Boc modifies the holoprosencephaly spectrum of Cdo mutant mice

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SUMMARY
Holoprosencephaly (HPE) is caused by a failure to form the midline of the forebrain and/or midface. It is one of the most common human birth defects, but clinical expression is extremely variable. HPE is associated with mutations in the sonic hedgehog (SHH) pathway. Mice lacking the Shh pathway regulator Cdo (also called Cdon) display HPE with strain-dependent penetrance and expressivity, implicating silent modifier genes as one cause of the variability. However, the identities of potential HPE modifiers of this type are unknown. We report here that whereas mice lacking the Cdo paralog Boc do not have HPE, Cdo;Boc double mutants on a largely Cdo-resistant genetic background have lobar HPE with strong craniofacial anomalies and defects in Shh target gene expression in the developing forebrain. Boc is therefore a silent HPE modifier gene in mice. Furthermore, Cdo and Boc have specific, selective roles in Shh signaling in mammals, because Cdo;Boc double-mutant mice do not display the most severe HPE phenotype seen in Shh-null mice, nor do they have major defects in digit patterning or development of vertebrae, which are also Shh-dependent processes. This is in contrast to reported observations in Drosophila, where genetic removal of the Cdo and Boc orthologs Ihog and Boi results in a complete loss of response to the hedgehog ligand. Therefore, there is evolutionary divergence between mammals and insects in the requirement of the hedgehog pathway for Cdo/Ihog family members, with mammalian development involving additional factors and/or distinct mechanisms at this level of pathway regulation.

INTRODUCTION
Holoprosencephaly (HPE) is characterized by failure to define the midline of the embryonic forebrain and/or midface and is one of the most common human birth defects (Cohen, 2006; Geng and Oliver, 2009; Muenke and Beachy, 2001; Yamada et al., 2004). Clinical presentation of HPE is remarkably variable, and the range of defects extends from most to least severe in a continuum known as the HPE spectrum. Based on the degree of midline cleavage of the telencephalon, classical HPE is commonly classified into three categories (Cohen, 2006; Muenke and Beachy, 2001). The most severe form, alobar HPE, is characterized by a complete failure to divide the forebrain into left and right hemispheres; semilobar and lobar HPE are progressively less severe subtypes. Craniofacial midline anomalies range from cyclopia with proboscis in the most severe cases to single nostril, cleft lip and palate, hypotelorism (close-spaced eyes) and solitary median maxillary central incisor in milder cases (Cohen, 2006; Muenke and Beachy, 2001). Mild facial midline abnormalities can occur in the absence of brain malformations and are sometimes called microforms of HPE.

The etiology of HPE is heterogeneous, with both genetic and environmental causes (Cohen and Shiota, 2002; Dubourg et al., 2007; Geng and Oliver, 2009; Krauss, 2007; Ming and Muenke, 2002). Non-syndromic HPE occurs in both inherited (familial) and sporadic forms, and nine genes have been linked to the former and/or the latter: SHH, ZIC2, SIX3, TGIF, PTCH1, GLI2, TDGF1, FOXH1 and DISP1 (Dubourg et al., 2007; Geng and Oliver, 2009). All mutations are found in the heterozygous state and are usually loss-of-function mutations, although some might possess dominant-negative properties (Domené et al., 2008; Roessler et al., 2009a; Roessler et al., 2009b; Singh et al., 2009). The extensive phenotypic variability that characterizes human HPE is seen in both sporadic cases and within pedigrees; in the latter, more than one-third of mutation carriers are without clinical expression (Cohen, 1989; Ming and Muenke, 2002). These observations suggest that heterozygous mutation of HPE genes might be insufficient to produce severe anomalies and that HPE arises from a complex interplay of developmental, genetic and environmental factors (Krauss, 2007; Ming and Muenke, 2002). Potential explanations include: (1) multiple genetic-hit models, such as heterozygous mutation of two HPE genes (digenic mutation) or interaction between mutation of an HPE gene and otherwise silent modifier genes; (2) environmental insults during specific stages of fetal development, with timing of the insult dictating severity of outcome; (3) stochastic events within a haploinsufficient HPE gene background; and (4) gene-environment interactions (Krauss, 2007).

Disruption of sonic hedgehog (SHH) signaling is a major common theme in the development of HPE. Studies with model organisms demonstrate that Shh produced by the prechordal mesendoderm (PCM) is required for initiating development of the midline of the forebrain and midface (Kiecker and Niehrs, 2001; Muenke and Beachy, 2000; Muenke and Beachy, 2001; Rubenstein and Beachy, 1998). This occurs by a progressive mechanism
expression in a successive midline structure. Shh produced by the
PCM induces expression of pathway target genes in the rostral
diencephalon ventral midline of the developing forebrain, including
Ptk1, Gli1 and Shh itself (Aoto et al., 2009; Cordero et al., 2004;
Geng et al., 2008; Marcucio et al., 2005; McMahon et al., 2003;
Rubenstein and Beachy, 1998). Shh produced by the rostral
diencephalon ventral midline is required for maintenance of Fgf8
expression in the rostral commissural plate (Aoto et al., 2002;
Ohkubo et al., 2002). Shh and Fgf8, in conjunction with dorsally
derived bone morphogenetic proteins (BMPs), ultimately dictate
dorsalventral patterning, cell proliferation and cell death in the
developing forebrain (Fernandes and Hébert, 2008; Ohkubo et al.,
2002; Wilson and Houart, 2004). As embryogenesis proceeds, Shh
produced by the forebrain induces expression of Shh in the
tectoderm of the frontonasal and maxillary processes of the
developing face. Shh produced by these latter two structures
patterns neural-crest-derived facial primordia to produce specific
cartilaginous structures and bones of the craniofacial midline
(Helms et al., 2005; Hu and Helms, 1999; Hu and Marcucio, 2009;
Marcucio et al., 2005). Most human HPE genes are either
components of the SHH pathway, or directly or indirectly affect
SHH expression in either the PCM or the developing ventral
forebrain (Dubourg et al., 2007; Geng and Oliver, 2009; Helms et
al., 2005; Hu and Helms, 1999; Hu and Marcucio, 2009; Marcucio
et al., 2005).

Shh activates a conserved signal transduction pathway in which
binding of Shh to its membrane receptor Ptc (Ptch1) results in
signaling by a second membrane protein, Smo (Smoothened) and
induction of pathway target genes via the Gli family of
transcription factors (Jiang and Hui, 2008; Varjosalo and Taipale,
2008). The levels and activity of Shh are tightly regulated, in part
via a feedback network that acts at the level of ligand binding (Kang
et al., 2007). Ptc1 and another cell surface protein, Hip1, are
induced by Shh signaling, and both bind and sequesters Shh,
consequently limiting the magnitude and range of signaling (Jeong
and McMahon, 2005). Cdo (also called Cdon) and Boc are closely
orthologs of Hedgehog (Hh) mutants, revealing that these
modifiers of Hh signaling in the fruit fly (Camp et al., 2010; Zheng et al., 2010).

RESULTS

Boc–/– mice are viable and do not display HPE phenotypes

To identify developmental functions of Boc in the mouse, gene
targeting in embryonic stem (ES) cells was used to disrupt the
Boc gene. The targeting strategy produced two different alleles
(Fig. 1A-C). BocAP-1 has a deletion of the first coding exon (which
encodes the start codon and signal sequence) and an insertion of
a cassette containing a selectable marker and an IRES human
placental alkaline phosphatase reporter gene in the second coding
exon; BocAP-2 contains only the insertion. No Boc protein was
observed in western blot analyses of embryos from mice
homozygous for either allele, suggesting they are likely to be null
(Fig. 1D). Embryos carrying either allele displayed alkaline
phosphatase activity in a pattern that mirrored the one seen with
RNA in situ hybridization with probes against Boc (shown in Fig.
1E for BocAP-1 at E11.5) (Mulieri et al., 2002). BocAP-1/– and
BocAP-2/– mice had similar phenotypes and were used interchangeably in this study. The mutant alleles are referred to
collectively as Boc– for convenience.

Boc–/– mice were interbred, and offspring were genotyped at
postpartum day 10 (P10). All three genotypes (Boc+/+, Boc–/– and
Boc–/–) were detected at the expected mendelian ratios in both a
mixed 129×B6 background and a congenic 129 background.
Boc–/– animals were viable and did not display HPE-related
phenotypes.
HPE phenotypes in Cdo<sup>−/−</sup>;Boc<sup>+/−</sup> and Cdo<sup>−/−</sup>;Boc<sup>−/−</sup> mice on a 129 background

Because Boc and Cdo have similar (although not identical) expression patterns and both bind Shh to promote signaling, we reasoned that some aspects of their function might be redundant or compensatory, similarly to what is observed with the Drosophila orthologs Ihog and Boi (Camp et al., 2010; Zheng et al., 2010). To evaluate this possibility, Boc and Cdo mutant mice on a 129 background were intercrossed to construct double mutants. The 129 background was chosen because Cdo<sup>−/−</sup> mice on this background are viable and display only microforms of HPE (Zhang et al., 2006). Unless otherwise noted in the figures, all results are from animals of the 129 background.

Cdo<sup>+/−;Boc<sup>+/−</sup> mice are fertile and appeared normal. These animals were intercrossed and offspring genotyped at P10. Mice of all possible genotypes were found at approximately the predicted mendelian ratio except Cdo<sup>−/−;Boc<sup>−/−</sup> mice, which were never recovered at P10 (supplementary material Table S1). Cdo<sup>−/−;Boc<sup>−/−</sup> embryos were obtained at various developmental stages, including E11.5, E13.5 and E15.5, suggesting that these mice died perinatally and/or were selectively culled by their mothers. Cdo<sup>−/−;Boc<sup>−/−</sup> mice were present at slightly less than the expected frequency (supplementary material Table S1). Some embryos of this genotype (but not embryos of the Cdo<sup>+/−;Boc<sup>−/−</sup> genotype) had craniofacial patterning defects that were nearly as severe as Cdo<sup>−/−;Boc<sup>−/−</sup> embryos (vide infra), and it is therefore likely that a small fraction of Cdo<sup>−/−;Boc<sup>−/−</sup> mice also die perinatally.

To address whether removal of Boc enhanced phenotypes of mice lacking Cdo, craniofacial and forebrain development was analyzed in mice of various genotypes. Whole Cdo<sup>−/−;Boc<sup>−/−</sup> embryos at E11.5, E13.5 and E15.5 had strong midfacial HPE defects with high penetrance. At E11.5, Cdo<sup>−/−;Boc<sup>−/−</sup> embryos displayed hypoplasia of the facial midline with somewhat variable expressivity (Fig. 2A);
the most severe cases included fusion of the nasal processes (Fig. 2Ae), a phenotype similar to that of Cdo−/−;Shh+/− embryos (Tenzen et al., 2006). At E13.5 and E15.5, double-mutant animals had a single pointed nostril, fused upper lip and hypotelorism (Fig. 2B). Cdo−/−;Boc+/− embryos showed similar defects but with lower penetrance (Table 1). At these stages, Boc−/− embryos always resembled the wild-type, and Cdo−/− embryos displayed related but much milder phenotypes at low frequency (Zhang et al., 2006) (our unpublished results). E18.5 cranial bone and cartilage preparations revealed that, although Boc−/− and Cdo+/−;Boc−/− embryos had normal cranial and palatal bone patterning, Cdo−/−, Cdo−/−;Boc+/− and Cdo−/−;Boc−/− embryos displayed progressively more severe midline defects in these bones, with increasing penetrance (Fig. 3, Table 1) (Cole and Krauss, 2003). Defects included fused premaxillary bones; dysmorphic maxillary bones with absent or diminished maxillary shelves; reduced size of the basisphenoid bone; and foramen in the basisphenoid bone. No defects of the mandible were seen in mice of any genotype studied.

Somewhat surprisingly, double-mutant embryos had relatively mild ventral forebrain midline defects, which were similar to lobar HPE. Frontal sections of E13.5 forebrains revealed that, although Cdo−/− and Boc−/− mice had normally patterned forebrains, Cdo−/−;Boc−/− embryos displayed continuity across the ventral midline, and medial and lateral ganglionic eminences that were either reduced in size or, occasionally, fused (Fig. 2C). Accordingly, expression of the direct Shh pathway target genes Ptch1 and Nkx2.1 was reduced in the ventral forebrains of E13.5 Cdo−/−;Boc−/− embryos (Fig. 2D). The reduction in Ptch1 and Nkx2.1 expression was not simply a consequence of loss of ventral forebrain structure, because the embryos shown here (Fig. 2Dd,h) did not have the most severe phenotype observed and had defined medial ganglionic eminences.

Expression of Shh and Fgf8 correlates with the strain-dependent severity of forebrain and facial HPE phenotypes in mice lacking Cdo and/or Boc

Fgf8 expression in the commissural plate is initiated independently of Shh at E8.5, but by E9.0 its maintenance requires Shh produced by the ventral forebrain (Ohkubo et al., 2002). Shh−/− embryos have the most severe form of HPE, and Fgf8 hypomorphs are also
holoprosencephalic (Chiang et al., 1996; Storm et al., 2006). Furthermore, Fgf signaling is downstream of Shh signaling in midline patterning, and at least some of the midline defects in Shh-deficient mice are likely caused by abnormal Fgf signaling (Gutin et al., 2006; Okada et al., 2008). Shh expression is delayed and reduced in the ventral forebrain of E8.75-E9.5 Cdo–/– embryos on the B6 background, and Fgf8 expression is strongly diminished in Fgf8-deficient mice are likely caused by abnormal Fgf signaling (Chiang et al., 1996; Storm et al., 2006; Gutin et al., 2006; Okada et al., 2008). Shh expression is delayed and reduced in the ventral forebrain of E8.75-E9.5 Cdo–/– embryos on the B6 background, and Fgf8 expression is strongly diminished in Fgf8-deficient single- and double-mutant embryos on the 129 background. The pattern of Shh and Fgf8 expression in the ventral forebrain of 129.Cdo–/– and 129.Boc–/– was similar to that of control embryos (Fig. 4Ba-c,Ca-c). The level of Shh expression in 129.Cdo–/–;Boc–/– embryos was clearly lower than in controls in the ventral telencephalon but, unlike B6.Cdo–/– embryos (Zhang et al., 2006), expression extended anterior to the developing eye (Fig. 4Bd). Also unlike B6.Cdo–/– embryos (Zhang et al., 2006), 129.Cdo–/–;Boc–/– embryos expressed Fgf8 in the commissural plate at this stage (Fig. 4Cd). These results suggest that a threshold level of Shh expression in the anterior ventral forebrain is required to maintain Fgf8 expression in the commissural plate, and strain 129 mice maintain such a level even in the absence of both Cdo and Boc, whereas B6 mice fail to maintain this threshold amount in the absence of Cdo alone. As Fgf8 hypomorphs are themselves holoprosencephalic and Fgf proteins act downstream of Shh in this system (Gutin et al., 2006; Okada et al., 2008; Storm et al., 2006), differential Fgf8 expression might be sufficient to explain the varying severity of forebrain phenotypes seen in B6.Cdo–/– embryos (incomplete cleavage) and 129.Cdo–/–;Boc–/– embryos (cleavage occurs; lobar HPE-type ventral midline defects).

The development of the forebrain and face are intimately connected, because the forebrain secretes signaling molecules that help pattern the face and also provides a supporting structure on

### Table 1. Frequency of craniofacial defects in 129.Cdo–/–;Boc–/– and 129.Cdo–/–;Boc+/– mice

| Defect                                      | 129.Cdo–/–;Boc–/– | 129.Cdo–/–;Boc+/– |
|---------------------------------------------|-------------------|-------------------|
| Fused upper lip                             | 12/38 (31.6%)     | 38/40 (95%)       |
| Single nostril                               | 4/38 (10.5%)      | 16/40 (40%)       |
| Primary palate malformed or missing         | 2/4 (50%)         | 10/11 (90.9%)     |
| Mispatterned secondary palate               | 1/4 (25%)         | 10/11 (90.9%)     |
| Open secondary palate                       | 1/4 (25%)         | 1/1 (9.1%)        |
| Fused premaxillary bones                    | 3/5 (60%)         | 2/2 (100%)        |
| Underdeveloped maxillary shelves            | 3/5 (60%)         | 2/2 (100%)        |
| Basisphenoid bone defect (notch or hole)    | 5/5 (100%)        | 2/2 (100%)        |
| Dysmorphic maxillary bones                  | 1/5 (20%)         | 2/2 (100%)        |
| Open palatal shelf                          | 2/5 (40%)         | 1/2 (50%)         |

To gain insight into the differential strain sensitivity of Cdo–/– mice, as well as into the genetic interaction between Cdo and Boc, Shh and Fgf8 expression were analyzed at E9.5 in Cdo and Boc single- and double-mutant embryos on the 129 background. The pattern of Shh and Fgf8 expression in the ventral forebrain of 129.Cdo–/– and 129.Boc–/– was similar to that of control embryos (Fig. 4Ba-c,Ca-c). The level of Shh expression in 129.Cdo–/–;Boc–/– embryos was clearly lower than in controls in the ventral telencephalon but, unlike B6.Cdo–/– embryos (Zhang et al., 2006), expression extended anterior to the developing eye (Fig. 4Bd). Also unlike B6.Cdo–/– embryos (Zhang et al., 2006), 129.Cdo–/–;Boc–/– embryos expressed Fgf8 in the commissural plate at this stage (Fig. 4Cd). These results suggest that a threshold level of Shh expression in the anterior ventral forebrain is required to maintain Fgf8 expression in the commissural plate, and strain 129 mice maintain such a level even in the absence of both Cdo and Boc, whereas B6 mice fail to maintain this threshold amount in the absence of Cdo alone. As Fgf8 hypomorphs are themselves holoprosencephalic and Fgf proteins act downstream of Shh in this system (Gutin et al., 2006; Okada et al., 2008; Storm et al., 2006), differential Fgf8 expression might be sufficient to explain the varying severity of forebrain phenotypes seen in B6.Cdo–/– embryos (incomplete cleavage) and 129.Cdo–/–;Boc–/– embryos (cleavage occurs; lobar HPE-type ventral midline defects).

The development of the forebrain and face are intimately connected, because the forebrain secretes signaling molecules that help pattern the face and also provides a supporting structure on
which the face develops (Marcucio et al., 2005). 129.\textit{Cdo}^{+/}\textit{Boc}^{−/−} embryos show craniofacial defects that are more severe than those in B6.\textit{Cdo}^{−/−} embryos, but the 129.\textit{Cdo}^{−/−};\textit{Boc}^{−/−} embryos show a milder forebrain midline defect than B6.\textit{Cdo}^{−/−} embryos, which is an unusual phenotype. As shown above, 129.\textit{Cdo}^{−/−};\textit{Boc}^{−/−} embryos expressed \textit{Shh} in a more rostral forebrain domain than did B6.\textit{Cdo}^{−/−} embryos (Zhang et al., 2006), and this was accompanied by \textit{Fgf8} expression in the former but not latter animals (Zhang et al., 2006) (Fig. 4). Although this might explain the milder forebrain phenotype in 129.\textit{Cdo}^{−/−};\textit{Boc}^{−/−} embryos, it is less consistent with the more severe facial defects seen in these mice, relative to the B6.\textit{Cdo}^{−/−} embryos. We therefore analyzed expression of \textit{Shh} and \textit{Shh} target genes, including \textit{Fgf8}, in the ventral forebrain at E10.5, a stage at which the forebrain is involved in patterning of the midface.

At E10.5, \textit{Shh} expression in the ventral telencephalon was almost completely absent in \textit{Cdo}^{+/−};\textit{Boc}^{−/−} embryos (Fig. 5A). Similarly, expression of \textit{Fgf8} and \textit{Nkx2.1} at E10.5 was also strongly reduced in the rostral midline of these mice (Fig. 5D,E). This progressive temporal reduction in \textit{Shh} and \textit{Fgf8} expression in the double mutants correlates with the roles of these factors in facial patterning, and \textit{Cdo} and \textit{Boc} appear to cooperate to regulate their expression in this process. To apply a more quantitative approach to these results, RNA was extracted from dissected heads of E10.5 control and \textit{Cdo}^{+/−};\textit{Boc}^{−/−} embryos, and quantitative RT-PCR (qPCR) was performed. mRNA levels of \textit{Shh}, \textit{Fgf8} and \textit{Nkx2.1} were each significantly reduced in the \textit{Cdo}^{−/−};\textit{Boc}^{−/−} embryonic heads (Fig. 6); in similar analyses, \textit{Ptch1} and \textit{Gli1} mRNA levels were not significantly different between control and double-mutant embryos (data not shown). It is likely that the qPCR results underestimate the reduction in expression of these genes in the most affected region of \textit{Cdo}^{−/−};\textit{Boc}^{−/−} embryos (the rostroventral midline) because more caudal structures where changes in expression are not obvious were by necessity included in the dissected embryos.

### Palatogenesis defects in mice lacking \textit{Cdo} and \textit{Boc}

Palatogenesis is regulated by \textit{Shh} signaling and defects in secondary palate formation sometimes present as part of the HPE spectrum (Gritli-Linde, 2008; Muenke and Beachy, 2001). Analysis of E17.5 embryos showed that although \textit{Cdo}^{−/−}, \textit{Boc}^{+/−} and \textit{Cdo}^{+/−};\textit{Boc}^{−/−} embryos had normal secondary palates, \textit{Cdo}^{−/−};\textit{Boc}^{+/−} and \textit{Cdo}^{−/−};\textit{Boc}^{−/−} embryos had malformations of the secondary palate, including in some cases a cleft (Fig. 7A and Table 1). Furthermore, the primary palates of one \textit{Cdo}^{−/−};\textit{Boc}^{+/−} and both \textit{Cdo}^{−/−};\textit{Boc}^{−/−} embryos were misshapen (Fig. 7A).

The secondary palate arises from the medial sides of the maxillary processes flanking the embryonic oral cavity. Development of the secondary palate involves vertical growth of palatal shelf primordia into the oral cavity, along the sides of the developing tongue. At E14.0, the bilateral palatal shelves elevate to a horizontal position above the tongue and grow toward each other.

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**Fig. 4. Expression of \textit{Shh} and \textit{Fgf8} correlate with the strain-dependent severity of forebrain phenotypes in \textit{Cdo} and \textit{Boc} mutants.** (A-C) Whole-mount RNA in situ hybridization analyses. (A) \textit{Fgf8} expression in E8.75 \textit{Cdo}^{+/+} and \textit{Cdo}^{−/−} embryos on a B6 background. Note that \textit{Fgf8} expression is induced properly in the \textit{Cdo}^{−/−} embryo in both the anterior neural ridge (arrow) and isthmus (arrowhead). Scale bar: 0.5 mm. (B) Lateral view of \textit{Shh} expression in E9.5 embryos of the indicated genotype on a 129 background. Note that \textit{Shh} is expressed in the ventral telencephalon of the \textit{Cdo}^{−/−};\textit{Boc}^{−/−} embryo (d, arrow) but signal strength is reduced compared with the control embryo (a). Scale bar: 0.2 mm. (C) Lateral view of \textit{Fgf8} expression in E9.5 embryos of the indicated genotype on a 129 background. Note that \textit{Fgf8} is expressed in the commissural plate of the \textit{Cdo}^{−/−};\textit{Boc}^{−/−} embryo (d, arrow). Scale bar: 0.2 mm.
At E14.5, the opposing palatal shelves then adhere along their medial edge epithelia to create a transient medial epithelial seam (MES), which starts to disappear at E15.5. At E16.5, the MES disappears completely to form the intact secondary palate, which separates the nasal cavity from the oral cavity, a condition that is required for simultaneous breathing and feeding. Disturbance of any of these processes can result in palate defects.

*Cdo*;*Boc* double-mutant embryos were analyzed at different stages of palatogenesis. Because a range of phenotypes was observed in these embryos, three different double mutants are presented at each stage. At E13.5, control embryos showed two vertically oriented palatal shelves (Fig. 7Ba). In double-mutant embryos, the shelves appeared to have initiated properly, but vertical growth was abnormal; instead of growing downward alongside the tongue, the palatal shelves usually grew medially towards each other (Fig. 7Bc,d). At E14.5, the control palatal shelves had elevated into a horizontal position above the tongue and came into contact to form the MES (Fig. 7Be). In *Cdo*;*Boc* double-mutant embryos, in most cases, the palatal shelves either resembled those at E13.5 (Fig. 7Bf) or, if some degree of growth had occurred by this stage, failed to elevate and form a MES (Fig. 7Bg). In a milder case, as shown in Fig. 6Bf, the palatal shelves showed relatively normal growth at E14.5; however, only one of the shelves had elevated normally. At this stage, it was also evident that the precartilage primordium of the nasal septum is hypoplastic in double mutants (Fig. 7Bf,g). At E15.5, the control palatal shelves had already fused, and the MES had started to disappear (Fig. 7Bi). The double-mutant palatal shelves had also begun to fuse with each other but, rather than fusing at the tips of the elevated palatal shelves as normally occurs, the mutant shelves appeared to fuse medially.
Boc modifies the HPE spectrum

In a milder case, where the palatal shelves had elevated appropriately, the tips of the shelves failed to fuse because they did not align properly for fusion to occur (Fig. 7Bj).

Palatogenesis is dependent on SHH signaling, and cleft palate has been observed in human HPE associated with SHH mutations (Gritli-Linde, 2008; Muenke and Beachy, 2001). Analysis of mice in which Shh is conditionally removed from the palatal shelf epithelium, or Smo is conditionally removed from the palatal mesenchyme, demonstrated that Shh signals directly from the epithelium to the mesenchyme to promote outgrowth of the palatal shelves; such mice have cleft palate (Lan and Jiang, 2009; Rice et al., 2004). Consistent with the notion that Shh signaling is diminished during palatogenesis in Cdo–/–;Boc–/– embryos, expression of Ptch1 in the palatal shelves of E13.5 double-mutant mice was reduced compared with levels in control animals (Fig. 7Ca vs 7Cb and 7Cc). The reduction in Ptch1 expression was not

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**Fig. 7. Cdo–/–;Boc–/– mice display defects in palatogenesis.** (A) Ventral views of E17.5 palates of embryos of the indicated genotype on a 129 background. Lower jaws have been removed for visualization of palates. PP, primary palate; SP, secondary palate. Black arrows indicate an open palate in Cdo–/–;Boc–/– and Cdo–/–;Boc–/– embryos (f,h). The red arrow indicates abnormal rugae in the secondary palate of a Cdo–/–;Boc–/– embryo (g). The red arrowheads indicate misshapen primary palates in Cdo–/–;Boc–/– and Cdo–/–;Boc–/– embryos (e-h). Scale bar: 1 mm. (B) H&E staining of palate sections from E13.5, E14.5 and E15.5 embryos of a control embryo and three different Cdo–/–;Boc–/– embryos; note that the double-mutant embryos display various palatogenesis defects. See text for detailed description. ps, palatal shelves; T, tongue; asterisk, molar; arrow in e indicates the medial epithelial seam (MES). Scale bars: 0.5 mm. (C) RNA in situ hybridization of Ptch1 expression in E13.5 palate sections. Scale bar: 0.3 mm.
simply a consequence of abnormal development, because a palate from a Cdo-/-;Boc-/- embryo that was relatively normally patterned (Fig. 7Cb) also displayed diminished Ptc1 signal.

**Cdo-/-;Boc-/- mice do not display significant defects in limb and axial skeleton development**

Cdo and Boc are both expressed in the anterior two-thirds of the limb bud mesenchyme, with expression excluded from the posterior region, including the zone of polarizing activity (ZPA), because of Shh-mediated repression of expression of these genes (Mulieri et al., 2000; Mulieri et al., 2002; Tenzen et al., 2006). Although Shh-/- mice have severe defects of the limbs and digits (McMahon et al., 2003), Cdo-/- mice display normal limb and digit patterning, even on the sensitized B6 background (Chiang et al., 1996; Zhang et al., 2006). We speculated that the reason that Cdo-/- animals showed normal limb and digit formation was that Boc had a redundant or compensatory role in this process. However, analysis of Cdo-/-;Boc-/- embryos revealed that they also had normal limbs and digits (Fig. 8A).

Shh is also required for formation of the axial skeleton, including vertebrae and ribs (McMahon et al., 2003). Skeletal preparations of E18.5 embryos revealed that cervical vertebrae were largely normal in Cdo-/-;Boc-/- embryos, although they lacked ossification of intervertebral discs (Fig. 8B). Rib development was also normal in double-mutant embryos (data not shown). Therefore, similarly to limb and digit patterning, development of the axial skeleton occurs without major disruption in the absence of Cdo and Boc.

That 129.Cdo-/-;Boc-/- mice displayed neither the most severe HPE phenotypes seen in Shh-null mice, nor major defects in limb and axial skeleton development, is distinct from the situation in *Drosophila*, where Ihog and Boi are essential but redundant for Hh pathway activity (Camp et al., 2010; Zheng et al., 2010). One possible mitigating factor is the 129 genetic background, which is largely resistant to HPE phenotypes induced by genetic removal of *Cdo* (Zhang et al., 2006). To assess this possibility, Boc-/- mice were back-crossed onto the B6 background. Boc-/- animals on this background were viable and had a normal exterior appearance at E14.5, lacking HPE and digit phenotypes (data not shown).

B6.Cdo-/-;Boc-/- mice were then generated and intercrossed to produce double-mutant mice on this sensitive background. B6.Cdo-/-;Boc-/- mice had a range of HPE phenotypes, including a proboscis-like nose and hypotelorism; they also had cleft lip, a trait not seen in single mutants on either background or double mutants on the 129 background (supplementary material Fig. S2). However, even B6 double mutants did not have cyclopia or defects in digit patterning (supplementary material Fig. S2). Therefore, although the genetic background can modify the phenotype of *Cdo;Boc* double mutants, it is not sufficient to explain the incomplete loss of Shh activity in the absence of all Cdo family function.

**DISCUSSION**

We report here analysis of mice lacking the related Shh pathway regulators Cdo and Boc; this is the first report of a vertebrate model lacking function of all Cdo/Ihog family members. The phenotypes of mice deficient in either gene singly or the two together have implications for the common birth defect HPE and provide insight into evolutionary differences in the Hh pathway between insects and mammals.

**Boc is a silent HPE modifier gene in mice**

Human HPE is associated with heterozygous mutations in genes that encode components of the SHH pathway or regulators of SHH expression in the rostroventral midline. However, the observations that many carriers of such mutations are without clinical phenotype and that heterozygous mutations in these genes do not generally produce HPE in model organisms (such as mice), has led to the notion that HPE arises as a consequence of several genetic and/or environmental insults (Krauss, 2007; Ming and Muenke, 2002). We have previously reported that mice lacking Cdo display HPE with strain-dependent severity: Cdo-/- mice on a 129 background have semilobar HPE with low penetrance, whereas Cdo-/- mice on a B6 background have semilobar HPE with high penetrance (Zhang et al., 2006). Therefore, strain-specific silent modifier genes determine the penetrance and expressivity of HPE in Cdo mutant mice. Furthermore, it is likely that modifier alleles of this type have a similar role in the human HPE spectrum (Krauss, 2007; Roessler et al., 2009c). The modifier loci involved in the differential sensitivity of 129 versus B6 mice to HPE are not known, and such alleles have
not yet been identified in human HPE. Boc<sup>−/−</sup> mice do not display HPE phenotypes, yet genetic removal of Boc strongly enhances such phenotypes in Cdo<sup>−/−</sup> mice, in a Boc gene dosage-sensitive manner. Therefore, Boc functions as a silent HPE modifier gene in mice and might have a similar role in humans, because numerous human BOC single-nucleotide polymorphisms (SNPs) are reported in the NCBI database, including missense and frame-shift changes (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=91653).

Although Boc might contribute to the differential sensitivity of 129 and B6 mice to loss of Cdo (and other HPE genes) (Geng et al., 2008; Petryk et al., 2004), it is very unlikely to be the major modifier gene in this system because B6.Cdo<sup>−/−</sup> mice (which express Boc) have a more severe forebrain phenotype than 129.Cdo<sup>−/−</sup>;Boc<sup>−/−</sup> mice. Therefore, additional modifiers remain to be identified.

Cdo is expressed at low levels in the PCMs but not in the developing ventral forebrain (Mulieri et al., 2000; Mulieri et al., 2002; Tenzen et al., 2006; Zhang et al., 2006). The PCMs are required for induction of the ventral forebrain and subsequent patterning of the forebrain and midfacial midline. The PCMs serve as a source of Shh in this process, but Shh is also required for normal development of the PCM itself (Aoto et al., 2009; Muenke and Beachy, 2001; Rubenstein and Beachy, 1998). Although we did not observe obvious defects in the PCM of B6.Cdo<sup>−/−</sup> mice (Zhang et al., 2006), the lack of Cdo expression in the developing ventral forebrain (the affected structure) argues that loss of Cdo function in the PCM is the likely cause of HPE in B6.Cdo<sup>−/−</sup> mice (Zhang et al., 2006). Mice that lack Boc do not display HPE, despite the fact that, similarly to Cdo, Boc binds Shh and promotes Shh signaling (Okada et al., 2006; Tenzen et al., 2006; Zhang et al., 2006). Unlike Cdo, Boc is expressed in the ventral forebrain (Mulieri et al., 2002; Okada et al., 2006) and transiently at low levels in the PCM (unpublished results). It is possible that the genetic interaction between Cdo and Boc in the production of HPE in mice arises from a combination of loss of Cdo function in the PCM and Boc function in the PCM and/or developing ventral forebrain. Cdo and Boc are also both expressed in the developing facial mesenchyme (Mulieri et al., 2000; Mulieri et al., 2002), and this might also contribute to HPE phenotypes. Conditional removal of these factors from specific structures will be required to definitively address this point.

The reduction of expression of Shh and pathway target genes in Cdo<sup>−/−</sup>;Boc<sup>−/−</sup> mice worsens between E9.5 and E10.5, revealing that Cdo and Boc are required together in these regions for robust temporal maintenance of the autoinduction system whereby Shh regulates its own expression in successive structures of the rostralventral midline. Apparently, one copy of Cdo is sufficient for this process to occur because we have not observed any HPE phenotypes in Cdo<sup>−/−</sup>;Boc<sup>−/−</sup> mice, whereas a fraction of Cdo<sup>−/−</sup>;Boc<sup>−/−</sup> mice do display such phenotypes. It should be noted that Cdo and Boc are coexpressed at high levels in the developing dorsal forebrain (Mulieri et al., 2000; Mulieri et al., 2002), but Shh is not required to pattern the dorsal CNS at these stages of development (Herbert and Fishell, 2008; Wilson and Houart, 2004), and the phenotypes seen in the double-mutant mice are consistent with the view that HPE arises from defects in ventral forebrain patterning (Muenke and Beachy, 2001).

129.Shh<sup>+/−</sup> and 129.Boc<sup>−/−</sup> mice do not have HPE, yet removal of one copy of Shh or dosage-dependent loss of Boc each synergize with loss of Cdo on the 129 background to produce HPE spectrum phenotypes (Tenzen et al., 2006) (and this study). These results, plus the fact that B6.Cdo<sup>−/−</sup> mice have semi-lobar HPE with high penetrance, suggest that 129.Cdo<sup>−/−</sup> mice have a largely subthreshold defect of Shh signaling in the rostroventral midline that renders these mice sensitive to additional, often silent, insults. Therefore, we propose that 129.Cdo<sup>−/−</sup> mice serve as a model system for testing the multifactorial hypothesis of human HPE and for identification of additional ‘second hits’. Consistent with this possibility, preliminary results indicate that 129.Cdo<sup>−/−</sup> embryos are sensitized to environmental exposures implicated in HPE (unpublished results).

**Evolutionary divergence in the requirement of the Hh pathway for Cdo/Ihog family members**

Although Hh ligands activate a largely evolutionarily conserved signaling pathway, there are some important differences between Hh signaling mechanisms in *Drosophila* and mice (for reviews, see Ingham and McMahon, 2009; Jiang and Hui, 2008). It was recently reported that combined genetic removal of *Ihog* and *Boi*, the *Drosophila* Cdo and Boc orthologs, results in complete loss of response to Hh ligand, including pathway target gene expression and segmental patterning (Camp et al., 2010; Zheng et al., 2010). Flies carrying mutations in either gene singly are viable, so *Ihog* and *Boi* apparently provide a redundant or compensatory function in the Hh pathway. Furthermore, a complex of *Ihog* or *Boi* and Patched is required for Hh receptor function (Camp et al., 2010; Zheng et al., 2010). By contrast, Cdo and Boc have specific, selective roles in Shh signaling in mammals. For example, they are obviously involved in development of the rostroventral midline, but are dispensable for digit patterning and formation of the axial skeleton, both Shh-dependent processes (McMahon et al., 2003).

The results reported here therefore reveal an evolutionary divergence of the Hh pathway in its reliance on Cdo/Ihog family members. One likely explanation for this is the existence in vertebrates, but apparently not *Drosophila*, of Gas1, a Shh-binding protein that is structurally unrelated to Cdo and Boc, but which appears to function similarly to them and has an overlapping expression pattern (Allen et al., 2007; Kang et al., 2007; Martinelli and Fan, 2007). Consistent with this possibility is that, unlike Cdo<sup>−/−</sup>;Boc<sup>−/−</sup> mice, mice lacking Gas1 display a defect in digit patterning, although it is not as severe as that seen in *Shh*<sup>−/−</sup> mice (Allen et al., 2007; Kang et al., 2007; Martinelli and Fan, 2007). The existence of additional putative co-receptors might relate to a need in mammals for additional ability to modulate Shh signaling during developmental events that play out over a longer period of time and are potentially more complex than in insects. This possibility might also be relevant to the somewhat variable facial HPE phenotypes seen in 129.Cdo<sup>−/−</sup>;Boc<sup>−/−</sup> mice and in the differences between *Cdo*Boc double mutants on 129 and B6 backgrounds: reliance on Gas1 as the only (known) putative co-receptor might result in somewhat variable HPE phenotypes in *Cdo*Boc double-mutant mice, dependant on both stochastic events and genetic background. Interestingly, *Cdo*Gas1 double-mutant mice have a much more severe HPE phenotype than either single mutant (Allen et al., 2007).

A second, non-mutually exclusive explanation for the evolutionary divergence in the requirement for Cdo/Ihog proteins...
is that, even though all family members bind Hh ligands and promote pathway activity, Cdo and Boc might function somewhat differently than Ihog and Boi. Hh and Shh bind non-orthologous FnIII repeats in Ihog and Cdo, respectively (McLellan et al., 2006; McLellan et al., 2008; Tenzen et al., 2006; Yao et al., 2006). Additionally, Ihog and Boi cooperate with Drosophila Patched to produce a high-affinity Hh binding receptor complex, but there are varying reports as to whether Cdo and Ptch1 cooperate or compete in ligand binding (Martinelli and Fan, 2007; McLellan et al., 2008). Therefore, reliance on Cdo/Ihog family members is not fully evolutionarily conserved, with mammalian development involving additional factors and/or distinct mechanisms at this level of regulation of the Hh pathway.

METHODS

Generation of Boc mutant mice, Cdo;Boc double-mutant mice and genotyping
A 500 bp mouse Boc cDNA fragment was used to probe a mouse PAC library (RPCI21, generously provided by Jonathan Licht, Northwestern University Feinberg School of Medicine, Chicago, IL). The targeting vector was designed to delete the first coding exon and insert a 4.3kb IRES-HAP-loxP/PGK-neo/loxP selectable marker and reporter gene cassette (generously provided by Thomas Lufkin, Genome Institute of Singapore, Singapore) into the second coding exon. The targeting vector generated a 540 bp deletion (encompassing the entire first coding exon) by linking the 5’ flanking 6 kb EcoRI-Scal fragments and 3’ flanking 2.5 kb KpnI-Xhol fragments. The targeting vector was linearized with NotI and electroporated into TCT1 ES cells (derived from 129S6/SvEvTac mice). G418-resistant ES cell clones were screened for homologous recombination events by Southern blot analysis with 5’ and 3’ external probes. ES cell clones of the two different mutant Boc alleles generated by the target vector (see Results) were used to generate chimeric males by injection into C57BL/6 blastocysts, and germline transmission from two chimeric males was confirmed by coat color and Southern blot analysis. These Boc^{+/-} mice on a mixed background were placed on a B6 background by back-crossing with wild-type B6 mice for six generations. Each Boc allele was placed directly onto the 129 background by crossing chimeric males with strain 129 females and screening progeny by Southern blotting. The phenotypes observed from intercrosses of strain 129 heterozygotes of each allele were similar, so the two alleles were used interchangeably in this report.

Cdo^{+/+} (also known as Cdon^{+/-}) mice on a 129 background, described previously (Cole and Krauss, 2003; Zhang et al., 2006), were crossed with Boc^{+/-} mice of the same background to generate double heterozygotes; these latter animals were intercrossed and offspring analyzed. All mice were genotyped by PCR analysis of genomic DNA isolated from tail biopsies performed at P9 or P10. Cdo genotyping was as described (Zhang et al., 2006). Boc genotyping was with a three-primer PCR: 5'-AGAACGAGATCAGCAGCCTGTTCC-3' (forward primer, mutant alleles), 5'-GGAGCCACATGGAAGCTGACTTGCG-3’ (forward primer, wild-type allele), and 5’-GCTATGACAAACACGCTAGGGTACGGA-3’ (reverse primer, wild-type and mutant alleles).

Skeletal analysis, E18.5 embryos were dissected and fixed in 95% ethanol for 1-2 hours before removing skin, adipose tissue and internal organs, except the brain. The embryos were then further

Southern and western blot analyses
Genomic DNA was isolated, digested and Southern blotted from ES cell clones, tail tips and visceral yolk sacs by standard procedures. Protein extracts were isolated from the head region of E13.5 embryos for western blot analysis as previously described (Cole and Krauss, 2003), with an anti-Boc antibody (R&D Systems).

Histology, alkaline phosphatase staining, in situ hybridization, qRT-PCR and skeletal analysis
Embryos for histology were dissected into 4% paraformaldehyde in phosphate-buffered saline (PBS) and fixed overnight. After dehydration, embryos were embedded in paraffin and sectioned at 8 μm. Hematoxylin and eosin staining was performed by standard protocols as described (Cole and Krauss, 2003). Briefly, after dewaxing in xylene and rehydrating in graded ethanol, slides were stained with Harris’ Hematoxylin (Fisher SH26) for 1 minute and washed in running tap water for 20 minutes. This was followed by differentiation in acid alcohol (1% HCl in 70% ethanol) and blueing in Scott’s tap water substitute (0.2% Na2CO3, 1% MgSO4). The slides were then counterstained in alcoholic eosin (Fisher SE22) supplemented with glacial acetic acid for 4 minutes. Finally, slides were dehydrated through graded ethanol and xylene and mounted in Cytoseal-60 (Richard-Allan Scientific).

For qRT-PCR analysis of Shh, Fgf8 and Nkx2.1 expression was performed on E10.5 control (Cdo^{+/+};Boc^{+/-}, Cdo^{+/-};Boc^{+/+} or Cdo^{+/+};Boc^{+/-} and Cdo^{+/-};Boc^{+/+}) embryos. Embryonic heads were dissected out and transferred into 0.4 ml Trizol. cDNA was synthesized from total RNA using SuperScript III First Strand Synthesis System (Invitrogen). qPCR was performed using PerfeCta SYBR Green FastMix for iQ (Quanta Biosciences) with a Bio-Rad iCycler iQ5. Data were normalized to Gapdh as fold change over control. qPCR primers for Gapdh, Shh and Fgf8 were from the Harvard PrimerBank (PrimerBank Ids: 6679937a1, 21617861a and 22094903a1, respectively) (Spandidos et al., 2010). qPCR primers for Nkx2.1 were from a published paper (Zhang et al., 2007).

For skeletal analysis, E18.5 embryos were dissected and fixed in 4% paraformaldehyde and fixed in 0.2% glutaraldehyde in PBS for 50 minutes at 4°C followed by fixation in 0.2% glutaraldehyde, 0.02% NP-40, and 0.01% sodium deoxycholate in PBS for 60 minutes at 4°C. Embryos were rinsed in PBS and then incubated in PBS at 72°C for 30 minutes to inactivate endogenous alkaline phosphatases. The embryos were then washed in NTM buffer (100 mM NaCl and 50 mM MgCl2 in 100 mM Tris-HCl, pH 9.5) for 10 minutes and stained with BM purple alkaline phosphatase substrate (Roche).

For whole-mount in situ hybridization, embryos were dissected and fixed in 4% paraformaldehyde in PBS at 4°C overnight and processed for RNA in situ hybridization as previously described (Mulieri et al., 2000) with probes against Shh, Fgf8, Gli1 and Ptch1. For thin-section in situ hybridization, embryos were embedded in paraffin after fixation and dehydration and sectioned at 8 μm. The sections were then dewaxed, rehydrated and processed for RNA in situ hybridization as previously described (Mulieri et al., 2000) with probes against Ptch1 and Nkx2.1.
 fixed for 3-5 days in 95% ethanol followed by treatment with acetone for 3 days. Embryos were then rinsed with water, stained for cartilage with Alcian Blue staining solution (30 mg Alcian Blue in 20 ml glacial acetic acid and 80 ml of 95% ethanol) for 24 hours, rinsed twice with 95% ethanol, and incubated in 95% ethanol for 20 ml glacial acetic acid and 80 ml of 95% ethanol) for 24 hours, fixed for 3-5 days in 95% ethanol followed by treatment with acetone for 3 days. Embryos were then rinsed with water, stained for cartilage with Alcian Blue staining solution (30 mg Alcian Blue in 20 ml glacial acetic acid and 80 ml of 95% ethanol) for 24 hours, rinsed twice with 95% ethanol, and incubated in 95% ethanol for 2 days. The embryos were then treated with 1% KOH for 1 hour rinsed twice with 95% ethanol, and incubated in 95% ethanol for 20 ml glacial acetic acid and 80 ml of 95% ethanol) for 24 hours, fixed for 3-5 days in 95% ethanol followed by treatment with acetone for 3 days. Embryos were then rinsed with water, stained for cartilage with Alcian Blue staining solution (30 mg Alcian Blue in 20 ml glacial acetic acid and 80 ml of 95% ethanol) for 24 hours, rinsed twice with 95% ethanol, and incubated in 95% ethanol for 2 days. The embryos were then treated with 1% KOH for 1 hour rinsed twice with 95% ethanol, and incubated in 95% ethanol for 20 ml glacial acetic acid and 80 ml of 95% ethanol) for 24 hours, fixed for 3-5 days in 95% ethanol followed by treatment with acetone for 3 days. Embryos were then rinsed with water, stained for cartilage with Alcian Blue staining solution (30 mg Alcian Blue in 20 ml glacial acetic acid and 80 ml of 95% ethanol) for 24 hours, rinsed twice with 95% ethanol, and incubated in 95% ethanol for 20 ml glacial acetic acid and 80 ml of 95% ethanol) for 24 hours, fixed for 3-5 days in 95% ethanol followed by treatment with acetone for 3 days. Embryos were then rinsed with water, stained for cartilage with Alcian Blue staining solution (30 mg Alcian Blue in 20 ml glacial acetic acid and 80 ml of 95% ethanol) for 24 hours, rinsed twice with 95% ethanol, and incubated in 95% ethanol for 2 days. The embryos were then treated with 1% KOH for 1 hour.
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