okada et al., 2006). Gas1 is a GPI-anchored Hh-binding protein whose extracellular domain shares homology to GDNF receptors (Cabrera et al., 2006; Schueler-Furman et al., 2006; Stebel et al., 2000). Gas1 functions in Shh signaling in multiple tissues during embryogenesis (Allen et al., 2007; Lee et al., 2001; Martinelli and Fan, 2007a). Strikingly, mice deficient in both *Gas1* and *Cdo* display severe patterning defects in Shh-dependent processes (Allen et al., 2007), suggesting that these molecules may cooperate in the promotion of Shh signaling during embryonic development.

Given the combined genetic data in flies and mice, we sought to assess the relative contribution of Gas1, Cdo and Boc to vertebrate Hh signal transduction during embryogenesis. Specifically, we examined Hh-dependent neural patterning in mice lacking Gas1, Cdo and Boc function individually, or in combination. We find that while removal of any one component individually has either no effect, or relatively mild effects on Shh-dependent neural patterning, removal of any two Hh pathway components severely affects both neural progenitor specification and subsequent maintenance of motor neuron progenitors. In contrast to *Drososphila*, Cdo and Boc alone are not essential for Shh signal transduction. Genetic removal of both Cdo and Boc activity abrogates Shh signaling in the developing neural tube; however, the loss of these components does not affect signaling in other Hh-responsive tissues, including the developing limb. Double mutant analyses indicate that in the limb, Gas1 and Boc, but not Cdo are required for an appropriate Hh response during digit specification. Strikingly, removal of all three Hh-binding proteins results in a near complete loss of Hh signaling, including a complete failure of ventral neural tube patterning, defective heart looping and early embryonic lethality, similar to $Smo^{-/-}$ embryos. Overall, these data support a model in which Gas1, Cdo and Boc function as

essential Hh co-receptors in vertebrate Hh signal transduction. Further, our findings provide additional insights into the dynamic requirement for Shh signaling in neural patterning.

Results

Gas1, Cdo and Boc all promote ectopic Shh signaling during neural patterning

Previous studies suggested that Gas1, Cdo and Boc promote Shh signaling during neural patterning (Allen et al., 2007; Martinelli and Fan, 2007a; Tenzen et al., 2006). To confirm these data and directly compare the ability of Gas1, Cdo and Boc to activate Shh signaling, we performed overexpression studies in the developing chick neural tube (Figure 1). Ectopic expression of GFP alone has no effect on ventral neural patterning (Figures 1A–1D), while expression of Gas1 (Figures 1E–1H), Cdo (Figures 1I–1L) or Boc (Figures 1M–1P) all promote ectopic Shh-dependent ventral neural patterning in a cell-autonomous manner, including ectopic specification of the class-II transcription factor Nkx6.1 (Figures 1E–1F, 1I–1J, 1M–1N, and 1Q–1R), which requires low-level Hh signaling, and the V3 interneuron progenitor (pV3) marker Nkx2.2 (Figures 1G–1H, 1K–1L, 1O–1P, and 1S–1T), which requires high-level Hh signaling (Briscoe and Ericson, 2001). Notably, despite strong ectopic expression of Gas1, Cdo and Boc in the dorsal neural tube, ectopic Nkx6.1 (Figures 1F, 1J, and 1N) and Nkx2.2 (Figures 1H, 1L, and 1P) are spatially restricted either to the ventral neural tube, or the dorsal-ventral intersect in embryos expressing these constructs. In contrast, expression of a constitutively active Smo construct (SmoM2; Xie et al., 1998) that activates Hh signaling independent of ligand results in ectopic expression of Nkx6.1 and Nkx2.2 even in dorsal positions where Shh signaling is absent (Figures 1Q–1T, arrowheads). Taken together, these data suggest that Gas1, Cdo and Boc all share a similar ability to promote Shh signaling in a ligand-dependent manner.

Normal neural patterning in *Boc*$^{-/-}$ embryos

Given the functional equivalence of Gas1, Cdo and Boc in the promotion of Shh signaling *in ovo*, and previous work demonstrating a role for Gas1 and Cdo action in neural patterning *in vivo* (Allen et al., 2007), we examined whether genetic removal of Boc function also alters Shh-dependent specification of ventral neural progenitors. Surprisingly, *Boc*$^{-/-}$ mice are viable and fertile, with no overt defects in Hh-dependent tissues (data not shown), as reported for a different null mutant (Okada et al., 2006). Detailed examination of neural patterning in these mice at embryonic day 10.5 (E10.5) revealed no differences in Shh expression (Figures S1A, 1D, and 1G), or that of distinct targets linked to low (Nkx6.1 and Dbx1; Figures S1B, 1E, and 1H), moderate (Olig2, which denotes motor neuron progenitors; pMNs), or high (Nkx2.2 and the floor plate marker FoxA2; Figures S1C, 1F, and 1I) levels of Shh signaling. Earlier analysis at E8.5-E9.5 also failed to reveal any difference in Shh patterning between *Boc*$^{-/-}$embryos and *wild type* (*wt*) embryos (data not shown).

Cdo$^{-/-}$*; Boc*$^{-/-}$ double mutants display severe and unusual neural patterning defects

One explanation for the lack of a phenotype in *Boc*$^{-/-}$ mice is that the structurally and functionally similar protein Cdo compensates for the loss of Boc in the embryo. To test this, we examined neural patterning in *Cdo*$^{-/-}$*; Boc*$^{-/-}$ double mutant embryos (Figure 2). *Cdo*$^{-/-}$ mutants display floor plate patterning defects at E10.5 (Tenzen et al., 2006), compared to *wt* embryos (Figures 2A, 2I, and 2Q), including reduced numbers of FoxA2+ cells, and increased expression of FoxA2 and Nkx2.2 double positive cells (Figures 2C, 2K, and 2S, arrowheads). Similarly to *Boc*$^{-/-}$ embryos (Figures 2B, 2J, and 2R), *Cdo*$^{+/-}$*; Boc*$^{-/-}$ embryos appear phenotypically normal (Figures 2D, 2L, and 2T). Importantly, *Cdo*$^{+/-}$*; Boc*$^{-/-}$ embryos are also viable and fertile, with no obvious defects (data not shown). In contrast, *Cdo*$^{-/-}$*; Boc*$^{-/-}$ embryos display severe patterning defects, including the loss of Shh (Figure 2E), and FoxA2 (Figure 2M) in the floor plate. Additionally, Nkx2.2+ and

Olig2+ populations were markedly reduced (Figure 2U), with Olig2+ pMNs more severely depleted than Nkx2.2+ V3 interneuron progenitors (pV3). Strikingly, this phenotype is almost identical that produced by reducing *Shh* levels in *Gas1* mutant embryo (*Gas1*$^{-/-}$; *Shh*$^{+/-}$; Allen et al., 2007). Overall, these results suggest a significant, yet redundant role for Cdo and Boc function during neural patterning; however, unlike *Drosophila* (Camp et al., 2010; Zheng et al., 2010), genetic removal of *Cdo* and *Boc* in mice does not result in complete loss of Hh signaling, since some Shh-dependent neural progenitors are established in double mutant embryos (Figure 2U). Thus, the requirement for Cdo and Boc function in Hh signaling appears to be distinct from ihog and boi function in *Drosophila*.

Gas1, Cdo and Boc are essential for Shh-dependent maintenance of motor neuron progenitors

In vitro Shh patterning assays have clearly demonstrated a lower threshold requirement for Olig2+ versus Nkx2.2+ progenitor cell specification (Dessaud et al., 2007; Ericson et al., 1997). Therefore, the differential sensitivity of Olig2+ cells (Figure 2U) is quite surprising as this population is expected to be less affected by reduced Shh input. Since *Cdo*$^{-/-}$; *Boc*$^{-/-}$ embryos lack Shh+ and FoxA2+ floor plate (Figures 2E and 2M), one possibility is that the lack of floor plate is sufficient to explain the phenotype of these mutants. However, at E10.5 *Gli2*$^{-/-}$ mutants that also lack floor plate (Figures 2F and 2N) still retain Olig2+ pMNs, but display a complete loss of Nkx2.2+ pV3 progenitors (Figure 2V). Thus, the phenotype of *Cdo*$^{-/-}$; *Boc*$^{-/-}$ embryos cannot be explained by the loss of floor plate expression of Shh alone and is not reconciled with a simple concentration dependent patterning model. A second possibility is that *Cdo* and *Boc* double mutant phenotypes reveal changing roles for Shh signaling in the developing neural tube. To test this hypothesis, we examined neural patterning a day earlier in *Cdo*$^{-/-}$; *Boc*$^{-/-}$ embryos.

Analysis of *Cdo*$^{-/-}$; *Boc*$^{-/-}$ embryos at E9.5 reveals several intriguing results (Figure 3). Compared to *Boc*$^{-/-}$ embryos (Figures 3A, 3D, and 3G), where neural patterning is normal, *Cdo*$^{-/-}$; *Boc*$^{-/-}$ double mutants resemble *Gli2*$^{-/-}$ mutants and lack expression of Shh or FoxA2 in the presumptive floor plate (Figures 3B–3C and 3E–3F). Surprisingly, although Nkx2.2+ pV3 progenitors are reduced in *Cdo*$^{-/-}$; *Boc*$^{-/-}$ embryos as observed at E10.5, Olig2+ pMN progenitor specification appears quite normal (Figure 3H). Thus Cdo and Boc are required at early stages to promote Shh-dependent specification of Nkx2.2+ pV3 progenitors, and at later stages to mediate Shh-dependent maintenance of Olig2+ pMN progenitors after their initial specification. Importantly, these data are consistent with previously published analyses suggesting an ongoing role for Shh signaling in motor neuron progenitor maintenance (Allen et al., 2007; Dessaud et al., 2010; Ericson et al., 1996).

Since previous work also indicated a potential role for Gas1 in motor neuron maintenance, and that Gas1 and Cdo cooperate to promote Shh-dependent neural patterning (Allen et al., 2007), we generated *Gas1*$^{-/-}$; *Boc*$^{-/-}$ mice to determine whether Gas1 and Boc might also cooperate in these processes (Figure 2). Gas1, Cdo and Boc proteins are expressed in largely overlapping domains in the neural tube of E10.5 mouse embryos (Figures S2A, 2C, and 2I), although Cdo protein is also detected in the notochord and floorplate (Figure S2C, arrow and asterisk). In agreement with previous data (Allen et al., 2007), and similar to *Cdo*$^{-/-}$; *Boc*$^{-/-}$embryos (Figures 2E, M, U), *Gas1*$^{-/-}$ embryos display a loss of Shh+ floor plate (Figure 2G), and co-expression of Nkx2.2 and FoxA2 (Figures 2O and 2W) in E10.5 *Gas1*$^{-/-}$ embryos. Additionally, the number of Olig2+ cells is also significantly reduced in these embryos (Figure 2W). As with *Cdo*$^{+/-}$; *Boc*$^{-/-}$ mice, *Gas1*$^{+/-}$; *Boc*$^{-/-}$ embryos are viable and fertile with no overt phenotype (data not shown). In contrast, *Gas1*$^{-/-}$; *Boc*$^{-/-}$ embryos have a more severe phenotype (Figures 2H, 2P, and 2X); in addition to a loss of Shh (Figure 2H) and FoxA2 (Figure 2P), there is a complete absence of Nkx2.2+ and

Olig2+ cells (Figure 2X) at E10.5, phenocopying the neural patterning deficiency of *Gas1*$^{-/-}$; *Cdo*$^{-/-}$ embryos at this developmental stage (Allen et al., 2007).

Although *Gas1*$^{-/-}$; *Cdo*$^{-/-}$ and *Gas1*$^{-/-}$; *Boc*$^{-/-}$ embryos have no Nkx2.2+ or Olig2+ cells at E10.5, the phenotype could result from either a failure of initial specification of these progenitors or ongoing maintenance of specified cell types. To distinguish between these alternative explanations, we examined neural patterning at E9.5 in both *Gas1*$^{-/-}$; *Cdo*$^{-/-}$ and *Gas1*$^{-/-}$; *Boc*$^{-/-}$embryos (Figure 3). As with *Cdo*$^{-/-}$; *Boc*$^{-/-}$ embryos (Figure 3B), *Gas1*$^{-/-}$; *Cdo*$^{-/-}$ and *Gas1*$^{-/-}$; *Boc*$^{-/-}$embryos do not express Shh in the presumptive floor plate (Figures 3J and 3K, respectively), though a few FoxA2+ cells (Figures 3L and 3M), and Nkx2.2+ cells (Figures 3N and 3O) are specified, indicating some initial Shh input, but insufficient signaling for continued ventral specification (cf. Figures 2H, 2P, and 2X). Consistent with these results, examination of Olig2 expression at E9.5 indicates relatively normal numbers of Olig2+ cells in *Gas1*$^{-/-}$; *Cdo*$^{-/-}$ embryos (Figure 3N), and a reduced, but significant number of Olig2+ pMN cells in *Gas1*$^{-/-}$; *Boc*$^{-/-}$embryos (Figure 3O). From these data we can conclude that the complete loss of Olig2+ cells in *Gas1*$^{-/-}$; *Cdo*$^{-/-}$ and *Gas1*$^{-/-}$; *Boc*$^{-/-}$ embryos at E10.5 is due to a failure to maintain these progenitors following specification. Further, removal of any two Shh-binding proteins (Cdo and Boc, Gas1 and Cdo, or Gas1 and Boc) results in severe patterning defects, but is not sufficient to abolish the Hh response at early developmental stages.

The significant reductions in the number of Olig2+ pMNs from E9.5 to E10.5 in *Gas1*, *Cdo* and *Boc* double mutant embryos raises the question of whether the requirement for Shh in motor neuron progenitor maintenance is restricted to the E9.5/E10.5 time window, or if this requirement extends throughout embryogenesis. To test this, we examined neural patterning at later developmental stages in *Gas1*$^{-/-}$ and *Gli2*$^{-/-}$ embryos (Figures 3P–3X). Olig2+ pMNs are present in both *Gas1*$^{-/-}$ and *Gli2*$^{-/-}$ embryos at E10.5 (Figures 2V and 2W). In *wt* embryos at E12.5, Shh is still strongly expressed in both the notochord and FP (Figure 3P). In contrast, in both *Gas1*$^{-/-}$ and *Gli2*$^{-/-}$ embryos, Shh is only present in the notochord (Figures 3Q and 3R). Compared with *wt* embryos (Figure 3S), which still display significant numbers of Olig2+ cells, examination of *Gas1*$^{-/-}$ embryos reveals that Nkx2.2+ pV3 progenitors are maintained, while only a few Olig2+pMN progenitors remain at E12.5 (Figure 3T). Similarly, reduced numbers of Olig2+ pMNs are also observed in *Gli2*$^{-/-}$ embryos (Figure 3U); the lack of Nkx2.2+ pV3 progenitors in *Gli2*$^{-/-}$ embryos is due to the earlier failure to initially specify these cells (cf. Figure 2V and Figure 3I; Matise et al., 1998; Mo et al., 1997). Overall, these data suggest that the requirement for Shh signaling in motor neuron progenitor maintenance extends for at least several days during embryogenesis, and that Gas1, Cdo and Boc all play a role in this process.

Late stage inhibition of Shh signaling during neural patterning specifically affects motor neuron, but not pV3 progenitor cell maintenance

Despite strong evidence from *Gas1*, *Cdo* and *Boc* double mutant analyses that there is a selective effect of prolonged Shh signaling on motor neuron maintenance, an indirect effect of Gas1, Cdo and Boc on other pathways that regulate motor neuron progenitors cannot be ruled out. To directly test a distinct requirement for ongoing Shh signaling in the maintenance of motor neuron progenitors, we examined the effects of blocking Hh signaling after the initial specification of neural progenitors in the developing chick neural tube, using the selective Hh pathway inhibitor, cyclopamine (Cooper et al., 1998). Cyclopamine treatment of developing chick embryos at early embryonic stages (i.e. Hamburger-Hamilton [HH] stage 10–11) effectively blocks Hh-dependent neuronal specification (Incardona et al., 1998). We examined inhibition of Hh signaling at later embryonic stages (Figure 4) through cyclopamine administration at HH stage 17–18, a developmental stage where initial patterning events, including specification of Olig2+ cells has already occured (Novitch et al.,

2001). Vehicle (ethanol) treated embryos display normal activation of the low level Hh target Nkx6.1 and repression of Pax 3 (Figures 4A–4D), as do cyclopamine treated embryos (Figures 4E–4H). Additionally, specification of the high level target, Nkx2.2, is unaffected in ethanol (Figure 4J) and cyclopamine treated embryos (Figure 4N). Strikingly, however, the number of Olig2+ pMN cells is significantly reduced in cyclopamine (Figure 4O), but not ethanol treated embryos (Figure 4K). In fact, some cyclopamine treated embryos display near total loss of Olig2+ cells (Figure 4N, inset), with no significant affect on Nkx2.2 cell number. Quantitation of less severely affected embryos (Figure 4Q) reveals a highly significant loss of Olig2+ pMN progenitors in late stage cyclopamine-treated embryos, confirming an ongoing and specific role for Shh in the maintenance of motor neuron progenitors that is conserved across species. Importantly, identical results are obtained following treatment with a second Hh pathway antagonist, SANT-1 (Figure S3), and these data are corroborated by recent studies in chick and mouse (Dessaud et al., 2010).

Gas1 and Boc, but not Cdo affect Shh-dependent digit specification during limb patterning

To determine whether Gas1, Cdo and Boc play equally important roles in other Hh-responsive tissues, we examined Shh-dependent digit specification in the developing limb at E18.5. *Gas1*, *Cdo* and *Boc* display overlapping expression in the anterior two-thirds of the forelimb bud in E10.5 mouse embryos (Figures S4B–S4D; Tenzen et al., 2006). Although digit specification is overtly normal in $Cdo^{-/-}$ (Figures 5A and 5H), $Boc^{-/-}$ (Figures 5B and 5I), and $Cdo^{-/-}; Boc^{-/-}$ E18.5 embryos (Figures 5C and 5J), examination of limb patterning in $Gas1^{-/-}; Boc^{-/-}$ embryos reveals an unexpected phenotype. As previously reported (Allen et al., 2007; Martinelli and Fan, 2007a), $Gas1^{-/-}$ embryos lack digit 2 or 3 in both the forelimb and hindlimb (Figures 5D and 5K); an identical phenotype is seen in $Gas1^{-/-}; Cdo^{-/-}$ embryos (Figures 5E and 5L). However, while $Gas1^{-/-}; Boc^{+/-}$ embryos appear identical to $Gas1^{-/-}$ mutants (Figures 5F and 5M), $Gas1^{-/-}; Boc^{-/-}$ embryos (Figures 5G and 5N) display a more severe defect in digit patterning; these embryos not only appear to lack digit 2 but also have an apparent fusion of digits 3 and 4 in both the forelimb and hindlimb, and a digit pattern where anterior and posterior halves exhibit a near mirror image symmetry most notably in the forelimb. These digit specification and digit patterning defects correlate with decreased expression of the Hh pathway targets *Ptch1* and *Gli1* in the forelimbs of E11.5 $Gas1^{-/-}; Boc^{-/-}$ embryos (Figures S4H–S4M). Significantly, there are no overt effects on *Shh* transcript levels in $Gas1^{-/-}; Boc^{-/-}$ double mutants (Figures S4E–S4G). Overall, these results indicate that Gas1 and Boc play a major role in Shh-dependent organization of the mammalian limb.

Gas1, Cdo and Boc are essential co-receptors for Shh signaling during neural patterning

The striking neural patterning defects observed in $Cdo^{-/-}; Boc^{-/-}$, $Gas1^{-/-}; Cdo^{-/-}$ and $Gas1^{-/-}; Boc^{-/-}$ embryos suggest that all three cell surface Hh pathway components play a role in mediating the Hh response during neural patterning. Importantly, however, Shh-dependent patterning still occurs to some extent in these embryos (Figure 3). One possibility is that Gas1, Cdo and Boc act as necessary, but partially redundant co-factors to promote Shh signaling. To test this model, we generated mice lacking all Gas1, Cdo and Boc activity. Importantly, $Gas1^{+/-}; Cdo^{+/-}; Boc^{-/-}$ mice are viable and fertile with no overt phenotype (data not shown). Embryos with only one allele of either *Cdo* or *Gas1* ($Gas1^{-/-}; Cdo^{+/-}; Boc^{-/-}$ or $Gas1^{+/-}; Cdo^{-/-}; Boc^{-/-}$) survive to birth and resemble double mutants (data not shown). In contrast, $Gas1^{-/-}; Cdo^{-/-}; Boc^{-/-}$embryos die by E9.5 (Figure 6), and display severe forebrain and cardiovascular defects at E8.5 (Figures 6C and 6D), including heart looping defects and pericardial edema. Triple mutant embryos fail to complete the turning process (Figures 6G and 6H), and additionally display cyclopia, and holoprosencephaly (Figures 6K and 6L), similar to $Shh^{-/-}; Ihh^{-/-}$ double mutant embryos and $Smo^{-/-}$ embryos (Zhang et al., 2001). Consistent with the gross morphological defects of these embryos,

detailed examination of E9.5 *Gas1*$^{-/-}$; *Cdo*$^{-/-}$; *Boc*$^{-/-}$ neural tubes reveals severe defects in ventral cell fate specification (Figure 7). Shh protein is expressed in the notochord of E9.5 *Gas1*$^{-/-}$; *Cdo*$^{-/-}$; *Boc*$^{-/-}$ embryos (Figure 7C), and secreted Shh protein can be detected in the lateral neural tube (Figure 7C, arrowhead). However, within the neural tube no Shh, FoxA2, Nkx2.2, Olig2, or Nkx6.1 are present (Figures 7C, 7G, 7K, and 7O), and Pax6, normally repressed by Shh signaling, extends to the ventral limit of the neural tube (Figure 7S). Strikingly, the neural patterning defects observed in *Gas1*$^{-/-}$; *Cdo*$^{-/-}$; *Boc*$^{-/-}$ embryos phenocopy *Shh*$^{-/-}$ embryos (Figures 7D, 7H, 7L, 7P, and 7T). Overall, the data presented here suggest that Gas1, Cdo and Boc indeed function as essential co-receptors for Hh signaling in multiple Hh-responsive tissues.

Discussion

Ongoing requirement for Shh signaling and the co-receptors Gas1, Cdo and Boc in motor neuron maintenance

The specification of Olig2+ pMN progenitors during neural patterning is a highly regulated and complex process that requires inputs from not only Shh (Roelink et al., 1994), but also Retinoic Acid (RA) and Fibroblast Growth Factors (FGFs; Novitch et al., 2003). Later, combinatorial Hox gene expression regulates the subsequent generation of motor neuron diversity along the rostro-caudal axis (Dasen et al., 2003; Dasen et al., 2005). These data suggest that multiple signaling pathways are required at specific times and discrete locations for the proper formation of motor neurons. Early studies from the Jessell lab indicated two roles for Shh in motor neuron specification: an early role in motor neuron progenitor specification and a later role in progenitor differentiation into motor neurons (Ericson et al., 1996). Here we present evidence for a third role of Shh in the maintenance of Olig2+ motor neuron progenitors during later stages of neural patterning. Our data suggest that Shh pathway activity is selectively required to maintain Olig2 expression over several days (E9.5-E12.5) of embryogenesis, and that Gas1, Cdo and Boc play significant roles in mediating this signaling.

Recent work from Dessaud et al. also examined the maintenance of Shh-dependent ventral cell identities during neural patterning (Dessaud et al., 2010); through floor plate-specific deletion of *Shh* around E9.5 (following initial specification of Olig2 and Nkx2.2), they demonstrated that Olig2 maintenance requires ongoing Shh signaling. Significantly, they also identified defects in Nkx2.2 maintenance, a result not duplicated in *Gas1*$^{-/-}$ embryos at the same stage (E12.5). Several possible explanations for this discrepancy include differences in the level of notochord derived Shh between these mice, the timing and completeness of Shh loss in the neural tube, or an altered response to Shh ligand in Nkx2.2+ cells in *Gas1*$^{-/-}$ embryos.

Notably, at later developmental stages (from E12.5 onward), Olig2+ pMNs no longer give rise to motor neurons, but instead produce oligodendrocytes (Ligon et al., 2006; Rowitch, 2004). Thus, our data implicate Gas1, Cdo and Boc not only as regulators of motor neuron formation, but also as mediators of oligodendrocyte formation during later embryogenesis. Importantly, these data are consistent with genetic analyses of *Shh*$^{-/-}$; *Gli3*$^{-/-}$ embryos that link Shh signaling to the precise timing of oligodendrocyte appearance during embryogenesis (Oh et al., 2005; Oh et al., 2009).

Gas1, Cdo and Boc are essential Hh co-receptors

The recent identification of Gas1, Cdo and Boc as ligand-binding components of the Hh pathway raises the question of their combinatorial roles in Shh signaling. This study demonstrates an absolute requirement for Gas1, Cdo and Boc in Shh-dependent neural

patterning. Our data indicate that Gas1, Cdo and Boc are all equally capable of promoting Shh signaling during neural patterning; overexpression of any individual component results in ectopic ventral cell fate specification in a cell-autonomous and ligand-dependent manner. Additionally, while genetic removal of *Gas1*, *Cdo* or *Boc* individually has only modest effects on Shh signaling, removal of any two components results in significantly reduced Shh-dependent ventral neural patterning. Most strikingly, simultaneous removal of *Gas1*, *Cdo* and *Boc* results in a complete loss of Shh-dependent neural progenitors, mirroring the loss of *Shh* itself. Additionally, $Gas1^{-/-}; Cdo^{-/-}; Boc^{-/-}$ embryos display phenotypes such as cyclopia, holoprosencephaly and linearized heart tubes, phenotypes observed in $Shh^{-/-}; Ihh^{-/-}$ double mutant embryos, and $Smo^{-/-}$ embryos, suggesting that most Hh signaling is abrogated when all co-receptors are absent (Zhang et al., 2001). However, that the linearized heart tube phenotype is not completely penetrant in $Gas1^{-/-}; Cdo^{-/-}; Boc^{-/-}$ embryos, suggests that some transient, early Hh signaling is possible in the absence of these receptors. Whether this early Hh signal is transduced solely through Shh binding to Ptch1 is an open question. Weak interactions of Shh with other extracellular factors such as vitronectin may be sufficient to promote some basal level of Hh signaling through Ptch1 (Pons and Marti, 2000; Pons et al., 2001).

Importantly, the findings presented here are corroborated by an independent study examining Hh-dependent cerebellar progenitor proliferation (Izzi et al., submitted); this work demonstrates an equally vital role for Gas1, Cdo and Boc in reception of the Hh signal in cerebellar granular neuron progenitor proliferation. Additionally, this study explores physical interactions between Gas1, Cdo and Boc with the canonical Hh receptor, Ptch1. Together these studies support a model in which Gas1, Cdo and Boc are essential co-receptors that mediate multiple cellular responses to Hh ligands in multiple tissues, and at multiple developmental stages.

Differential requirement for Hh co-receptors in vertebrates

Recent data suggest that the *Drosophila* homologs of Cdo and Boc (ihog and boi) are essential co-receptors for Hh in the developing wing imaginal disc (Camp et al., 2010; Zheng et al., 2010). However, despite many highly conserved core elements of the Hh pathway across species, significant differences exist in Hh signal transduction between vertebrates and invertebrates. Perhaps the most striking of these is the requirement for the primary cilium in vertebrate Hh signaling (Huangfu et al., 2003). Other differences exist though, including the function of a vertebrate-specific Hh antagonist Hhip1 (Chuang and McMahon, 1999), and, of greatest relevance to this study, the mode of Hh ligand binding to ihog/boi in flies, and Cdo/Boc in mice (McLellan et al., 2008). This report highlights two additional differences in Hh signaling. First, genetic data presented here indicate that mice lack an absolute requirement for Cdo and Boc function during embryogenesis. In fact, while genetic removal of Cdo and Boc function does result in significant neural patterning defects, Shh-mediated digit specification occurs normally in $Cdo^{-/-}; Boc^{-/-}$ embryos; thus, Cdo and Boc are dispensable for normal limb development. These data are corroborated by a recent study examining the spectrum of holoprosencephaly in *Cdo* and *Boc* mutant mice (Zhang et al., 2010). Second, we present evidence that, in mice, Gas1 plays a significant role in reception of the Shh signal, and that together, Gas1, Cdo and Boc function as essential co-receptors for the vertebrate Hh pathway.

An outstanding question is what role, if any does Gas1 play in Hh signal reception in *Drosophila*? Recent work has identified structural similarities between Gas1 and glial cell derived neurotrophic factor receptors (GFRs; Cabrera et al., 2006). While *Drosophila* lacks both GDNF ligands (GFLs), as well as a clear Gas1 homolog, a Gas1 homolog does exist in Honey bees (*Apis mellifora*). There is also a single GDNF receptor-like (GFRL) homolog present in *Drosophila* (Airaksinen et al., 2006; Hatinen et al., 2007; Schueler-Furman et al.,

2006). Future studies will be important to determine whether this GFRL protein plays any role in mediating Hh signaling in *Drosophila* or if it functions more similarly to GFRs as a modifier of Drosophila Ret signaling (Ibanez, 2010).

Additional cell surface proteins in Hh signal transduction

Gas1, Cdo and Boc are essential components of the Hh pathway that promote Shh signaling in a cell-autonomous manner through direct interactions with ligand. Since Gas1 is a GPI anchored protein (Stebel et al., 2000), and previous work demonstrated that the cytoplasmic domains of Cdo and Boc are dispensable for the promotion of Shh signaling (Tenzen et al., 2006), these proteins must utilize other cell surface or membrane-associated molecules to transduce extracellular Hh signals across the plasma membrane. A likely model, that Gas1, Cdo and Boc form a physical complex with Ptch1, and that engagement of this complex by Hh ligand is essential for signal transduction is supported by recent biochemical data (Zheng et al., 2010), and by functional data indicating that ligand binding is necessary, but not sufficient for the Hh-promoting function of Cdo and Boc (Allen et al., 2007; Tenzen et al., 2006).

While recent structural studies address the precise physical interactions of Hh ligands with Cdo and Boc (reviewed by Beachy et al., 2010), future work assessing the co-receptor function of Gas1, Cdo and Boc must also consider a growing number of additional Hh cell surface proteins. This includes the Hh-binding cell surface antagonists Ptch2 and Hhip1 (Carpenter et al., 1998; Chuang et al., 2003; Chuang and McMahon, 1999; Motoyama et al., 1998) and Hh pathway components that regulate the trafficking and turnover of Hh ligands, as has been described for megalin (McCarthy and Argraves, 2003; McCarthy et al., 2002) and recently proposed for Dispatched1 (Etheridge et al., 2010). Recent studies have also identified opposing roles for different glypican family members (six glypicans exist in mouse and human) in Hh signal transduction (Capurro et al., 2008; Williams et al., 2010); whether Gas1, Cdo and Boc functionally cooperate or compete with these proteins for Hh binding at the cell surface is unknown. Finally, a number of secreted and extracellular matrix proteins also regulate Shh, including *Drosophila* Shifted, (Glise et al., 2005; Gorfinkiel et al., 2005), zebrafish Scube2 (Glise et al., 2005; Hollway et al., 2006; Kawakami et al., 2005; Woods and Talbot, 2005), *Drosophila* trol (Park et al., 2003), and vitronectin (Martinez-Morales et al., 1997; Pons and Marti, 2000; Pons et al., 2001). Taken together, the total number of cell surface regulators of the Hh pathway consists of well over a dozen components, highlighting the complex and tightly regulated nature of Hh signaling during embryogenesis.

Experimental Procedures

Mice—*Gas1*^{LacZ} (Martinelli and Fan, 2007b), *Cdo*$^{LacZ-2}$ (Cole and Krauss, 2003), Gli2^{zfd} (Mo et al., 1997) and *Shh* (St-Jacques et al., 1998) mice have all been described previously. The generation of *Boc*$^{AP-2}$ (referred to as *Boc*) mice is described elsewhere (Zhang et al., 2010). *Gas1*, *Cdo* and *Shh* mice were maintained on a C57BL/6 background. *Gli2* mice were maintained on a mixed C57BL/6; 129S4/SvJaeJ background. *Gas1; Cdo; Boc* mice were maintained on a mixed C57BL/6; 129S6/SvEvTac background. Noon of the day on which a vaginal plug was detected was considered as E0.5. For all subsequent analyses, a minimum of three embryos of each genotype was examined; representative images are shown.

***In situ* hybridization and immunofluorescence—**Whole-mount digoxigenin in situ hybridization was performed as described (Wilkinson, 1992). Immunofluorescent analyses of mouse neural tubes were performed essentially as described previously (Jeong and McMahon, 2005). The antibodies used were as follows: 1:20 Mouse IgG1 anti Nkx6.1 (DSHB; F55A10), 1:20 mouse IgG2b anti-Nkx2.2 (DSHB; 74.5A5), 1:20 mouse IgG1 anti-

Shh (DSHB; 5E1), 1:20 mouse IgG1 anti-FoxA2 (DSHB; 4C7), 1:20 mouse IgG2a Pax3 (DSHB), 1:20 mouse IgG1 anti-Pax6 (DSHB); Rabbit IgG anti-Olig2 antibody was purchased from Millipore (AB9610) and used at a dilution of 1:1000; Rabbit IgG anti-Dbx1 antibody was a gift from Yasushi Nakagawa and used at a dilution of 1:1000. Gas1, Cdo and Boc were detected with the following antibodies: 1:1000 goat IgG anti-Gas1 (R&D systems), 1:1000 goat IgG anti-Cdo (R&D systems), and 1:1000 goat IgG anti-Boc (R&D systems). All secondary antibodies were obtained from Invitrogen and used at a dilution of 1:500. Primary antibodies were incubated overnight at 4°C, followed by incubation with secondary antibodies for 1hr at room temperature. Images were collected with a Zeiss LSM 510 confocal microscope and Leica SP5X confocal microscope.

Chick electroporations—Chick electroporations were performed essentially as described previously (Tenzen et al., 2006). Briefly, pCIG, Gas1-pCIG, Cdo-pCIG, Boc-pCIG, and SmoM2-pCIG were injected into the neural tubes of Hamburger-Hamilton (HH) stage 10–12 chicken embryos at concentrations of 1.0μg/μl in PBS with 50 ng/μl Fast Green. Approximately 48 hours following electroporation embryos were recovered and fixed in 4% paraformaldehyde for subsequent immunofluorescent analysis. Fertile eggs were obtained from both Charles River and the Michigan State University Poultry Farm.

Hh antagonist administration—Cyclopamine (purchased from both Sigma and Alexis Biochemicals) was dissolved to a concentration of 1mg/ml in 100% ethanol. SANT-1 was purchased from Tocris Biosciences, dissolved in DMSO to a concentration of 10mM, and used at a concentration of 1uM. Neural tubes of HH stage 18 chick embryos were injected with each compound, ethanol alone, or untreated, followed by incubation for 24 hours. Embryos were then dissected and processed for subsequent immunofluorescent analysis of neural patterning.

Skeletal preparations—Skeletons of E18.5 mouse embryos were prepared according to a modified Alcian Blue (Sigma; A5268) and Alizarin Red (Sigma; A5533) staining protocol (Kessel et al., 1990; Wallin et al., 1994). Briefly, E18.5 embryos were dissected, skinned and eviscerated. Subsequently, embryos were fixed in ethanol, followed by acetone and stained for four days in an Alcian Blue/Alizarin red stain solution. Remaining tissue was digested with 1% potassium hydroxide, and embryos were cleared by incubation with progressively increasing concentrations of glycerol.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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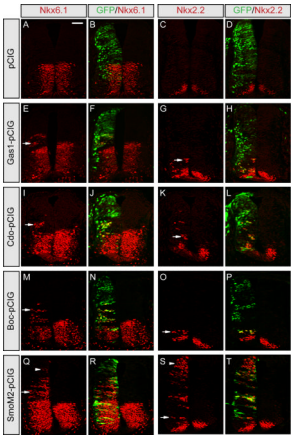

Figure 1. Gas1, Cdo and Boc equally promote Shh-dependent specification of ventral neural progenitors

HH stage 21–22 chick neural tubes electroporated with pCIG (A–D), Gas1-pCIG (E–H), Cdo-pCIG (I–L), Boc-pCIG (M–P), or SmoM2-pCIG (Q–T) were sectioned at the forelimb level and stained with antibodies raised against Nkx6.1 (red; A, E, I, M, Q) and Nkx2.2 (red; C, G, K, O, S). GFP expressing cells (green; B, F, J, N, R and D, H, L, P, T) indicate electroporated cells on one side, while the un-electroporated half of the neural tube serves as an internal negative control. Arrows denote ectopic expression of Nkx6.1 (E, I, M, Q) and Nkx2.2 (G, K, O, S). Arrowheads indicate ectopic Nkx6.1 and Nkx2.2 expression throughout the dorsal neural tube in embryos expressing SmoM2 (Q–T), whereas ectopic induction of these markers following Gas1 (E–H), Cdo (I–L) and Boc (M–P) electroporation was restricted to the ventral neural tube. Scale bar: A, 50μm.

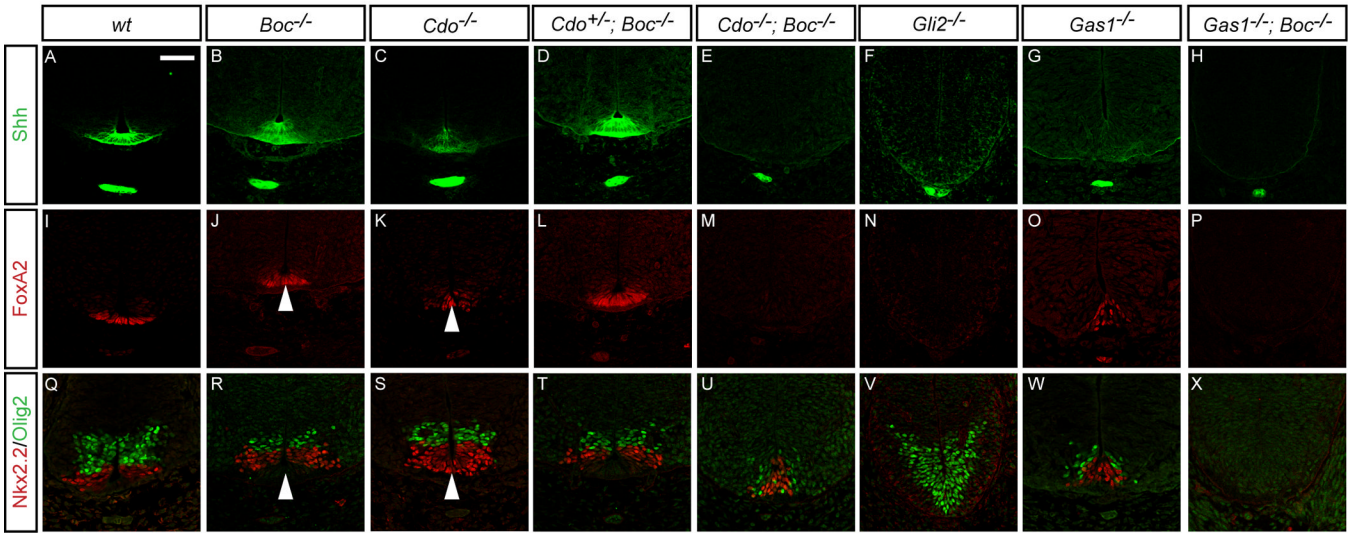

Figure 2. Defective ventral neural patterning in E10.5*Cdo*$^{-/-}$; *Boc*$^{-/-}$ and *Gas1*$^{-/-}$; *Boc*$^{-/-}$ mouse embryos

Immunofluorescent analysis of E10.5 forelimb level sections detects expression of Shh (green; A–H), FoxA2 (red; I–P), Nxk2.2 and Olig2 (red and green, respectively; Q–X) in *wt* (A, I, Q), *Boc*$^{-/-}$ (B, J, R), *Cdo*$^{-/-}$ (C, K, S), *Cdo*$^{+/-}$; *Boc*$^{-/-}$ (D, L, T), *Cdo*$^{-/-}$; *Boc*$^{-/-}$ (E, M, U), *Gli2*$^{-/-}$ (F, N, V), *Gas1*$^{-/-}$ (G, O, W), and *Gas1*$^{-/-}$; *Boc*$^{-/-}$ (H, P, X) embryos. Arrowheads denote FoxA2 and Nkx2.2 double positive floorplate cells in *Cdo*$^{-/-}$ embryos (S), but not *Boc*$^{-/-}$ embryos (R). Despite the complete loss of FoxA2+ FP cells in both *Cdo*$^{-/-}$; *Boc*$^{-/-}$, and *Gli2*$^{-/-}$ embryos (M and N, respectively), there is a selective loss of Olig2+ but not Nkx2.2+ cells in *Cdo*$^{-/-}$; *Boc*$^{-/-}$embryos (U). In contrast, *Gli2*$^{-/-}$ embryos lack Nkx2.2+ cells, but maintain Olig2 expression (V). *Gas1*$^{-/-}$; *Boc*$^{-/-}$ embryos display complete loss of FoxA2 (P), Nkx2.2 and Olig2 (X) at E10.5. Scale bar: A, 50μm. See Figure S1 for a more detailed analysis of neural patterning in *Boc*$^{-/-}$embryos. Refer to Figure S2 for Gas1, Cdo and Boc protein distribution in E10.5 mouse neural tubes.

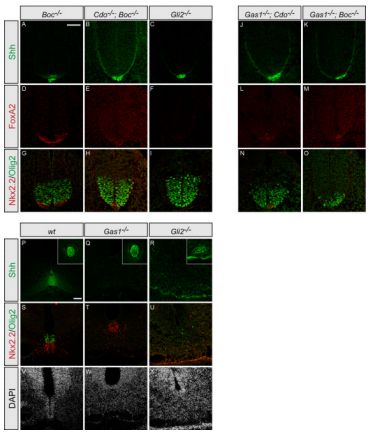

Figure 3. Motor neuron progenitors are specified, but not maintained in embryos with reduced Shh signaling

Antibody detection of Shh (green; A–C, J–K), FoxA2 (red; D–F, L–M), Nkx2.2 and Olig2 (red and green, respectively; G–I, N–O) in forelimb level sections of E9.5 *Boc*$^{-/-}$ (A, D, G), *Cdo*$^{-/-}$*; Boc*$^{-/-}$ (B, E, H), *Gli2*$^{-/-}$ (C, F, I), *Gas1*$^{-/-}$*; Cdo*$^{-/-}$ (J, L, N), and *Gas1*$^{-/-}$*; Boc*$^{-/-}$(K, M, O) embryos. At E9.5 *Cdo*$^{-/-}$*; Boc*$^{-/-}$ embryos contain similar numbers of Olig2+ progenitors to *Boc*$^{-/-}$ or *Gli2*$^{-/-}$ embryos (G and I, respectively) in marked contrast to E10.5 embryos. Note the variable presence of a few FoxA2+ and Nkx2.2+ cells in *Cdo*$^{-/-}$*; Boc*$^{-/-}$, *Gas1*$^{-/-}$*; Cdo*$^{-/-}$ and *Gas1*$^{-/-}$*; Boc*$^{-/-}$ embryos at the forelimb level (E, H, L–O). Neither cell type appears to be specified in *Gli2*$^{-/-}$ embryos (F, I). Immunofluorescent detection of Shh (green; P–R), Nkx2.2 and Olig2 (red and green, respectively; S–U) in forelimb level sections of E12.5 *wt* (P, S, V), *Gas1*$^{-/-}$ (Q, T, W), and *Gli2*$^{-/-}$ (R, U, X) embryos. Nuclei are identified with DAPI (G–I). Insets (A–C) indicate notochord expression of Shh. Note that in *Gas1*$^{-/-}$ embryos (E), only a few Olig2+ cells are present, while the number of Nkx2.2+ cells is comparable to *wt* (D). Olig2 + cells are reduced and fail to cluster normally in *Gli2*$^{-/-}$ embryos (F). Scale bar: A,P, 50μm.

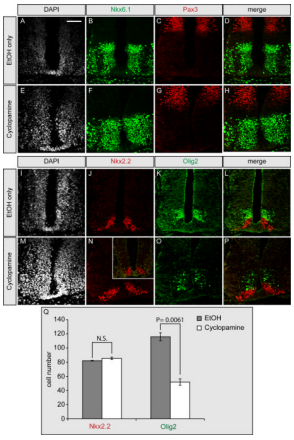

Figure 4. Delayed cyclopamine administration selectively affects motor neuron maintenance in HH stage 22 chick embryos

Cyclopamine (E–H, M–P) or ethanol (A–D, I–L) were administered to HH stage 17–18 chick embryos for 24 hours, followed by immunofluorescent detection of nuclei (DAPI; A, E, I, M), Nkx6.1 (green; B, D, F, H), Pax3 (red; C, D, G, H), Nkx2.2 (red; J, L, N, P), and Olig2 (green; K, L, O, P). Note the normal maintenance of Nkx6.1, Pax3, and Nkx2.2 in cyclopamine-treated embryos (F–H, N–P). In contrast, there is a significant reduction in the number of Olig2+ cells following cyclopamine administration (N–P). The number of Olig2 cells varies from moderate (O, P) to severe (inset, N). Comparison of Nkx2.2 and Olig2 cell numbers in EtOH-treated embryos and moderately affected cyclopamine-treated embryos is quantitated in Q. Error bars represent the mean +/− SD calculated from analysis of sections from three different embryos. P-values calculated by two-tailed Student's t-test are listed. NS, not significant. Scale bar: A, 50μm. Refer to Figure S3 for a similar analysis with a second Hh pathway antagonist, SANT-1.

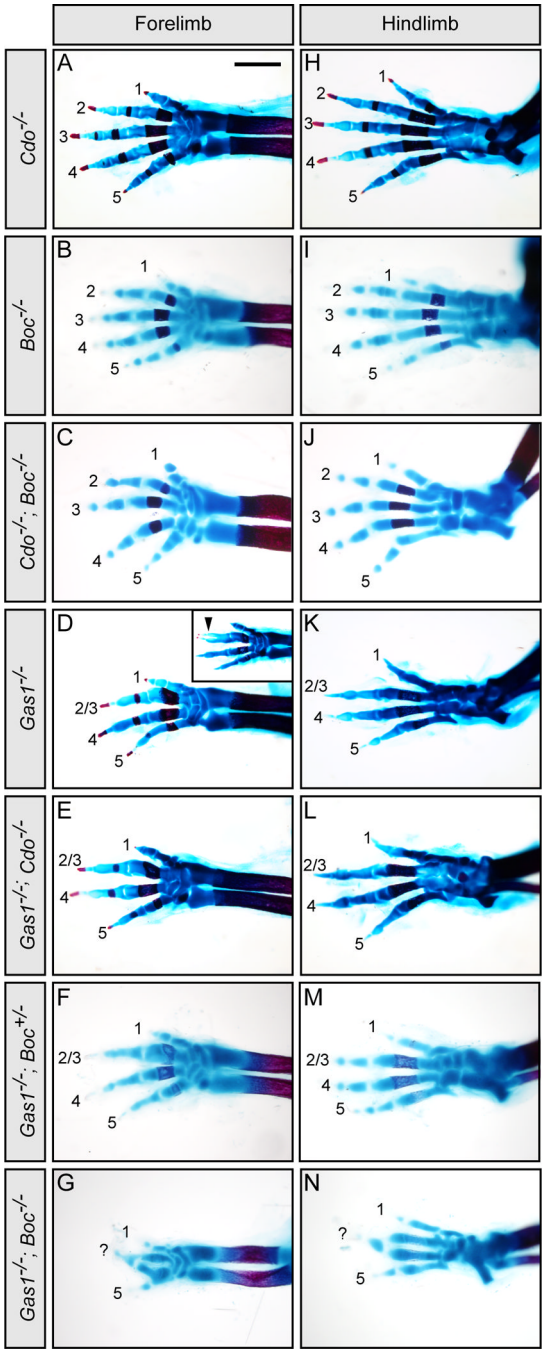

Figure 5. Analysis of *Cdo*$^{-/-}$; *Boc*$^{-/-}$ and *Gas1*$^{-/-}$; *Boc*$^{-/-}$ limb development

Forelimbs (A–G) and hindlimbs (H–N) of E18.5 embryos stained with alcian blue and alizarin red to visualize cartilage and bone, respectively, in the limb skeleton. Numbers denote specific digits (1 most anterior, and 5 most posterior). *Cdo*$^{-/-}$ (A, H), *Boc*$^{-/-}$ (B, I) and *Cdo*$^{-/-}$; *Boc*$^{-/-}$ (C, J) embryos display normal digit patterning. In contrast, *Gas1*$^{-/-}$ embryos display fusion and loss of digits 2/3 (D, K). A similar phenotype is seen in *Gas1*$^{-/-}$; *Cdo*$^{-/-}$ (E, L) and *Gas1*$^{-/-}$; *Boc*$^{+/-}$ (F, M). In contrast, *Gas1*$^{-/-}$; *Boc*$^{-/-}$ embryos display a significantly more severe digit patterning defect; only digits 1 and 5 are identifiable in both the forelimb (G) and hindlimb (N), and a third digit (labeled as "?"), possibly a fusion of digits 3 and 4, is at the anterior-posterior intersect. Scale bar: A, 1mm.

Gas1, *Cdo* and *Boc* expression in the developing limb is provided in Figure S4, along with analysis of *Shh*, *Ptch1*, and *Gli1* transcript levels in E10.5 $Gas1^{-/-}$; $Boc^{-/-}$ forelimb buds.

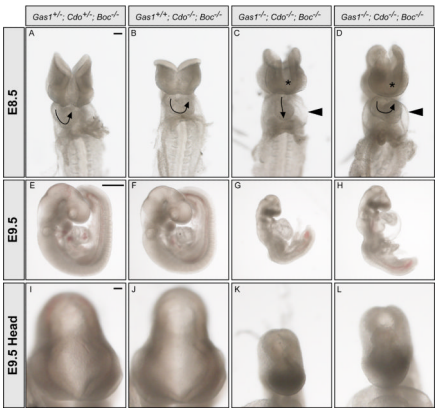

Figure 6. Cyclopia, holoprosencephaly and heart looping defects in E8.5 and E9.5 *Gas1*$^{-/-}$; *Cdo*$^{-/-}$; *Boc*$^{-/-}$ embryos*Gas1*$^{+/-}$; *Cdo*$^{+/-}$; *Boc*$^{-/-}$ (A, E, I), *Gas1*$^{+/+}$; *Cdo*$^{-/-}$; *Boc*$^{-/-}$ (B, F, J) and two *Gas1*$^{-/-}$; *Cdo*$^{-/-}$; *Boc*$^{-/-}$

(C–D, G–H, K–L) embryos are shown. En face images of E8.5 (10–12 somite) embryos (A–D). Arrows indicate the direction of heart looping, while arrowheads denote pericardial edema that is present in *Gas1*$^{-/-}$; *Cdo*$^{-/-}$; *Boc*$^{-/-}$ embryos (C, D). 50% of *Gas1*$^{-/-}$; *Cdo*$^{-/-}$; *Boc*$^{-/-}$ embryos display a linear heart tube (N = 4/8 embryos). Asterisks indicate abnormal forebrain development that is a hallmark of the failure to specify ventral midline cell fates. Examination of embryos of the same genotype at E9.5 (20–25 somites; E–H) demonstrates a failure to complete the turning process in *Gas1*$^{-/-}$; *Cdo*$^{-/-}$; *Boc*$^{-/-}$ embryos (G, H). Higher magnification views of the heads of E9.5 embryos (I–L) reveal holoprosencephaly in *Gas1*$^{-/-}$; *Cdo*$^{-/-}$; *Boc*$^{-/-}$ embryos (K, L). Scale bars: A, 100um; E, 500μm; I, 100μm.

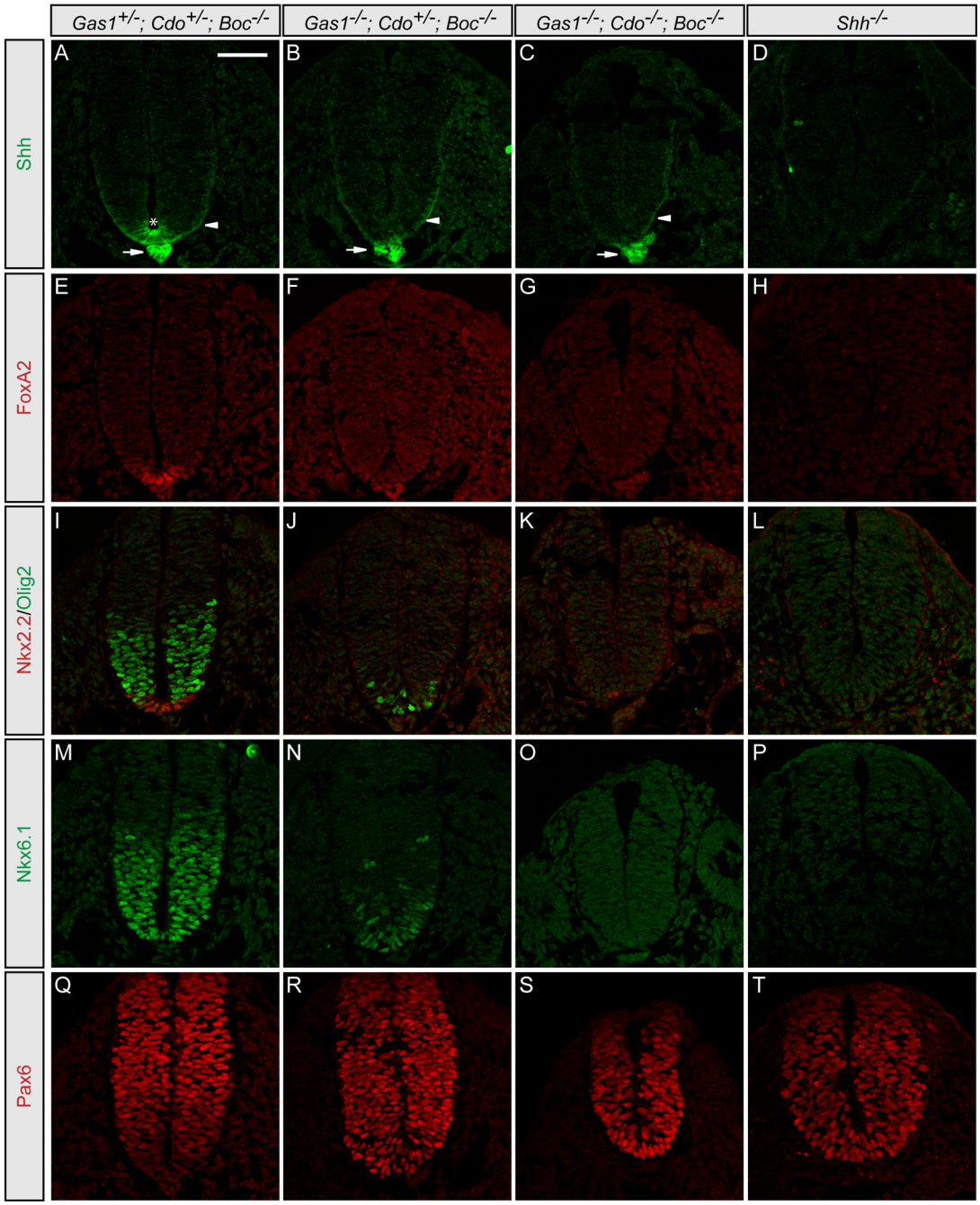

Figure 7. Simultaneous genetic removal of *Gas1*, *Cdo* and *Boc* results in complete loss of Shh-dependent ventral cell specification

Antibody detection of Shh (green; A–D), FoxA2 (red; E–H), Nkx2.2 and Olig2 (red and green, respectively; I–L), Nkx6.1 (green; M–P), and Pax6 (red; Q–T) in *Gas1*$^{+/-}$*; Cdo*$^{+/-}$*; Boc*$^{-/-}$ (A, E, I, M, Q), *Gas1*$^{-/-}$*; Cdo*$^{+/-}$*; Boc*$^{-/-}$ (B, F, J, N, R), *Gas1*$^{-/-}$*; Cdo/-; Boc*$^{-/-}$ (C, G, K, O, S), and *Shh*$^{-/-}$ (D, H, L, P, T) E9.5 embryos. Arrows (A–C) indicate Shh expression in the notochord. Arrowheads (A–C) denote secreted Shh protein. Shh expression in the floorplate is detected only in *Gas1*$^{+/-}$*; Cdo*$^{+/-}$*; Boc*$^{-/-}$ embryos (asterisk in A). Scale bar: A, 50μm.